Functional characterization of mammalian NDR kinase deficiency: Novel functions and insights into downstream signaling mechanisms

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(Dekan)

Table of content

A. List of abbreviations

Less frequently used abbreviation are defined upon their first use in the text.

AGC	Protein kinase A/G/C like
ATP	Adenosine triphosphate
C. elegans	*Caenorhabditis elegans* (roundworm)
Cdk	Cyclin-dependent kinase
CKI	Cyclin-dependent kinase inhibitor
D. melanogaster	*Drosophila melanogaster* (fruit fly)
DP/SP	single/ double positive
ENU	*N*-ethyl-*N*-nitrosourea
FACS	Fluorescence-activated cell sorter
HM	Hydrophobic motif
IRES	Internal ribosomal entry site
KD	Kinase-dead (mutation of ATP binding pocket)
LATS	Large tumor suppressor
MEF	Mouse embryonic fibroblast
MOB	Mps-one binder
MST	Mammalian Ste20 like kinase
NDR	Nuclear Dbf2 related kinase
NTR	N-terminal regulatory domain
OA	Okadaic acid
RASSF	Ras-association domain family
S/Ser	Serine
S. cerevisiae	*Saccharomyces cerevisiae* (budding yeast)
S. pombe	*Schizosaccharomyces pombe* (fission yeast)

STK	Serine/threonine kinase
T/Thr	Threonine
TA	Mutation of hydrophobic motif phosphorylation site to alanine
Trc	Tricornered
WT	Wild-type
YAP	Yes-associated protein

B. Summary

Protein kinases are important players in signal transduction. They are involved in the regulation of almost every aspect of biological regulation. Given their central role in signal transduction, aberrant protein kinase activities are involved in a variety of human diseases, such as diabetes or cancer. The human genome encodes for 518 protein kinases, which are further classified into groups, families and sub-families based on their catalytic domain. The NDR kinase family belongs to the AGC group (protein kinase A/G/C like) of serine/threonine kinases. Members of this family are highly conserved from yeast to men. Genetical and biochemical work on NDR kinases in yeast and invertebrates revealed, that NDR kinases are involved in the regulation of important biological processes such as mitotic exit, morphogenesis, neuronal and epithelial morphology, growth, proliferation and apoptosis amongst others. Although NDR kinases are implicated in a variety of biological processes amongst species, the topology of the signaling pathways regulating NDR kinases are remarkably conserved. NDR kinases from yeast to men are regulated by members of the Ste20 like kinases, MOB adaptor proteins and scaffolding proteins. The human genome encodes for 4 members of the NDR kinase family: NDR1, NDR2, LATS1 and LATS2. Although many of the biochemical mechanisms regulating NDR kinases have been worked out using human NDR1 and NDR2, functions for these two kinases have only been reported recently: Human NDR has been shown to function in centrosome duplication, the alignment of mitotic chromosomes and apoptosis signaling. Defects in regulation of these processes have been linked to tumor development. However, these functions were investigated using cell culture systems and physiological functions for NDR1 and NDR2 remain to be defined. Interestingly, NDR1 in these contexts has been shown to be regulated by components of the HIPPO tumor

suppressor pathway. The tumor suppressor proteins Rassf1a, MST1/2 and hMOB1 have been shown to not only regulate NDR kinase activity, but also the activity of the other NDR family members LATS1 and LATS2. LATS kinases function as tumor suppressors by restricting the activity of the YAP oncogene. Given the interaction with known components of the HIPPO pathway, a tumor suppressive function for NDR1 and NDR2 seems possible. However, although first functions for human NDR kinases have been defined recently, signaling mechanisms downstream of NDR1 and NDR2 remain elusive. Addressing two of the major questions relating to mammalian NDR kinases, we define a first physiological function for NDR kinases in mice in tumor suppression. Deficiency and heterozygosity of NDR1 predisposes mice to T-cell lymphoma development. Reduction of NDR kinase expression results in increased resistance to pro-apoptotic stimuli. Furthermore we identify a novel role for NDR1 and NDR2 in the regulation of cell cycle progression. NDR1/2 directly regulate the protein stability of the proto-oncogene c-myc and the cyclin-dependent kinase inhibitor p21. Mammalian NDR kinases therefore seem to play important roles in tumor and cell biology by regulating proliferation and apoptosis.

1. Introduction

1.1 Protein kinases: central players in health and disease

Protein kinases represent central players in signal transduction. This class of enzymes catalyzes the transfer of the gamma-phosphate residue from ATP to the hydroxyl-groups of the amino-acids serine, threonine or tyrosine (*1*). Protein kinases thereby create phosphorylation-marks on proteins which serve diverse functions in signal transduction such as activation/inactivation of enzymes, binding sites or localization signals. Protein kinases are therefore involved in almost every aspect of signal transduction and the proper regulation of their activity is crucial for the normal physiology of organisms (*2*).

The human genome encodes for approximately 518 different protein kinases which represents ~2% of all human genes (*2*). This protein family, also referred to as kinome, therefore constitutes one of the biggest enzyme families in the human genome. Based on the catalytic domain, Manning et al. grouped the protein kinases into various groups, families and sub-families, with tyrosine kinases representing the biggest group, followed by the CAMK, the CMGC and the AGC group of kinases (Figure 1). The kinase domain consists of 250-300 amino-acids, which contains conserved residues that contribute to nucleotide binding, metal-binding, substrate binding,

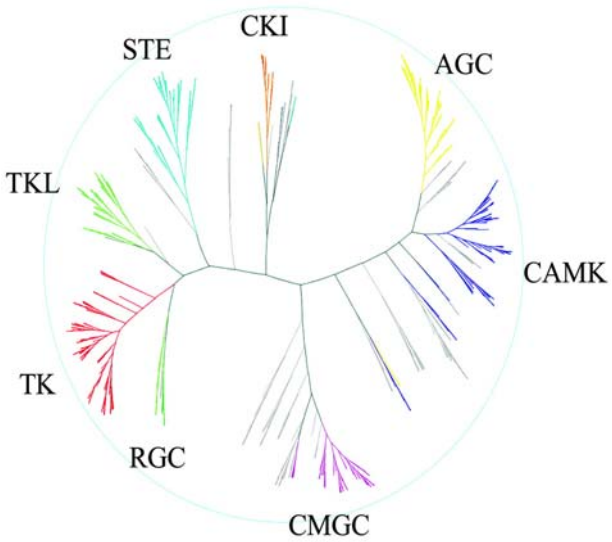

Figure 1. Dendrogram of 491 eukaryotic protein kinase domains from 478 genes. Major groups are labeled and colored. (Taken from (2)).

and phosphoryl- transfer. These residues are located within functional domains (subdomains) that are used to describe structural details of protein kinases (*1*). Given their central role in physiology, de-regulated kinase signaling has long been associated with the development of human diseases, such as diabetes, immune-defects, inflammation and cancer (*3*).

Diabetes mellitus is a disease which is characterized by the inability of the organism to either produce or properly respond to the hormone insulin, resulting in the inability to regulate blood glucose levels (*4*). In 1963 it was shown that a central enzyme important for insulin action, glycogen synthase is regulated by phosphorylation. These initial findings were followed by a long series of studies revealing that insulin exerts its function largely by initiating a kinase signaling cascade involving prominent members of the kinase family such as the insulin receptor, PI3Ks, PKB and GSK3 amongst others. Conversely, main players in the insulin-signaling cascade are under investigation for the treatment of diabetes (*4*).

Cancer is a heterogeneous disease evoked by the uncontrolled growth and homeostasis of cells. There are more than 100 distinct types of cancer, and subtypes of tumors can be found within specific organs. Despite this plethora of cancers, the principle alterations a cell has to acquire to become a tumor cell have been described in a ground-breaking review by Hanahan and Weinberg (*5*). Tumor cells are characterized by self-sufficiency in growth signals, insensitivity to growth-inhibitory signals, evasion of programmed cell death (apoptosis), limitless replicative potential, sustained angiogenesis, and tissue invasion and metastasis (Figure 2). Not surprisingly, protein kinases encounter central nodes in the regulation of each of these processes. Indeed, several kinases have been shown to act as oncogenes, such as BCR-ABL, Her2 or Src or are activated by oncogenes such as RAS. Interestingly,

recent research revealed that the PI3K pathway, a well known kinase signaling cascade represents the most highly mutated pathway in human cancers (*6*). Given the central roles of kinases in the development and onset of tumors, several kinases have emerged as drug targets over the years and successful therapies have been developed targeting these aberrant kinase activities. One famous example of these targeted therapies is the use of Gleevec to inhibit BCR-ABL in human chronic myeloid leukemia (CML). More than 90% of all cases of CML show a reciprocal chromosome translocation between chromosome 9 and 22 known as Philadelphia-Chromosome, resulting in the expression of a fusion protein kinase known as BCR-ABL. More than 20 years ago, Novartis in Basel, Switzerland started to use small molecules to inhibit protein kinases. This initiative let to the development of an inhibitor of the BCR-ABL kinase termed Gleevec, which got the FDA approval 2001 and stands as a powerful example of the possibilities of targeted therapies not only addressing aberrant kinase-activities but also other enzymes (*3*).

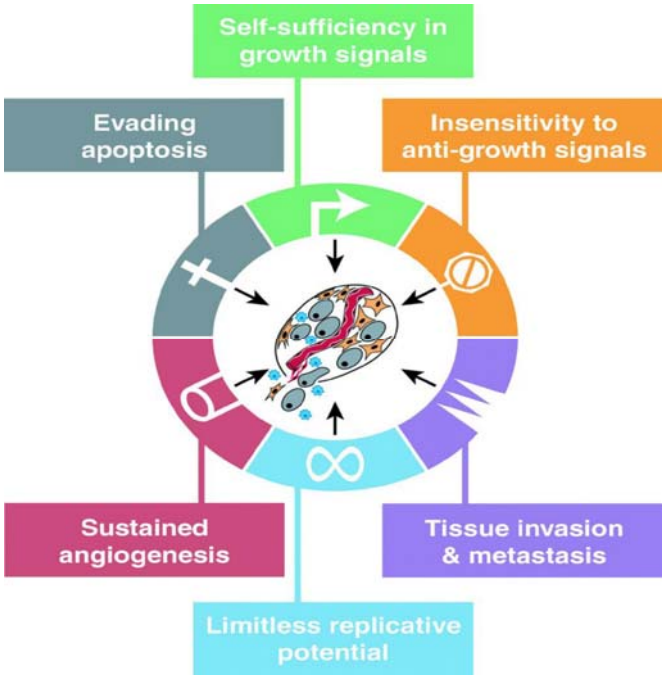

Figure 2. Acquired capabilities of cancer. It was suggested that most if not all cancers have acquired the same set of functional capabilities during their development, albeit through various mechanistic strategies. (Taken from 5)

These two examples of the roles of protein kinases in health and disease should highlight the importance of protein kinases as possible drug-targets. Investigating the functions of protein kinases therefore continues to present an important field of

research not only to provide novel targets for drug-discovery but also to understand fundamental processes in biology.

1.2 The NDR kinase family

The AGC (protein kinase A/G/C-like) group of protein kinases represents the third largest group of protein kinases in the human genome and consists of 63 members organized into 14 families (Figure 3) (*2*). Members of the AGC group are serine/threonine kinases. Notable representatives of this group are the protein kinase A (PKA), protein kinase C (PKC) and the protein kinase B (PKB) isoforms.

The NDR kinases represent one family within the AGC group. The human genome encodes four related NDR kinases: NDR1 (also known as serine/threonine kinase

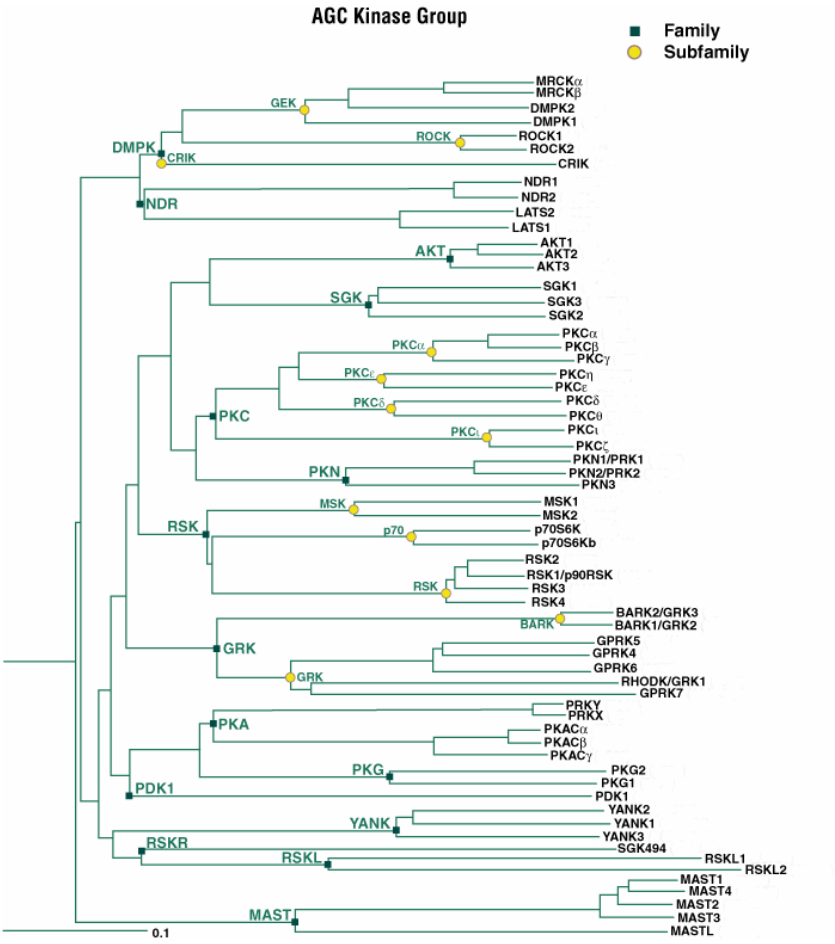

Figure 3. Dendrogram of the AGC-group of serine/threonine kinases. The AGC-group is divided into families and subfamilies as shown. Taken from www.cellsignal.com

(STK) 38/STK38), NDR2 (or STK38-like/ STK38L), LATS1 (Large-tumor suppressor 1) and LATS2 (Large-tumor suppressor 2) (*7*). NDR kinases are highly conserved from yeast to men and members can be found in yeast (Dbf-2/Dbf20p and Cbk1p in *S. cerevisiae* and Sid2p and Orb6 in *S. pombe*), *C. elegans* (SAX-1 and LATS) and *D. melanogaster* (Trc and LATS) as well as in other fungi, protozoan and plant genomes (*7*). Genetic and biochemical studies have shown that NDR kinases regulate various processes ranging from mitosis, morphogenesis, cell growth and proliferation, apoptosis, centrosome duplication, as well as various developmental processes (*8*). Taken together, NDR kinases in different species have been implicated in central important nodes in cellular signal transduction and research is ongoing to further define the biological roles of NDR kinases.

1.2.1 Structural characteristics of NDR kinases

The primary structure of NDR kinase family members is conserved from yeast to men (Figure 4) (*7*). As mentioned above, the kinase domain can be subdivided into 12 different subdomains based on the existence of conserved key residues (*1*). Although NDR kinases show typical characteristics of AGC group kinases such as the activation segment and the hydrophobic-motif phosphorylation sites, they exhibit two defining features: an insertion of 30-60 amino-acids in the kinase domain between subdomain VII and VIII and an N-terminal regulatory domain (NTR) (Figure 4).

The NTR contains a significant number of conserved basic and hydrophobic residues and has been shown to be responsible for the interaction with regulatory proteins of the MOB family as well as S100B (*9, 10*). Mutation of the conserved basic residues on NDR resulted in severely impaired binding of NDR kinases to MOB1 (*9, 11, 12*). Further insight was given by the analysis of the MOB1 structure in combination with

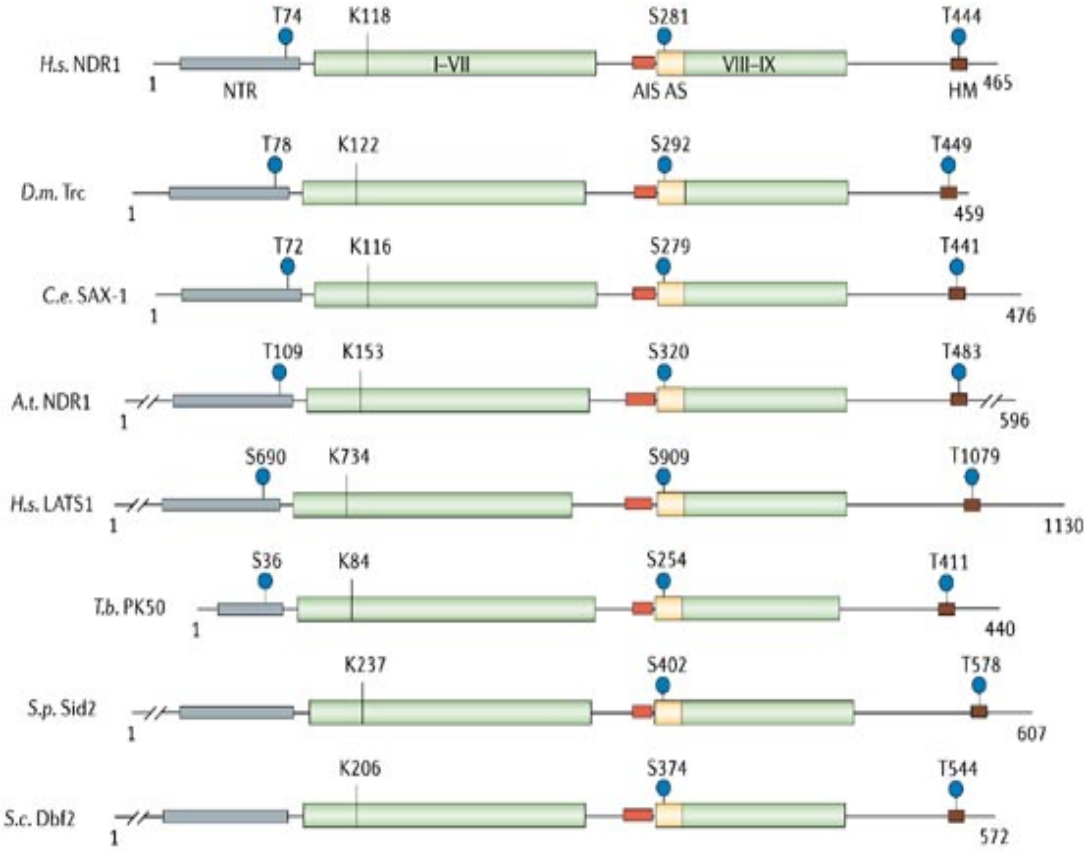

Figure 4. Primary structure of selected NDR family kinases. Eight members of the NDR kinase family are depicted from unicellular and multicellular organisms (*H.s. Homo sapiens, D.m. Drosophila melanogaster, C.e. Caenorhabditis elegans, A.t. Arabidopsis thaliana, T.b. Trypanosoma brucei*). The NTR (grey), the kinase domain (green) with the activation segment (yellow) and the hydrophobic motif (brown) are indicated. In addition, the auto-inhibitory sequence (red) and conserved phosphorylation sites (blue dots) are highlighted. Taken from (7).

an NDR N-terminal peptide (*13*). Interestingly the interaction area on MOB1 has been shown to be largely negatively charged, further supporting a NDR/MOB complex formation based on electrostatic interactions.

Although the 30-60 amino-acid insert between kinase subdomain VII and VIII is not conserved on the primary sequence between NDR family members, all show a stretch of basic amino-acids towards the C-terminus (*9*). As the insert precedes the activation segment, the positive charges of the cluster seem to impact on NDR kinase activity, since mutation of the basic amino-acids to alanines in NDR1 resulted in increased kinase activity (*9*). Therefore, the short stretch of basic amino-acids is referred to as an auto-inhibitory sequence (AIS) (*9*).

1.2.2 Regulation of NDR kinases

Regulation by Phosphorylation

Being members of the AGC group NDR kinases contain two conserved phosphorylation sites required for kinase activity: one in the activation loop (S281 in human NDR1) and one in the hydrophobic motif (T444 in human NDR1) (*7*). However, opposed to other AGC group kinases the activation loop phosphorylation of NDR kinases seems to be performed by auto-phosphorylation (*14, 15*). Still the hydrophobic motif (HM) is targeted by upstream kinases (*7*). The importance of the HM-motif for NDR kinase activation is further highlighted by the possibility to create a constitutively active NDR kinase by exchanging the HM of NDR2 with that of the constitutively active kinase PRK2 (*14*). Consistently, mutation of the hydrophobic motif phosphorylation site to alanine results in a complete loss of kinase activity (*14*).

Both phosphorylation sites are targeted by PP2A. Treatment of cells with okadaic acid or calyculin A (potent inhibitors of PP2A) results in increased phosphorylation and activation of yeast, fly and mammalian NDR kinases (*16-19*). In addition, recombinant PP2A is able to completely inactivate human NDR1 in vitro (*19*).

In higher eukaryotes a third phosphorylation site in the NTR is conserved (T74 in human NDR1 or S690 in human LATS1) (*14, 15*). The functional relevance of this site has not been evaluated. Mutation of this site to alanine has been shown to decrease both basal NDR kinase activity and NDR activation after okadaic acid treatment. However, this mutation also severely impairs NDR binding to MOB proteins and S100B (*9*). It remains to be shown, whether the effects of mutating T74 are a result of impaired binding to MOB1 or whether this phosphorylation regulates the kinase activity itself and whether phosphorylation of this site occurs in vivo.

Regulation of NDR kinases by Ste20 like kinases

First insights into the identity of upstream kinases for NDR kinase family members came from genetic studies in yeast. The yeast Ste20-like kinases Kic1p, Nak1p, Sid1p and Cdc15p have been placed upstream of the yeast NDR kinases Cbk1p, Orb6p, Sid2p and Dbf2p (*18, 20, 21*), however only Cdc15p was shown to directly phosphorylate and activate Dbf2p (*18*). Further evidence was gathered from work in *Drosophila*. Genetic and biochemical studies revealed that the Ste20-like kinase Hippo functioned upstream of LATS (*22-27*). Furthermore Hippo was able to interact with LATS and phosphorylate it in vitro. Interestingly, later it was shown that Hippo is also capable of phosphorylating and activating the NDR1/2 homologue trc in *Drosophila*, already indicating a diversion of Ste20-like kinase signaling on the level of NDR kinases (*28*).

The first upstream kinase solely phosphorylating the HM of NDR kinases was identified as the mammalian Ste20 like kinase 3 (MST3) (*29*). MST3 phosphorylates human NDR1/2 on T444/442 both in vitro and in vivo. The MST family of kinases consists of 5 members: MST1, MST2, MST3, MST4 and MST5 (also termed SOK1 or YSK1). Recent work has shown that also other members of the MST family represent in vivo upstream kinases for NDR family kinases. MST1 and MST2 have been shown to activate LATS1 and LATS2 in vivo (*16*). Interestingly MST1 is also able to regulate NDR1/2 in vivo (*30, 31*). Also MST2 was shown to phosphorylate NDR1/2 in vitro (*31*), but it has also been suggested to function as upstream kinase in vivo (*32*). Taken together, genetic and biochemical work from different organisms have established members of the Ste20-like kinase family as upstream kinases for NDR family members and indicated conserved signaling pathways across species. However, the existence of several members of Ste20-like kinases being able to

phosphorylate one given NDR kinase and the possibility of one Ste20-like kinase regulating 2 or even 4 different NDR kinase family members already implicates a significant complexity in NDR kinase signaling.

Regulation of NDR kinases by MOB proteins

The N-terminal region (NTR) represents one defining feature of NDR kinases. It has been shown to interact with members of the MOB family of adaptor proteins. MOB stands for Mps one binder. In *S. cerevisiae* Mob1p has been identified as regulator of Mps1 and Dbf2 localization and activity (*33, 34*). In addition yeast genomes encode for a second MOB isoform, Mob2p, which controls Cbk1p activity and localization in polarized growth (*35, 36*).

The human genome encodes 6 MOB related genes: MOB1A/B, MOB2 and MOB3A/B/C (*30*). So far only MOB1A/B and MOB2 have been shown to interact with NDR-family kinases and impact on NDR kinase activity (*30*). Whereas MOB1A/B has been shown to bind to and regulate the kinase activity of all four human NDR kinases, MOB2 solely binds to NDR1/2 (*9, 11, 37, 38*).

Most insight into the regulation of NDR kinases by MOB proteins has been gathered from work on human MOB1A/B. MOB1 isoforms interact with NDR and LATS in the N-terminal region and it seems likely that positively charged residues in the NTR interact with a negatively charged surface on MOB1 (*9, 12, 13, 38*). Recently it has been shown that interfering with MOB1-NDR1 complex formation also functionally impairs NDR1 in the context of centrosome duplication (*30*). Furthermore, mutation of the conserved residues important for MOB1 binding in LATS1 resulted in impaired kinase activity (*38*). Recent work showed that in addition to NDR kinase binding, MOB1 proteins are also capable of binding to MST1/2, possibly functioning as a

bridge between upstream kinase and NDR kinase (*31, 39, 40*). Indeed, the existence of a complex between Ste20-like kinase, MOB1 protein and NDR kinase has been reported (*31*). MST1/2 are also able to phosphorylate MOB1 and thereby strengthen complex formation with NDR kinases (*39*). However, although the interaction with MOB1 is required for NDR1/2 HM phoshorylation (*30, 31*), LATS1/2 HM-phosporylation seems to be regulated differently (*39*). In addition, exactly how MOB1-NDR association, apart from proximity to the upstream kinase, impacts on NDR activity has not been clarified yet. One study indicates that binding of MOB1 releases NDR1/2 from auto-inhibition by the AIS (*9*). More work and especially crystal structures of the MOB1-NDR kinase complexes are needed to further define the interplay between Ste-20 kinase, MOB1 protein and NDR kinase. However the data obtained so far indicate a model, in which MOB1 is phosphorylated by MST1/2 resulting in efficient complex formation between NDR kinase and MOB1 (*41*). Consecutively NDR kinases are phosphorylated by the upstream kinase resulting in full NDR kinase activation (Figure 5).

Much less is known about the role of MOB2 in NDR kinase signaling. However, it is interesting, that the NDR kinase-family is divided in terms of MOB2 binding. The NDR part of the family (such as human NDR1/2, trc, Cbk1 and Orb6) binds to MOB2 (*35, 37, 42, 43*), whereas the LATS part (LATS1/2, dLATS, Dbf2 and Sid2) does not. Conflicting data exists about the function of the NDR-MOB2 interaction. Work in budding and fission yeast indicates, that the MOB2-Cbk1/Orb6 interaction is important for NDR kinase localization and activity (*35, 43*). In *Drosophila*, work from the Adler lab has shown that dMOB2 interacts and co-localizes with tricornered, however, no kinase activity was measured in this study (*42*). Interestingly, overexpression of dMOB2 in this system resulted in a weak tricornered phenotype,

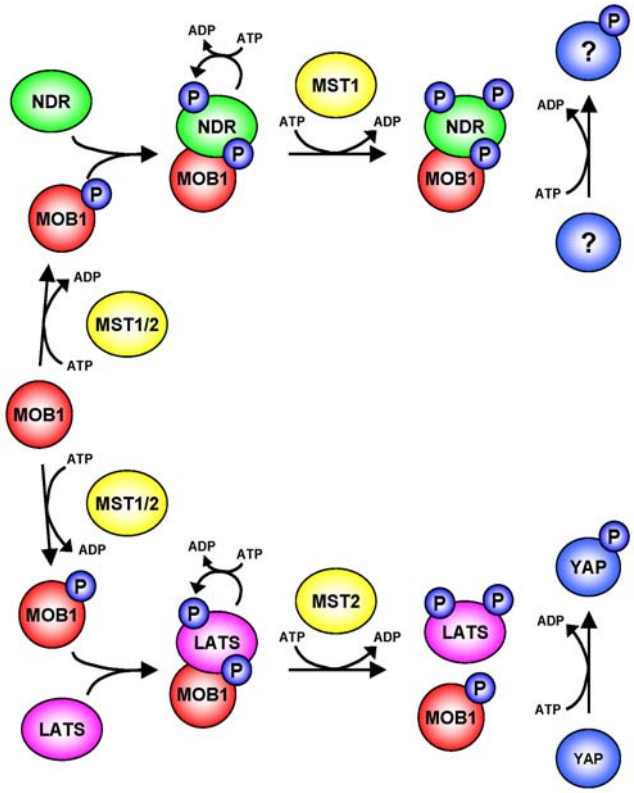

Figure 5. Current model of human NDR family kinase activation by MST kinases and MOB1 proteins. As an initial step, MST1/2 phosphorylate MOB1 which results in efficient complex formation with NDR kinases. Binding of MOB1 to NDR kinases facilitates activation loop auto-phosphorylation and hydrophobic motif phosphorylation by MST1/2. Fully active NDR kinases subsequently phosphorylate substrates such as YAP. Note that for substrate phosphorlyation NDR1/2 seem to stay in a complex with MOB1, whereas LATS1/2 do not seem to depend on MOB1 binding for continued activity. Taken from (41).

indicating, that dMOB2 could function as an inhibitor of tricornered. In higher eukaryotes, it has been shown, that MOB2 could activate NDR1/2 in vitro, however, the in vivo role of MOB2 for NDR1/2 has not been addressed so far. Recent work from the Hemmings lab, showed a different role for MOB2 for NDR kinase activity (Kohler et al. unpublished data). Addressing MOB2 function in vivo, the authors could show that MOB2 does not function as an activator, but rather an inhibitor of NDR kinase activation, both in apoptosis and centrosome duplication. Furthermore, although the exact binding site of MOB2 on NDR1 could not be mapped, the NTR seemed to be important and MOB2 was capable of competing with MOB1 for NDR1 binding (Kohler et al. unpublished data). Taken together, MOB proteins have been shown to be important regulators of NDR family kinases across species. With MOB1 proteins being activators of all NDR kinase family members tested so far, the role of

MOB2 proteins seems to be more complex; not all NDR family members bind to MOB2 and the function of MOB2-NDR complexes needs to be further defined.

Regulation of NDR kinases by scaffolding proteins

A third class of proteins represent conserved regulators of NDR kinase activity. In yeast, *C. elegans*, *D melanogaster* and humans, large scaffolding proteins have been identified, which impact on NDR kinase activity and function (*21, 32, 44-47*). In yeast the proteins Tao3p and Mor2p impact on Cbk1p and Orb6p respectively (*21, 47*), whereas in *C. elegans* SAX-2 has been shown to genetically interact with SAX-1 (*45*). In drosophila the scaffolding protein furry genetically and biochemically interacts with tricornered and was shown to be essential for trc kinase activity (*46*). Recently murine furry has been cloned, which interacts with NDR1 and seems to regulate its activity, although the functional context of this regulation remains unclear, as this has not been tested in terms of NDR1/2 signaling in the context of apoptosis and centrosome duplication (*32*).

For LATS1/2 in humans and LATS in *Drosophila* different scaffolding proteins have been described. The relatively small WW domains containing WW45 in mammals and Salvador in flies, have been shown to be important for LATS function (*16, 25, 26, 48*). Interestingly, these proteins are less conserved and so far no clear yeast orthologue has been described.

However, both classes of scaffolding proteins seem to function by linking NDR kinases with their respective upstream kinases. Work is still needed to define the role of scaffolding proteins for NDR kinase activity and function, especially with respect to MOB-NDR interaction.

1.2.3 Functions of NDR kinases

Functions for NDR kinases have first been worked out using genetic approaches in yeast. In *S. cerevisae* and *S. pombe* Dbf2p and Sid2p have been implicated in the regulation of the mitotic exit network (MEN) and the septation initiation network (SIN) (*49*), whereas the other members Cbk1 (in *S. cerevisae*) and Orb6 (in *S. pombe*) function in the regulation of morphogenesis (*50, 51*). Since then NDR kinases have been implicated in a variety of important biological functions such as the regulation of proliferation, cell growth and apoptosis, tumor suppression, neuronal growth, differentiation and tiling, centrosome duplication and embryonic development (Table 1). Although in lower eukaryotes the functions of NDR kinases have been worked out in a quite detailed manner using genetical approaches, only recently biochemical approaches identified substrates for NDR kinases. In addition, biochemical work in higher eukaryotes provided insights into the mechanisms of NDR kinase regulation, but only recently the physiological functions of NDR kinases in these systems begin to unravel. In the following several functions for NDR kinases in different model organisms are highlighted.

Regulation of mitotic exit and septation initiation in yeast

The mitotic exit network ensures that each daughter cell receives only one copy of each chromosome (*49*). Only then exit from mitosis and cytokinesis are initiated. Intensive research in budding and fission yeast revealed a conserved signaling network in control of this process, which is termed mitotic exit network (MEN) in *S. cerevisiae* and septation initiation network (SIN) in *S. pombe* (*49, 52, 53*). The NDR family kinases Dbf2 and Sid2p play important roles in the regulation of MEN and SIN. In *S. cerevisiae* the activation of MEN involves the small GTPase Tem1p, which

Kinase	Species	Functional information
Dbf2p	*S. cerevisiae*	Controls mitotic exit and cytokinesis#
Cbk1p	*S. cerevisiae*	Centerpiece of RAM network; couples cell morphology with the cell cycle
Sid2p	*S. pombe*	Regulates septum formation and cytokinesis#
Orb6p	*S. pombe*	Links morphological changes with the cell cycle
TBPK50	*T. brucei*	Coordinates cell shape and cell cycle
SAX-1	*C. elegans*	Important for neurite outgrowth and dendritic tiling#
Trc	*D. melanogaster*	Controls epidermal outgrowths, and dendritic tiling and branching#
Lats/Warts	*D. melanogaster*	Central player in the Hippo pathway; required for dendritic maintenance#
LATS1	*H. sapiens/M. musculus*	Regulates G2/M cell cycle transition, apoptosis and mitotic progression; part of the G1 tetraploidy checkpoint; the LATS1 cDNA can rescue the loss of *D. melanogaster* Lats/Warts#
LATS2	*H. sapiens/M. musculus*	Controls cell proliferation, genomic stability and mitotic progression; linked to the G1 tetraploidy checkpoint; essential gene, since LATS2 null mice die before embryonic day 12.5#
NDR1/STK38	*H. sapiens/M. musculus*	Required for centriole duplication and FAS mediated apoptosis. Implicated in the regulation of mitotic chromosome alighment. The NDR1 cDNA can compensate for the loss of *D. melanogaster* Trc#
NDR2/STK38L	*H. sapiens/M. musculus*	Functions in the regulation of neuronal growth and differentiation. Overexpression of NDR2 can induce centrosome duplication and can rescue defects in centriole dublication upon RNAi mediated knock-down of NDR1 #

Table 1. Selected functions of NDR kinase family members. Modified from (8). #References for the indicated functions can be found in the text.

stimulates the activation of the Cdc15p protein kinase, which subsequently activates the NDR kinase Dbf2. Dbf2 in turn phosphorylates the phosphatase Cdc14p, which results in re-localization of Cdc14p from the nucleolus into the cytoplasm, where it de-phosphorylates and inactivates the Cyclin B-Cdk complex, which allows exit from mitosis. The SIN is organized in a similar manner, consisting of the small GTPase Spg1p, the protein kinases Sid1p, Cdc7 and Sid2p, as well as the coactivator MOB1p regulating the phosphatase Clp1p.

Interestingly, LATS1 has been implicated in the regulation of mitotic exit in human cells (*11*). However, the network and the mechanisms regulating mitotic exit in human cells remain poorly defined and future studies are needed to show, whether the function of NDR kinases in regulating mitotic exit are conserved in higher eukaryotes.

Neuronal morphology and differentiation in C. elegans and D. melanogaster

Work in *C. elegans* and *D. melanogaster* revealed a function for NDR kinases in the regulation of neuronal growth, morphology and differentiation. The group of C. Bargmann could show that in *C. elegans* the NDR family kinase SAX-1 together with the large scaffolding protein SAX-2 regulates neurite outgrowth and dendritic tiling (*45, 54*). Dendritic tiling is a neuronal phenomenon in which the dendrites of a group of neurons with the same response characteristics completely innervate a tissue in a non-redundant manner (*55*). This process ensures that receptive fields of neurons do not overlap and prevents misconnections in neuronal systems. The involvement of NDR family kinases in this process has been shown to be conserved in *Drosophila* (*17*). A study performed in the Jan Laboratory revealed that the NDR kinase tricornered (trc) together with the large scaffolding protein furry controls dendritic tiling in fly neurons (*17*). Importantly, they could also show that this process is dependent on the kinase activity of trc, which is directly regulated by furry (*17, 46*). Further insight into the function of NDR kinases in neuronal morphology came from the same lab (*28*). Using biochemical and genetic approaches Emoto and colleagues could show that both tricornered and LATS are regulated by the Ste20 like kinase Hippo, revealing for the first time, that one given Ste20-like kinase could regulate two different NDR kinase family members, which was subsequently also shown in mammalian systems (*28*). While Hippo regulated trc for proper dendritic tiling and neurite outgrowth, Hippo signaling to LATS was important for maintenance of neurite structures (*28*). Interestingly, this indicated that two distinct NDR kinase family members could regulate two different aspects of the same biological process. In addition, recent work implicated the *Drosophila* target of rapamycin complex 2 (TORC2) in the regulation of trc activity in the process of dentritic tiling (*56*). Taken

together, work in invertebrates revealed an important function for the NDR kinases tricornered and LATS in neuronal morphology and differentiation. However, downstream signaling mechanisms of LATS and tricornered in the process of dendritic tiling and the maintenance of neurite structures remain to be described. It will be important in future studies to test, whether this function is conserved in higher eukaryotes. Interestingly, one report identified the murine NDR2 gene as being upregulated in amygdala neurons from fear-conditioned mice (*57*). In cultured cells NDR2 was involved in neuronal growth and differentiation, especially in neurite outgrowth, indicating a conserved role for NDR kinases in neuronal morphology (*57*).

Tumor suppression in Drosophila and higher eukaryotes: The HIPPO pathway

The NDR kinase LATS (large tumor suppressor) has first been identified in screens for putative tumor suppressors performed in *Drosophila* by two independent labs (*58, 59*) and subsequently been found to also function as tumor suppressor in mice (*60*). In addition, the Ste20-like kinase Hippo was placed upstream of LATS in tumor suppression (*22, 27*). Since then more than 10 different proteins have been implicated in the regulation of the emerging tumor suppressor pathway arranged around Hippo and LATS (*41*). This tumor suppressor pathway, which also seems to be conserved in mammalian systems, is nowadays collectively called the Hippo-pathway.

The major players in this pathway in flies are the kinase Hippo, the co-activator/scaffold proteins Salvador and Mats, and the NDR family kinase Warts/LATS, which negatively regulates the Yorkie proto-oncogene, thereby controlling cell growth, proliferation and apoptosis (*61*) (Figure 6). Various stimuli from upstream molecules, such as Fat/Expanded and Merlin (*62-65*), activate the Hippo kinase, which forms a complex with salvador (*27*). This complex then

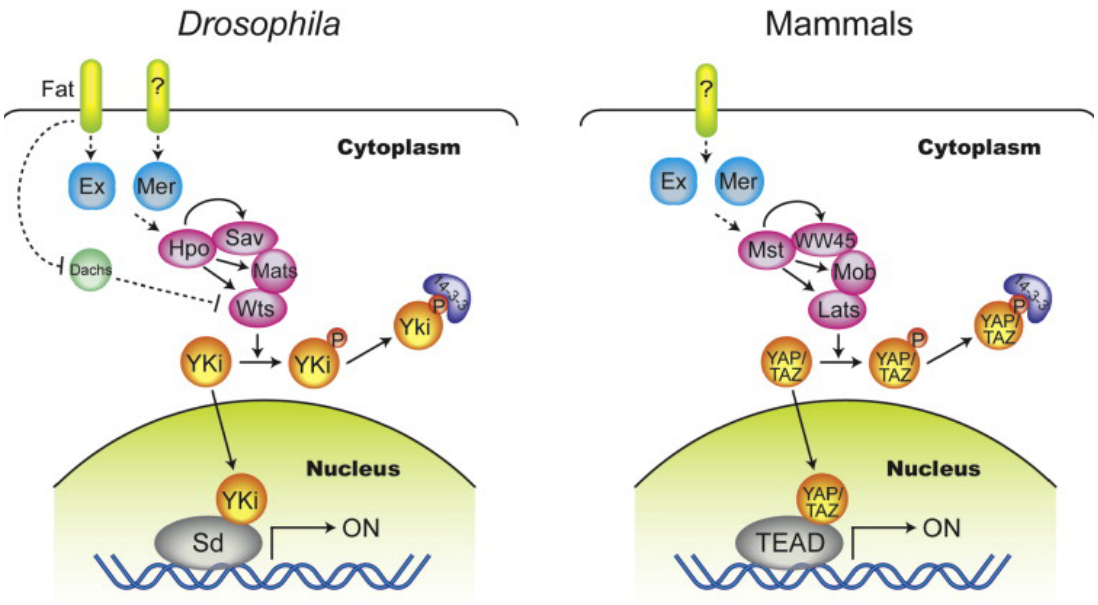

Figure 6. Schematic representation of the HIPPO pathway in *Drosophila* and mammals. Corresponding components are shown in the same color. Question marks denote unknown components and dashed lines indicate unknown biochemical mechanisms. Abbreviations are as follows: Ex (Expanded), Mer (Merlin, also called NF2), Hpo (Hippo), Sav (Salvador), Mats (Mob as tumor suppressor), Wts (Warts), Yki (Yorkie). Taken from (61).

phosphorylates LATS, which in turn phosphorylates Yorkie (*23*). Importantly Yorkie represents the first in vivo substrate for an NDR kinase family member identified. Yorkie is phosphorylated by LATS on Serine 168, which results in 14-3-3 binding and nuclear exclusion (*66*). Yorkie functions as a transcriptional co-activator which interacts with the TEAD transcription factor scalloped to regulate the expression of dCyclinE, dE2F1, dIAP and the bantam microRNA (*67-73*).

Recent results from mammalian cells indicate that this pathway is conserved in higher eukaryotes (*74*). The mammalian HIPPO pathway is composed of the Hippo homologues MST1/2, the mammalian salvador protein WW45, the Mats homologues MOB1A/B as well as the mammalian LATS kinases LATS1/2, and the Yorkie-related transcriptional coactivators YAP and TAZ (*41*). Interestingly, the mammalian HIPPO pathway differs in some components from its *Drosophila* counterpart. First on YAP 5 LATS1/2 responsive phosphorylation sites have been identified (in addition to S127, the site homologous to S167 in Yorkie, S61, S109, S164 and S397) (*75*). Although S127 seems to be main site regulating YAP nuclear localization (*76*), one study

reported that all serines have to be mutated to affect YAP phosphorylation at least in cell culture systems (*75*). In addition, the activity of MST1/2 in mammalian systems is regulated by the RASSF1A tumor suppressor protein (*77*). RASSF1A binds to MST1/2 through their SARAH domains and thereby stimulates MST1/2 kinase activity (*77*). In *Drosophila* however, dRASSF, although functioning as a tumor suppressor by inhibiting dRaf1, inhibits Hippo activation by competing with salvador for Hippo binding (*78*). Recently, another RASSF isoform, RASSF6, has been indicated to function in a similar manner than dRASSF in mammalian cells (*79*). Another difference so far is due to mammalian YAP being able to bind to other transcription factors than TEAD1-4. In apoptosis signaling YAP stimulates the transcriptional activity of the p53 isoform p73 (*80, 81*). In addition, YAP is able to bind to RUNX1/2 and other PPXY-motif-containing transcription factors as well as the ErbB4 cytoplasmatic domain (*82, 83*).

The evaluation of the conserved tumor suppressive function of the HIPPO pathway in mammals has so far mainly been performed in cell-culture systems and evidence for a tumor-suppressor function for some crucial components from gene-knockout studies in mice is still lacking. However, for some components of the mammalian HIPPO pathway a tumor suppressive function in mice has been validated. One of the earliest components which was targeted in mice was LATS1 (*60*). LATS1 deficient mice are viable and fertile (although an increased rate of infertility was noted) and develop soft tissue sarcoma and ovarian tumors. Interestingly, LATS2 deficient mice are embryonic lethal (*84, 85*). Although LATS2$^{-/-}$ MEFs display loss of contact inhibition and genomic instability, due to the embryonic lethality, tumor spectrum and penetrance in these mice have not been analyzed so far. Similar to LATS2 mice, mice lacking WW45 are embryonic lethal (*48*). No mice lacking MOB1A/B have been

described so far. RASSF1A and Merlin/Nf2 have been targeted in mice as well and their tumor-suppressive role has been confirmed (*86, 87*). Recently Zhou and colleagues confirmed the tumor suppressive role of the Hippo homologues MST1 and MST2 (*88*). Mice deficient for MST1 have been described earlier; however they did not exhibit increased tumor susceptibility, but rather defects in T-cell proliferation and homing (*89, 90*). Subsequently, mice deficient for MST2 have been described, which did not show any phenotype (*91*). Mice double deficient for MST1 and MST2 have been shown to be embryonic lethal, confirming a redundant role for MST1 and MST2 in embryonic development (*91*). Using a floxed allele for MST2 on an MST1 deficient background and adenoviral mediated cre-recombinase delivery to the liver, Zhou and colleagues could show that mice lacking MST1/2 in the liver develop tumors in a YAP-dependent manner (*88*). However it is important to note, that although YAP phosphorylation upon deletion of MST1/2 was severely impaired, no changes in LATS1/2 phosphorylation were observed. In addition, fractionation experiments revealed a YAP-kinase activity different from LATS1/2 to be responsive to MST1/2 deletion, which led the authors to conclude that a different YAP kinase apart from LATS1/2 exists in the liver. Interestingly, hydrophobic motif phosphorylation of the other NDR family kinases in mice, NDR1/2, was clearly reduced, suggesting a possible role for NDR1/2 in suppressing liver-cancer development downstream of MST1/2. In cell culture systems NDR1/2, however, failed to phosphorylate YAP (*75*). More work and more sophisticated mouse models are needed to further define the components of the mammalian HIPPO pathway. It will also be important in the future to define the transcriptional targets regulated by the mammalian HIPPO pathway. Still, work from *Drosophila* and mammalian

systems defines the HIPPO pathway as an important regulator and integrator of cell growth, proliferation and apoptosis.

The functions of NDR1 and NDR2 in mammalian systems

Although the major mechanisms of biochemical regulation for NDR family members have been worked out using human NDR1 and NDR2, functions for NDR1/2 have only recently been identified. By looking at the subcellular localization of NDR1 Hergovich and colleagues identified a subfraction of NDR1 binding to the centrosomes (*92*). Overexpression of NDR1 and NDR2 in this system resulted in centrosome overduplication. Later it was shown that the role of NDR1/2 in centrosome duplication was dependent on MOB1 and MST1 (*30*). Furthermore, another study from the Hemmings lab described the activation of NDR1/2 downstream of RASSF1A and MST1 in apoptosis signaling (*31*). Interestingly, RNAi mediated knock-down of NDR1/2 resulted in apoptosis defects upon FAS-induced apoptosis. Recently a function for NDR1/2 in the alignment of mitotic chromosomes has been described (*32*). By studying the mammalian homologue of furry, Chiba and colleagues indicated a function for NDR1/furry in microtubule stability and dynamics. However, the results of this study remain to be confirmed, as the well established centrosome phenotype of NDR1 depleted cells could not be reproduced. In addition, the authors fail to reproduce the stimulating effect of MOB1 on NDR kinase activity. Taken together, although characterized well in terms of biochemical activation, the physiological functions of mammalian NDR1 and NDR2 remain poorly defined, which can be mainly attributed to the lack of *in vivo* substrates identified for NDR1 or NDR2. Future studies using mice carrying targeted alleles for NDR1 and NDR2 are warrant to further define physiological functions of NDR1 and NDR2.

2. Scope of the thesis

The aim of this study was to further investigate the functional roles of NDR1 and NDR2 in cell-culture systems and mice. Although in the meantime NDR1/2 have been implicated in the process of centrosome duplication, apoptosis and the alignment of mitotic chromosomes, we describe the first physiological function for NDR kinases in mice. Loss of NDR1 predisposed mice to the development of T-cell lymphoma both upon age and after carcinogen treatment. Although loss of NDR1 was compensated by increased levels of NDR2 in healthy tissues, total NDR kinase levels were found to be reduced in murine as well as human T-cell lymphoma samples. Interestingly, interfering with NDR2 upregulation in healthy, untransformed cells resulted in increased resistance to pro-apoptotic stimuli, indicating that by ensuring proper apoptotic responses NDR kinases function as tumor suppressors in mice.

In addition a novel function of NDR1/2 was identified. By analyzing NDR kinase activation in a cell cycle dependent manner, we found that NDR1/2 were activated in G1-phase of the cell cycle. RNAi mediated knock-down of both NDR1 and NDR2 resulted in proliferation defects due to a G1-block. Importantly, NDR kinases were found to directly impact on the stability of c-myc and p21 by different mechanisms. Whereas c-myc directly bound to NDR1/2 in a kinase-activity independent manner, p21 was found to be directly phosphorylated by NDR1/2. These findings represent the first downstream signaling mechanisms of NDR1/2 in a functional context.

3. Results

The results obtained during this thesis are separated into two manuscripts. Manuscript one entitled "Ablation of mammalian NDR1 kinase predisposes mice to T-cell lymphoma development" describes the work performed with NDR1 targeted mice and the role of NDR kinases in tumor development. The second manuscript describing the role of NDR kinases in G1-progression/S-phase entry and the work on signaling mechanisms downstream of NDR is entitled "Human NDR kinases control G1 progression/S-phase entry by regulating p21 and c-myc stability". The numbering of figures and references in these manuscripts is restricted to each manuscript, meaning that for each manuscript the numbering of figures and references starts with 1.

3.1 Ablation of mammalian NDR1 kinase predisposes mice to T-cell lymphoma development

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3.1.1 Abstract

Defective apoptosis has been shown to contribute to the development of a variety of human malignancies. The kinases NDR1 and NDR2 have been implicated in the regulation of apoptosis downstream of the tumor necrosis factor-receptor family member FAS. FAS signaling is crucial for lymphocyte homeostasis with defects being linked to lymphoproliferative disorders, autoimmunity and cancer. To further analyze the role of NDR1 downstream of FAS, NDR1-deficient mice were generated. Mice lacking NDR1 appeared normal and NDR1-deficient T-cells exhibited normal responses to different pro-apoptotic stimuli. Analysis of NDR1 and NDR2 expression revealed that NDR1 loss is functionally compensated by an increase in NDR2 protein levels. Despite this compensation NDR1-deficient and heterozygous mice showed significantly increased T-cell lymphoma development. Analysis of NDR1 and NDR2 expression in T-cell lymphoma samples revealed that tumor development in mice and human is accompanied by a decrease in NDR kinase levels. Thus, reduction of NDR1 triggers a decrease in total NDR kinase expression. Taken together, our data suggests that low expression of NDR kinases results in defective response to pro-apoptotic stimuli, thereby facilitating tumor development.

3.1.2 Introduction

Apoptosis signaling in the immune system has been implicated in a variety of biological processes ranging from the regulation of immune cell development to lymphoid homeostasis and the clearing of cells after immune responses (1). Defects in apoptosis signaling predispose to the development of diseases such as autoimmunity or cancer (1). FAS signaling in immune cells has been shown to be important for the control of lymphocyte homeostasis with defects resulting in lymphadenopathy, autoimmunity and lymphoma development (2). Signaling downstream of FAS is initiated upon binding of its ligand FASL to the extracellular domain of the receptor resulting in receptor clustering and recruitment of the adaptor protein FADD and procaspase 8 with subsequent activation and cleavage of procaspase 8. Active caspase 8 in turn cleaves other proteins such as the effector caspases 3 and 7 leading to full activation of the apoptotic program (2).

Among the substrates of active caspases following FAS activation is the mammalian Ste20- like-kinase 1 (MST1) (3). MST1 has been shown to be a pro-apoptotic kinase activated by cleavage upon FAS activation. Members of the NDR/LATS family of protein kinases are among the substrates phosphorylated by MST1 (4, 5).

Four members of the NDR/LATS family exist in mammalian genomes (LATS1/2 and NDR1/2) (6). The NDR kinase family members LATS1/2 function as tumor suppressors as part of the HIPPO pathway in *Drosophila* and mice (7, 8). Work using cell culture systems has implicated the other mammalian NDR kinase family members NDR1 and NDR2 in the regulation of centrosome duplication, mitotic chromosome alignment and apoptosis induction (4, 9, 10). NDR1 and NDR2 display high sequence identity and so far no differences in biochemical regulation or function have been

described, although both kinases show a distinct expression pattern in mammalian organisms (11). While NDR1 is highly expressed in immunological organs and especially in the thymus, NDR2 is mainly expressed in tissues of the digestive tract indicating tissue specific functions of both isoforms. Given the predominant expression of NDR1 in lymphocytes and its suggested role in apoptosis signaling downstream of FAS, we generated mice lacking NDR1 to analyze its role in apoptosis signaling in lymphocytes.

Here we present evidence that NDR1 functions as a haploinsufficient tumor suppressor. Reduction in NDR1 gene-dose is functionally compensated by increased NDR2. Despite this apparent compensation, complete loss or heterozygosity of NDR1 predisposes mice to T-cell lymphoma development, which is accompanied in mice by a reduction in both NDR1 and NDR2 levels. Consistent with this finding, NDR1 levels in human T-cell lymphoma samples are frequently reduced indicating a conserved tumor suppressor function for NDR1 in T-cell lymphoma.

3.1.3 Results

Generation of NDR1-deficient mice

To obtain mice deficient for NDR1, exon 4 of the gene encoding NDR1 was targeted (Figure S1A). Successful recombination was demonstrated by Southern blotting and PCR (Figure S1B, C). The absence of NDR1 protein was confirmed in the knock-out mice using an antibody specific for murine NDR1 (Figure S1D). There was also a decrease in NDR1 protein in heterozygous mice, suggesting a gene-dose effect. Since complete ablation of the gene encoding the respective NDR orthologue (tricornered; trc) in Drosophila results in embryonic lethality (*12*), variation in genotype ratio among the

progeny of NDR1 heterozygous matings was analyzed (Figure S1E). As the progeny showed the expected Mendelian ratio, the deletion of NDR1 did not lead to obvious developmental defects. In addition organ weights and architecture in young $NDR1^{+/+}$, $NDR1^{+/-}$ and $NDR1^{-/-}$ mice were analyzed without showing significant differences. Thus, ablation of NDR1 in mice results in viable, fertile animals without obvious defects.

NDR1 is activated in thymocytes in response to different apoptotic stimuli

An earlier report described the activation of NDR kinases in transformed cells in response to FAS and TNFα treatment. Furthermore, shRNA-mediated knock-down of both isoforms resulted in increased apoptosis resistance (4). To confirm these findings in untransformed cells, freshly isolated thymocytes were treated with stimuli to activate extrinsic and intrinsic apoptotic pathways (Figure 1A). Two hours after induction of apoptosis with anti-FAS antibody, there was an increase in hydrophobic motif phosphorylation of NDR (T444-P) which coincided with MST1 activation as monitored by cleavage and phosphorylation of MST1 (Figure 1A). A similar activation of NDR was observed after gamma irradiation and dexamethasone treatment (Figure 1A). These results showed, that NDR1/2 were not only activated in response to extrinsic apoptotic stimuli, but also to intrinsic stimuli.

Next, we tested whether loss of NDR1 would result in increased apoptosis resistance of thymocytes. Freshly isolated thymocytes from $NDR1^{+/+}$, $NDR1^{+/-}$ and $NDR1^{-/-}$ mice were treated with gamma irradiation, etoposide, dexamethasone or anti-FAS antibody and apoptosis was measured 10 h after induction (Figure 1B). Furthermore thymocyte cell death upon treatment with anti-CD3/CD28 antibodies and cytokine withdrawal was

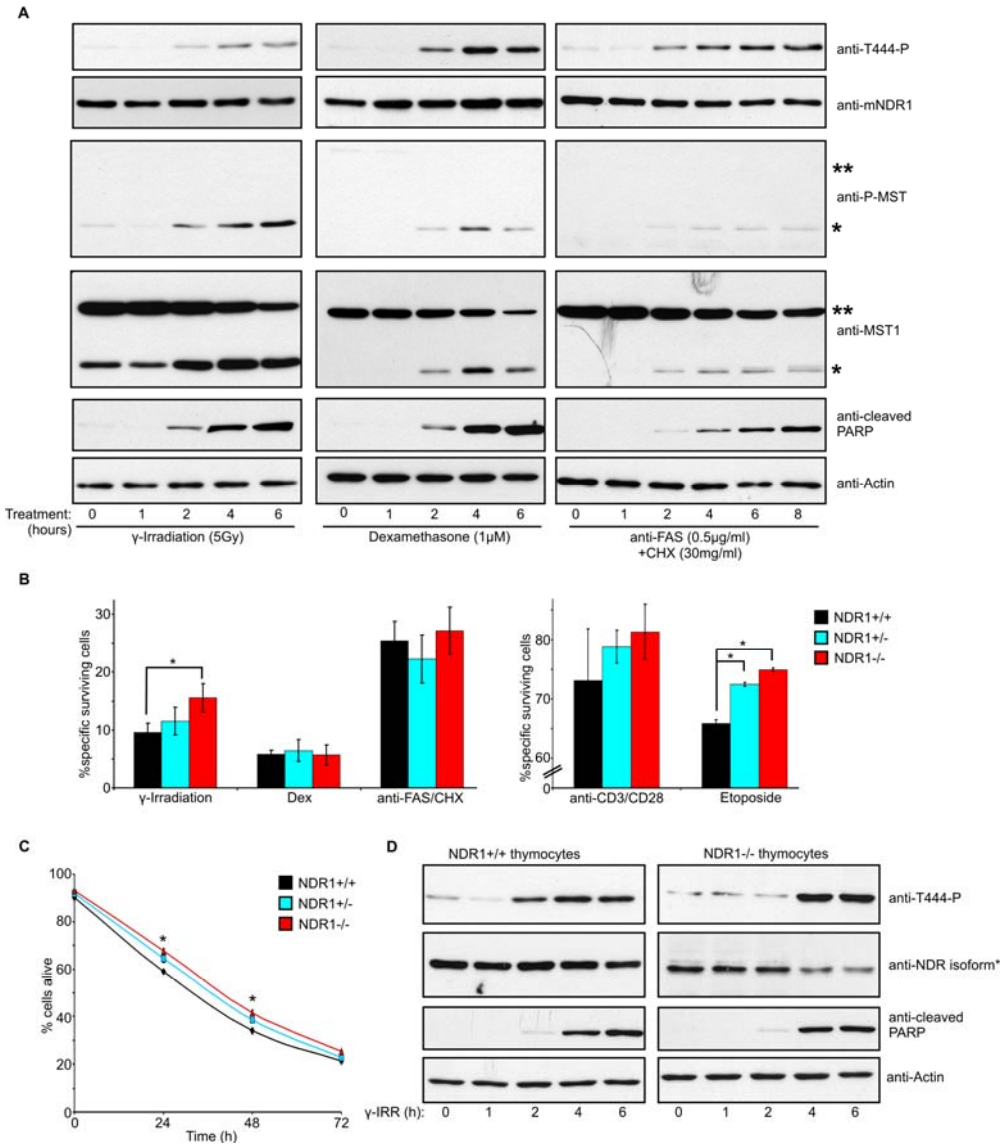

Figure 1. NDR1 is activated in response to different pro-apoptotic stimuli, but largely dispensable for apoptosis induction.
A: Thymocytes were isolated from young wild-type mice (4-6 weeks old) and apoptosis was induced by gamma irradiation, dexamethasone or treatment with anti-FAS antibody+ cycloheximide. Cells were lysed after the indicated incubation times and analyzed for the activation of NDR1/2 and MST1 (** uncleaved form of MST1; * cleaved form). **B:** Thymocytes were isolated and treated with gamma irradiation, dexamethasone, anti-FAS+CHX, etoposide or anti-CD3/CD28 and apoptosis was measured 10 h later (or 24h later for anti-CD3/CD28) by PI/AnnexinV staining. Specific survival of cells was calculated as the ratio of PI/AnnexinV double-negative cells with and without treatment (n = 3, * P<0.005). **C:** Isolated thymocytes were seeded into IMDM+10%FCS and analyzed after the indicted time using a Vicell automated cell counter. **D:** Activation of NDR kinases in NDR1$^{+/+}$ and NDR1$^{-/-}$ thymocytes. Freshly isolated thymocytes were treated with gamma irradiation and lysed after the indicated times. Activation of NDR was assessed using the anti-T444-P antibody; total kinase was analyzed using the NDR1 antibody for wild-type thymocytes and the NDR2 antibody for NDR1-deficient samples.

accessed (Figure 1B/C). No statistically significant differences in the number of cells surviving after dexamethasone, anti-FAS or anti-CD3/CD28 treatment were detected (Figure 1B). Although not statistically significant, NDR1$^{+/-}$ and NDR1$^{-/-}$ thymocytes displayed a tendency of increased survival after anti-CD3/CD28 treatment. However, a

statistically significant decrease in the number of surviving $NDR1^{+/+}$ when compared to $NDR1^{-/-}$ cells was detected after gamma irradiation and etoposide treatment (Figure 1B). Furthermore, NDR1 deficient thymocytes showed increased survival after cytokine withdrawal (Figure 1C). Treatments which resulted in increased survival of NDR1 deficient thymocytes seemed to also result in increased survival of $NDR1^{+/-}$ cells. In addition, mature T-cells were tested for a defect in response to activation-induced cell death (AICD) and cytokine withdrawal (Figure S2). Again, loss of NDR1 and in the case of cytokine withdrawal also heterozygosity resulted in slight, but statistically significant resistance of NDR1 targeted cells towards apoptosis induction.

These results were confirmed by analyzing NDR activation in $NDR1^{-/-}$ thymocytes following apoptosis induction; a delay in NDR activation in knock-out thymocytes was only observed after gamma irradiation (Figure 1D and S3). Taken together, these experiments showed that although NDR was activated in thymocytes upon treatment with several apoptotic stimuli, loss of NDR1 alone did not result in strong apoptotic resistance.

NDR2 compensates for NDR1 deficiency

NDR1 and NDR2 show >80% identity at the protein level (11). Thus, the absence of developmental phenotypes and the overall normal apoptotic responses in $NDR1^{+/-}$ and $NDR1^{-/-}$ mice might be due to compensation by NDR2. The effect of NDR1 heterozygosity and deficiency on NDR2 expression was analyzed using tissues with high (thymus, spleen and lymph nodes) and low (colon) NDR1 expression (Figure 2A; Figure S4). NDR2 protein levels were up-regulated upon NDR1-knockout in a tissue specific manner (Figure 2A). Compared with a minor up-regulation of ~1.5-fold in

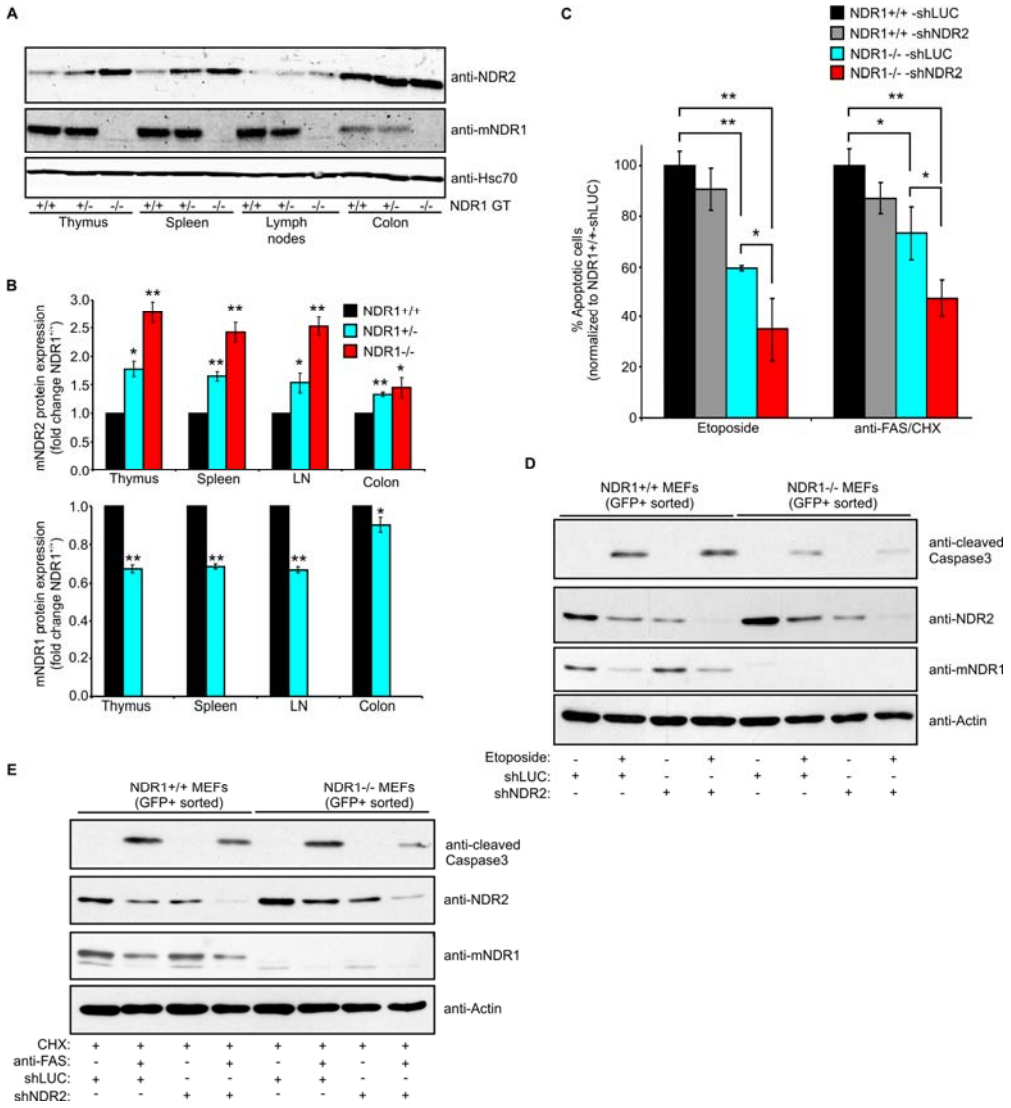

Figure 2. Reduction in NDR1 gene-dose is compensated by increased NDR2 protein levels.
A: Analysis of NDR1/2 expression in representative tissues from NDR1$^{+/+}$, NDR1$^{+/-}$ and NDR1$^{-/-}$ mice using specific antibodies against NDR2 and NDR1. **B:** Quantification of changes in NDR1 and NDR2 protein levels upon heterozygosity and deficiency of NDR1 protein (n = 3). Changes are expressed as fold changes in the NDR/Hsc70 ratio as compared with the wild-type level (* P<0.03; ** P<0.005). **C:** Primary MEFs expressing shRNA against firefly luciferase (shLUC) or NDR2 (shNDR2) were seeded for apoptosis induction 72 h after transfection. Apoptosis was induced 24 h later using 200µM etoposide or 1µg/ml anti-FAS-Ab in the presence of 1µg/ml CHX. Apoptosis in GFP^{+} cells was measured 22 h for etoposide and 18h for anti-FAS/CHX after induction by FACS. Results obtained were normalized to NDR1$^{+/+}$-shLUC for better comparison of the two treatments (* P<0.03; ** P<0.005). **D:** Primary MEFs were transfected as described in C, sorted for GFP^{+} and further expanded for 48h. Apoptosis was induced using 200µM etoposide for 22h. **E:** MEFs were pretreated as in D, but apoptosis was induced using 1µg/ml anti-FAS-Ab in the presence of 1µg/ml CHX for 18h.

colon, there was a 2.5- to 3-fold increase in tissues with high NDR1 expression (Figure 2B). NDR2 was slightly increased in NDR1$^{+/-}$ mice compared with NDR1$^{+/+}$, whereas NDR1 protein levels were lower, reflecting the gene-dosage effect observed earlier (Figure S1D). These changes, however, did not result from increased transcription of the NDR2 gene or increased NDR2 protein stability (Figure S5). Taken together, NDR1

deficiency in mice appears to be compensated by an increase in NDR2 protein levels via a posttranscriptional mechanism.

We analyzed next whether interfering with the observed compensation would result in apoptosis defects. Early passage NDR1$^{+/+}$ and NDR1$^{-/-}$ MEFs expressing shRNA against NDR2 (shNDR2) or luciferase control (shLUC) were treated with both etoposide and anti-FAS. Knock-down of NDR2 in NDR1$^{-/-}$ MEFs resulted in a significant decrease in apoptotic cells compared to wild-type MEFs expressing shLUC (NDR1$^{+/+}$-shLUC) (Figure 2C; Figure S6). Although not consistently observed in thymocytes (Figure 1B/C), NDR1 deficiency in MEFs resulted in increased resistance towards both stimuli. This might indicate that compensation efficiency was cell-type dependent or disturbed upon *ex vivo* culture. However, reduction of NDR2 in the NDR1-deficient background further reduced apoptosis in this setting (Figure 2C). In addition, analysis of apoptotic extracts revealed that reduction of NDR2 in NDR1-deficient MEFs resulted in significantly reduced cleavage of caspase 3 after both anti-FAS and etoposide treatment (Figure 2D/E). This data showed that NDR2 functionally compensated NDR1 reduction and deficiency in untransformed cells, with defects in this compensation resulting in increased resistance towards apoptotic stimuli.

Aged NDR1$^{+/-}$ and NDR1$^{-/-}$ animals develop high-grade peripheral T-cell lymphoma

Although our initial analysis revealed that loss of NDR1 is functionally compensated by NDR2 in a gene-dosage dependent manner, with only minor apoptotic defects resulting from NDR1 deficiency and heterozygosity, it seemed possible that targeting NDR1 could contribute to the development of age-related diseases. To this end, we analyzed aged (17-27months) NDR1$^{+/+}$, NDR1$^{+/-}$ and NDR1$^{-/-}$ mice (Figure 3A). Approximately

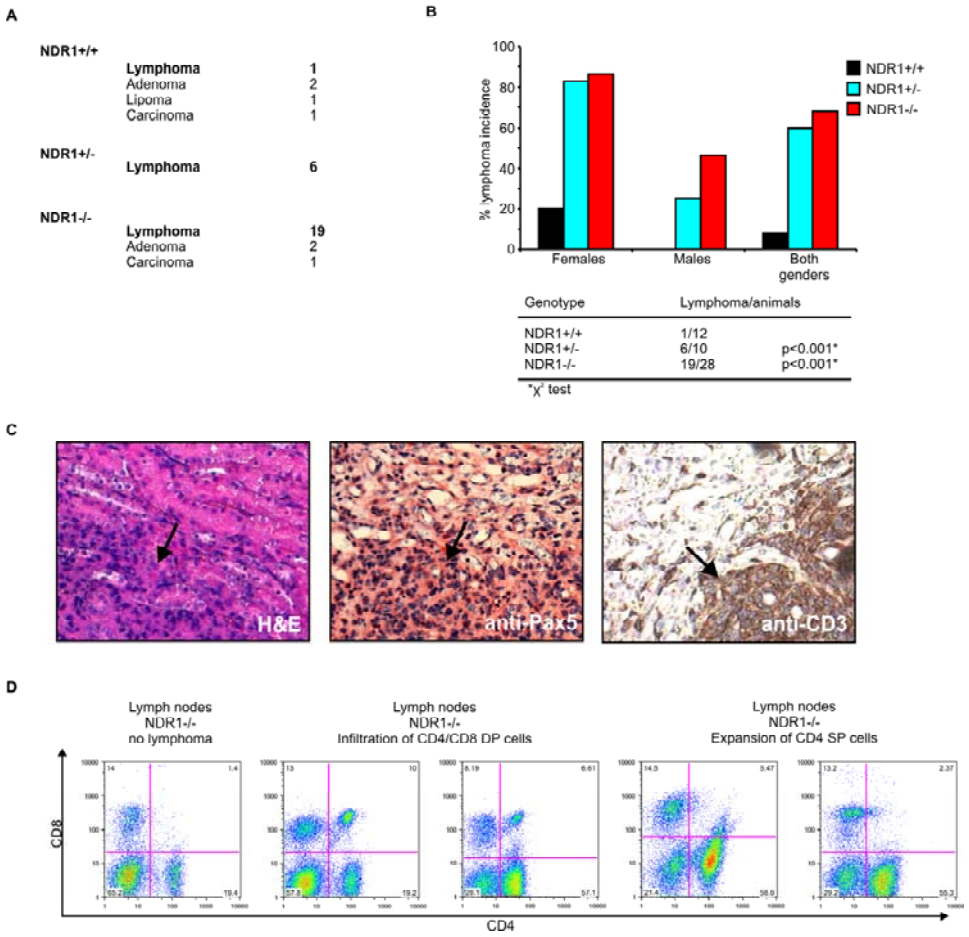

Figure 3. Aged NDR1$^{+/-}$ and NDR1$^{-/-}$ animals develop high-grade peripheral T-cell lymphoma
A: Tumor spectrum in aged NDR1$^{+/+}$, NDR1$^{+/-}$ and NDR1$^{-/-}$ mice (age 18-27 months). Mice were dissected and H&E-stained tissue sections were analyzed for the development of tumors. **B:** Bar chart representing the rates of lymphoma detected in aged NDR1$^{+/+}$, NDR1$^{+/-}$ and NDR1$^{-/-}$ mice. In addition, the gender-specific rates of lymphoma development are shown. Note that female mice seem to be more prone to develop lymphoma than male. The numbers of total mice used for analysis are given in the lower panel. **C:** Example of immunohistochemical characterization of identified lymphatic lesions. Tumor cells (arrow) infiltrating kidney tissue were stained with antibodies against Pax-5 and CD3 to discriminate between B- and T-cells. **D:** Further characterization of T-cell lymphoma. Single-cell suspensions from lymph nodes of aged animals were stained with anti-CD4 and anti-CD8 and analyzed by FACS. Examples are given of a normal FACS profile from an aged NDR1$^{-/-}$ mouse (left panel), of the infiltration of CD4/8 double-positive cells into lymph nodes (middle panel) and of the expansion of the CD4 single-positive population (right panel).

70% of all NDR1$^{+/-}$ and NDR1$^{-/-}$ mice examined exhibited lymphomas in various tissues analyzed (Figure 3B; Table S1). Female mice were more lymphoma-prone (80%) than male mice (Figure 3B). Immune phenotyping defined the lesions as high-grade peripheral T-cell lymphomas, with all tumors being CD3 positive (Figure 3C). FACS analysis of T-cell lymphomas from aged NDR1$^{+/-}$ and NDR1$^{-/-}$ mice revealed that the lesions were characterized either by infiltration of CD4/CD8 double-positive (DP) cells into peripheral immunological organs or by expansion of CD4 single-positive (SP)

cells; ~60% of analyzed T-cell lymphomas were CD4/8 DP and 40% CD4 SP (Figure 3D). Thus, deficiency of NDR1 or heterozygosity appeared to predispose mice to T-cell lymphoma development later in life, indicating that NDR1 seemed to act as a haploinsufficient tumor suppressor in T-cell lymphoma.

$NDR1^{+/-}$ and $NDR1^{-/-}$ mice are highly susceptible to carcinogen-induced lymphomagenesis

To examine whether younger mice show increased susceptibility to carcinogen-induced lymphoma development, N-ethyl-N-nitrosourea (ENU) was chosen as a carcinogen since it has been reported to induce mainly T-cell lymphomas in the C57BL/6 background (*13*). Mice 4-5 weeks old were injected intraperitonally with a single dose of ENU at 100mg/kg and monitored for a period of 9 months for cancer development. Nine months after injection, 88% of $NDR1^{-/-}$ and 79% of $NDR1^{+/-}$ mice had developed tumors, compared with only 50% of wild-type mice (Figure 4A, Table S2). Analogous to aged mice heterozygosity in NDR1 resulted in a similar rate of tumor development than deficiency. Hematopoietic tumors were mainly observed, with T-cell lymphoma being most frequent. In addition, myeloproliferative diseases (MPD) were also found at later time-points (Table S2). Focusing on T-cell lymphomas, 53% of the treated $NDR1^{-/-}$ and 47% of the $NDR1^{+/-}$ mice developed tumors. In contrast, as already reported (*13*, *14*) , only 30% of the wild-type mice developed T-cell lymphomas after ENU treatment (Figure 4B). $NDR1^{+/-}$ and $NDR1^{-/-}$ animals not only showed increased penetrance but also earlier onset of T-cell lymphoma development after carcinogen treatment; tumor development in $NDR1^{-/-}$ began ~2 months earlier than in wild-type mice (Figure 4B). The T-cell lymphomas were characterized by a massive increase in thymus size and

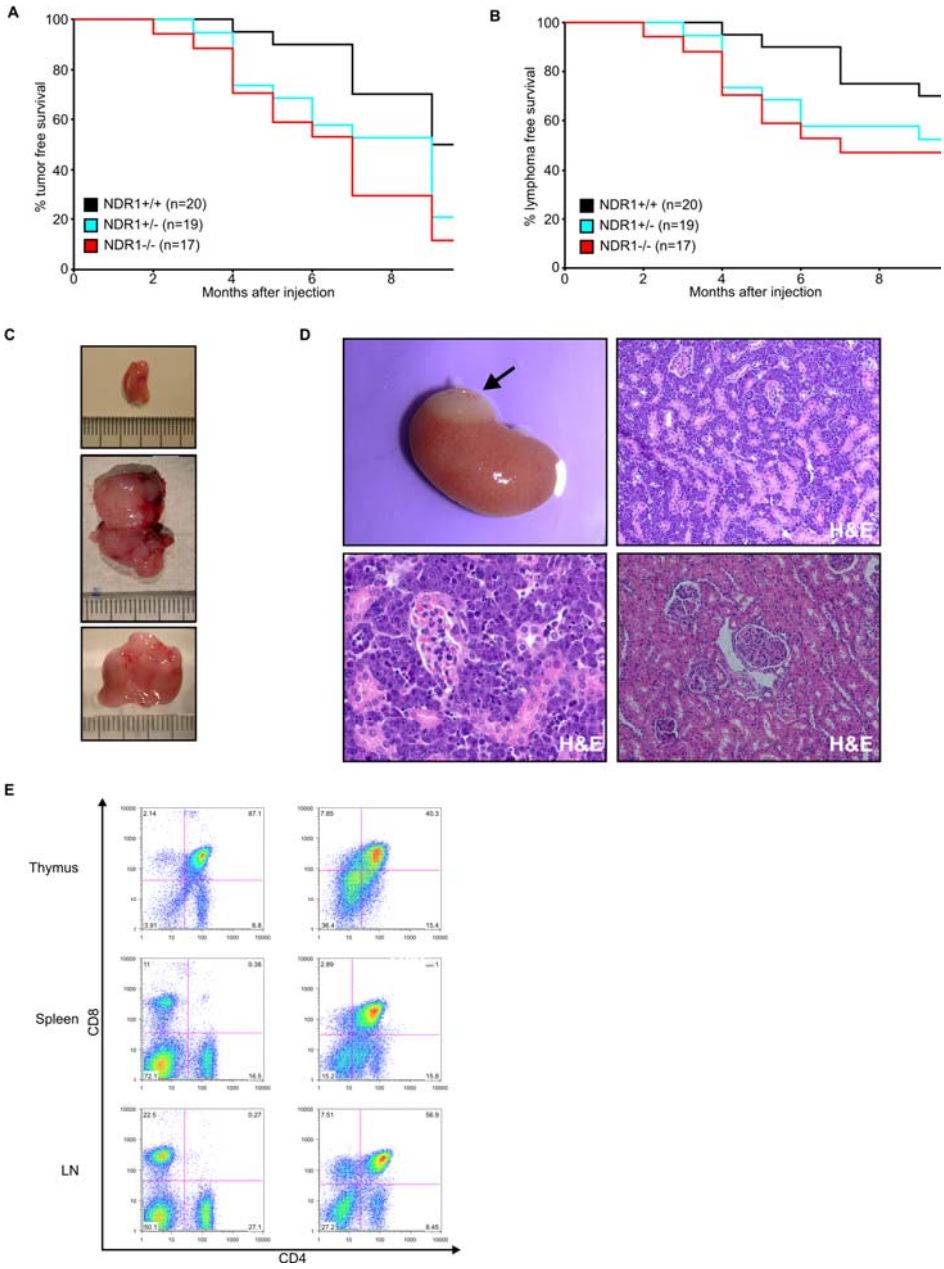

Figure 4. NDR1$^{+/-}$ and NDR1$^{-/-}$ show a high susceptibility to ENU-induced lymphomagenesis
A: Kaplan-Meier tumor-free survival curve of ENU-treated mice starting after injection with 100µg/g ENU at age 4 weeks and followed for up to 10 months. **B:** Kaplan-Meier lymphoma-free survival curve of ENU-treated mice. Fatal thymic T-cell lymphoma occurred at the highest frequency and at the earliest time points. Other hematopoietic malignancies occurred at later time-points (see Figure S1; Table S2). **C:** Examples of T-cell lymphoma from an ENU-treated NDR1$^{-/-}$ (middle panel; 25x enlarged) and an NDR1$^{+/-}$ mouse (lower panel; 20x enlarged). For comparison, a normal untreated thymus from a wild-type mouse is given (upper panel). **D:** Example of macroscopic (upper left panel) and microscopic infiltration of kidney tissue. Sections from affected organs were stained with H&E and analyzed for infiltrating tumor cells (20x upper right panel; 40x lower left panel). For comparison, an H&E-stained section from an unaffected wild-type kidney is given (lower right panel). **E:** CD4/CD8 profiles from thymus (upper panel), spleen (middle panel) and lymph nodes (lower panel) of ENU-treated NDR1$^{-/-}$ animals, without T-cell lymphoma (left) or with fatal T-cell lymphoma (right). Note that CD4/8 double-positive cells infiltrate spleen and lymph nodes.

weight (Figure 4C). Infiltration of other organs, such as lymph nodes, spleen and kidneys, leading to disruption of normal tissue architecture was frequently observed (Figure 4D). FACS analysis of the tumors showed them to be characterized by CD4/CD8 double-positive cells thereby confirming the T-cell lineage origin of the tumors (Figure 4E). Thus, although development of precursor and not peripheral T-cell lymphoma occurred after ENU treatment, $NDR1^{+/-}$ and $NDR1^{-/-}$ mice had a higher susceptibility to carcinogen-induced lymphomagenesis.

Myeloproliferative diseases (MPD) were observed in addition to T-cell lymphomas (*14*). Again $NDR1^{+/-}$ and $NDR1^{-/-}$ mice were more prone to these diseases (32% and 35%, respectively, vs. 20% in the wild type) (Figure S7A). Some mice affected by MPDs showed massive splenomegaly (Figure S7B). Analysis of these lesions by FACS showed an increase in cells belonging neither to the T-cell nor to the B-cell lineage. This population was found be composed of erythroid ($Ter119^{+}/CD71^{+}$) and myeloid ($Gr\text{-}1^{+}/Mac1^{+}$) cells (Figure S7C). In summary, loss or heterozygosity of NDR1 predisposed younger mice to the development of T-cell lymphoma and MPDs after carcinogen treatment which further confirmed that NDR1 functioned as a haploinsufficient tumor suppressor.

Lymphoma development is associated with a decrease in NDR1 and NDR2 expression

The apparent gene-dosage effect of NDR1 on NDR2 expression and the predisposition of NDR1 targeted mice to T-cell lymphoma development prompted us to analyze tumor material from ENU-treated mice for the expression of NDR1 and NDR2. As changes in NDR2 protein upon loss of NDR1 were not reflected at the mRNA level (Figure S5), the analysis focused on the protein levels using NDR1 and NDR2 specific antibodies.

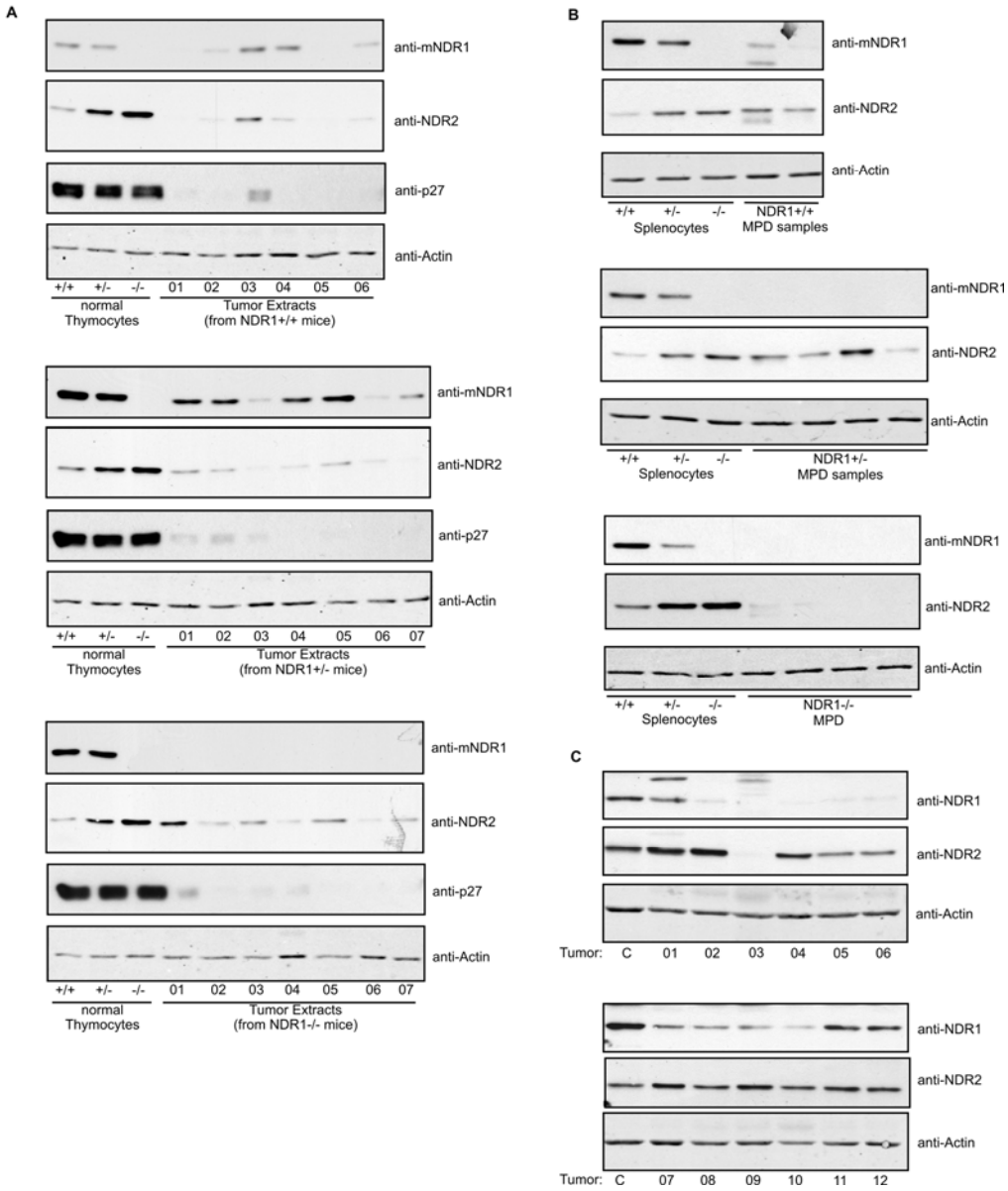

Figure 5. Expression of NDR1 and NDR2 in murine and human tumors
A: Analysis of NDR1 and NDR2 expression in tumor material from ENU-treated $NDR1^{+/+}$ (upper panel), $NDR1^{+/-}$ (middle panel) and $NDR1^{-/-}$ (lower panel) mice. Single-cell suspensions from normal thymi of the indicated genotype or tumors were lysed and blotted for NDR1/2 expression using isoform-specific antibodies with Actin as loading control. Note that the expression of both isoforms is down-regulated in most of the tumors compared with expression in normal thymocytes of the respective genotypes. The samples were also blotted for p27 as a tumor marker. **B:** Expression of NDR1 and NDR2 in MPD samples obtained from $NDR1^{+/+}$ (upper panel), $NDR1^{+/-}$ (lower panel) and $NDR1^{-/-}$ mice. Single-cell suspensions from spleens were lysed and blotted for NDR1/2 expression with Actin as loading control. **C:** Expression of NDR1 and NDR2 in human T-cell lymphoma samples. Tumor tissue (malignant T-cells >70% as judged by histopathology) was extracted and blotted for NDR1 and NDR2 using specific antibodies; purified $CD3^+$ human T-cells purified from whole blood were used as control.

Thymocyte extracts from healthy and untreated $NDR1^{+/+}$, $NDR1^{+/-}$ and $NDR1^{-/-}$ mice were used as expression controls since tumor cells were mainly CD4/8 DP (Figure 5A). Loss of p27 expression was used as a tumor marker (*15, 16*). Comparison of the

expression of NDR1 and NDR2 in tumors and in normal thymocytes of the respective genotypes showed in most cases that both kinases were down-regulated (Figure 5A). For example, in NDR1-deficient tumors there was a dramatic decrease in NDR2 protein levels, with only one of seven tumors retaining NDR2 protein levels similar to that of untransformed thymocytes (Figure 5A, lower panel). The same was true for the majority of $NDR1^{+/+}$ and $NDR1^{+/-}$ tumors (Figure 5A, upper/middle panel). Tumor material from MPDs obtained during the ENU experiment was also analyzed for NDR1 and NDR2 expression (Figure 5B). Again, there was a reduction in total NDR kinase levels in the tumor samples. However, in tumors from $NDR1^{+/+}$ and $NDR1^{+/-}$ mice, NDR1 levels were mainly affected. In samples lacking NDR1, there was a striking reduction in NDR2 protein (Figure 5B, lower panel). In summary, most murine tumors showed a reduction in at least one NDR kinase isoform suggesting that tumor development was accompanied by a reduction in total NDR kinase.

To determine NDR1/2 expression in human T-cell lymphoma samples with different characteristics (Table S3), tumor biopsies with >70% malignant T-cells or purified $CD3^{+}$ human T-cells were isolated and their lysates blotted for NDR1 and NDR2 expression using specific antibodies (Figure 5C). Interestingly, most human tumors showed a significant decrease in NDR1 expression, similar to the situation observed in murine MPD samples. Thus, total NDR protein levels were reduced in murine and human T-cell lymphoma samples suggesting a conserved role for NDR kinases in tumor biology.

Reduced expression of NDR1/2 in tumors correlates with changes in the expression of genes implicated in cell death regulation

Analysis of NDR1/2 expression in tumors revealed that tumor development was associated with down-regulation of NDR kinases in most tumor samples (Figure 5A). Therefore we grouped the tumors according to their NDR expression into those with approximately normal NDR expression (NDR-high), those with slightly reduced NDR (NDR-middle), and those with strong reduction (NDR-low) and performed microarray analysis (Table S4). 46 genes showed at least a 1.5-fold change ($P<0.05$) in NDR-low tumors compared with normal thymocytes, NDR-high and NDR-middle tumors (Figure 6A; Table S5). The correlations between NDR expression in tumors and changes in gene expression at the protein level were validated using Pou2af1 expression, as Pou2af1 mRNA exhibited a strong and consistent up-regulation in NDR-low tumors (Figure S8).

Application of the Ingenuity Pathway Analysis Program to functionally annotate the genes correlating to low NDR expression, revealed enrichment of genes implicated in the regulation of cell death and the cell cycle (Figure 6B). Indeed, up-regulation of genes which have been shown to exert anti-apoptotic functions such as CD5L, MEF2c or Pou2af1 was observed (*17-19*). Furthermore, decreased expression of genes such as CDKN2C, TOP2A and CCND3 with pro-apoptotic function was noted (*20-23*). As we identified genes also correlating with low NDR level in tumors implicated in the regulation of the cell cycle, we tested, whether loss of NDR1 would impact on the proliferation of T-cells (Figure S9). Surprisingly, loss of NDR1 resulted in reduced proliferation of T-cells after stimulation with 1.0ug CD3 alone or 1.0ug CD3+1.0ug CD28, however stimulation with a higher dose of anti-CD3 antibody abolished this

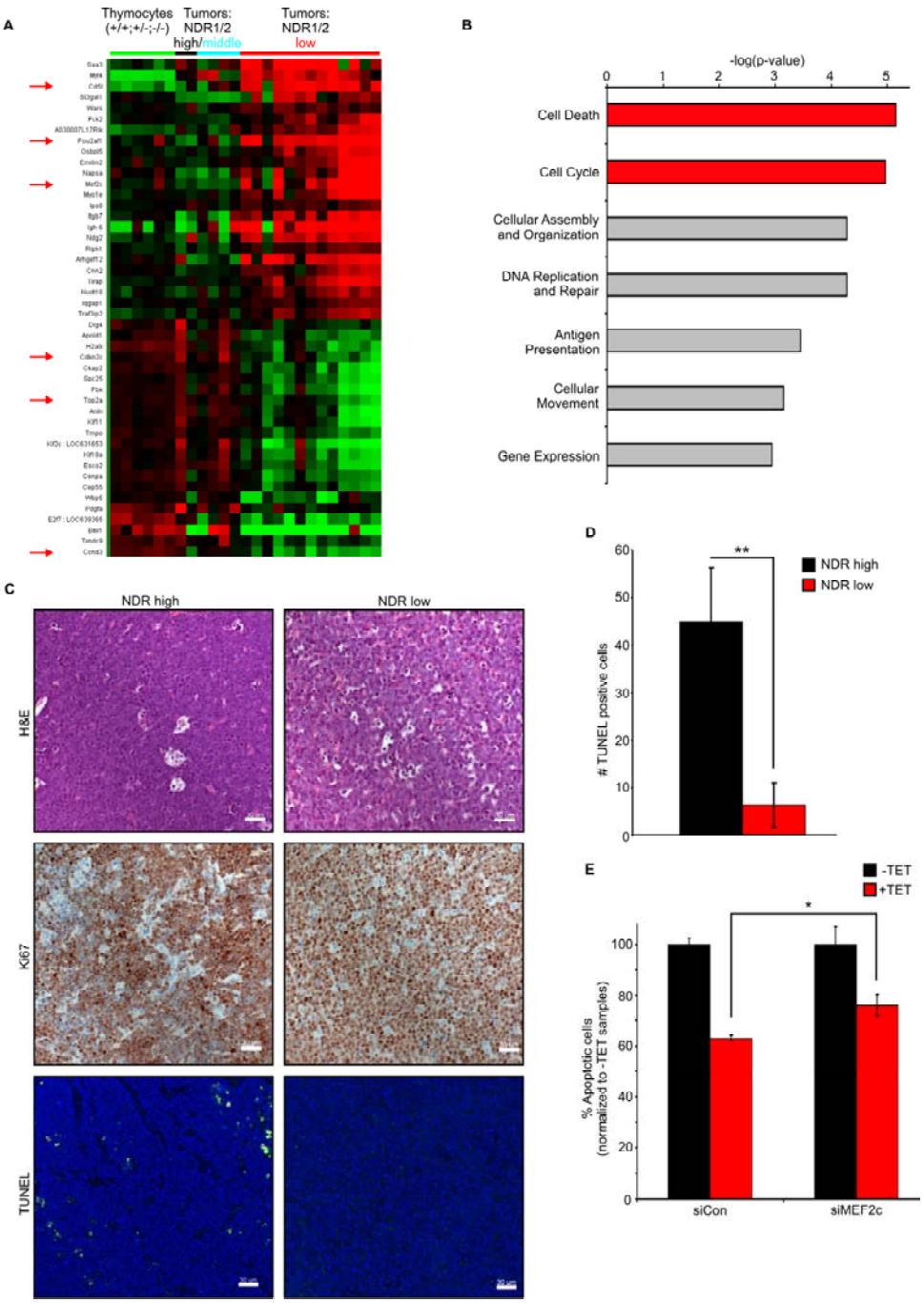

Figure 6. Low NDR1/2 expression in tumors correlates with changes in the expression of genes associated with cell death
A: Expression profiles of genes that were changed 1.5 fold (P<0.05) in NDR-low tumors (Table S4. Red arrows indicate selected genes discussed in the text). **B:** Functional analysis of genes associated with NDR-low in tumors. The list of genes obtained in B was imported into the Ingenuity Pathway Analysis Program and functional analysis performed. Functions with a -log(P-value) of >3 are shown. **C:** Analysis of Ki67 and TUNEL in NDR-high and NDR-low tumors. Colocalization (red) of DAPI (blue) and TUNEL (green) was analyzed using IMARIS imaging software. **D:** Quantification of TUNEL positive cells in NDR-high (n=2) and NDR-low tumors (n=3). For each sample 27 field of view were analyzed. (** P<0.001). **E:** HeLa cells expressing inducible shRNA against NDR1 and NDR2 were treated with tetracycline for 72h and transfected with siRNA against MEF2c (siMEF2c) or control siRNA (siCon). 24h later cells were treated with 100μM etoposide for 48h and afterwards analyzed using AnnexinV/PI staining (* P<0.002; n=3).

effect. Next we attempted to confirm our findings in NDR-high and NDR-low tumor samples (Figure 6C/S10). Addressing both proliferation and apoptosis we observed no major differences in Ki67 staining between NDR-high and NDR-low tumors. However, the numbers of apoptotic cells were significantly decreased in NDR-low tumors (Figure 6D), confirming the apoptosis resistance in cells with decreased NDR level as observed earlier.

Increased MEF2c expression has been reported to correlate with increased apoptosis resistance (*19*). We therefore tested whether siRNA mediated knock-down of MEF2c would rescue the apoptosis defects described for knock-down NDR1/2 HeLa cells (Figure 6E). Decreasing MEF2c-level in NDR1/2 depleted cells resulted in a slight but statistically significant rescue effect after etoposide treatment but not after FAS induced apoptosis (Figure S11), indicating that additional factors mediated the effects downstream of NDR. Taken together, low levels on NDR kinases in tumors correlated with changes in the expression of genes implicated in cell death and proliferation. However analysis showed that mainly the apoptotic response seemed to be affected by low NDR levels in tumors, thereby fully confirming the results obtained earlier.

Decrease in E47 expression mediates apoptosis resistance upon decrease in NDR level

To further understand the impact of NDR kinases on the expression of genes correlating with low levels on NDR in tumors, we performed an analysis to identify common upstream factors regulating gene expression in our settings (Figure 7A). E2A (E12/E47) targets were significantly enriched in our dataset. Loss or functional inactivation of E2A has been shown to result in T-cell lymphoma and leukemia development in mice and men (*24-26*). We therefore tested whether E2A protein expression would be reduced in

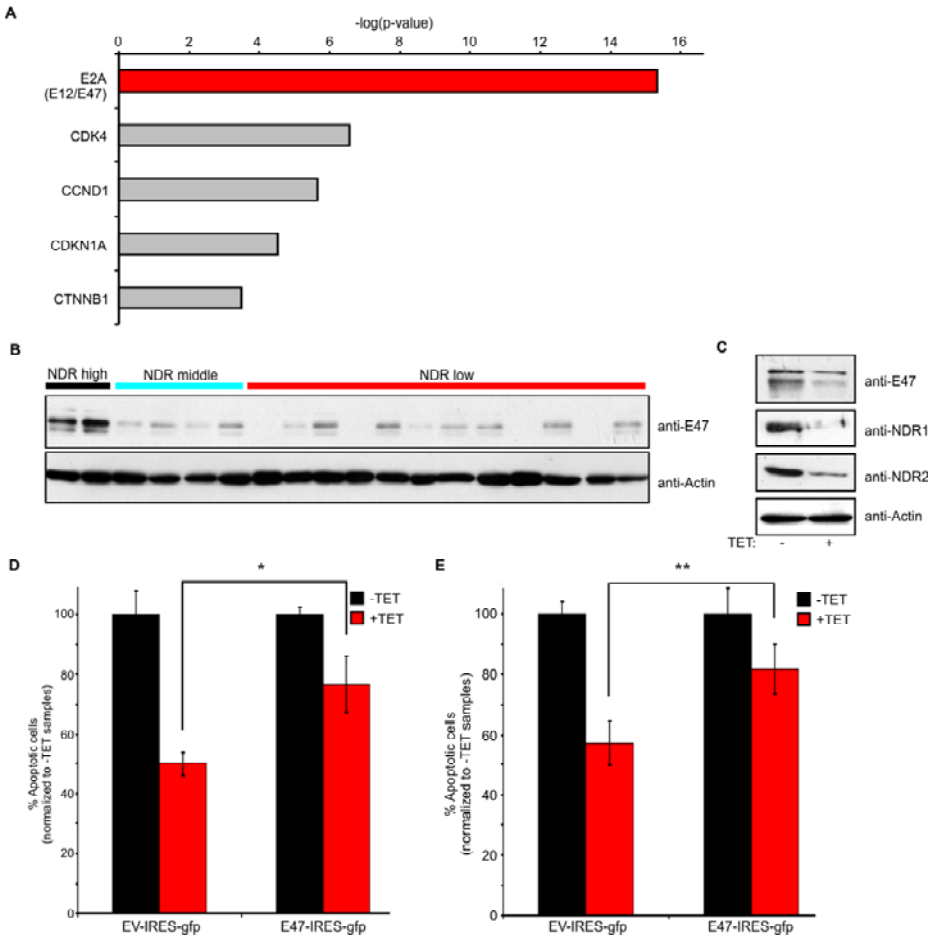

Figure 7. Reduction of NDR kinases result in decreased levels of E47 in tumors and cells leading to apoptosis resistance
A: Analysis of common upstream regulators of correlating genes. Using the Ingenuity Pathway Analysis Program Knowledge Base, upstream regulators of the expression of each correlating gene and the total known regulated genes were extracted. P-values were calculated using hypergeometric distribution. Regulators with a -log(*P*-value) of >3 are shown. **B:** Analysis of E47 expression in ENU induced tumors. **C:** Reduced expression of E47 upon knock-down of NDR1/2 in HeLa cells. Knock-down in HeLa cells stably expressing shRNA against NDR1 and NDR2 was induced for 4 days using tetracycline. **D:** Rescue of apoptotic defects in NDR1/2 depleted HeLa cells by overexpression of E47. Knock-down of HeLa cells was induced for 72h and cells were transfected with E47 cDNA coupled to an IRES-gfp to monitor transfection. Apoptosis was induced 24h later using 100µM etoposide for 48h. Apoptosis was accessed using AnnexinV/PI staining gated on GFP-positive cells (* $P<0.005$; n=3). **E:** HeLa cells were pretreated and transfected as in D. Apoptosis was induced as described (*4*) and accessed as described in D (** $P<0.025$; n=3).

tumors exhibiting reduction of NDR kinases. Only tumors with normal NDR kinase levels retained high expression of the E2A isoform E47, whereas E47 expression was significantly reduced upon decreased NDR expression and even absent in approximately 40% of the NDR-low tumors (Figure 7B). To validate the impact of diminished NDR kinase expression on E47 levels, we analyzed the expression of E47 in HeLa cells stably expressing validated shRNA against NDR1 and NDR2 (Figure 7C). Indeed, E47 expression was reduced upon knock-down of NDR1/2 in HeLa cells (Figure 7C).

Finally, we attempted to rescue the apoptosis defects in NDR depleted cells by overexpression of E47 (Figure 7D/E). Indeed, overexpression of E47 rescued the apoptotic defects in NDR1/2 knock-down cells to ~50% after both etoposide and anti-FAS treatment (Figure 7D/E). Taken together, reduced levels of NDR kinases in tumors and cells impacted on the expression of the E2A gene-product E47. Even more importantly, restoring E47 level reduced the apoptosis defects after Etoposide and anti-FAS treatment, thereby giving mechanistic insight into the tumor suppressive function of NDR kinases.

3.1.4 Discussion

The NDR family kinases NDR1 and NDR2 have been implicated in the regulation of apoptosis downstream of FAS (4). We generated mice deficient for NDR1 to further analyze the role of NDR1 in apoptosis signaling, mainly in lymphocytes, where NDR1 is highly expressed. We show that NDR1 in thymocytes is not only activated upon FAS stimulation, but also after apoptosis induction using intrinsic stimuli, such as DNA damage and dexamethasone. This finding attributes to NDR1 a broader role in apoptosis signaling and shows that NDR1 activation is not limited to death receptor stimulation. However, although apoptosis induction results in NDR kinase activation, loss of NDR1 does not result in major defects in apoptosis in thymocytes and T-cells. Interestingly, in conditions in which loss of NDR1 results in apoptosis defects heterozygosity in NDR1 also increased the resistance to apoptosis induction (Figure 1/S2). Analyzing the expression of the second NDR kinase isoform in mammals, NDR2, we find that NDR2 is up-regulated in NDR1-deficient and heterozygous tissues. Interfering with the up-regulation results in increased apoptotic defects. Thus, NDR2 can functionally

compensate NDR1 reduction indicating a tight, gene-dosage dependent regulation of total NDR kinase levels *in vivo*.

Although NDR1 loss is compensated in young healthy mice, we still observe that loss of NDR1 predisposes mice to the development of T-cell lymphoma, both in older mice and upon carcinogen treatment of younger mice (Figure 3/4). Given that NDR1 levels are gene-dose sensitive, it is not surprising that even NDR1 heterozygous mice display a similar predisposition to tumor development, indicating that NDR1 functions as a haploinsufficient tumor suppressor in T-cell lymphoma (*27*). Indeed, under several conditions already loosing one allele of NDR1 results in apoptosis defects comparable to those observed in NDR1 deficient cells (Figure 1/S2). Although decreased levels in NDR1 are compensated by increased NDR2 levels, our experiments show that at least in terms of apoptosis the compensation is not always complete. Under certain conditions loss or heterozygosity of NDR1 results in increased resistance towards apoptosis induction. Could the increased tumor development in heterozygous and knock-out mice be explained by a predisposition to decrease the expression of NDR2 and thereby increasing the resistance towards pro-apoptotic stimuli?

Addressing this hypothesis, we analyzed tumor material from $NDR1^{+/+}$, $NDR1^{+/-}$ and $NDR1^{-/-}$ mice for the expression of NDR1 and NDR2. In T-cell lymphoma and MPD samples deficient for NDR1, the expression of the remaining isoform NDR2 is strongly decreased, as compared to the normal expression of NDR2 in NDR1-deficient cells (Figure 5). This indicates that tumor development is accompanied with defects to compensate for NDR1 loss. Importantly, T-cell lymphoma samples from heterozygous mice also display a decrease in NDR2 expression in most of the cases analyzed. Interestingly, T-cell lymphoma development in these mice is also accompanied by a

decrease in NDR1 expression (Figure 5). Even in tumors obtained from wild-type mice, a decrease in NDR1 and NDR2 amounts is observed in most cases. Thus, we can conclude that T-cell lymphoma development in mice is accompanied by a decrease in both NDR kinase isoforms. Given, that in healthy tissue the reduction of NDR1 by loss of one allele is already compensated by increased NDR2, one might argue that T-cell lymphoma development is accompanied by a decrease in total NDR kinase levels. From this point of view, a reduction in one isoform without up-regulation of the other would yield a reduction of total NDR kinase levels and thereby facilitating defects in the response to pro-apoptotic stimuli. In line with this interpretation, NDR1 expression is significantly reduced without an accompanying NDR2 up-regulation in 75% of analyzed human T-cell lymphoma samples. Thus, human T-cell lymphoma development is also associated with a decrease in NDR kinase expression. Interestingly, NDR kinase expression is reduced in MPD samples, arguing for a tumor suppressive function of NDR kinases in different cell types and organs. Taken together, T-cell lymphoma development in mice and humans is accompanied by a reduction in total NDR kinase levels. Apparently, loss of one allele of NDR1 predisposes mice to tumor development by further down-regulation of NDR1 and NDR2 expression. Recent publications have shown that predisposition to tumorigenesis can be strongly affected by even small changes in the expression and protein abundance of tumor suppressors (*28-30*), which in the case of DAPK1 in Chronic Lymphocytic Leukemia has been shown to impact the response of tumor cells to pro-apoptotic stimuli (*28*). Decrease of NDR kinase level could therefore similarly predispose to tumor development by impacting on the response of cells to apoptotic stimuli.

Evading apoptosis is considered to be one of the major hallmarks of cancer (*31*). We show here that NDR kinases are activated not only after extrinsic death signals, but also after intrinsic signals such as DNA damage. Importantly, down-regulation of total NDR kinase in untransformed cells results in increased resistance of these cells towards both extrinsic and intrinsic apoptotic stimuli. In line with this observation, tumors in which both NDR kinases are down-regulated exhibit a specific gene expression profile associated with cell death. Furthermore this genes expression profile is enriched for genes implicated in the regulation of the cell cycle. However, NDR1 deficient T-cells do not exhibit increased proliferation after stimulation. Interestingly, proliferation defects upon knock-out of several well known tumor suppressor proteins such as BRCA1, SMAD4 or VHL have been described (*32-34*). In addition, whereas tumors with low levels of NDR show a significant decrease in apoptotic cells, proliferation in these tumors does not seem to be significantly affected. During tumor development the cells probably acquire changes in other signaling pathways to overcome the defects in proliferation upon loss of NDR1. Still, it will be interesting for future studies to address the effect of NDR1 deficiency on proliferation in more detail.

The genes correlating with low expression of NDR1/2 in T-cell lymphoma are enriched for targets of the E2A gene products E12/E47. Deficiency in E2A has been shown to result in T-cell lymphoma development in mice (*24, 26*). In addition, functional inactivation of E47 has been described in human T-cell acute lymphoblastic leukemia/lymphoma (*25*). Importantly, it has been shown that re-expression of E12/E47 results in cell death in E2A deficient lymphoma cells (*35*). Strikingly, knock-down of NDR1/2 in HeLa cells results in decreased E47, a situation also observed in the tumors analyzed. Importantly, overexpression of E47 in HeLa cells depleted of NDR1/2

significantly rescued the apoptosis defects after both etoposide and anti-FAS treatment, confirming the functional link between NDR kinases and E47 expression in apoptosis. Reduction of NDR1/2 thus results in apoptosis resistance of tumors at least partially by impacting E47 protein levels. Now, future studies are needed to further define the impact of NDR kinases on E47 protein expression.

The data presented here suggest that NDR kinases have a tumor-suppressive function. Indeed, recent publications have reported loss of heterozygosity of the genomic region containing the NDR1 gene in diffuse large B-cell non-Hodgkin's lymphoma, cervical cancer, colorectal carcinoma, lung cancer, ovarian cancer and renal cell carcinoma (*36-41*). However, no deletions of the NDR2 locus have been reported so far. In addition, loss/reduction of NDR1 or NDR2 mRNA expression has not been associated so far with the development of T-cell lymphoma in humans (*42*). This might be explained by our results, as we observe changes in NDR1 and NDR2 expression mainly at the protein levels. Firstly, loss of NDR1 does not result in increased expression of NDR2 mRNA. Secondly, although we observe a strong reduction in NDR1 and NDR2 protein in tumors, their genes do not show up in the gene expression analysis. Therefore, it will be relevant in future studies to develop immuno-histological methods to analyze the expression of NDR1/2 in tumors at the protein level.

In conclusion, we have identified NDR1 as a novel tumor suppressor protein in T-cell lymphoma. Interestingly, NDR1 shows features of haploinsufficiency, as loss of one allele of NDR1 predisposes mice to T-cell lymphoma development. Loss and heterozygosity of NDR1 results in increased apoptosis resistance of thymocytes and T-cells under several conditions. Furthermore, our analysis shows that reduction in NDR1 triggers the reduction of the expression of the compensating NDR2 isoform by a yet to

be identified post-transcriptional mechanism resulting in a total decrease in NDR kinases in tumors. Given that NDR1 is expressed predominantly in lymphocytes and that its expression is frequently reduced in T-cell lymphoma samples of murine and human origin, the assessment of NDR kinase protein expression in other lymphoid neoplasias could likely show a reduction of NDR1 (and NDR2) in these tumors as well. Thus, addressing the protein levels of NDR1 (and NDR2) in tumors of different origins will broaden the role of NDR kinases in counteracting tumor development.

3.1.5 Material and Methods

Generation of NDR1-deficient mice and treatment

A ~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 into the XhoI site of exon 4. The targeting vector was linearized using the SalI site and electroporated into 129/Ola ES cells. An external probe was used for ES cell Southern screening following KpnI digestion. An internal probe and a lacZ-Neo probe were used to characterize ES clones positive for homologous recombination. 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 was genotyped by multiplex PCR with three primers: (1) Ex4checkb, 5'-GTCTTCTCATCGCTGTCACAGCT-3'; (2) Neo-2, 5'-GCTGCCTCGTCCTGCAGTTCATTC-3'; and (3) 6540bk, 5'- GCTCCCGCTCAGT-TACCTGCTCC-3'. To induce lymphoma development, young female $NDR1^{+/+}$, NDR1

NDR1$^{+/-}$ and NDR1$^{-/-}$ mice were injected intraperitonally at the age of 4 weeks with a single dose of N-ethyl-N-nitrosourea (ENU) at 100 mg/kg dissolved in PBS (pH 6.0) and were monitored up to 9 months after injection. All mouse experiments were performed according to the Swiss Federal Animal Welfare Law.

Reagents and antibodies

The following reagents were used for experiments: dexamethasone, N-ethyl-N-nitrosourea (Sigma), anti-CD3e, anti-CD28 (both from eBioscience), anti-CD4, anti-CD8, anti-B220 (from Immunotools), anti-p27 and anti-Actin (Santa Cruz), anti-E47 (BD Bioscience), anti-cleaved PARP (BD Bioscience), anti-FAS (Jo-2; BD Bioscience), anti-cleaved Caspase 3, anti-MST1 and anti-P-MST (Cell Signaling), anti-Hsc70 (Stressgen). Anti-P-Thr-444/442-NDR1/2 (referred to as anti-T444-P), anti-NDR1 (human) and anti-NDR2 have been described elsewhere (*4, 43*). To obtain antibodies specific to murine NDR1, a peptide corresponding to the C terminal part of murine NDR1 (ILKPTVTTSSHPETDYKNKD) was used to immunize rabbits. Antibodies were purified by immunoaffinity purification and tested for specificity (Figure S12). Antibodies against Pou2af1 were a kind gift from P. Matthias and have been described elsewhere (*44*).

Cell culture and transfections

The generation of HeLa cells expressing shRNA against NDR1 and NDR2 has been described previously (*4*). To induce knock-down of NDR1 and NDR2 cells were treated with tetracycline for 72h. Cells were transfected using Lipofectamine2000 (Invitrogen) as described by the manufacturer. Validated siRNA against MEF2c was obtained from

Ambion, control siRNA was from Qiagen (Figure S13A). E47 cDNA was a kind gift from C. Gallegos (University of Lleida, Spain) and constructs expressing E47 together with an IRES-gfp was obtained by subcloning E47 into a pcDNA3 vector containing an IRES-gfp cassette using *BamHI/EcoRI* (Figure S13B).

Protein extraction and immunoblotting

Proteins were extracted from freshly isolated or cultured cells as previously described (*45*). For the extraction of frozen tissues, freshly isolated organs were flash frozen and minced with a tissue homogenizer using 6 µl of lysis buffer per mg tissue. Extracts were incubated for 1 h at 4°C and cellular debris were removed by centrifugation at 14'000 rpm for 15 min at 4°C. Western blot was performed as described previously, except that blots analyzed using the Licor Odyssey System were incubated with secondary antibodies coupled to fluorescent dyes. Quantifications were carried out using the Licor Odyssey software.

Apoptosis Assays

For the analysis of thymocyte apoptosis, freshly isolated thymocytes from 4- to 6-week-old mice were isolated and 1 x 10^6 cells were seeded into 48-well plates. Apoptosis was induced using gamma irradiation (TORREX 120D, Astrophysics Research Corp.; 5 mA/120 kV and 0.13 Gy/s), etoposide, dexamethasone, anti-CD3/CD28 treatment or treatment of cells with anti-Fas (Jo2; BD Bioscience) antibody in the presence of cycloheximide. The response to cytokine withdrawal was accessed by seeding the cells in IMDM+10% FCS for the indicated time and analyzed using a Vicell automated cell counter. For the induction of apoptosis in MEF cells, cells were seeded at consistent

densities 24 h before treatment. Apoptosis was induced by treating cells with etoposide or anti-Fas antibody in the presence of cycloheximide. Apoptosis was assessed using either Annexin V staining (BD Bioscience) or a cationic lipophilic dye DilC1(5) assay kit (Invitrogen). Cells were analyzed by FACS. Assays to assess activation-induced cell death (AICD) were performed as described previously (*46*). In short, T-cells were purified from spleens using T-cell enrichment columns (eBioscience) and activated for 72 h on plates coated with 5µg/ml anti-CD3 antibody in the presence of 1µg/ml anti-CD28 (both eBioscience). Activated viable cells were obtained using Lympholyte-M (Cedarlane Labs) and replated for 20 h on plates coated with increasing concentrations of anti-CD3 antibody in the presence of 50 U/ml IL-2 (Immunotools). Analysis of AICD was done as described previously (*46*, *47*).

Retrovirus-mediated knockdown of NDR2

To knock down NDR2 protein in mouse embryonic fibroblasts (MEFs), oligonucleotides targeting murine NDR2 were inserted into pTER(*48*) and tested in transient transfections of IMCD-3 cells (Figure S14). shNDR2#13 and shLUC (sequence provided upon request) were cloned into the pSUPER-retro.gfp.neo vector (Oligoengine) and used for further experiments. For virus production, Phoenix-Eco cells were transfected with jetPEI transfection reagent (polyplustransfection) and virus was harvested 48 h and 72 h later. Virus-containing supernatant was used to spin infect MEFs in the presence of 5 µg/ml polybrene (1000 *g*, 30°C, 1 h). Medium was changed after 6 h and the cells left to recover for 48 h.

FACS analysis

Single-cell suspensions from lymphatic organs or tumor tissue were obtained by pressing the organ through a 70-µm nylon mesh. Resulting suspensions were depleted of erythrocytes by incubation in Gey's solution and subsequently stained with antibodies covalently coupled to FITC, PE or APC. The antibodies used for FACS staining were anti-CD3e, Gr-1, Ter119, CD71 (BD Bioscience), anti-CD4, anti-CD8 and anti-B220 (Immunotools), and Mac-1 (Southern Biotech). Flow cytometric analysis was performed using a FACScalibur cell analyzer (BD Bioscience). Cell sorting was performed using a MoFlo device (DakoCytomation).

Histopathological analysis

Tissue specimens were fixed in 4% neutral buffered formalin and embedded in paraffin. Paraffin sections (4 µm) were stained with hematoxylin and eosin (H&E) or with antibodies against Pax-5 (BD Bioscience), CD3 (Dako) or Ki67 (Neomarkers). TUNEL staining was performed using the ApoAlert DNA Fragmentation Assay Kit (Clontech) as described by the manufacturer. Acquired images were analyzed using the IMARIS imaging software.

RNA isolation and quantitative RT-PCR

Total RNA from flash frozen organs or tumor cells was isolated with TRIzol reagent (Invitrogen) and further purified using RNeasy kit (Qiagen). cDNA from samples was generated from 2µg of total RNA using M-MuLV reverse transcriptase (NEB) and Oligo-dT primers. Quantitative RT-PCR to detect mNDR2 (primer sequences upon

request) was carried out using SYBR green technology in an ABI Prism 7000 detection system (Applied Biosystems).

Microarray analysis

RNA extracted as described above was processed and hybridized on to Affymetrix 430v2 chips as described by the manufacturer. Data was analyzed using Expressionist (GenData AG). Normalized data was analyzed using N-way ANOVA ($p<0.05$). Only changes greater than 1.5-fold were analyzed.

Statistical Analysis

Statistical analyses were performed with Student's t-test for the comparison between two samples. N-way ANOVA was used for the analysis of micro-array data.

3.1.6 References

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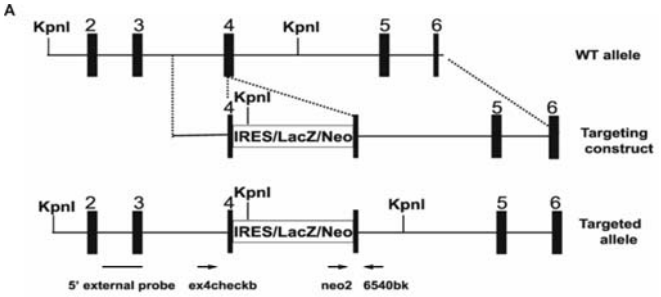
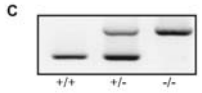
3.1.7 Supplemental Material





E

Genotype	+/+	+/-	-/-
Number of animals	63	119	60
Obtained ratio	26%	49%	24%
Expected ratio	25%	50%	25%

Figure S1. Generation of NDR1-deficient mice
A: Schematic representation of the targeting strategy. The murine *Ndr1* locus was disrupted by insertion of an IRES/lacZ/neo cassette into exon 4 of the kinase. Binding sites of the 5' external probe used for Southern blot analysis as well as the primers used for genotyping are indicated. **B:** Southern blot analysis of KpnI-digested tail DNA samples from a heterozygous mating. **C:** Analysis of tail DNA samples using PCR genotyping. **D:** Validation of successful disruption of the *Ndr1* locus at the protein level. Thymus extracts from NDR1$^{+/+}$, NDR1$^{+/-}$ and NDR1$^{-/-}$ mice were analyzed using an NDR1-specific antibody. Note that NDR1 protein expression was already reduced in NDR1$^{+/-}$ samples. **E:** Ratio of genotypes obtained from NDR1 heterozygous matings, compared with the expected Mendelian ratios.

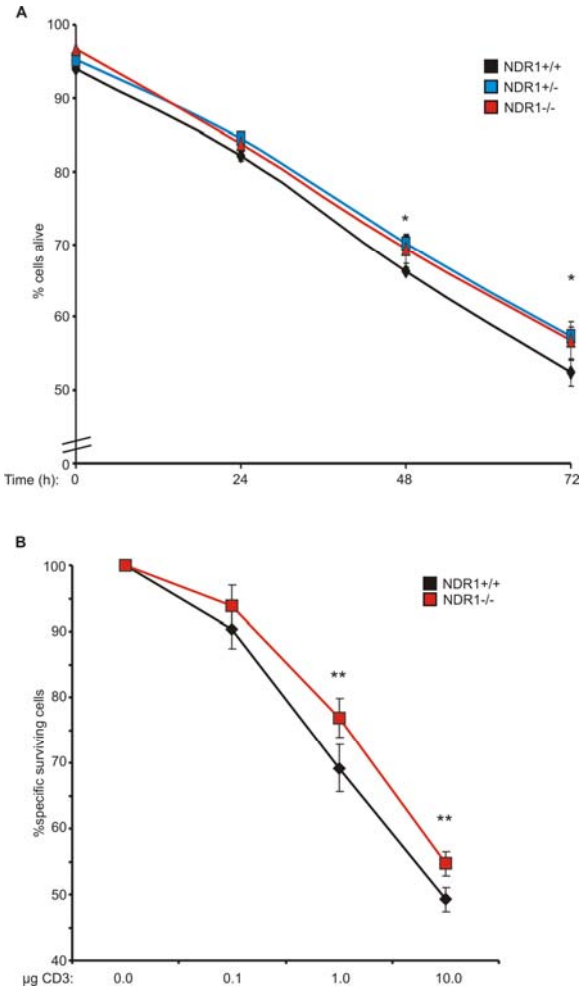


Figure S2. Mature T-cells from NDR1$^{+/-}$ and NDR1$^{-/-}$ mice show slightly increased apoptosis resistance
A: Mature T-cells were isolated and seeded in IMDM+10% FCS. After the indicated time cells were harvested and cell viability was accessed using a Vicell automated cell counter (* P<0.04). **B:** Analysis of the apoptotic response of NDR1$^{+/+}$ and NDR1$^{-/-}$ mature T-cells to activation-induced cell death (AICD, n = 3). Calculations were carried out as in B (** P<0.025).

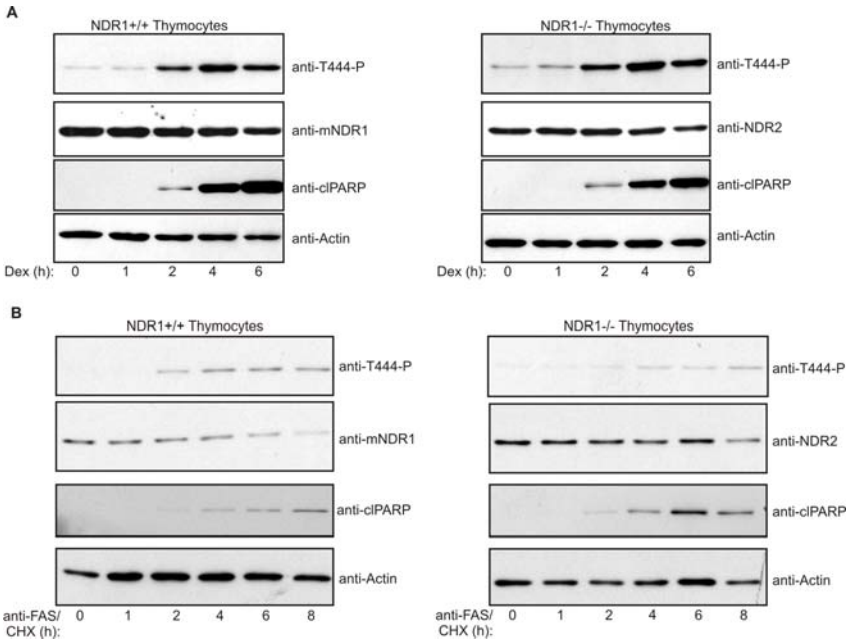


Figure S3. No change in NDR activation in NDR1-deficient thymocytes upon dexamethasone and anti-FAS/CHX-induced apoptosis

A/B: Freshly isolated thymocytes from NDR1$^{+/+}$ and NDR1$^{-/-}$ mice were treated with 1 µM dexamethasone (A) or anti-FAS antibody (0.5 µg/ml) + CHX (30 µg/ml) (B) and lysed after the indicated incubation times. Activation of NDR kinases was assessed using the anti-T444-P antibody; total kinase was analyzed using NDR1 or NDR2 isoform-specific antibodies. Induction of apoptosis was analyzed by PARP cleavage.

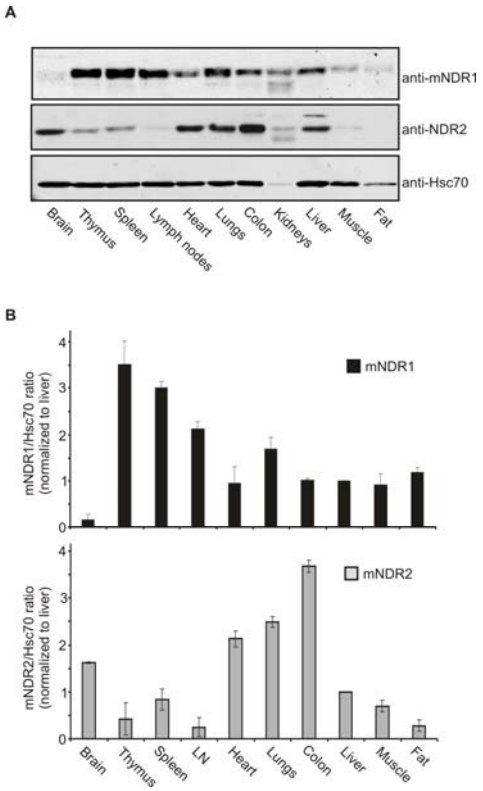


Figure S4. NDR1 and NDR2 show distinct protein expression pattern in mice
A: Representative expression of NDR1 and NDR2 proteins in different tissues from wild-type mice. Tissues extracts were blotted with specific antibodies against NDR2 and reblotted for NDR1 after stripping using the Licor Odyssey technology. Hsc70 expression was used as loading control. **B:** Quantification of NDR1 and NDR2 tissue distribution. Blots were scanned using Licor Odyssey technology and the respective NDR kinase/Hsc70 ratio was normalized to the expression in liver ($n = 3$). Note that kidney samples were omitted from the quantification due to obvious degradation.

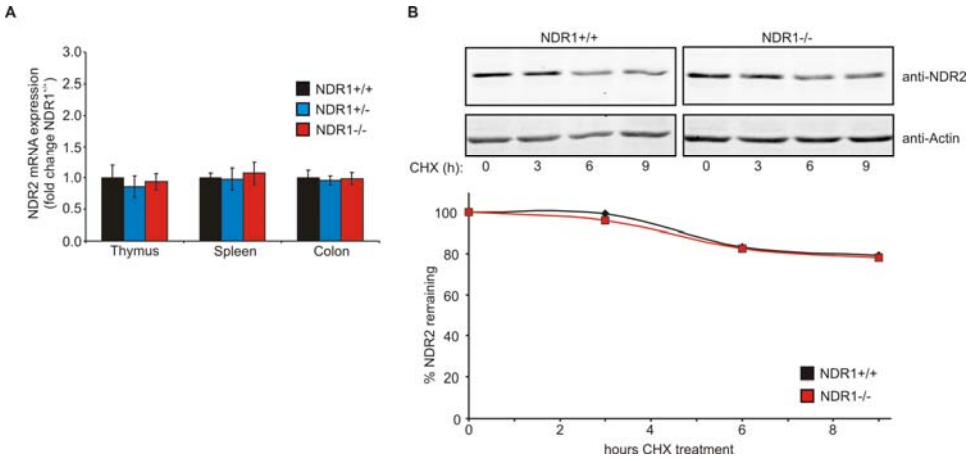


Figure S5. No difference in NDR2 gene expression and protein stability upon loss of NDR1
A: Analysis of NDR2 gene expression in $NDR1^{+/+}$, $NDR1^{+/-}$ and $NDR1^{-/-}$ thymus, spleen and colon extracts using qRT-PCR. **B:** Analysis of NDR2 protein stability in $NDR1^{+/+}$ and $NDR1^{-/-}$ MEFs. MEFs were treated for the indicated time with CHX (50ug/ml) and lysed. Blots for NDR2 and Actin were scanned using the Licor Odyssey technology.

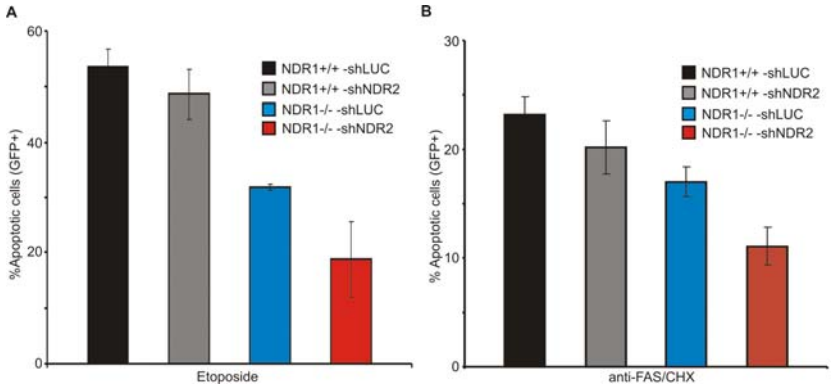


Figure S6. Reduction of NDR2 level in NDR1 deficient MEFs result in increased apoptosis resistance after etoposide and anti-FAS/CHX treatment
A: Primary MEFs were transfected with a retrovirus coding for shRNA against firefly luciferase (shLUC) or NDR2 (shNDR2). The cells were seeded for apoptosis induction 72 h after transfection. Apoptosis was induced 24 h later using 200 µM Etoposide and apoptosis in GFP^+ cells was measured 22 h after induction by FACS. Results are given for a representative experiment, which was repeated twice with a similar outcome. **D:** MEFs were treated as in A, but apoptosis was induced using or 1 µg/ml anti-FAS-Ab in the presence of 1 µg/ml CHX. Apoptosis was determined 18 h later. Results are given for a representative experiment, which was repeated twice with a similar outcome.

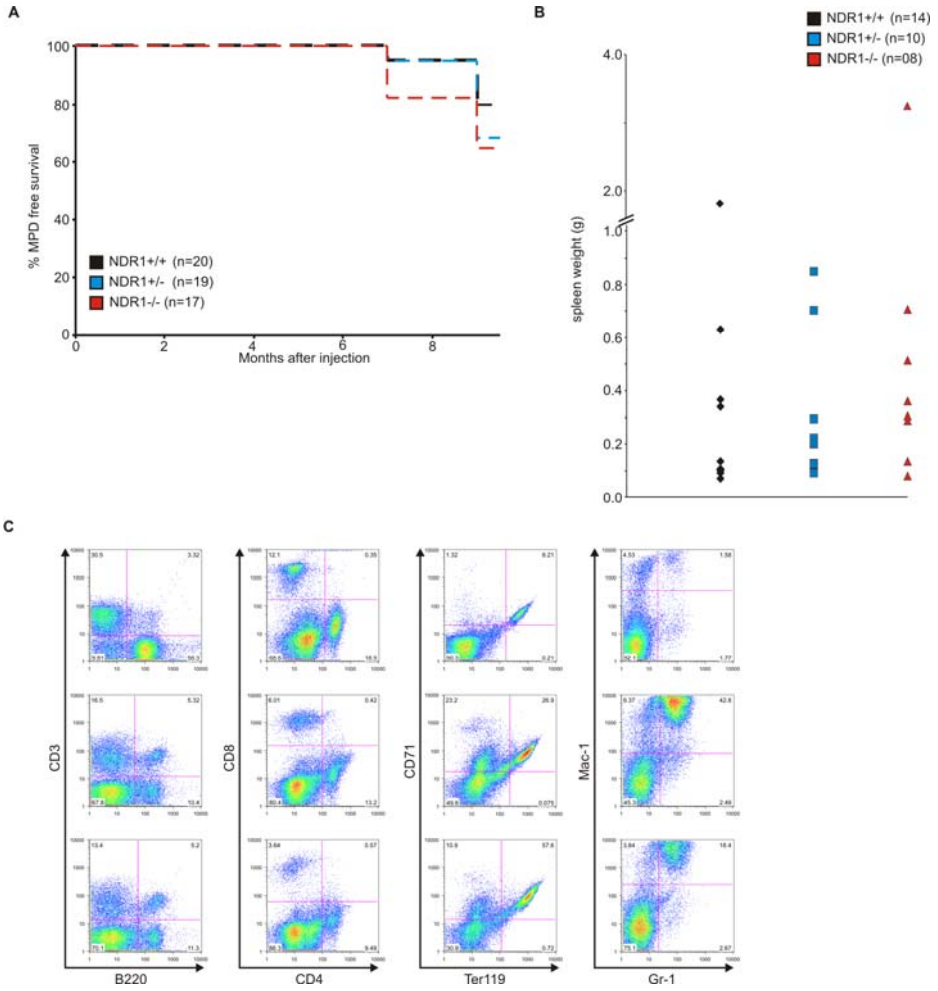


Figure S7. Increased development of myeloproliferative diseases (MPD) in NDR1$^{+/-}$ and NDR1$^{-/-}$ mice after ENU treatment
A: Kaplan-Meier survival plot showing the incidence of MPDs after ENU treatment. **B:** Spleen weights of mice not suffering from T-cell lymphoma upon dissection. The average weight of unaffected spleens was 0.11 ± 0.02 g. **C:** Characterization of MPDs found in ENU-treated mice. Splenocytes from an unaffected (upper panel) and two mice with MPD (middle and lower panel) were stained with antibodies against B220, CD3 (B- vs. T-cells), CD4, CD8 (T-cell marker), CD71, Ter119 (erythroid marker), Mac-1 and Gr-1 (granulocyte/ monocyte marker). Note that in mice with MPD, the B220^{-}/CD3^{-} population was markedly increased, which may be accounted for by an increase in either c- KIT^{+}/Ter119^{+} cells or Mac-1^{+}/Gr-1^{+} cells.

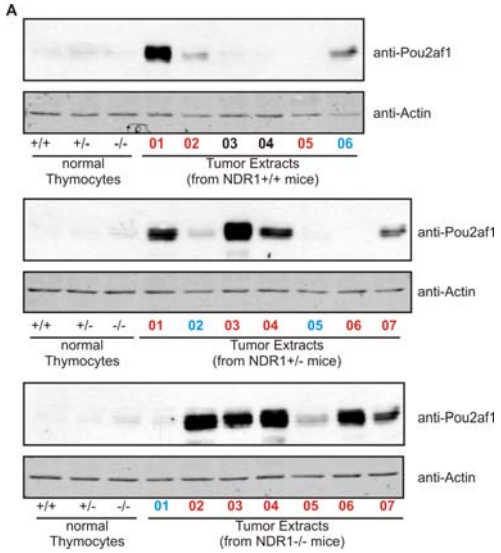


Figure S8. Increase in Pou2af1 correlates with low NDR levels in tumors
A: Confirmation of change in NDR-low-associated gene expression for the example of Pou2af1. Tumor extracts from all ENU-induced tumors were blotted for Pou2af1 expression using a murine Pou2af1-specific antibody

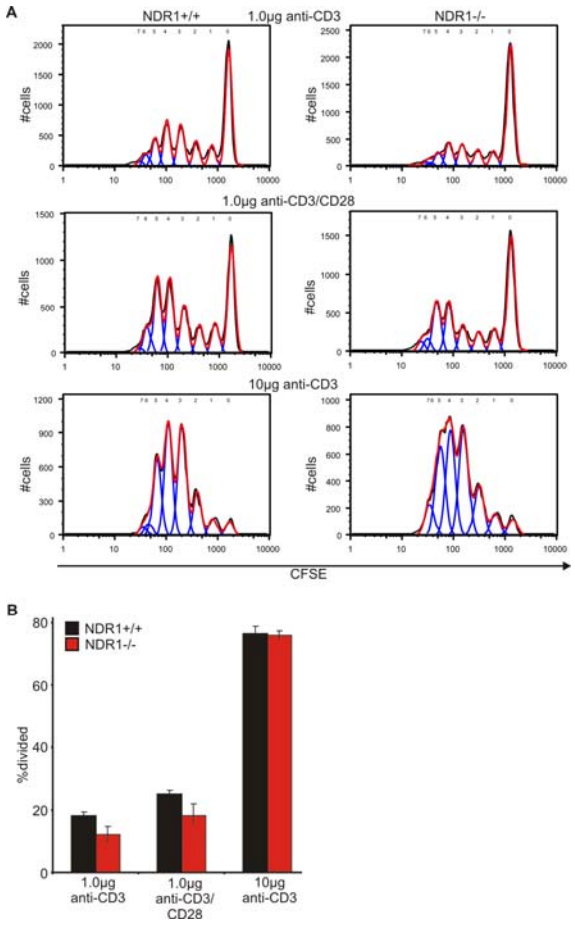


Figure S9. Loss of NDR1 results in proliferation defects in mature T-cells after TCR-stimulation
A: Mature T-cells from NDR1+/+ and NDR1-/- mice were isolated, labeled with CFSE and seeded into wells coated with the indicated concentrations of anti-CD3 antibody in the presence of anti-CD28 (1µg/ml) where indicated. 72h later cells were harvested and analyzed using FACS. Results were analyzed using the proliferation platform of the FlowJo analysis software (Number of cycles and model sum are shown). **B:** Quantification of the number of divided cells as accessed using the proliferation platform of the FlowJo analysis software.

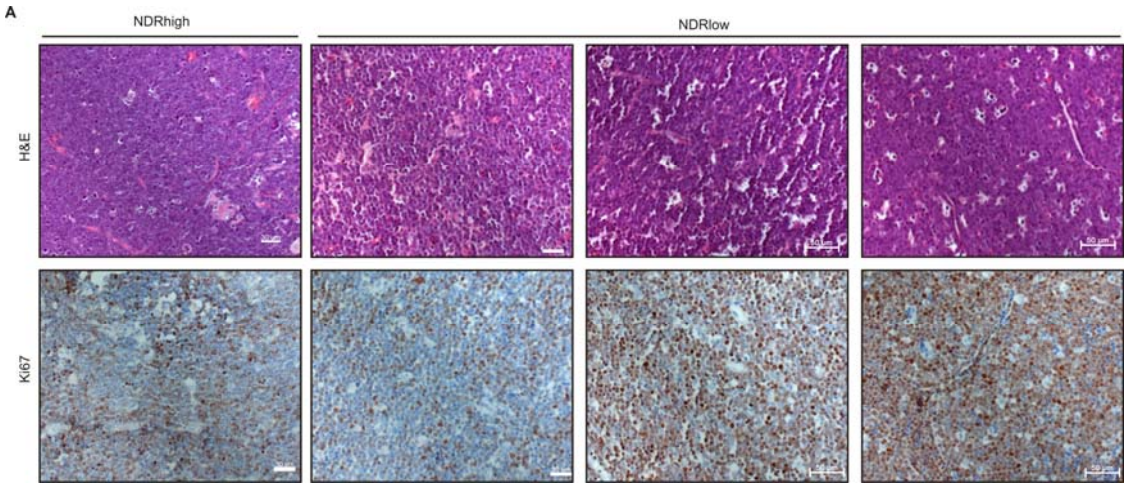


Figure S10. No consistent differences in Ki67 staining between NDR-high and NDR-low tumors
A: Analysis of Ki67 staining in NDR-high and NDR-low tumors (see also Figure 6C).

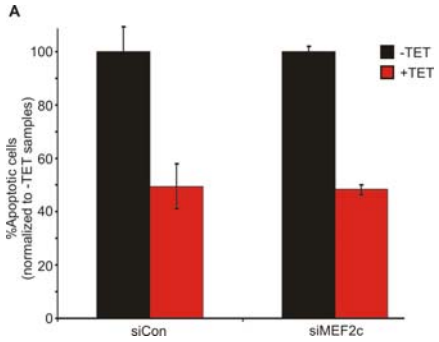


Figure S11. siRNA against MEF2c does not rescue apoptosis defects in NDR1/2 depleted cells after anti-FAS/CHX treatment
A: HeLa cells expressing inducible shRNA against NDR1 and NDR2 were treated with tetracycline for 72h and transfected with siRNA against MEF2c (siMEF2c) or control siRNA (siCon). 24h later cells were treated with anti-FAS antibody (0.5µg/ml) in the presence of 10µg/ml CHX for 6h and afterwards analyzed using AnnexinV/PI staining (n=3).

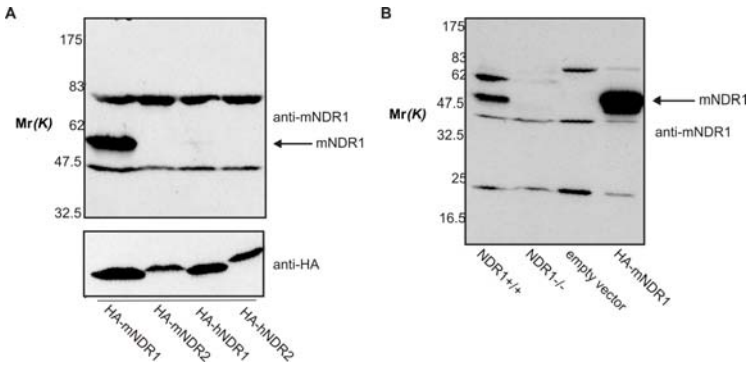


Figure S12. Characterization of a murine NDR1-specific antibody
A: Whole cell extracts from HEK293 cells transfected with the indicated HA-tagged murine and human NDR isoforms were probed with anti-mNDR1 and anti-HA antibody. **B:** Whole cell/ tissue extracts from $NDR1^{+/+}$ and $NDR1^{-/-}$ thymus, untransfected and HA-mNDR1-transfected HeLa cells were probed with anti-mNDR1.

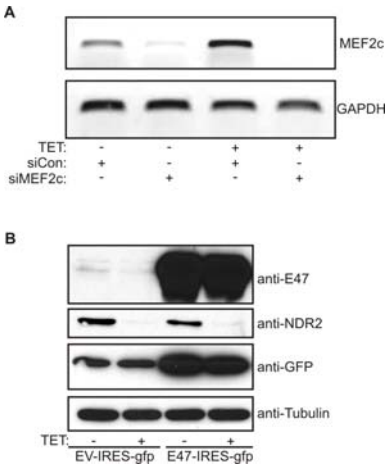


Figure S13 Confirmation of siRNA against MEF2c and overexpression of E47
A: HeLa cells expressing inducible shRNA against NDR1 and NDR2 were treated with tetracycline for 72h and subsequently transfected with siRNA against MEF2c (siMEF2c) or control siRNA (siCon). 24h later cells were lysed and MEF2c RNA level were analyzed using semi-quantitative RT-PCR with GAPDH level serving as control. Note the increase in MEF2c RNA level upon knock-down of NDR1/2. **B:** HeLa cells expressing inducible shRNA against NDR1 and NDR2 were treated with tetracycline for 72h and subsequently transfected with cDNA for E47. The cells were lysed 24h later and E47 level were analyzed using western-blotting.

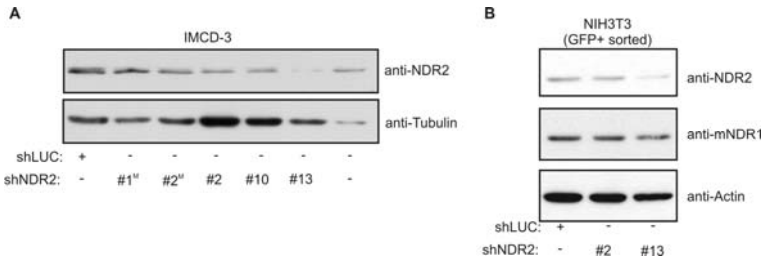


Figure S14. Testing of different shNDR2 constructs
A: IMCD-3 cells were transfected with pTER vectors containing different shRNA constructs against murine NDR2 using Lipofectamine 2000. Knock-down efficiency was analyzed after 72 h using an NDR2-specific antibody. Sequences #2 and #13 were chosen for subsequent cloning into pSUPER. **B:** NIH3T3 cells were infected with retrovirus-containing supernatant expressing the indicated shRNAs. GFP^+ cells were sorted 48 h after infection and expanded for another 36 h. Knock-down efficiency and specificity were tested using antibodies against NDR1 and NDR2.

Table S1. Aged mice analyzed for tumor development

No.	Genotype	Gender	Age at dissection	Lymphoma[a]
258	$NDR1^{+/+}$	female	24.2	-
440	$NDR1^{+/+}$	female	23.7	+
449	$NDR1^{+/+}$	female	23.9	-
451	$NDR1^{+/+}$	female	25.6	-
486	$NDR1^{+/+}$	female	20.0	-
353	$NDR1^{+/+}$	male	25.2	-
424	$NDR1^{+/+}$	male	23.4	-
437	$NDR1^{+/+}$	male	23.7	-
441	$NDR1^{+/+}$	male	23.9	-
482	$NDR1^{+/+}$	male	20.5	-
544	$NDR1^{+/+}$	male	21.2	-
568	$NDR1^{+/+}$	male	19.9	-
237	$NDR1^{+/-}$	female	24.7	+
290	$NDR1^{+/-}$	female	27.1	+
454	$NDR1^{+/-}$	female	25.7	+
515	$NDR1^{+/-}$	female	24.1	-
517	$NDR1^{+/-}$	female	24.1	+
610	$NDR1^{+/-}$	female	18.9	+
455	$NDR1^{+/-}$	male	24.6	-
472	$NDR1^{+/-}$	male	24.5	+
481	$NDR1^{+/-}$	male	20.5	-
533	$NDR1^{+/-}$	male	21.1	-
93	$NDR1^{-/-}$	female	24.2	-
198	$NDR1^{-/-}$	female	24.8	+
237	$NDR1^{-/-}$	female	25.1	+
260	$NDR1^{-/-}$	female	25.2	+
276	$NDR1^{-/-}$	female	23.4	+
291	$NDR1^{-/-}$	female	27.1	+
325	$NDR1^{-/-}$	female	26.5	+
369	$NDR1^{-/-}$	female	24.8	+
425	$NDR1^{-/-}$	female	23.4	+
448	$NDR1^{-/-}$	female	23.9	+
457	$NDR1^{-/-}$	female	22.1	-
553	$NDR1^{-/-}$	female	21.2	+
586	$NDR1^{-/-}$	female	24.9	+
635	$NDR1^{-/-}$	female	17.5	+
694	$NDR1^{-/-}$	female	23.1	+
57	$NDR1^{-/-}$	male	26.8	+
243	$NDR1^{-/-}$	male	24.4	-
281	$NDR1^{-/-}$	male	21.5	+
282	$NDR1^{-/-}$	male	21.5	-
303	$NDR1^{-/-}$	male	25.5	-
360	$NDR1^{-/-}$	male	24.1	-

369	NDR1$^{-/-}$	male	24.8	+
479	NDR1$^{-/-}$	male	20.7	-
483	NDR1$^{-/-}$	male	20.5	-
496	NDR1$^{-/-}$	male	22.1	+
507	NDR1$^{-/-}$	male	23.7	-
521	NDR1$^{-/-}$	male	24.0	+
644	NDR1$^{-/-}$	male	25.6	+

[a] Detection of lymphoma was done by evaluating H&E stained paraffin sections of various organs (- = negative for lymphoma/ + = positive for lymphoma). T-cell origin of tumor cells was verified by subsequent staining of sections with anti-Pax-5 and anti-CD3. In addition analysis of samples by FACS was done by staining single cell suspensions of lymphatic organs with antibodies against CD3, CD4, CD8 and B220.

Table S2. Overview of tumors identified in ENU-treated mice

Genotype	Number of injected mice	Number of mice with hematopoietic tumors[a]	Number of mice with T-cell lymphoma	Number of mice with MPD
$NDR1^{+/+}$	20	10 (50%)	6 (30%)	4 (20%)
$NDR1^{+/-}$	19	15 (79%)	9 (47%)	6 (32%)
$NDR1^{-/-}$	17	15 (88%)	9 (53%)	6 (35%)

[a] Hematopoietic tumors were comprised of T-cell lymphoma and myeloproliferative diseases as described in Fig.4 and S1.

Table S3. Classification of human T-cell lymphoma samples

Number	Classification[a]	NDR expression reduced:
01	T-NHL:AITL	-
02	T-NHL:AITL	+
03	T-NHL: NOS	+
04	T-NHL: NOS	+
05	T-NHL: NOS	+
06	T-NHL: AITL	+
07	T-NHL: NOS	+
08	T-NHL: NOS	+
09	T-NHL: AITL	+
10	T-NHL: AITL	+
11	T-NHL: NOS	-
12	T-NHL: NOS	-

[a] Abbreviations used: NHL: non Hodkins lymphoma; NOS: not otherwise specified; AITL: angioimmunoblastic T-cell lymphoma

Tumors were classified according to the current WHO-classification.

Table S4. Grouping of tumors according to their NDR1/2 expression

NDR1/2 expression	**Tumor sample**
NDR1/2 high	NDR1+/+ 03
	NDR1+/+ 04
NDR1/2 middle	NDR1+/+ 06
	NDR1+/- 02
	NDR1+/- 05
	NDR1-/- 01
NDR1/2 low	NDR1+/+ 01
	NDR1+/+ 02
	NDR1+/+ 05
	NDR1+/- 01
	NDR1+/- 03
	NDR1+/- 04
	NDR1+/- 06
	NDR1+/- 07
	NDR1-/- 02
	NDR1-/- 03
	NDR1-/- 04
	NDR1-/- 05
	NDR1-/- 06
	NDR1-/- 07

Table S5. Genes statistically significant up-/down-regulated in NDR-low tumors

Gene Symbol	Description	NDR-high/ normal[a]	NDR-middle/ normal[a]	NDR-low/ normal[a]
Igh-6	Immunoglobulin heavy chain 6 (heavy chain of IgM)	0.41	3.45	115.79
Mef2c	**myocyte enhancer factor 2C**	**0.67**	**0.58**	**80.07**
Saa3	serum amyloid A 3	2.12	1.44	41.73
Napsa	napsin A aspartic peptidase	0.47	0.61	35.00
Pou2af1	**POU domain, class 2, associating factor 1**	**0.45**	**0.87**	**32.54**
Cd5l	**CD5 antigen-like**	**1.91**	**2.64**	**30.52**
Myo1e	myosin IE	0.93	1.04	22.44
Myl4	myosin, light polypeptide 4	5.31	9.11	18.46
Emilin2	elastin microfibril interfacer 2	0.79	0.93	12.34
Arhgef12	Rho guanine nucleotide exchange factor (GEF) 12	1.27	0.64	7.55
Ndg2	Nur77 downstream gene 2	2.13	1.09	6.22
A030007L17Rik	RIKEN cDNA A030007L17 gene	1.72	1.68	5.98
Tirap	toll-interleukin 1 receptor (TIR) domain-containing adaptor protein	1.18	1.01	5.90
Itgb7	integrin beta 7	0.45	1.02	5.40
Osbpl5	oxysterol binding protein-like 5	1.07	0.96	4.83
Pck2	phosphoenolpyruvate carboxykinase 2 (mitochondrial)	0.86	0.80	3.52
Traf3ip2	Traf3 interacting protein 2	1.15	1.32	2.81
Nudt18	nudix (nucleoside diphosphate linked moiety X)-type motif 18	0.76	1.11	2.41
Cnn2	calponin 2	0.70	0.84	2.33
St3gal1	ST3 beta-galactoside alpha-2,3-sialyltransferase 1	0.50	0.41	2.16
Ripk1	receptor (TNFRSF)-interacting serine-threonine kinase 1	0.91	0.89	1.82
Ipo8	importin 8	1.32	1.04	1.62
Iqgap1	IQ motif containing GTPase activating protein 1	0.97	1.05	1.62
Wars	tryptophanyl-tRNA synthetase	1.24	0.97	1.51
Dlg4	discs, large homolog 4 (Drosophila)	1.86	0.95	0.65
Wbp5	WW domain binding protein 5	0.75	1.00	0.65
Pdgfa	platelet derived growth factor, alpha	8.18	1.05	0.60
Cep55	centrosomal protein 55	1.15	1.08	0.53
Txndc9	thioredoxin domain containing 9	1.01	0.98	0.52
Tmpo	thymopoietin	1.06	0.88	0.51
Top2a	**topoisomerase (DNA) II alpha**	**1.24**	**1.00**	**0.50**
Cdkn2c	**cyclin-dependent kinase inhibitor 2C (p18, inhibits CDK4)**	**1.36**	**1.30**	**0.49**

Kif11	kinesin family member 11	1.46	1.08	0.47
Apold1	apolipoprotein L domain containing 1	1.26	1.19	0.46
Kif18a	kinesin family member 18A	1.23	1.23	0.45
Anln	anillin, actin binding protein (scraps homolog, Drosophila)	1.47	1.13	0.45
Cenpa	centromere protein A	0.95	1.01	0.44
Esco2	establishment of cohesion 1 homolog 2 (S. cerevisiae)	1.24	1.27	0.44
Spc25	spindle pole body component 25 homolog (S. cerevisiae)	1.07	1.11	0.42
Pbk	PDZ binding kinase	1.00	1.19	0.35
H2afx	H2A histone family, member X	1.07	0.80	0.34
Ckap2	cytoskeleton associated protein 2	0.94	0.91	0.33
Kif2c : LOC631653	kinesin family member 2C /// similar to Kinesin-like protein KIF2C (Mitotic centromere-associated kinesin) (MCAK)	1.56	1.25	0.30
Ccnd3	**cyclin D3**	**0.57**	**0.67**	**0.29**
E2f7 : LOC639365	E2F transcription factor 7	0.58	0.44	0.21
Bex1	brain expressed gene 1	0.55	2.45	0.14

[a] Expression values for each tumor type was divided by the respective value for normal thymocytes.

3.2 Human NDR kinases control G1 progression/S-phase entry by regulating p21 and c-myc stability

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3.2.1 Abstract

The G1-phase of the cell cycle is an important integrator of internal and external cues, allowing a cell to decide whether to proliferate, differentiate or die. We identify here a role for mammalian NDR kinases in regulating G1-progression and S-phase entry. Although characterized well terms of biochemical regulation and upstream signaling, signaling downstream of mammalian NDR kinases remains largely unknown. Here we describe downstream signaling mechanisms by which NDR kinases regulate cell cycle progression. NDR kinases directly impact on the protein stability of the c-myc proto-oncogene and the Cyclin-Cdk inhibitor protein p21. Addressing the mechanisms behind the regulation of c-myc and p21 stability, we identify p21 as the first *in vivo* substrate for mammalian NDR kinases. Whereas p21 levels are regulated in a kinase activity dependent manner, c-myc levels are regulated independently of NDR kinase activity: NDR kinases bind to c-myc and interfere with c-myc ubiquitination and degradation.

3.2.2 Introduction

In order to duplicate, cells have to proceed through a defined order of events collectively called the cell cycle. The eukaryotic cell cycle consists of 4 phases, which can roughly be defined by growth and preparation for the duplication of the genetic material in G1-phase, duplication of the genetic material in S-phase, preparation for separation in G2-phase and finally separation of the genetic material into two daughter cells in M-phase. Proper progression through a given cell cycle phase and unidirectional transition between the phases are highly controlled on multiple levels (*1*). Defects in the mechanisms controlling the cell cycle have been shown to result in accumulation of genetic alterations and subsequent cancer development (*2*). The G1-phase of the cell cycle is a very important integrator of internal and external cues, allowing cells to grow, process outside information or repair damage before entering S-phase. In G1 a cell decides whether to self renew, differentiate or die (*1*). Entry into S-phase is mediated by the action of Cyclin-Cdk complexes. Initially Cyclin D-Cdk4/6 and later Cyclin E-Cdk2 complexes phosphorylate the Rb tumor suppressor protein allowing dissociation of Rb from E2F transcription factors and subsequent transcription of genes required for S-phase entry (*3*).

The activity of Cyclin-dependent kinases is controlled on multiple levels (*4*). The association of Cdks with Cylin subunits is a prerequisite for Cdk activation. This process is controlled firstly by the availability of the Cyclin sub-unit, which abundance is regulated both by transcriptional and post-transcriptional processes (*4*). Furthermore, Cyclin-Cdk inhibitor (CKI) proteins of the Cip/Kip and INK4 family control Cyclin-Cdk activity by different mechanisms. Whereas members of the Cip/Kip family such as p21, p27 and p57 directly inhibit the Cdk activity, the INK4 family members p15, p16, p18 and p19 actively promote Cyclin-Cdk complex

disassembly. Interestingly, the Cip/Kip family members p21 and p27 are needed for efficient assembly of Cyclin D-Cdk4/6 complexes (*5*). Importantly, loss of several CKI proteins has been associated with tumor development (*2*).

Members of the NDR family of Ser/Thr kinases are highly conserved from yeast to men and have been implicated in the regulation of a variety of biological processes (*6*). With the regulation of mitotic exit, cell growth, proliferation, centrososme duplication and morphogenesis, NDR kinases across species have been shown to function in processes tightly linked to the cell cycle (*6*). The human genome encodes four different NDR kinase family members: NDR1/2 and LATS1/2 (*7*). The kinases LATS1/2 function as part of the HIPPO pathway thereby controlling the localization and function of the YAP oncogene (*8*). The HIPPO pathway thereby controls cell growth, cell size, proliferation and apoptosis. Furthermore, roles for LATS1 and LATS2 in controlling mitotic exit and genomic stability have been described (*9, 10*). Although well characterized in terms of biochemical regulation, functions for the other two NDR family kinases in the human genome NDR1 and NDR2 only recently started to unravel. In cellular systems NDR kinases have been implicated in the regulation of centrosome duplication, apoptosis and the alignment of mitotic chromosomes (*11-13*). Furthermore, a recent study indicated a tumor suppressive function for NDR1/2 in mice by controlling proper apoptotic responses (Cornils, et al. submitted). Interestingly, with the involvement of MST1 and hMOB1 in the regulation of NDR1/2 in centrosome duplication and apoptosis and MST2 in the alignment of mitotic chromosomes, several components of the HIPPO pathway also function in the regulation of NDR1/2 (*11, 13, 14*). However, although first functions for NDR1/2 were defined recently, downstream signaling remained elusive. Furthermore, although NDR kinases have been implicated in cell-cycle dependent

processes such as centrosome duplication and the alignment of mitotic chromosomes, discrepancies exist whether NDR kinases are activated in M or S-phase of the cell cycle (*11, 14*). Here we addressed the activation of NDR1/2 throughout the cell cycle. We show that NDR1/2 are activated in G1-phase by MST3, the third MST-family kinase shown to function upstream of NDR1/2. Even more importantly, with the direct regulation of c-myc and p21 stability, we define first downstream signaling mechanisms by which NDR kinases control G1-progression and S-phase entry.

3.2.3 Results

NDR kinases are activated in G1-phase by MST3

Earlier reports have implicated NDR family kinases in the regulation of cell-cycle dependent processes such as mitotic chromosome alignment and the regulation of centrosome duplication (*11, 14*). However it has been reported that the activation of NDR kinases can take place in M-phase or in S phase of the cell-cycle. In order to address this discrepancy we analyzed NDR activity changes during the cell-cycle. M-phase arrested HeLa S3 cells were washed free from nocodazole, replated in fresh medium and followed for up to 18h after release (Figure 1A). NDR kinase activation was assessed using an antibody specific for the hydrophobic motif (HM) phosphorylation of NDR1 and NDR2 (referred to as anti-T444P). HM-phosphorylation of NDR1/2 was nearly absent in M-phase, but increased after 3h and peaked around 6-8h after release. The activation of NDR1/2 coincided with accumulation of the G1-phase marker CyclinD1 and PI-staining confirmed that 4-6h after release the cells were mainly in G1. HM-phosphorylation of NDR1/2 started to gradually decrease after 12h up to the end of the experiment, which coincided with the accumulation of the S/G2-phase marker CyclinA. Thus, NDR kinases were activated

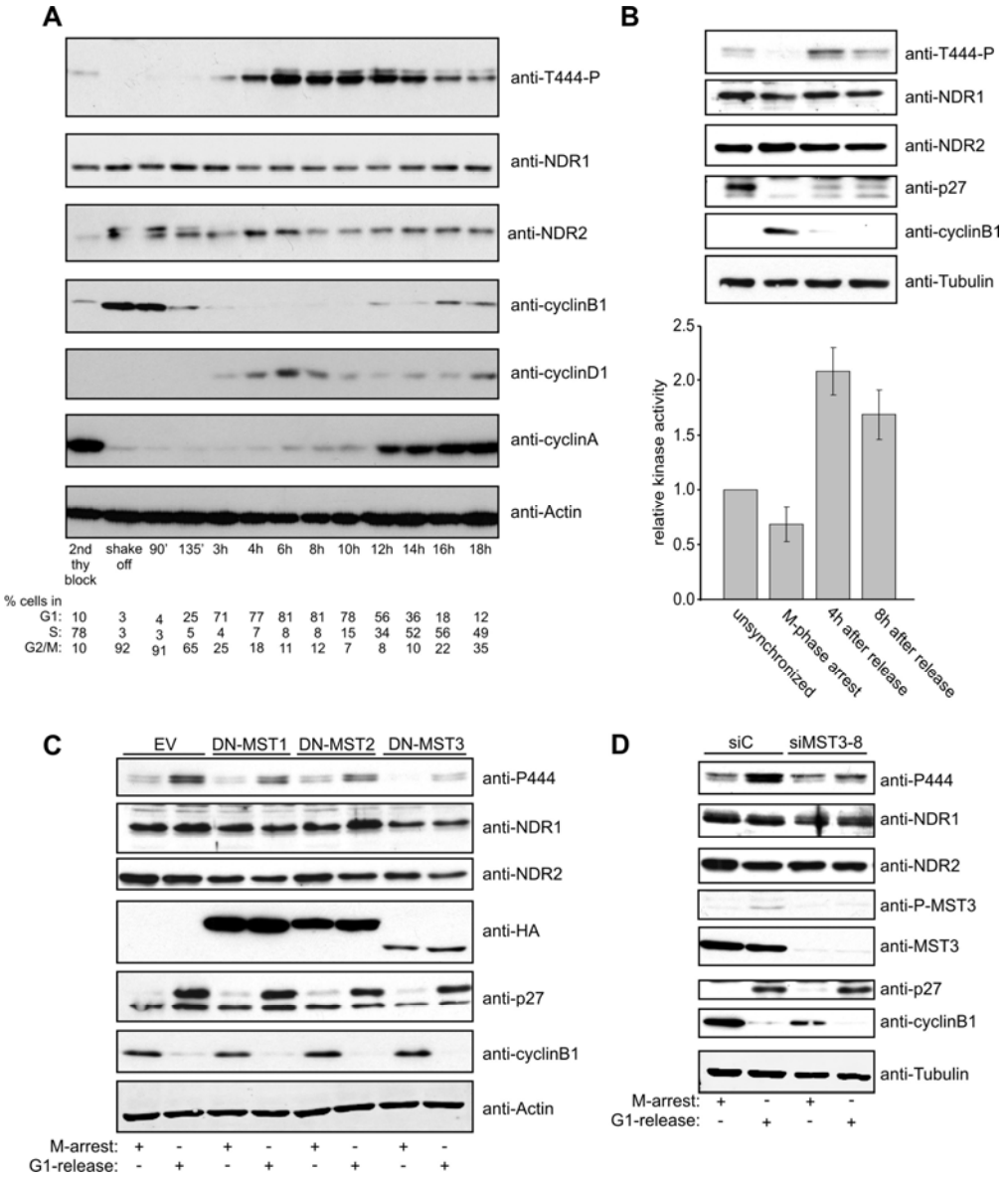

Figure 1. NDR kinases are activated by MST3 in G1-phase of the cell-cycle.
A: HeLa S3 cells were synchronized using a double thymidine block followed by nocodazole arrest and mitotic shake-off. Floating cells were collected, washed free from nocodazole and replated in fresh medium. After the indicated time, cells were harvested and processed for immunoblotting and FACS analysis. Activation of NDR1/2 was accessed using anti-T444-P, NDR1 and NDR2 antibodies. Cell cycle distribution was accessed using PI staining and FACS analysis. Cell cycle phases were confirmed using Cyclin B1, Cyclin D1 and Cyclin A expression. **B:** HeLa cells were arrested at G2/M-border using nocodazole treatment for 14h. Cells were released into M/G1 phase for the indicated time before harvesting. Lysates were subjected to immunoblotting and immuno-precipitation of endogenous NDR species using a mixture of isoform specific antibodies. NDR kinase activity was accessed using peptide kinase assay (n=3). **C:** HeLa cells were transfected with dominant-negative MST1, MST2 or MST3 and 24h later arrested with nocodazole for 14h. Arrested cells were harvested or released into G1 for 8h before harvesting. NDR activation was accessed using T-444-P antibody. Cell cycle phases were confirmed by analyzing Cyclin B1 and p27 expression. **D:** HeLa cells were transfected with control siRNA (siC) or siRNA against MST3 (siMST3) and treated and analyzed as described in C. MST3 activation was accessed by using a P-MST4-T178/-MST3-T190/-STK25-T174 specific antibody (anti-P-MST3). Note that the P-MST3 signal disappears in the siMST3 treated samples.

in G1-phase of the cell cycle with the activation persisting until the end of S-phase. The G1-activation of NDR1/2 was confirmed by analyzing endogenous NDR1/2 activity using a peptide kinase assay (Figure 1B). HeLa cells were arrested at G2/M-

border and either harvested directly or released for 8h into fresh medium. Endogenous NDR species were immuno-precipitated and their activity measured using a specific peptide kinase assay. Direct assessment of NDR kinase activity confirmed that NDR kinases were activated in G1, whereas the activity was decreased in M-phase as compared to unsynchronized cells (Figure 1B).

Three members of the mammalian STE20 like kinases have been implicated in the regulation of NDR1/2: MST1, MST2 and MST3 (*11, 13, 14, 19*). For each MST kinase it has been shown that the kinase-dead variant can act in a dominant negative manner (*14*). To test which of the MST kinases was important for NDR1/2 activation in G1, we analyzed G1 activation of NDR upon over-expression of DN-MST1-3 (Figure 1C). Strikingly, although over-expression of DN-MST1 and DN-MST2 resulted in a moderate decrease in NDR activation, the expression of DN-MST3 significantly reduced NDR phosphorylation in this setting. To validate this finding we knocked-down endogenous MST3 using siRNA-technology and analyzed NDR activation (Figure 1D, Figure S1A). Depletion of endogenous MST3 resulted in decreased NDR activation in G1, confirming the role of MST3 for G1-activation of NDR1/2. To test whether MST3 phosphorylation would be increased in G1, MST3 activity was analyzed in our setting (Figure 1D, Figure S1A). Importantly, when comparing P-MST3 levels in G1 phase cells versus M-phase arrested cells, an increase of P-MST3 was observed in G1, which was not observed in cells depleted from MST3. Taken together, NDR kinases were activated in G1-phase of the cell cycle, with the activation persisting until late S-phase. Furthermore, our experiments revealed MST3 as the responsible upstream kinase for NDR1/2 in this setting, providing the first functional link between NDR and MST3.

Decrease in NDR1/2 results in cell proliferation defects due to a G1 block

To analyze whether NDR kinases functioned in cell cycle progression and proliferation we made use of HeLa cells expressing shRNA against NDR1 and NDR2 (Figure 2A). Both isoforms were targeted to avoid any compensatory effects as described earlier (Cornils, et al.; submitted). Knock-down of NDR kinases resulted in consistent proliferation defects of around 50%, which were not observed in control clones expressing shRNA against firefly luciferase (Figure 2B/C). In addition the effects of single knock-down of either NDR1 or NDR2 were tested (Figure S2). Interestingly, no compensatory effects of knocking down one isoform were observed (Figure S2C). Although compensation of NDR1 deficiency by NDR2 in healthy tissues was described earlier (Cornils, et al.; submitted), this data suggested that in our setting the compensatory mechanisms regulating NDR kinase isoforms were disturbed. Next we tested whether the observed proliferation defects would be a result of a cell-cycle block. Using BrdU incorporation we observed an increase in cells in G1 accompanied by a decrease in S-phase in NDR1/2 depleted cells (Figure S3). As normal proliferating HeLa cells were mainly in G1, we employed a method described by Mikule et al. to verify G1 blockade (*18*). Before cell-cycle analysis, nocodazole was added for 14h to accumulate cycling cells at the G2/M-border (Figure 2D). Indeed, cells deprived of NDR mostly stayed in G1, confirming that knock-down of endogenous NDR kinase species resulted in G1-arrest. An earlier report showed that NDR kinases were important for centrosome duplication (*12*). Disturbances in centrosome assembly have been reported to trigger the activation of a centrosome integrity checkpoint resulting in the activation of p38 and p53 with subsequent upregulation of p21 and G1-arrest (*18, 20*). Furthermore, inhibiting p38 in this setting decreased G1-arrest. To test whether the centrosome integrity checkpoint was

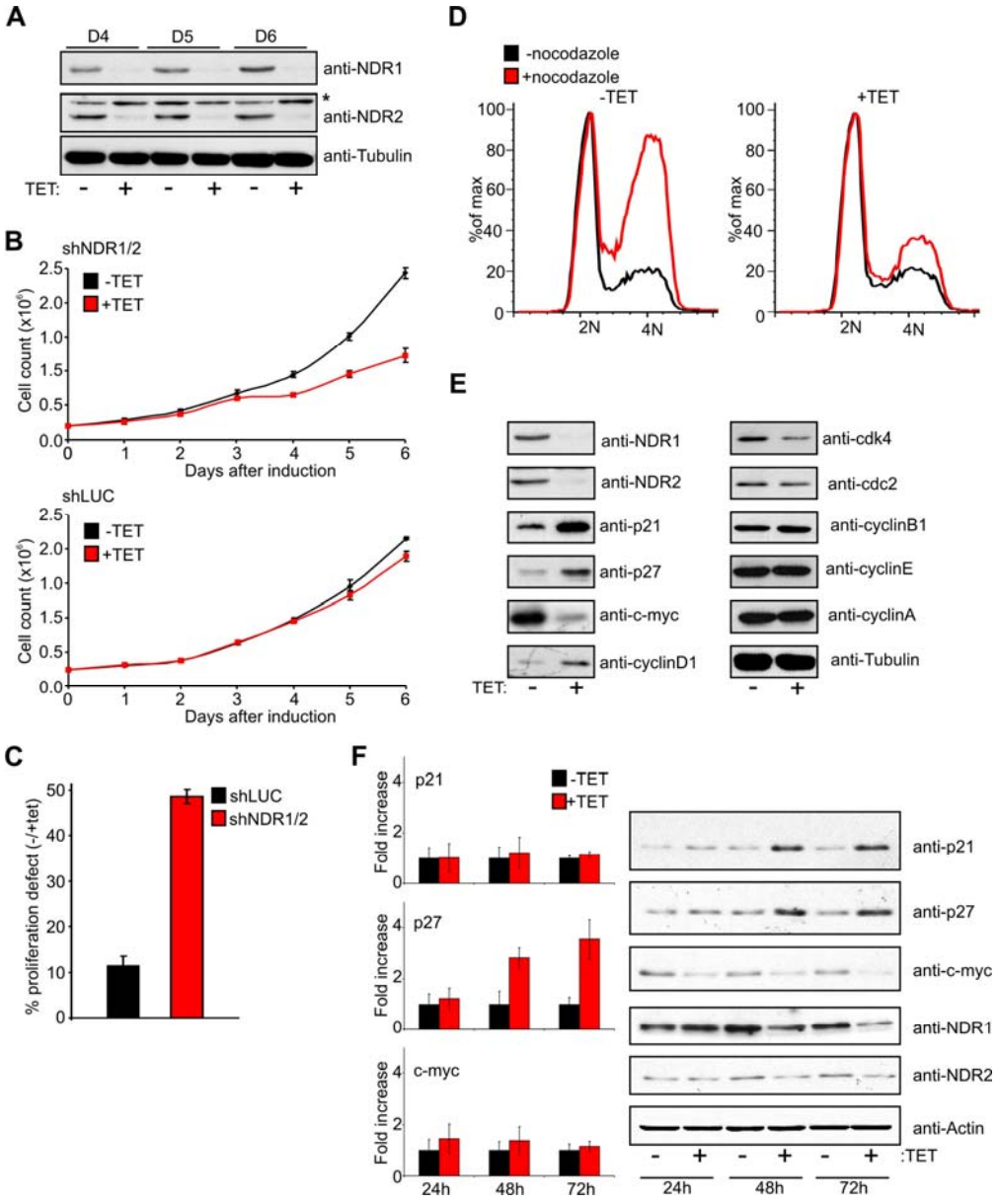

Figure 2. shRNA mediated knock-down of NDR1/2 results in defects in cellular proliferation due to a G1-block.
A: Characterization of HeLa-Trex cells stably expressing shRNA against NDR1 and NDR2. Cells were seeded in 10cm dishes and shRNA expression was induced by the addition of tetracycline (TET) for the indicated time. Lysates from harvested cells were analyzed for NDR1 and NDR2 expression using isoform-specific antibodies. **B:** HeLa cells expressing shRNA against NDR1/2 (shNDR1/2) or firefly luciferase (shLUC) as a control were seeded in triplicates and tetracycline was added to induce shRNA expression. After the indicated time, cells were harvested by trypsination and counted using a Vicell-automated cell counter. **C:** Validation of proliferation defects in different clones stably expressing shNDR1/2 or shLUC (n=3). Experiments were performed as in B, differences in proliferation were calculated as percentage of cells without tetracycline to cells with tetracycline counted on day6 after induction of shRNA expression. **D:** Knock-down of NDR1/2 was induced for 4 days using tetracycline. 14h before harvesting and processing for FACS analysis, cells were treated with 2.5µg/ml nocodazole to induce G2/M-accumulation. Fixed cells were stained with PI and analyzed by FACS. Histograms were overlaid to facilitate better comparison of cells in a given cell cycle phase. **E:** Knock-down of NDR1/2 was induced for 4days and cell-lysates were analyzed for the expression of the indicated cell-cycle regulators using western-blotting. **F:** Knock-down of NDR1/2 was induced for the indicated timepoints and protein and RNA extracts were prepared to analyze p21, p27 and c-myc mRNA by quantitative RT-PCR and protein expression by western blotting. Values for p21, p27 and c-myc mRNA are given a fold change to untreated samples (n=3).

activated by depleting NDR1/2, we treated the cells with inhibitors for p38 prior to G1-arrest assessment. Adding p38 inhibitors to cells deprived of NDR did not result in release from G1 block, indicating that the observed G1 arrest in this setting was not

due to activation of the centrosome integrity checkpoint (Figure S4). Consistent with the activation of NDR1/2 in G1, reduction of NDR1/2 levels resulted in proliferation defects due to a G1-block. However, the observed G1-block was not mediated by a p38 dependent centrosome integrity checkpoint.

Loss of NDR kinases results in upregulation of the cell cycle inhibitors p21 and p27 and a decrease in c-myc protein levels.

Next we analyzed the mechanisms underlying the G1-block by investigating the levels of known regulators of G1 progression/S-phase entry. The activity of Cyclin-Cdk complexes important for G1-progression/S-phase entry is regulated by Cyclin-Cdk inhibitors such as p21 and p27 (*5*). Indeed, the expression of p21 and p27 was elevated in NDR1/2 knock-down cells without significant decrease in the expression of cyclins and their respective cdks (Figure 2E). Interestingly, Cyclin D1 was slightly increased. In addition the expression of the c-myc oncogene was reduced. These results were confirmed in HeLa cells expressing shRNA against NDR1 and NDR2 alone, as well as in transiently transfected HCT116 (Figure S5A-E). Furthermore validated U2OS cells expressing shRNA against NDR1 together with a rescue construct for wild-type NDR1 were analyzed (Figure S5F/G) (*12*). Importantly, re-expression of NDR1 wt not only counteracted the upregulation of p21 and p27, but also restored cell proliferation (Figure S5G). This suggested that the observed G1-block upon depletion of NDR1/2 was due to the inhibition of Cyclin-Cdk complexes by increased levels of p21 and p27.

It has been shown that c-myc is able to repress p21 and p27 expression (*21, 22*); therefore we tested whether for both proteins changes on the mRNA level were detectable (Figure 2F). Interestingly, although p27 mRNA level were clearly

increased, we did not observe any elevation of p21 mRNA. Recently it has been reported, that NDR1 functioned as a regulator of c-myc protein stability in B-cells, with decreased NDR1 level resulting in a decrease in c-myc protein, but not mRNA (*23*). In line with this observation, we did not observe decreased c-myc mRNA levels in our setting. Taken together, these experiments indicated that the G1-block detected in NDR depleted cells seemed to be a result of increased levels of p21 and p27 accompanied by a decrease in c-myc protein. Interestingly, only the p27 mRNA was increased in this setting, indicating that NDR1/2 presumably impacted directly on both p21 and c-myc protein turnover.

Hydrophobic motif phosphorylation of NDR kinases increases binding to c-myc and c-myc protein stability

A recent report analyzing post-transcriptional modifiers of c-myc in B-cells implicated NDR1 in the regulation of c-myc protein stability (*23*). However, although an interaction between NDR1 and c-myc was described, no mechanistic studies were performed to understand how NDR1 impacted on c-myc stability. In full agreement with this report, c-myc protein level were rescued by the addition of the proteasomal inhibitor MG132 (Figure 3A). In addition, our experiments confirmed an interaction between NDR1 and c-myc both with overexpressed and endogenous proteins (Figure S6A/C). Furthermore NDR2 was also capable of binding to c-myc with similar efficiency than NDR1 (Figure S6B).

As we observed an increase in NDR1/2 kinase activation and HM-phosphorylation in G1-phase, we tested whether NDR kinase activity would influence NDR-myc complex formation. First we analyzed whether c-myc would be a substrate for NDR kinases. However, in *in vitro* kinases assays no specific phosphorylation of c-myc by

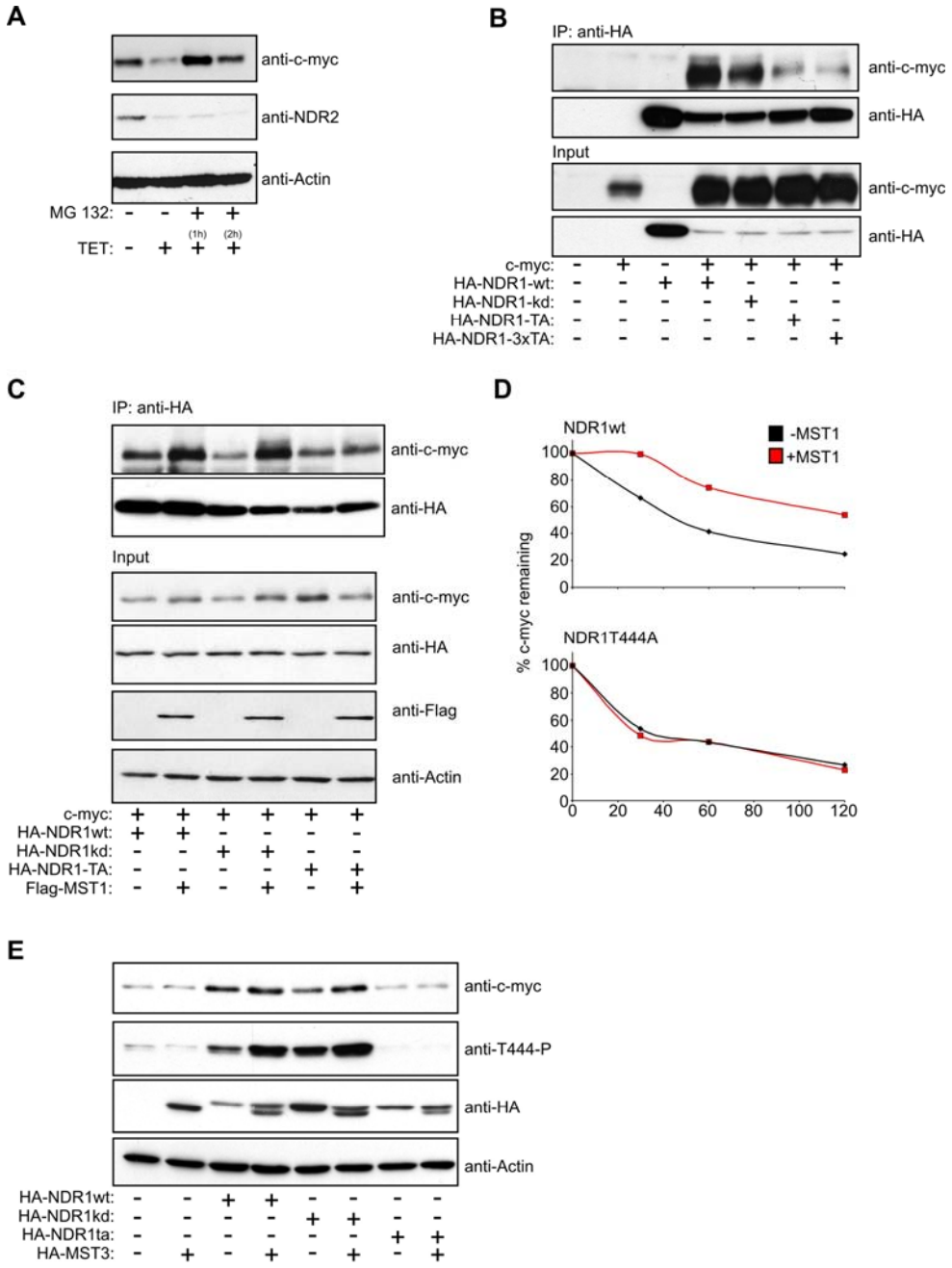

Figure 3. NDR1/2 interact with c-myc in a HM-phosphorylation dependent manner impacting on c-myc stability.
A: Knock-down of NDR1/2 was induced for 48h. To inhibit proteasome dependent degradation of c-myc MG132 was added for the indicated time. **B:** HEK cells were transfected with c-myc together with the indicated HA-tagged NDR1 constructs. NDR1 species were immuno-precipitated using anti-HA antibody and binding to c-myc was analyzed by SDS-page. **C:** HEK cells were transfected with c-myc and the indicated NDR1 cDNAs together with a vector encoding FLAG-MST1. Complex formation was analyzed as in B. **D:** HEK cells transfected with c-myc, NDR1wt or NDR1ta in the presence of absence of FLAG-MST1 were treated with cycloheximide for the indicated time and lysates were analyzed for the expression of c-myc using the Licor Odyssey system. **E:** HEK cells were transfected with HA-tagged variants of NDR1 together with HA-MST3 where indicated and lysates were analyzed for the expression of endogenous c-myc 24h later.

NDR kinases was observed (data not shown). Next, NDR1wt, NDR1kd and NDR1-T444A (referred to as NDR1ta), a mutant where the HM phosphorylation site was mutated to alanine, were tested for complex formation by co-immunoprecipitation (Figure 3B). Interestingly, no differences in c-myc binding were observed between

NDR1wt and NDR1kd, indicating that kinase activity of NDR1 did not influence binding to c-myc. However, binding was impaired upon expression of the T444A mutant. In addition, expression of a mutant in which all phospho-sites (T74A, S281A, T444A; referred to as NDR1-3xTA) have been mutated to alanine exhibited similar binding as the T444A mutant, confirming the importance of the HM-phosphorylation site for interaction with c-myc.

Next we tested whether phosphorylating the HM would increase complex formation (Figure 3C). Indeed when MST1 as an upstream kinase was co-expressed, increased complex formation was observed for NDR1wt and NDR1kd, but not for NDR1ta, again arguing, that the effects of NDR kinases on c-myc were not dependent on NDR kinase activity, but on hydrophobic motif phosphorylation. MST1 was used as an upstream kinase in some assays, as it was assumed to be responsible for NDR activation in G1 before the identification of MST3 as responsible kinase. To test whether increased binding of NDR to c-myc would result in increased c-myc protein stability, c-myc turnover was analyzed in the presence of NDR1wt and NDR1ta together with overexpression of MST1 as an upstream kinase. Strikingly, c-myc stability was greatly enhanced upon overexpression MST1 together with NDR1wt. The non-phosphorylateable NDR1ta mutant showed no response to overexpression of MST1, confirming that increased binding of NDR kinase to c-myc due to HM-phosphorylation indeed increased c-myc stability. In addition, NDR1kd showed a similar stabilizing activity than NDR1wt in these assays (Figure S6D), confirming, that NDR kinases impact on c-myc stability independently of their kinase activity. Finally, the effect of NDR1 overexpression and HM-phosphorylation on the stability of endogenous c-myc was analyzed (Figure 3E). Only overexpression of NDR1wt and NDR1kd increased endogenous c-myc protein level. In addition, increasing NDR1-

HM phosphorylation by overexpressing MST3 resulted in a further increase in c-myc level. Taken together, these experiments confirmed the stabilizing effect of NDR1 and NDR2 on c-myc protein level. Furthermore our data provided mechanistic insight into the regulation of c-myc stability by NDR kinases: NDR kinases bind to c-myc in a kinase-activity independent but HM-phosphorylation dependent manner, thereby increasing c-myc protein stability.

NDR kinases compete with the ubiquitin-ligase Skp2 for c-myc binding, thereby interfering with Skp2 mediated ubiquitination of c-myc.

To further understand the mechanism behind the stabilizing effect of NDR on c-myc, we analyzed the NDR binding site on c-myc, using deletion mutants of c-myc (Figure 4A). Interestingly, NDR1 bound both to the C-terminus as well as the N-terminus of c-myc (Figure 4B). Two Ubiquitin-ligases, Fbw7 and Skp2, have been reported to target c-myc for proteasomal degradation (*24-27*). Strikingly, Skp2 has also been shown to interact with c-myc both on the N- and the C-terminus (*24, 25*). Furthermore the MB2 domain of c-myc was important for interaction with Skp2 (*24, 25*). We therefore deleted the MB1 or the MB2 domain in c-myc and tested for NDR1 binding (Figure 4C). Strikingly, only deletion of MB2 resulted in impaired binding to NDR1, suggesting that NDR kinases bound to sites on c-myc overlapping with those described for Skp2 interaction. Therefore we postulated that if NDR kinases could compete with Skp2 for c-myc binding, overexpression of NDR1 would decrease Skp2-mediated ubiquitination of c-myc. Indeed, overexpression of NDR1wt impaired c-myc ubiquitination (Figure 4D). Furthermore overexpression of NDR1 interfered with increased c-myc ubiquitination upon overexpression of Skp2 (Figure 4E). Taken together our analysis suggested a role for NDR kinases in the regulation of c-myc

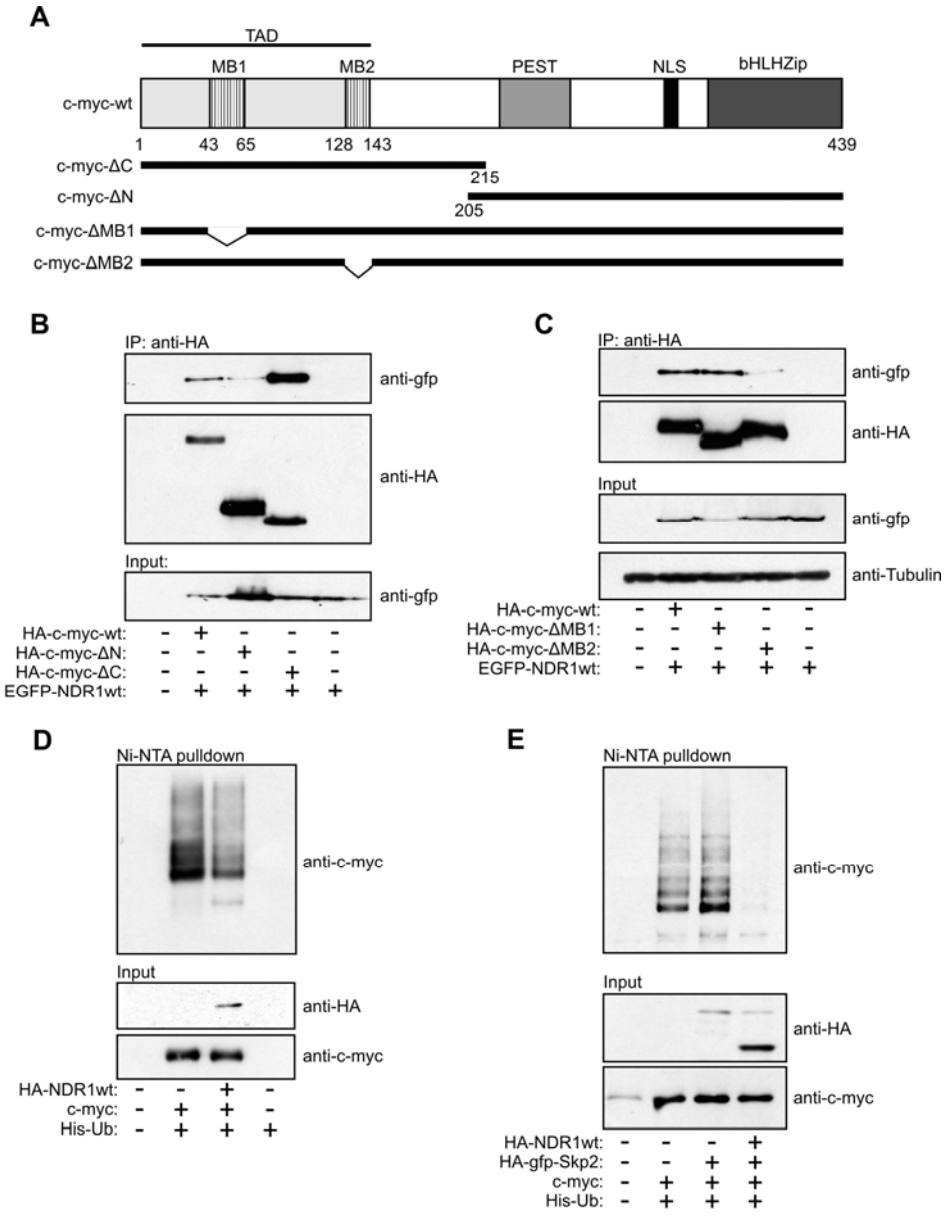

Figure 4. NDR1/2 interfere with Skp2-mediated ubiquitination and degradation of c-myc.
A: Schematic representation of c-myc structure and deletion mutants used for interaction studies. **B:** EGFP-tagged NDR1wt together with the indicated c-myc mutants were transfected into HEK cells and binding was analyzed by co-immunoprecipitation. Note, that although binding was reduced upon deletion of the N-terminus, still a fraction of NDR1 bound to c-myc-ΔN. **C:** Analysis of the interaction of c-myc-ΔMB mutants with NDR1wt. Experiments were performed as in B. **D:** HEK cells were transfected with c-myc and His-tagged ubiquitin (His-Ub) together with HA-NDR1wt where indicated. Ubiquitinated proteins where pulled-down from cell lysates using Ni-NTA sepharose and analyzed by SDS-page. Note the appearance of a mono-ubiquitinated form c-myc upon NDR1 co-expression. **E:** Impact of NDR1wt on Skp2 mediated ubiquitination of c-myc. Experiment was performed as in D, but where indicated HA-gfp-Skp2 was co-expressed.

protein stability by interfering with Skp2 mediated ubiquitination. NDR kinases were phosphorylated in G1-phase of the cell cycle, which would result in increased binding of NDR kinases to c-myc competing with Skp2. NDR phosphorylation would gradually decrease in S-phase, allowing increased degradation of c-myc by Skp2.

Interestingly, so far no other factors regulating Skp2 mediated degradation of c-myc have been described.

NDR kinases regulate p21 protein stability by phosphorylating serine 146

Our analysis of the consequences of NDR kinase depletion on proliferation not only revealed a downregulation of c-myc protein, but also showed an increase in p21 protein, without accompanying upregulation of p21 mRNA. We therefore analyzed, whether knock-down of NDR1/2 would impact on p21 protein stability (Figure 5A). As predicted, in cells depleted of NDR1/2 the stability of p21 was markedly increased (Figure 5A). No binding of NDR1/2 to p21 could be detected (data not shown), indicating a mechanism distinct from the regulation of c-myc. Earlier studies have shown that p21 stability is regulated at least partially by phosphorylation. Several phosphorlyation sites on p21 have been implicated in regulating p21 stability (*28*). We performed *in vitro* kinase-assays to test whether NDR was capable of phosphorylating p21. Indeed using recombinant and immuno-purified NDR2 phosphorylation of p21 was detected (Figure 4B/Figure S7A). Importantly, both NDR1 and NDR2 were able to phosphorylate p21 in these assays, while the respective kinase-dead variants failed to do so (Figure S7B). Although no substrates of NDR1/2 have been described in the literature, one report showed that NDR1 required a stretch of basic amino acids for phosphorylation. Analysis of the p21 primary sequence identified a stretch of 4 basic amino-acids upstream of the known phosphorylation sites T145 and S146 (*29*). To map the exact phospho-acceptor site we mutated T145 or S146 or both to alanine and performed *in vitro* kinase assays (Figure 5C/Figure S7C). Strikingly, mutation of S146 to alanine abolished phosphorylation by NDR in these assays, showing that

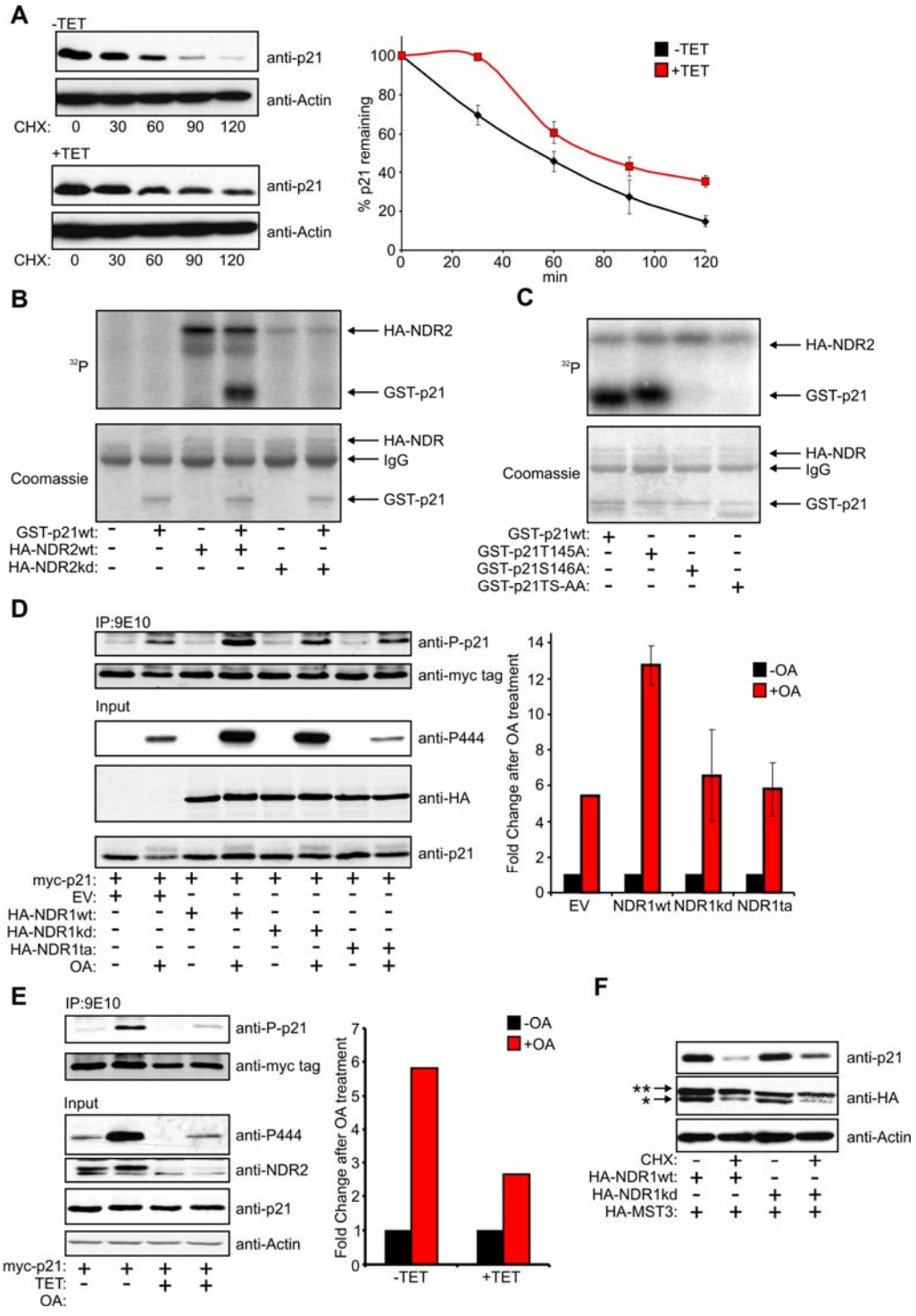

Figure 5. NDR kinases phosphorylate p21 on S146 *in vitro* and *in vivo*.
A: HeLa-shNDR1/2 cells were treated with tetracycline for 72h. Prior to harvesting, cells were treated for the indicated time with cycloheximide. p21 level were analyzed using the Licor Odyssey system (n=3). **B:** HA-tagged NDR2wt or HA-NDR2kd was immuno-precipitated from okadaic acid stimulated HEK cells and after pre-incubation with cold ATP used for *in vitro* kinase assays with GST-p21 as substrate. **C:** *In vitro* kinase assay to determine NDR phosphorlyation site on p21. GST-p21 with mutated T145, S146 or doubly mutated T145/S146 phospho-acceptor sites were used as substrates for *in vitro* kinase assays as described in B. **D:** HeLa cells were transfected with the indicated HA-tagged NDR1 species together with myc-tagged p21. Samples were stimulated with OA for 1h before lysis where indicated. myc-tagged p21 was immunoprecipitated from lysates and P-p21-S146 levels were analyzed and quantified using the Licor Odyssey system (n=3). P-p21-S146 levels were normalized to –OA controls. **E:** HeLa-shNDR1/2 cells were induced for 72h with tetracycline and transfected with cDNA encoding myc-tagged p21. 24h after transfection cells were stimulated with OA for 1h and lysed afterwards. P-p21-S146 levels were analyzed as in D. **F:** HeLa cells were transfected with cDNAs encoding HA-NDR1wt or HA-NDR1kd in the presence of HA-MST3. Prior to lysis, cells were treated with CHX for 60min, where indicated. **HA-NDR1; *HA-MST3

NDR kinases phosphorylate p21 on S146 (Figure S7C/D). NDR kinases are efficiently activated by okadaic acid treatment, therefore we tested whether over-expression of NDR1wt or NDR2wt would increase okadaic acid induced phosphorylation of p21 *in vivo* (Figure 5D/ Figure S7E). Indeed, overexpression of NDR1/2wt but not kinase dead or a HM mutant increased phosphorylation of p21 upon OA treatment, confirming p21 as an *in vivo* substrate for NDR kinases. Furthermore, reduction of NDR kinases in cells by shRNA reduced OA mediated phosphorylation of p21 on S146 (Figure 5E). It has been shown that phosphorylation of S146 by atypical PKCs could destabilize p21 (*30*). Therefore we tested the effect of overexpressing NDR1 together with MST3 on the stability of p21 (Figure 5F). Although overexpression of NDR1wt or kinase dead did not significantly impact on p21 steady-state level, treatment of cells with CHX to inhibit translation, revealed that overexpression of NDR1wt but not NDR1kd decreased p21 stability in this setting. Taken together, NDR kinases directly phosphorylated p21 on S146 *in vitro* and *in vivo*. Furthermore, phosphorylation of S146 by NDR1/2 resulted in destabilization of p21, whereas in the absence of NDR1/2 p21 protein levels were stabilized.

Increased p21 level upon knock-down of NDR1/2 mediate G1-block and senescence

Our analysis revealed a role for NDR kinases in the regulation of p21 and c-myc stability by different mechanisms. To confirm the effects of NDR on c-myc and p21 we performed experiments to rescue the effects of depletion of NDR2 by transient overexpression of NDR2 mutants refractory to shRNA (Figure 6A). Indeed, whereas overexpression of NDR2wt in this setting rescued both the effects on c-myc and p21 level, overexpression of NDR2kd only restored the c-myc level. Importantly, NDR2ta failed to rescue either of the effects. Next we analyzed whether overexpression of c-

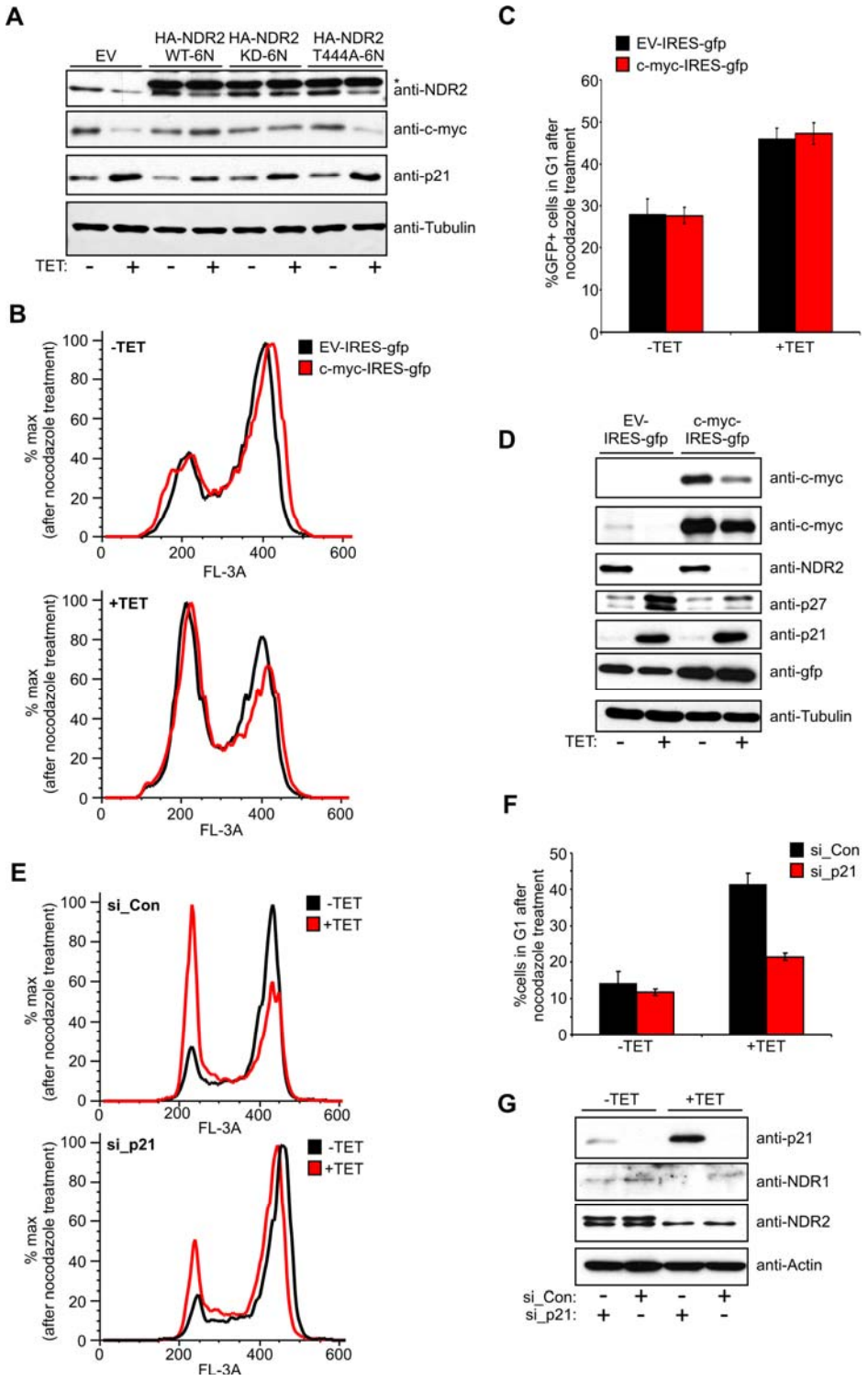

Figure 6. G1-arrest upon depletion of NDR1/2 is dependent on NDR kinase activity and mediated by increase in p21-level.
A: HeLa cells expressing shRNA against NDR2 were treated for 48h with tetracycline and subsequently transfected with the indicated NDR2 mutants refractory to shNDR2. Lysates were analyzed for the expression of NDR2, c-myc and p21. *HA-tagged NDR2. **B:** HeLa cells expressing shRNA against NDR1/2 were induced for 72h with tetracycline and transfected with vectors expressing c-myc together with an IRES-gfp as a transfection marker. G1-arrest analysis was performed as described. Cell cycle profiles of gfp-positive cells were overlaid to allow better comparison. **C:** Analysis of gfp-positive cells in G1 after nocodazole arrest upon depletion of NDR1/2 and overexpression of c-myc (n=3). **D:** Analysis of c-myc, p21 and p27 in NDR1/2 depleted cells upon overexpression of c-myc. Cells were treated as described in B, but lysates were prepared before G1-arrest analysis. **E:** HeLa cells expressing shRNA against NDR1/2 were treated with tetracycline for 48h before being transfected with siRNA against p21 on two consecutive days. The cells were treated for G1-arrest analysis as decribed 24h after the second transfection. **F:** Quantification of cells in G1 after nocodazole treatment (n=3). **G:** Cells obtained in E were analyzed for the expression of p21 and NDR1/2. Note that two rounds of siRNA treatment were sufficient to reduce increased p21 level.

myc would be sufficient to rescue the G1-arrest observed in NDR1/2 depleted cells (Figure 6B/C). However overexpression of c-myc failed to relieve the cells from G1-blockade. Interestingly, although overexpression of c-myc resulted in levels above endogenous, we observed that even overexpressed c-myc was destabilized in NDR1/2 depleted cells (Figure 6D). Restoring c-myc level did not affect p21 upregulation, but the increase in p27 levels. The upregulation of p27 mRNA upon knock-down of NDR1/2 therefore seemed to be mediated by reduced repression of the p27 promoter by decreased c-myc level in this setting (*22*). As restoring c-myc level failed to rescue cells from shNDR1/2 induced G1-arrest, we tested the effect of siRNA mediated depletion of p21 (Figure 6E-G). Decreased p21 level resulted in a significant relieve of NDR1/2 depleted cells from G1-blockade. To further confirm the role of p21 in mediating the G1-block upon depletion of NDR1/2, p21 levels and G1-arrest were analyzed in a timecourse dependent manner (Figure 7A). This analysis showed that p21 levels increased gradually starting from 48h after tetracycline induction, which correlated with increased amounts of cells in G1 after nocodazole treatment. Although p27 levels, a possible read-out for decreased c-myc level (*22*), were increased generally in NDR1/2 depleted cells, no accumulation of p27 was observed. Increased p21 levels have been shown to not only mediate G1-arrest of cells, but also to result in increased senescence (*31*). Indeed, NDR1/2 depleted cells show morphological changes of senescent cells, such as flattening, increase in cell-size and filopodia (Figure 7B) (*31*). Quantification of senescence-associated beta-Galactosidase stained cells confirmed an increase in senescent cells upon depletion of NDR1/2 (Figure 7C). Taken together, although knock-down of NDR1/2 resulted in a decrease of c-myc and an increase of p21 levels, only downregulation of p21 affected G1-arrest in NDR1/2 depleted cells. Furthermore increase in p21 levels correlated with increased G1-

blockade and increased senescence, confirming, that the upregulation of p21 upon knock-down of NDR1/2 seemed to be the major mechanism to regulate the G1-arrest.

3.2.4 Discussion

We show here that NDR1/2 are activated in a cell-cycle dependent manner, with the activation starting in G1 and persisting until late S-phase. Interfering with NDR activation results in proliferation defects and G1-arrest indicating an important function for NDR kinases in regulating G1-progression and S-phase entry. Interestingly, we identify MST3 as responsible upstream kinase for NDR activation in G1. Although being the first upstream kinase described for NDR1/2, the functional context of NDR activation by MST3 remained elusive (*19*). In the context of apoptosis signaling and centrosome duplication NDR activity has been shown to be regulated by MST1 (*13, 14*), whereas MST2 has been implicated in NDR kinase regulation in the alignment of mitotic chromosomes (*11*). MST3 signaling has been implicated in the regulation of axon outgrowth, cellular migration and stress induced-apoptosis (*32-34*). However, an involvement of MST3 in cell-cycle progression has not been described so far. Although we observe an increase of active MST3 in G1 as compared to M-phase, the mechanisms activating MST3 in this context remain to be analyzed.

Depletion of NDR kinases results in proliferation defects and G1-arrest (Figure 2). The activity of Cyclin-Cdk complexes important for G1-progression and S-phase entry is regulated the action of Cdk inhibitors (CKIs), such as p21 and p27 (*1*). Although depletion of NDR1/2 does not affect Cyclin levels (apart from a slight increase in Cyclin D1), the levels of the Cyclin-Cdk inhibitors p21 and p27 are significantly increased. The proto-oncogene c-myc has been shown to regulate both

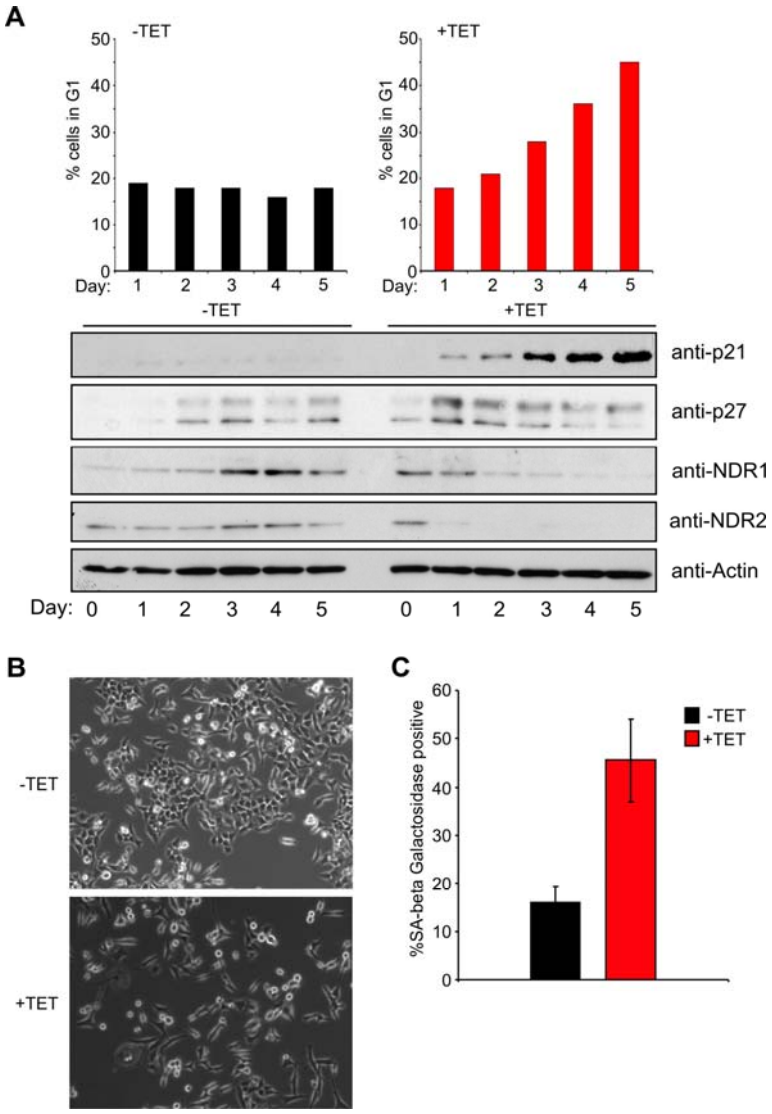

Figure 7. Increased p21 level upon knock-down of NDR1/2 correlate with G1-arrest and mediate increased senescence.
A: HeLa cells expressing shRNA against NDR1/2 were induced with tetracycline for the indicated time before harvest and lysis. Cells used for cell-cycle analysis were treated with 2.5µg/ml nocodazole for 14h before harvest. **B:** HeLa cells expressing shRNA against NDR1/2 were treated for 4 days with tetracycline and photomicrographs were taken. **C:** Cells treated as in B were stained for senescence-associated-beta galactosidase activity and counted. A total of 250 cells were counted for each condition and experiment (n=3).

p21 and p27 level by repression of their respective promoters (*21, 22*). Depletion of NDR1/2 also results in decreased c-myc level. Indeed, the upregulation of p27 protein level is also reflected in increased p27 mRNA. However p21 mRNA levels are not elevated, indicating a regulation of p21 level independent of c-myc. Furthermore, overexpression of c-myc in NDR1/2 depleted cells affects p27 level, whereas p21 levels remain unaffected. Importantly, the G1-block in NDR1/2 depleted cells is not affected by overexpression of c-myc, but by knock-down of p21. Furthermore, our experiments reveal, that the increase in p21 level is due to increased p21 protein

stability. The G1-arrest in NDR kinase depleted cells is therefore a consequence of increased p21 protein levels.

Degradation of p21 is mediated by both ubiquitin-dependent and independent mechanisms (*35*). In addition, phosphorylation of p21 has been shown to modulate a variety of p21 functions by impacting on p21 localization, complex formation and degradation (*28*). We show that NDR1 and NDR2 phosphorylate p21 on S146 *in vitro* and *in vivo* identifying p21 as first *in vivo* substrate for human NDR1/2. Phosphorylation of p21 on serine 146 has been reported to both increase and decrease p21 protein stability, depending on the cellular context and whether endogenous or over-expressed p21 was analyzed (*30, 36-38*). In our setting we can confirm the destabilizing effect of phosphorylated serine 146 on p21 protein turnover. Overactivation of NDR1/2 results in increased p21 degradation, whereas knock-down of NDR1/2 increases p21 protein stability, resulting in G1-arrest and increased senescence.

Although we can show that decreased NDR kinase level not only impact on p21 level, but also on c-myc, the effects of decreased c-myc level in our setting remain to be defined. NDR1 has been shown previously to bind to and increase c-myc stability. However, no studies have been performed to further analyze the mechanisms by which NDR impacts on c-myc stability (*23*). c-myc protein levels are strictly regulated in cells. Apart from a pronounced regulation on the level of transcription, protein levels of c-myc are controlled by the ubiquitin-ligases FBW-7 and Skp2 (*39*). Whereas the mechanisms regulating c-myc degradation by FBW7 proteins have been well described (*26, 27, 40*), factors regulating c-myc degradation by Skp2 remained elusive. Reports show, that Skp2 binds to c-myc on two sites in both the N-terminus and the C-terminus, thereby mediating ubiquitination and degradation (*24, 25*). Our

analysis suggests that NDR binding sites on c-myc overlap with those described for Skp2. NDR kinases possibly compete with Skp2 for c-myc binding, thereby reducing Skp2 mediated ubiquitination and degradation. Indeed, NDR kinases can reduce ubiquitination of c-myc by Skp2. Therefore our data suggest a model for the regulation of c-myc protein stability by NDR1/2. NDR kinases bind to c-myc dependent on its HM-phosphorylation. Increased phosphorylation of NDR on its HM by MST3 results in increased binding to c-myc competing with Skp2. Reduced binding of Skp2 results in increased c-myc stability. However, although Skp2 mediates c-myc ubiquitination, it has also been shown, that mono-ubiquitination of c-myc by Skp2 is important for efficient transcription of genes such as Cyclin D2 and ornithine decarboxylase important for S-phase entry (*24, 25*). Interestingly, although overexpression of NDR kinases reduces c-myc poly-ubiquitination and degradation, it seems as if the mono-ubiquitinated form of c-myc is stabilized. It remains to be addressed, whether the transcription of Cyclin D2 and ornithine decarboxylase is affected in this setting. Furthermore the functional role of NDR induced c-myc stabilization remains to be determined, as a role for c-myc in the G1-arrest induced by decreased NDR1/2 could be ruled out. It will be interesting to investigate, whether overexpression of NDR1/2 in combination with active MST3 could increase cellular transformation. Although the other members of the MST family (MST1/MST2) implicated in the regulation of NDR1/2 have been shown to functions as tumor suppressors as part of the HIPPO pathway (*8*), a role for MST3 in tumor biology has not been reported so far. Recently a role for NDR kinases in tumor suppression was described (Cornils, et al.; submitted). However, the finding that increased NDR kinase level could result in increased levels of the proto-oncogene c-myc seems contradictory. On the other hand, several established tumor suppressors have been

described to also function as oncogenes or facilitating tumor development in a context dependent manner (*41*). Interestingly, the other downstream factor identified in this study, p21, has been reported to function as a context dependent tumor-suppressor (*41*). Although loss of p21 predisposes mice to the development of tumors together with activating oncogenes such as RAS (*42*), loss of p21 also decreases cancer incidence in c-myc transgenic mice (*43*). This dualism has also been described on the cellular level. On the one hand, p21 regulates cell-cycle arrest for example after p53 activation (*44*). On the other hand, it functions in an anti-apoptotic manner by binding to and inhibiting caspase3 (*45, 46*). Depending on the context p21 can act as a tumor suppressor by inhibiting growth or as oncogene by inhibiting apoptosis (*41*). It seems likely that also NDR kinases could function as context dependent tumor suppressors. As mentioned above, p21 represents the first substrate identified so far for mammalian NDR kinases. Interestingly, increased levels of p21 have also been described to inhibit apoptosis as well as centrosome duplication (*45-47*). It is tempting to speculate that the increased level of p21 upon knock-down of NDR1/2 could also be responsible for the defects in apoptosis and centrosome duplication described earlier. However this possibility remains to be addressed and together with the potential oncogenic function of the MST3-NDR1/2-c-myc connection provides an interesting field for future research.

Taken together, in this study we identify a novel function for NDR1 and NDR2 in the regulation of G1-progression and S-phase entry. We provide evidence for the first functional context of NDR kinase activation by MST3. In addition the first mechanisms of signaling downstream of NDR kinases are defined: NDR kinases impact on both c-myc and p21 protein stability by different mechanisms. On the one hand NDR interacts with and stabilizes c-myc in a kinase-activity independent, but

HM-phosphorylation dependent manner. On the other hand p21 is defined as first *in vivo* substrate for NDR kinases. NDR kinases phosphorylate p21 resulting in decreased p21 protein stability. Decrease in NDR kinases results in accumulation of p21 and arrest of cells in G1, with subsequent accumulation of cells in senescence. Thus NDR kinases seem to play a pivotal role in regulating cellular proliferation by impacting on central players in G1-phase progression.

3.2.5 Material and Methods

Cell Culture, transfections and treatments

HeLa, HeLa S3, HEK293, U2OS and HCT116 cells were maintained in DMEM supplemented with 10% fetal calf serum (FCS). Cells were transfected using Fugene 6 (Roche), Lipofectamine2000 (Invitrogen) or jetPEI (Polyplus Transfection) as described by the manufacturer. For siRNA mediated knock-down of MST3 or p21 cells were transfected with pre-designed siRNA (Qiagen) using Lipofectamine2000. Validated control siRNAs were from Qiagen and used according to the manufacturer's instructions. For rescue experiments targeting elevated p21 level, cells were transfected twice in 24h intervals with siRNA against p21 or control siRNA. HeLa cells expressing tetracycline inducible shRNA against NDR1 and NDR2 and U2OS cells stably expressing shRNA against NDR1 together with an NDR1wt rescue construct have been described elsewhere (*12, 13*). HeLa and U2OS cells stably expressing shRNA against NDR1 or NDR2 alone were generated as described in (*12, 13*). To access protein stability, cells were treated with 50µg/ml cycloheximide (CHX) or 10µM MG132 for the indicated time. Staining for cellular senescence was performed as described in (*15*).

Reagents and Antibodies

The generation of antibodies against T-444-P, NDR1, NDR2 and NDR1/2 have previously been described (*13*). Antibodies against Cyclin A, Cyclin E, Cyclin B1, cdc2, p27, gfp, c-myc (N262), HA (Y11) and actin were from Santa Cruz. Antibodies to detect p21, Cyclin D1, Cdk4 and myc-tagged proteins (71D10) were from Cell Signalling. Antibodies against HA-tag (12CA5, 42F13), Tubulin (Y1/2) and c-myc (9E10) were used as hybridoma supernatants. Additional antibodies used included: anti-P-p21-S146 (Abgent), anti-P-MST4-T178/-MST3-T190/-STK25-T174 (referred to as P-MST3; Epitomics), anti-MST3 (BD Bioscience) and anti-FLAG (M2) (Sigma). Nocodazole, Thymidine, PI and Cycloheximide were from Sigma. SB203580 and SB202190 were from Alexis. MG132 was from Calbiotech and the BrdU and the anti-BrdU antibody was from BD Bioscience.

Construction of plasmids

The construction of plasmids encoding cDNAs for tagged variants of NDR1, NDR2, MST1, MST2 and MST3 has been described elsewhere (*13, 14, 16*). RNAi-rescue constructs for NDR2 were obtained by introducing silent mutations into the shRNA target sites using PCR-mutagenesis. For constructs expressing cDNAs fused to an IRES-gfp, the IRES-gfp cassette was excised from the pMIG-vector (a kind gift from W. Hahn, Dana-Farber Cancer Institute, Boston) using XhoI/SalI digestion and inserted into pcDNA3 containing the indicated cDNAs using XhoI. Constructs for pGEX2T-GSTp21, pcDNA3-p21 and pcDNA-myc-p21 were obtained by PCR cloning attaching BamHI/XhoI sites to p21-cDNA (a kind gift from N. Lamb, Institut de Génétique Humaine, Montpellier) and insertion into the BamHI/XhoI sites of the respective vector. Mutation of T145, S146 and T145/S146 to alanine was done by

PCR-mutagenesis. cDNA encoding c-myc was a kind gift from N. Hynes (Friedrich-Miescher Institute, Basel) and HA-tagged c-myc was obtained similar to myc-p21 by PCR-cloning into a pcDNA3-HA vector. HA-tagged variants of c-myc containing only the first 215 amino-acids (c-myc-ΔC) or the last 234 amino-acids (c-myc- ΔN) were obtained by PCR cloning. Deletion of the MB1 or MB2 domain was performed by mutagenesis-PCR. Primer sequences are available upon request. Vectors encoding cDNA for Skp2 or ubiquitin (Ub) were kind gifts from W. Krek (Institute of Cell Biology, ETH Zürich, Zürich) and W. Filipowicz (Friedrich-Miescher Institute, Basel).

Protein extraction, immunoprecipitation, immunoblotting and ubiquitination analysis

Proteins extraction from cultured cells, immunoprecipitation and immunoblotting were done as described previously (*16*). The following antibodies were used for immunoprecipitation: anti-HA (12CA5), anti-c-myc (9E10, N262) and a mixture of NDR1 and NDR2 specific antibodies to access endogenous NDR species. For quantification using the Licor Odyssey System, western blots were incubated with secondary antibodies conjugated with fluorescent dyes. Quantifications were carried out using the Licor Odyssey software. Analysis of c-myc ubiquitination was performed as described in (Cold Spring Harb. protocols; 2006, doi:10.1101/pdb.prot4616)

Cell cycle analysis

HeLa and HeLa S3 cells were synchronized using either a double thymidine block with subsequent nocodazole arrest and mitotic shake-off (*17*) or a single treatment with 100ng/ml nocodazole for 14h. Cells were washed free from nocodazole with ice-

cold PBS and released into fresh medium for the indicated time before harvesting. Cell cycle distribution was accessed using either BrdU labeling, as described by the manufacturer or PI staining as described (*12*). To detect cells blocked in G1 a method described in (*18*) was used. In short, cells were seeded at defined densities into 10cm dishes. 24h later 2.5ug/ml nocodazole was added for 14-16h to terminally arrest cells at G2/M border. Cells were harvested by trypsination and processed for FACS analysis.

Kinase Assays

Methods to determine the activity of endogenous NDR kinases have been described earlier (*13*). To assay p21 phosphorylation by NDR1/2 in vitro, HEK293 cells were transfected with cDNAs encoding for HA-tagged NDR kinase isoforms and mutants. Cells were stimulated with 1μM okadaic acid for 60h prior to lysis and immunoprecipitation. In vitro kinases assay using purified GST-tagged p21 isoforms were performed as described (*16*) with minor modifications. Before adding ^{35}P-labelled ATP and GST-p21, the immuno-precipitated kinases were pre-incubated for 90min at 30°C in reaction buffer without ^{35}P-labelled ATP. The labeling reaction was stopped after 60' by boiling the samples in sample buffer for 5' at 95°C. Samples were resolved on SDS-PAGE, stained with Coomassie and exposed to a phosphor-imager (Amersham Biosciences).

Proliferation Assays

For the analysis of cell proliferation, cells were seeded at defined densities in triplicates, for experiments including inducible shRNAs fresh tetracycline was added

each day starting with cell seeding. After the indicated time, cells were harvested by trypsination and counted using a ViCell-automated cell counter (Beckman-Coulter).

RNA isolation and quantitative Real Time PCR

Total RNA from cells was isolated with TRIzol reagent (Invitrogen) and further purified using RNeasy kit (Qiagen). cDNA from samples was generated from 2µg of total RNA using M-MuLV reverse transcriptase (NEB) and Oligo-dT primers. Quantitative RT-PCR to detect p21, p27 and c-myc (primer sequences upon request) was carried out using SYBR green technology in an ABI Prism 7000 detection system (Applied Biosystems).

3.2.6 References

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3.2.7 Supplemental material

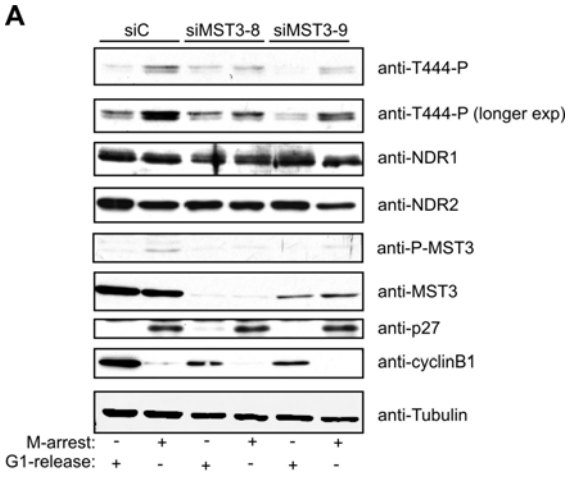

Figure S1. Validation of siMST3 effect on NDR activation in G1-phase.
A: HeLa cells were transfected with control siRNA (siC) or siRNAs against MST3 (siMST3-8/9 and 24h later arrested with nocodazole for 14h. Arrested cells were harvested or released into G1 for 8h before harvesting and analysis.

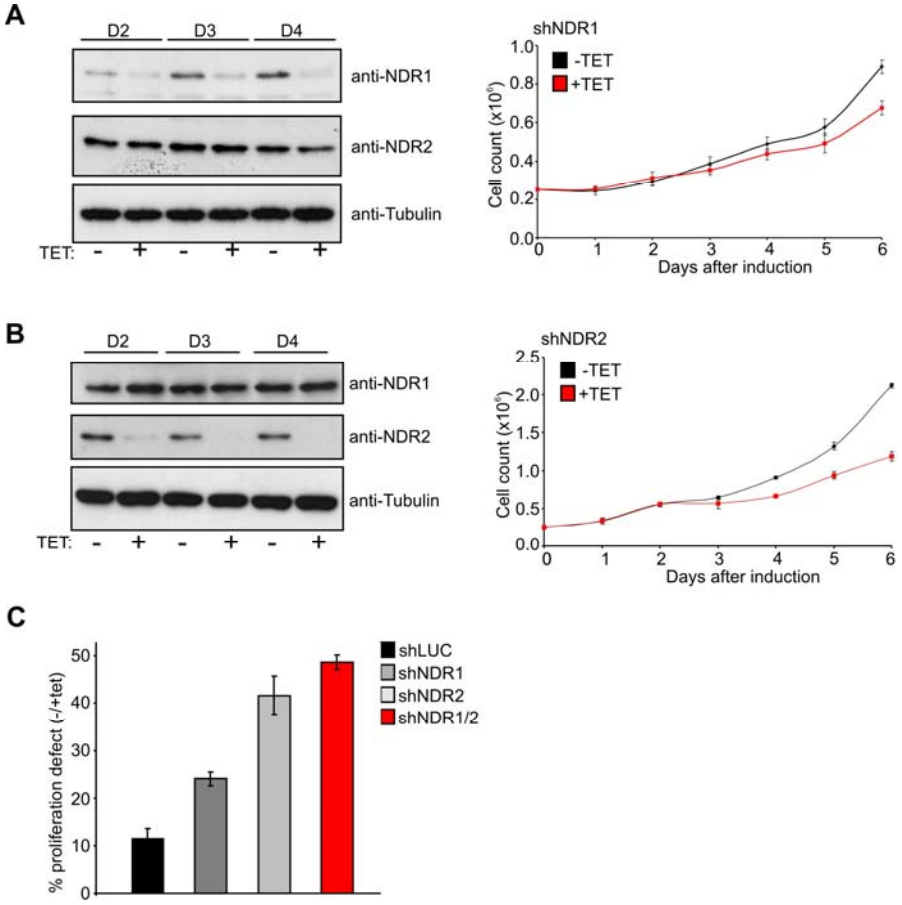

Figure S2. Single NDR1 or NDR2 knock-down cells also show proliferation defects.
A: HeLa cells expressing shRNA against NDR1 alone were seeded and treated with tetracycline for the indicated time. Cells were either processed for immuno-blotting (left panel) or counted using a Vicell-automated cell counter. **B:** HeLa cells expressing shRNA against NDR2 alone were treated and analyzed as in A. **C:** Validation of proliferation defects in different clones stably expressing shNDR1, shNDR2 or shNDR1/2 or shLUC (n=3). Experiments were performed as in A differences in proliferation were calculated as percentage of cells without tetracycline to cells with tetracycline counted on day6 after induction of shRNA expression.

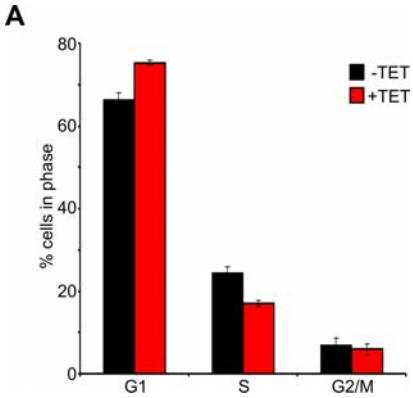

Figure S3. Depletion of NDR1 and NDR2 results in an increase in G1-cells accompanied by a decrease in S-phase cells.
A: HeLa cells expressing shRNA against NDR1/2 were induced for 4 days with tetracycline. BrdU was added directly to the cell medium for 30min before harvesting and processing for FACS analysis (n=3).

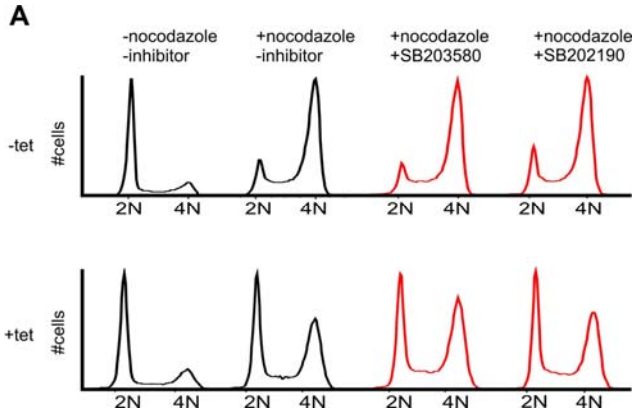

Figure S4. Treatment of NDR1/2 depleted cells with inhibitors against p38 does not suppress G1-arrest.
A: HeLa cells expressing shRNA against NDR1/2 were induced for 4 days with tetracycline. 24h before analysis SB203580 or SB202190 (10μM final) were added to the cells. G1-arrest analysis was performed as described.

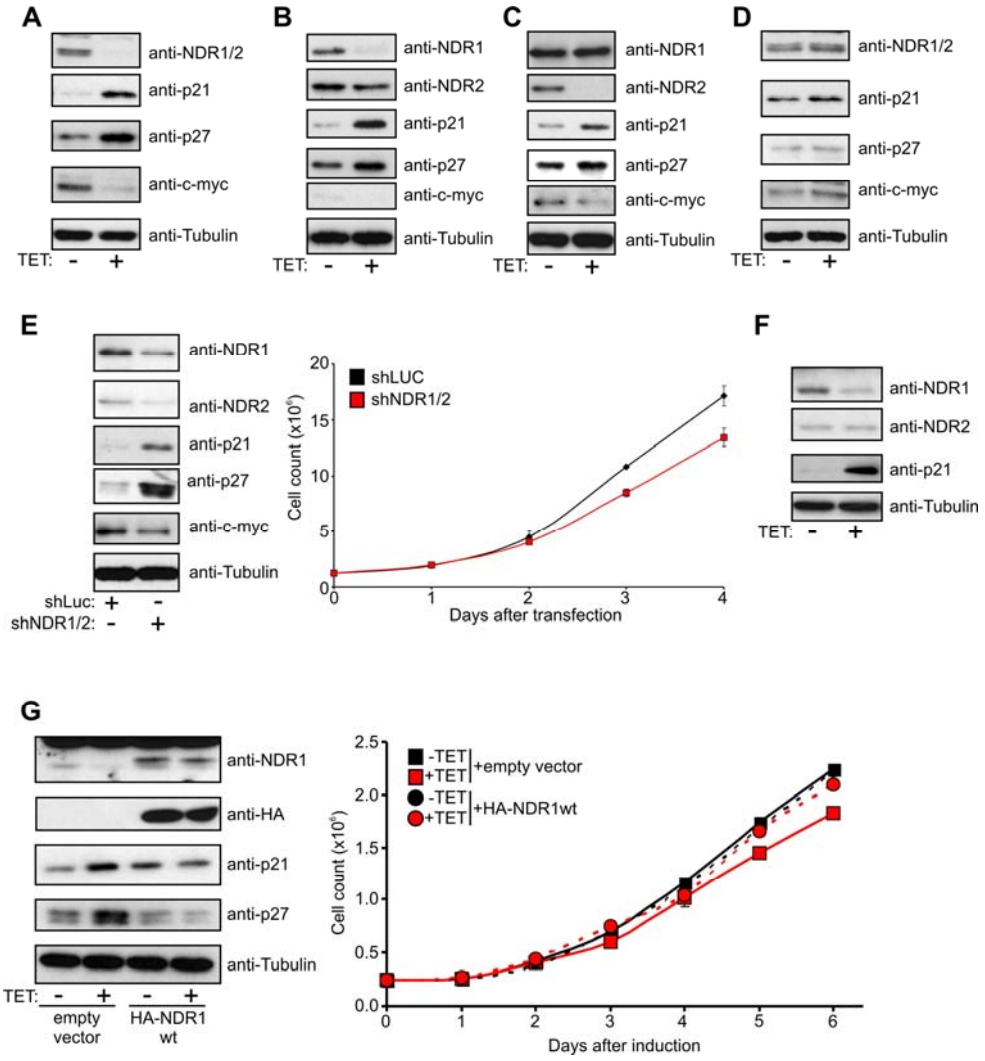

Figure S5. Validation of the effects of depletion of NDR kinases on p21, p27 and c-myc level.
A: Analysis of p21, p27 and c-myc level in a different HeLa clone expressing shRNA against NDR1/2. shRNA expression was induced for 72h before harvest and analysis. **B:** Analysis of p21, p27 and c-myc upon knock-down of NDR1 alone. Experiment was performed as in A. **C:** Analysis of p21, p27 and c-myc upon knock-down of NDR2 alone. Experiment was performed as in A. **D:** Analysis of p21, p27 and c-myc in a control clone expressing shLUC. Experiment was performed as in A. **E:** Validation of findings in HCT116 cells. Cells were transiently transfected with vectors expressing shLUC or shNDR1/2. Protein lysates were obtained 3 days after transfection. Cells were seeded in triplicates and counted after the indicated time using a Vicell-automated cell counter. **F:** Analysis of p21 level in U2OS cells expressing shNDR1. Cells were treated as described in A. **G:** Effects of NDR1 depletion on p21 and p27 level and on proliferation were rescued by re-expression of an NDR1wt rescue contruct. U2OS cells expressing shNDR1 and rescue constructs were described and validated in (12). Protein lysates were obtained 72h after tetracycline induction and cell counts were obtained as described in E.

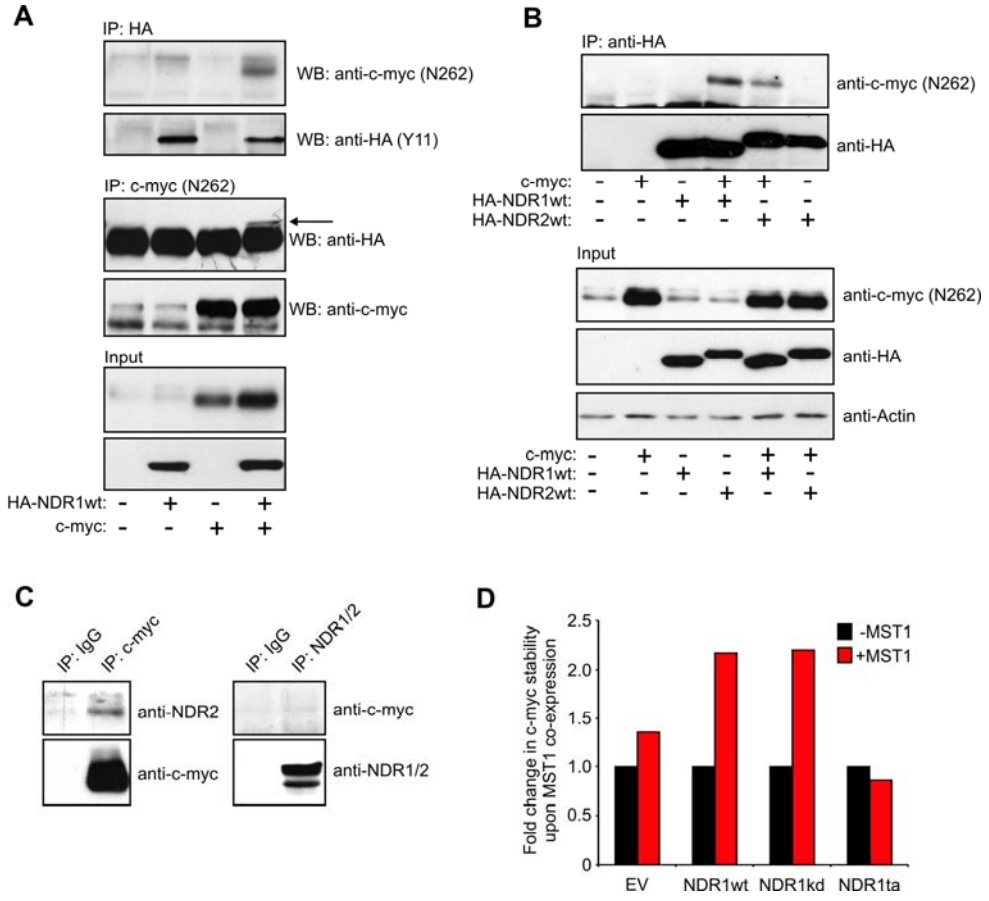

Figure S6. NDR1/2 interact with c-myc both on over-expressed and endogenous protein.
A: HEK cells were transfected with cDNAs for c-myc and HA-NDR1wt. NDR1 was immunoprecipitated using anti-HA antibodies and c-myc was immunoprecipitated using anti-c-myc (N262) antibody. Precipitates were analyzed by SDS-page. **B:** HEK cells were transfected with HA-NDR1, HA-NDR2 and c-myc and complexes were analyzed as in A. Note that the loading for the immunoprecipitations differed from the input. **C:** Endogenous NDR kinases or c-myc were precipitated from HeLa cell lysates and complex formation was analyzed using SDS-page. **D:** Effect of MST1 expression on NDR1 mediated c-myc stability. HEK cells were transfected and treated as described in Figure 3D. Fold change in c-myc level 120' after CHX addition upon MST1 co-expression were analyzed.

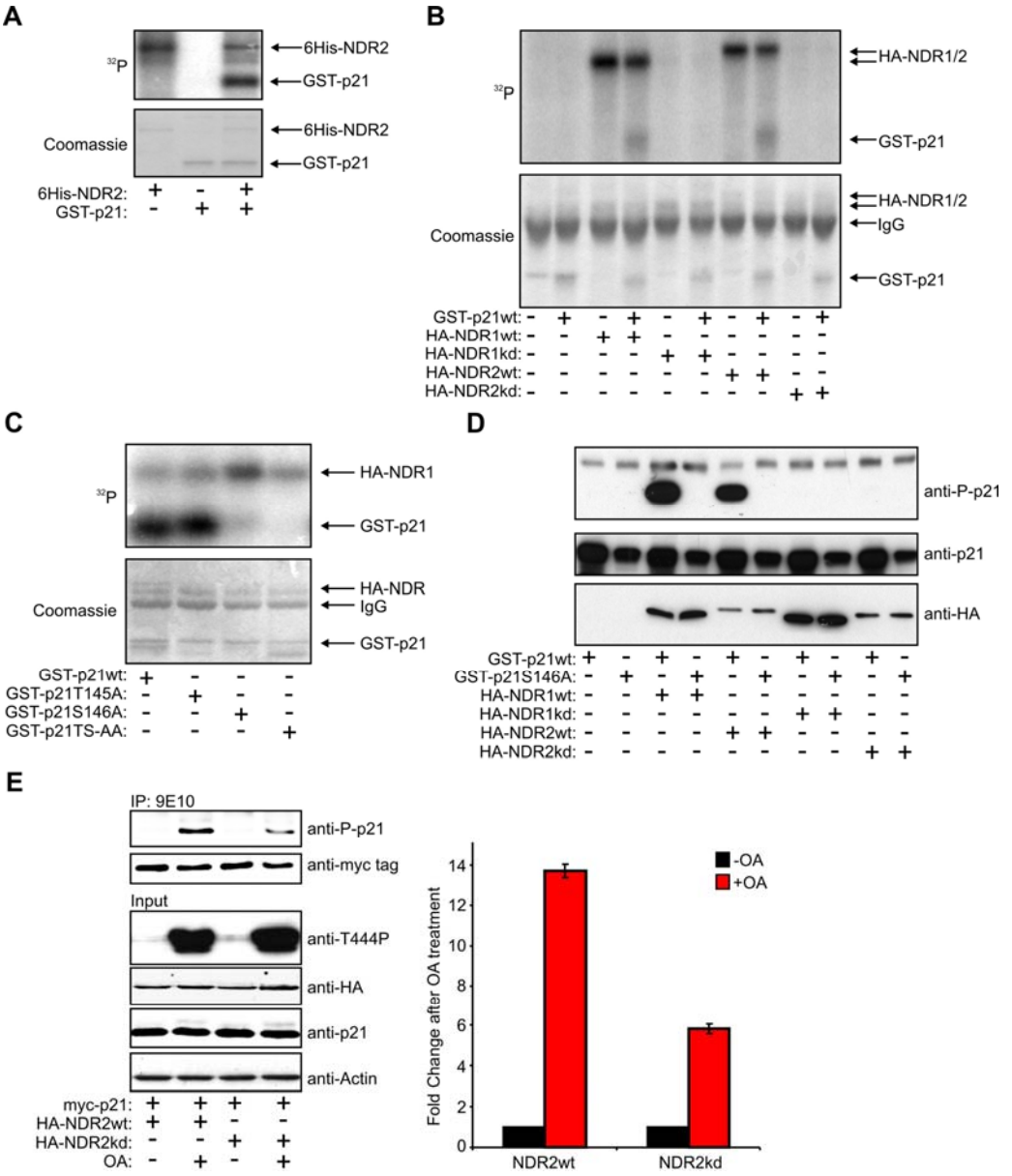

Figure S7. Validation of p21 as substrate for both NDR1 and NDR2 in vitro and in vivo.
A: His-tagged NDR2 was purified from okadaic acid treated Sf9 cells using Ni-NTA sepharose and used for in vitro kinase assay using GST-p21 as a substrate. **B:** HA-tagged NDR1/2wt or HA-NDR1/2kd was immuno-precipitated from okadaic acid stimulated HEK cells and after pre-incubation with cold ATP used for in vitro kinase assays with GST-p21 as substrate. **C:** In vitro kinase assay to determine NDR phosphorlyation site on p21. GST-p21 with mutated T145, S146 or doubly mutated T145/S146 phospho-acceptor sites were used as substrates for in vitro kinase assays as described in B. Here NDR1 was immunoprecipitated from cells. **D:** Cold kinase assay confirming the results obtained from "hot" kinase-assays and characterizing P-p21-S146 antibody. HA-tagged NDR1/2wt or HA-NDR1/2kd was immuno-precipitated from okadaic acid stimulated HEK cells and used for in vitro kinase assays with GST-p21wt or GST-p21S146A as substrate. **E:** HeLa cells were transfected with the HA-tagged NDR2wt or NDR2kd together with myc-tagged p21. Samples were stimulated with OA for 1h before lysis where indicated. myc-tagged p21 was immunoprecipitated from lysates and P-p21-S146 levels were analyzed and quantified using the Licor Odyssey system (n=3). P-p21-S146 levels were normalized to –OA controls.

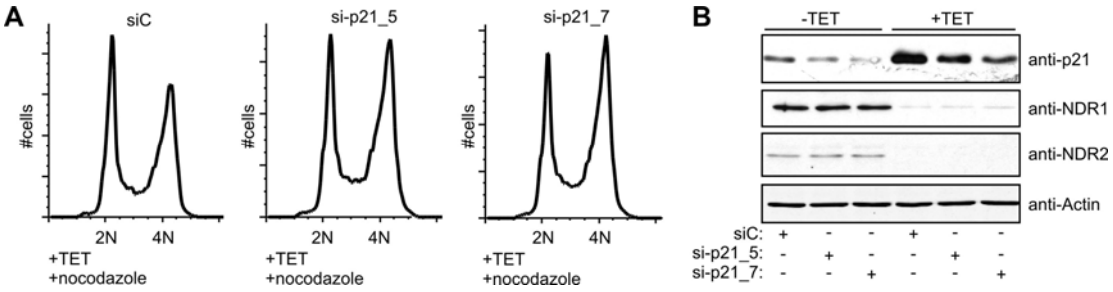

Figure S8. Characterization of siRNA against p21 in NDR1/2 depleted cells after one round of siRNA transfection.
A: HeLa cells expressing shRNA against NDR1/2 were induced for 48h and transfected once with siRNAs against p21. 48h later G1-arrest and p21 level were analyzed. Note that although siRNAs reduce p21 level in NDR1/2 depleted cells, one round of siRNA transfection was not sufficient to reduce level to untreated cells. In addition, amount of G1-arrest seemed to depend on p21 level. si-p21_7 reduced p21 level the strongest and shows the highest relieve from G1 arrest.

4. General discussion

The aim of this thesis was to characterize the consequences of NDR kinase deficiency in a mouse model and in cell culture systems. Addressing this question we were able to define a first in vivo function for NDR kinases in tumor suppression. Furthermore, using cell culture systems we characterized a novel function for NDR kinases in the regulation of G1-progression and S-phase entry. In addition, the first signaling mechanisms downstream of NDR1/2 could be defined.

Concerning the role of NDR kinases in tumor suppression, we could show that loss of one or two alleles of NDR1 predisposes mice to the development of T-cell lymphoma, both upon age and upon carcinogen treatment (Figure 7). In addition, our analysis revealed that the role of NDR kinases in preventing tumor development seems to be mediated by the regulation of proper apoptotic responses to various pro-apoptotic signals. Indeed, we could identify a pattern of genes correlating to low NDR kinase levels in tumors. Further analysis revealed a significant enrichment for targets of the E2A gene products E12/E47 in our data-set. Mechanistic analysis provided further insight: Downregulation of NDR1/2 in cells resulted in decreased levels of E47. Furthermore, overexpression of E47 could partially suppress the defects in apoptosis observed in NDR1/2 depleted cells (Figure 7).

A role of NDR kinases in tumor suppression was suggested before. First depletion of NDR1/2 in transformed cells resulted in defects to FAS mediated apoptosis (*31*). In addition decreased NDR activity has been shown to result in defects in centrosome duplication (*92*) and the alignment of mitotic chromosomes (*32*), with both processes being linked to genomic instability and tumor development. Secondly, it has been shown that several upstream regulators of NDR kinases in the above-mentioned contexts have been implicated in tumor suppression as components of the HIPPO

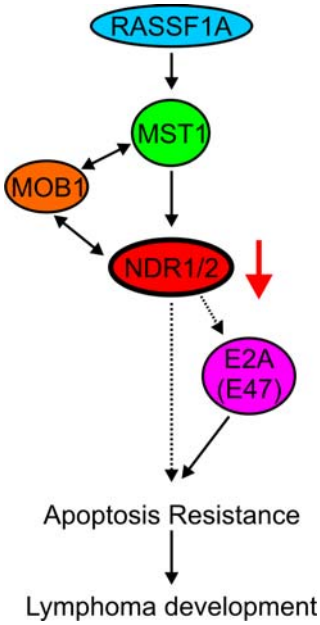

Figure 7. Model summarizing NDR kinase function as a tumor suppressor in T-cell lymphoma. Reduced levels of NDR kinases in cells of the T-cell lineage decrease the levels of the tumor suppressor protein E47, resulting in increased resistance to pro-apoptotic stimuli. (Dashed lines indicate unknown involvement or unknown biochemical mechanisms.)

tumor suppressor pathway (*61*). Specifically, the proteins RASSF1A, MST1/2 (as Hippo homologues) and hMOB1 have been shown to function as part of the HIPPO pathway. Our data therefore confirms the proposed role for NDR1/2 in tumor suppression. It was shown earlier, that a given Ste20 like kinase could regulate two different NDR kinase family members in a given context (*28*). In the process of dendritic tiling and maintenance of neuronal connectivity in drosophila, Hippo was shown to regulate both the NDR1/2 homologue tricornered and the LATS1/2 homologue LATS to control the outgrowth of neurites via trc and the maintenance of neurites via LATS (*28*). It is tempting to speculate that a similar mode of action exists in the context of apoptosis signaling. Whereas the HIPPO pathway controls the YAP oncogene and thereby the expression of anti-apoptotic genes such as cIAP via LATS, the HIPPO pathway could "split" on the level of the NDR kinases to also control YAP independent functions via NDR, possibly by regulating the levels of other transcription factors such as E47. Another possibility for NDR1/2 functioning as tumor suppressors was given by the analysis of MST1/2 deficiency in the liver (*88*).

Deletion of both MST1 and MST2 as Hippo homologues resulted in the development of liver cancers dependent on YAP. However, the role of LATS1/2 as connection between MST1/2 and YAP was questioned in this setting (*88*). Firstly, LATS phosphorylation was not changed in this setting and secondly, although LATS correlated with a YAP kinase activity in fractionated liver extracts, this activity was not changed upon deletion of MST1/2. Could the other two NDR family members in mammals NDR1 and NDR2 fill this empty spot? It has been shown, that NDR1/2 could phosphorylate YAP in vitro, but failed to phosphorylate YAP in cells upon overexpression (*75*). However, it has to be noted that overexpression of NDR1/2 in cells does not result in a significant increase in NDR kinase activity. Our data revealed a tumor suppressive function for NDR1/2 in vivo and it will be important for future studies to address the role of NDR1/2 in tumor suppression using mice doubly deficient for NDR1/2. For example it will be interesting to the test the effect of deleting NDR1/2 in liver and to analyze whether NDR1/2 would correspond to the MST1/2 responsive YAP-kinase activity defined earlier (*88*). Interestingly, analysis of animals doubly deficient for NDR1 and NDR2 revealed an embryonic lethality phenotype very similar to MST1/2 doubly deficient animals (Schmitz, et al.; unpublished). Taken together, our experiments revealed a tumor suppressive function for NDR kinases in T-cell lymphoma. It will now be important to further address this function in different organs and tumor-settings. It will also be important to investigate the relation of this finding to the HIPPO tumor suppressor pathway and also to further define downstream signaling mechanisms important for NDR kinases as tumor suppressors.

Somewhat contradictory to the above described tumor suppressive function we observed that loss of NDR1/2 impairs cell proliferation by imposing a G1-arrest

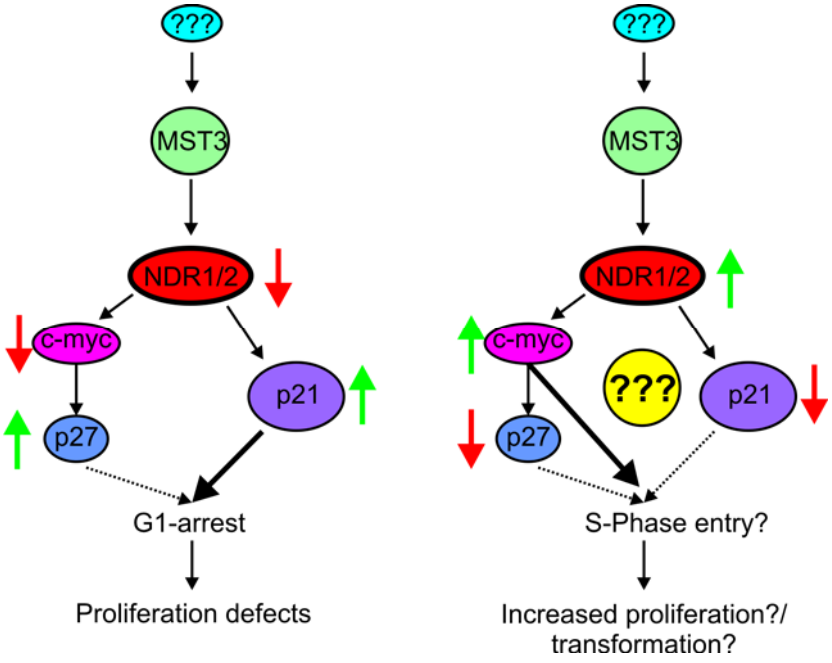

Figure 8. Model summarizing the role of NDR1/2 in cell cycle progression. Decrease in NDR1/2 levels results in G1-arrest due to upregulation of p21 levels (left panel). Over-expression/ -activation of NDR1/2 could potentially contribute to increased proliferation/ transformation by increased c-myc levels. (Dashed lines show unknown contribution to the indicated biological read-out)

mediated by increased p21 level (Figure 8). Furthermore, decreased NDR kinase levels result in reduced levels of the well known proto-oncogene c-myc, whereas overexpression of NDR1/2 results in increased c-myc protein levels. Could NDR kinases actually function as oncogenes under certain circumstances? Indeed this seems possible. Firstly, the impact of NDR1 on c-myc was also identified in B-cell lymphoma and B-cell leukemia samples, while analyzing possible novel factors regulating c-myc protein stability (*93*). In addition NDR2 has been found downstream of a pro-viral integration site in B-cell lymphoma (*94*). Another report identified NDR1 as a target gene of the tumor and metastasis promoting matrix-metalloproteinase MT1-MMP (*95*). Interestingly no increase in NDR1 or NDR2 mRNA levels in tumors deriving from the T-cell lineage have been described so far. Therefore our data and data from other labs indicate a possible oncogenic function for NDR kinases (Figure 9). How could a certain protein function both as tumor suppressor and as oncogene? Interestingly for several well known oncogenes and tumor suppressors this dual function has already been described (*96*). KLF4, p21, the

TGF-beta pathway, Runx, NOTCH1, E2F1, RAS, LKB1 and VHL have all been shown to either promote or oppose tumor development in different contexts. For example, the tumor suppressor protein VHL has been identified as mutated in the von Hippel Landau syndrome in humans (*97*). In mice deletion of VHL causes vascular tumors and VHL deficient cells proliferate in low serum conditions (*98, 99*), however, deletion of VHL also results in decreased proliferation of cells via the upregulation of p21 and p27 (*100*). The TGF-beta pathway represents another example. In humans the TGF-beta pathway is inactivated in pancreatic and colon tumors, but also overexpressed in solid tumors, where it is implicated in epithelial to mesenchymal transition (EMT) and metastasis (*101*). In mice it has been shown, that inactivation of the pathway results in tumor development, whereas inactivation also impairs tumor invasiveness and metastasis (*101*). Furthermore, the Cyclin-Cdk inhibitor p21 has also been placed in this list (*96, 102*). Mutations in the p21 gene are rare in human tumors (*103*); however, polymorphisms in p21 have been associated with oral and breast cancer (*104-106*). In human pancreatic tumors increased p21 expression occurs during progression and re-localization of p21 to the cytoplasm is linked with poor prognosis (*107-109*). p21 targeted mice show an increased rate of spontaneous tumor development and deficiency in p21 also increases the rate of tumor development in the background of Apc heterozygosity and overexpressed oncogenic RAS (*110-112*). On the other hand, loss of p21 decreases tumor development in myc-transgenic mice, Atm deficient mice and upon gamma irradiation treatment (*111, 113, 114*). Interestingly, p21 has been shown to partially mediate the tumor suppressive effects of the TGF-beta pathway and the context dependent tumor suppressor Klf4 (*96*). Cellular analysis revealed the mechanisms behind this dual activity of p21. Upon overexpression of p21, cells arrest in the cell cycle due to an inhibition of Cyclin-Cdk

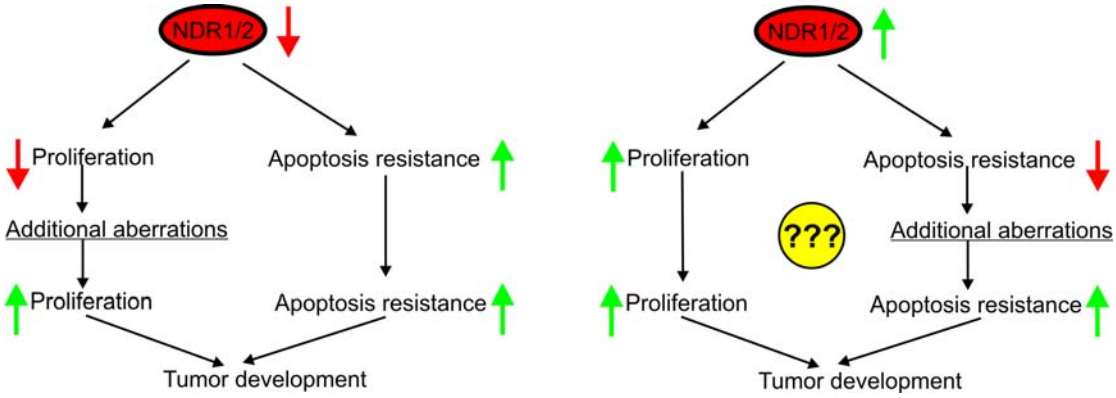

Figure 9. Models summarizing the potential dual role of NDR kinases in tumor development. Decrease in NDR kinases results in decreased proliferation and increased apoptosis resistance (left panel). During tumor development additional aberrations in signaling pathways overcome the defects in proliferation. Overexpression of NDR potentially results in increased proliferation, but decreased apoptotic thresholds. Acquiring additional changes in anti-apoptotic pathways could result in tumor development under these conditions.

complexes (*115*). However, increased levels of p21 have also been shown to promote cell proliferation by increasing the assembly of CyclinD-Cdk 4/6 complexes (*116*). In addition an anti-apoptotic function for p21 has been described (*117*, *118*). p21 has been shown to directly bind to caspase 3 and to interfere with caspase 3 cleavage and activation. However, so far the anti-apoptotic function of p21 has been limited to cytoplasmatic p21. Interestingly, in breast cancer cell lines overexpressing Her2, increased activity of PKB has been found to phosphorylate p21 on T145 resulting in cytoplasmatic localization, potentially increasing p21's anti-apoptotic activity (*119*). As we identified increased p21 level in NDR1/2 depleted cells as being responsible for cell cycle arrest, it is tempting to speculate that increased p21 also (at least partially) mediate the apoptotic resistance observed in NDR depleted cells and tumors. We already identified the E2A gene product E47 as being partially responsible for the apoptotic defects in NDR depleted cells and tumors. A functional connection between E47 and p21 in this setting remains to be addressed. Interestingly, our analysis also revealed that apart from E2A targets, targets of the G1-phase regulators Cdk4, Cyclin D1 and p21 were also significantly enriched in the genes correlating with low NDR levels in tumors. It will be important in future studies to

further dissect the interplay between NDR kinases, p21, c-myc and E47 in apoptosis signaling and proliferation. As the consequences of NDR kinase overexpression on apoptosis signaling, proliferation and transformation remain to be defined, future research is warrant to define a potential oncogenic function of NDR kinases (Figure 9). Similar to the c-myc oncogene, which induces cellular proliferation, but also lowers the apoptotic threshold, decreased NDR lowers proliferation, but increases apoptotic resistance (Figure 9). Both proteins need a certain context to exert its tumor promoting or suppressing functions. Whereas c-myc overexpressing tumors depend on changes in signaling pathways to lower the apoptotic threshold (*120*), NDR deficient tumor cells need to overcome the proliferation defects resulting from low NDR kinase level. Indeed our analysis revealed that tumors with low NDR kinase levels did not show a consistent decrease in proliferation, whereas apoptosis was significantly decreased. Conversely, elevated levels of NDR could increase proliferation by stabilizing c-myc, but could also result in increased apoptosis. Pro-apoptotic signaling in this context could therefore be the target of additional changes, while the proliferative response of elevated NDR level might prove advantageous for tumor development (Figure 9). Future research therefore could indeed define mammalian NDR kinases as true context dependent tumor suppressors and it will be of utter interest to further define the signaling pathways and mechanisms upstream and downstream of NDR under these conditions to gain insight into the functions of NDR kinases *in vivo*.

Taken together, by further defining the consequences of NDR kinase deficiency in mammals, the results obtained during this thesis shed light onto the mechanisms of NDR1/2 function in the context of cell cycle regulation, apoptosis and tumor suppression, indicating an important role for NDR1 and NDR2 in signal transduction.

5. References

(This section contains the references cited in the introduction and the general discussion. For the references important for the results, please see the corresponding manuscript)

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Publications

Cornils, H., Stegert, M.R., Hergovich, A., Hynx, D., Schmitz, D., Dirnhofer, S., Hemmings, B.A.; Ablation of mammlian NDR1 kinase predisposes mice to T-cell lymphoma development. Science Signaling (submitted)

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Fuller, S.J., Pikkarainen, S., Tham el, L., Cullingford, T.E., Molkentin, J.D., **Cornils, H.**, Hergovich, A., Hemmings, B.A., Clerk, A., Sugden, P.H.; Nuclear Dbf2-related protein kinases (NDRs) in isolated cardiac myocytes and the myocardium: activation by cellular stresses and by phosphoprotein serine-/threonine-phosphatase inhibitors. Cellular Signaling **20**, 1564-1577 (2008).

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Patents

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Other skills

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8. Appendix

This section contains three additional manuscripts and one review to which I contributed during the course of the thesis. The review covers NDR kinases in the context of centrosome duplication and tumor development published in BBA 2008. The manuscripts contain work on NDR kinases in the context of apoptosis signaling ("NDR kinase is activated by RASSF1A/MST1 in response to FAS receptor stimulation and promotes apoptosis" by Vichalkovsky, et al.; Current Biology 2008) and centrosome duplication ("The MST1 and hMOB1 tumor suppressors control human centrosome duplication by regulating NDR kinase phosphorylation" by Hergovich, et al.; Current Biology 2009). The third manuscript contains work on NDR1/2 activation by stress and PP2A inhibitors in isolated rat myocytes ("Nuclear Dbf2-related protein kinases (NDRs) in isolated cardiac myocytes and the myocardium: activation by cellular stresses and by phosphoprotein serine-/threonine-phosphatase inhibitors" by Fuller, et al.; Cellular Signaling 2008) where we contributed to work performed in the laboratory of Peter Sugden (Imperial College London; London). The first two manuscripts are intensively discussed in the main text and are also placed in relation to the work obtained during this thesis. Although the work described in the third manuscript describes a possible implication of NDR kinases in heart function, it did not significantly increase our knowledge about the biochemical mechanisms regulating NDR kinases. Therefore this work is not intensively discussed in this thesis.

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Review

Mammalian NDR protein kinases: From regulation to a role in centrosome duplication

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Abstract

The NDR (nuclear Dbf2-related) family of kinases is highly conserved from yeast to human, and has been classified as a subgroup of the AGC group of protein kinases based on the sequence of the catalytic domain. Like all other members of the AGC class of protein kinases, NDR kinases require the phosphorylation of conserved Ser/Thr residues for activation. Importantly, NDR family members have two unique stretches of primary sequence: an N-terminal regulatory (NTR) domain and an insert of several residues between subdomains VII and VIII of the kinase domain. The kinase domain insert functions as an auto-inhibitory sequence (AIS), while binding of the co-activator MOB (Mps-one binder) proteins to the NTR domain releases NDR kinases from inhibition of autophosphorylation. However, despite such advances in our understanding of the molecular activation mechanism(s) and physiological functions of NDR kinases in yeast and invertebrates, most biological NDR substrates still remain to be identified. Nevertheless, by showing that the centrosomal subpopulation of human NDR1/2 is required for proper centrosome duplication, the first biological role of human NDR1/2 kinases has been defined recently. How far NDR-driven centrosome overduplication could actually contribute to cellular transformation will also be discussed.

Keywords: NDR/LATS protein kinase; Activation mechanism; MOB binding; Subcellular localization; Centriole duplication; Tumour suppressor; Cellular transformation

1. Introduction

Protein kinases are crucial for the regulation of various cellular processes and have emerged as important therapeutic targets over the last decade, since many protein kinases are linked to human diseases. In human cells, 518 protein kinases have been identified so far that could catalyse transfer of phosphates (phosphorylation) to serine (Ser), threonine (Thr) and tyrosine residues, thereby defining protein kinases as one of the largest superfamilies found in the human genome [1]. In general, these enzymes have a catalytic domain of about 250 to 300 amino acids (in most cases combined with regulatory domains) which contains conserved residues that contribute to nucleotide binding, metal-binding, substrate binding, and phosphoryl transfer. These conserved residues are located within functional domains (subdomains) that can be used to describe structural details of protein kinases [2]. The phosphorylation of certain key residues – i.e. within the activation segment (subdomain VIII) – can also result in the modulation of kinase activity [3].

The human genome encodes about 70 protein kinases that are classified as members of the serine/threonine AGC (protein kinase A (PKA)/PKG/PKC-like) class of protein kinases. Importantly, AGC kinases – such as PKA, protein kinase B (PKB, also known as AKT), p70 ribosomal S6 kinase (S6K) or p90 ribosomal S6 kinase (RSK) – require phosphorylation of a conserved Ser/Thr residue within the activation segment for

Abbreviations: AGC, PKA/PKG/PKC-like; NDR, nuclear Dbf2-related; MOB, Mps-one binder; LATS, large tumour suppressor; Trc, tricornered; SAX-1, sensory axon guidance-1; PP2A, protein phosphatase type 2A; MST, Mammalian Ste20-like kinase; SWH, Salvador/Warts/Hippo; Hpo, Hippo; Sav, Salvador; Mer, Merlin; Ex, Expanded; Ft, Fat; Yki, Yorkie; RASSF1A, RAS-association domain family protein 1A; PLK4, Polo-like kinase 4; Cdk2, cyclin-dependent kinase 2; NTR, N-terminal regulatory; AIS, auto-inhibitory sequence; SPB, spindle pole bodies (SPB); MEN, mitotic exit network; SIN, septation initiation network

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activation, and have crucial functions in the regulation of cellular processes that are important for cell metabolism, proliferation and survival.

2. Members of the NDR protein kinase family

Members of the NDR (nuclear Dbf2-related) family of kinases are highly conserved from yeast to man [4]. The NDR family has been classified as a subgroup of the AGC group of protein kinases, based on the sequence of the catalytic domain [1]. Human cells express four related NDR kinases, NDR1 (also known as serine/threonine kinase 38 or STK38), NDR2 (or STK38L), LATS1 (large tumour suppressor-1) and LATS2. Members of the NDR family can also be found in *Drosophila melanogaster* [Trc (tricornered) and Warts/Lats], *Caenorhabditis elegans* [SAX-1 (sensory axon guidance-1) and LATS], *Saccharomyces cerevisiae* (Dbf2p, Dbf20p and Cbk1p), *Schizosaccharomyces pombe* (Sid2p and Orb6p), and some other fungi, protozoan and plants [4].

While genetic studies have unraveled pathways (discussed in Section 3.4) and functions (discussed briefly in Section 4) that center around NDR kinases in yeast and invertebrates, the precise mechanisms of NDR regulation have been established in mammalian systems (summarized in Section 3). To date, NDR kinases have been shown to regulate mitosis, cell growth, embryonic development, and neurological processes [4]. Moreover, NDR kinases have recently been shown to control centrosome duplication [5]. A brief description of key NDR family members is included below. Table 1 provides a brief summary of the available, functional information on selected NDR kinases.

3. Regulation of the NDR family

In contrast to studies of the physiological roles of NDR kinases (mainly undertaken in yeast and flies), the regulatory mechanisms of NDR kinases at the molecular level have been mostly studied in mammalian cells over the past decade [4]. Nevertheless, we summarize in this section our current understanding of these processes with respect to the entire NDR family, with particular focus on the role of phosphorylation by Ste20-like kinases (Section 3.2) and the regulation of NDR kinases through their association with MOB (Mps-One-Binder) proteins (Section 3.3). At the end of this section, these components are discussed in the context of signaling cascades (Section 3.4).

3.1. Regulation by phosphorylation

All NDR kinases contain two main regulatory phosphorylation sites: the activation segment (Ser281 of human NDR1; or Ser909 of human LATS1) and the hydrophobic motif phosphorylation sites (Thr444 of human NDR1; or Thr1079 in human LATS1). While in other AGC kinases the activation loop is targeted by an upstream kinase (i.e. PDK1 phosphorylates PKBα on Thr308), the activation of NDR kinases is regulated by autophosphorylation on the activation segment [6,7]. Importantly, unlike other AGC kinases, NDR kinases also have a unique insert of several residues between subdomains VII and VIII of the kinase domain [4]. All kinase domain inserts contain a region that is rich in basic residues, located near the C-terminal end of this insert, hence this positively charged cluster immediately precedes the activation segment. Interestingly, this stretch of primary sequence seems to negatively affect NDR kinase activity, since a significant increase in kinase activity of human NDR1/2 was observed when the positive residues in the kinase domain insert were mutated to alanines [8]. Therefore, the insert motif is also referred to as an auto-inhibitory sequence (AIS), a typical feature of NDR family members.

Similar to other AGC kinases, the hydrophobic motif of NDR kinases is targeted by an upstream kinase (discussed in Section 3.2). The best illustration of the importance of this phosphorylation event has been provided by a study of a distant relative of NDR kinases, namely PKB [9]. Phosphorylation of the

Table 1
NDR kinase family members

Kinase	Species	Functional information
Dbf2p	*S. cerevisiae*	Controls mitotic exit and cytokinesis
Cbk1p	*S. cerevisiae*	Centerpiece of RAM network; couples cell morphology with the cell cycle
Sid2p	*S. pombe*	Regulates septum formation and cytokinesis
Orb6p	*S. pombe*	Links morphological changes with the cell cycle
TBPK50	*T. brucei*	Coordinates cell shape and cell cycle
SAX-1	*C. elegans*	Important for neurite outgrowth and dendritic tiling
Trc	*D. melanogaster*	Controls epidermal outgrowths, and dendritic tiling and branching
Lats/Warts	*D. melanogaster*	Central player in the Hippo pathway; required for dendritic maintenance
LATS1	*H. sapiens/M. musculus*	Regulates G2/M cell cycle transition, apoptosis and mitotic progression; part of the G1 tetraploidy checkpoint; the LATS1 cDNA can rescue the loss of *D. melanogaster* Lats/Warts
LATS2	*H. sapiens/M. musculus*	Controls cell proliferation, genomic stability and mitotic progression; linked to the G1 tetraploidy checkpoint; essential gene, since LATS2 null mice die before embryonic day 12.5
NDR1/STK38	*H. sapiens/M. musculus*	Required for centriole duplication; the NDR1 cDNA can compensate for the loss of *D. melanogaster* Trc
NDR2/STK38L	*H. sapiens/M. musculus*	Functions in the regulation of neuronal growth and differentiation

hydrophobic motif of PKB results in structural reordering, or more specifically in a disorder-to-order transition. In contrast to PKB, a single change of the phospho-acceptor Thr to a phospho-mimicking residue is not sufficient to augment NDR kinase activity [10], however, the exchange of the entire hydrophobic motif of human NDR2 by the hydrophobic motif of PRK2 kinase (that mimics hydrophobic motif phosphorylation) leads to a constitutively active form of human NDR2 kinase [6]. Of note, mutating either the activation segment or the hydrophobic motif phospho-acceptor residue to alanine residues nearly completely abolishes the kinase activity of yeast, fly, mouse and human NDR kinases [4], thereby showing that the phosphorylation of both the activation segment and the hydrophobic motif is essential for NDR kinase activity. Furthermore, these activating phosphorylation events can be removed by protein phosphatase type 2A (PP2A) in mammalian, fly and yeast cells [4]. Overall, current evidence strongly indicates that NDR kinases are activated by multi-site phosphorylation and inactivated by dephosphorylation.

3.2. Ste20-like upstream regulators

Several genetic studies have already shown that Ste20-like kinases function upstream of NDR kinases (summarized in [4]). However, MST3 (mammalian serine/threonine Ste20-like kinase 3) was the first Ste20-like kinase shown to specifically phosphorylate the hydrophobic motif, but not the activation segment [11]. Another report provided further evidence that MST1/2 phosphorylate human LATS1/2 [12]. Moreover, several yeast Ste20-like kinases have been demonstrated to operate upstream of NDR kinases in budding and fission yeast. In flies, Hpo (one of two Ste20-like kinase in *D. melanogaster*) has been shown to function biochemically and genetically as a direct upstream regulator of Lats and Trc [13]. Importantly, the Jan laboratory could show that Hpo specifically phosphorylates the hydrophobic motif of Lats and Trc. Therefore, considering that human and fly NDR kinases are selectively phosphorylated on their hydrophobic motif phosphorylation site by Ste20-like kinases, it is very likely that the majority of NDR family members are targeted on this motif by their respective Ste20-like upstream regulators (i.e. phosphorylation of human LATS1 on Thr1079 by MST1/2 or phosphorylation of Dbf2p on Thr544 by Cdc15p). Studies are now needed that test whether this prediction is valid in other model systems in order to allow a full assessment of the conservation of the Ste20-like/NDR pathway. Current open questions include whether Sid2p is phosphorylated on Thr578 by Sid1p, or whether SAX-1 is regulated by phosphorylation on Thr441 by any of the two Ste20-like kinases in *C. elegans*. How far human LATS1/2 is specifically targeted on the hydrophobic motif phosphorylation site by MST1/2 also remains to be determined in cells.

3.3. MOB proteins as co-activators

Besides the unique insert between subdomains VII and VIII of the kinase domain, all NDR kinases have an N-terminal regulatory (NTR) domain that contains a number of conserved basic hydrophobic residues [4]. This positively charged cluster mediates the interaction between NDR kinases and a negatively charged area on the surface of the co-activator MOB (Mps-one binder). Genetic, structural, and biochemical studies strongly indicate that this interaction is essential for the regulation of NDR kinases. In *S. cerevisiae* and in *S. pombe*, Mob1p is crucial for the localization and kinase activity of Dbf2p and Sid2p, while Mob2p regulates Cbk1p and Orb6p, respectively (summarized in [4]). In *Drosophila*, expression of human hMOB1A can rescue the lethality associated with dMob1/Mats loss of function [14], thereby restoring the tumour suppressor activity of the Mats/Lats complex. However, different MOBs (dMob1–4) have been shown to interact with Trc and Lats in flies [15], whereby the Adler laboratory could demonstrate that residues conserved between yeast Mob1p and fly dMob2 were essential for the interaction of Trc with dMob2 [15]. Importantly, the same conserved residues in Mob1p are also important for the functionality of the Mob1p/Dbf2p complex in yeast [16]. Furthermore, substitutions of key residues by alanines in the NTR domain of human NDR1/2 and human LATS1 completely abolished their interaction with hMOB1A and severely impaired kinase activity [8,17,18]. Taking into further consideration the structures of human, yeast and frog MOB1 [16,19,20], together with our knowledge of key residues in human NDR1/2 and LATS1 that are required for hMOB1A association [8,18,21], it is extremely likely that the positively charged NTR domain of NDR kinases is responsible for the direct interaction with a negatively charged surface on MOBs.

Current data actually suggest several functions for the binding of MOBs to NDR kinases: (1) one study indicates that this binding releases human NDR1/2 kinases from inhibition mediated by the AIS within the kinase domain insert [8], (2) MOBs are thought to activate NDR kinases by the disruption of an inhibitory self-association in yeast [22], and (3) recent evidence further suggests that the targeting of MOBs to the plasma membrane is sufficient to fully activate human NDR1/2 and LATS1—most likely by allowing efficient autophosphorylation on the activation segment, and simultaneously recruiting NDR kinases into the proximity of its upstream kinase that phosphorylates the hydrophobic motif phosphorylation site [11,17,18]. However, more work is needed in order to understand these regulatory processes in more detail. Nevertheless, it is intriguing that all NDR kinases contain the AIS motif [4] and that hMOB1A contains also a cluster of positively charged residues [16] which would allow its association with negatively charged phospholipids in membranous structures. Still, the mechanism(s) regulating the membrane association of MOBs are yet to be elucidated.

3.4. Signaling cascades involving NDR kinases

Lats kinase is the centerpiece of a pathway that balances the relationship between cell size, cell proliferation and cell death in flies—the Hippo (Hpo) or SWH (Salvador/Warts/Hippo) signaling cascade (please see also Section 4). In *Drosophila*, an unknown extracellular stimulus transmits a signal through Ft (Fat), Mer (Merlin) and Ex (Expanded) to a complex containing

Hpo, Sav (Salvador), dMob1 and Lats, which in turn phosphorylates and inactivates the transcriptional co-activator Yki (Fig. 1). Normally, Yki stimulates the transcription of cyclin E (a driver of cell proliferation), DIAP1 (an inhibitor of apoptosis) and the bantam microRNA. Thus, loss of Hpo signaling components results in tissue overgrowth that is associated with increased cell proliferation and decreased cell death. Current evidence strongly indicates that Hpo phosphorylates and activates Lats in a Sav/Hpo/Lats complex by a Sav-mediated manner (summarized in more detail by [4,23,24]). Recent data further suggest that dMob1 associates with Hpo and is phosphorylated by Hpo [25]. Phosphorylated dMob1 then has a higher affinity for Lats, and this activated dMob1/Lats complex (both molecules are phosphorylated by Hpo) can target the downstream factor Yki more efficiently [25]. Interestingly, further evidence suggests that the Hpo pathway can be activated by radiation in a p53-dependent manner and that Hpo is required for p53-induced cell death in flies [26]. Therefore, the Hpo pathway might be kept in check by p53 under conditions of aberrant activity in *Drosophila* cells.

Of importance, it appears that the entire Ft/Ex/Mer/Sav/Hpo/Lats/dMob1/Yki *Drosophila* cascade is conserved in mammals as the FAT/NF2/hWW45/MST/LATS/hMOB1/YAP pathway (Fig. 1). hWW45, MST1/2, and LATS1 (the human relatives of *Drosophila* Sav, Hpo, and Lats, respectively) have been reported to form a complex [12,27,28]. Furthermore, MST1/2 phosphorylates and activates human LATS1/2 [12], and hMOB1A associates with human LATS1 [18,21], LATS2 [29], as well as MST1 [25]. However, it remains to be determined whether the proto-oncogene YAP (the human orthologue of Yki; [30]) is regulated by the Hpo pathway in human cells, and whether the p53 tumour suppressor protein is required for the activation of MST1/2 by radiation. Of equal importance will be more studies that address whether the tumour suppressor RASSF1A (RAS-association domain family protein 1A) plays an activating or inhibiting role in the Hpo/Sav/Lats complex. Current reports suggest that dRASSF functions as an antagonist of the Hpo pathway in *Drosophila* [31], while in human cells RASSF1A activates MST2, thereby promoting the phosphorylation of LATS1 [28]. Overall, providing sound evidence for the conservation and

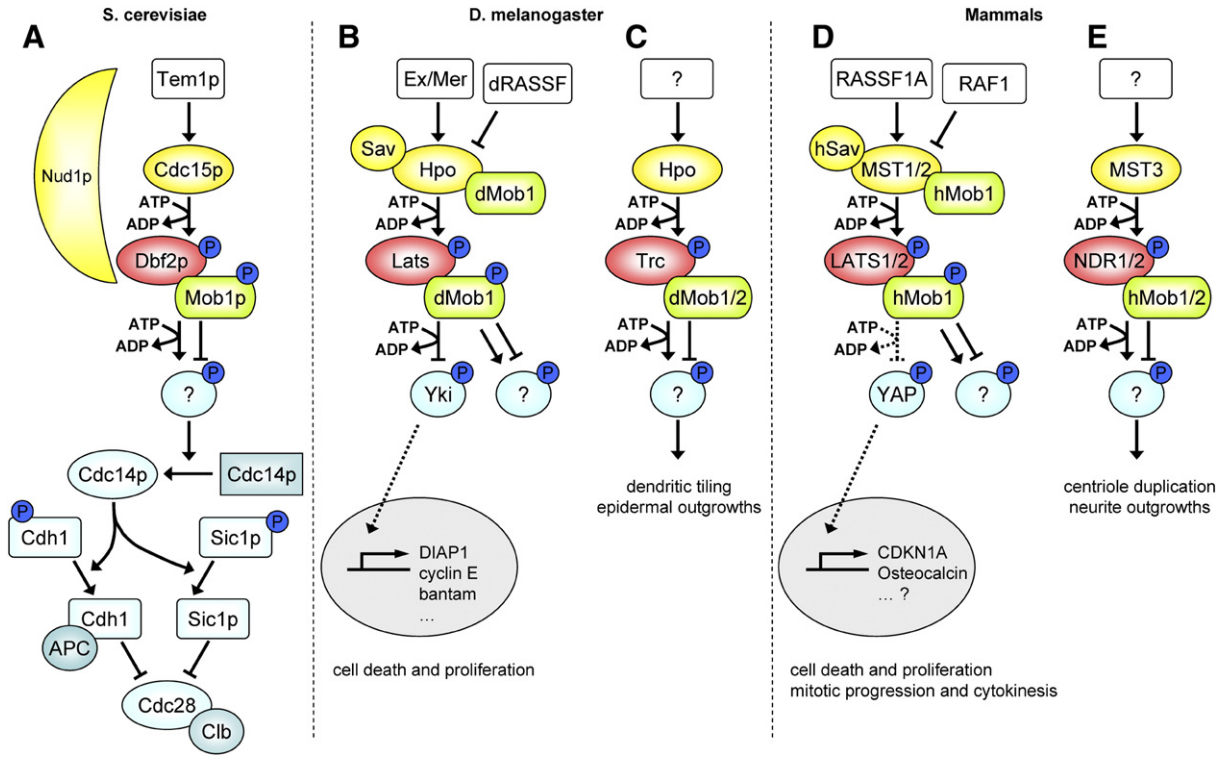

Fig. 1. NDR/LATS signaling pathways in yeast, flies and mammals. (A) The mitotic exit network in *S. cerevisiae* [32,33]. Tem1p activates the kinase Cdc15p (yellow), which phosphorylates Dbf2p (red) and Mob1p (green), whereby Nud1p (light yellow) serves as a scaffold. It is not known how the Dbf2p/Mob1p complex activates the phosphate Cdc14. (B) The Hippo/SWH pathway in *D. melanogaster* [23,24]. Expanded (Ex) and Merlin (Mer) stimulate a complex containing Sav (Salvador, light yellow); Hpo (Hippo, yellow); Lats (red) and dMob1 (also termed Mats, green), whereby Hpo phosphorylates both Lats and dMob1. The active Lats/dMob1 complex then inactivates Yorkie (Yki) by phosporylation. Consequently, Yki cannot stimulate the expression of DIAP1 and cyclin E, which results in decreased cell proliferation and increased cell death. (C) The Hpo/Trc pathway in control of morphological changes. Hpo (yellow) activates Trc (tricornered; red), and the active Trc/dMob1 (green) and Trc/dMob2 complexes are required for the control of dendritic tiling in sensory neurons and epidermal outgrowths. (D) The putative Hpo pathway in mammals [4,23,24]. Mammalian sterile 20-like kinases 1 and 2 (MST1/2; yellow) associate with human Salvador (hSav also termed hWW45; light yellow) and activate LATS1/2 by phosphorylation. The LATS/hMOB1 (green; whereby hMOB1 can also be phosphorylated by MST2) then potentially activates specific gene expression programs through phosphorylation of YAP (the putative human orthologue of Yki). Of note, the existence of the entire mammalian signaling cascade remains to be documented. (E) The NDR/MST pathway. MST3 (yellow) phosphorylates NDR1/2 (red), and the NDR/hMOB (green) complex then potentially controls centriole duplication and neurite outgrowths. Noteworthy, it is currently unclear whether MST3 or hMOB1/2 contribute to any of those two NDR-related functions. Question marks highlight unknown upstream and downstream factors.

physiological role of the entire Hpo pathway still remains a significant challenge in mammals.

In the budding and fission yeast, the NDR kinases Dbf2p and Sid2p are essential for the functionality of MEN (mitotic exit network)/SIN (septation initiation network) networks [32,33]. In *S. cerevisiae*, after the initial activation of Tem1p, the Cdc15p protein kinase is stimulated, followed by increased activity of the Mob1p/Dbf2p complex, consequent release of the protein phosphatase Cdc14p from the nucleolus into the cytoplasm, thereby leading to a dephosphorylation wave through which cyclin B/cdc34p is inactivated, finally allowing the exit from mitosis (Fig. 1). A very similar pathway consisting of Spg1p, Sid1p, Cdc7, Sid2, Mob1p, and Clp1p has also been described in fission yeast. Strikingly, it seems that several components of the yeast MEN/SIN pathway are conserved in human cells [4]. Some human proteins (for example CDC14) are functionally so conserved that they can compensate for the loss of their yeast counterpart [34]. Furthermore, human LATS1 and LATS2 have recently been reported to play a role in mitotic exit [21,29]. Nevertheless, any detailed analysis of mammalian MEN/SIN pathways is yet to be undertaken.

4. Functions of NDR kinases in yeast and invertebrates

As already pointed out, extensive genetic studies in budding and fission yeast have unraveled that NDR kinases play crucial roles in the regulation of MEN in budding yeast and SIN in fission yeast. Considering that both pathways (centering around Dbf2p and Sid2p, respectively) have already been reviewed in detail, we refer herein to selected reviews for further reading [32,33].

The second NDR kinase in yeast (Cbk1p in budding yeast and Orb6p in fission yeast) is part of a morphogenesis network (see also [4]). In *S. cerevisiae*, Cbk1p is an essential component of the RAM (regulation of Ace2p activity and cellular morphogenesis) signaling cascade that regulates polarized growth. In *S. pombe*, Orb6p is required for the coordination of morphological changes with cell cycle progression. Interestingly, the existence of the RAM pathway with CBK1 as centerpiece has also been described in the human pathogenic fungus *Cryptococcus neoformans* [35]. Whether such morphogenesis networks also function in multicellular eukaryotes is not clear, although studies in the worm and fly showed that NDR family members have important roles concerning morphological changes in metazoans.

In *D. melanogaster*, the NDR Kinase Trc is important for the integrity of outgrowths (such as epidermal hair) and essential for the control of non-redundant innervations (dendritic tiling) and branching of sensory neurons (summarized in more detail in [4]). The second NDR kinase in flies, Lats, has recently been reported to regulate the maintenance of dendrites by genetically interacting with Polycomb group genes [13,36]. The homologue of Trc in *C. elegans*, termed SAX-1, is also important for neurite growth and neurite tiling [37], indicating that NDR kinases play important roles during dendritic outgrowth in invertebrates.

Lats is also part of a novel signaling network in *Drosophila*, known as the Hpo/SWH pathway [23,24]. Some components of this kinase cascade are: Salvador (Sav), Lats (also known as Warts), Hpo (a Sav binding partner that functions as upstream kinase of Lats), dMob1 (also termed Mats; a co-activator of Hpo and Lats), Yorkie (Yki; a substrate of Lats), as well as Expanded, Merlin and Fat. Currently, eleven proteins have been implicated as members of this pathway that are responsible for the coordination of cell growth, survival, and proliferation in *D. melanogaster* [23,24].

5. Roles of mammalian NDR kinases

Mammalian cells express four related NDR kinases, NDR1, NDR2, LATS1 and LATS2, whereas the latter two kinases have been shown to function as tumour suppressor proteins. LATS1-deficient mice exhibited ovarian and soft-tissue tumours, in addition to hypersensitivity to carcinogenic treatments [38]. Downregulation of LATS1/2 mRNA levels has been reported in association with human sarcomas, ovarian carcinomas, aggressive breast cancers, human astrocytomas and acute lymphoblastic leukemia [39–42]. Furthermore, LATS1 overexpression suppressed growth of MCF-7 cells in soft agar and tumour formation in nude mice [43]. In addition, the ectopic expression of LATS2 in v-Ras transformed NIH/3T3 cells suppressed the development of tumours in nude mice and inhibited cell proliferation in vitro [44]. The tumour suppressor activity of LATS2 has as yet not been addressed in knock-out animals, since LATS2-null mice die before embryonic day 12.5 due to proliferation defects and genomic instability [29,45]. In contrast, LATS1-deficient animals can develop normally and be fertile, although about 60% of female knock-out animals displayed infertility [38]. Moreover, male LATS1-null mice also appear to have reduced fertility [38].

Mammalian NDR1 is also not essential for development because NDR1-deficient mice are viable and fertile. However, loss of NDR1 seems to be compensated by the elevation of NDR2 protein levels in a tissue-specific manner (Cornils, H., Stegert, M.R., Dirnhofer, S., and Hemmings, B.A., unpublished data). Nevertheless, aged NDR1-null animals develop T-cell lymphomas, and preliminary results suggest that knock-out animals might be hypersensitive to carcinogenic treatment (Cornils, H., Stegert, M.R., Dirnhofer, S., and Hemmings, B.A, unpublished data). The ongoing generation of NDR2-null and NDR1/NDR2 double knock-out mice will be essential to elucidate the full spectrum of the physiological roles of NDR1/2 kinases in mammals.

Overall, LATS1/2 and NDR1 appear to be tumour suppressor proteins, although some evidence indicates that mammalian NDR1/2 kinases could function as proto-oncogenes. NDR1 mRNA levels are up-regulated in progressive ductal carcinoma in situ [46], lung adenocarcinoma [47,48], and ovarian cancer [49,50]. NDR2 mRNA levels are elevated in germ cell tumours [51,52], ovarian cancer [53], and a highly metastatic, non-small cell lung cancer cell line [54]. In addition, human NDR protein levels are increased in some melanoma cell lines [55], and the upstream integration of a retrovirus in the murine NDR2 locus resulted in B-cell lymphoma [56]. In contrast, NDR1 and NDR2 mRNA levels have been reported to be down-regulated in

samples of patients suffering from prostate cancer [57–60]. Therefore, mammalian NDR1/2 kinases might have opposing roles in cancer, by either functioning as tumour suppressor proteins or oncogenes, as already reported for factors such as KLF4, CDKN1A, TGFβ, NOTCH1 or Ras [61]. Many more studies are now needed to tackle the role(s) of mammalian NDR kinases in cancer biology, in order to fully comprehend the potential importance of these kinases in cellular transformation. Perhaps already described functions of mammalian LATS/NDR kinases could be translated into the analysis of cancer development, in particular roles of mammalian LATS1/2 kinases that have been studied more intensively over the past years.

On the cellular level, LATS1 has been shown to regulate the G2/M cell cycle transition and apoptosis [43,62], and to interact with the serine protease Omi/HtrA2 that can control cell proliferation and cell death [63,64]. Recently, it was reported that LATS1 seems to play a role in cellular senescence [65]. The LATS1 kinase has also been implicated in the regulation of mitotic progression [66], whereby evidence was provided that LATS1 regulates both mitotic exit [21] and cytokinesis [67]. Strikingly, LATS2 knock-out cells exhibit also acceleration of exit from mitosis [29], as well as defects in cell proliferation control and maintenance of genomic stability [45]. Similar to LATS1, LATS2 also controls cell cycle progression and apoptosis [44,68]. Both, LATS1 and LATS2, have further been linked to the G1 tetraploidy checkpoint [66,69], whereas LATS2 has been shown to be part of a positive feedback loop between p53 and LATS2 in order to prevent tetraploidisation [69]. Oren and colleagues reported that LATS2 protects p53 from Mdm2-mediated degradation in a kinase activity dependent manner, while elevated p53 protein levels stimulated the expression of LATS2 [69]. Interestingly, another study has documented that two microRNAs, miR-372 and miR-373 (that can function as novel oncogenes in the development of human testicular germ cell tumours), are implicated in the p53-mediated inhibition of Cdk (cyclin-dependent kinase), possibly by down-regulating the expression of LATS2 [70]. These findings suggest that the LATS2 and p53 tumour suppressor pathways can strongly interact and are also dependent on each other (at least to a certain degree). However, how far the putative mammalian Hpo pathway is involved in this collaboration with the p53 signaling cascade is as yet unclear (please see also Section 3.4 for a discussion of this point).

In contrast to our detailed understanding of mammalian LATS1/2 kinases, only two functions have so far been attributed to mammalian NDR1/2 kinases: (1) mammalian NDR2 has been implicated in fear conditioning [71], and (2) human NDR1/2 kinases play a role in the regulation of centrosome duplication (discussed in detail in Section 6). Stork and colleagues observed that mouse NDR2 was up-regulated in the amygdala of fear-conditioned animals and proposed that NDR2 contributes to the coupling of morphological changes of neurons with fear-memory consolidation [71]. At least in cultured cells, they could show that the overexpression of NDR2 alters neuronal growth and differentiation, as well as facilitates neurite outgrowth. Therefore, mammalian NDR2 could potentially regulate dendritic tiling and branching, as already reported for invertebrate NDR kinases, Trc [72] and SAX-1 [37]. However, the physiological role(s) of mammalian NDR family members remain to be studied with respect to such dendritic interactions.

6. Centrosome duplication and NDR kinases

In animal cells, the centrosome (comprised of two centrioles surrounded by pericentriolar material) functions as a primary microtubule-organising center, regulates cell polarity, motility and adhesion, is required for cilia/flagella formation, and also plays a role in cell cycle regulation [73]. Centrosomal abnormalities occur in many cancer types and have been observed in association with genomic instability [74,75]. In addition, several centrosomal components are required for the assembly of cilia and flagella, which have essential functions in development and physiology [76–78]. Therefore, it is crucial for a healthy organism to maintain centrosomes and centriole numbers under strict control. However, little is known about the co-ordination and control of centriole duplication.

Electron microscopy studies have defined the main phases of the centrosome cycle: (1) centriole disengagement, (2) centriole duplication/formation of procentrioles, (3) elongation of procentrioles, (4) centrosome separation [73,74]. Genetic and proteomic screens have allowed the identification of proteins including ZYG-1, SPD-2 SAS-4, SAS-5, and SAS-6, that are part of a conserved centriole assembly protein module [73,79]. Recent evidence strongly indicates that PLK4 (Polo-like kinase 4; the mammalian homologue of ZYG-1) and SAS-6 are key regulators of centriole duplication since their absence abolishes centriole duplication, while their overexpression results in an increase of centrosome/centriole numbers in human and invertebrate cells [80–86]. The depletion or inhibition of cyclin-dependent kinase 2 (Cdk2) also results in a block of centrosome (re-)duplication [87–92]. Therefore, centriole duplication is orchestrated by different protein kinases.

Interestingly, NDR family members have been detected on spindle pole bodies (SPB; the equivalent of centrosomes in lower eukaryotes) and centrosomes. In yeast, Dbf2p and Sid2p reside on the SPB and at the cell division site (summarized in [4]), and in mammals, LATS1 and LATS2 have been detected on interphase and mitotic centrosomes [45,93–97]. Furthermore, mammalian NDR1/2 kinases have been found on centrosomal structures throughout the entire cell cycle [5]. Noteworthy, phospho-species of NDR1/2 were detectable on one out of two centrioles/centrosomes in G1/S phase, while NDR species were equally distributed between both centrosomes in G2/M phase (Fig. 2). Although NDR family members might utilize the SPB/centrosome “only” as signaling platforms [98,99], LATS2 knock-out cells displayed centrosome fragmentation and amplification [29,45]. Nevertheless, mammalian LATS1/2 kinases are most likely not directly involved in the control of centrosome duplication, because (1) the overexpression of LATS1/2 wild-type kinases did not cause an increase in centrosome numbers, and (2) the overproduction of LATS1/2 kinase-dead forms (which can function as dominant-negative molecules) did not interfere with centrosome overamplification [5].

Interestingly, the first known molecular function of mammalian NDR1/2 kinases has been defined as regulators of centriole duplication [5]. Overexpression of NDR1/2 resulted in centrosome/centriole overduplication in a kinase-activity-dependent manner, while expression of kinase-dead NDR1/2 or depletion of NDR1/2 by small interfering RNA (siRNA) negatively affected centrosome duplication (Fig. 3). Furthermore, by selectively targeting NDR species to the centrosome, our laboratory could show that the centrosomal pool of active NDR is sufficient to generate supernumerary centrosomes. In addition, our study revealed that NDR-driven centrosome duplication requires Cdk2 activity and that Cdk2-induced centrosome amplification is affected upon reduction of NDR kinase activity. Altogether, our recent data indicate that mammalian NDR1/2 kinase activity regulates centriole duplication directly on the centrosome in a Cdk2-dependent manner.

Studies are now needed that address the mechanism of this function by testing the co-operation of NDR1/2 with other known regulators of centriole duplication. Possibly, tackling the relationship of NDR1/2 with the two master regulators of centriole duplication, PLK4 and SAS-6, will be of great interest. Considering that NDR1 immobilised on centrosomes still is able to affect centrosome duplication [5], it is very likely that direct substrates of NDR1/2 are present on centrosomes. Potentially, the identification and characterization of such substrates could reveal novel regulators of centriole duplication. However, any physiological substrates of mammalian NDR kinases remain to be defined [4]. Furthermore, it is as yet unclear at which step NDR1/2 kinases control centrosome duplication: (1) directly at the centrosome duplication machinery, (2) as licensing molecules, (3) or by co-coordinating DNA and centriole duplication [73]. Potentially, mammalian NDR1/2 are “general” regulators of cell cycle progression and DNA replication. Therefore, several different lines of research are now necessary to establish the precise role(s) of mammalian NDR1/2 at the molecular level.

7. NDR kinases as therapeutic targets

Abnormal centrosome amplification occurs frequently during cellular transformation (summarized in [100,101]), hence, factors contributing to the regulation of centriole duplication are likely to play a role in cancer development [74,102]. Furthermore, the deregulation of some regulators of centrosome duplication has already been shown to contribute to tumour formation. Elderly PLK4 heterozygote mice are prone to develop liver and lung cancers [103], while PLK4 null mice die very early at E7.5 during embryogenesis [104]. PLK4 knockout embryos displayed increased cell death and an elevated mitotic index [104], similar to human cells depleted of PLK4 [80]. Interestingly, mitotic abnormalities and an increase in cell death were also reported after depletion of SAS-6 in human cells [82]. Furthermore, this link between centrosome duplication, embryonic survival, and haplo-insufficiency for tumour suppression has also been observed with nucleophosmin [105], another regulator of centrosome duplication [106,107]. NDR1+/− animals also develop tumours with increased age (Cornils, H., Stegert, M.R., Dirnhofer, S., and Hemmings, B.A., unpublished

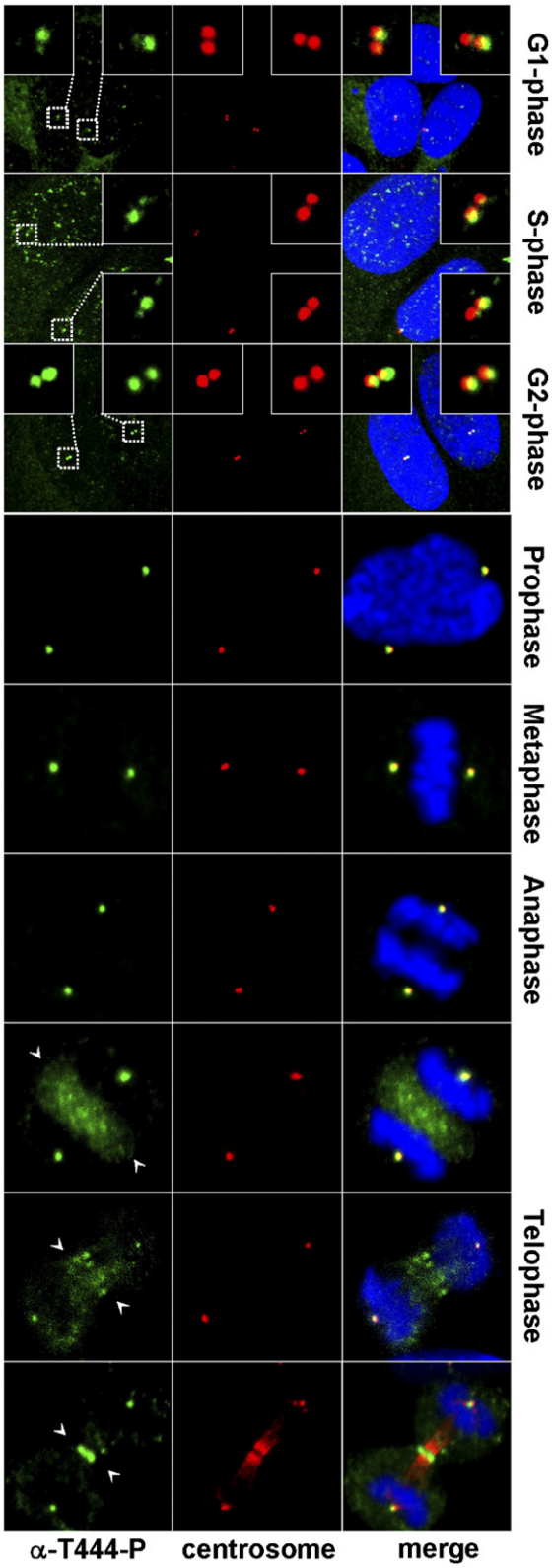

Fig. 2. Cell cycle-dependent localization of phospho-NDR1/2 species. U2-OS cells were processed for immunofluorescence using indicated markers. Phospho-NDR1/2 molecules are shown in green, and centrosomes are indicated in red. DNA is stained in blue. Insets show enlargements of centrosomes. White arrowheads indicate midzone staining of phospho-NDR1/2. (Figure originally published in Hergovich et al., Molecular Cell [5]).

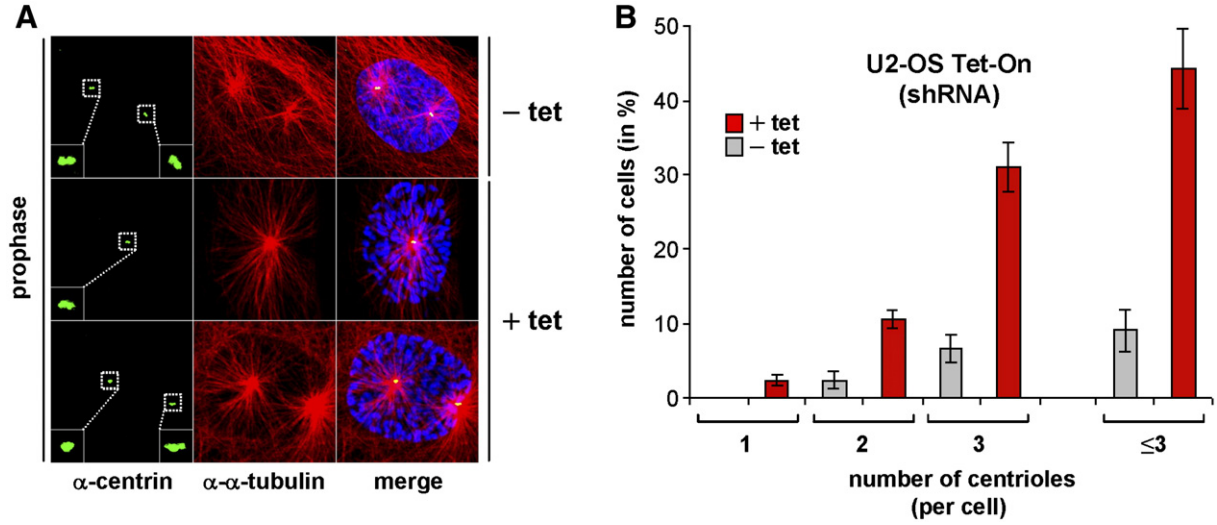

Fig. 3. Defective centriole duplication after knock-down of NDR1 by RNAi in human cells. (A) U2-OS cells expressing tetracycline-inducible short-hairpin RNA (shRNA) directed against human NDR1 were incubated without (−) or with (+) tetracycline for 4 days, before analysis by immunofluorescence. Centrioles are shown in green, the tubulin network in red, and DNA is stained blue. Insets show enlargements of centrioles. (B) Histograms show the percentage of mitotic cells (prophase and prometaphase) that displayed the indicated numbers of centrioles. Noteworthy, nearly 50% of cells with decreased NDR1 levels lacked at least one centriole, while less than 10% of control cells displayed such a characteristic. (Figure originally published in Hergovich et al., Molecular Cell [5]).

data), however, it is currently unclear how NDR's role in centriole duplication [5] contributes to this phenotype. Nevertheless, current data suggest that studies of mammalian NDR1/2 kinases might provide a further link between centrosome duplication and haploinsufficiency for tumour suppression. Altogether, these studies strongly support a link between centrosome defects and oncogenesis (summarized in Table 2) that has already been proposed nearly a century ago [108]. However, whether centrosome alterations are a cause or consequence of tumour development is as yet unclear [74,102].

Although, one is tempted by our recent work to speculate that NDR kinases can function as oncogenes by driving centrosome overduplication [5], the existing animal data rather suggest that mammalian NDR1 has a role as a tumour suppressor protein (Cornils, H., Stegert, M.R., Dirnhofer, S., and Hemmings, B.A., unpublished data). However, since the loss of NDR1 appears to be compensated by the elevation of NDR2 protein levels in some, but not all, tissues (Cornils, H., Stegert, M.R., Dirnhofer, S., and Hemmings, B.A., unpublished data), one cannot yet exclude the possibility that the development of T-cell lymphomas in homo- and heterozygote NDR1 animals is simply the result of overcompensation by increased NDR2 protein levels. The analysis of NDR2-null and NDR1/NDR2 double knock-out mice will be essential to analyse this role of mammalian NDR1/2 as potential tumour suppressor proteins in more detail. Altogether, it will be crucial to address the role(s) of NDR1/2 kinases in cancer biology by taking into account that these kinases might show context-dependent, opposing roles in cellular transformation, as already reported for factors such as TGFβ, Notch, or Ras [61].

Irrespective of its direct role in cellular transformation, mammalian NDR1/2 kinases might also be a useful target in

Table 2
Regulators of centrosome duplication and oncogenesis in mammals

Protein	Species	Study	Phenotype
PLK4	*H. sapiens*	RNAi	No centriole duplication
		Overexpression	Centriole amplification
PLK4/SAK	*M. musculus*	Knock-out	Null mice die at E7.5; heterozygotes develop liver and lung cancers
SAS-6	*H. sapiens*	RNAi	No centriole duplication
		Overexpression	Centriole amplification
NPM1/B23	*H. sapiens/M. musculus*	RNAi	Centrosome amplification
		Overexpression	No centrosome duplication
NPM/B23	*M. musculus*	Knock-out	Null mice die at E11.5–E16.5; heterozygotes develop blood disorders
p53	*H. sapiens*	Mutations	Centrosome amplification
p53	*M. musculus*	Knock-out	Centrosome amplification in p53 null cells
NDR1/STK38	*H. sapiens*	RNAi	Impaired centriole duplication
		Overexpression	Centriole amplification
NDR1/STK38	*M. musculus*	Knock-out	Hetero- and homozygote mice develop lymphomas

cancer therapy on a different level. As already speculated earlier with respect to PLK4 [80,81], inhibition of a kinase that is involved in centriole duplication most likely will prove incompatible with cell proliferation. Therefore, our finding that human NDR1/2 kinases are required for proper centriole duplication [5] has potential implications for the development of anti-cancer compounds. However, two points are to be taken into consideration with respect to this approach: (1) murine NDR1 is not essential for development, because NDR1-deficient mice are viable and fertile [however, due to the potential compensation by NDR2 a full assessment of the role of mammalian NDR1/2 kinases in development will require the analysis of NDR1/NDR2 double knock-out animals], and (2) the increased tumourigenesis in NDR1 homo- and heterozygote mice may raise concerns as to the safety of an NDR kinase inhibitor. Nevertheless, we believe that it is worth testing whether diminished centriole duplication due to the down-regulation of human NDR1/2 kinases negatively interferes with tumour cell proliferation. Experiments addressing the consequences of NDR1/2 knock-down on cell proliferation are currently ongoing in tumour-derived cell lines, in order to determine the potential value of NDR1/2 kinases as targets in cancer treatment. Another challenge is going to be whether researchers trained in drug design will be able to take advantage of the unique structure of NDR kinases (see Section 3). Furthermore, additional studies are to be undertaken that address the long-term consequences of blocked centriole duplication in normal and tumour tissues. Before the investment of more resources, one will first have to exclude the following scenario. After an initial wave of cell death and cell cycle arrest due to blocked centriole duplication, some cells with increased DNA content (possibly generated by abortion of mitosis or ongoing DNA replication) survive and proliferate even faster than the tumour cells before drug treatment. As a consequence of DNA amplification during the "centriole arrest", cells might also have acquired an increased potential to form metastasis. In spite of these concerns, inhibitors of kinases that regulate centriole duplication might prove to be valid anti-cancer therapeutics in the future, in particular when applied in combination with other anti-proliferative cancer drugs.

Besides being a potential drug target in human disease, another NDR family member could serve as target for anti-parasitic agents [109]. TBPK50, the NDR kinase in *Trypanosoma brucei*, has been shown to co-ordinate cell shape and division [110], whereby this function appears to be performed together with MOB1 [111]. Mottram and colleagues reported that TBPK50 forms an active complex with MOB1 in vivo, and more importantly, could show that MOB1 is required for cytokinesis in blood stream forms of *T. brucei* [111]. This finding that the TBPK50/MOB1 complex is essential for efficient proliferation makes the TBPK50 kinase a promising drug target candidate in trypanosomes. Therefore, further research on NDR kinases from different species could provide important leads in the discovery and validation of NDR kinases as targets for the development of anti-cancer as well as anti-parasitic therapeutics.

8. Conclusions and perspectives

Over the past decade, studies of NDR family members have revealed essential functions for NDR kinases in uni- and multicellular organisms. In yeast, NDR kinases regulate mitotic exit and cytokinesis, and their mammalian relatives appear to have similar roles. In flies, the Hpo/SWH signaling pathway coordinates cell death and proliferation, and this essential signaling cascade in *Drosophila* might also be conserved in vertebrates. Furthermore, NDR kinases have been shown to regulate morphological changes on the cellular level, in particular SAX-1 and TRC are important regulators of dendritic outgrowth in invertebrates. The corresponding physiological function, however, is yet to be addressed in more detail in mammals.

The regulation of NDR kinases at the molecular level has also been studied in much detail over the past decade. Phosphorylation events on the activation segment and the hydrophobic motif have been shown to be essential for kinase activity as well as functionality of various NDR family members. The phosphorylation on the activation segment is controlled by two stretches of primary sequence that are unique for NDR kinases: (1) binding of MOB proteins to the NTR domain stimulates autophosphorylation, and (2) basic residues in close proximity to the activation segment phosphorylation site contribute to inhibition of phosphorylation. In contrast, genetic and biochemical evidence strongly indicates that the hydrophobic motif is targeted by members of the Ste20-like kinase family. Nevertheless, several lines of research are still necessary to define the precise roles of these NDR interactors. In particular, all five MST kinases and six MOB proteins are to be tested with respect to their involvement in the regulation of mammalian NDR/LATS kinases. Moreover, in spite of our advanced understanding of the molecular activation mechanism and function(s) of NDR kinases, the biological substrates still remain to be identified. Only one substrate of the entire NDR family is currently known, namely *Drosophila* Yki. Potentially, molecular studies of Yki phosphorylation by Lats will be able to provide general insights into the substrate specificity of NDR kinases, but many more NDR/LATS substrates are to be identified and characterized in order to complement our understanding of NDR-regulated morphological changes, mitotic exit or centrosome duplication. Any extracellular stimulus that activates NDR kinases is also unknown to date. In particular, research addressing the activating trigger for the Hpo/SWH pathway is currently of great interest.

Altogether, one challenge is to identify more components of the already established Hpo/SWH and MEN/SIN networks in uni- and multicellular model organisms such as yeast, worms or flies. A second task is the translation from our knowledge gained in yeast and invertebrates to mammals. Studies addressing the regulation of dendritic outgrowth by NDR/LATS kinases as well as the composition of the still putative mammalian Hpo/SWH or MEN/SIN signaling cascades will be essential in order to broaden our knowledge with respect to mammalian NDR/LATS kinases. The phenotypical analysis of NDR1 and NDR2 single and double knock-out animals is also to be reported. The

generation and detailed characterization of mice that carry a tissue-specific deletion of the desired kinase gene (i.e. LATS2) should be of great help in this respect. Considering the important role of NDR family members play in the control of mitotic events, cell death and proliferation in yeast and invertebrates, it is likely that their human counterparts play similar roles in the control of these central processes. Actually, ongoing cancer research is aiming to identify and characterize novel regulators of cell survival and proliferation that are dysfunctional in transformed mammalian cells. However, studies testing the contributions of mammalian NDR/LATS kinases (in particular of NDR1/2) and their regulators (i.e. MOB proteins) to human diseases such as cancer appear only now on the horizon. Over the coming years, it will be essential to understand where, how and why mammalian NDR1/2 and LATS1/2 kinases are required in embryonic development and tissue homeostasis, in order to establish how far NDR/LATS pathways are suitable therapeutic targets in the fight against human diseases.

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Report

NDR Kinase Is Activated by RASSF1A/MST1 in Response to Fas Receptor Stimulation and Promotes Apoptosis

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Summary

Human NDR1 and 2 (NDR1/2) are serine-threonine protein kinases in a subgroup of the AGC kinase family [1]. The mechanisms of physiological NDR1/2 activation and their function remain largely unknown. Here we report that Fas and TNF-α receptor stimulation activates human NDR1/2 by promoting phosphorylation at the hydrophobic motif (Thr444/442). Moreover, NDR1/2 are essential for Fas receptor-induced apoptosis as shown by the fact that NDR knockdown significantly reduced cell death whereas overexpression of the NDR1 kinase further potentiated apoptosis. Activation of NDR1/2 by death receptor stimulation is mediated by the tumor suppressor RASSF1A. Furthermore, RASSF1A-induced apoptosis largely depends on the presence of NDR1/2. Fas receptor stimulation promoted direct phosphorylation and activation of NDR1/2 by the mammalian STE20-like kinase 1 (MST1), a downstream effector of RASSF1A. Concurrently, the NDR1/2 coactivator MOB1 induced MST1-NDR-MOB1 complex formation, which is crucial for MST1-induced NDR1/2 phosphorylation upon induction of apoptosis. Our findings identify NDR1/2 as novel proapoptotic kinases and key members of the RASSF1A/MST1 signaling cascade.

Results and Discussion

Apoptotic pathways, including those induced by death receptors, are often suppressed in cancer cells through the inactivation of corresponding tumor suppressors, which normally resist cell overgrowth [2–4]. RASSF1A (Ras association domain family 1 isoform A) is a well-known tumor suppressor protein encoded by a gene on human chromosome 3 at p21.3 and is often silenced by promoter hypermethylation in a wide variety of human cancers [2, 5]. Reintroduction of RASSF1A was reported to induce cell-cycle inhibition via inactivation of the APC/Cdc20 complex [6] or apoptosis via MST1 or MST2 [7, 8]. Although MST2 is known to signal through LATS1/2 [8–10], the apoptotic signaling cascade downstream of MST1 in mammalian cells remains obscure.

NDR1/2 are serine-threonine protein kinases belonging to an AGC kinase subfamily implicated in many essential processes such as cell division and proliferation [1]. So far the only known function implicates NDR1 in the regulation of centrosome duplication [11]. Two phosphorylation sites of human NDR1/2, Ser281/282 and Thr444/442, are essential for full kinase activation in vitro and in vivo [12, 13]. The binding of coactivator MOB1A to the N terminus of NDR1/2 stimulates kinase activity, which results in autophosphorylation on Ser281/282 at the activation segment [14].

We reported earlier that MST3 kinase can phosphorylate NDR1/2 at Thr444/442 in the hydrophobic motif [15]. Although no physiological stimulus has been reported for the MST3 kinase, close members within the MST kinase family, such as MST1 and MST2, are actively involved in apoptosis and stress response [16–20]. To determine whether NDR1/2 are regulated by apoptotic stimuli, we treated HeLa cells with the Fas antibody or TNF-α. We observed a robust induction of NDR1/2 phosphorylation at Thr444/442, but total NDR1/2 did not change. This NDR1/2 phosphorylation was paralleled by activation of MST1/2 and by PARP cleavage, confirming the onset of apoptosis (Figure 1A, top). Similar results were obtained with Jurkat cells (Figure S1A available online). The kinase activity of endogenous NDR1/2 increased significantly (more than 8-fold) in cells undergoing Fas and TNF-α receptor stimulation, indicating NDR1/2 activation during apoptosis (Figure 1A, bottom). These data are the first indication of a physiological stimulation of NDR kinases.

It has been reported that Fas receptor stimulation triggers RASSF1A-dependent activation of the downstream signaling cascade [7, 8]. Indeed, expression of RASSF1A was sufficient to promote NDR1/2 activation, comparable to induction by the Fas receptor (Figure 1B, top). Moreover, localization of activated NDR1/2 and RASSF1A partially overlapped in the cytoplasm (Figure 1B, bottom). Decreased levels of RASSF1A protein in cells treated with the Fas antibody resulted in impaired NDR Thr444/442 phosphorylation, indicating that the Fas-dependent apoptotic signaling cascade activating NDR kinases is mediated by RASSF1A (Figure 1C).

RASSF1A protein was shown to exert its proapoptotic effects via MST1 and MST2 kinases [7, 8, 21]. In Fas receptor-induced apoptosis, MST2 transmits an apoptotic signal to LATS1; however, no downstream target of MST1 has yet been proposed. To investigate whether MST1 is involved in the regulation of NDR Thr444/442 phosphorylation, the level of the endogenous MST1 kinase was decreased by shRNA. Stimulation of NDR Thr444/442 phosphorylation upon Fas antibody treatment failed in cells with low MST1 content (Figure 1D), demonstrating the importance of MST1 for the NDR1/2 activation during apoptosis. Transient knockdown of MST2 resulted in only moderate downregulation of NDR1/2 phosphorylation under the same experimental conditions (Figure S1B). Consistent with these data, we also found reduced NDR Thr444/442 phosphorylation triggered by the expression of RASSF1A in MST1 knockdown cells (Figure 1E). In addition, NDR1/2 kinase activity was stimulated by the nonspecific inducer of apoptosis, actinomycin D (Figure S1C). Knockdown of MST1 efficiently prevented NDR1/2 phosphorylation, induced by actinomycin D, whereas knockdown of MST3 did not change the level of phosphorylated NDR (Figure S1D).

Because MST1 kinase was crucial for the activation of NDR1/2 during apoptosis, we tested its ability to induce NDR1 activation in a series of kinase assays. MST1, but

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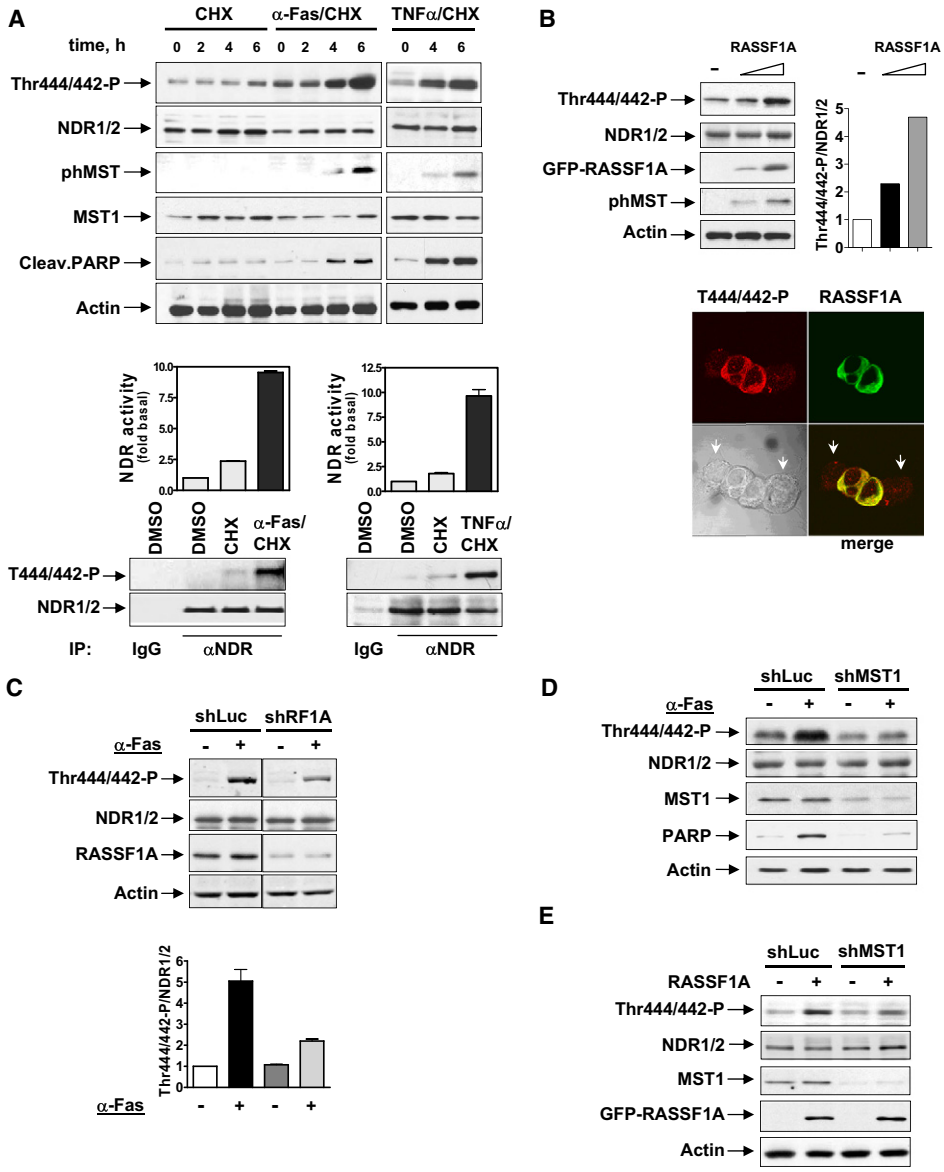

Figure 1. Death Receptor-Induced NDR Activation Is Mediated by RASSF1A and MST1

(A) Top: HeLa cells were treated with cycloheximide (CHX) alone or in combination with Fas antibody or TNF-α for the indicated time periods. Cell lysates were analyzed for the phosphorylation of NDR1/2 (Thr444/442) and MST1/2 (Thr183/Thr180) and PARP cleavage by western blotting. Bottom: Immunoprecipitated NDR1/2 from HeLa cells treated with CHX, Fas antibody, or TNF-α for 6 hr was assayed for kinase activity. The results are from duplicate assays from two independent experiments; data show mean ± standard deviation.

(B) GFP-RASSF1A was overexpressed in H1299 cells with subsequent analysis of NDR Thr444/Thr442 phosphorylation either by western blotting with Odyssey quantification (top) or by confocal microscopy (bottom). Note that cells expressing GFP-RASSF1A stained more intensely for Thr444/442 phosphorylated NDR1/2 than did untransfected cells (arrows).

(C) HeLa cells expressing shRNA against RASSF1A and treated with a combination of CHX and Fas antibody for 6 hr were lysed and processed for western blotting. The results are from three independently quantified experiments; data show mean ± standard deviation.

(D) HeLa cells expressing shRNA against MST1 and treated with a combination of CHX and Fas antibody for 6 hr were lysed and assessed by western blotting for the occurrence of the indicated proteins.

(E) GFP-RASSF1A was overexpressed in H1299 cells along with the shRNA against MST1, NDR Thr444/Thr442 phosphorylation was analyzed by western blotting.

not its kinase dead variant, phosphorylated MBP-NDR1 protein in vitro (Figure 2A, left). Next, we confirmed that MST1 was able to directly phosphorylate MBP-NDR1 at the hydrophobic motif (Thr444) (Figure 2A, top right), raising the question of whether this phosphorylation also induced NDR1/2 activation. Indeed, NDR1 phosphorylated at Thr444 by MST1 displayed greatly (7-fold) enhanced protein kinase activity (Figure 2A, bottom right). In order to verify the specificity of the kinase reaction, we performed experiments with recombinant MST1 (Figures S2A and S2B). Finally, we determined whether MST2 and MST4 are also potential upstream kinases for NDR. Although MST1, MST2, and MST3 potently activated NDR1 in vitro, MST4 had only a minor effect (Figure S2C).

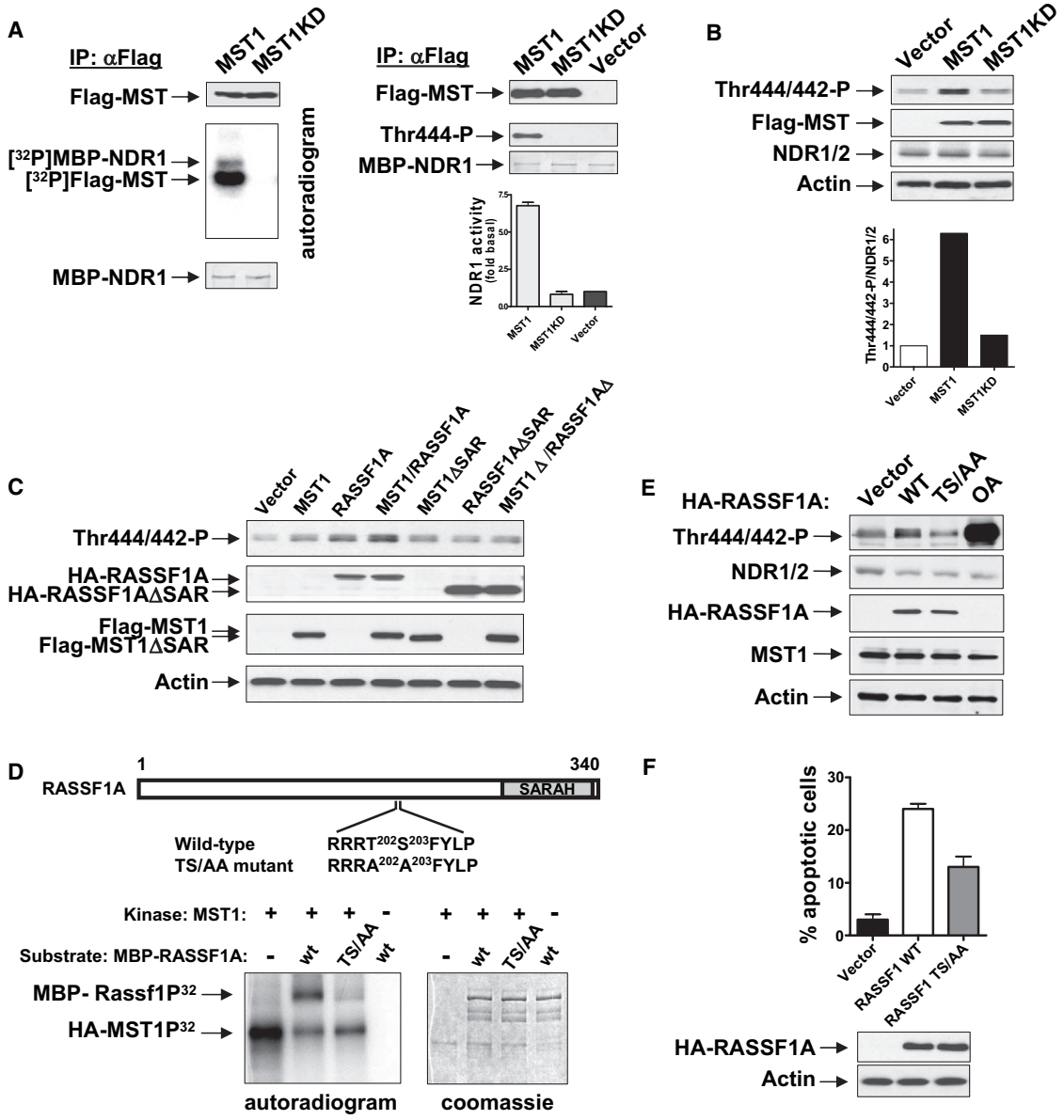

Figure 2. MST1 Phosphorylates and Activates NDR In Vitro and In Vivo

(A) MBP-NDR1 was in vitro phosphorylated in the presence of [γ-^{32}P]ATP by Flag-MST1 and its kinase dead variant (MST1KD) immunoprecipitated from HEK293 lysates. Kinase reactions were resolved on the SDS-PAGE (left). Experiment performed as above but in the absence of radiolabelled ATP and NDR1 Thr444 phosphorylation was analyzed by western blotting (right). MBP-NDR1 activated by MST1 was used in a subsequent in vitro kinase assay on the NDR substrate peptide (right). The results are from duplicate assays from two independent experiments: data show mean ± standard deviation.

(B) HEK293 cells were transfected with Flag-MST1 or Flag-MST1KD, cell lysates were analyzed for phosphorylated active NDR1/2 by western blotting and quantified.

(C) HA-RASSF1A and Flag-MST1 as well as their SARAH domain deletion mutants were expressed in H1299 cells; western blotting of the indicated proteins is shown.

(D) Putative phosphorylation sites (Thr^{202}Ser^{203}) in the sequence of RASSF1A are displayed (top). HA-MST1, immunoprecipitated from the HEK293 cells, was used in an in vitro radioactive kinase assay with MBP-RASSF1A or its phospho-deficient mutant Thr/Ala^{202}Ser/Ala^{203}.

(E) HeLa cells were transfected with wild-type HA-RASSF1A or phosphorylation-deficient mutant, and activation of NDR1/2 by Thr444/Thr442 phosphorylation was assessed by western blotting.

(F) Apoptosis of HeLa cells expressing wild-type RASSF1A versus a RASSF1A-Thr/Ala^{202}Ser/Ala^{203} mutant upon Fas antibody treatment. Data including standard deviations from three independent experiments are displayed.

The contribution of MST1 to in vivo phosphorylation and activation of NDR was studied by expressing the kinase in HEK293 cells. MST1 induced significant phosphorylation of NDR1/2 on Thr444/442 (Figure 2B), comparable with that induced by RASSF1A expression (Figure 1B), Fas antibody, or TNF-α treatment (Figure 1A). Next, we examined whether the observed increase in NDR Thr444/442 phosphorylation was mediated by RASSF1A-MST1 binding via their SARAH domains [7, 22]. As expected, wild-type MST1 coimmunoprecipitated with RASSF1A, whereas the MST1 mutant lacking the SARAH domain (ΔSAR) as well as MST3 (naturally lacking a SARAH domain) failed to bind RASSF1A (Figure S2D).

Comparison of NDR1/2 phosphorylation in cells expressing wild-type or SARAH domain-deletion variants (ΔSAR) of RASSF1A, MST1, or their combination showed that interaction of RASSF1A and MST1 is required for a positive effect on NDR Thr444/442 phosphorylation (Figure 2C).

Our data place mammalian NDR1/2 directly downstream of the MST1 kinase. In *Drosophila*, the ortholog of NDR (Trc), as well as of LATS (Warts/Lats), are also regulated by MST (Hpo), a core member of Hippo tumor suppressor pathway [23].

We examined whether RASSF1A may be a substrate of NDR or MST1. The direct phosphorylation of MBP-RASSF1A fusion protein in in vitro kinase assays with the wild-type or kinase dead variants revealed a marked effect of MST1 but not of NDR1 (Figure S2E). We then mutated two putative phosphorylation sites in the MBP-RASSF1A construct (Figure 2D, top). MST1 efficiently phosphorylated MBP-RASSF1A, whereas MBP-RASSF1A Thr/Ala^{202}Ser/Ala^{203} displayed only a residual signal (Figure 2D, bottom). To investigate the effects of putative RASSF1A phosphorylation in vivo, mutant RASSF1A was expressed along with the wild-type species. Interestingly, analysis of NDR Thr444/442 phosphorylation demonstrated a reduced capacity of the RASSF1A mutant to activate NDR1/2 (Figure 2E). It has been reported previously that overexpression of RASSF1A is sufficient to induce apoptosis [4, 7, 8]. We found that the mutant RASSF1A was less potent in triggering apoptosis than the wild-type protein (Figure 2F). MST1 and RASSF1A form a complex via association of their SARAH domains [24]. This interaction could facilitate MST1-mediated RASSF1A phosphorylation upon induction of apoptosis [25]. The same motif (Thr^{202}Ser^{203}) was previously shown to be targeted by Aurora-A [26] and CDK4 [27]. However, phosphorylation of RASSF1A by these kinases results mostly in effects on the cell cycle and no apoptotic function has been proposed.

Previously, we established that full activation of NDR1/2 depends on the MOB1 coactivator, which elicits its effect through direct binding to the N termini of NDR1 and NDR2 [14, 28]. Human MOB1 protein exists in two isoforms that share more than 95% sequence similarity: MOB1A and B (collectively termed as MOB1 hereafter). In order to study the role of MOB1 in NDR activation during apoptosis, we knocked down both MOB1 isoforms in HeLa cells and treated with the Fas antibody. In these experimental settings, a decrease in MOB1 protein level correlated with reduced Thr444/442 phosphorylation of NDR1/2 (Figure 3A). Further, coexpression of MOB1A and MST1 resulted in enhanced NDR1 activity (Figure 3B). In vitro kinase assays with MBP-NDR1 as a substrate revealed a stimulatory effect of MOB1A on MST1-induced NDR phosphorylation (Figure S3A).

We showed earlier that MOB1A binds to NDR1 kinase and that this association is increased upon okadaic acid (OA) treatment, which preferentially inhibits protein phosphatase 2A [14]. Recent studies also demonstrated association of *Drosophila* Mats (MOB) and Warts/Lats (LATS) when Mats is phosphorylated by Hpo (MST) [29]. Based on these results, we examined whether human MOB1A and MST1 interact in the presence of OA. Indeed, MOB1A, MST1, and activated NDR1/2 were found in a complex upon OA treatment (Figures S3B and S3C). Moreover, all three proteins displayed cytoplasmic colocalization visualized by confocal microscopy (Figure 3C, top). The inducible knockdown of MOB1 almost completely abolished binding between endogenous MST1 and HA-NDR1 (Figure 3C, bottom).

Because Fas receptor stimulation activated both MST1 and NDR1/2, we examined whether this stimulus could serve as a physiological signal for complex formation. Indeed, we observed association of endogenous MOB1-MST1-NDR proteins, triggered by the Fas antibody treatment. In addition, shRNA against MOB1 was sufficient for disruption of the complex (Figure 3D), implying that MOB1 was its driving force. Our results support a model in which MOB1 binding not only releases the autoinhibitory conformation of NDR1/2 [14, 30], but also promotes maximal activation by inducing a triple complex formation, essential for MST1-mediated phosphorylation. During the preparation of this manuscript, a report was published that describes the association between MST2, MOB1, and NDR1 [31]. However, it is not known whether these proteins form an endogenous complex or which physiological stimulus is required for this association.

Given that activation of NDR1/2 occurred in response to death receptor stimulation and involved several prominent proapoptotic molecules, we were prompted to investigate the functional relevance of NDR1/2 in the apoptotic process. To address this issue, we made use of HeLa cells expressing inducible shRNA directed against NDR1/2. Cells depleted of NDR1/2 were more resistant in developing morphological signs of apoptosis such as membrane blebbing and cellular shrinkage upon addition of the Fas antibody (Figure 4A, top). In the same experimental settings, knockdown of NDR1/2 resulted in a significant reduction of cleaved PARP and cytochrome c release in cells undergoing apoptosis (Figure 4A, bottom). The proapoptotic effect of NDR1/2 was further confirmed by assessing the mitochondrial membrane potential ($\Delta\Psi$), which is a critical factor in the irreversible phase of apoptosis. Loss of $\Delta\Psi$ induced by the Fas antibody was significantly lower in cells depleted of NDR1/2 than in control HeLa cells (Figure 4B). Transient knockdown of NDR1/2 in HeLa and MCF7 cells had a similar effect (Figures S4A and S4B). The expression of siRNA-resistant wild-type NDR1 or NDR2 allowed Fas-induced apoptosis, whereas reintroduction of siRNA-resistant kinase dead (or even more so Thr444Ala) mutants did not restore efficient induction of apoptosis (Figure 4C). These data confirm that phosphorylation of the hydrophobic motif as part of NDR activation mechanism is crucial for proapoptotic function of NDR kinases. In addition, it shows that both NDR1 and NDR2 isoforms mediate Fas-induced apoptosis.

We addressed the relevance of NDR1/2 for RASSF1A-mediated apoptosis. Even though both NDR1/2 knockdown and control cells expressed comparable RASSF1A levels, apoptosis was not efficiently induced in the absence of NDR1/2, indicating that these kinases are essential for RASSF1A-mediated cell death (Figure 4D). Overexpression of NDR1 significantly enhanced the release of the apoptotic markers, in particular cytochrome c (Figure S4C, top), and further potentiated apoptosis triggered by the Fas antibody (Figure S4C, bottom). Furthermore, overexpression of NDR1wt but not NDR1Thr444Ala mutant promoted apoptosis in MCF7 cells (Figure S4D). Significantly, depletion of endogenous MOB1 resulted in similar reduction in apoptotic response as knockdown of NDR (Figures S4A and S4B). Our data identify NDR1/2 as key apoptotic players downstream of RASSF1A/MST1/MOB1 and provide evidence for this novel NDR kinase function, as summarized schematically in Figure 4F.

Our results, together with the data on MST2-mediated activation of LATS [8, 10], suggest that the analog of the Hippo pathway in mammals splits at the level of MST kinases to activate LATS and NDR.

Whereas MST1 and 2 have been assigned a proapoptotic function and MOB1 and LATS1/2 are known tumor

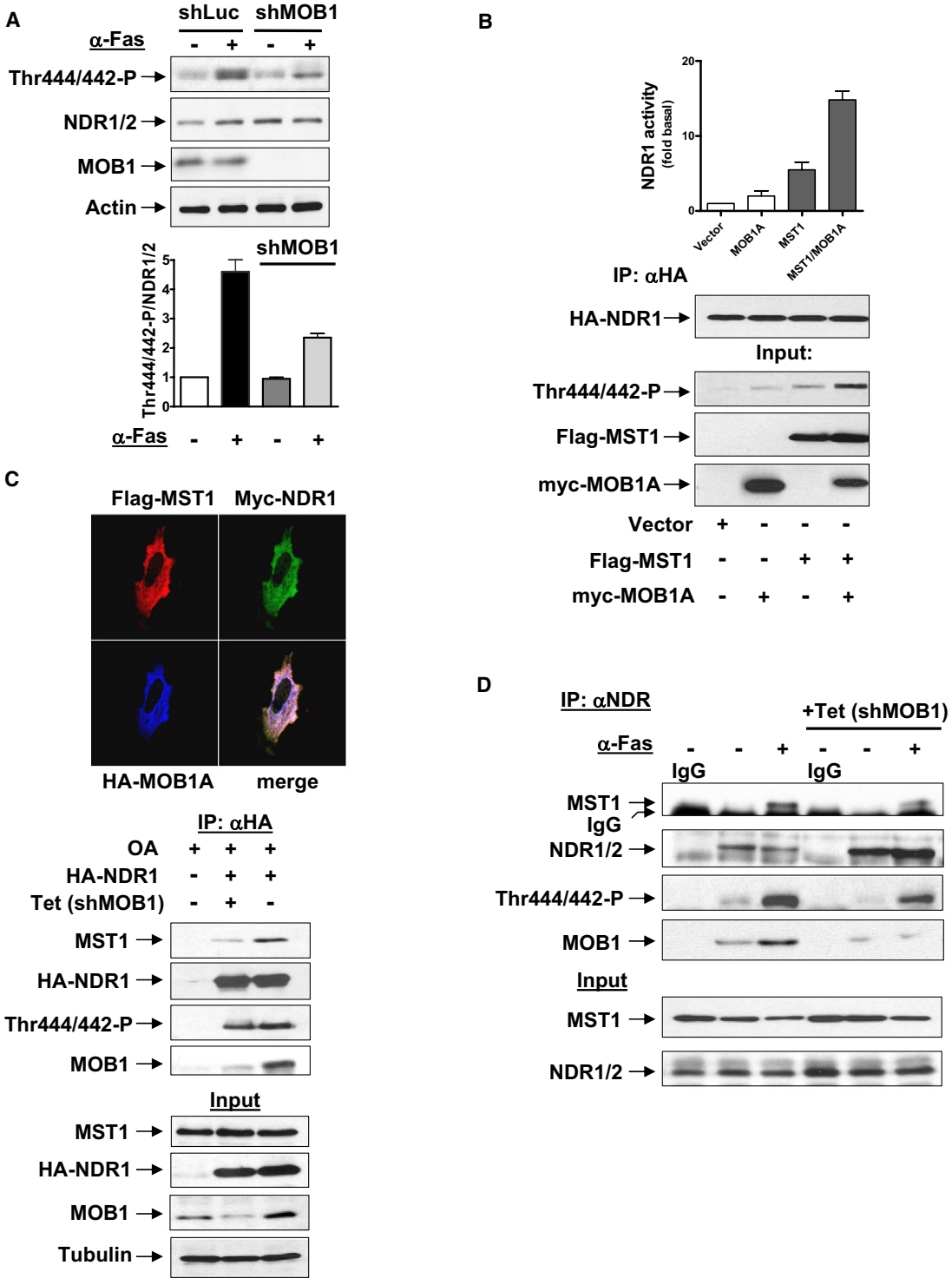

Figure 3. MOB1 Is Required for the Association of NDR and MST1 and for NDR Activation during Fas-Induced Apoptosis

(A) HeLa cells expressing shRNA against MOB1 and treated with a combination of CHX and Fas antibody for 6 hr were lysed and processed for western blotting with the indicated antibodies. The results are from three independently quantified experiments: data show mean ± standard deviation.

(B) HA-NDR1 precipitated from HEK293 cells transfected with either MST1 and MOB1A alone or their combination and was assayed for kinase activity on NDR substrate peptide. The results are from duplicate assays from two independent experiments; data show mean ± standard deviation.

(C) Indicated proteins were expressed in HeLa cells and their colocalization analyzed by confocal microscopy (top). HA-NDR1 expressed in U2OS with tetracycline-regulated shRNA against human MOB1 stimulated with okadaic acid (OA) was coimmunoprecipitated with endogenous MST1 in the absence of inducible MOB1 knockdown (bottom).

(D) Endogenous NDR1/2 were coimmunoprecipitated with endogenous MST1 after stimulation with Fas antibody from U2OS cells expressing inducible knockdown of MOB1 (compare lines 3 and 6).

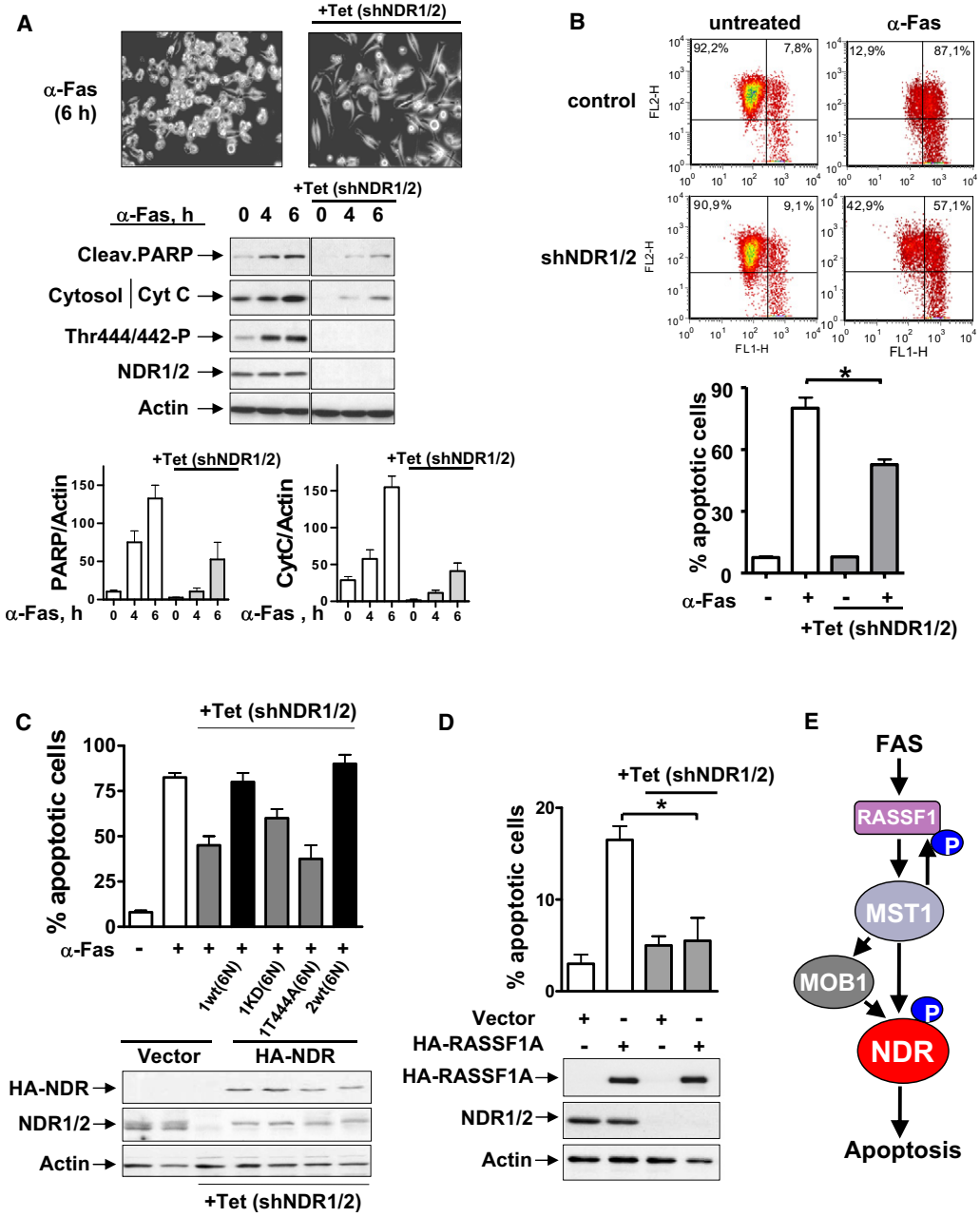

Figure 4. NDR Mediates Fas-Induced Apoptosis

(A) Images of HeLa cells expressing tet-inducible shRNA against NDR1/2 after 6 hr of stimulation with CHX alone or in combination with Fas antibody. Cells were lysed and analyzed by quantitative western blotting with the antibodies against total and active NDR1/2 (phospho-Thr444/Thr442), cleaved PARP, and cytochrome c. The results are from three independent experiments; data show mean ± standard deviation.

(B) Cells treated as in (A) were analyzed for depolarization of mitochondrial membrane potential by flow cytometry. Note that knockdown of NDR1/2 significantly reduced the number of cells undergoing apoptosis. Data including standard deviations from three independent experiments are displayed; *$p < 0.005$.

(C) HeLa cells expressing tet-inducible shRNA against NDR1/2 were transfected with empty vector, HA-NDR1 wild-type, kinase dead, Thr444Ala mutant, or HA-NDR2 wild-type that is refractory to siRNA [1wt(6N), 1kd(6N), 1T444A(6N), or 2wt(6N)]. Cells were treated as in (A) and analyzed for depolarization of mitochondrial membrane potential by flow cytometry. Data including standard deviations from three independent experiments are displayed.

(D) Apoptotic response to HA-RASSF1A overexpression was measured by flow cytometry in HeLa cells expressing tet-inducible shRNA against NDR1/2. Knockdown of NDR1/2 and expression levels of HA-RASSF1A were monitored by western blotting. Data including standard deviations from three independent experiments are displayed; *$p < 0.005$.

(E) Schematic representation of the signaling pathway triggered by Fas receptor stimulation with the subsequent activation of NDR1/2 via the RASSF1A/MST1.

suppressors [32–34], the possible involvement of NDR1/2 in cell transformation has not yet been addressed. The pathway we describe highlights the importance of NDR1/2 in the regulation of apoptosis and contributes to the mechanism of death receptor signaling, which is a part of antitumor defense and is often compromised in cancer.

Supplemental Data

Supplemental Data include Supplemental Experimental Procedures and four figures and can be found with this article online at http://www.current-biology.com/supplemental/S0960-9822(08)01430-9.

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Article

The MST1 and hMOB1 Tumor Suppressors Control Human Centrosome Duplication by Regulating NDR Kinase Phosphorylation

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Summary

Background: Human MST/hSAV/LATS/hMOB tumor suppressor cascades are regulators of cell death and proliferation; however, little is known about other functions of MST/hMOB signaling. Mob1p, one of two MOB proteins in yeast, appears to play a role in spindle pole body duplication (the equivalent of mammalian centrosome duplication). We therefore investigated the role of human MOB proteins in centrosome duplication. We also addressed the regulation of human centrosome duplication by mammalian serine/threonine Ste20-like (MST) kinases, considering that MOB proteins can function together with Ste20-like kinases in eukaryotes.

Results: By studying the six human MOB proteins and five MST kinases, we found that MST1/hMOB1 signaling controls centrosome duplication. Overexpression of hMOB1 caused centrosome overduplication, whereas RNAi depletion of hMOB1 or MST1 impaired centriole duplication. Significantly, we delineated an hMOB1/MST1/NDR1 signaling pathway regulating centrosome duplication. More specifically, analysis of shRNA-resistant hMOB1 and NDR1 mutants revealed that a functional NDR/hMOB1 complex is critical for MST1 to phosphorylate NDR on the hydrophobic motif that in turn is required for human centrosome duplication. Furthermore, shRNA-resistant MST1 variants revealed that MST1 kinase activity is crucial for centrosome duplication whereas MST1 binding to the hSAV and RASSF1A tumor suppressor proteins is dispensable. Finally, by studying the PLK4/HsSAS-6/CP110 centriole assembly machinery, we also observed that normal daughter centriole formation depends on intact MST1/hMOB1/NDR signaling, although HsSAS-6 centriolar localization is not affected.

Conclusions: Our observations propose a novel pathway in control of human centriole duplication after recruitment of HsSAS-6 to centrioles.

Introduction

Centrosomes function as the main microtubule-organizing centers in animal cells. Each centrosome is composed of two centrioles surrounded by pericentriolar material [1–3]. They play an important part in organizing the bipolar spindle during mitosis, ensuring equal distribution of genetic material between the two daughter cells. Centrosomal components are further required for the assembly and maintenance of cilia and flagella, two structures with essential functions in mammalian development and physiology [4, 5]. Therefore, the doubling of centrosomes during S phase (termed centrosome duplication) is under strict control. Studies with mammalian cells have shown that centriole duplication is orchestrated by different protein kinases, such as polo-like kinase 4 (PLK4), cyclin-dependent kinase 2 (Cdk2), and NDR kinases [6–9].

The NDR/LATS family is a subgroup of AGC serine/threonine protein kinases and consists of four related kinases (NDR1/STK38, NDR2/STK38L, LATS1, and LATS2) in the mammalian genome [10]. Although members of the NDR family have been detected on spindle pole bodies (SPB) and centrosomes, only human NDR1/2 kinases have been attributed a role in centrosome duplication [11]. Although LATS1/2 kinases are found on centrosomes, they are not involved directly in the regulation of centrosome duplication in human cells [9]. In multicellular organisms, LATS kinases play a central role in Hippo/SWH (Salvador/Warts/Hippo) signaling, which coordinates cell proliferation and apoptosis [12–14]. Initially delineated in flies as the Hpo/Sav/Lats/dMOB1/Yki network, mammalian MST/hSAV/LATS/hMOB/YAP tumor suppressor signaling was also defined recently [15]. In mammalian cells, this machinery regulates tissue homeostasis by balancing cell proliferation and apoptotic events, where hSAV, MST1/2, LATS1/2, and hMOB1 form complexes (summarized in [11]). However, very little is known about other molecular functions of MST/hMOB signaling.

Intriguingly, one study has already suggested that Mob1p (the yeast counterpart of human hMOB1A/B proteins) plays a role in SPB duplication [16]. Therefore, we analyzed in this study all six human MOB proteins (hMOBs: hMOB1A, hMOB1B, hMOB2, hMOB3A, hMOB3B, and hMOB3C) for a potential involvement in centrosome duplication. Given that MOB proteins can function together with Ste20-like kinases in yeast, fly, and human cells [10], we further expanded our study by addressing all human mammalian serine/threonine Ste20-like kinases (MSTs: MST1, MST2, MST3, MST4, and SOK1) in centrosome duplication. Significantly, we found that MST1/hMOB1 signaling is required for centrosome duplication. Furthermore, we show here that centriole formation depends on intact MST1/hMOB1/NDR signaling, although the association of HsSAS-6 with centrioles appears to be normal.

Results

Overexpression of hMOB1A/B Results in Centrosome Overduplication

Given the intriguing observation with Mob1p [16], we initially addressed hMOBs in human centrosome duplication by overexpression studies (Figure 1). All six human MOB proteins were overexpressed and the numbers of centrosomes per mononucleated cell were determined by immunofluorescence microscopy (Figure 1A). Except for hMOB2, all hMOBs were detected mainly in the cytoplasm (Figure 1A). Overexpression of hMOB1A/B caused a significant increase in cells displaying extra centrosomes (three or more centrosomes per cell), whereas expression of hMOB2, hMOB3A, and hMOB3B had no effect (Figure 1B). Overexpression of hMOB3C resulted in slightly increased centrosome amplification in U2-OS cells (Figure 1B) but did not cause centrosome amplification in

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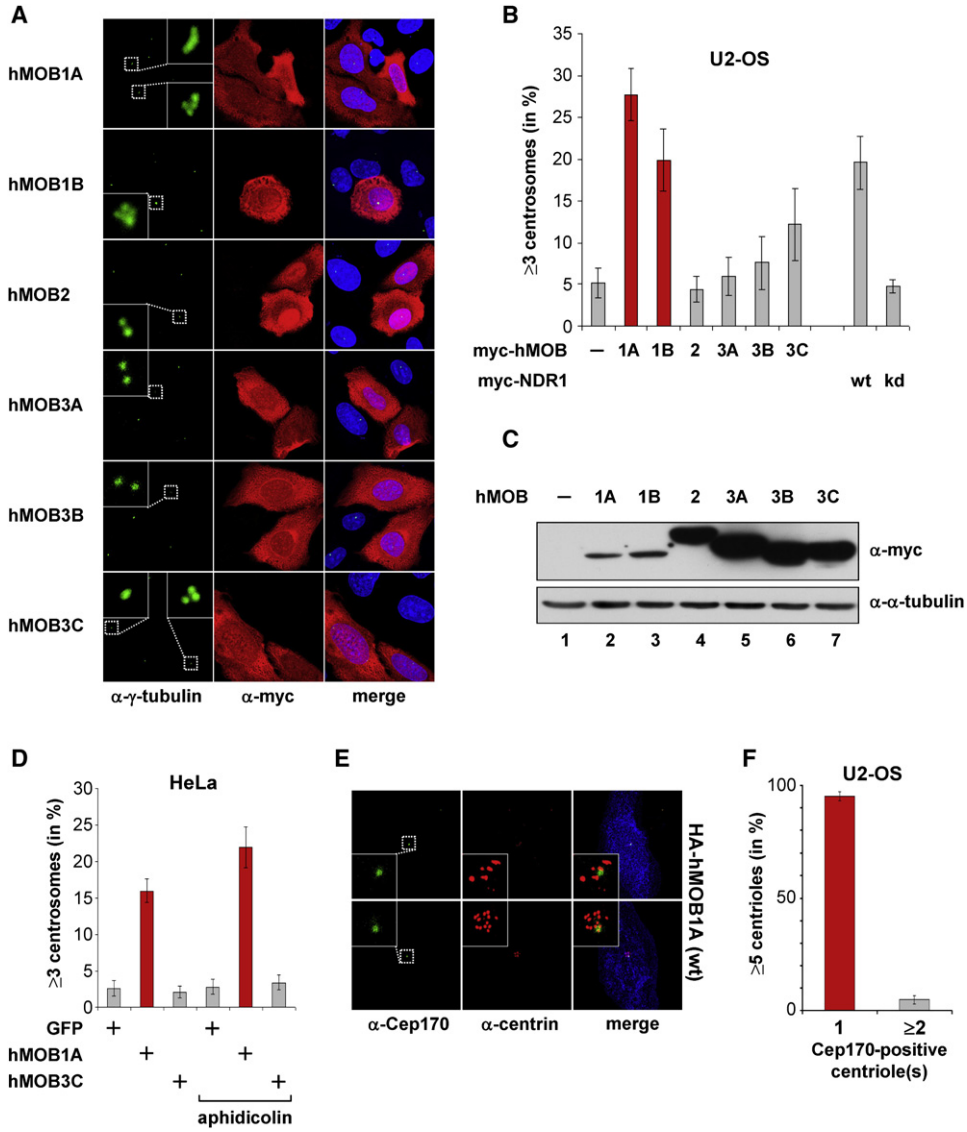

Figure 1. Overexpression of hMOB1A/B Leads to Centrosome Overduplication

(A and C) U2-OS expressing indicated human MOB proteins (1A, 1B, 2, 3A, 3B, or 3C) for 48 hr were processed for immunofluorescence (A) or immunoblotting (C) with the indicated antibodies.

(A) Insets show enlargements of centrosomes in green. DNA is stained blue.

(B) Histograms showing percentages of cells with excess centrosomes (more than three per mononucleated cell; ≥ 3). Cumulative data from two independent experiments with at least two replicates of 100 cells counted per experiment. Error bars indicate standard deviations.

(D) Histograms showing percentages of cells with supernumerary centrosomes in HeLa cells. Cells were incubated with aphidicolin (2 μg/ml) for 8 hr before being transfected with indicated cDNAs. Cells were incubated for a further 48 hr before processing for immunofluorescence. Cumulative data from two independent experiments with at least two replicates of 100 cells counted per experiment. Error bars indicate standard deviations.

(E) Staining of U2-OS cells expressing HA-hMOB1A(wt) with antibodies against Cep170 (green), centrin (red), and HA (blue). Enlargements of centrioles are shown.

(F) Quantification analysis of the experiment shown in (E). Cumulative data from three independent experiments with at least 100 cells counted per experiment. Error bars indicate standard deviations.

HeLa cells (Figure 1D). Notably, of the six hMOBs, expression levels of hMOB1A and hMOB1B were consistently the lowest for unknown reasons (Figure 1C; data not shown).

To investigate whether the generation of supernumerary centrosomes resulting from hMOB1A/B overexpression is a consequence of centriole/centrosome overduplication or failure of cytokinesis, we arrested HeLa cells in S phase by aphidicolin treatment and compared the induction of centrosome amplification to that in untreated normally cycling cells. Significantly, hMOB1A/B overexpression triggered centrosome amplification regardless of the presence or absence of aphidicolin (Figure 1D; data not shown). Centrosomes do not overduplicate spontaneously during prolonged S phase arrest in HeLa [17, 18], so these data suggest that hMOB1A/B overexpression causes centrosome amplification by an overduplication mechanism. To verify this finding, we analyzed hMOB1A-overexpressing cells with increased centrosome number for Cep170 staining (Figure 1E). If

supernumerary centrosomes are a result of overduplication, the majority of cells should contain only one Cep170-positive centriole, whereas failure of cell division would cause the accumulation of at least two mature Cep170-positive centrioles [19]. As seen in Figures 1E and 1F, the majority of hMOB1A-expressing cells with supernumerary centrioles displayed only one Cep170-positive centriole, suggesting that hMOB1A/B overexpression causes centrosome overduplication in our experimental system.

Endogenous hMOB1A/B Is Required for Centrosome (Over)duplication

To study endogenous hMOB1A/B, centrosome overduplication assays [20] were performed in U2-OS cells depleted of hMOB1A/B (Figure 2). In parallel to the generation of a hMOB1A/B antibody that selectively recognized hMOB1A and hMOB1B (Figure S1 available online), stable cell lines were generated expressing tetracycline-inducible short hairpin RNA (shRNA) directed against hMOB1A/B (Figure 2A). Of note, hMOB1A and hMOB1B mRNAs had to be targeted simultaneously by two different shRNAs, to allow efficient knockdown of total hMOB1A/B protein levels (Figure 2A). Significantly, centrosome amplification was altered in hMOB1A/B-depleted cells upon S phase arrest (Figures 2B and 2C). To ensure the specificity of our RNAi experiments, wild-type hMOB1A cDNAs refractory to shRNA were introduced into U2-OS cells expressing inducible vector-based RNAi (Figure 2D). Expression of shRNA-resistant hMOB1A restored centrosome overduplication upon depletion of endogenous hMOB1A/B (Figure 2E), indicating that the failure of hMOB1A/B-depleted cells to efficiently overduplicate centrosomes is due to specific knockdown of endogenous hMOB1A/B.

To address hMOB1A/B in normal centriole duplication, we analyzed centriole numbers at the end of the centriole duplication cycle [9, 21]. Nearly 50% of hMOB1A/B-depleted cells lacked at least one centriole in early mitotic stages (Figures 2F and 2G; control: 9.3% ± 1.7%; hMOB1A/B knockdown: 47.2% ± 8.7%). About 34% of hMOB1A/B-depleted cells displayed bipolar spindles containing only three centrioles instead of the normal four centrioles per cell (untreated control background: 8%), and 13% of hMOB1A/B-depleted cells contained only one or two centrioles (control: 2%). Depletion of endogenous hMOB1A/B in HeLa and diploid untransformed RPE1 cells also resulted in decreased centriole numbers (Figure S2). U2-OS cells expressing shRNA-resistant hMOB1A did not display a significant loss of centrioles upon depletion of endogenous hMOB1A/B (Figure 2G; control: 10.2% ± 2.9%; hMOB1A/B knockdown with shRNA-resistant hMOB1A: 13.5% ± 4%), suggesting that endogenous hMOB1A/B contributes to normal centriole duplication in human cells.

hMOB1A/B Regulates Hydrophobic Motif Phosphorylation of NDR Kinase

hMOB1A/B proteins have already been reported to interact with NDR kinases [22–27]; hence, NDR1/2 protein levels were analyzed in hMOB1A/B-depleted cells upon S phase arrest. Significantly, phosphorylation of NDR1/2 on Thr444 (the hydrophobic motif of NDR1) was strongly diminished upon hMOB1A/B knockdown (Figure 2A) and partially restored by expression of shRNA-resistant hMOB1A (Figure 2D). This finding was surprising, because recombinant hMOB1A did not cause an increase in NDR1/2 phosphorylation on Thr444 in vitro [22] and hMOB1A/B is not required for hydrophobic motif phosphorylation of LATS1 in cells [26]. Nevertheless, our findings suggest that hMOB1A/B regulates the phosphorylation of NDR1/2 on Thr444 by upstream kinase(s) upon S phase arrest of cells.

hMOB1/NDR Complex Formation Is Essential for Efficient Centrosome Duplication and NDR Phosphorylation

Before addressing the nature of the upstream kinase(s), we determined whether the interaction of hMOB1A/B with NDR1/2 is required for the regulation of centrosome duplication. First, the effects of selected NDR1 mutants (initially defined in [22, 25]) on the centrosome cycle were examined (Figure 3). These NDR1 mutants displayed intact hMOB2 binding, although interactions with hMOB1A/B (also termed hMOB1) were undetectable (Figure S3; data not shown). All NDR1 variants expressed at comparable levels and displayed similar subcellular distribution (Figures 3A and 3B; data not shown). However, only overexpression of NDR1(wt) resulted in centrosome amplification (Figure 3C). Overexpression of NDR1 kinase-dead (kd) or NDR1 deficient in hMOB1 binding did not increase centrosome numbers (Figure 3C). Expression of shRNA-resistant NDR1 mutants did not restore centrosome overduplication upon depletion of endogenous NDR1 (Figure 3E). These mutants also displayed dramatically decreased phosphorylation on Thr444 (Figure 3D), suggesting that NDR1/hMOB1 complex formation is required for Thr444 phosphorylation and centrosome amplification.

To further address the role of hMOB1/NDR complex formation, we generated a hMOB1A(E51K) mutant deficient in NDR1/2 binding (Figure S4). Overexpression of hMOB1A(E51K) did not lead to centrosome overduplication, even though expression and localization were not significantly changed (Figure S5). Moreover, shRNA-resistant hMOB1(E51K) did not compensate for the depletion of endogenous hMOB1A/B (Figure S5). Furthermore, although NDR1 expression and subcellular localization were not obviously affected upon knockdown of hMOB1A/B, NDR1-driven centrosome overduplication was impaired in hMOB1A/B-depleted cells (Figure S6; data not shown). Inversely, centrosome amplification resulting from overexpression of hMOB1A was decreased in NDR1-depleted cells (Figure S6; data not shown). Overall, the findings described in Figure 3 and Figures S5 and S6 strongly suggest that a functional hMOB1/NDR complex is indispensable for centrosome overduplication.

MST1 Kinase Regulates Centrosome (Over)duplication and NDR Phosphorylation in a hMOB1A/B-Dependent Manner

Our data shown in Figure 2 and Figure S5 suggested that hMOB1A/B regulates the phosphorylation of NDR1/2 by upstream kinase(s). Therefore, we investigated whether any of the postulated upstream activators (the entire group of MST kinases [10]) is responsible for Thr444 phosphorylation in a hMOB1A/B-dependent manner (Figure 4). In U2-OS cells, overexpression of MST1(wt) increased phosphorylation of endogenous NDR1/2 the most efficient (Figure 4A and Figure S7), although MST1, MST2, and MST3 can phosphorylate NDR1/2 on Thr444 in vitro [27, 28]. This increase in NDR1/2 phosphorylation was dependent on MST1 kinase activity (Figure 4A) and was blocked in hMOB1A/B-depleted cells but restored by expression of shRNA-resistant hMOB1A(wt) upon hMOB1A/B knockdown (Figure 4B). In full agreement with our previous observations (Figure 2 and Figure S5) these results suggest that MST1 phosphorylates NDR1/2 on Thr444 in a hMOB1A/B-dependent manner. Furthermore, they suggest that MST1 kinase might be involved

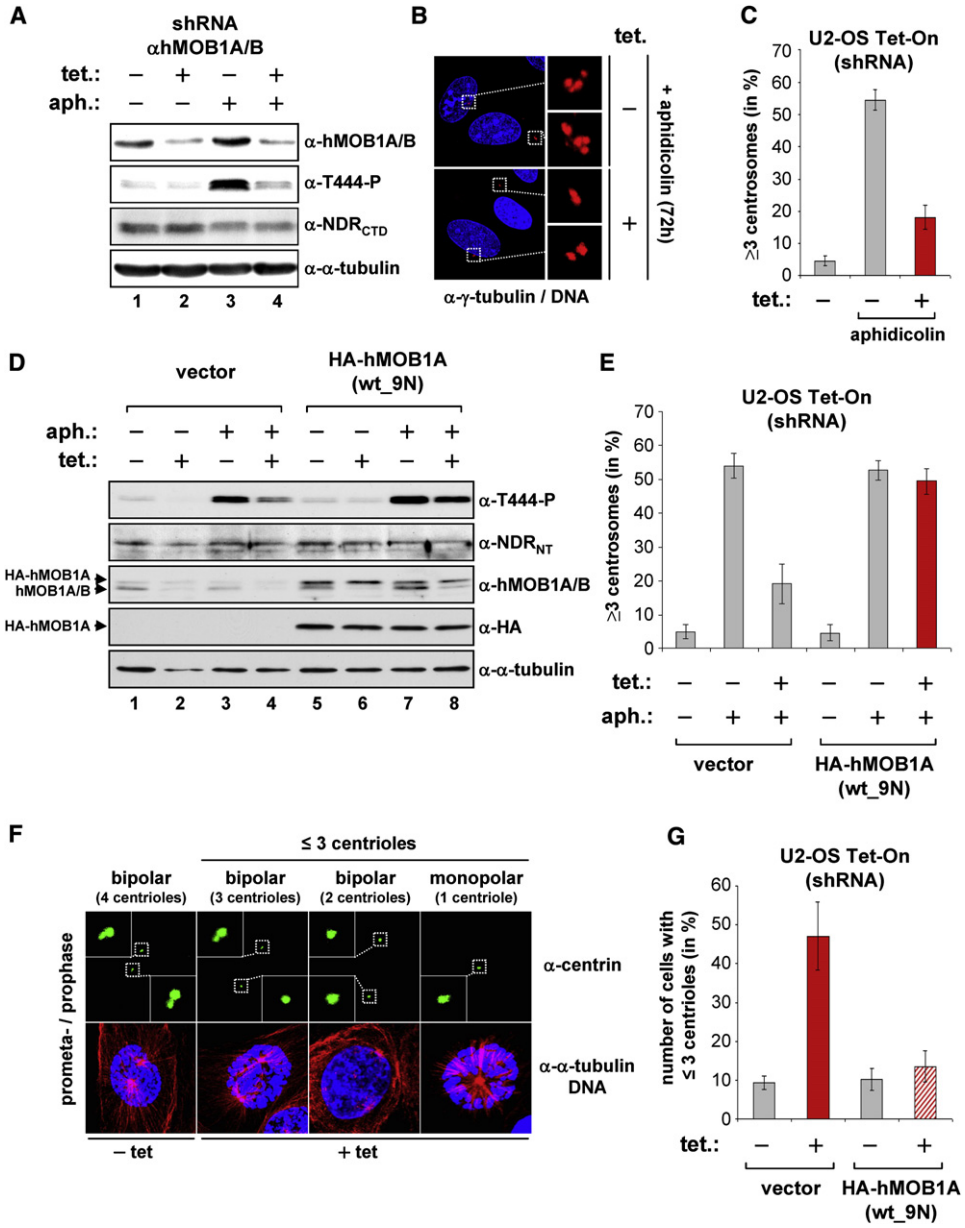

Figure 2. Endogenous hMOB1A/B Is Required for Centrosome Duplication

(A and B) U2-OS cells stably expressing tetracycline-regulated short-hairpin RNA (shRNA) directed against hMOB1A/B were incubated for 72 hr without (−) or with (+) tetracycline (2 μg/ml) and for a further 72 hr with aphidicolin (2 μg/ml), before processing for immunoblotting (A) or immunofluorescence (B) with the indicated antibodies.

(B) DNA is in blue. Insets show centrosome enlargements in red.

(C) Histograms showing percentages of cells with excess centrosomes (≥3) incubated without (−) or with (+) tetracycline, followed by incubation with aphidicolin. Cumulative data from three independent experiments with at least two replicates of 100 cells counted per experiment. Error bars indicate standard deviations.

(D) U2-OS stably expressing shRNA against hMOB1A/B were infected with empty vector (lanes 1–4) or HA-hMOB1A wild-type cDNA (lanes 5–8) that is refractory to shRNA [wt_9N]. After incubation for 72 hr with (+) or without (−) tetracycline and for an additional 72 hr with aphidicolin, cells were processed for immunoblotting with the indicated antibodies.

(E) In parallel, cells were processed for immunofluorescence to determine centrosome numbers per cell. Histograms show the percentage of cells with excess centrosomes (≥3) incubated without (−) or with (+) tetracycline, followed by incubation with aphidicolin. Cumulative data from two independent experiments with at least two replicates of 100 cells counted per experiment. Error bars indicate standard deviations.

(F) U2-OS cells expressing shRNA directed against hMOB1A/B were incubated without (−) or with (+) tetracycline for 96 hr, before processing for immunofluorescence with centrin (green) and α-tubulin (red) antibodies. DNA is shown blue.

(G) Histograms showing percentages of mitotic cells in prophase and prometaphase that displayed the loss of at least one centriole (≤3 centrioles per cell) in the presence (+) or absence (−) of tetracycline. Cumulative data from three independent experiments with at least 100 cells counted per experiment. Error bars indicate standard deviations.

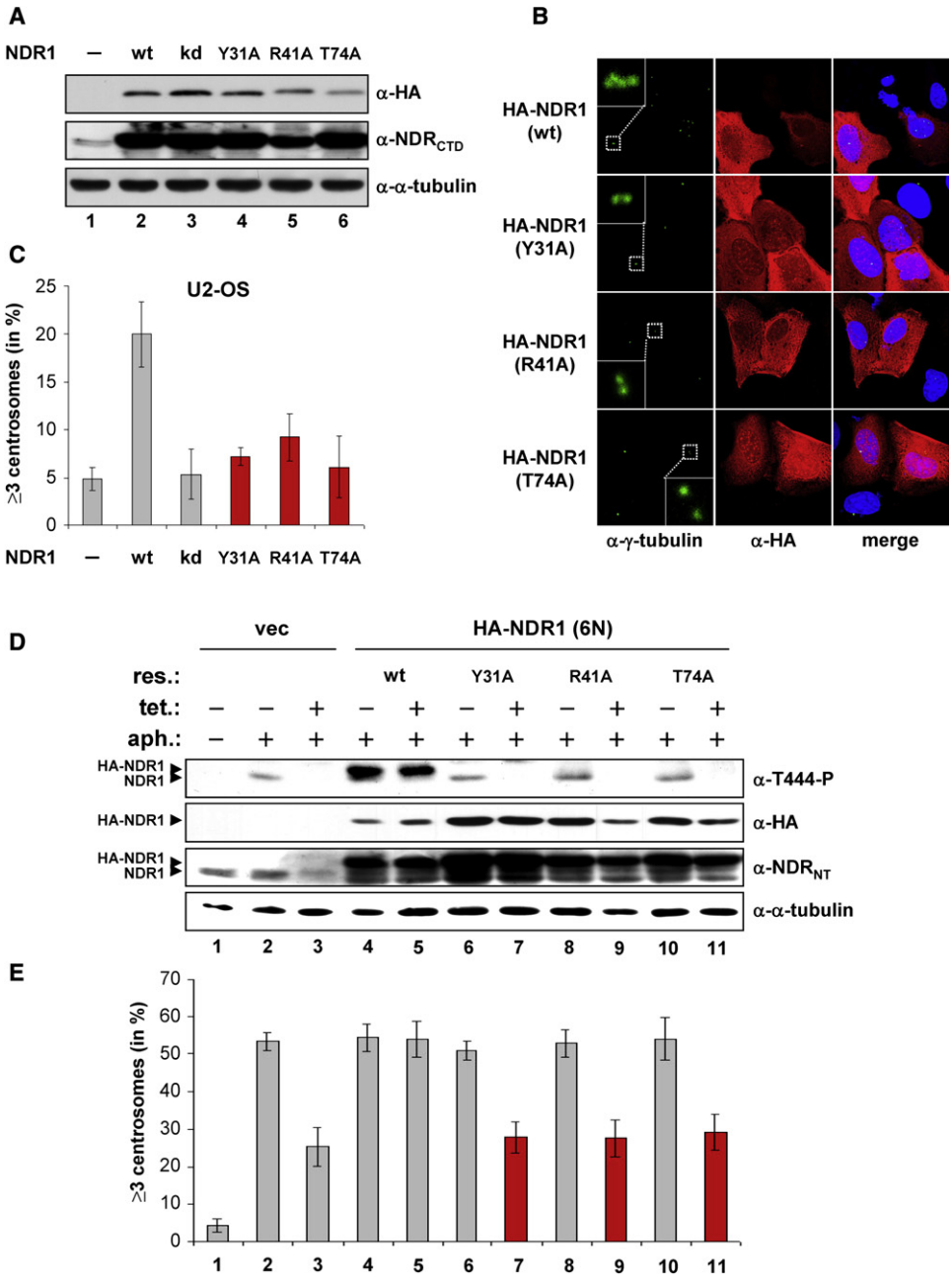

Figure 3. NDR1 Kinase Deficient in hMOB1A/B Binding Does Not Support Centrosome Amplification

(A and B) U2-OS cells expressing HA-NDR1 wild-type(wt), kinase-dead(kd), or hMOB1A/B binding mutants (Y31A, R41A, or T74A) for 48 hr were processed for immunoblotting (A) or immunofluorescence (B) with the indicated antibodies.

(B) Insets show enlargements of centrosomes in green. DNA is stained blue.

(C) Histograms showing percentages of cells with excess centrosomes (≥3). Cumulative data from three independent experiments, with at least 150 cells counted per experiment. Error bars indicate standard deviations.

(D and E) U2-OS cells stably expressing tetracycline-regulated short-hairpin (shRNA) directed against human NDR1 were infected with empty vector (lanes 1–3), HA-NDR1 wild-type (lanes 4 and 5), or hMOB1A/B binding mutants (lanes 6–11) that are refractory to shRNA [HA-NDR1(6N)]. After incubation for 72 hr without (–) or with (+) tetracycline and for a further 72 hr with aphidicolin, cells were analyzed by immunoblotting with the indicated antibodies (D) or by immunofluorescence for centrosome numbers (E).

(E) Histograms showing percentages of cells with excess centrosomes (≥3). Cumulative data from two independent experiments, with at least two replicates of 100 cells counted per experiment. Error bars indicate standard deviations.

in the regulation of centrosome (over)duplication and Thr444 phosphorylation upon S phase arrest.

To address this experimentally, stable cell lines were generated expressing tetracycline-inducible shRNA directed against MST1 (Figure 4C). Phosphorylation of NDR1/2 on Thr444 was decreased in MST1-depleted cells (Figure 4C), indicating that MST1 is the main upstream kinase under these conditions. Significantly, centrosome overduplication was decreased upon MST1 knockdown (Figure 4D). By analyzing the number of centrioles at the end of the centriole duplication

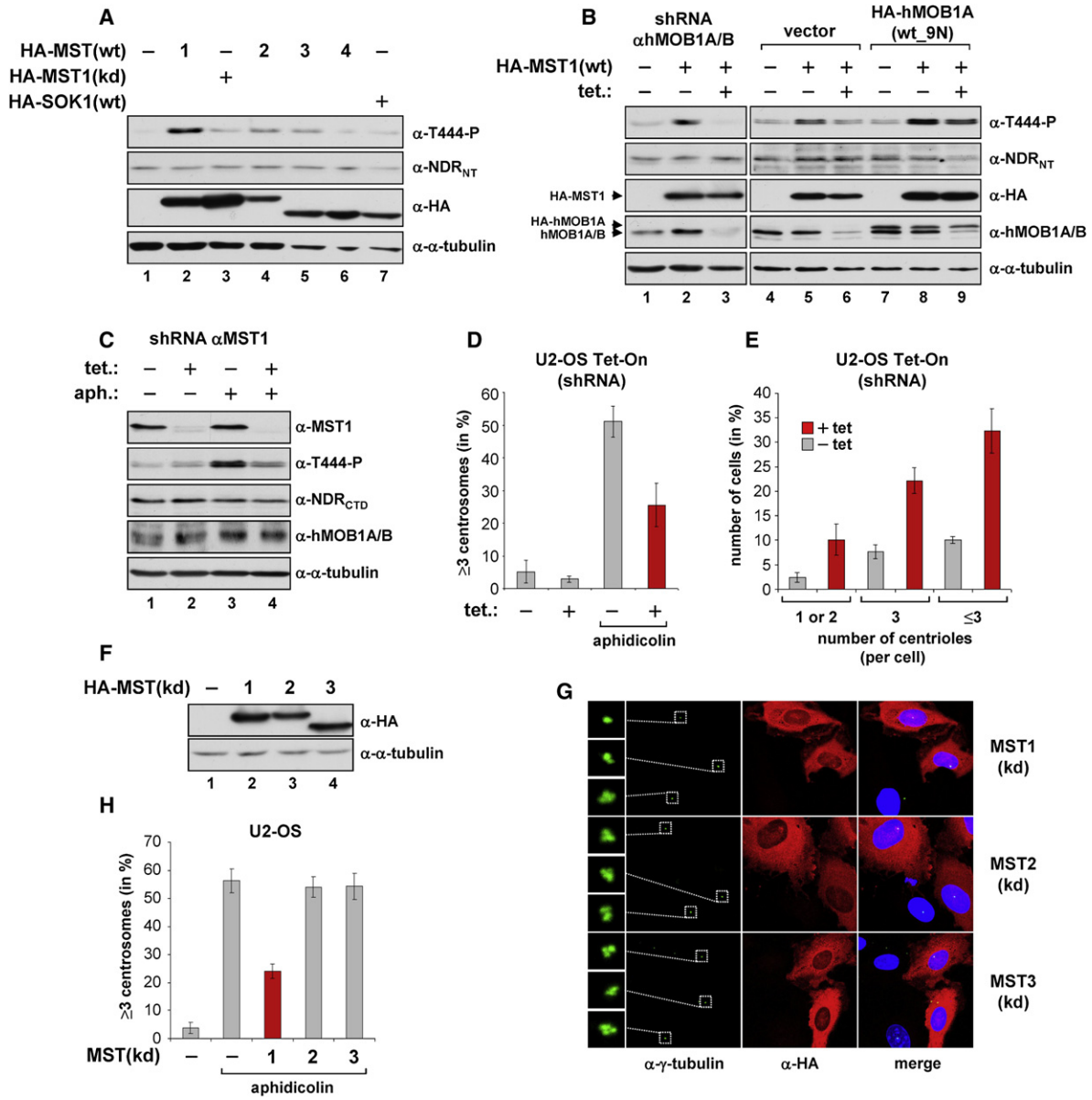

Figure 4. Human MST1 Kinase Is Crucial for Centrosome (Over)duplication

(A) U2-OS transfected with indicated cDNAs for 20 hr were processed for immunoblotting with the indicated antibodies.

(B) U2-OS stably expressing shRNA against hMOB1A/B (lanes 1–3), or the same cells infected with empty vector (lanes 4–6) or shRNA-resistant HA-hMOB1A (wt_9N) (lanes 7–9) were incubated without (−) or with (+) tetracycline for 72 hr before being transfected with HA-MST1(wt) overnight. Subsequently, cells were processed for immunoblotting with the indicated antibodies.

(C) U2-OS cells stably expressing tetracycline-regulated short-hairpin RNA (shRNA) directed against human MST1 were incubated for 72 hr without (−) or with (+) tetracycline and for a further 72 hr with aphidicolin, before processing for immunoblotting.

(D) In parallel, cells were analyzed by immunofluorescence microscopy. Histograms show the percentages of cells with excess centrosomes ($\geq$3) incubated without (−) or with (+) tetracycline, followed by incubation with aphidicolin. Cumulative data from three independent experiments with at least two replicates of 100 cells counted per experiment. Error bars indicate standard deviations.

(E) U2-OS stably expressing shRNA against MST1 were analyzed after 96 hr with (+) or without (−) tetracycline. Histograms show the percentage of mitotic cells in prophase and prometaphase that lost at least one centriole ($\leq$3 centrioles per cell). Cumulative data from three independent experiments with at least 100 cells counted per experiment. Error bars indicate standard deviations.

(F and G) U2-OS cells expressing the indicated cDNAs were processed for immunoblotting (F) or immunofluorescence (G) with indicated antibodies.

(G) Insets show centrosome enlargements in green. DNA is shown blue.

(H) Histograms showing percentages of cells with excess centrosomes ($\geq$3). Cells expressing the indicated kinase-dead(kd) MST kinases were incubated with aphidicolin for 72 hr. Cumulative data from four independent experiments with at least two replicates of 100 cells counted per experiment. Error bars indicate standard deviations.

cycle (see Figure 2F), we found that 32% of MST1-depleted cells lacked at least one centriole (Figure 4E; control: 10.1% ± 0.7%; MST1 knockdown: 32.3% ± 4.5%). Knockdown of endogenous MST1 in HeLa and RPE1 cells also caused loss of centrioles (Figure S2), indicating that endogenous MST1 contributes to normal centriole duplication in different human cells.

Next, we analyzed the consequence of MST1 kinase-dead (kd) overexpression (Figures 4F–4H), because MST1(kd) can function as a dominant-negative kinase [29]. Significantly, centrosome overduplication was impaired in U2-OS cells expressing MST1(kd), whereas overexpression of MST2(kd), MST3(kd), MST4(kd), or YSK1/SOK1(kd) had no effect, despite similar expression and subcellular localization patterns (Figures 4F–4H; data not shown). Because MST2(kd), MST3(kd), MST4(kd), and SOK1(kd) can also function as dominant-negative kinases [30], these findings suggest that mainly MST1 contributes to centrosome overduplication in our settings. However, although MST1 plays a role in NDR1/2 phosphorylation and centrosome duplication (Figure 4), these findings did not necessarily demonstrate that NDR-driven centrosome duplication requires MST1. Thus, we determined the effect of NDR1(wt) or hMOB1A overexpression on centrosome amplification in MST1-depeleted cells, revealing that NDR1- or hMOB1A-driven centrosome overduplication was impaired in MST1-depleted cells (Figure S8). Therefore, it is very likely that centrosome duplication is regulated by MST1/hMOB1/NDR signaling in our experimental systems.

MST1 Kinase Activity, but Not RASSF1A or hSAV Binding, Is Required for Centrosome Amplification

MST1 kinase is controlled by various mechanisms [30], most importantly by binding to RASSF1A, hSAV, or to itself via a C-terminally located SARAH (Sav/Rassf/Hippo) domain [31–35]. Therefore, we generated a C-terminally truncated form of MST1 (residues spanning 1–433; termed ΔC) that was deficient in hSAV-, RASSF1A-, and homodimer-complex formation (Figure S9). Surprisingly, overexpressed MST1(ΔC) phosphorylated NDR1/2 on Thr444 similarly to MST1(wt) kinase (Figure S9).

Given this observation, we introduced MST1(wt), (kd), and (ΔC) cDNAs refractory to shRNA into U2-OS-expressing inducible vector-based RNAi against MST1 (Figure 5A). Although expression of shRNA-resistant MST1(wt) restored centrosome overduplication and Thr444 phosphorylation of NDR1/2 in MST1-depleted cells, shRNA-resistant MST1(kd) did not compensate for MST1 depletion, despite similar localization and expression levels (Figure 5; data not shown). Significantly, expression of shRNA-resistant MST1(ΔC) supported centrosome amplification upon depletion of endogenous MST1 (Figure 5B) and phosphorylation of Thr444 (Figure 5A). These data show that binding of MST1 to RASSF1A, hSAV, or homodimer formation through the SARAH domain is dispensable for centrosome overduplication, whereas MST1 kinase activity is crucial.

The MST1/hMOB1/NDR Cascade Is Required for Human Centriole Duplication, but Dispensable for Centriolar "Seed" Formation

To understand in more detail the role of MST1/hMOB1/NDR signaling in human centriole duplication, we investigated whether the MST1/hMOB1/NDR machinery is required for PLK4-driven centriole biogenesis (Figure 6). As already reported [21], overexpression of PLK4 is sufficient to trigger centriole amplification, where two types of procentriole arrangements have been observed: centrioles arranged either in (1) flower-like structures around parental centrioles, or (2) clusters of centrioles after disengagement [36]. Significantly, PLK4-driven centriole amplification was impaired in hMOB1A/B-, MST1-, or NDR1-depleted cells, although PLK4 expression and centriole localization were not obviously

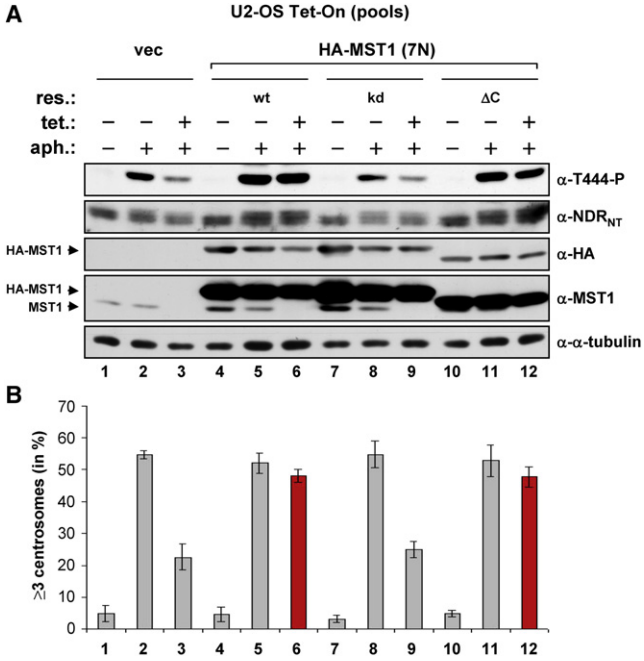

Figure 5. MST1 Kinase Activity, Not the SARAH Domain, Is Required for Centrosome Overduplication in Human Cells

(A) U2-OS stably expressing shRNA against MST1 were infected with empty vector (lanes 1–3), HA-MST1 wild-type (lanes 4–6), kinase-dead (lanes 7–9), or a C-terminally truncated mutant cDNA (ΔC; lanes 10–12) that is refractory to shRNA [wt_7N, kd_7N, or ΔC_7N]. After incubation for 72 hr with (+) or without (−) tetracycline and for an additional 72 hr with aphidicolin, cells were processed for immunoblotting with indicated antibodies.

(B) In parallel, the number of centrosomes per cell was determined. Histograms show the percentage of cells with excess centrosomes. Cumulative data from three independent experiments with at least two replicates of 100 cells counted per experiment. Error bars indicate standard deviations.

affected (Figure 6; Figure S10; data not shown). Moreover, no significant differences in cell cycle profiles were observed upon depletion of MST1/hMOB1/NDR signaling components and/or PLK4 overexpression (Figure 6D; Figures S10 and S11), suggesting that the observed defect in centriole amplification is not simply a consequence of a general cell cycle arrest. Overall, reduction of MST1/hMOB1/NDR signaling appears to negatively affect PLK4-driven centriole biogenesis without any direct effect on PLK4 expression, subcellular localization, and cell cycle profiles.

Next, we addressed whether MST1/hMOB1/NDR signaling might play a role in other steps of the centriole assembly pathway conserved from lower to higher eukaryotes [37–52]. In human cells, after the activation of PLK4 on the parental centriole, γ-tubulin, CPAP, Cep135, and HsSAS-6 are rapidly recruited to the centriole [36]. Then, CP110 forms a cap on the newly forming procentriole, and finally, the centriole grows by addition of tubulin. To determine any involvement of MST1/hMOB1/NDR signaling in this pathway, we focused our analysis on centrosomes and centrioles at the end of the centriole duplication cycle (as already defined in Figure 2F). Cell lines expressing inducible shRNA directed against hMOB1A/B, MST1, or NDR1 were cultured in the absence or presence of tetracycline without apparent changes in cell cycle profiles and protein expression, except for the targeted proteins of interest (Figures 7A and 7B; Figures S11 and S12). As expected [53, 54], normal prophase cells (with condensed DNA and

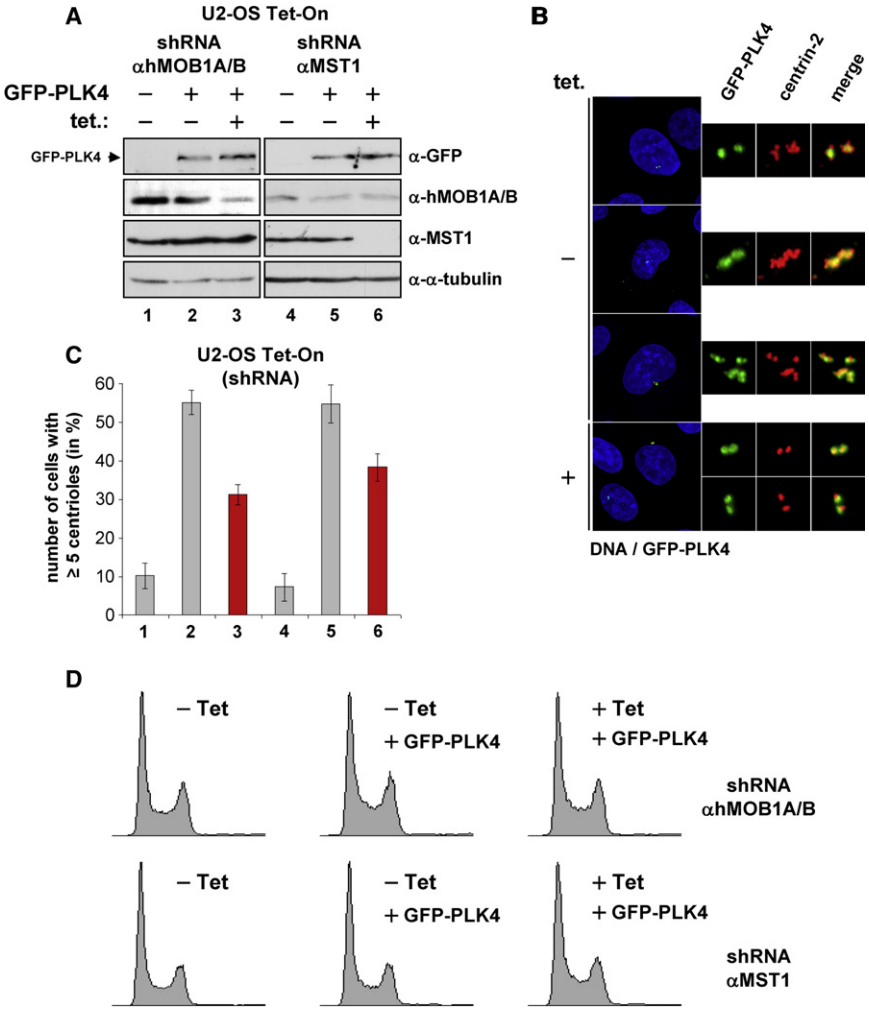

Figure 6. PLK4-driven centriole biogenesis is impaired upon hMOB1A/B or MST1 depletion

(A and B) U2-OS stably expressing shRNA against hMOB1A/B (lanes 1–3), or MST1 (lanes 4–6) were incubated without (–) or with (+) tetracycline for 72 hr before being transfected with GFP-PLK4(wt) overnight. Subsequently, cells were processed for immunoblotting (A) or immunofluorescence (B) with the indicated antibodies.

(B) Insets show enlargements of centrioles. GFP-PLK4 is in green and centrioles are shown in red. DNA is stained blue.

(C) Histograms showing percentages of cells with excess centrioles ($\geq$5). Cumulative data from two independent experiments, with at least two replicates of 100 cells counted per experiment. Error bars indicate standard deviations.

(D) In parallel, cells from the same samples were analyzed for DNA content by FACS.

separated centrosomes) displayed one single HsSAS-6 dot or two CP110 signals at each spindle pole (Figures 7C and 7E). Interestingly, the association of HsSAS-6 with prophase centrosomes was not altered upon hMOB1A/B, MST1, or NDR1 depletion (Figure 7C; data not shown). Irrespective of the decreased centriole number per spindle pole, HsSAS-6 associated with centriole pairs or single centrioles (Figure 7D; data not shown).

Next, to address in more detail whether the cell cycle-dependent centriole localization of HsSAS-6 [54] relies on MST1/hMOB1/NDR signaling components, we determined the cell cycle stages of individual cells by PCNA staining (Figures S13 and S14). In full agreement with existing literature [54], HsSAS-6 was not detected on centrioles in most U2-OS cells during G1 phase, but was present on the majority of centrioles in S and G2 phase (Figure S14). It is noteworthy that depletion of hMOB1A/B, MST1, or NDR1 did not affect this cell cycle-regulated HsSAS-6 localization pattern (Figure S14; data not shown). Taken together, these findings suggest that the initiation of procentriole formation (also termed centriolar "seed" formation; see [36, 54]) is independent of MST1/hMOB1/NDR signaling.

In contrast, despite unaffected DNA condensation and centrosome separation, a significant portion of hMOB1A/B-, MST1-, or NDR1-depleted cells lacked at least one CP110 centriole signal in prophase (Figure 7E; control [without tetracycline induction], 8% [n = 130]; hMOB1A/B knockdown, 44% [n = 142]; control, 7% [n = 121]; MST1 knockdown, 30% [n = 127]; control, 9% [n = 131]; NDR1 knockdown, 41% [n = 116]). As already described for centrin-2 (see Figures 2F, 2G, and 4E), CP110 signals displayed reduced numbers of centrioles in depleted cells (Figure 7F; data not shown). Two additional centriole markers (glutamylated-tubulin and acetylated-α-tubulin) further confirmed that centriole numbers are decreased upon hMOB1A/B, MST1, or NDR1 depletion (Figure S15; data not shown). Overall, the analysis of depleted cells with five independent centriole markers—HsSAS-6, CP110, centrin-2, glutamylated-tubulin, and acetylated-α-tubulin—revealed that MST1/hMOB1/NDR signaling is required for normal centriole duplication in human cells, although the association of HsSAS-6 with centrioles does not appear to be affected.

Discussion

Taken together, our findings indicate that MST1/hMOB1/NDR signaling contributes to centriole duplication in human cells. Endogenous hMOB1A/B and MST1 are required for normal centriole duplication (Figures 2, 4, and 5). The association of hMOB1A/B with NDR1/2 kinases is essential for centrosome duplication (Figure 3; Figure S5). Moreover, centrosome overduplication requires MST1 kinase activity but is independent of the SARAH domain of MST1 (Figure 5). Because MST1 binding to hSAV, RASSF1A, NORE1, and CNK1 depends on the SARAH domain of MST1 [30], this suggests that all currently known activators/inhibitors of MST1 are unlikely to contribute to MST1 signaling in centrosome duplication.

Our data would indicate that MST1 kinase activity plays a role in human centrosome duplication, although MST1 is best known as a proapoptotic kinase [30] whose activity is enhanced by RASSF1A/MST1 complex formation [34]. RASSF1A binding to MST1 through the SARAH domain also increased NDR1/2 kinase activity in apoptotic cells [27]. In contrast, MST1 signaling in centrosome duplication is SARAH

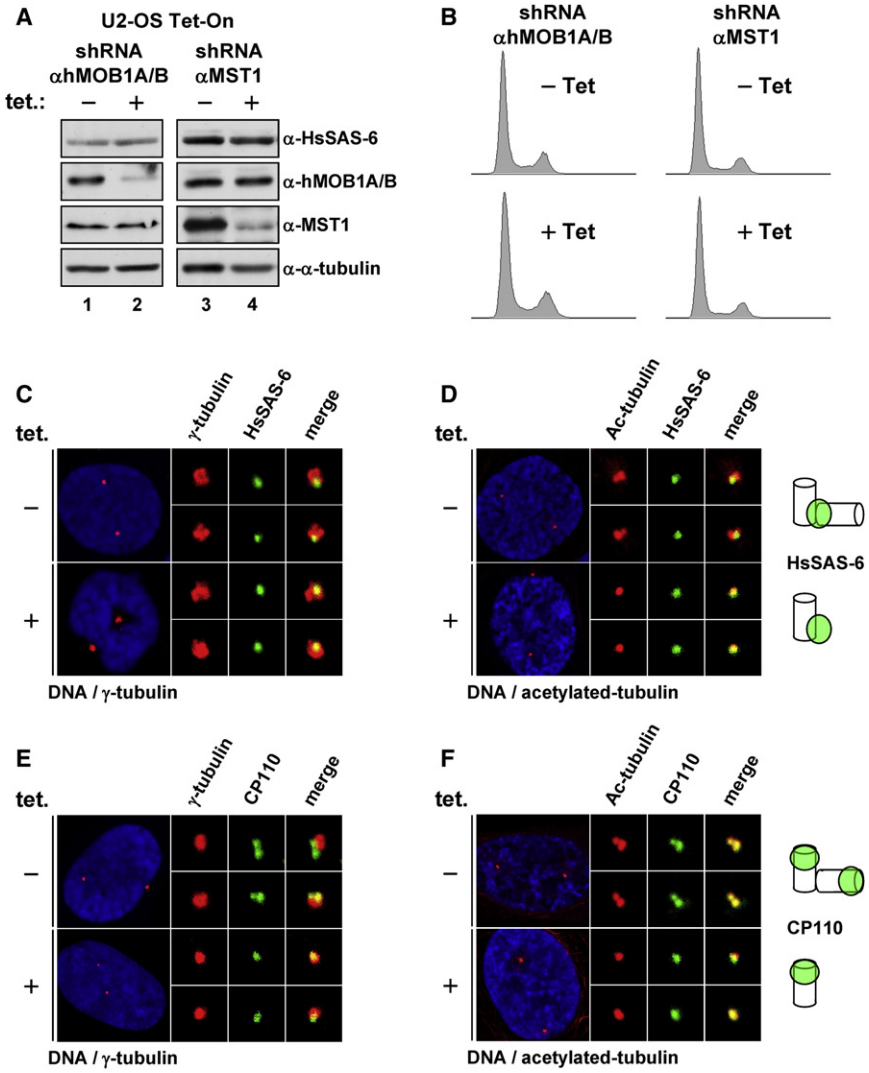

Figure 7. Centriole Localization of CP110, but Not of HsSAS-6, Depends on MST1/hMOB1 Signaling

(A and B) U2-OS cells expressing shRNA directed against hMOB1A/B (lanes 1 and 2) or MST1 (lanes 3 and 4) were incubated without (–) or with (+) tetracycline for 96 hr before processing for immunoblotting (A) or DNA content analysis by FACS (B).

(C–F) In parallel, U2-OS cells expressing shRNA directed against hMOB1A/B were processed for immunofluorescence with indicated antibodies. γ-tubulin (centrosome) and acetylated-α-tubulin (centriole) stainings are in red. DNA is shown blue. Insets show enlargements of centrosomes/centrioles. Schemes on the right indicate HsSAS-6 and CP110 localization on centrioles in green.

domain independent (Figure 5). This suggests that the SARAH domain of MST1 might exemplify the means by which human cells utilize similar signaling systems for the regulation of very different biological processes (e.g., programmed cell death versus centrosome duplication in the case of MST1/hMOB1/NDR signaling). Considering further that centrosome duplication occurs in S phase, it is tempting to speculate that MST1 kinase regulates NDR1/2 kinases in a cell cycle-dependent manner. Intriguingly, we could confirm the reported [55] S-phase-induced phosphorylation of MST1 (Figure S16), suggesting that MST1 kinase activity could oscillate during the cell cycle. As a result, a new line of research will be required to elucidate how MST1 activity is regulated (in)dependently of its SARAH domain during the cell cycle. Hence, future research addressing the role of MST1/hMOB1/NDR signaling in cell cycle progression is warranted.

Our data suggest that MST1 regulates human centrosome duplication through the phosphorylation of endogenous NDR1/2 in S phase (Figures 4 and 5). However, human LATS1/2, histone 2B (H2B), and FoxO have also been identified as MST1 substrates [56–58]. In this context, it is noteworthy that LATS1/2 are not involved in centrosome duplication [9]. Of further importance, caspase-cleaved MST1 phosphorylates H2B in the nucleus, whereas full-length MST1 targets cytosolic FoxO1 in a SARAH domain-dependent manner [59]. Therefore, considering that cytoplasmic MST1 lacking the SARAH domain is capable of driving centrosome overduplication (Figure 5; data not shown), it is rather unlikely that the phosphorylation of H2B or FoxO by MST1 plays a role in the centrosome cycle. Taking into further account that shRNA-resistant NDR1(T444A) cannot restore centrosome overduplication in NDR1-depleted cells (Figure S17), our data indicate that the phosphorylation of NDR1/2 on Thr444 by MST1 is a key event in the regulation of centrosome duplication in this setting.

We also found that hydrophobic motif phosphorylation of NDR1/2 requires endogenous hMOB1A/B in addition to MST1 (Figures 2 and 4). The analysis of NDR1 and hMOB1A mutants (Figure 3; Figure S5) showed that a functional NDR/hMOB1 complex is critical for the phosphorylation of NDR on the hydrophobic motif by MST1, which in turn is required for human centrosome duplication (Figure S18).

Significantly, we also addressed at which step MST1/hMOB1/NDR signaling controls centriole duplication in human cells. Although PLK4-driven centriole amplification is impaired upon depletion of MST1/hMOB1/NDR components, the association of PLK4 with centrioles is not affected (Figure 6). The recruitment of HsSAS-6 to centrioles is also independent of the MST1/hMOB1/NDR cascade (Figure 7). Therefore, the two first steps of the human centriole assembly pathway [36, 54], namely PLK4 and HsSAS-6 localization to centrioles, appear to be normal upon knockdown of MST1/hMOB1/NDR signaling components. However, recruitment of centrin-2 and CP110 to procentrioles appears to depend on an intact MST1/hMOB1/NDR cascade (Figures 2, 4, and 7). Although the incorporation of CP110 and centrin-2 into nascent procentrioles occurs rapidly [36], we observed that a significant fraction of MST1/hMOB1/NDR knockdown cells displayed reduced centriole staining (Figures 2, 4, and 7). Collectively, these observations suggest that MST1/hMOB1/NDR signaling is important for efficient centriole duplication (daughter centriole formation), even though the initiation of procentriole

formation (as monitored by HsSAS-6 association with centrioles) appears to be normal (Figure S18).

The stabilization of a first centriolar seed is very likely to be a rate-limiting step in human centriole duplication [36, 54]. However, our data would suggest that human centriole duplication can also be regulated after the initiation step involving PLK4/HsSAS-6. Currently, we do not know precisely at which step centriole duplication is blocked in MST1-, hMOB1-, or NDR1-depleted cells (Figure S18). Most likely, a combination of live cell imaging and electron microscopy will be required to decipher exactly at which stage daughter centriole formation depends on MST1/hMOB1/NDR signaling. Given that the MST1/hMOB1/NDR cascade also plays a role in the regulation of apoptosis [27], future research will also be needed to address how MST1/hMOB1/NDR signaling can be fine-tuned to allow the regulation of different biological aspects by the same signaling modules. In this context, another future challenge will be the identification of NDR substrates that play a direct role in centriole duplication and/or apoptosis. A further challenge will be to test how far the role of MST1/hMOB1/NDR signaling in centrosome duplication is conserved from yeast to man. Taken together, the elucidation of a role for the MST1/hMOB1/NDR pathway in centrosome duplication reported here might open novel avenues in the pursuit of centriole duplication signaling as well as molecular function(s) that might contribute to tumor-suppressing activities of MST1 and hMOB1.

Experimental Procedures

Cell Culture, Transfections, and Chemicals

U2-OS, HeLa, PT67, COS-7, and RPE1-hTert cells were maintained in DMEM supplemented with 10% fetal calf serum. U2-OS, HeLa, COS-7, and RPE1-hTert cells were plated at a consistent confluence and transfected with Fugene 6 (Roche), jetPEI (PolyPlus Transfection), or Lipofectamine 2000 (Invitrogen) as described by the manufacturer. Aphidicolin was from Calbiochem.

Generation of Stable Cell Lines

To generate tetracycline-inducible cell lines, U2-OS T-Rex cells were transfected with pTER constructs [60] expressing shRNA against hMOB1A/B or MST1. Cell clones were selected and maintained as described previously [9]. Retroviral pools of rescue cell lines were generated as described elsewhere [9]. U2-OS Tet-On cells expressing tetracycline-regulated shRNA against NDR1 have been described already [9].

Immunoblotting, Immunoprecipitation, Cell Fractionation, Immunofluorescence Microscopy, and FACS

Immunoblotting, coimmunoprecipitation, and cell fractionation experiments were performed as described [25]. Cells were processed for FACS and immunofluorescence as defined elsewhere [9].

Supplemental Data

Supplemental Data include Supplemental Experimental Procedures (construction of plasmids; antibody sources) and 21 figures and can be found with this article online at http://www.cell.com/current-biology/supplemental/S0960-9822(09)01698-4.

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Nuclear Dbf2-related protein kinases (NDRs) in isolated cardiac myocytes and the myocardium: Activation by cellular stresses and by phosphoprotein serine-/threonine-phosphatase inhibitors

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ABSTRACT

The nuclear Dbf2-related protein kinases 1 and 2 (NDR1/2) are closely-related AGC family kinases that are strongly conserved through evolution. In mammals, they are activated *inter alia* by phosphorylation of an hydrophobic domain threonine-residue [NDR1(Thr-444)/NDR2(Thr-442)] by an extrinsic protein kinase followed by autophosphorylation of a catalytic domain serine-residue [NDR1(Ser-281)/NDR2(Ser-282)]. We examined NDR1/2 expression and regulation in primary cultures of neonatal rat cardiac myocytes and in perfused adult rat hearts. In myocytes, transcripts for NDR2, but not NDR1, were induced by the hypertrophic agonist, endothelin-1. NDR1(Thr-444) and NDR2(Thr-442) were rapidly phosphorylated (maximal in 15–30 min) in myocytes exposed to some phosphoprotein Ser-/Thr-phosphatase 1/2 inhibitors (calyculin A, okadaic acid) and, to a lesser extent, by hyperosmotic shock, low concentrations of H_2O_2, or chelerythrine. In myocytes adenovirally-transduced to express FLAG-NDR2 (which exhibited a mainly-cytoplasmic localisation), the same agents increased FLAG-NDR2 activity as assessed by *in vitro* protein kinase assays, indicative of FLAG-NDR2(Ser-282/Thr-442) phosphorylation. Calyculin A-induced phosphorylation of NDR1(Thr-444)/NDR2(Thr-442) and activation of FLAG-NDR2 were inhibited by staurosporine, but not by other protein kinase inhibitors tested. In *ex vivo* rat hearts, NDR1(Thr-444)/NDR2(Thr-442) were phosphorylated in response to ischaemia-reperfusion or calyculin A. From a pathological viewpoint, we conclude that activities of NDR1 and NDR2 are responsive to cytotoxic stresses in heart preparations and this may represent a previously-unidentified response to myocardial ischaemia *in vivo*.

1. Introduction

The nuclear Dbf2-related protein kinases (NDRs) are members of the AGC protein serine-/threonine-kinase family and are strongly conserved between species as diverse as yeasts and *Homo sapiens* [1,2]. Human (and mouse) cells express two isoforms encoded by separate genes, namely NDR1 (or serine-/threonine kinase 38, STK38) and NDR2 (or serine-/threonine kinase 38-like, STK38L) [1,2]. These cells also express two NDR1-/NDR2-related protein kinases, large tumour suppressor-1 (LATS1) and LATS2 [1,2]. The NDRs are widely-expressed in mouse [3] and human [4,5] tissues. In mouse, NDR1 transcripts are most-highly expressed in spleen and are also moderately expressed in brain, lung, thymus, testis and adipose tissue whereas NDR2 transcripts are most-highly expressed in the gastro-intestinal tract (large and small intestine, stomach) and moderately expressed in testis [3]. The heart also expresses NDR1 and NDR2 transcripts, albeit at lower levels [3]. In human tissues, NDR1 and NDR2 are most highly expressed in thymus [4]. NDR1 is also highly expressed in human skeletal muscles whereas NDR2 is highly expressed in heart and brain [4]. It is not known whether these apparent differences in expression profiles between mouse and *Homo sapiens* are real or reflect inter-laboratory variations. The consensus sequence phosphorylated by this group of kinases has been variously reported to be (K/R)XX(S/T) (human NDR1 [6]), RXXS (preferred to RXXT) (yeast Dbf2 [7]) or HX(R/H/K)XX(S/T) (human LATS1 [8]). The physiological functions of the NDRs in mammals are obscure. NDR2 may be involved in the organisation of the actin cytoskeleton in pheochromocytoma 12 (PC12) cells and its transcript is also rapidly but transiently induced in mouse amygdalae following Pavlovian fear conditioning training [9]. Recently, NDR1/NDR2 have been implicated in centrosome duplication with overexpression inducing centrosome overduplication [10].

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In their inactive state, NDRs are autoinhibited by an insert between the catalytic domain subdomains VII and VIII (the autoinhibitory sequence) that is rich in basic residues [11] and was originally thought to represent a nuclear localisation signal [5]. Activation of NDRs involves regulatory phosphorylations and/or interactions with other proteins. As with many AGC kinases, NDRs are phosphorylated on two conserved residues, one in the C-terminal hydrophobic domain (Thr-444 in human, mouse or rat NDR1, Thr-442 in human or mouse NDR2) and one in the activation loop (or T loop) of the catalytic domain (Ser-281 in human, mouse or rat NDR1, Ser-282 in human or mouse NDR2) [3,12]. One protein kinase which phosphorylates NDR1(Thr-444)/NDR2(Thr-442) is the mammalian serine-/threonine-STE20-like kinase 3 (MST3) [13] with dephosphorylation catalysed by phosphoprotein serine-/threonine-phosphatase (PPP) PP2A [3,12], now known as PPP2. In contrast (and unusually for AGC kinases), phosphorylation of NDR1(Ser-281)/NDR2(Ser-282) is thought to represent an autophosphorylation [3,13,14]. In addition, activation may involve interactions with other proteins. Initially, the S100 Ca^{2+} binding protein was thought to be of major importance and to account for the Ca^{2+}-sensitivity of NDR activation [3,6,14]. One suggestion is that phosphorylation of NDR2(Thr-75) in a S100/Ca^{2+}-dependent manner leads to phosphorylation of NDR2(Thr-442) in the phospho-NDR2 (Thr-75) [NDR2(*P*Thr-75)] species by an unknown kinase [14,15]. The NDR2(Ser-281) residue in the NDR2(*P*Thr-75/*P*Thr-442) species is then autophosphorylated leading to activation [14,15]. However, the emphasis has now switched to the Mps-one binder (MOB) proteins which interact with basic residues in the N-terminal regulatory domains of the NDRs [4,11]. It is not entirely clear how MOB proteins activate NDRs. One view is that they bring NDRs to the plasma membrane for interaction with the upstream kinases for the hydrophobic domain phosphorylation, thus allowing enhanced autophosphorylation of the activation loop [16]. However, other mechanisms proposed are the release of the inhibition imposed by the autoinhibitory sequence [11] or, in yeast, perturbation and disruption of inhibitory self-associations [17].

Our interests in NDRs were stimulated by the observation that a microarray screen of the changes in transcript abundances following exposure of neonatal rat cardiac (ventricular) myocytes to the Gq protein-coupled receptor agonist, endothelin-1 (ET-1), revealed that NDR2 transcript abundances were increased >2-fold after 4 h [18]. In these cells, which become terminally differentiated during the perinatal period and are thus unable to undergo complete cycles of cell division, ET-1 is a powerful stimulator of hypertrophic growth, and this response has pathophysiological relevance [19–21]. These effects of ET-1 are brought about by rapid (within minutes) but transient activation of intracellular signalling pathways, particularly the extracellular signal-regulated kinase 1/2 (ERK1/2) cascade [22,23], and rapid but phasic activation of successive waves of gene expression, the earliest of which involve expression of immediate early genes [18]. Many of the early changes in gene expression are sensitive to inhibition of the ERK1/2 cascade [18,24]. Ultimately, these early changes lead to expression of genes associated with the established hypertrophic response [25]. Here, we have characterised the acute activation of NDRs in cardiac myocytes and have found that, whilst the expression of NDR2 was stimulated by ET-1, their activities were stimulated by cytotoxic interventions and particularly by PPP inhibitors.

2. Materials and methods

2.1. Materials

With the exception of ET-1 (which was from Bachem), biochemical reagents were from Sigma-Aldrich, Calbiochem or Alexis/Axxora, and laboratory chemicals were from VWR. Anti-NDR antibodies were raised to residues 59–86 of *Homo sapiens* NDR1 (for the measurement of the total pool) [16], and to a phosphopeptide (residues 436–450) encompassing NDR1(*P*Thr-444) for measurement of the species phosphorylated in the hydrophobic domain [14]. These antibodies also recognise human NDR2 and NDR2 (*P*Thr-442), respectively [13]. Since rat NDR1 is identical to human NDR1 and rat NDR2 is identical to human NDR2 in these immunogen regions, (see Supplementary Material, Fig. 1), these antibodies should and do recognise rat NDR1 and rat NDR2. Rabbit polyclonal anti-OctA (FLAG®) Probe (D-8) (sc-807) and mouse monoclonal anti-OctA (FLAG®) Probe (H-6) (sc-7787) were from Santa Cruz Biotechnology. Secondary antibodies and fluorescent mounting medium were from Dako. [γ-^{32}P]ATP, rainbow molecular weight markers, streptavidin-Texas red and Hyperfilm MP were from GE Healthcare.

2.2. Primary culture of neonatal rat cardiac myocytes

Myocytes were dissociated from the ventricles of 1- to 3-day-old Sprague-Dawley rat hearts by an adaptation of the method of Iwaki et al. [26] as previously described [27]. Unless stated otherwise, cells were plated in gelatin-coated Primaria culture dishes (BD Biosciences) at a density of 2×10^6 cells/35 mm dish or 4×10^6 cells/60 mm for 18 h in 15% (v/v) foetal calf serum. Unless experiments involved adenoviral infection, serum was withdrawn for the 24 h before experimentation.

2.3. Infections with adenoviral vectors

Details of the construction of the adenoviral vectors (Adv) encoding rat N-terminally FLAG-tagged wild-type NDR2 (FLAG-NDR2) and N-terminally FLAG-tagged NDR2-PIFtide {rat NDR2(1-432), i.e. lacking the hydrophobic domain residues 433-464, with the human PIFtide [3-phosphoinositide-dependent kinase 1 (PDK1) interacting fragment] from protein kinase N2 (PKN2, residues 969–983) spliced to the C-terminus, FLAG-NDR2-PIFtide} are provided in the 'Supplementary Material' section. For Adv infections other than those for immunofluorescence staining, serum was withdrawn from myocytes (2×10^6 cells/35 mm dish) for approximately 6 h before infection with Adv encoding FLAG-NDR2 or FLAG-NDR2-PIFtide. In initial experiments, the multiplicities of infection were titrated so that the relative levels of expression of FLAG-NDR2 and endogenous NDR1/NDR2 were similar, as assessed by immunoblotting using the rabbit polyclonal anti-NDR1/NDR2 antibody. Likewise, the multiplicity of infection of Adv.FLAG-NDR2-PIFtide was adjusted so that expression of FLAG-NDR2-PIFtide and FLAG-NDR2 was approximately equal as shown using the rabbit polyclonal anti-FLAG antibody. Myocytes were used for experimentation (no change of medium) at 20 h after they had been infected.

2.4. Sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) and immunoblotting

Following exposure to agonists, myocytes were washed twice with ice-cold phosphate-buffered saline (PBS). For whole cell extracts, myocytes were scraped into 150 μl/dish of Buffer A [20 mM β-glycerophosphate pH 7.5, 2 mM EDTA, 5 mM dithiothreitol (DTT), 50 mM NaF, 0.2 mM Na_3VO_4, 2 μM microcystin LR, 10 mM benzamidine, 300 μM phenylmethylsulphonyl fluoride (PMSF), 200 μM leupeptin, 10 μM *trans*-epoxy-succinyl-l-leucylamido-(4-guanidino)-butane (E64), containing 1% (v/v) Triton X-100]. Samples were incubated on ice for 10 min and then centrifuged (10,000 ×*g*, 5 min, 4 °C). The supernatants were retained and protein contents measured by the Bradford assay (BioRad) [28]. Samples were boiled with 0.33 vol of SDS-PAGE sample buffer [10% SDS (w/v), 13% glycerol (v/v), 300 mM Tris–HCl pH 6.8, 130 mM DTT, 0.2% bromophenol blue (w/v)]. Proteins (20–50 μg/lane, but equal for each blot) were separated by SDS-PAGE using 10% (w/v) resolving gels and 4% (w/v) stacking gels, and transferred to nitrocellulose as described previously [29]. Non-specific binding sites were blocked with 5% (w/v) non-fat milk powder in TBST [20 mM Tris–HCl pH 7.5, 137 mM NaCl, 0.1% (v/v) Tween 20]. Membranes were incubated (overnight, 4 °C) with primary antibodies [1:300 for total NDR1/NDR2 and for NDR1(*P*Thr-444)NDR2(*P*Thr-442)] diluted in TBST containing 5% (w/v) bovine serum albumin. The NDR1(*P*Thr-444) NDR2(*P*Thr-442) antibody in TBST/albumin could be used repeatedly providing it was stored at 4 °C in the interim. Membranes were washed in TBST, incubated (60 min, room temperature) with horseradish peroxidase-conjugated secondary antibodies (1/5000) in TBST containing 1% (w/v) nonfat milk powder, and were then washed in TBST. Bands were detected by enhanced chemiluminescence (Santa Cruz Biotechnology) using Hyperfilm MP and were quantified by scanning densitometry.

2.5. Assay of FLAG-NDR2 activity (immunoprecipitation kinase assays)

Myocytes were washed twice with ice-cold PBS and extracted into 100 μl of Buffer B [20 mM Tris–HCl pH 7.5, 10% (v/v) glycerol, 100 mM KCl, 5 mM $MgCl_2$, 1 mM EDTA, 0.05% (v/v) 2-mercaptoethanol, 5 mM DTT, 5 mM NaF, 0.2 mM Na_3VO_4, 2 μM microcystin LR, 300 μM PMSF, 200 μM leupeptin, 10 μM E64, containing 1% (v/v) Triton X-100]. Samples were incubated on ice for 10 min and then centrifuged (10,000 ×*g*, 5 min, 4 °C). The supernatants were retained and samples were taken for immunoprecipitation (75 or 90% of the supernatant used), protein determination by Bradford assay [28] and occasionally for immunoblotting. For immunoprecipitation, samples were added to Eppendorf tubes containing 20 μl of protein A-Sepharose (1:1 slurry in Buffer B) and 5 μl of rabbit anti-FLAG antibody that had been pre-incubated together overnight at 4 °C with rotation. Samples were rotated overnight at 4 °C and the pellets collected by centrifugation (10,000 ×*g*, 1 min, 4 °C). The pellets were washed three times in Buffer B (0.7 ml), once with kinase assay buffer (150 μl, 20 mM Tris–HCl pH 7.5, 1 mM DTT, 1 μM microcystin LR, 4 μM leupeptin, 1 mM benzamidine) and finally resuspended in 45 μl of

Table 1
Quantitative PCR primers

Gene	Accession no./probe set	Size (bp)	Forward primer	Reverse primer
NDR1	NM_001015025	176	TTCGAGGGCCTGACAGCCAG (807–826)	TCAGAGACTTGACTAGGACGCAGT (960–983)
NDR2	EF444939.1	238	AGGTCATCCGTTCTTTGAGGGTGTG (1284–1308)	ACGTGGGGATGGAGCCTCGCTG (1501–1522)
Gapdh	NM_017008	83	GCTGGCATTGCTCTCAATGACA (1738–1759)	TCCACCACCCTGTTGCTGTA (1801–1820)

Nucleotide positions in transcripts are shown in parentheses for each primer. mRNA sequences for established genes were obtained from the Rat Genome Database (http://rgb.mcw.edu, viewed at http://www.ncbi.nlm.nih.gov/entrez).

kinase assay buffer containing 1 mM KKRNRRLSVA peptide substrate [6]. The recovery of FLAG-NDR2 or FLAG-NDR2-PIFtide (estimated by immunoblotting using the monoclonal anti-FLAG antibody following immunoprecipitation with the rabbit polyclonal anti-FLAG antibody) was about 75% of the input.

FLAG-NDR2 or FLAG-NDR2-PIFtide assays were initiated by the addition of 5 μl of 1 mM [γ-^{32}P]ATP(1 μCi/assay)/100 mM $MgCl_2$ followed by incubation at 30 °C for 30 min. Reactions were terminated by the addition of 5 μl 0.5 M EDTA, pH7.5, followed by cooling on ice. Samples were centrifuged (10,000 ×*g*, 1 min, 4 °C) and duplicate samples (20 μl) of the supernatants were spotted onto P81 paper squares which were then washed in 75 mM H_3PO_4 (4×15 min) on a rocking platform. Radioactivity was measured by Čerenkov radiation counting. Blanks (one with kinase assay buffer+[γ-^{32}P]ATP/$MgCl_2$ and one involving assay of KKRNRRLSVA kinase activity with immunopreciptates from cells infected with 'empty' FLAG-tag Adv) were routinely included.

2.6. Ex vivo heart preparations

Hearts of female rats (279±6 g body wt.) that had littered 7–10 days previously were perfused retrogradely at 10 kPa with Krebs–Henseleit bicarbonate-buffered saline as described previously [30]. In each set of experiments, following a 15 min retrograde stabilisation perfusion, hearts were either (i) perfused retrogradely for a further 20 min, (ii) rendered globally-ischemic for 10 min by occlusion of the aortic inflow, (iii) reperfused retrogradely for 10 min following 10 min of global ischaemia, or (iv) perfused retrogradely with 40 nM calyculin A for 5 min. The hearts were then frozen between tongs cooled in liquid N_2 and powdered by grinding in a pestle and mortar cooled in liquid N_2.

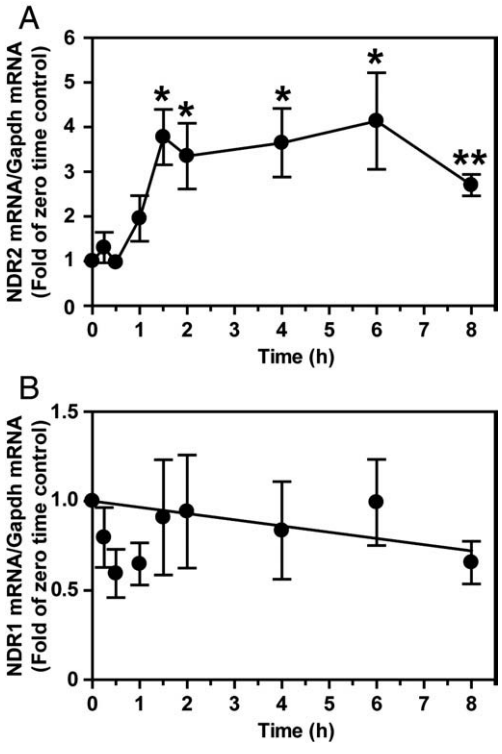


Fig. 1. Quantitative PCR of (A) NDR2 and (B) NDR1 mRNA abundances in cardiac myocytes exposed to endothelin-1 (ET-1). Myocytes (4 independent preparations for NDR2, 5 independent preparations for NDR1) were exposed to 100 nM ET-1 for the times indicated and RNA extracted. Transcript levels were normalised to those of Gapdh, then expressed relative to the zero-time controls. Results are expressed as means±SEM. Statistical significance *versus* the zero-time control: *$P<0.05$; **$P<0.01$ by a paired two-tailed Student's *t* test.

2.7. Immunofluorescence staining

Myocytes were cultured on glass coverslips [precoated with laminin (20 μg/ml in PBS) and 1% (w/v) gelatin] placed in 35 mm Primaria culture dishes at a density of 1.5×10^6 cells/dish for 18 h in the presence of 15% (v/v) foetal calf serum. Myocytes were infected with Adv.FLAG-NDR2 (as described under '*Infection with adenoviral vectors*' above) for 16 h in serum-free medium. The medium was discarded and the cells were washed with ice-cold PBS (3×0.5 ml). Cells were fixed in 4% (w/v) formaldehyde (10 min), permeabilised with 0.3% (v/v) Triton X-100 in PBS, and non-specific antibody binding was blocked with 10% (w/v) horse serum in PBS containing 0.3% (v/v) Triton X-100. FLAG-NDR2 was detected by incubation of cells with mouse anti-FLAG antibody (1:25 dilution, 2 h, 37 °C), a biotinylated goat anti-mouse IgG secondary antibody (1:200 dilution, 30 min, room temperature) and streptavidin-Texas Red (1:200 dilution, 30 min, room temperature). Sarcomeric structures were identified by FITC-phalloidin (1:100, 1 h, room temperature). Nuclei were stained with Hoechst 33258 (50 μg/ml, 10 min, room temperature). Cells were washed with PBS (3×0.5 ml) between each incubation. Slides were mounted and examined using a Zeiss Axioskop microscope with a 100× oil immersion objective and photographed using a digital camera.

2.8. Quantitative PCR (qPCR)

RNA was extracted and cDNA synthesised using reverse transcription as previously described [31]. qPCR was performed using a Real-Time PCR System model 7500 (Applied Biosystems). Amplifications were carried out in optical 96-well reaction-plates (Applied Biosystems) with each well containing 12.5 μl of SYBR Green Jump Start Taq Readymix (Sigma Aldrich Chemical Co.), 5 μl of oligonucleotide primers (5 pmol each of forward and reverse primers) and 7.5 μl of cDNA template (diluted 15-fold in water). Details of the primers used are given in Table 1. qPCR analysis of Gapdh was performed as a control and the relative quantification protocol was used. PCR conditions for all primer pairs were 50 °C for 2 min, 95 °C for 10 min (Jump-Start Taq polymerase activation step), followed by 40 cycles of 95 °C for 15 s and 59 °C for 60 s. Following qPCR, dissociation curve analysis was routinely performed to check for aberrant amplification products (e.g. primer-dimers).

2.9. Calculation of results and statistical methods

Within a single experiment (i.e. a single preparation of cardiac myocytes), results are expressed relative to the maximum signal for the quantified immunoblots or FLAG-NDR2 activities. Statistical significance was tested using two-tailed Student's *t* test for paired samples or one-way ANOVA with Tukey's multiple comparison test as appropriate. A statistically significant difference required that $P<0.05$.

3. Results

3.1. Molecular cloning of rat NDR2 cDNA

In an Affymetrix rat 230 2.0 array screen of primary cultures of neonatal rat cardiac myocytes exposed to ET-1 for 4 h, two probe-sets

Table 2
Potencies of PPP1/PPP2 inhibitors

Inhibitor	IC_{50} against		Reference
	PPP1	PPP2	
Calyculin A	0.3 nM	0.13 nM	[65]
Okadaic acid	3.4 nM	0.07 nM	[65]
Microcystin LR	0.1 nM	0.1 nM	[65]
Fostriecin	4 μM	40 nM	[66]
Tautomycetin	1.6 nM	62 nM	[67]

(1372955_at and 1383299_at) identified an expressed sequence tag (EST) that was significantly upregulated to 2.28-fold (average of both probe-sets) of the zero-time control [18]. A BLAST search revealed that this rat EST corresponded to the mouse NDR2 nucleotide sequence. Rat NDR2 cDNA was cloned as described in 'Supplementary Material' and the sequence obtained (bases 1-2406) deposited in Genbank (accession no. EF444939.1). The translational open reading frame (bases 151-1542, 464 amino acids) was aligned against human, mouse and rat NDR1, and human and mouse NDR2 using the ClustalW program (www.ebi.ac.uk/clustalw) (Supplementary Material, Fig. 1). The primary structure of rat NDR2 (calculated molecular mass: 53.6 kDa) is identical to mouse NDR2 except for replacement of mouse Asn-15 and Val-216 with Ser-15 and Ile-216, respectively, in rat NDR2.

Using qPCR, we verified that NDR2 mRNA was significantly upregulated (3- to 4-fold of zero-time control) by ET-1 at 1.5 h and remained upregulated at this level until at least 8 h (Fig. 1A). In contrast, NDR1 mRNA was not significantly altered over the same time period (Fig. 1B). However, using the antibody which recognises both NDR1 and NDR2, we were unable to detect any change in the abundances of NDR proteins by ET-1 over the same time period (results not shown). The primary structures of NDR1 and NDR2 show a high degree of identity (Supplementary Material, Fig. 1) and the proteins are very similar in molecular mass (NDR1 is 54.0 kDa), so that it is possible that any newly-synthesised NDR2 would not necessarily be readily detectable against the pre-existing NDR1/NDR2 background.

3.2. Phosphorylation of NDR1(Thr-444)/NDR2(Thr-442) and activation of FLAG-NDR2 by calyculin A

Calyculin A is an inhibitor of PPP1 and PPP2 (formerly known as PP2A), inhibiting each approximately equipotently (Table 2). Calyculin A (200 nM) induced a rapid phosphorylation (detectable by 1 min) of NDR1(Thr-444)/NDR2(Thr-442) in cardiac myocytes, reaching a maximum at 10–20 min and remaining stable for at least 90 min

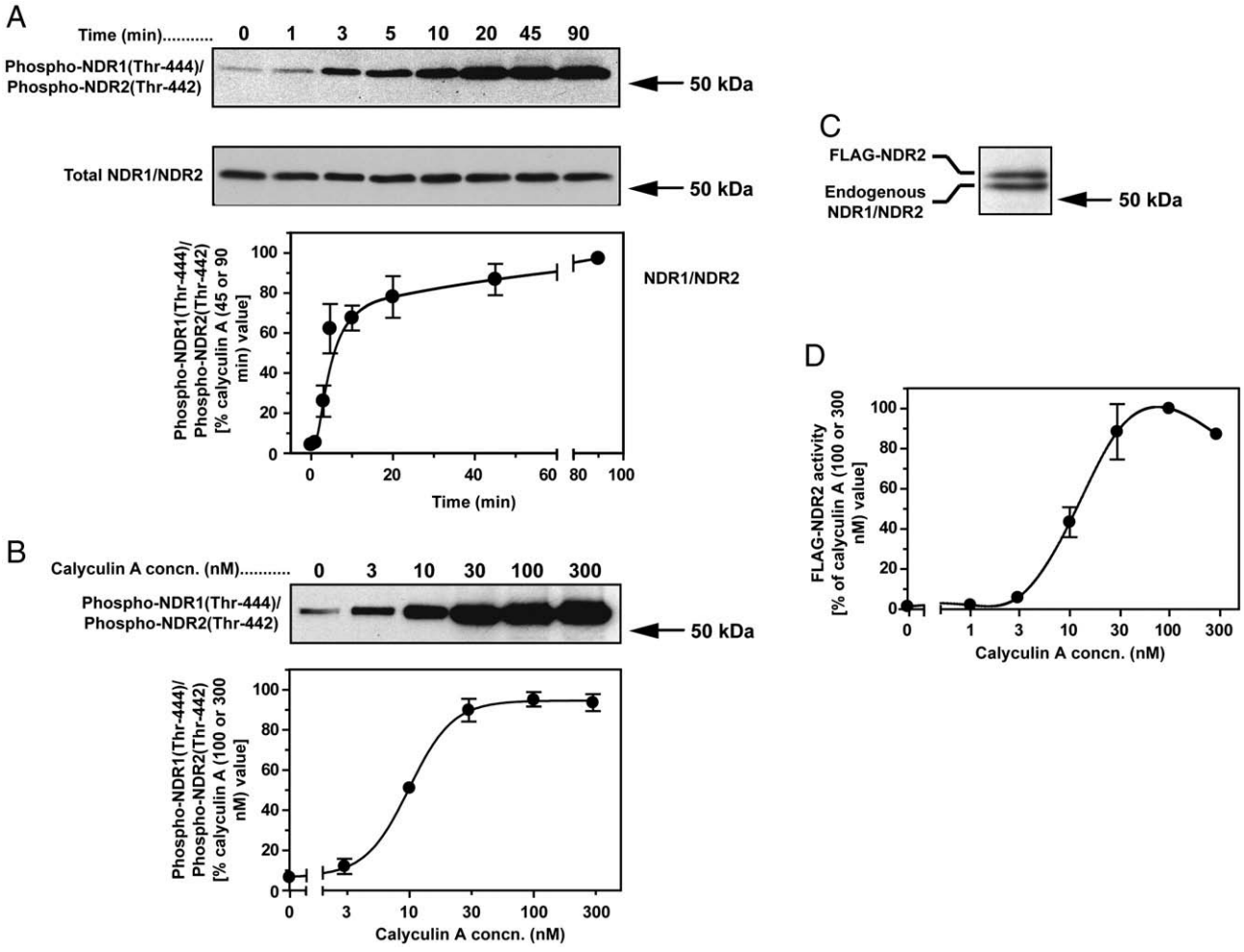


Fig. 2. Phosphorylation of NDR1(Thr-444) and NDR2(Thr-442), and activation of FLAG-NDR2 in cardiac myocytes exposed to calyculin A. All results are expressed as means ± SEM. (A) Myocytes (3 independent preparations) were exposed to 200 nM calyculin A for the times indicated and phosphorylation of NDR1(Thr-444)/NDR2(Thr-442) assessed by immunoblotting with an anti-NDR phosphopeptide antibody. There were no changes in the abundances of total NDR1/NDR2. The intensities of the NDR1(*P*Thr-444)/NDR2(*P*Thr-442) signals were assessed by scanning densitometry and expressed relative to the maximum values which occurred after either 45 or 90 min of exposure of myocytes to calyculin A, depending on the time point at which the greater extent of phosphorylation was observed. (B) The dependence of NDR1(Thr-444)/NDR2(Thr-442) phosphorylation on calyculin A concentration was assessed in myocytes (4 independent preparations) exposed to calyculin A for 20 min. The intensities of the NDR1(*P*Thr-444)/NDR2(*P*Thr-442) signals were assessed as described under (A) and expressed relative to the maximum values which occurred at either 100 or 300 nM calyculin A, depending on the concentration at which the greater extent of phosphorylation was observed. (C) Myocytes were infected with Adv.FLAG-NDR2. Immunoblotting with an anti-NDR1/NDR2 antibody showed that endogenous NDR1/NDR2 and FLAG-NDR2 were expressed in approximately equal amounts. (D) The dependence of FLAG-NDR2 activity on calyculin A concentrations was assessed in infected myocytes (4 independent preparations) exposed to calyculin A for 20 min. Activities were expressed relative to the maximum values which occurred at either 100 or 300 nM calyculin A, depending on the concentration at which the greater activity was observed.

(Fig. 2A). There was no change in total NDR1/NDR2 abundance (Fig. 2A). Because of their similarities in molecular mass, NDR1 and NDR2 usually co-migrate though, if samples are electrophoresed for longer, occasionally two closely-migrating bands are detectable with the antibody to NDR1(*P*Thr-444)/NDR2(*P*Thr-442). Using this anti-phospho-NDR antibody, the EC_{50} for the calyculin A-mediated phosphorylation of NDR1(Thr-444)/NDR2(Thr-442) was computed to be approximately 10 nM (Fig. 2B). The activation of NDR1 or NDR2 requires dual phosphorylation of NDR1(Ser-281/Thr-444) or NDR2 (Ser-282/Thr-442), the phosphorylation of NDR1(Ser-281)/NDR2(Ser-282) representing autophosphorylations following the NDR1(Thr-444)/NDR2(Thr-442) phosphorylations [3,13,14]. However, the available antibody [14] raised against NDR1(*P*Ser-281)/NDR2(*P*Ser-282) is of relatively low affinity/efficacy and we could not examine the phosphorylation of these sites. We did investigate whether, following adenovirally-mediated transfer of FLAG-NDR2, the activity of FLAG-NDR2 as measured by immunoprecipitation kinase assays was commensurate with phosphorylation of NDR1(Thr-444)/NDR2(Thr-442). We adjusted the adenoviral infections so that we achieved essentially equal expression of FLAG-NDR2 and endogenous NDR1/NDR2 (Fig. 2C). Immunprecipitation kinase assays using the anti-FLAG antibody showed that, as for the phosphorylation of NDR1(Thr-444)/NDR2(Thr-442) (Fig. 2B), the activity of FLAG-NDR2 was also stimulated by calyculin A with an EC_{50} of approximately 10 nM (Fig. 2D). We noticed that calyculin A also appeared to be positively chronotropic in these myocyte cultures and thus considered the possibility that contractile activity might be responsible for NDR phosphorylation. However, contractile arrest by blockade of the L-type- and T-type Ca^{2+} channels with 10 μM nifedipine and 1.8 μM mibefradil did not reduce the effects of calyculin A (results not shown).

3.3. Phosphorylation of NDR1(Thr-444)/NDR2(Thr-442) by hyperosmotic shock and oxidative stress

Exposure of myocytes to hyperosmotic shock (0.5 M sorbitol) resulted in a rapid (maximal in 10 min or less) phosphorylation of NDR1(Thr-444)/NDR2(Thr-442) (Fig. 3A) though, unlike phosphorylation elicited by 200 nM calyculin A (Fig. 2A), phosphorylation had declined by 90 min. Oxidative stress (0.5 mM H_2O_2) caused a somewhat slower phosphorylation (maximal at about 20 min) and this again declined by 90 min (Fig. 3B). In neither condition was there any change in the abundance of total NDR1/NDR2 (Fig. 3A–B). The phosphorylation elicited by oxidative stress was detectable at 0.1 mM H_2O_2 and showed a distinct peak occurring at about 0.5 mM (Fig. 3C). At higher concentrations (1–10 mM H_2O_2), phosphorylation declined. There were no losses of myocytes from the dishes at up to 3 mM H_2O_2, as shown by the immunoblots against total NDR1/NDR2 (Fig. 3C), though it is possible that some losses occurred at 10 mM H_2O_2.

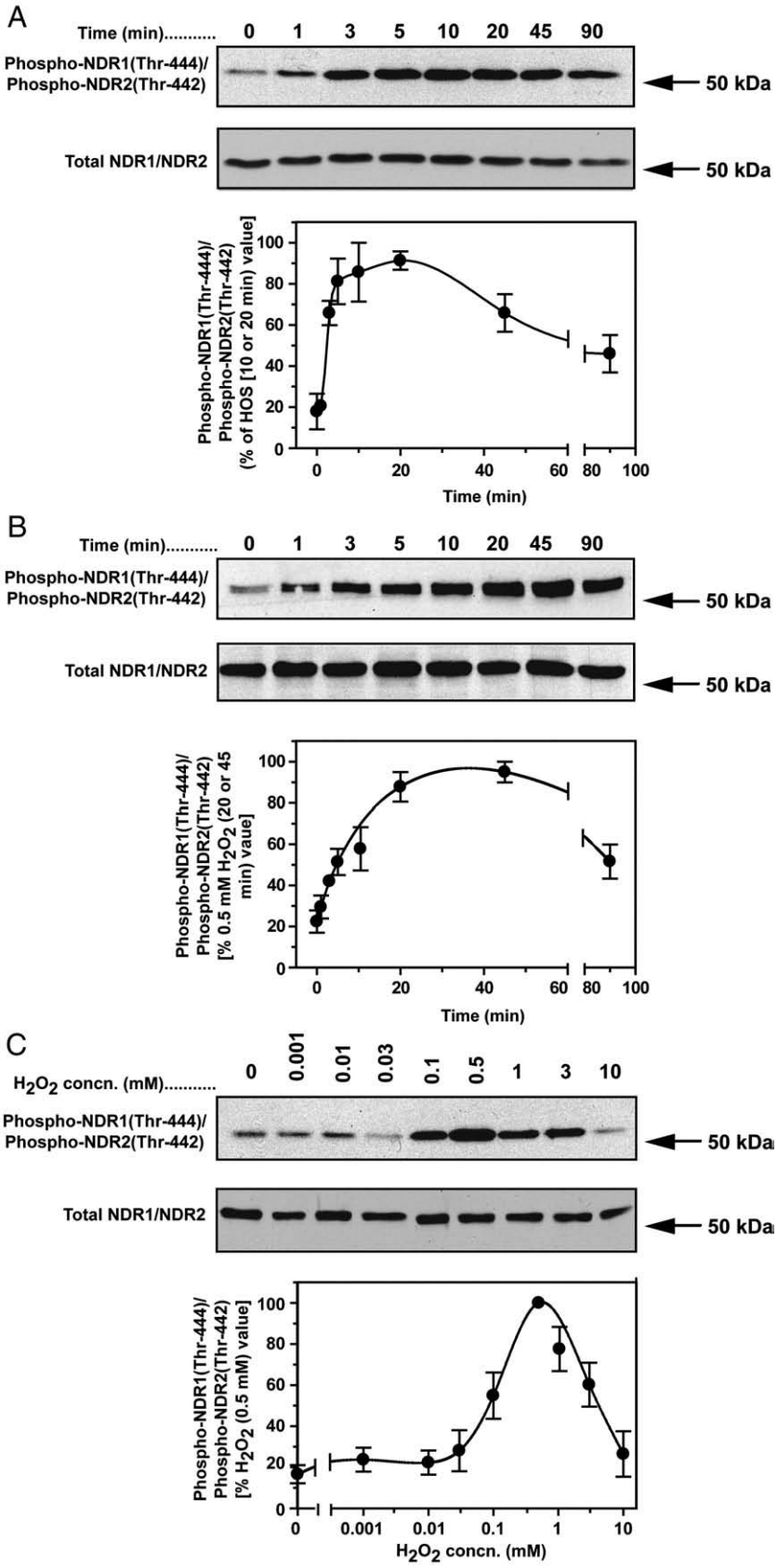
3.4. Relative potencies of phosphorylation of NDR1(Thr-444)/NDR2(Thr-442) and activation of FLAG-NDR2 by calyculin A, hyperosmotic shock and H_2O_2, and identification of additional modulators

We examined the ability of a wide range of known modulators of myocardial processes to mediate phosphorylation of NDR1(Thr-444)/NDR2(Thr-442) and/or activation of FLAG-NDR2 (see Supplementary Material, Table 2 for details of agonists/interventions without effect). We also set any modulators identified as being effective in a hierarchy in comparison with calyculin A. Chelerythrine is allegedly a protein kinase C (PKC) inhibitor [32], though this has been questioned at least in the case of the PKCα isoform [33]. In cardiac myocytes, chelerythrine causes conventional and novel PKC-independent pyknosis, shrinkage and death that may result from the induction of oxidative stress [34]. In comparison with 200 nM calyculin A (NDR1/NDR2 control not subtracted), chelerythrine is about 45 to 50% as effective in inducing phosphorylation of NDR1(Thr-444)/NDR2(Thr-442) (Fig. 4A), but is only about 20% (control FLAG-NDR2 activity not subtracted) as effective in inducing activation of FLAG-NDR2 (Fig. 4B). Likewise, 0.5 mM H_2O_2 is about 40 to 45% as effective as 200 nM calyculin A in inducing phosphorylation of NDR1(Thr-444)/NDR2(Thr-442) but is only about 5 to 10% as effective in inducing activation of FLAG-NDR2 (Fig. 4A–B). The PPP2 inhibitor okadaic acid (OKA), which also inhibits PPP1 though less effectively (Table 2), is known to induce phosphorylation and activation of NDR1/NDR2 [3,12]. In our hands, after a 60 min incubation, 1 μM OKA is almost as effective as incubation with 200 nM calyculin A for 20 or 60 min in inducing phosphorylation of NDR1(Thr-444)/NDR2(Thr-442) (Fig. 4A). However, following a 20 min incubation, 1 μM OKA does not elicit any statistically significant phosphorylation of NDR1(Thr-444)/NDR2(Thr-442) compared with a control incubation (Fig. 4A), though there is a statistically-insignificant increase to about 30% of the level observed with 200 nM calyculin A for 20 or 60 min. For activation of FLAG-NDR2, OKA (1 μM, 20 min) was <10% as effective as calyculin A (200 nM, 20 min) (Fig. 4C). However, after a 60 min incubation with OKA, this had increased to 45 to 50% (Fig. 4C). The general conclusions are (i) calyculin A is the most powerful stimulator of NDR1(Thr-444)/NDR2(Thr-442) and activation of FLAG-NDR2 that we have identified, (ii) generally, activation of FLAG-NDR2 lags behind phosphorylation of NDR1(Thr-444)/NDR2(Thr-442). Because it could be argued that this difference was related to the different species of NDRs examined (endogenous NDR1/NDR2 or FLAG-NDR2), we examined the phosphorylation of endogenous NDR1/NDR2 and FLAG-NDR2 in FLAG-NDR2-transduced cardiac myocytes (Fig. 4D). This was feasible because of the lower mobility of FLAG-NDR2. No differences between the phosphorylation of endogenous NDR1(Thr-444)/NDR2(Thr-442) and FLAG-NDR2(Thr-442) could be identified for any agonist. We also calculated the approximate ratio of expression of FLAG-NDR2(*P*Thr-442) to endogenous NDR1(*P*Thr-444)/NDR2(*P*Thr-442) using the anti-NDR1(*P*Thr-444)/NDR2(*P*Thr-442) antibody. If it can be assumed that the most significant factor influencing this ratio is the levels of expression, the value should approximate to the FLAG-NDR2/endogenous NDR1/NDR2 ratio (Fig. 2C). The FLAG-NDR2(*P*Thr-442)/endogenous NDR1(*P*Thr-444)/NDR2(*P*Thr-442) ratio is approximately 1.5, i.e. similar to that observed in Fig. 2C.

Given that calyculin A is an equipotent PPP1/PPP2 inhibitor whereas OKA inhibits PPP2 more potently (Table 2), the spectrum of NDR1/NDR2 phosphorylation (Fig. 4A,D) and FLAG-NDR2 activation (Fig. 4C) suggests that the effects of calyculin A could be exerted primarily through PPP1. We therefore investigated the effects of tautomycetin (200 nM for up to 4 h) which is primarily a PPP1 inhibitor (Table 2) and fostriecin (200 nM for up to 4 h) which is primarily a PPP2 inhibitor (Table 2) on activity of FLAG-NDR2. Neither

Fig. 3. Phosphorylation of NDR1(Thr-444) and NDR2(Thr-442) in cardiac myocytes exposed to hyperosmotic shock or oxidative stress. All results are expressed as means ± SEM. (A and B) Myocytes (3 independent preparations) were exposed to hyperosmotic shock (HOS; 0.5 M sorbitol) (A) or 0.5 mM H_2O_2 (B) and phosphorylation of NDR1(Thr-444)/NDR2(Thr-442) assessed by immunoblotting with an anti-NDR phosphopeptide antibody. There were no changes in the abundances of total NDR1/NDR2. The intensities of the NDR1(*P*Thr-444)/NDR2(*P*Thr-442) signals were assessed by scanning densitometry and were expressed relative to the maximum values which occurred after either 10 or 20 min of exposure of myocytes to hyperosmotic shock (A) or 20 or 45 min of exposure to 0.5 mM H_2O_2 (B), depending on the time point at which the greater extent of phosphorylation was observed. (C) The dependence of NDR1(Thr-444)/NDR2(Thr-442) phosphorylation on H_2O_2 concentration was assessed in myocytes (5 independent preparations) exposed to H_2O_2 for 20 min. The intensities of the NDR1(*P*Thr-444)/NDR2(*P*Thr-442) signals were assessed as described under (A and B) and were expressed relative to the maximum values which occurred at 0.5 mM H_2O_2.

inhibitor had any detectable effect (results not shown). Furthermore, the potent PPP1/PPP2 inhibitor (Table 2), microcystin LR (2 μM), did not activate FLAG-NDR2, nor did it induce phosphorylation of endogenous NDR1(Thr-444)/NDR2(Thr-442) or FLAG-NDR2(Thr-442). The effects of PPP inhibitors on the NDRs are therefore limited largely to calyculin A, with lesser effects of OKA.



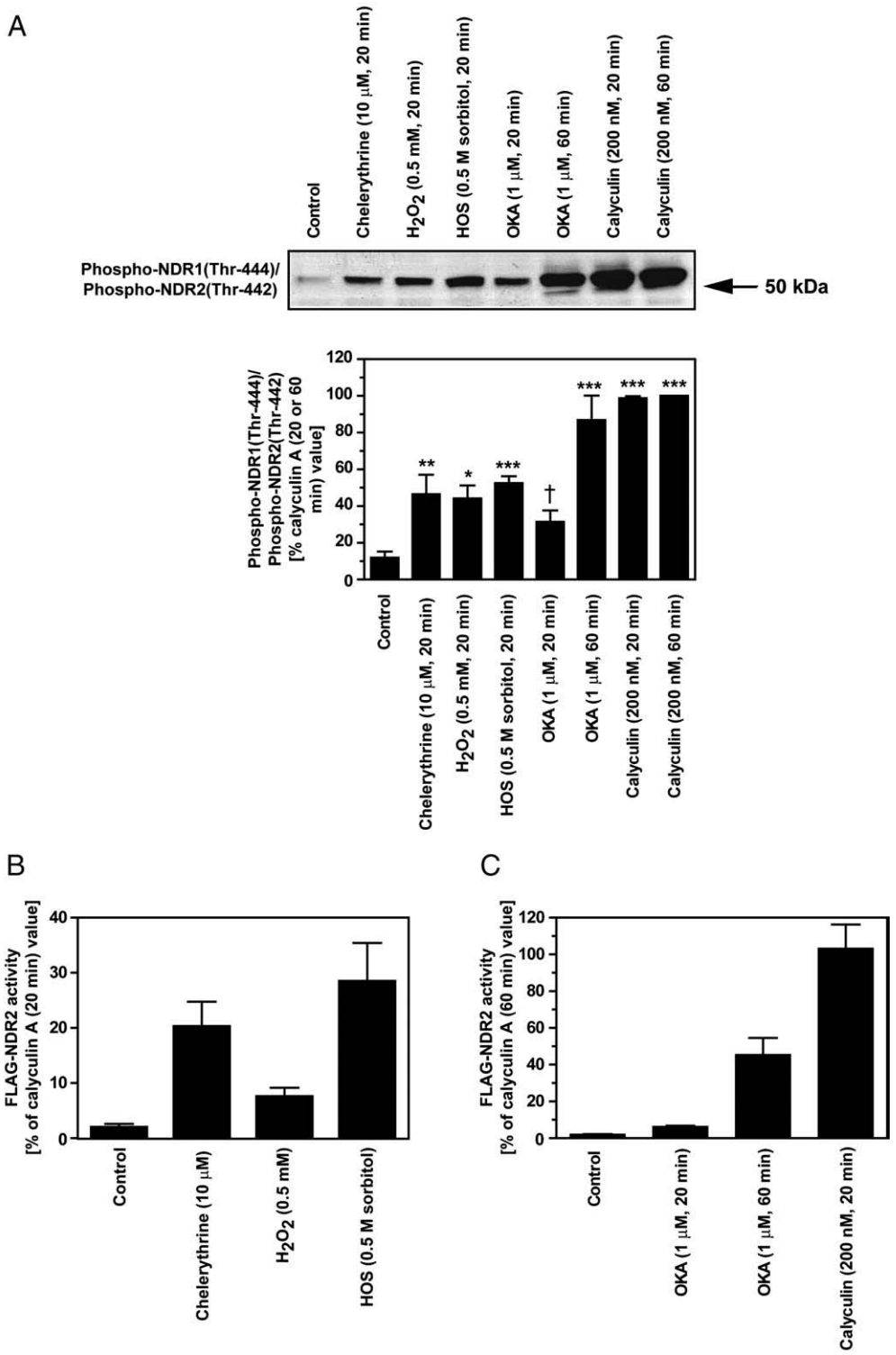


Fig. 4. Direct comparison of induction of phosphorylation of NDR1(Thr-444) and NDR2(Thr-442), and activation of FLAG-NDR2 in cardiac myocytes exposed to a range of interventions. HOS, hyperosmotic shock; OKA, okadaic acid. (A) Myocytes (3 to 8 independent preparations) were exposed to NDR modulators at the concentrations and for the times indicated and phosphorylation of NDR1(Thr-444)/NDR2(Thr-442) assessed by immunoblotting with an anti-NDR phosphopeptide antibody. The intensities of the NDR1(*P*Thr-444)/NDR2(*P*Thr-442) signals were assessed by scanning densitometry. Results are expressed relative to maximum phosphorylation which was observed with 200 nM calyculin A at either 20 min or 60 min, depending on the time point at which the greater extent of phosphorylation was observed, and are means ± SEM. Statistical significance: $^{*}P<0.05$; $^{**}P<0.01$, $^{***}P<0.001$ versus control or $\dagger P<0.01$ versus OKA (1 μM, 60 min) by a 1-tailed ANOVA with Tukey's multiple comparison test. (B and C) FLAG-NDR2 activity following exposure of myocytes [8 independent preparations in (B), 3 independent preparations in (C)] to the interventions indicated and expressed relative to the maximum activity which was observed with 200 nM calyculin A at either 20 min or 60 min, depending on the time point at which the greater activity was observed. Results are means ± SEM. (D) Myocytes expressing the FLAG-NDR2 transgene (2 independent preparations of myocytes) were exposed to NDR modulators at the concentrations and for the times indicated and phosphorylation of NDR1 (Thr-444)/NDR2(Thr-442) assessed by immunoblotting with an anti-NDR phosphopeptide antibody. The intensities of the NDR1(*P*Thr-444)/NDR2(*P*Thr-442) signals were assessed as under (A). The upper band represents FLAG-NDR2(*P*Thr-442) and the two lower bands represent NDR1(*P*Thr-444)/NDR2(*P*Thr-442). The unfilled bars in the histogram represent FLAG-NDR2(*P*Thr-442) whereas the filled bars represent endogenous NDR1(*P*Thr-444)/NDR2(*P*Thr-442). Results are expressed relative to maximum phosphorylation which was observed with 200 nM calyculin A at 60 min and are means ± SD.

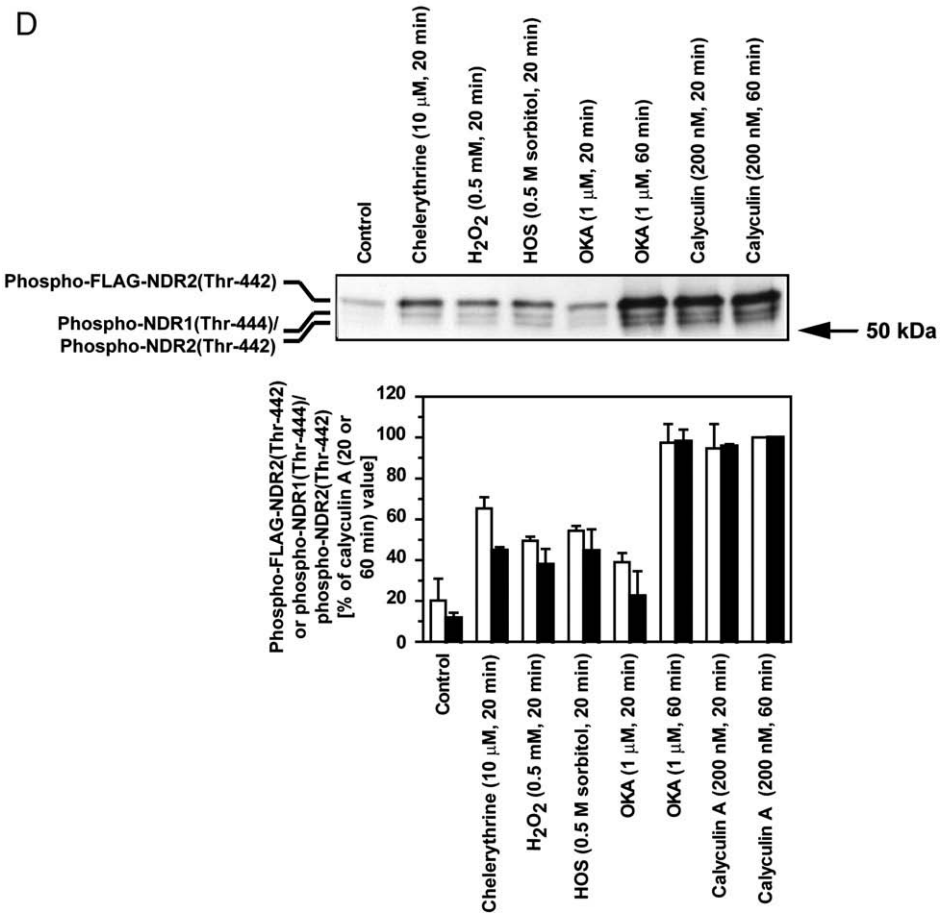


Fig. 4 (*continued*).

3.5. Inhibitors of calyculin A-mediated activation of FLAG-NDR2

We investigated the ability of a number of known protein kinase inhibitors to reduce the activation of FLAG-NDR2 resulting from exposure of cardiac myocytes to 50 nM calyculin A. Of the inhibitors examined, the only inhibitor with any effect was staurosporine. Staurosporine was originally identified as a PKC inhibitor [35] but is now recognised to be a non-selective protein kinase inhibitor [36] (see also Reference [33] for 7-hydroxystaurosporine). Staurosporine exhibited an IC_{50} of approximately 40 nM for the inhibition of the activation of FLAG-NDR2 by 50 nM calyculin A (Fig. 5A). This inhibition was not attributable to 'carry-over' of staurosporine from the cell incubations into the assay because addition of staurosporine up to 1 μM for the last 5 min of a 20 min exposure of myocytes to 50 nM calyculin A [i.e. after FLAG-NDR2 would have been essentially completely activated by calyculin A (Fig. 2A) but prior to extraction and immunoprecipitation] did not result in any significant reduction in FLAG-NDR2 activity. It is possible that staurosporine may not inhibit phosphorylation of NDR1(Thr-444)/NDR2(Thr-442) but could selectively inhibit the autophosphorylation of NDR1(Ser-281)/NDR2(Ser-282). We therefore also examined its effects on the calyculin A-stimulated phosphorylation of NDR1(Thr-444)/NDR2(Thr-442) and FLAG-NDR2(Thr-442) (Fig. 5B). Staurosporine also inhibited the calyculin A-stimulated phosphorylation of endogenous NDR1(Thr-444)/NDR2(Thr-442) and of exogenous FLAG-NDR2(Thr-442) (Fig. 5B), and this in itself should prevent phosphorylation of FLAG-NDR2(Ser-282) and its activation (Fig. 5A). We do not know whether staurosporine also inhibits the autophosphorylation of NDR1(*P*Thr-444)/NDR2(*P*Thr-442) at the T loop residues.

The following were ineffective in inhibiting activation of FLAG-NDR2 by calyculin A: 100 μM genistein (a generalised Tyr-protein kinase inhibitor [37]), 10 μM GF109203X (generalised PKC inhibitor [38] which also inhibits a number of other protein kinases [33]), 20 μM HA1077 (a PKN inhibitor [39] which inhibits a number of protein kinases including protein kinase A and Rho kinase [33]), 10 μM SB203580 [inhibitor of the α and β isoforms of p38-mitogen-activated protein kinase (p38-MAPK) [40]] or 10 μM Y27632 (Rho kinase inhibitor [41] which also inhibits PKN [33]). Equally, no inhibitor activated FLAG-NDR2 in the absence of calyculin A (results not shown). The phosphoinositide 3-kinase (PI3K) inhibitor, LY294002 [42] (50 μM), did not inhibit NDR1(Thr-444)/NDR2(Thr-442) phosphorylation or activation of FLAG-NDR2 (results not shown). [We ensured that the LY294002 preparation used was active by demonstrating that it prevented stimulation of protein kinase B/Akt (PKB/Akt) phosphorylation by 300 nM insulin.]

3.6. Activity of FLAG-NDR2-PIFtide

Mutation of the two phosphorylation sites in the catalytic and hydrophobic domains of NDR1 (i.e. Ser-281 and Thr-444) to acidic residues is relatively-ineffective in producing a kinase with increased constitutive activity [3]. A constitutively-activated species can be produced by splicing NDR2(1-432) to PKN2(969-983) to produce NDR2-PIFtide, as has been previously described for human NDR2 [3]. We examined the activities of FLAG-NDR2 (rat) and FLAG-NDR2-PIFtide (rat-human chimera) in the absence or presence of calyculin A and approximately matching the abundances of FLAG-NDR2 and FLAG-NDR2-PIFtide (Fig. 6). After subtracting the activity seen

following infection with the adenoviral blank (which was not accurately matched for multiplicity of infection with Adv.FLAG-NDR2 and Adv.FLAG-NDR2-PIFtide) and correcting for differences in relative abundance, the constitutive activity of FLAG-NDR2-PIFtide was found to be approximately 10-fold greater than that of FLAG-NDR2 (Fig. 6). Not unexpectedly, FLAG-NDR2-PIFtide (which lacks the Thr-442 phosphorylation site) could not be activated by calyculin A (Fig. 6).

3.7. Intracellular localisation of NDR2 in cardiac myocytes

Using the antibodies available, we were unsuccessful in staining for endogenous dephospho-NDR (as were Hergovich et al. [16] in U2-OS cells), nor were we successful in staining for phospho-NDRs. We therefore examined the intracellular localisation of NDR2 by infecting myocytes with Adv.FLAG-NDR2 and staining for the FLAG epitope. FLAG-NDR2 localised to the cytoplasm in cardiac myocytes and was clearly excluded from the nucleus (Fig. 7). The pattern of staining was somewhat uneven in the cytoplasm and there was a suggestion of some partition of NDR2 to the plasma membrane. There was no evidence of any change in localisation following exposure to 30 nM calyculin A for 20 min (results not shown).

3.8. Phosphorylation of NDR1(Thr-444)/NDR2(Thr-442) in intact heart by calyculin A or by ischaemia and reperfusion

Calyculin A proved to be the most powerful stimulator of NDR1 (Thr-444)/NDR2(Thr-442) phosphorylation and activator of FLAG-NDR2 in neonatal rat cardiac myocytes that we identified here (see Fig. 4A–D). Some of the interventions resulting in phosphorylation of NDR1(Thr-444)/NDR2(Thr-442) and activation of FLAG-NDR2 in myocytes are established cytotoxic stresses in neonatal cardiac myocytes (chelerythrine [34], H_2O_2 [43] and hyperosmotic shock [44]). However, it is important to show that these findings are not confined to neonatal cells. We therefore investigated the effects of calyculin A and of pathologically-relevant cardiac stresses, namely ischaemia and ischaemia-reperfusion in intact adult rat hearts. As expected, calyculin A (40 nM, 5 min) induced a highly-significant ($P<0.0001$) increase in the phosphorylation of NDR1(Thr-444)/NDR2 (Thr-442) in perfused hearts (Fig. 8). Although there was some

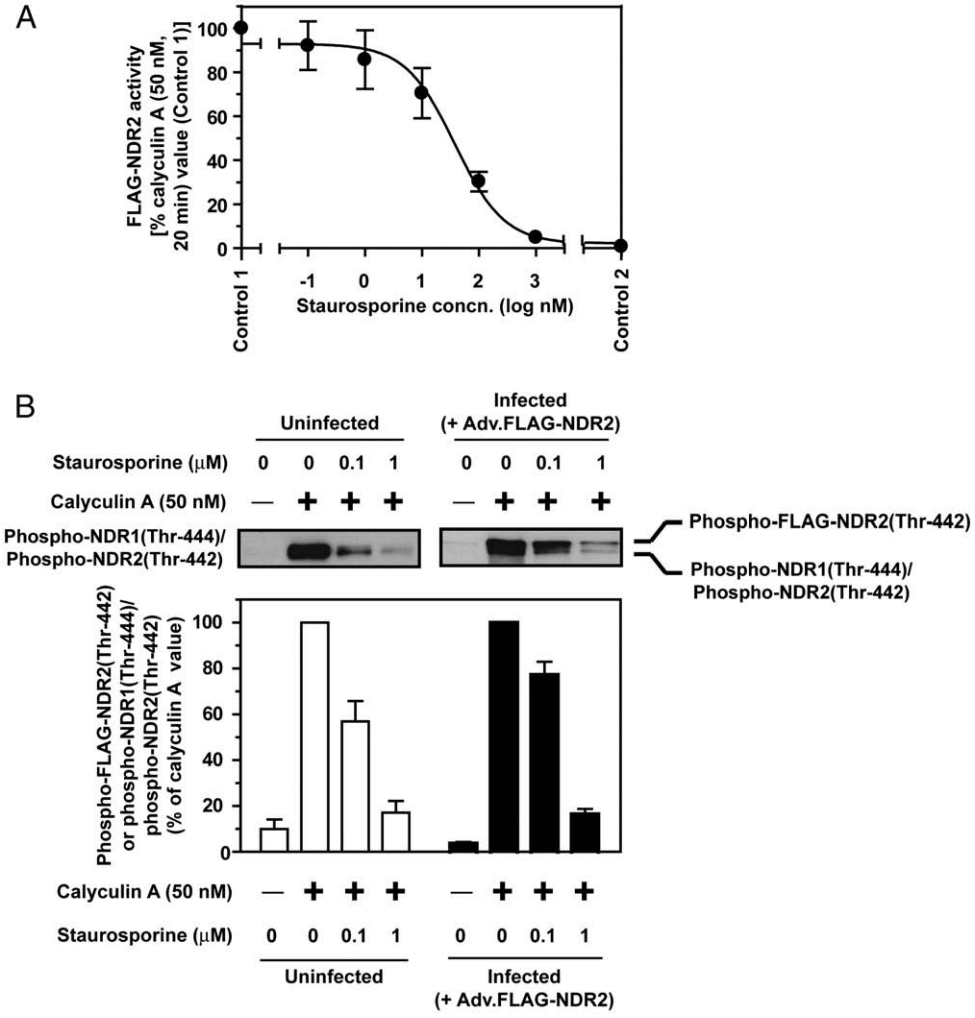


Fig. 5. Inhibition of calyculin A-induced activation of FLAG-NDR2 and phosphorylation of endogenous NDR1(Thr-444)/NDR2(Thr-442) and FLAG-NDR2(Thr-442) by staurosporine. Adv, adenoviral vector. (A and B) Myocytes were preincubated for 20 min with staurosporine at the concentrations indicated, then were exposed to 50 nM calyculin A for a further 20 min. (A) Inhibition of calyculin A-induced stimulation of FLAG-NDR2 activity by staurosporine. FLAG-NDR2 activity in control 1 was assessed at 50 nM calyculin A (20 min) in the absence of staurosporine and results were expressed relative to this. FLAG-NDR2 activity in control 2 was assessed in the absence of 50 nM calyculin A and staurosporine. (B) Inhibition of calyculin A-induced phosphorylation of endogenous NDR1(Thr-444)/NDR2(Thr-442) (left hand immunoblot panel and unfilled bars), and of endogenous NDR1(Thr-444)/NDR2(Thr-442) and FLAG-NDR2(Thr-442) (right hand immunoblot panel and filled bars) was assessed by immunoblotting with an anti-NDR phosphopeptide antibody. Intensities of the endogenous NDR1(*P*Thr-444)/NDR2(*P*Thr-442) signals (left hand immunoblot panel) or endogenous NDR1(*P*Thr-444)/NDR2(*P*Thr-442) + FLAG-NDR2(*P*Thr-442) (right hand immunoblot panel) were quantified by scanning densitometry and expressed relative to the values obtained with calyculin A (50 nM, 20 min) for each single experimental grouping. Results are means±SEM for 3 (A) or 4 (B) independent preparations of myocytes.

suggestion that global ischaemia (10 min) increases phosphorylation of NDR1(Thr-444)/NDR2(Thr-442), this was not significantly different from control normoxic perfusions. In contrast, global ischaemia (10 min) followed by reperfusion (10 min) resulted in significant ($P<0.0001$) phosphorylation of NDR1(Thr-444)/NDR2(Thr-442) which, after subtraction of the control, was equivalent to about 30% of the level induced by 40 nM calyculin A for 5 min (Fig. 8).

4. Discussion

4.1. General comments

The NDRs are highly-conserved protein Ser-/Thr-kinases that are members of the AGC kinase superfamily [1,2]. In terms of their activation, the current scheme is that NDR1(Thr-444) and NDR2(Thr-442) (the hydrophobic domain residues) are first phosphorylated by an upstream kinase and then NDR1(*P*Thr-444) and NDR2(*P*Thr-442) each autophosphorylate their own T loop residues in their catalytic domains to produce the active species NDR1(*P*Ser-281/*P*Thr-444) and NDR2(*P*Ser-282/*P*Thr-442) [1,2]. The fact that activation of FLAG-NDR2 tends to lag behind phosphorylation of NDR1(Thr-444)/NDR2 (Thr-442) (for example, seen clearly for OKA in Fig. 4A, C) suggests that this ordered phosphorylation occurs in cardiac myocytes. The agonists that lead to activation of NDRs are poorly-characterised and their biological functions in higher organisms are poorly-understood. Given that NDR1/NDR2 are highly-conserved through evolution, it seems likely that they possess important functions. NDR2 may be involved in organisation of the actin cytoskeleton [9] and, recently, a role for NDR1/NDR2 in centrosome duplication has been identified [2,10]. Although populations of myocytes range from the mononucleate to the multinucleate and thus must possess some capacity for karyokinesis, they are generally considered to be terminally-differentiated and incapable of cytokinesis. It therefore seems unlikely that centrosome duplication is of major importance in their case.

4.2. Expression of the NDR1 and NDR2 genes

At the level of mRNA, NDR2 transcripts are rapidly but transiently induced in amygdalae of mice following emotional stress [9] and, here, we show that NDR2 mRNA is also rapidly induced by ET-1 in neonatal rat cardiac myocytes (Fig. 1A). In contrast, NDR1 mRNA is not induced by ET-1 (Fig. 1B). Presumably, their promoter regions are sufficiently different to allow differential transcription or else transcript stability may be involved. Using microarrays (Affymetrix rat 230 2.0 array at 30 min or the more-restricted Affymetrix rat U34A microarrays at 2 or 4 h) in conjunction with semi-quantitative PCR and/or qPCR, we have shown that, for ET-1, the activation of the ERK1/2 cascade is required for the increased expression of the majority of transcripts upregulated at 30 min, 2 h or 4 h [18,24]. We have not yet studied in detail whether this is the case for NDR2 mRNA [the U34A microarrays did not have a probe set for rat NDR2 [24] and we only studied the 30 min time point in our more recent study [18] when NDR2 transcripts are not upregulated (Fig. 1A)].

4.3. Involvement of PPPs in NDR phosphorylation and activation

The PPP superfamily, of which there are seven sub-families, is encoded by 20 genes in *Homo sapiens* and these encode about 28 catalytic subunits [45]. There is also a multiplicity of regulatory subunits (particularly for PPP1) which are involved in targeting (to control subcellular localisation and substrate specificity) and regulation (e.g. inhibitory subunits). Previously, OKA has been identified as an agent that stimulates phosphorylation and activation of NDR1/NDR2 [3,12]. This is presumably achieved by inhibiting a PPP, allowing unknown NDR1/NDR2 kinases to phosphorylate NDRs. Here, we show that, at maximally-effective concentrations, calyculin A is more effective than OKA in inducing NDR1(Thr-444)/NDR2(Thr-442) phosphorylation and activation of FLAG-NDR2 in neonatal rat cardiac myocytes after 20 min (Fig. 4A, C). Although calyculin A or OKA treatment result in equivalent NDR1(Thr-444)/NDR2(Thr-442) phosphorylation after 60 min,

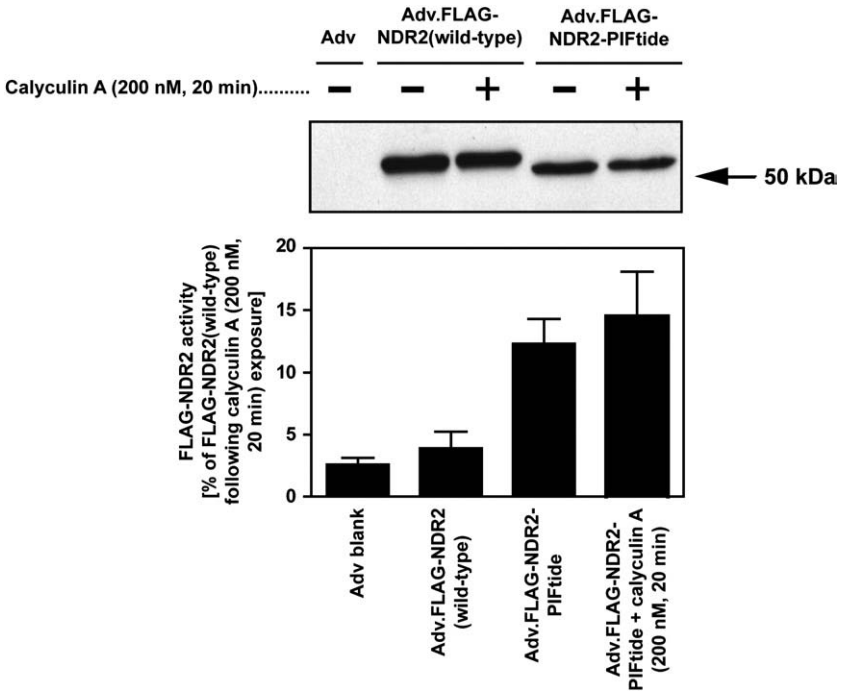


Fig. 6. Stimulation of NDR2 activity in the FLAG-NDR2-PIFtide chimera. Myocytes were infected with an adenovirus (Adv) empty vector, with Adv.FLAG-NDR2(wild-type) or with Adv. FLAG-NDR2-PIFtide. The relative levels of expression of FLAG-NDR2(wild-type) and FLAG-NDR2-PIFtide were assessed with an antibody to the FLAG epitope (upper panel). Myocytes expressing the transgenes were exposed to 200 nM calyculin A for 20 min and the activities of FLAG-NDR2(wild type) or FLAG-NDR2-PIFtide were measured (lower panel). Results are expressed relative to the FLAG-NDR2(wild-type) infected myocytes exposed to 200 nM calyculin A for 20 min and are means ± SEM for 6 independent preparations of myocytes. The results shown were not corrected for expression levels and the Adv blank was not subtracted (but see the Results section for these calculations).

activation of FLAG-NDR2 with OKA is still less than with calyculin A (Fig. 4A, C). A similar hysteresis between phosphorylation of NDR1(Thr-444)/NDR2(Thr-442) and activation of FLAG-NDR2 is seen in the individual comparisons of all interventions (Fig. 4). It probably reflects the ordered nature of the phosphorylation of first NDR1(Thr-444)/NDR2(Thr-442) followed by NDR1(Ser281)/NDR2(Ser-282). In previous work in which COS cells were transfected with tagged NDRs, OKA (1 μM, 60 min) stimulated NDR activity by 12- to 25-fold [3,12]. This compares with 21-fold in our analogous experiments (Fig. 4C), but we find that calyculin A (200 nM, 60 min) stimulates activity by about 45-fold (Fig. 4C). We do not know whether these differences result from trivial causes (e.g. differences in permeability) or whether there are mechanistic differences. Importantly, calyculin A stimulates phosphorylation of NDR1(Thr-444)/NDR2(Thr-442) in adult perfused heart (Fig. 8), confirming that the findings in neonatal myocyte are not confined to this particular stage of maturation.

Though calyculin A and OKA may show differing selectivities for PPP1 and PPP2 [OKA has a 50-fold greater IC_{50} for PPP1 than PPP2 (Table 2)], they are both relatively potent inhibitors of both PPPs at least *in vitro* (Table 2). We had hoped possibly to discriminate between PPP1 and PPP2 using the more selective inhibitors fostriecin and tautomycetin (Table 2), and we also studied the effects of microcystin LR. However, we were never able to activate FLAG-NDR2 with these, nor did microcystin LR cause phosphorylation of endogenous NDR1(Thr-444)/NDR2(Thr-442) or FLAG-NDR2(Thr-442). We do not understand the reasons for this. They could be trivial (e.g. permeability), but all ineffective inhibitors have been used in intact cell preparations previously. Equally, there may be PPP1/PPP2 holoenzyme species which are insensitive to fostriecin, tautomycetin or microcystin LR, or there may be PPP species other than PPP1 and PPP2 which are sensitive to calyculin A and OKA but which are not sensitive to fostriecin, tautomycetin or microcystin LR. Furthermore, it may be incorrect to assume that the actions of calyculin A or OKA are exerted at the level of the PPP(s) directly responsible for dephosphorylating NDRs. The point(s) of action could lie further upstream. The current predominating view that PPP2 is responsible for the dephosphorylation of NDR1(*P*Ser-281/*P*Thr-444) and NDR2(*P*Ser-282/*P*Thr-442) is based on experiments in intact cells with OKA [3,12], and on *in vitro* experiments involving dephosphorylation of NDR1(*P*Ser-281/*P*Thr-444) with recombinant PPP2 [12]. However, the concentration of OKA used in these experiments was 1 μM or 300-times greater than the IC_{50} even for PPP1. Whilst there may be unassessed permeability problems related to the access of OKA to the intracellular compartment, these experiments are not unequivocal. Equally, *in vitro* experiments with recombinant PPP2 are not unequivocal because of the possibility that any PPP might dephosphorylate the phospho-NDRs if present in high enough excess. The PPP(s) responsible for dephosphorylating NDR1 (*P*Ser-281/*P*Thr-444) and NDR2(*P*Ser-282/*P*Thr-442) remain somewhat obscure.

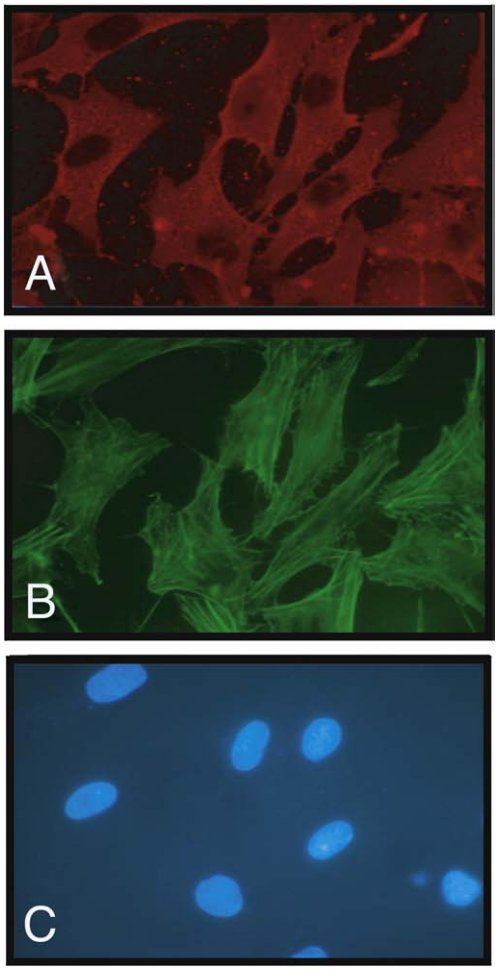


Fig. 7. Subcellular localisation of FLAG-NDR2. Myocytes were infected with Adv.FLAG-NDR2 and were stained with (A) monoclonal anti-FLAG, (B) phalloidin, or (C) Hoechst 33258 and a representative image of a single field is shown.

4.4. Phosphorylation and activation of NDR1/NDR2

Here, we have shown that NDR1/NDR2 are phosphorylated and activated by a variety of stimuli in cardiac myocytes and intact heart. Though we have not identified the kinases responsible, these experiments show that the endogenous upstream signalling is intact. With respect to the established kinases, PDK1 is often involved in phosphorylation of a T loop Ser-/Thr-residue in AGC kinases, i.e. a Ser-/Thr-residue in the catalytic domain [46,47]. Activation of PDK1, which is probably potentially active in the basal state by virtue of its high affinity interactions with 3-phosphoinositides [48], is mediated by PI3K-dependent phosphorylation of phosphatidylinositol 4,5-*bis*phosphate [PtdIns(4,5)P_2] to PtdIns(3,4,5)P_3 and lies downstream from the insulin or insulin-like growth factor 1 (IGF1) receptor protein tyrosine kinases (RPTKs) as well as other RPTKs [47,49]. These T loop phosphorylations are either regulatory (i.e. respond to external stimuli to change phosphorylation state and activity of the kinase involved) or are essentially facilitative (i.e. are constitutive rather than regulatory, but are necessary for activity). Evidence has been presented that neither PDK1 nor insulin or IGF1 is involved in the regulation of NDR1 [12] though, equally, contradictory evidence has been presented that IGF1-activated PDK1 is an NDR2(Thr-442) kinase and this phosphorylation precedes the autophosphorylation of the T loop residue [15]. We did not find any evidence that insulin (which does cause PI3K/PDK1-dependent phosphorylation of PKB/Akt in cardiac myocytes [50]) stimulated phosphorylation of NDR1(Thr-444)/NDR2(Thr-442) (Supplementary Material, Table 2A) nor did LY294002 inhibit the phosphorylation of NDR1(Thr-444)/NDR2(Thr-442) or activation of FLAG-NDR2 by calyculin A. Our data thus do not support a role for PDK1. A second mode of activation proposed by Suzuki et al. [15] involves a S100/Ca^{2+}-dependent phosphorylation of NDR2(Thr-75), then phosphorylation of NDR2(Thr-442) in the NDR2(*P*Thr-75) species by an unknown kinase, and finally autophosphorylation of NDR2(Ser-281) in the NDR2(*P*Thr-75/PThr-442) species leading to activation [14,15]. The necessary increase in Ca^{2+} concentrations is mediated through phospholipase Cγ (PLCγ) and hydrolysis of PtdIns(4,5)P_2 to the Ca^{2+}-mobilising 'second messenger', inositol 1,4,5-*tris*phosphate [Ins(1,4,5)P_3] [15]. However, in cardiac myocytes, platelet-derived growth factor stimulates PtdIns(4,5)P_2 hydrolysis and Tyr-phosphorylation of PLCγ1 [50], but does not stimulate NDR1(Thr-444)/NDR2(Thr-442) phosphorylation (Supplementary Material, Table 2A). Furthermore, ET-1 stimulates PLCβ-mediated hydrolysis of PtdIns(4,5)P_2 in cardiac myocytes [51] but does not stimulate phosphorylation of NDR1(Thr-444)/NDR2(Thr-442) or activation of FLAG-NDR2 (Supplementary Material, Table 2A and B).

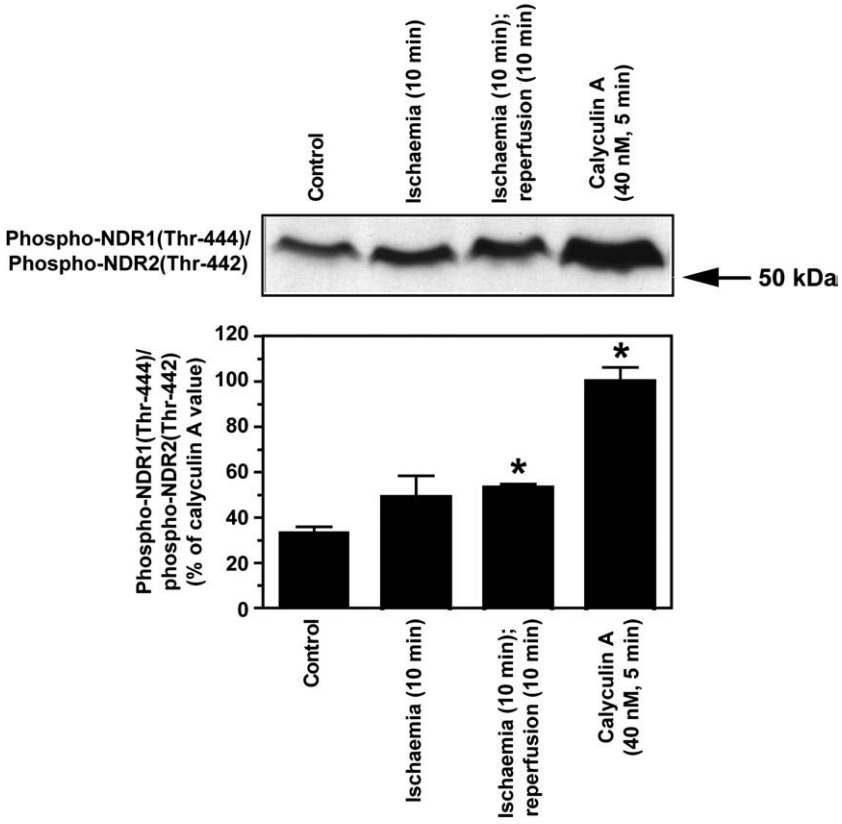


Fig. 8. Phosphorylation of NDR1(Thr-444)/NDR2(Thr-442) in intact hearts. In each separate set of experiments, following a 15 min retrograde stabilisation perfusion, *ex vivo* rat heart preparations were either (i) perfused retrogradely for a further 20 min, (ii) rendered globally-ischemic for 10 min by occlusion of the aortic inflow, (iii) reperfused retrogradely for 10 min following global ischaemia (10 min), or (iv) perfused retrogradely with 40 nM calyculin A for 5 min. Phosphorylation of NDR1(Thr-444)/NDR2(Thr-442) was assessed by immunoblotting with an anti-NDR phosphopeptide antibody, and intensities of the NDR1(*P*Thr-444)/NDR2(*P*Thr-442) signals were quantified by scanning densitometry and expressed relative to the values obtained with perfusions with calyculin A (40 nM, 5 min) for each single experimental grouping. Results are means±SEM for 8 complete sets of experiments. Statistical significance versus the control perfusions: $*P<0.0001$ *versus* control by an unpaired two-tailed Student's *t* test.

One upstream kinase that may be involved in the phosphorylation of NDR1(Thr-444)/NDR2(Thr-442) is mammalian STE20-like kinase 3 (MST3) [13], also known as STK24, a member of the germinal centre kinase family GCK-III subclass [52,53]. The regulation of these kinases in terms of the agonists that lead to their activation is not well-understood and indeed Pombo et al. [53] state that 'To date, no stimuli have been shown to activate MST3'. In fact, like the NDRs, MST3 is activated by OKA [13] and calyculin A [54]. Overexpression of MST3 in HEK293 cell causes fragmentation of DNA indicative of apoptosis/cell death [55]. If the NDRs are involved, this would suggest that their activation by MST3 should be pro-apoptotic. It will be of interest to examine whether, in addition to calyculin A and OKA, pro-apoptotic stimuli such as chelerythrine, hyperosmotic shock and H_2O_2 activate MST3. One characteristic of the upstream NDR1(Thr-444)/NDR2(Thr-442) kinase that we did identify is that it is inhibited by staurosporine (Fig. 5A–B). Staurosporine and its derivatives inhibit a broad spectrum of Ser-/Thr-protein kinases (including PDK1), though a number of kinases are relatively insensitive [33,36]. A prerequisite of the NDR1(Thr-444)/NDR2(Thr-442) kinase is that its activity or activation should be inhibited by staurosporine.

4.5. Pathophysiological relevance of interventions which stimulate phosphorylation of NDR1(Thr-444)/NDR2(Thr-442) and activation of FLAG-NDR2

The interventions which stimulate phosphorylation of NDR1(Thr-444)/NDR2(Thr-442) and activation of FLAG-NDR2 most clearly are calyculin A (Figs. 2A–B, D, 4A, C, 8) and OKA (Fig. 4A, C). Other than possibly contributing to the understanding of diarrhetic shellfish poisoning for which OKA is responsible, these findings have very little pathophysiological significance. The phosphorylation of NDR1(Thr-444)/NDR2(Thr-442) and activation of FLAG-NDR2 are also stimulated by a relatively-limited subset of cell stress stimuli, notably hyperosmotic shock (Figs. 3A, 4A–B) and oxidative stress (Figs. 3B–C, 4A–B) in neonatal cardiac myocytes. Perhaps most notably, global ischaemia/reperfusion, a pathologically-important pro-apoptotic stress in the heart [56], stimulates phosphorylation of NDR1(Thr-444)/NDR2(Thr-442) in isolated adult rat hearts (Fig. 8). Although there is a suggestion that ischaemia alone also resulted in phosphorylation of NDR1(Thr-444)/NDR2(Thr-442), this result did not achieve statistical significance (Fig. 8). In isolated hearts, the stress-activated protein kinases of the MAPK family are activated (by phosphorylation) by ischaemia and ischaemia/reperfusion [57]. Thus, ischaemia activates p38-MAPKs and this is increased on reperfusion [56,57]. Ischaemia alone does not activate the c-Jun N-terminal kinases (JNKs), but they are activated on reperfusion [57]. However, p38-MAPKs and JNKs are activated by a wider range of cellular stresses (e.g. anisomycin) in cardiac myocytes than those that lead to phosphorylation of NDR1(Thr-444)/NDR2(Thr-442) (Supplementary Material, Table 2A) or activation of FLAG-NDR2 (Supplementary Material, Table 2B), and they are also activated by some G protein receptor agonists, e.g. ET-1 and phenylephrine [58–60]. As with phosphorylation of NDR1(Thr-444)/NDR2(Thr-442) and activation of FLAG-NDR2 (Figs. 3 B–C, 4A–B), JNKs and p38-MAPKs are activated by oxidative stress in cardiac myocytes and the perfused heart [30,61], and our findings indicate that the increases in oxidative stress that the heart experiences during ischaemia and, more particularly, on reperfusion [62] contribute to their activation [30]. A similar mechanism could apply to the NDRs.

4.6. Subcellular localisation of NDR2

The subcellular localisation of the NDRs as assessed by either immunofluorescence or subcellular fractionation is controversial. NDR1 was first thought to be localised to the nucleus [5]. Tagged NDR1 overexpressed in HeLa cells also appears to localise primarily to the nucleus with evidence of an additional cytoplasmic localisation at higher levels of expression [4]. Somewhat surprisingly in view of the similarity in their primary sequences (see Supplementary Material, Fig. 1), endogenous or overexpressed tagged NDR2 appears to localise primarily to the cytoplasm (sometimes in a punctate manner) with evidence of nuclear localisation or perinuclear concentration when expression at higher levels [3,4]. However, Hergovich et al. [16] (see also Reference [10]) have now demonstrated that both endogenous NDR1 and overexpressed NDR1 are primarily cytoplasmic and suggest that some previous studies demonstrating a nuclear localisation resulted from misinterpretation of immunofluorescence data. In our hands, FLAG-NDR2 expressed ectopically to about the same level as total endogenous NDRs localises to the cytoplasm of cardiac myocytes, though there is some evidence of increased concentration at the plasma membrane (Fig. 7). This might perhaps be in keeping with the need for NDR2, on association with MOB proteins, to translocate to the plasma membrane for activation by phosphorylation [11,16]. We did not assess the localisation of NDR1.

4.7. NDR2-PIFtide

When protein kinases are activated by Ser-/Thr-phosphorylation, the normal tactic to increase the level of constitutive activity is to mutate the Ser-/Thr- residues to Asp- or Glu-. However, mutation of NDR1(Ser-281) does not affect activity and mutation of NDR1(Thr-444) caused only a 1.5- to 2-fold increase in activity [12]. In PKB/Akt, phosphorylation of the homologous hydrophobic domain Ser-(Ser-473, Ser-474 or Ser-472) depending on the isoform (α, β or γ, respectively)] results in full activation of PKB/Akt phosphorylated on the PDK1 site in the T loop (Thr-308 in PKBα, or Thr-309 in PKBβ and PKBγ) [49]. Crystallographic studies show that phosphorylation of PKBβ/Akt2(Ser-474) results in a disorder-to-order transition of the αC helix and interaction with PKB/Akt(*P*Thr-309) [63]. This ordered structure can be mimicked by the hydrophobic motif C-terminal region of PKN2 which contains the so-called PIFtide sequence which is highly ordered [64]. In NDR2, replacement of residues 433-464 with the PKN2 PIF domain (human PKN2 residues 969-983) produces an NDR2 species which was estimated as possessing a 20-fold increase in activity over the wild type NDR2 and in which the T loop phosphorylation site is phosphorylated [3]. The specific activity of tagged NDR2-PIFtide is about 2-fold greater than that of the tagged NDR2(wild type) from COS cells treated with 1 μM OKA for 60 min, and its activity might be slightly further increased by exposure of the cells to OKA [3]. We estimate here that the activity of FLAG-NDR2-PIFtide was about 10-fold greater than FLAG-NDR2(wild-type) (Fig. 6), and this was still much less (about 15–20%) than that of FLAG-NDR2 (wild-type) that had been activated by 200 nM calyculin A for 20 min. Calyculin A did not further activate FLAG-NDR2-PIFtide (Fig. 6), presumably because Thr-442 is absent. We were hoping that ectopic expression of the activated FLAG-NDR2-PIFtide might cause some obvious changes in its subcellular localisation and/or myocyte morphology. However, we were not able to detect any such changes and a more-detailed examination of any prospective phenotypes is currently being undertaken.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.cellsig.2008.04.013.

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