Functional Characterization of Human NDR Kinases: Novel Regulatory Mechanisms and a Path towards Direct Substrates

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Reto Kohler
aus Landiswil (BE)

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Prof. Dr. Martin Spiess

(Dekan)

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A. List of abbreviations

AGC	Protein kinase A (PKA)/PKG/PKC-like
ATP	Adenosine triphosphate
C. elegans	*Caenorhabditis elegans* (roundworm)
D. melanogaster	*Drosophila melanogaster* (fruit fly)
HM	Hydrophobic motif
Hpo	Hippo
KD	Kinase-dead
LATS	Large tumor suppressor
M/Met	Methionine
MOB	Mps-one binder
MST	Mammalian sterile-20 like
NDR	Nuclear Dbf2 related
NTR	N-terminal regulatory domain
OA	Okadaic acid
S. cerevisiae	*Saccharomyces cerevisiae* (budding yeast)
S. pombe	*Schizosaccharomyces pombe* (fission yeast)
STK	Serine/Threonine kinase
S/Ser	Serine
T/Thr	Threonine
Trc	Tricornered
WT	Wild-type
YAP	YES-associated protein

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

B. Summary

Protein kinases are important regulators of signal transduction pathways in both unicellular and multicellular organisms. They play critical roles in biological processes such as cell growth, division, differentiation, adhesion, motility and cell death. Given their central role in mediating cellular function and physiological responses, perturbation of protein kinase signaling can cause many diseases, including cancer and diabetes. Out of the 518 protein kinases encoded by the human genome, approximately 60 belong to the AGC group of Ser/Thr protein kinases, including the NDR kinase family. Members of this family are highly conserved from yeast to men and regulate important processes such as mitotic exit, cell polarity, neuronal and epithelial morphology, growth, proliferation and apoptosis. Despite the fact that NDR kinase family members regulate important cellular processes, direct downstream targets have only been identified recently. Furthermore, the regulation of NDR kinase signaling by upstream kinases of the Ste20-like family or co-activator proteins of the MOB family is also remarkably conserved. The human genome encodes for four NDR kinases: NDR1, NDR2, LATS1 and LATS2. Whereas the molecular mechanisms of NDR kinase regulation have mostly been worked out using human NDR1/2 kinases, biological functions have just started to emerge. Human NDR kinases were implicated in regulating centrosome duplication, mitotic chromosome alignment and apoptosis signaling. Additionally, the human MOB family consists of six distinct members (hMOB1A, -1B, -2, -3A, -3B and -3C), with hMOB1A/B the best studied due to their tumor suppressive functions through regulation of NDR/LATS kinases. The roles of the other MOB proteins are not as well defined.

We investigated the role of human MOB proteins in NDR/LATS kinase regulation. We found that three hMOB proteins did not bind to or activate all human NDR kinases and that hMOB2 was an NDR-specific binder. Furthermore, we describe competitive binding of hMOB1A/B and hMOB2 towards the NTR of human NDR1/2. Interestingly, in contrast to hMOB1A/B, hMOB2 is bound to unphosphorylated NDR1/2. Moreover, RNAi-mediated depletion of hMOB2 protein resulted in increased NDR activity. Consistent with these findings, hMOB2 overexpression impaired not only okadaic acid-induced activation of NDR but also the functional roles of NDR in death receptor-induced apoptosis and centrosome duplication. In summary, our data indicate that hMOB2 is a negative regulator of human NDR1/2 kinases.

Additionally, we established a basis for the discovery of additional human NDR kinase substrates. We employed the chemical genetic method developed by Shokat and colleagues to create analog-sensitive variants of NDR1/2 kinases. Subsequently, we have tried to identify direct targets of analog-sensitive NDR1(M166G) by performing *in vitro* kinase assays on cell lysates and immunocomplexes in the presence of a radiolabeled ATP analog and observed a specific and reproducible pattern of labeled bands in reactions containing NDR1(M166G) immunocomplexes. Our data together with the recent identification of the first *in vivo* substrate of human NDR1/2 kinases, p21, should stimulate further efforts to dissect the downstream signaling of mammalian NDR kinases.

1. Introduction

1.1. Protein kinases: Critical regulators of signal transduction

Protein kinases are key regulators of intracellular signaling pathways and mediate most of the signal transduction in eukaryotic cells. All protein kinases catalyze the transfer of the γ-phosphate group of ATP to the hydroxyl group of serine, threonine or tyrosine amino acid side chains [1]. The phosphorylation by protein kinases can alter the substrates properties such as activation/deactivation of enzyme activity, change in subcellular localization or increase/decrease in protein stability. Therefore, protein kinases play critical roles in cell growth, division, differentiation, motility, adhesion and death in unicellular and multicellular organisms and proper regulation of their activity is crucial for the normal physiology of organisms [2]. Hence, perturbation of protein kinases by mutation, altered expression, or general dysregulation can cause many human diseases such as cancer or diabetes [3].

The human protein kinase complement of the human genome, also known as the kinome, encodes for ~518 protein kinases and constitutes one of the biggest gene family of the human genome [2]. To gain insight into kinase function and evolution, all 518 protein kinases were classified into a hierarchy of phylogenetic groups, families and subfamilies based on the catalytic domain sequence [2]. The biggest kinase group is represented by tyrosine kinases, followed by the

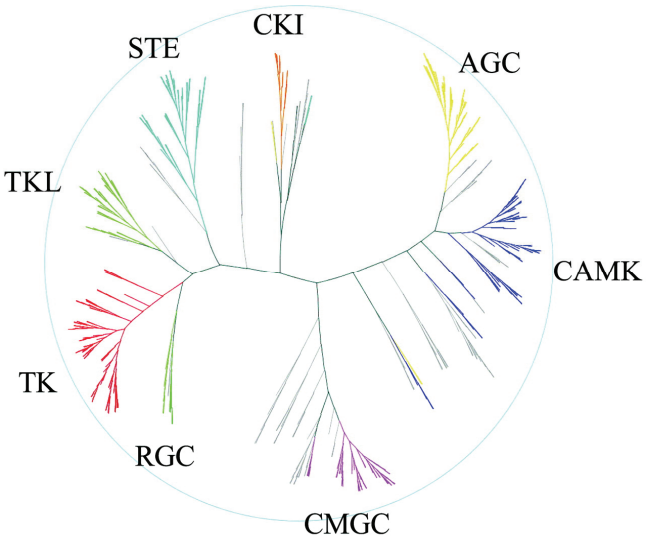

Figure 1. Dendrogram of 491 eukaryotic protein kinase domains from 478 genes. Major groups are labeled and colored (Taken from (2)).

CAMK, the AGC and then the CMGC group of kinases (Figure 1). The catalytic domain (kinase domain) consists of 250-300 amino acids defining 12 conserved subdomains which are thought to fold into a common bilobal catalytic core structure since residues that contribute to nucleotide binding, phosphoryl transfer or ion binding are highly conserved [1]. Furthermore, since deregulated protein kinase signaling is associated with the development of human diseases, members of this class of enzymes are emerging as attractive drug targets [4].

1.2. The NDR kinase family

The human genome encodes 63 protein kinases that are classified as members of the AGC group of serine/threonine kinases [2]. These kinases, along with their regulatory mechanisms by phoshporylation, are highly conserved throughout eukaryotic evolution and contain notable members such as isoforms of protein kinase A (PKA), PKC or PKB [5].

NDR kinases represent a less well-studied family of the AGC group. The human genome encodes four related NDR kinases: NDR1 (STK38), NDR2 (STK38L), LATS1 and LATS2 [6]. Members of the NDR family are highly conserved throughout evolution and can be found in organisms such as *S. cerevisiae* (Dbf2p, Dbf20p and Cbk1p), *S. pombe* (Sid2p and Orb6p), *C. elegans* (SAX-1 and LATS) and *D. melanogaster* (Warts and Trc) as well as other fungi, plants and protozoans [6]. Genetic and biochemical studies showed that NDR kinases amongst all species are crucial regulators of important functions such as mitosis, cytokinesis, cell polarity and morphogenesis, apoptosis, proliferation, centrosome duplication and other developmental processes [7].

1.2.1. Characteristics of NDR kinases

The primary structure of NDR kinases is conserved from yeast to men (Figure 2) [6]. The highly conserved kinase domain can be subdivided into 12 subdomains based on structural features and conserved residues [1]. AGC kinases share typical features such as phosphorylation sites in the activation segment located at subdomain VIII of the kinase domain and at the hydrophobic motif which is found in a non-catalytic region positioned C-terminally of the kinase domain [5]. All NDR kinases contain both typical characteristics of AGC kinases required for activation but are unique among the AGC group because they exhibit two distinct features only present in the NDR family: an N-terminal regulatory domain (NTR) also known as the S100B/hMOB1 association domain (SMA) and an insert of about 30-60 amino acids

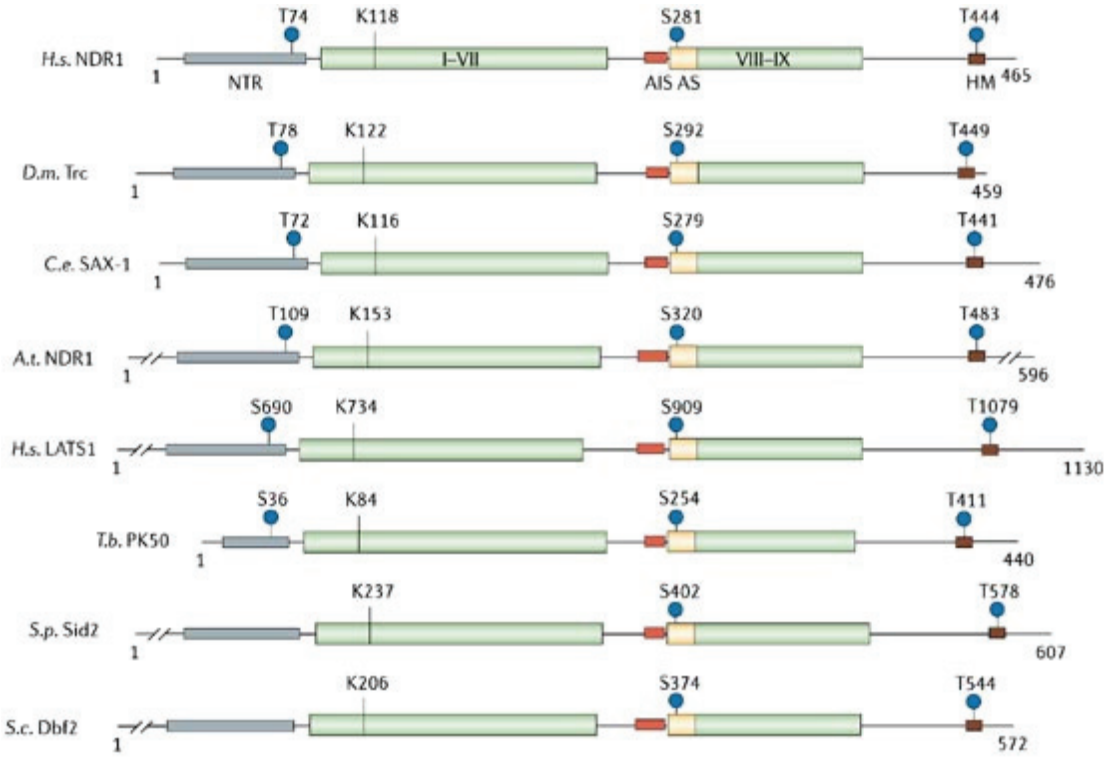

Figure 2. Primary structure of selected NDR kinases. Eight members of NDR family kinases from unicellular to multicellular organisms are shown (*H.s. Homo sapiens, D.m. Drosophila melanogaster, C.e. Caenorhabditis elegans, A.t. Arabidopsis thaliana, T.b. Trypanosoma brucei*). The N-terminal regulatory domain (NTR, grey), the kinase domain (green) with the activation segment (yellow) and the hydrophobic motif (brown) are shown. In addition, the auto-inhibitory sequence (red) and conserved phosphorylation sites (blue dots) are indicated. Taken from (7).

between subdomains VII and VIII of the kinase domain (Figure 2). The NTR is responsible for the interaction with S100B and hMOB1 proteins [8, 9]. Furthermore, the NTR contains a significant number of basic and hydrophobic residues which were shown to be essential for the interaction of hMOB1A with human NDR1/2 and LATS1/2 [8, 10-12]. Interestingly, MOB1 proteins contain a negatively charged area suggesting that the NDR/MOB, LATS/MOB complex formation is based on electrostatic interactions, which was supported by the analysis of a MOB1 structure in complex with an N-terminal peptide of NDR [13]. However, this structure did not include the full-length NTR and therefore the exact mechanism of how hMOB1 proteins interact with NDR remains largely unknown.

The primary sequence of the 30-60 residues insert between kinase subdomains VII and VIII seems to differ between NDR family members. However, all the inserts contain a stretch of basic residues located towards subdomain VIII [6]. These positively charged residues precede the activation segment and seem to inhibit NDR kinase activity since mutations of these residues to alanine lead to a significant increase in NDR1/2 kinase activity [8]. Therefore, this motif is also referred to as an auto-inhibitory sequence (AIS).

1.2.2. Regulation of NDR kinases

Regulation by phosphorylation

As mentioned above, many AGC kinases require phosphorylation on two highly conserved regulatory motifs for full activation [5]. Thus, all NDR kinases contain those main regulatory phosphorylation sites: the activation segment (Ser281 of human NDR1) and the hydrophobic motif phosphorylation site (Thr444 of human NDR1). In contrast to other AGC kinases, the activation segment is not targeted by an upstream

kinase but is regulated by autophosphorylation [14, 15]. Whether this autphosphorylation is mediated by a cis-acting or trans-acting mechanism has yet to be investigated, although work on the budding yeast NDR kinase Cbk1p suggests that the activation loop modification occurs through intramolecular autophosphorylation [16]. Still, similar to other AGC kinases, the hydrophobic motif of NDR kinases is targeted by upstream kinases [6]. The importance of phosphorylation of the hydrophobic motif is best illustrated by a structural study performed on PKB, another AGC kinase [17, 18]. The phosphorylated hydrophobic motif engages a hydrophobic groove within the N-terminal lobe of the kinase domain and this intramolecular association results in a global disorder-to-order transition and ultimately kinase activation [17, 18]. Furthermore, this mechanism of activation is likely to be transferrable to other AGC kinases, since replacement of the entire hydrophobic motif of human NDR2 kinase by the hydrophobic motif of PRK2 (which mimics hydrophobic motif phosphorylation) results in a constitutively active NDR kinase [14]. Congruently, the activation loop phosphorylation site and the HM phosphorylation site are essential for kinase activity, since mutation to alanine abolishes kinase activity of human NDR kinases [14]. Both phosphorylation sites are targets of the serine/threonine phosphatase PP2A. Treatments with okadaic acid (OA, potent inhibitor of PP2A) increase phosphorylation of yeast, fly and human NDR kinases [19-22]. In addition, recombinant PP2A completely inactivates human NDR [21].

Interestingly, in higher eukaryotes, a third phosphorylation site located within the NTR is conserved (Thr74 of human NDR1) [14, 21]. Mutation of this site to alanine reduces kinase activity of human NDR1 and also abolishes binding to hMOB1A [8]. Therefore, it remains to be shown whether this phosphorylation site has intrinsic

functions on kinase activity or whether the effect is a consequence of MOB1-binding loss.

Regulation by NDR kinases by the Ste20-like kinase family

Genetic studies in yeast lead to the first insights in the nature of upstream kinases regulating NDR kinase family members. It was shown that the Ste20-like kinases Cdc15p, Kic1p, Sid1p and Nak1p function upstream of the NDR kinases Dbf2p, Cbk1p, Sid2p and Orb6p [20, 23-26]. Yet, only Cdc15p was shown to directly phosphorylate and activate Dbf2p [20]. Genetic and biochemical studies in *D. melanogaster* also demonstrated that the Ste20-like kinase Hippo (Hpo) functions upstream of the NDR family kinase Warts (Wts) and that Hpo interacts and phosphorylates Wts [27-32]. Interestingly, Hippo also phosphorylates and activates in fruit flies the second NDR family kinase, Tricornered (Trc), on the HM [33]. This finding indicates that in higher eukaryotes multiple NDR kinases can be regulated by a single Ste20-like kinase.

The first Ste20-like kinase that was shown to phosphorylate specifically the hydrophobic motif phosphorylation site of NDR kinases was identified as mammalian sterile20-like kinase 3 (MST3) [34]. MST3 phosphorylates the HM of NDR1/2 kinases (Thr444/442) *in vitro* and *in vivo* [34]. The human MST family of protein kinases consists of 5 members: MST1, MST2, MST3, MST4 and YSK/SOK1. Recent work also demonstrated that kinases other than MST3 can act as HM-kinases of human NDR/LATS kinases. MST1 and MST2 were shown to phosphorylate human LATS1/2 [19]. Interestingly, MST1 also phosphorylates and activates NDR1/2 [35, 36]. Furthermore, MST2 phosphorylates NDR1/2 *in vitro* [36] and was suggested to function as the upstream kinase in cell culture [37]. Overall, genetic and biochemical

work undertaken in different organisms has linked Ste20-like kinases and NDR family kinase which indicates that the function of Ste20-like kinase as upstream activators of NDR kinase is a part of conserved signaling pathways across species. However, given that one Ste20-like kinase can regulate more than one NDR kinase and that one NDR family kinase can be regulated by multiple Ste20-like kinases indicates a significant complexity of the Ste20-like-NDR signaling pathways in higher eukaryotes. Therefore, multiple Ste20-like kinases might govern the different functions of a single NDR kinase.

Regulation of NDR kinases by MOB proteins

The N-terminal regulatory domain represents a unique feature of the NDR kinase family. The NTR is essential for the interaction with the MOB (Mps one-binder) family of proteins. MOB proteins are small globular proteins with no enzymatic activity and can be found in unicellular organisms to human (Phylogenetic tree of MOB proteins from yeast, Drosophila and humans can be found on page XX). Also conserved is the interaction of MOB proteins with the NTR of NDR kinases [6]. Structures of yeast, human and frog MOB1 proteins have been resolved [13, 38] and in combination with the knowledge of key residues in human NDR1/2 and LATS1/2 that are required for hMOB1A binding [8, 11, 39], it is likely that the positively charged NTR of NDR kinases interacts with a negatively charged surface on MOB1 proteins. In yeast, Mob1p binds to and is necessary for the activation of Dbf2p/Dbf20p and Sid1p kinases [20, 40, 41]. Similarly, the second MOB protein in yeast Mob2p binds to and activates Cbk1p and Orb6p [42, 43]. Interestingly, yeast MOB proteins and NDR kinases form restricted heterodimers in which the subunits are not interchangeable [42, 44]. In multicellular organisms, binding of MOB proteins

is not restricted to a single NDR kinase. In *D. melanogaster*, MOB as tumor suppressor (Mats)/dMOB1 physically interacts with Wts and is necessary for Wts activity [45, 46]. Mats/dMOB1 function seems to be evolutionary conserved since the human homolog hMOB1A can rescue the loss of Mats/dMOB1 in *Drosophila* [46]. In addition, Mats interacts genetically with the second NDR kinase, Trc [47]. Moreover, Trc physically interacts with a second MOB protein in flies, dMOB2 [47].

The human genome encodes six MOB genes, human MOB1A/B, hMOB2 and hMOB3A/B/C [35] and one closely related gene (Phocein) which is part of a phosphatase complex [48]. Whereas hMOB1A/B and hMOB2 were shown to physically interact and impact human NDR/LATS kinase activity [8, 11, 12, 39, 49], recent studies provide evidence that hMOB3A/B/C proteins do not interact with or activate all four NDR/LATS kinases [10, 35, 50]. Interestingly, whereas hMOB1A/B interact and activate all four human NDR/LATS kinases, hMOB2 was shown to interact only with NDR1/2 [8, 10-12, 49, 50].

Whereas it is known that MOB proteins interact with and are essential for the function of yeast and fly NDR kinase family members, most insight into the regulation of NDR activity and functions by MOB proteins was gained by studies on hMOB1A/B. Key residues in the NTR of NDR1/2 and LATS1/2 important for hMOB1A/B binding were identified [8, 11, 39]. hMOB1A/B are essential for the functions of human NDR1/2 in apoptosis and centrosome duplication [35, 36]. Furthermore, interfering with hMOB1A/B-NDR complex formation impaired activation and NDR1/2 function in the context of centrosome duplication [35]. Moreover, mutation of the conserved basic residues in the NTR of LATS1 negatively affects LATS1 kinase activity and activation [11]. In addition to the association with NDR kinases, hMOB1A/B proteins are also capable to interact with human MST1/2,

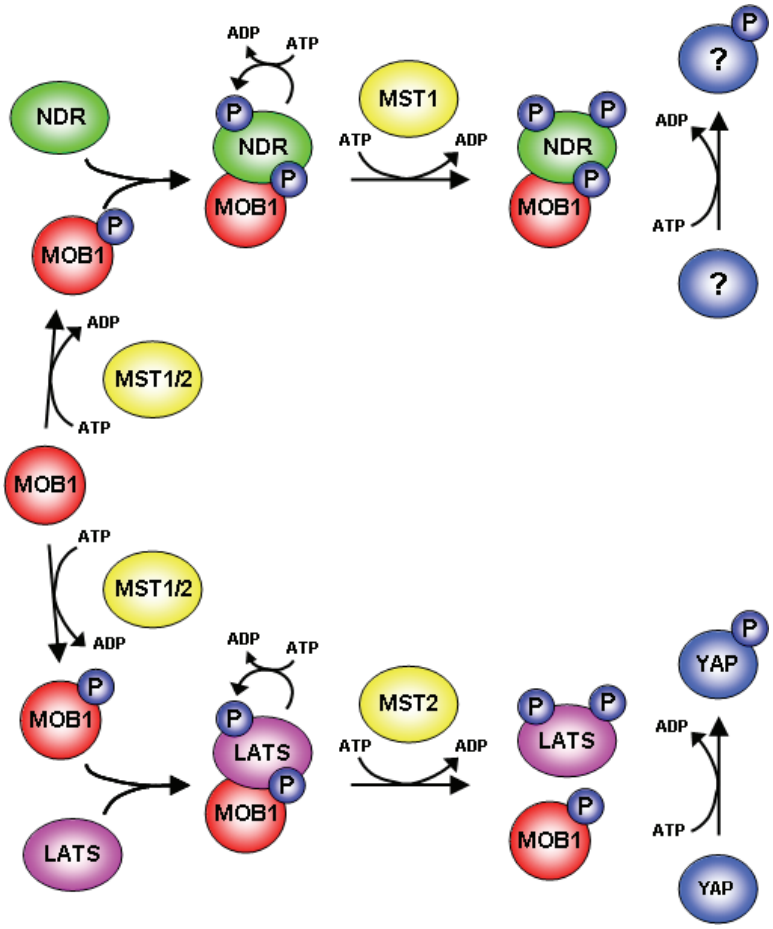

Figure 3. Current model of human NDR family kinase activation by MST kinases and hMOB1 proteins. Initially, MST1/2 phosphorylate hMOB1which results in efficient complex formation with NDR kinases. Binding of hMOB1 to NDR kinases facilitates activation-loop autophosphorylation and hydrophobic motif phosphorylation by MST1/2 kinases. Fully active NDR kinases then phosphorylate substrates as YAP for LATS1/2. Note that for phosphorylation by MST1, NDR1/2 reside in complex with hMOB1 whereas it is not necessary for LATS1/2. Taken from (52).

functioning possibly as adaptor proteins between NDR/LATS and MST kinases [36, 45, 51]. Moreover, hMOB1A/B proteins facilitate and are required for efficient HM phosphorylation of human NDR1/2 by MST kinases [34-36] .Accordingly, a tripartite complex of MST1, hMOB1A/B and NDR1/2 has been described [36]. Furthermore, human MST1/2 kinases as well as the fly Hpo kinase phosphorylate hMOB1A/B and Mats/dMOB1, respectively, thereby increasing MOB1 protein affinity towards NDR/LATS or Wts kinases [45, 51]. In addition, hMOB1A/B binding to NDR1/2 kinases is thought to release the kinase from autoinhibition by the AIS [8]. The obtained data so far suggest a model of NDR activation in which hMOB1A/B is

phosphorylated by MST1/2 which results in efficient complex formation of hMOB1 proteins and human NDR1/2 kinases, which in turn facilitates the phosphorylation of NDR1/2 by MST kinases [52] (Figure 3). Another level of human NDR kinase regulation by MOB1 proteins seems to be spatial relocalization. Artificial targeting of hMOB1 proteins to the plasma membrane results in rapid and robust activation of NDR1/2 or LATS1/2 [11, 39]. Indeed, membrane targeting of Mats/dMOB1 in *D. melanogaster* also leads to Wts activation and membrane-targeted Trc rescues the effect of *trc* mutant flies [22, 53]. These observations indicate that NDR/LATS kinase activation via relocalization to the plasma membrane, is an important step in the activation process and is most likely conserved *in vivo*. Interestingly, MOB1 proteins contain a positively charged surface [38] which would allow interaction with negatively charged phospholipids at the plasma membrane. However, the mechanisms by which NDR kinases are recruited to and activated at the plasma membrane by MOB1 proteins remain to be elucidated.

Much less is known about the role of MOB2 proteins in NDR kinase signaling. The NDR kinase family appears to be divided in terms of MOB2 binding. Whereas the NDR branch (human NDR1/2, Trc, Cbk1p and Orb6p) interacts with MOB2 proteins, the LATS branch (human LATS1/2, Wts, Dbf2p and Sid2p) does not associate with MOB2 [11, 12, 20, 42-44, 46, 47, 49]. Conflicting data exist about the function of NDR-MOB2 interactions. In yeast, the interaction between Cbk1p/Orb6p and Mob2p proteins are essential for yeast NDR kinase activity and functions [42, 54]. In *Drosophila,* the role of dMOB2 appears to be different. dMOB2 and trc physically interact and co-localize, yet, kinase activity was not addressed in this setting [47]. Moreover, mutations in the dMOB2 gene did not enhance the *trc* phenotype, whereas mutations in the Mats/dMOB1 gene did so [47]. Intriguingly, forced overexpression

of a truncated version of dMOB2, which shares high sequence similarity with the human MOB2 protein, resulted in a weak Trc dominant-negative phenotype [47]. This observation indicates that dMOB2 might have inhibitory properties towards Trc kinase. Apart from that, a recent study implicates dMOB2 in photoreceptor morphogenesis in *Drosophila* [55]. Whether this function is dependent on NDR kinases such as Trc has yet to be elucidated. Comparable to *Drosophila* MOB2, the role of mammalian MOB2 in the regulation of NDR kinases is not clear. Two studies suggest that human MOB2 serves as an activator of human NDR1/2 kinases *in vitro* [37, 49]. However, these findings were not confirmed in a cellular setting.

Altogether, MOB proteins are essential regulators of NDR kinases across species. While MOB1 proteins appear to be activators in all species tested so far, the role for MOB2 proteins in the course of NDR kinase activation in higher eukaryotes remains less defined. Furthermore, the function of the additional MOB proteins (such as human MOB3A/B/C) has yet to be clarified in multicellular organisms.

Regulation of NDR kinases by scaffolding proteins

Large scaffolding proteins are thought to provide higher specificity for signal transmission and are also conserved regulators of NDR kinase activity and function. In yeast, *C. elegans*, *D. melanogaster* and mammals, scaffold proteins which impact on NDR kinases were identified [24, 37, 56-59]. In yeast, scaffold proteins Tao3p and Mor2p are required for Cbk1p and Orb6p functions [24, 58]. In *C. elegans*, the scaffold protein SAX-2 was shown to genetically interact with the NDR kinase SAX-1 [57]. More is known about the role of Furry in the regulation of Trc in *D melanogaster*. Furry genetically and physically interacts with Trc and is essential for Trc kinase activity [56, 60, 61]. Furthermore, the region of Trc necessary for the

interaction with Furry was investigated [60]. More recently, murine Furry was cloned and was shown to physically interact with human NDR1/2 and hMOB2 and was suggested to regulate human NDR kinase activity [37]. However, the functional significance of this interaction has yet to be investigated in the context of NDR regulation during apoptosis or centrosome duplication.

In the case of LATS kinases in flies and humans, different scaffolding proteins are described. The relatively small scaffold protein human WW45 and its fly homolog Salvador (Sav) contain a WW domain necessary for its interaction with LATS kinases and were shown to be important for functions of human LATS1/2 and fly Warts [19, 29, 32, 62]. Although no clear yeast ortholog of WW45 proteins has been described, the Furry-like and WW45 proteins share the common function of linking the Ste20-like upstream kinases with the respective NDR kinases. Nevertheless, more work is needed to understand the role of large scaffolding proteins in the regulation of NDR kinase activity and function.

1.2.3. Functions of NDR kinases

The functions of NDR kinase family members were first identified using genetic studies in yeast. Dbf2p is a central player of the mitotic exit network (MEN) in *S. cerevisiae* and Sid2p regulates the septation initiation network (SIN) in *S. pombe* [63], while the other members Cbk1p and Orb6p are critical regulators of morphogenesis in *S. cerevisiae* and *S. pombe* [64, 65]. Since then, NDR kinases across all species were shown to be implicated in important biological functions including regulation of proliferation, cell growth, apoptosis, neuronal growth, differentiation and dendritic tiling, centrosome duplication and embryonic development (Table 1).

Functions of NDR kinases in yeast

As mentioned above, the NDR kinases Dbf2p and Sid2p are essential for the functionality of MEN and SIN in budding and fission yeast [63, 66]. Briefly, in *S. cerevisiae*, regulation of MEN includes activation of the small GTPase Tem1p, after which the Cdc15p protein kinase is stimulated, followed by activation of the Dbf2p/Mob1p complex. The Dbf2p/Mob1p complex then phosphorylates Cdc14p leading to Cdc14p phosphatase relocalization from the nucleolus to the cytoplasm [67], where it dephosphorylates CyclinB-Cdk complex finally allowing exit from mitosis. A very similar pathway exists in *S. pombe* consisting of the small GTPase Spg1p, the protein kinases Sid1p and Cdc7p, the Sid2p/Mob1p complex and the phosphatase Clp1p. Interestingly, also in fission yeast, the activated Sid1p/Mob1p complex phosphorylates Clp1p therefore controlling retention of Clp1p in the cytoplasm [68]. Human LATS1 has also been implicated in the regulation of mitotic exit [12]. However, the precise mechanisms remain undefined and more detailed work needs to be undertaken to assess whether NDR kinases are implicated in a mammalian mitotic exit network.

The second NDR kinases in yeast, Cbk1p in budding yeast and Orb6p in fission yeast, are part of a morphogenesis network In *S. cerevisiae*, Cbk1p is an essential component of the RAM (regulation of Ace2p activity and cellular morphogenesis) signaling cascade that regulates polarized growth. The Cbk1p/Mob2p complex directly phosphorylates the transcription factor Ace2p leading to its retention in the nucleus in the daughter cell, therefore controlling transcriptional asymmetry [69]. Orb6p in S. pombe is required for the coordination of morphological changes with cell cycle progression. It is thought that Orb6 controls cell morphogenesis by spatially restricting the localization and the activity of the GTPase Cdc42 [70].

NDR Kinase	Species	Functional information
Dbf2p	*S. cerevisiae*	Controls mitotic exit and cytokinesis
Cbk1p	*S. cerevisiae*	Centerpiece of the 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
SAX-1	*C. elegans*	Important for neurite outgrowth and dendritic tiling
Warts	*D. melanogaster*	Central player in the Hippo pathway; required for dendritic maintenance
Trc	*D. melanogaster*	Controls epidermal outgrowths, and dendritic tiling and branching
LATS1	*H. sapiens, M. musculus*	Regulates G2/M cell cycle transition, apoptosis and mitotic progression; part of the G1 tetraploidy checkpoint; LATS1 cDNA can rescue the loss of *Drosophila* 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 E12.5
NDR1/STK38	*H. sapiens, M. musculus*	Required for centrosome duplication and FAS mediated apoptosis; implicated in mitotic chromosome alignment; NDR1 cDNA can compensate for the loss of *Drosophila* Trc; NDR1 null mice develop T-cell lymphoma
NDR2/STK38L	*H. sapiens, M. musculus*	Functions in the regulation of neuronal growth and differentiation; NDR2 overexpression induces centrosome overduplication and NDR2 can compensate for depletion of NDR1 in centriole duplication

Table 1. Selected functions of NDR family kinases. Modified from (7).References for the indicated functions can be found in the text.

NDR kinases in neuronal growth and differentiation

Work in *C. elegans* and *D. melanogaster* identified a role for NDR kinases in the regulation of neuronal growth, morphology and differentiation. In *C. elegans*, the NDR kinase SAX-1 (sensory axon defects-1), in conjunction with the large scaffold protein SAX-1, regulates neurite outgrowth and dendritic tiling [57, 71]. Dendritic tiling is a organizational phenomenon for a same type of neurons to maximally cover a receptive field with minimal redundancy [72]. The role for NDR kinases in dendritic tiling is conserved in *Drosophila* [72]. The Jan lab has shown that Trc together with Furry regulate dendritic tiling in *D. melanogaster* [61]. Importantly, they could also demonstrate that this function is dependent on Trc kinase activity which is directly

regulated by Furry [56, 61]. Moreover, using genetic and biochemical approaches, they could demonstrate that Trc as well as Wts are phosphorylated and activated by Hippo in the context of dendritic tiling [33]. Hippo regulates Trc for proper neurite outgrowth and tiling, whereas Wts is regulated to ensure maintenance. Thus, they demonstrated for the first time that one single Ste20-like kinase can regulate two different NDR kinase family members [33]. Interestingly, this indicates that two distinct NDR family members can regulate two different aspects of the same biological process. Furthermore, the target of rapamycin complex 2 (TORC2) has been implicated in regulating Trc activation in the process of dendritic tiling [22]. Besides regulating dendritic tiling, the NDR kinase Trc, together with Furry, also control cell morphogenesis such as post-mitotic wing hair development [47, 56].

Overall, the work from invertebrates identified an important role for the NDR kinases such as Tricornered and Warts in neuronal growth and differentiation. Unfortunately, the downstream targets of Trc or Warts in the context of dendritic tiling are unknown. Furthermore, mammalian NDR kinases involvement in neuronal processes such as dendritic tiling has yet to be investigated. Interestingly, one report identified the NDR2 transcript to be upregulated in the amygdala in fear-conditioned mice and that in cultured cells NDR2 was involved in neuronal growth and neurite outgrowth therefore indicating a conserved role for NDR in neuronal morphology [73].

Tumor suppression from flies to humans: the Hippo pathway

The NDR kinase Wts was first identified in screens for tumor suppressors in *Drosophila* [74, 75] and subsequently been shown to act as a tumor suppressor in mice [76]. Later, the Ste20-like kinase Hippo was found to act upstream of Wts and

also the MOB protein Mats/dMOB1 was shown to regulate Wts tumor suppressive function [28, 31, 46]. Since then, numerous proteins were shown to be implicated in the regulation of this emerging tumor suppressor pathway collectively called the Hippo pathway (Figure 4). This pathway has attracted broad interest in both clinical and basic research. Most of the components of the pathway are highly conserved across species and have been extensively reviewed in the last years [52, 77-85].

Four tumor suppressors were found to constitute the core kinase cassette of hippo signaling in flies: the NDR family kinase Wts, the Ste20-like kinase Hippo and the adaptor proteins Sav and Mats/dMOB1 [78, 85]. Biochemically, these proteins form a cascade in which the Hippo-Salvador complex phosphorylates and activates the Warts/Mats complex which in turn phosphorylates and inactivates the transcriptional co-activator Yorkie [27, 28, 45]. Yorkie was the first identified substrate of all NDR kinases across species. Phosphorylation of Yorkie on Ser 168 leads to 14-3-3 mediated nuclear exclusion [86]. In the nucleus, Yorkie interacts with TEAD family transcription factor Scalloped and promotes the transcription of genes such as dCyclinE, diap1 (drosophila inhibitor of apoptosis 1) or the bantam microRNA to regulate apoptosis and cell proliferation [87-90]. Genetic screens or candidate gene-based approaches lead to the identification of many other genes involved in the regulation of the core cassette of the hippo pathway. These include the FERM domain containing proteins Merlin/Nf2 and Expanded, the protocadherins Fat and Dachsous, the apical transmembrane protein Crumbs, the WW and C2 domain containing protein Kibra, the protein phosphatase 2A complex dSTRIPAK and the CK1 family kinase Discs Overgrown ([78, 85] and Figure 4).

As mentioned before, most of the components of the fly Hippo pathway are conserved in mammals. The core cassette of the mammalian Hippo pathway consists

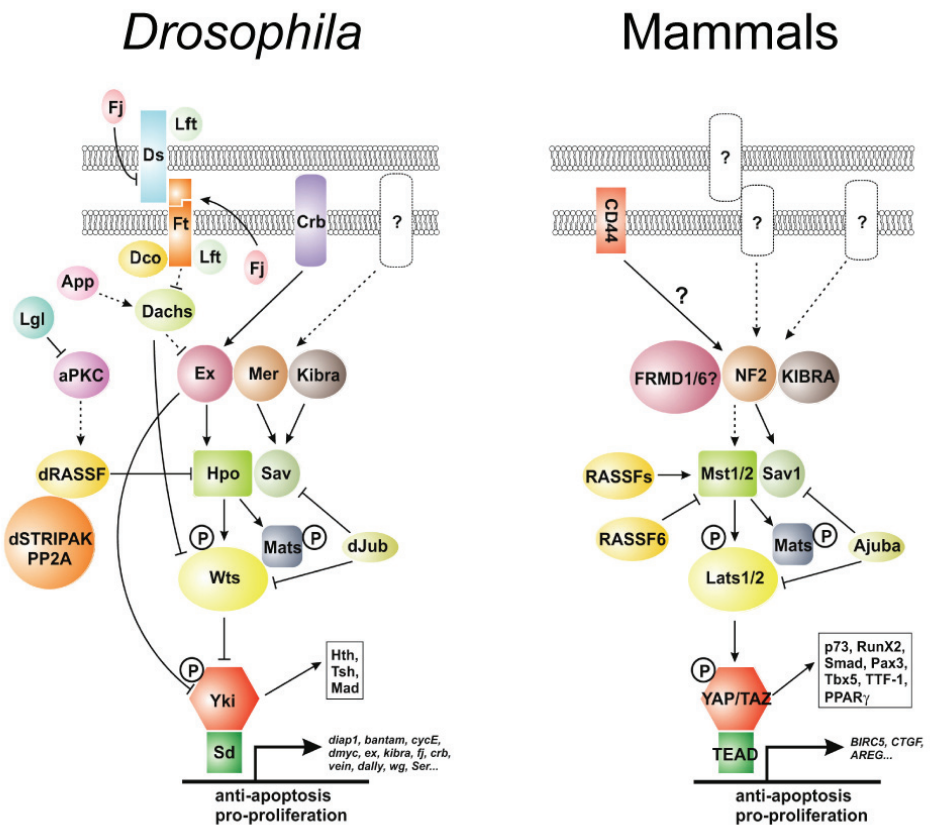

Figure 4. Signaling diagram of the hippo signaling network in *Drosophila* and mammals. Corresponding proteins in *Drosophila* and mammals are matched in colors and shapes. Direct biochemical interactions are indicated by solid lines or drawn as proteins in direct contact with each other. Dashed lines indicate genetic interactions for which no direct protein-protein interactions have been reported. Arrowed or blunted ends indicate activation or inhibition, respectively. Also shown are selected target genes. Yki- or YAP/TAZ-interacting transcription factors other than Scalloped (*Drosophila*) or TEAD (mammals) are collectively listed in a box. Adapted from (78).

of the Hippo orthologs MST1/2, the mammalian Salvador protein WW45, the Mats/dMOB1 homologs hMOB1A/B, the mammalian Wts kinases LATS1/2 and the transcriptional co-activator proteins and Yorkie homologs YAP and TAZ [52, 78, 85]. Although most of the additional regulators identified in *Drosophila* are conserved in mammals, their function in regulating the mammalian Hippo pathway has yet to be determined [85]. Furthermore, recent evidence revealed an interplay between the Hippo pathway and other established signaling cascades such as the Wnt, Notch and Hedgehog pathway [79, 85]. Interestingly, work on the Hippo pathway in mammalian systems revealed additional complexity and in some cases also divergence from the *Drosophila* network. For instance, LATS1/2 phosphorylate YAP and TAZ on five

sites homologous to Ser168 in Yorkie. Although Ser127 in YAP (the Ser168 homology site in Yorkie) seems to be the main regulator of nuclear translocation, there is also evidence that for certain functions all five phosphorylation sites are important [86, 91, 92]. Moreover, recent studies demonstrate a role for phosphorylated YAP and TAZ proteins in regulating Wnt and TGF-β-SMAD signaling in the cytoplasm [93, 94], mainly by direct binding to Dishevelled or SMAD proteins. Furthermore, YAP was shown to bind other transcription factors than the ones part of the TEAD family. In apoptosis signaling, YAP also binds to and activates the p53 family member p73 [95]. Also, RASSF (Ras-association domain family) proteins were shown to differentially regulate MST1/2 kinases in mammals. Whereas RASSF1A binds to MST1/2 through their SARAH domain and stimulates kinase activity of MST1/2 [96], another isoform, RASSF6, was shown to act similarly to the sole Drosophila ortholog dRASSF and was shown to inhibit MST1/2 signaling [97, 98].

Using genetically modified mice, tumor suppressive roles could be confirmed for some components of the mammalian Hippo pathway such as LATS1 [76], RASSF1A [99], Merlin/Nf2 [100, 101] and MST1/2 [102]. Interestingly, neither MST1 nor MST2 single knock-outs display cancer phenotypes and the MST1/2 double knock-out are embryonic lethal [103], indicating a functional redundancy between MST1 and MST2. However, the tissue-specific double knock-out in the liver results in tumor formation [102, 104]. Furthermore, the Hippo pathway might also diverge in mammals form *Drosophil*a in terms of tissue or cell type-dependent actions of the components. In mouse embryonic fibroblast (MEF) cells, ablation of MST1 and MST2 does not affect LATS1/2 phosphorylation [102]. More interestingly, an alternative YAP-Ser127 kinase might exist in mouse liver [105]. Genetic ablation of

MST1/2 in this tissue had no effect on LATS1/2 phosphorylation but abolished YAP Ser127 phosphorylation [102]. In addition, Zhou et al. tested the presence of additional Ser127 activity in biochemical fractions of MST1/2 knock-out livers and found that a second peak distinct from LATS1/2 existed in the liver [102]. This data indicate that in the mouse liver a different YAP kinase is present which probably could be NDR1/2 [105]. However, NDR1/2 phosphorylate YAP *in vitro* but fail to do so in tissue cultured cells [91]. More work is needed to decipher the tissue and cell dependent components of the mammalian Hippo pathway. Still, studies in *Drosophila* and mammals have firmly established the Hippo pathway as a central mechanism controlling organ size and tissue homeostasis and that dysregulation of this pathway underlies various diseases such as cancer.

In addition to the regulation of YAP and TAZ, mammalian LATS1 and LATS2 kinases have also been implicated in other functions independent of the Hippo pathway (Table 1) [7].

The roles of NDR1 and NDR2 in mammals

The mechanisms of biochemical regulation of NDR kinase family members have mainly been worked out using human NDR1/2 kinases. However, the biological functions of human NDR1/2 kinases have only been identified recently. Hergovich and colleagues identified a role for NDR1/2 kinases in centrosome duplication [106]. Significantly, overexpression of NDR1 or NDR2 lead to overduplication of centrosomes and RNAi (RNA interference)-mediated depletion of NDR1 resulted in impaired centrosome duplication [106]. Further work demonstrated that the function of NDR1/2 at the centrosome depends on intact interaction with hMOB1A/B and the regulation of HM phosphorylation by the MST1 kinase [35]. Moreover, another study

performed by the Hemmings lab provided evidence that NDR1/2 kinases are activated by hRASSF1A and MST1 upon FAS-mediated apoptosis [36]. RNAi-mediated depletion of NDR1/2 lead to reduced apoptosis upon stimulation of death receptors and also this function of NDR1/2 appears to be dependent of hMOB1A/B binding [36]. NDR1/2 were also implicated in the alignment of chromosomes during mitosis [37]. Chiba and colleagues investigated the role of the mammalian homolog of Furry and could indicate a role for human NDR1/2-Furry in microtubule dynamics and stability. Although this finding is potentially interesting, the study displays major inconsistencies with previously published work. They could not reproduce the centrosome duplication phenotype in NDR-depleted cells and hMOB1A was not able to stimulate NDR1/2 kinase activity in their assay [37]. More recently, Cornils and colleagues described for the first time a tumor-suppressive role for NDR kinases *in vivo* [107]. Firstly, mice homozygous for knock-outs of NDR1 do not have any major phenotypes possibly due to a functional redundancy of the two isoforms NDR1 and NDR2 [107]. Indeed, in tissues that express mainly NDR1under wild-type conditions, NDR2 is upregulated upon knock-out of NDR1. Significantly, mice lacking NDR1 are more prone to develop T-cell lymphomas than wild-type littermates. Furthermore, the study indicates that the analyzed lymphomas display loss of the second isoform NDR2. Therefore, the loss of both NDR isoforms in mammals results in resistance to apoptosis and ultimately leads to tumor development [107]. Based on the functional compensation of NDR1 and NDR2, expression of both isoforms in human cancers should be investigated in order to gain more insight on the role of NDR1/2 in tumor development.

Altogether, although the biochemical regulation of human NDR1/2 kinases has been well established, the physiological roles of mammalian NDR1/2 is just starting

to unravel. However, still more work using genetically modified mice carrying tissue-specific disruptions of NDR1/2 is needed in order to further define the roles of NDR1/2 in normal physiology and tumor development and therefore complement aforementioned studies undertaken in tissue-cultured cells.

1.2.4. Substrates of NDR Kinases

Although the functions of NDR kinase family members have been worked out in detail first in *S. cerevisiae* and *S. pombe*, and that it was subsequently shown across species that these kinases are regulators of important functions, direct substrates have only been recently identified (Table 2).

In budding yeast, Dbf2p phosphorylates three serines adjacent to the nuclear localization signal (NLS) of the protein phosphatase Cdc14p thereby abrogating its NLS activity leading to cytoplasmic localization [67]. All three phosphorylation sites match a previously identified consensus phosphorylation motif for the activated Dbf2p-Mob1p complex, RxxS [67, 108]. Similarly, in fission yeast the Sid2p-Mob1p complex phosphorylates Clp1p, the Cdc14p homolog in *S. pombe* [68]. However, Sid2p needs to phosphorylate six serines which however also lie within RxxS consensus sites. In contrast, Clp1p phosphorylation by Sid2p creates binding sites for the 14-3-3 protein Rad24, leading to its cytoplasmic retention during cytokinesis [68]. However, whether this kinase-substrate pair is conserved in higher eukaryotes has yet to be investigated since the phosphorylation sites in Cdc14p or Clp1p are not conserved in the two human homologs CDC14A/B.

Recently, direct substrates for the second NDR kinase in budding yeast, Cbk1p, were identified. The study performed by the Weiss lab suggests that Cbk1p exhibits strong preference to phosphorylate the sequence HxRRxS determined by positional

scanning peptide arrays [69]. Furthermore, they provide evidence that Cbk1p controls transcriptional asymmetry through phosphorylation of the transcription factor Ace2p on four sites containing the consensus motif HxK/RK/RXS. Phosphorylation of Ace2p by Cbk1p blocks its interaction with nuclear export machinery thereby resulting in accumulation in the nucleus and increased function [69]. Moreover, a second substrate of Cbk1p was described, a RNA-binding protein Ssd1. Cbk1p phosphorylates Ssd1 on at least eight sites matching the previously determined consensus for Cbk1p [109]. Thereby, Cbk1p regulates the translation and transcription of daughter cell-specific genes via inhibition of Ssd1. No substrate has been identified for the second NDR kinase in *S. pombe*. However, one study suggests that Orb6p could control cellular morphogenesis by directly phosphorylating the Cdc42 guanine nucleotide exchange factor Gef1 [70]. It still needs to be determined whether Gef1 is a direct substrate of Orb6p *in vivo*.

The first *in vivo* substrate of NDR kinases across species was identified by the investigation of interaction partners of the Wts kinase in *Drosophila* [27]. The transcriptional co-activator Yorkie is phosphorylated by Wts on Ser168. The phosphorylation site, as well as the underlying regulatory mechanisms, seems to be conserved in mammals [86]. *Drosophila* Wts phosphorylates Yorkie on Ser168 and LATS1/2 phosphorylate the Yorkie homologs YAP and TAZ on at least five sites, one of them, Ser127 in YAP and Ser89 in TAZ, is the Ser168 homology site of Yorkie [86, 91, 92, 110]. Phosphorylation on this site creates a 14-3-3 binding site and exclusion from the nucleus. Interestingly, all phosphorylation sites lie within the HxRxxS consensus [86, 91, 92, 110]. Although the fly NDR kinases Wts and Tricornered cooperate in establishment and maintenance of dendritic tiling, no substrates in this particular function have yet been identified (Table 2).

Species / NDR Kinase		Substrate	p-Motif	Consequence	Identification
S. cerevisiae	Dbf2	Cdc14	RxxS	release from nucleolus	candidate
	Cbk1	Ace2, Ssd1	HxR/KR/KxS	inhibiting nuclear export, inhibition	candidate, motif search
S. pombe	Sid2	Cdc14	RxxS	cytoplasmic retention	candidate
	Orb6	-	-	-	
D. melanogaster	Warts	Yorkie	HxRxxS	cytoplasmic retention	interactor
	Trc	-	-	-	
H. sapiens	LATS1/2	YAP, TAZ	HxRxxS	cytoplasmic retention	conservation
	NDR1/2	p21*	RRKRxxS	degredation	candidate

Table 2. Selected substrates of NDR kinases from budding yeast (*S. cerevisiae*), fission yeast (*S. pombe*), fruit fly (*D. melanogaster*) and human (*H. sapiens*). Phosphorylation motifs of the respective substrate are indicated (H, Histidine; R, Arginine; S, Serine; x; any aminoacid). Identification indicates the method used to identify the respective substrate. References can be found in the text. *this study, see Appendix.

In mammals, LATS1/2 phosphorylate YAP and TAZ as mentioned above. Recently, LATS2 was shown to phosphorylate the pro-apoptotic protein ASPP1 (apoptosis-stimulating protein of p53-I) [111]. However, the phosphorylation site as well as the functional relevance of this modification in the pro-apoptotic function of ASPP1 remains to be shown. Although functions of human NDR1/2 were identified, direct substrates for human NDR1/2 kinases have not be identified. Only very recently the first *in vivo* substrate of NDR1/2 in the context of cell cycle regulation was identified as the cyclin-dependent kinase inhibitor p21/Waf1/Cip1 (Cornils and Kohler, see Appendix). NDR1/2 directly phosphorylate p21 on Ser146 leading to its degradation and proper G1/S progression of the cell cycle (see Appendix).

Overall, the substrates of NDR kinases from yeast to men were identified mainly by a candidate substrate approach [112] (Table 2). No study so far has used an unbiased approach for the identification of NDR kinase substrates. Therefore, it seems important to apply such an approach in order to find additional NDR kinase targets which will further clarify our understanding of how NDR kinases execute their different roles across species.

2. Scope of the Thesis

The aim of the study was to functionally characterize the human MOB (hMOB) family in the context of human NDR kinase regulation. Although the regulation of human NDR kinase family members by hMOB1/A/B is well studied, the function of the remaining human MOB proteins is not that well characterized (hMOB2, hMOB3A/B/C). We describe differential interactions of human MOB proteins with NDR/LATS kinases. Whereas hMOB3 proteins did not interact with or activate NDR/LATS kinases, hMOB2 restrictively interacted with NDR1/2. Concentrating on the function of hMOB2, we demonstrated an inhibitory role for a MOB protein for the first time. hMOB2 competes with hMOB1A/B for binding to the NTR of NDR1/2 kinases thereby interfering with hMOB1A-mediated activation. Although NDR1/2 kinases were shown to form active complexes with hMOB1A/B proteins, we observed that hMOB2-NDR1/2 represents an inactive complex. Indeed, our data indicates that endogenous hMOB2 has inhibitory properties towards NDR1/2 since RNAi-mediated knock-down of hMOB2 resulted in increased NDR phosphorylation and activity. Congruently, overexpression of hMOB2 impaired activation and function of human NDR1/2 kinases in apoptosis and centrosome duplication.

An additional aim was the identification of novel substrates for human NDR1/2 kinases. We employed a chemical genetic strategy to create analog-sensitive NDR kinases which can be used to specifically label direct targets using an analog ATP. By performing kinase assays on immunocomplexes of analog-sensitive NDR1, we observed a specific and reproducible pattern of labeled bands. The development of such an unbiased method for NDR and the recent identification of the first *in vivo* substrate of human NDR1/2 kinases will certainly stimulate future efforts to discover novel downstream targets of human NDR kinases.

3. Results

3.1. Human MOB2 inhibits human NDR1/2 kinases

3.1.1. Summary

MOB proteins are integral components of signaling pathways controlling important cellular processes such as mitotic exit, centrosome duplication, apoptosis and cell proliferation in eukaryotes. The human MOB protein family consists of six distinct members (human MOB1A [hMOB1A], -1B, -2, -3A, -3B and -3C), with hMOB1A/B being the best studied due to their putative tumor suppressive functions through the regulation of NDR/LATS kinases. The roles of the other MOB proteins are less well defined. Accordingly, we characterized all six human MOB proteins in the context of NDR/LATS binding and their ability to activate NDR/LATS kinases. hMOB3A/B/C proteins neither bind nor activate any of the four human NDR/LATS kinases. We found that both hMOB2 and hMOB1A bound to the N-terminal region of NDR1. However, our data suggest that the binding modes differ significantly. Our work revealed that hMOB2 competes with hMOB1A for NDR binding. hMOB2, in contrast to hMOB1A/B, is bound to unphosphorylated NDR. Moreover, depletion of hMOB2 by RNA interference (RNAi) resulted in increased NDR kinase activity. Consistent with these findings, hMOB2 overexpression interfered with the functional roles of NDR in death-receptor signaling and centrosome overduplication. In summary, our data indicate that hMOB2 is a negative regulator of human NDR kinases in biochemical and biological settings.

3.1.2. Published Manuscript

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Reto S. Kohler, Debora Schmitz, Hauke Cornils, Brian A. Hemmings and Alexander Hergovich

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Differential NDR/LATS Interactions with the Human MOB Family Reveal a Negative Role for Human MOB2 in the Regulation of Human NDR Kinases[▽]

Reto S. Kohler, Debora Schmitz, Hauke Cornils, Brian A. Hemmings,* and Alexander Hergovich*

Friedrich Miescher Institute for Biomedical Research, Maulbeerstrasse 66, 4058 Basel, Switzerland

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MOB proteins are integral components of signaling pathways controlling important cellular processes, such as mitotic exit, centrosome duplication, apoptosis, and cell proliferation in eukaryotes. The human MOB protein family consists of six distinct members (human MOB1A [hMOB1A], -1B, -2, -3A, -3B, and -3C), with hMOB1A/B the best studied due to their putative tumor-suppressive functions through the regulation of NDR/LATS kinases. The roles of the other MOB proteins are less well defined. Accordingly, we characterized all six human MOB proteins in the context of NDR/LATS binding and their abilities to activate NDR/LATS kinases. hMOB3A/B/C proteins neither bind nor activate any of the four human NDR/LATS kinases. We found that both hMOB2 and hMOB1A bound to the N-terminal region of NDR1. However, our data suggest that the binding modes differ significantly. Our work revealed that hMOB2 competes with hMOB1A for NDR binding. hMOB2, in contrast to hMOB1A/B, is bound to unphosphorylated NDR. Moreover, RNA interference (RNAi) depletion of hMOB2 resulted in increased NDR kinase activity. Consistent with these findings, hMOB2 overexpression interfered with the functional roles of NDR in death receptor signaling and centrosome overduplication. In summary, our data indicate that hMOB2 is a negative regulator of human NDR kinases in biochemical and biological settings.

The first MOB (Mps one binder) protein was identified in *Saccharomyces cerevisiae* more than a decade ago (22, 25). Since then, members of the MOB protein family have been found in unicellular organisms to mammals. Initially, the biological roles of MOB proteins were mainly investigated using budding and fission yeasts, revealing that Mob1p plays a vital role in the control of mitotic exit (3, 8, 23). *Drosophila* MOB1 (dMOB1)/Mats (MOB as tumor suppressor) emerged as an integral part of the Hippo tumor-suppressing pathway controlling cell proliferation and apoptosis from recent work in *Drosophila melanogaster* (24, 37). Interestingly, the functions of MOB proteins seem to be evolutionarily conserved, since the lethality and overgrowth phenotypes in *Drosophila mats* mutants can be rescued by the human homolog human MOB1A (hMOB1A) (24). This suggests that the Hippo signaling pathway is highly conserved from flies to humans (9, 12, 30, 31, 40). However, the biological roles of hMOB1A/B seem to be more diverse, as they function in cellular proliferation (29), apoptosis (36), and centrosome duplication (13). Mob2p in budding and fission yeasts is an essential part of a signaling network responsible for polarized cell growth and transcriptional asymmetry (6, 20, 38). In flies, the biological functions of dMOB2 and dMOB3 are less understood. However, dMOB2 seems to play a role in wing hair morphogenesis (10). In mammals, the biological roles of MOB2 proteins have so far proved elusive.

A conserved property of MOB proteins is the association with and activation of the NDR (nuclear-Dbf2-related) kinases of the AGC family (16, 28). In yeast, Mob1p binds to and is necessary for the activation of Dbf2/Dbf20 and Sid2 kinases (19, 22, 26). Similarly, Mob2p binds to and activates Cbk1 and Orb6 (20, 38). Furthermore, yeast MOB proteins and NDR kinases form restricted heterodimers of signaling complexes in which the subunits are not interchangeable (18, 20). In contrast, in multicellular organisms, the binding of MOB proteins is not restricted to a unique NDR kinase. For example, three MOB proteins exist in flies: dMOB1/Mats, dMOB2, and dMOB3 (10). dMOB1/Mats was shown to interact physically with warts, the fly homolog of human LATS1/2, and to be necessary for warts activity (24, 37). Moreover, dMOB1/Mats also genetically interacts with the second NDR kinase in flies, tricornered (trc) (10). Furthermore, it was shown in coimmunoprecipitation experiments that dMOB2 physically associates with trc (10).

The molecular mechanisms by which MOB proteins bind to and activate NDR kinases are best understood in mammals. hMOB1A binds to and activates human NDR1/2 kinases by stimulating autophosphorylation on the activation segment (2). Similarly, hMOB1A also binds to and activates LATS1 and -2 (4, 15, 39). In contrast, hMOB2 was shown to bind to NDR1 and NDR2, but not to LATS1 (4, 15). Importantly, hMOB1A/B are also required for efficient phosphorylation of the hydrophobic motif (T444/442) of NDR1/2 kinases by MST1 kinase (mammalian STE-20-like 1) (13, 36). Spatial relocalization of NDR kinases seems to be a further level of regulation, because membrane targeting of hMOB1 proteins leads to rapid activation of NDR1/2 and LATS1 kinases (11, 15). Indeed, mem-

* Corresponding author. Mailing address for Brian A. Hemmings: Friedrich Miescher Institute for Biomedical Research, Maulbeerstrasse 66, CH-4058 Basel, Switzerland. Phone: 41 61 697 4872. Fax: 41 61 697 3976. E-mail: brian.hemmings@fmi.ch. Present address for Alexander Hergovich: UCL Cancer Institute, University College of London, London WC1E 6BT, United Kingdom. Phone: 44 20 7679 0723. Fax: 44 20 7679 6817. E-mail: a.hergovich@cancer.ucl.ac.uk.

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brane targeting of dMOB1/Mats in *Drosophila* activates warts kinase and inhibits tissue growth by increasing apoptosis and reducing proliferation (17). Further, membrane-targeted tricornered kinase rescues the dendritic tiling defect in trc mutant flies (21). These observations indicate that activation of NDR kinases by relocalization to the plasma membrane is an important step in NDR/LATS kinase activation and function.

Here, we study for the first time all six human MOB proteins (hMOB1A/B, hMOB2, and hMOB3A/B/C) with respect to their abilities to bind and activate all four human NDR kinases. Surprisingly, we found that three out of the six MOBs neither bind to nor activate human NDR1/2 or LATS1/2 kinases. By focusing on the NDR1/2-specific binder hMOB2, we found that hMOB2 competes with hMOB1A/B for NDR binding. Furthermore, we provide evidence that overexpression of hMOB2 impairs NDR1/2 activation in a binding-dependent manner and affects functions of NDR, such as centrosome duplication and apoptotic signaling. Significantly, RNA interference (RNAi)-mediated reduction of the hMOB2 protein resulted in increased NDR kinase activity. These data indicate that hMOB2, in contrast to hMOB1A/B, plays an inhibitory role in the regulation of human NDR1/2 kinases.

MATERIALS AND METHODS

Construction of plasmids. Human NDR1 and NDR2 and hMOB1A, hMOB1B, hMOB2, hMOB3A, hMOB3B, and hMOB3C cDNAs were subcloned into pcDNA3, pGEX-4T1, or pMal-2c using BamHI and XhoI restriction sites. Accession numbers for hMOB3 reference cDNAs are 3A, NM_130807; 3B, NM_024761; and 3C, NM_201403. The cloning of hMOB3 cDNAs has been described previously (13). Plasmids containing human LATS1 and LATS2 were described elsewhere (14). pcDNA3 derivatives contained a hemagglutinin (HA) or a myc epitope alone or the myristolyation/palmitylation motif of the Lck tyrosine kinase (MGCVCSSN) combined with a myc epitope (mp-myc). Mutants of NDR1 and hMOB2 were generated by site-directed mutagenesis according to the manufacturer's instructions (Stratagene). Deletion mutants of NDR1 were cloned via PCR. Individual PCR products were digested with BamHI and XhoI and cloned into pcDNA3 derivatives. To generate a construct expressing the N terminus of NDR1 or NDR2 with a C-terminal tag, the coding sequences for amino acids 1 to 83 of NDR1/2 were amplified by PCR, digested by NheI and KpnI, and cloned into pcDNA3.1-myc-RFP as described elsewhere (27). To generate hMOB3 proteins containing a C-terminal myc tag, hMOB3A/B/C cDNAs were cloned into pcDNA3.1-myc-RFP as described above, and the red fluorescent protein (RFP) was removed by PCR. To generate tetracycline-regulated mammalian expression vectors, cDNAs encoding myc-hMOB2(wt) or myc-hMOB2(H157A) were digested with KpnI and XhoI and ligated into pENTR 3C (Invitrogen). N-terminally tagged hMOB2 cDNAs were finally inserted into pT-Rex-DEST30 using Gateway technology (Invitrogen). To obtain pTER-shMOB2 vectors that express short hairpin RNAs (shRNAs) against human MOB2, the following oligonucleotide pairs were inserted into pTER using BglII and HindIII: 5′-GATCCCGCTGGTGACGGATGAGGACTTCAAGAGAGTCCTCATCCGTCACCAGCTTTTTGGAAA-3′ (targeting sequences of the hMOB2 coding sequence are underlined) and 5′-AGCTTTTCCAAAAAGCTGGTGACGGATGAGGACTCTCTTGAAGTCCTCATCCGTCACCAGCGG-3′. The generation of the pTER-shLuc control vector has been described previously (14). All constructs were confirmed by sequence analysis.

Cell culture, transfections, and chemicals. COS-7, HEK 293, U2-OS, and HeLa cells were maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal calf serum. Exponentially growing COS-7 cells were plated at consistent confluence (1×10^6 cells/10-cm dish) and transfected the next day using Fugene 6 (Roche) as described by the manufacturer. Exponentially growing HEK 293 cells were transfected in solution at consistent confluence (5×10^6 cells/10-cm dish) using jetPEI (PolyPlus Transfections) according to the manufacturer's instruction. Exponentially growing U2-OS cells were plated at consistent confluence and transfected the next day using Lipofectamine 2000 (Invitrogen) as described by the manufacturer. Aphidicolin was from Calbiochem, and okadaic acid (OA) was purchased from Alexis Biochemicals (Enzo Life Sciences). Apoptosis of U2-OS cells was induced by the addition of activating anti-Fas antibody clone CH-11 (0.5 μg/ml) in combination with cycloheximide (CHX) (10 μg/ml).

Generation of stable cell lines. To generate inducible cell lines, U2-OS T-Rex cells were transfected with pT-Rex-DEST30 vectors encoding hMOB2 variants. Cell clones were selected by growth in the presence of 1 mg/ml G418 (Gibco) and 50 μg/ml hygromycin B (Invivogen). Stable transformants were maintained in DMEM supplemented with 0.5 mg/ml G418 and 50 μg/ml hygromycin B. Expression of myc-hMOB2 variants was induced by the addition of 2 μg/ml tetracycline.

Antibodies. The generation and purification of anti-T444-P, anti-S281-P, anti-NDR2, anti-NDR_{NT}, anti-NDR_{CTD}, and anti-hMOB1A/B antibodies has been described previously (13, 14, 35, 36). It is important to note that the anti-T444-P antibody recognizes the phosphorylated hydrophobic motifs of both NDR isoforms, NDR1 (T444-P) and NDR2 (T442-P). Anti-HA 12CA5 and 42F13, anti-myc 9E10, and anti-α-tubulin YL1/2 were used as hybridoma supernatants. Further, anti-HA antibody (Y-11) and anti-β-actin were purchased from Santa Cruz and anti-Fas (CH-11) from Millipore. Anti-LATS1 antibody was purchased from Cell Signaling and anti-cleaved poly(ADP-ribose) polymerase (PARP) from BD Bioscience. Anti-p63(G1/296) antibody was from Alexis Biochemicals (Enzo Life Sciences). Anti-hMOB2 antibody was raised against purified, bacterially produced full-length hMOB2 fused C terminally to maltose-binding protein (MBP). Rabbit injections and bleed collections were done by Eurogentec. Anti-protein antibody was purified by preabsorbing the bleeds against ~10 mg of immobilized MBP and then binding them to 5 to 10 mg of GST-hMOB2 covalently coupled to glutathione-Sepharose 4B beads. Antibodies were eluted with 0.2 M glycine (pH 2.2).

Immunoblotting and immunoprecipitation. Immunoblotting experiments were performed as described previously (11). For immunoprecipitation, cells were harvested, pelleted at $1{,}000 \times g$ for 3 min, and washed with cold phosphate-buffered saline (PBS) before lysis in immunoprecipitation (IP) buffer (20 mM Tris, 150 mM NaCl, 10% glycerol, 1% NP-40, 5 mM EDTA, 0.5 mM EGTA, 20 mM β-glycerophosphate, 50 mM NaF, 1 mM Na_3VO_4, 1 mM benzamidine, 4 μM leupeptin, 0.5 mM phenylmethylsulfonyl fluoride [PMSF], 1 μM microcystine, and 1 mM dithiothreitol [DTT] at pH 8.0). Lysates were centrifuged for 10 min at $16{,}000 \times g$ at 4°C before being precleared with protein A-Sepharose. The beads were washed twice with IP buffer, once with IP buffer containing 1 M NaCl, and finally once with IP buffer before samples were analyzed by SDS-PAGE. To analyze the association of NDR1/2 or LATS1/2 and hMOB species by coimmunoprecipitation, cells coexpressing HA-NDR or HA-LATS and myc-hMOB species were subjected to immunoprecipitation using anti-HA 12CA5 antibody as described above, omitting the wash with 1 M NaCl IP buffer, before analysis by SDS-PAGE and immunoblotting. For immunoprecipitation of endogenous proteins, cells were processed for immunoprecipitation as described above. Lysates were preincubated with control rabbit IgG, anti-hMOB2, anti-NDR2, anti-LATS1, or anti-T444-P antibody overnight, and then protein A-Sepharose was added for 3 h and the beads were washed four times in IP buffer containing 150 mM NaCl before analysis by SDS-PAGE. To analyze the association of NDR1 mutants and hMOB2, coimmunoprecipitation experiments were performed as described above, including one wash with IP buffer containing 1 M NaCl. Characterization of hMOB2 mutants by IP was performed in low-stringency buffer (30 mM HEPES, pH 7.4, 20 mM β-glycerophosphate, 20 mM KCl, 1 mM EGTA, 2 mM NaF, 1 mM Na_3VO_4, 1% TX-100) supplemented with protease inhibitors.

HA-NDR kinase assay and HA-LATS kinase assay. Analysis of HA-NDR or HA-LATS kinase activities after immunoprecipitation was performed as described previously (11, 15).

HA-LATS autophosphorylation assay. Analysis of immunoprecipitated HA-LATS autophosphorylation was also carried out as reported previously (15).

Fractionation of cells. Cytosolic and membrane-associated proteins were separated by S100/P100 fractionation as described previously (11).

Immunofluorescence microscopy. Cells were processed for immunofluorescence analysis as defined elsewhere (14).

RESULTS

Human NDR and LATS kinases do not interact with hMOB3A, -B, or -C protein. MOB proteins are evolutionarily highly conserved from yeast to humans. Unfortunately, human MOB proteins have been named inconsistently in the literature (Table 1). Alignments, as well as phylogenetic analysis of the human MOB family (data not shown), revealed a close rela-

TABLE 1. Human MOB proteins

Protein	% identity	No. of amino acids	Alternative names
hMOB1A	100	216	MOB1α, MOBKL1B, MOBK1B, MOB4B, hMats1
hMOB1B	95	216	MOB1β, MOBKL1A, MOB4A, hMats2, MOB1
hMOB2	38	237	HCCA2, hMOB3
hMOB3A	50	217	MOBKL2A, MOB-LAK, MOB1C, hMOB2A
hMOB3B	51	216	MOBKL2B, MOB1D, hMOB2B
hMOB3C	49	216	MOBKL2C, MOB1E, hMOB2C

tionship of hMOB3 proteins with hMOB1A. Many biochemical properties of hMOB1A and -B have been described (2, 11, 15), suggesting that hMOB3A/B or -C proteins might display some of these properties. In order to test whether hMOB3 proteins can physically interact with human NDR or LATS kinases, HA-NDR1/2 or HA-LATS1/2 were coexpressed with N-terminally myc-tagged hMOB proteins prior to being processed for immunoprecipitation and subsequent immunoblotting (Fig. 1). As expected, we observed interactions between HA-NDR1 and myc-hMOB2 (Fig. 1A, top, lane 1) as well as HA-NDR2 and myc-hMOB2 (Fig. 1B, top, lane 1). To our surprise, none of the hMOB3 proteins interacted with HA-NDR1 (Fig. 1A, top, lanes 2 to 4) or HA-NDR2 (Fig. 1B, top, lanes 2 to 4) in these settings. In addition, hMOB3A, -B, and -C did not associate with HA-LATS1 or HA-LATS2 (Fig. 1C and D, top, lanes 2 to 4). In full agreement with the existing literature (4, 15), we confirmed that HA-LATS1 and myc-MOB2 do not interact in cells (Fig. 1C, top, lane 5) and also demonstrated that myc-hMOB2 cannot associate with HA-LATS2 (Fig. 1D, top, lane 5), thus illustrating that hMOB2 is a specific binder of NDR1/2. Significantly, these data were fully confirmed using hMOB proteins containing a C-terminal myc tag (data not shown).

Membrane-targeted variants of hMOB3 proteins do not activate human NDR and LATS kinases. We have demonstrated that hMOB3A, -B, and -C do not bind to NDR1/2 or LATS1/2 in our settings (Fig. 1). In order to exclude possible postlysis effects we applied a second experimental setting as described previously (11, 15). Briefly, fusion of the myristoylation/palmitoylation motif (mp) from the Lck kinase to the N terminus of myc-tagged hMOB1A (mp-myc-hMOB1A) led to efficient plasma membrane localization. Importantly, the resulting activation of NDR/LATS is dependent on hMOB1A-NDR/LATS interaction and takes place within the cells before subsequent manipulations, such as cell lysis and immunoprecipitation. To address whether membrane-targeted variants of hMOB3 proteins are able to activate either human NDR1/2 or LATS1/2 kinases, we transfected HEK 293 cells with the respective NDR/LATS kinase and membrane-targeted hMOBs. As reported previously (11), mp-myc-MOB1A robustly activated HA-NDR1, as reflected in increased Thr444 phosphorylation at the hydrophobic motif of NDR1 (Fig. 2A, top, lane 2), paralleled by increased kinase activity (Fig. 2B, lane 2). Coexpressing membrane-targeted hMOB3 variants produced no increase in phosphorylation (Fig. 2A, top panel, lanes 3 to 5) or kinase activity (Fig. 2B, lanes 3 to 5). Comparable results were obtained when cells were transfected with HA-NDR2 and mp-myc-hMOB3A, -B, or -C (Fig. 2C, top, lanes 3 to 5, and D, lanes 3 to 5). This is consistent with the coimmunoprecipitation

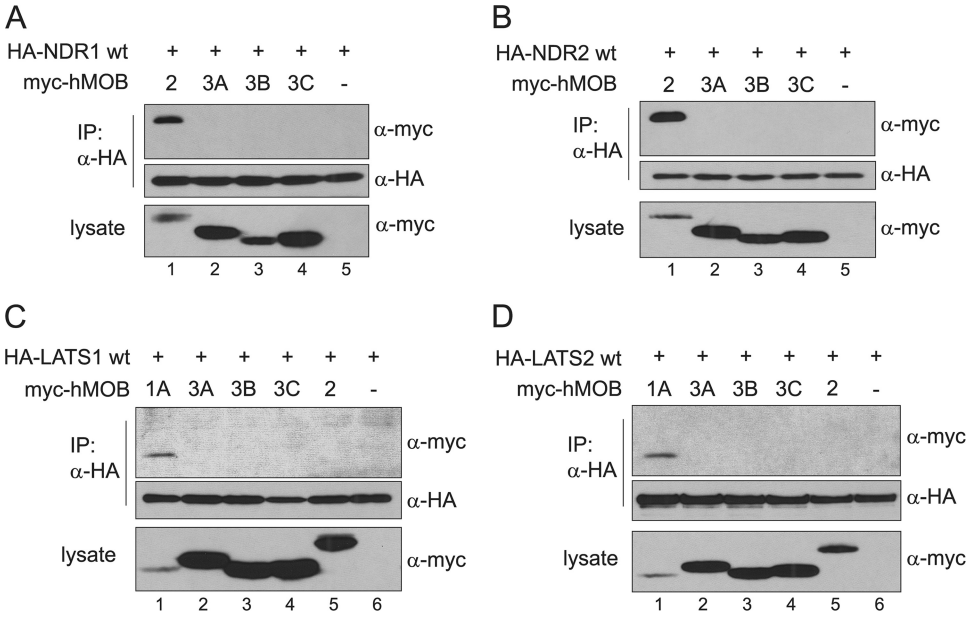

FIG. 1. Human NDR and LATS kinases do not interact with hMOB3A/B/C proteins. (A and B) Lysates of HEK 293 cells coexpressing the indicated combinations of HA-tagged NDR1 wild-type (wt), HA-tagged NDR2(wt), and myc-tagged hMOB species were analyzed by IP using anti-HA 12CA5 antibody. Complexes were assayed by immunoblotting using anti-myc antibody (top) or anti-HA antibody (middle). Input lysates were analyzed by immunoblotting using anti-myc antibody (bottom). hMOB2 served as a positive control. (C and D) Lysates of HEK 293 cells coexpressing the indicated HA-tagged LATS1(wt) or LATS2(wt) and myc-tagged human MOB species were analyzed as described for panels A and B, except that hMOB1A served as the positive control and hMOB2 as the negative control.

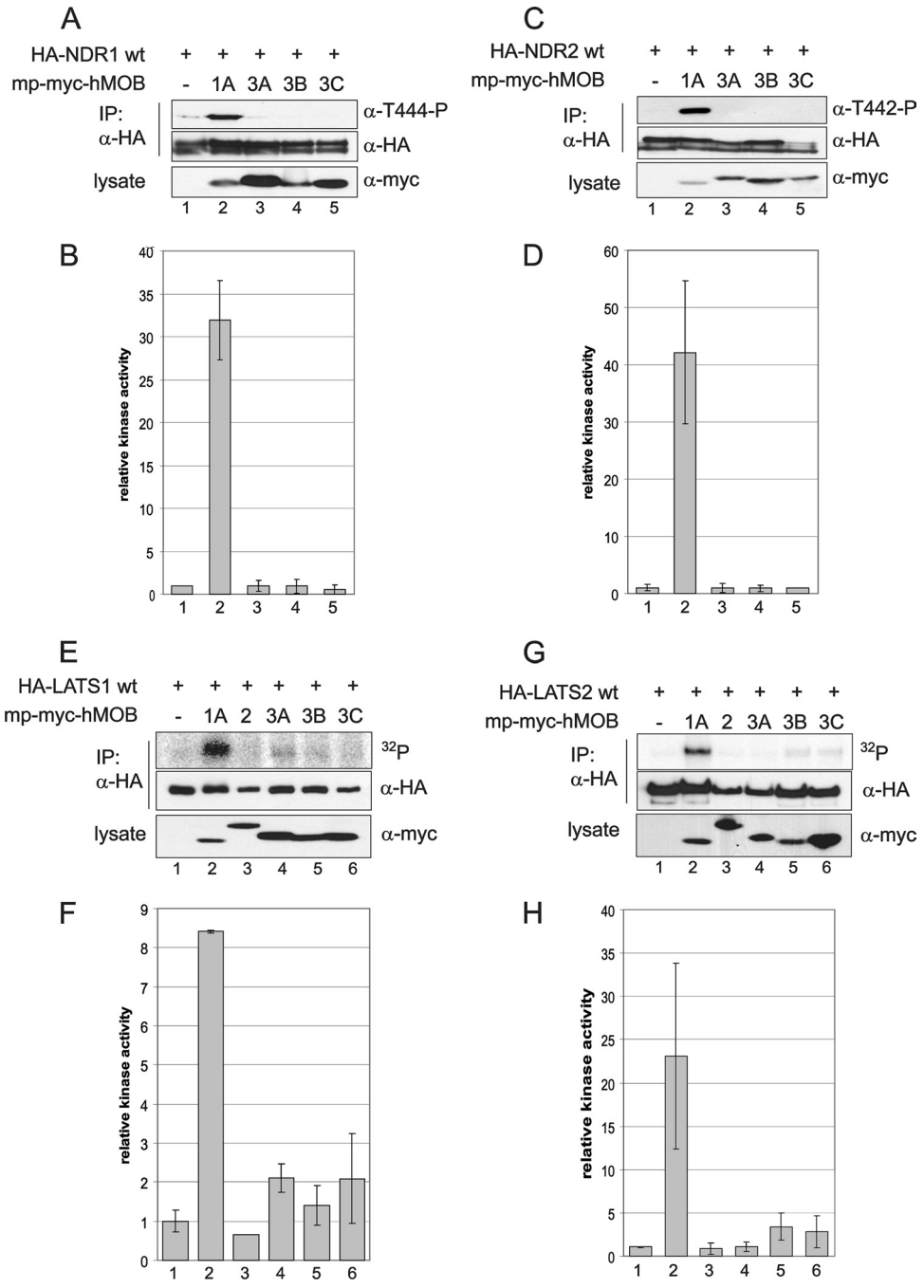

FIG. 2. Membrane-targeted variants of hMOB3A/B/C do not activate human NDR and LATS kinases. (A) Lysates of HEK 293 cells coexpressing the indicated combinations of HA-tagged NDR1(wt) and membrane-targeted human MOB proteins (mp-myc-hMOB) were analyzed by IP using anti-HA 12CA5 antibody. Complexes were assayed by immunoblotting using anti-T444-P antibody (top) or anti-HA antibody (middle). Input lysates were immunoblotted with anti-myc antibody (bottom). (B) In parallel, complexes were subjected to kinase assays. The results of two independent experiments are shown. The error bars indicate standard deviations. (C) Lysates of HEK 293 cells coexpressing the indicated combinations of HA-tagged NDR2(wt) and mp-myc-hMOB proteins were analyzed as described for panel A. (D) In parallel, complexes were subjected to kinase assays as described for panel B. (E) Lysates of HEK 293 cells coexpressing the indicated combinations of HA-tagged LATS1(wt) and mp-myc-hMOB species were analyzed by IP using anti-HA 12CA5 antibody before being assayed by immunoblotting using anti-HA antibody (middle) or an autophosphorylation assay (top). Input lysates were analyzed by immunoblotting with anti-myc antibody. (F) In parallel, complexes were subjected to peptide kinase assays. The result from one representative experiment performed in duplicate is shown. (G) Lysates of HEK 293 cells coexpressing the indicated combinations of HA-tagged LATS2(wt) and mp-myc-hMOB species were analyzed as described above for panel E. (H) In parallel, complexes were subjected to peptide kinase assays. The results from two independent experiments are shown. The error bars indicate standard deviations.

experiments (Fig. 1A and B) and indicates that hMOB3s cannot interact with human NDR1/2 kinases in cultured mammalian cells despite the significant similarity to hMOB1A. Furthermore, we addressed whether membrane-targeted hMOB3 variants are able to activate HA-LATS1 or HA-LATS2 (Fig. 2E to H). As already reported (15), HA-LATS1 was substantially activated by mp-myc-hMOB1A (Fig. 2E and F, lanes 2), as illustrated by increased autophosphorylation and kinase activity, whereas mp-hMOB3 proteins were unable to activate HA-LATS1 (Fig. 2E and F, lanes 4 to 6). We observed similar results in the case of HA-LATS2 (Fig. 2G and H). In combination with the coimmunoprecipitation experiments (Fig. 1), these findings strongly suggest that none of the three hMOB3 proteins physically interacts with or activates human NDR1/2 or LATS1/2 kinases.

hMOB2 binds to the amino terminus of human NDR1/2 kinases in a mode distinct from hMOB1A/B binding. We have shown that hMOB3s do not associate with human NDR1/2 kinases (Fig. 1 and 2), despite their higher degree of similarity to hMOB1A/B than to hMOB2 (Table 1). Interestingly, hMOB2 appears to be an NDR-specific binder, since it did not bind to human LATS1 (4, 15) or LATS2 (Fig. 1 and 2) but readily bound to NDR1 and 2 (Fig. 1). Therefore, we investigated the interaction of hMOB2 with NDR1/2 in more detail using a series of NDR1 mutants (Fig. 3A and Table 2). We deleted the N or C terminus of NDR1 to determine which region was necessary for the interaction with hMOB2 (Fig. 3B). NDR1(wt) and NDR1(1-380) coprecipitated hMOB2 (Fig. 3B, top, lanes 2 and 4), whereas NDR1 lacking the conserved N-terminal regulatory domain (NTR), HA-NDR1(ΔNTR), did not (Fig. 3B, top, lane 3). Conversely, we addressed whether the N terminus of NDR (amino acids 1 to 83) was sufficient for association with hMOB2. Indeed, NDR1(1-83)-myc-RFP interacted with HA-hMOB2 (Fig. 3C, top, lane 2), and NDR2(1-83)-myc-RFP also bound HA-hMOB2 (Fig. 3D, top, lane 2). Remarkably, hMOB1A/B binds to the same N-terminal region of NDR (2). Therefore, since the key residues essential for interaction between NDR1/LATS1 kinases and hMOB1A have been described (2, 15), we investigated whether hMOB2 utilized the same conserved binding motif. Interestingly, point mutations in the NDR1 N terminus that abolish or diminish the interaction with hMOB1A did not impair binding of hMOB2 (Fig. 3E, top, lanes 5 and 6, and Table 2). Since hMOB2 appeared to bind to NDR separately from hMOB1A, we aimed to define the N-terminal region on human NDR1 necessary for the hMOB2 interaction via N-terminal mutagenesis of NDR1 (Fig. 3F). We observed that NDR1 lacking the first 26 amino acids [NDR1(Δ26)] still interacted with hMOB2 (Fig. 3F, top, lane 3), whereas an NDR1 mutant lacking the first 33 residues [NDR1(Δ33)] was no longer able to bind to hMOB2 (Fig. 3F, top, lane 2), arguing that the amino acids between residues 27 and 33 of human NDR1 are necessary for the interaction. We sought to further analyze this region and mutated 5 residues within this stretch to alanines (HA-NDR1 5A: Leu27, Glu28, Asn29, Phe30, and Ser32, respectively) and examined whether this mutant was still able to bind to hMOB2 (Fig. 3G). Unexpectedly, the NDR1 mutant carrying 5 point mutations bound to hMOB2 but lost the ability to bind to hMOB1A (Fig. 3G, top, lanes 4 and 5). Neither single point mutations in this stretch nor multiple mutations led to the loss of hMOB2 interaction (Table 2). Therefore, we attempted to create an NDR1 mutant incapable of binding to hMOB2 by mutating residues in the N terminus that differ significantly from the N-terminal region of LATS1. However, this effort remained ineffective, since all tested mutants bound to hMOB2 (Table 2), leaving a defined binding motif of hMOB2 on NDR1 yet to be determined. Nevertheless, these data demonstrate that while hMOB2 and hMOB1 proteins utilize identical regions of human NDR1/2 kinases to bind, the interactions differ significantly between these two hMOB isoforms.

hMOB2 competes with hMOB1A for binding to NDR and interferes with the activation of endogenous NDR by okadaic acid. We showed that hMOB2, like hMOB1A, binds to the N terminus of NDR (Fig. 3), suggesting that hMOB1A and hMOB2 might function competitively in binding NDR kinases. Thus, we examined whether the coimmunoprecipitation of myc-tagged hMOB1A by HA-NDR1 is affected by expressing increasing amounts of myc-hMOB2 (Fig. 4A). In the absence of myc-hMOB2, a substantial amount of myc-hMOB1A coimmunoprecipitated with HA-NDR1 (Fig. 4A, top, lane 2). On the other hand, coexpression of increasing amounts of myc-hMOB2 led to a significant decrease in myc-hMOB1A coimmunoprecipitating with HA-NDR1 (Fig. 4A, lanes 3 to 6), despite the fact that the overall amount of expressed myc-hMOB1A was not changed (Fig. 4A, bottom, lanes 2 to 6). Interestingly, hMOB2 displaced hMOB1A even though it was expressed at a lower level than hMOB1A (Fig. 4A, lanes 3 and 4). This indicates that hMOB2 can efficiently compete with hMOB1A for binding to NDR1.

Activation of NDR kinases by the protein phosphatase 2A inhibitor OA was shown to depend on intact interaction of NDR1/2 and hMOB1 proteins (2). Since hMOB2 is able to partially displace hMOB1A from NDR, we investigated the effect of hMOB2 expression on OA-induced activation of endogenous NDR species (Fig. 4B and C). As expected, treatment of HEK293 cells with OA strongly increased Thr-444 phosphorylation of NDR (Fig. 4B, top, lane 3) and elevated the kinase activity of endogenous NDR2 (Fig. 4C, lane 3). Interestingly, expression of hMOB2(wt) impaired NDR phosphorylation (Fig. 4B, top, lane 4) and led to an ~50% reduction in endogenous NDR2 activity (Fig. 4C, lane 4). Overall, these data suggest that hMOB2 competes with hMOB1 for NDR binding and interferes with OA-induced activation of NDR, in contrast to hMOB1, which was previously shown to enhance OA-induced activation (2).

hMOB2 interferes with the activation of ectopic and endogenous NDR kinases by membrane-targeted hMOB1A in a binding-dependent manner. Next, we investigated whether the competition with hMOB1 and the inhibitory effect on NDR activation by hMOB2 depended on an intact NDR-hMOB2 interaction. For this, we generated an hMOB2 variant deficient in NDR binding (Fig. 5A). Mutating His157 to alanine abolished binding to NDR1 and -2 despite similar expression levels (Fig. 5A, top, compare lanes 2 and 3, 5 and 6). Subsequently, we investigated whether hMOB2 can interfere with the activation of NDR by membrane-targeted hMOB1A in an interaction-dependent manner (Fig. 5B and C). As previously reported (11), mp-myc-hMOB1A potently activates HA-NDR1 (Fig. 5B and C). Intriguingly, myc-tagged hMOB2(wt) expression almost completely abolished the activation of HA-NDR1

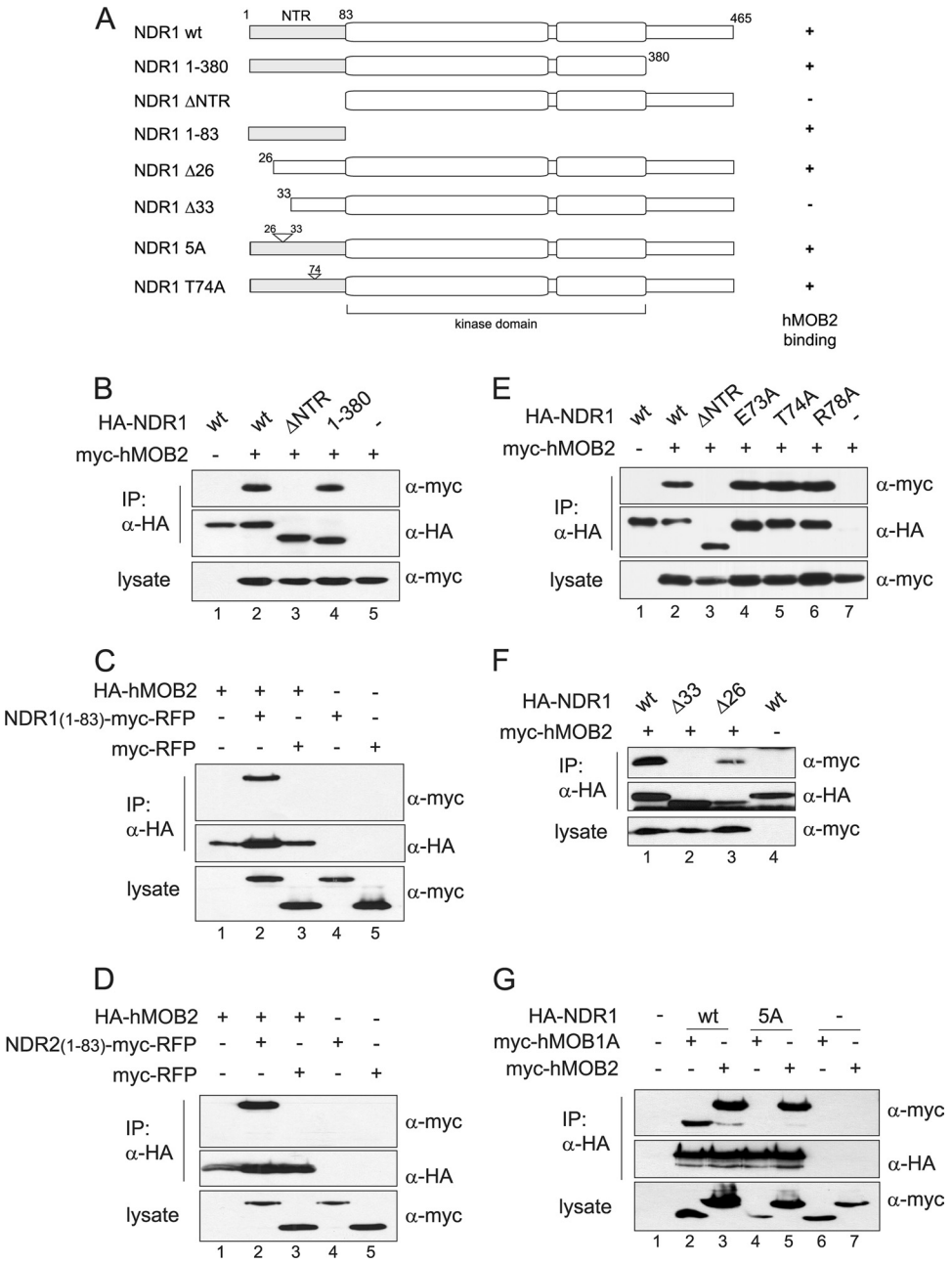

FIG. 3. hMOB2 binds to the N terminus of NDR but in a manner distinct from that for hMOB1A/B. (A) Primary structures of human NDR1 and overview of HA-tagged NDR1 mutant derivatives. (B) Lysates of COS-7 cells containing the indicated combinations of HA-tagged NDR1 forms and myc-tagged hMOB2(wt) were analyzed by IP using anti-HA antibody. Complexes were analyzed by immunoblotting using anti-myc (top) or anti-HA (middle) antibody. Input lysates were analyzed using anti-myc antibody. ΔNTR denotes deletion of amino acids 1 to 83 of NDR1, the NTR (N-terminal regulatory domain). (C) Lysates of HEK 293 cells coexpressing the indicated combinations of HA-tagged hMOB2(wt), the N terminus of NDR1 (amino acids 1 to 83) fused N terminally to myc-RFP, or myc-RFP alone were analyzed by IP using anti-HA antibody. Complexes were assayed by immunoblotting using anti-myc (top) and anti-HA (middle) antibodies. The lysates were analyzed using anti-myc antibody. (D) Lysates of HEK 293 cells coexpressing combinations of HA-tagged hMOB2(wt), the N terminus of NDR2 (amino acids 1 to 83) containing a C-terminal myc-RFP tag, or myc-RFP alone were analyzed as described for panel C. (E) Lysates of COS-7 cells coexpressing HA-tagged NDR1(wt), NDR1(ΔNTR), NDR1(E73A), NDR1(T74A), NDR1(R78A), and myc-tagged hMOB2(wt) were analyzed by IP using anti-HA antibody. Complexes were analyzed by immunoblotting using anti-myc or anti-HA antibody. Input lysates were assayed by immunoblotting using anti-myc antibody. (F) Lysates of HEK 293 cells coexpressing HA-tagged NDR1(wt), NDR1 containing a deletion of amino acids 1 to 33 [NDR1(Δ33)], NDR1(Δ26), and myc-tagged hMOB2(wt) were analyzed by IP using anti-HA antibody. Complexes were assayed using anti-myc or anti-HA antibody. Input lysates were assayed by immunoblotting using anti-myc antibody. (G) Lysates of HEK 293 cells containing the indicated combinations of HA-tagged NDR1(wt), NDR1 5A mutant, myc-tagged hMOB1A, or hMOB2 were analyzed by IP using anti-HA antibody. NDR1 5A mutant denotes mutation of amino acids Leu27, Glu28, Asn29, Phe30, and Ser32 to alanine. Complexes were assayed by immunoblotting using anti-myc (top) or anti-HA (middle) antibody. Input lysates were assayed by immunoblotting using anti-myc antibody (bottom).

TABLE 2. Summary of coimmunoprecipitation experiments

NDR1 mutation	Binding to[a]:	
	hMOB2	hMOB1A
Y31A	+	−
R41A	+	−
R44A	+	(+)
T74A	+	−
R78A	+	−
K24A	+	+
T26A	+	ND
T26F	+	ND
L27A	+	ND
E28A	+	+
N29A	+	ND
F30A	+	ND
S32A	+	ND
A36K	+	ND
E40A	+	+
V51E	+	ND
E54R	+	ND
D59A	+	ND
E60A	+	+
E61A	+	+
R63A	+	+
E73A	+	+
TVT23/25/26FFF	+	ND
FY30/31HV	+	ND
EE39/40AA	+	ND
Q45K/K47Q	+	ND
EEE53-55RRR	+	ND
EEKR60-63AAAA	+	ND
KRR62/63/65QDM	+	ND
H69D/R71D	+	ND
SAHAR67-71KMLCQ	+	ND

[a] HA-tagged NDR1 mutants were coexpressed with either myc-hMOB2 or myc-hMOB1A in HEK 293 or COS7 cells before coimmunoprecipitation experiments were performed. +, interaction; −, no interaction; (+), impaired interaction; ND, not determined.

by mp-myc-hMOB1A (Fig. 5B and C, lanes 3), even though the expression of mp-myc-hMOB1A remained unchanged (Fig. 5B, bottom, lane 3). However, coexpression of myc-tagged hMOB2(H157A), which cannot bind to NDR1/2 kinases, did not interfere with mp-myc-hMOB1A-driven activation of HA-NDR1 (Fig. 5B and C, lanes 4). In conclusion, the negative effect of hMOB2 on NDR1 activation by membrane-targeted hMOB1A is likely to be binding dependent.

We have previously shown that expression of membrane-targeted hMOB1A in U2OS cells leads to the membrane recruitment and activation of endogenous NDR1 species (11). To address the effect of hMOB2 on membrane recruitment and activation of endogenous NDR species, HEK 293 cells transfected with mp-myc-hMOB1A, myc-hMOB2(wt), or myc-hMOB2(H157A) were separated into cytoplasmic and membranous fractions prior to analysis by immunoblotting (Fig. 5D). While in untransfected cells native phospho-T444/442 proteins were found almost exclusively in the cytoplasmic fraction (Fig. 5D, lane 1), in cells expressing mp-myc-hMOB1A NDR, phosphospecies were enriched at the membrane (Fig. 5D, lane 4). Congruently, endogenous NDR1/2 was recruited to the membrane by mp-myc-hMOB1A (Fig. 5D, lane 4). Upon coexpression of myc-tagged hMOB2(wt) the phosphosignal of endogenous NDR species at the membrane disappeared (Fig. 5D, lane 6), although we still observed residual NDR2 in the membranous fraction (Fig. 5D, lane 6). To address whether this effect was dependent on the interaction between hMOB2 and endogenous NDR species, we coexpressed NDR binding-deficient hMOB2(H157A) with mp-myc-hMOB1A. Confirming the result with overexpressed HA-NDR1 (Fig. 5B), myc-hMOB2(H157A) did not interfere with

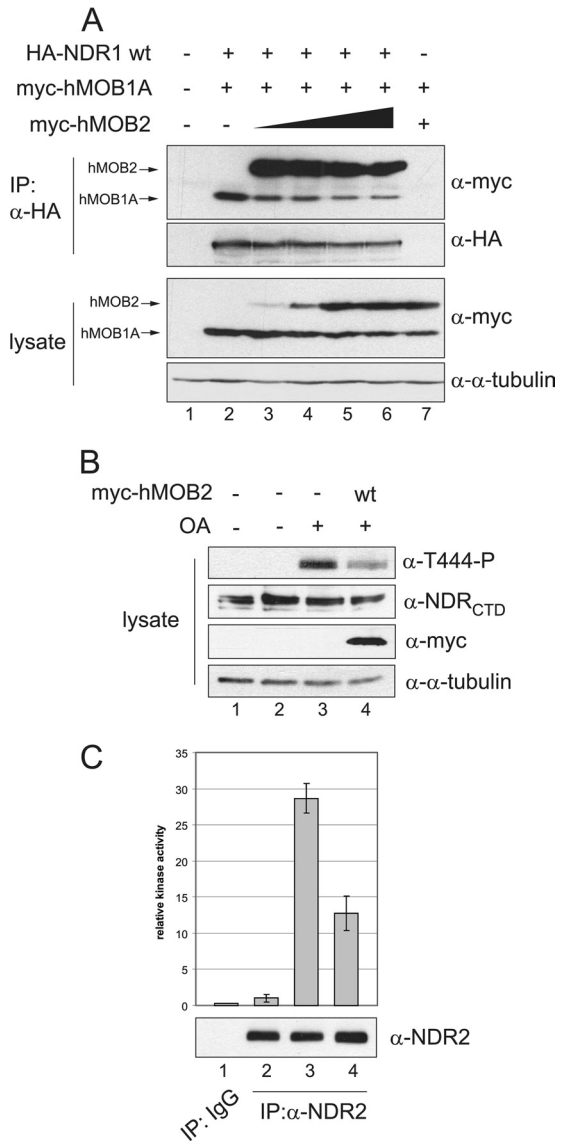

FIG. 4. hMOB2 competes with hMOB1A and interferes with okadaic acid-induced activation of endogenous NDR kinases. (A) hMOB1A-NDR1 and hMOB2-NDR1 interactions are mutually exclusive. Lysates of HEK 293 cells coexpressing HA-tagged NDR1(wt) and myc-tagged hMOB1A and hMOB2 were analyzed by IP using anti-HA antibody. Complexes were assayed by immunoblotting using anti-myc (top) and anti-HA (middle) antibodies. Input lysates were analyzed by immunoblotting using anti-myc antibody. (B) HEK 293 cells transfected with empty vector (−) or hMOB2(wt) were treated with 1 μM OA for 45 min before input lysates were processed for immunoblotting with the indicated antibodies. (C) In parallel, samples were subjected to immunoprecipitation using rabbit IgG or anti-NDR2 antibody before peptide kinase assays were performed. Data from at least two independent experiments with two replicates per experiment are shown. The error bars represent standard deviations.

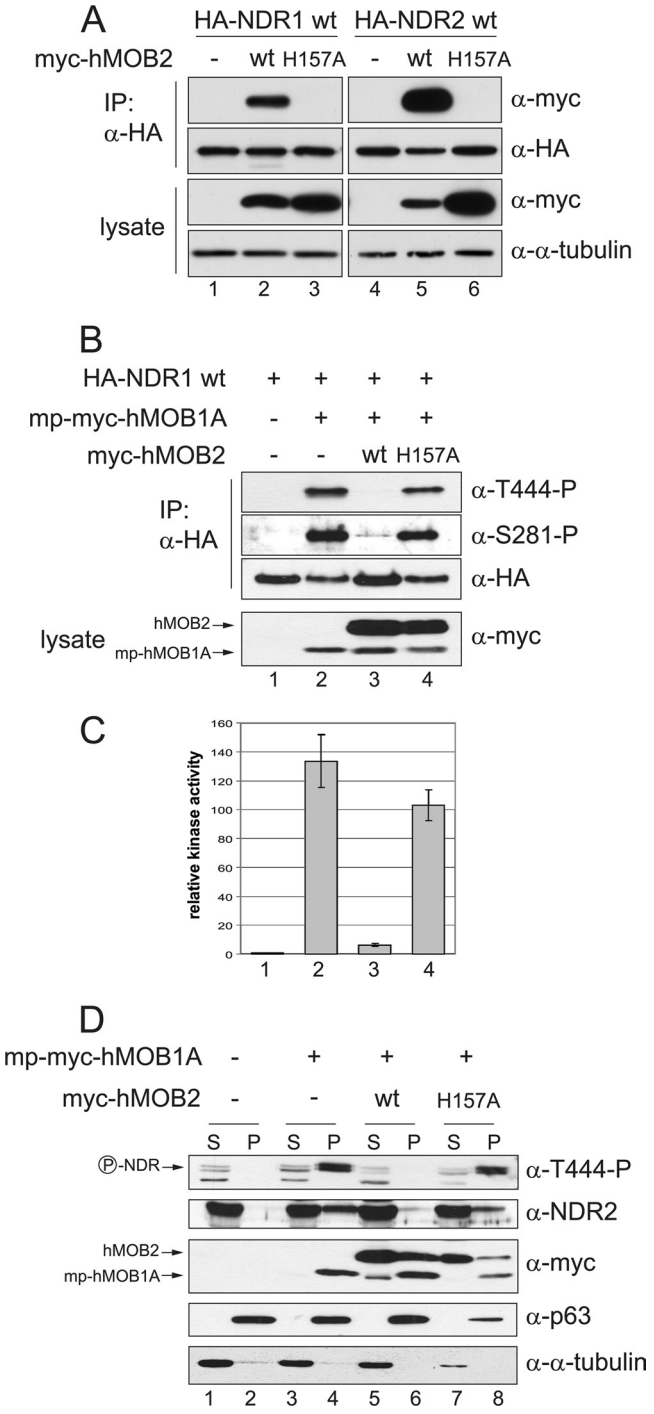

FIG. 5. hMOB2 interferes with the activation of human NDR kinases by membrane-targeted hMOB1A in an NDR binding-dependent manner. (A) COS-7 cell lysates expressing HA-tagged NDR1(wt) (lanes 1 to 3), HA-NDR2(wt) (lanes 4 to 6), myc-tagged hMOB2(wt), or myc-hMOB(His157Ala) were lysed in low-stringency lysis buffer and then subjected to IP using anti-HA 12CA5 antibody. Complexes were analyzed by immunoblotting with anti-HA (middle) and anti-myc (top) antibodies. Input lysates were analyzed by immunoblotting using anti-myc and anti-α-tubulin antibodies (bottom). (B) Lysates of COS-7 cells containing the indicated combinations of HA-tagged NDR1(wt), membrane-targeted hMOB1A (mp-myc-hMOB1A), and myc-tagged hMOB2(wt) and hMOB2(H157A) were analyzed by immunoprecipitation using anti-HA antibody. Complexes were assayed by immunoblotting and probed with anti-T444-P, anti-S281-P, and anti-HA antibodies. The input lysate was analyzed by immunoblotting using anti-myc antibody. (C) In parallel, complexes were subjected to peptide kinase assays. The results from two independent experiments are shown. The error bars indicate standard deviations. (D) HEK 293 cells transfected with membrane-targeted hMOB1A (mp-myc-hMOB1A) and the indicated myc-tagged hMOB2 constructs were subjected to S100/P100 (S, cytoplasm; P, membrane) fractionation before being immunoblotted with anti-T444-P, anti-NDR2, anti-myc, anti-CLIMP63 (p63) (a marker for membranous fraction), and anti-α-tubulin (a marker for the cytoplasmic fraction) antibodies.

either membrane recruitment of endogenous NDRs by mp-myc-hMOB1A (Fig. 5D, lane 8) or the activation of endogenous NDR at the membrane (Fig. 5D, top, lane 8). We conclude that hMOB2 competes with hMOB1A for NDR binding and can interfere with the activation of human NDR kinases in a binding-dependent manner.

Endogenous hMOB2 physically interacts with human NDR, but not with LATS1. In overexpression settings, hMOB2 readily coimmunoprecipitates with human NDR1/2 kinases (Fig. 1). In order to address the interaction of endogenous proteins, we raised a rabbit polyclonal antibody against hMOB2 (Fig. 6A and B). The affinity-purified anti-hMOB2 antibody detected a band at approximately 27 kDa, the predicted molecular size of the hMOB2 protein, which was reduced in cells expressing shRNA against hMOB2 (Fig. 6A, top). Furthermore, the anti-hMOB2 detected only recombinant glutathione *S*-transferase (GST)-hMOB2, but none of the other hMOBs (Fig. 6B, top). Endogenous hMOB2 coprecipitated with NDR2 when an anti-NDR2 antibody was used for immunoprecipitation, but not with control antibody (Fig. 6C, top). Conversely, when an anti-hMOB2 antibody was used to immunoprecipitate endogenous hMOB2, endogenous NDR2 coprecipitated in HEK293 cells (Fig. 6D, top). Similar results were observed using HeLa cell lysates (data not shown). Moreover, endogenous hMOB2 could not be coimmunoprecipitated using an anti-LATS1 antibody (Fig. 6E, top, lane 3). Therefore, our data show for the first time that endogenous hMOB2 is a specific binder of NDR1/2 kinases.

hMOB2 is found preferentially in unphosphorylated NDR complexes, while hMOB1A/B is associated with active NDR kinases. Given that we observed a putative negative role for hMOB2 in the course of NDR activation and that endogenous hMOB2-NDR complexes are readily detectable (Fig. 4, 5, and 6), we examined endogenous total NDR-hMOB complexes and active NDR-hMOB complexes by immunoprecipitation experiments using anti-NDR2 and anti-T444-P antibodies (Fig. 7). The anti-T444-P antibody recognizes only phosphorylated hydrophobic motifs of active NDR1/2 kinases (14). HEK 293 cells were subjected to immunoprecipitation with the two different anti-NDR antibodies described above and to subsequent immunoblotting experiments. When the anti-NDR2 antibody was used, a small fraction of the immunoprecipitated NDR2 protein was phosphorylated at the hydrophobic motif (T444-P), indicating that mostly inactive NDR species were immunoprecipitated (Fig. 7, lane 2). In contrast, using the anti-T444-P antibody to immunoprecipitate active NDR species, we obtained a significant amount of phospho-T444 species despite the small amount of total NDR2 pulled down (Fig. 7,

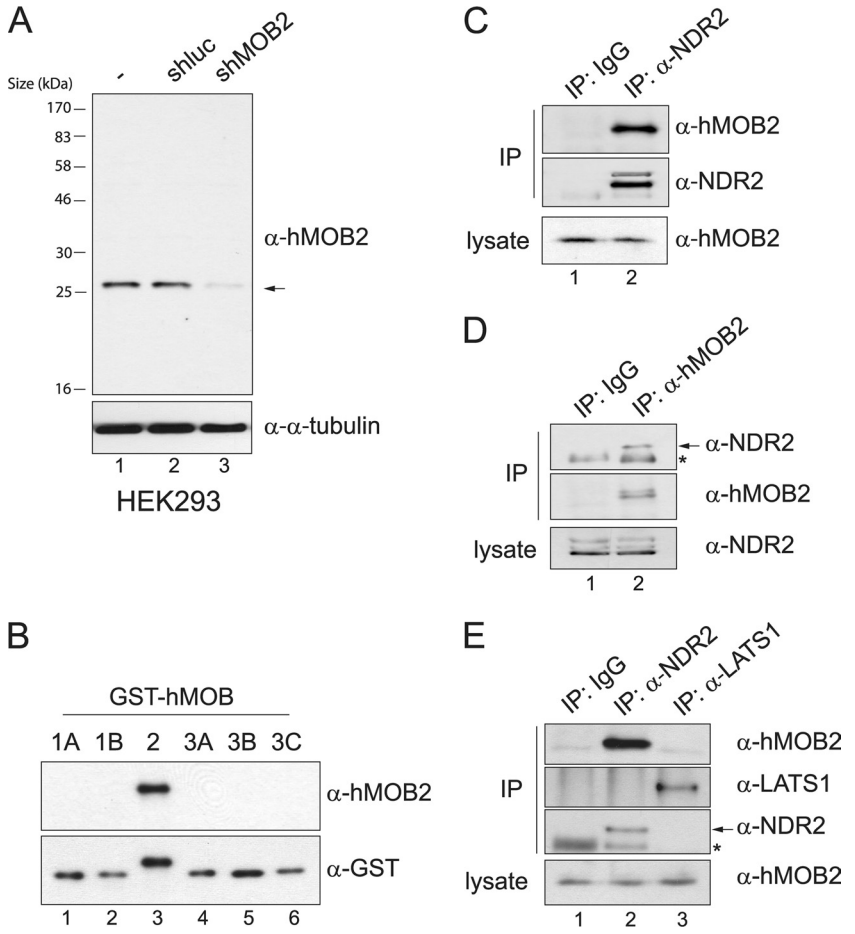

FIG. 6. Endogenous hMOB2 interacts with NDR, but not with LATS1, in tissue-cultured cells. (A) Characterization of anti-hMOB2 rabbit polyclonal antibody. HEK 293 cells transfected with short hairpin targeting either firefly luciferase (shLuc) or hMOB2 (shMOB2) were analyzed 72 h after transfection by immunoblotting using affinity-purified anti-hMOB2 antibody (top) and anti-α-tubulin antibody (bottom). Molecular masses are indicated. (B) Recombinant GST-tagged human MOB proteins were separated by SDS-PAGE and analyzed by immunoblotting using anti-hMOB2 (top) and anti-GST (bottom) antibodies. (C) Interaction of endogenous hMOB2 and NDR2. Whole-cell extracts of HEK 293 cells were subjected to immunoprecipitation using control rabbit IgG or anti-NDR2 antibody. Complexes were analyzed by immunoblotting using anti-hMOB2 (top) and anti-NDR2 (middle) antibodies. Input lysates were probed with anti-hMOB2 antibody. (D) Endogenous NDR2 was coimmunoprecipitated with hMOB2. Lysates of HEK 293 cells were assayed by immunoprecipitation using control rabbit IgG or anti-hMOB2 antibody. Complexes were assayed by immunoblotting using anti-NDR2 (top) or anti-hMOB2 (bottom) antibody. Antibody heavy chains are marked with an asterisk. Input lysates were analyzed with anti-NDR2 antibody. (E) Endogenous hMOB2 coimmunoprecipitates with NDR2, but not with LATS1. Lysates of HEK 293 cells were subjected to immunoprecipitation with the indicated antibodies and analyzed by immunoblotting using anti-NDR2, anti-LATS1, and anti-hMOB2 antibodies. Antibody heavy chains are marked with an asterisk.

lane 3). Interestingly, endogenous hMOB2 was enriched using anti-NDR2 antibody (Fig. 7, lane 2), whereas when anti-T444-P antibody was used to pull down active NDR species, hMOB2 was not detectable (Fig. 7, lane 3). On the other hand, hMOB1A was almost exclusively detected in phosphorylated complexes of NDR (Fig. 7, compare lanes 2 and 3). This finding is in agreement with previous reports demonstrating enhanced complex formation of hMOB1A/B and NDR kinases upon activation (36). Overall, we conclude that hMOB2 preferentially associates with unphosphorylated NDR and, in contrast, hMOB1A/B associates with phosphorylated NDR.

Reduction of hMOB2 protein results in increased NDR1/2 kinase activity. hMOB2 is found in complex with unphosphorylated NDR kinases (Fig. 7), and ectopically expressed hMOB2 competes with hMOB1A/B, interfering with the activation of human NDR kinases (Fig. 4 and 5). Therefore, we addressed the role of endogenous hMOB2 by RNAi. HEK293 cells, untransfected or transfected with plasmids encoding shRNAs against firefly luciferase (Fig. 8A, lane 2) or hMOB2 (Fig. 8A, lane 3), were analyzed by immunoblotting and kinase assays on endogenous NDR proteins performed in parallel (Fig. 8A and B). hMOB2 protein levels were reduced upon transfection with shMOB2, whereas hMOB1A/B levels were not changed (Fig. 8A, lane 3). Interestingly, knockdown of hMOB2 proteins resulted in an increase of phosphorylated NDR species (Fig. 8A, top, lane 3), despite a slight reduction in total NDR protein (Fig. 8A, lane 3). The increase of phosphorylated NDR was reflected in a significant increase in kinase activity when a peptide kinase assay using immunoprecipitated NDR2 was performed (Fig. 8B, lane 3). Therefore, we conclude that endogenous hMOB2 has inhibitory properties. However, the precise mechanism by which hMOB1A/B

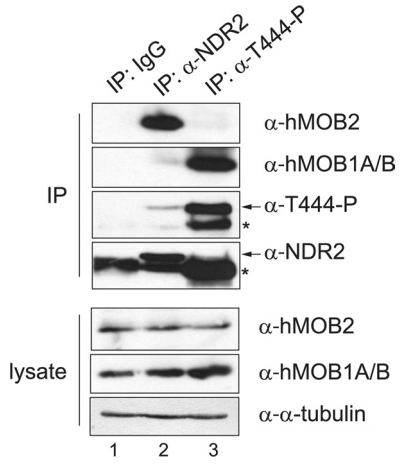

FIG. 7. Endogenous hMOB2 preferentially associates with inactive NDR kinases. Whole-cell extracts of HEK 293 cells were subjected to IP using the indicated antibodies and analyzed by immunoblotting with anti-T444-P, anti-NDR2, anti-hMOB2, and anti-hMOB1A/B. Input lysates were assayed by immunoblotting using the antibodies listed above. Antibody heavy chains are marked with an asterisk.

and hMOB2 complex formation with NDR is regulated remains unknown, since analysis of HEK293 cells treated with OA showed that hMOB2 protein levels remained unchanged (Fig. 8C) during the course of NDR activation (Fig. 8C, top) whereas hMOB1A protein levels increased with time during the treatment (Fig. 8C). Further, we did not observe significant changes in either hMOB2 or hMOB1A/B protein levels during the course of NDR activation upon induction of apoptosis (Fig. 8D).

hMOB2 expression affects biological functions of human NDR kinases. Recent studies suggest that binding of hMOB1A/B to human NDR1/2 kinases is necessary for apoptosis signaling (36) and efficient centrosome duplication (13) in human cells. Our findings show that hMOB2 is preferentially located in inactive complexes with NDR (Fig. 7) and competes with hMOB1A/B for NDR binding, thereby interfering with activation of NDR (Fig. 5 and 6). Therefore, we tested whether hMOB2 binding to NDR kinases affects NDR function in apoptosis and centrosome duplication. To examine the effect of hMOB2 on apoptotic signaling, we generated U2-OS cell lines expressing myc-hMOB2(wt) or myc-hMOB2(H157A) in a tetracycline-inducible manner (Fig. 9). Cells were treated with or without tetracycline for 24 h before anti-Fas antibody in combination with cycloheximide was added. Cells were harvested at the time points indicated and analyzed by immunoblotting (Fig. 9). Unexpectedly, the hMOB2(H157A) variant displayed reduced protein stability, since no residual protein could be detected after the addition of a combination of anti-Fas antibody and CHX or CHX alone (data not shown). Overexpression of hMOB2(wt) resulted in reduced phosphorylation of the hydrophobic motif of NDR1 (T444) after 4 and 6 h of treatment compared with control cells (Fig. 9, top, compare

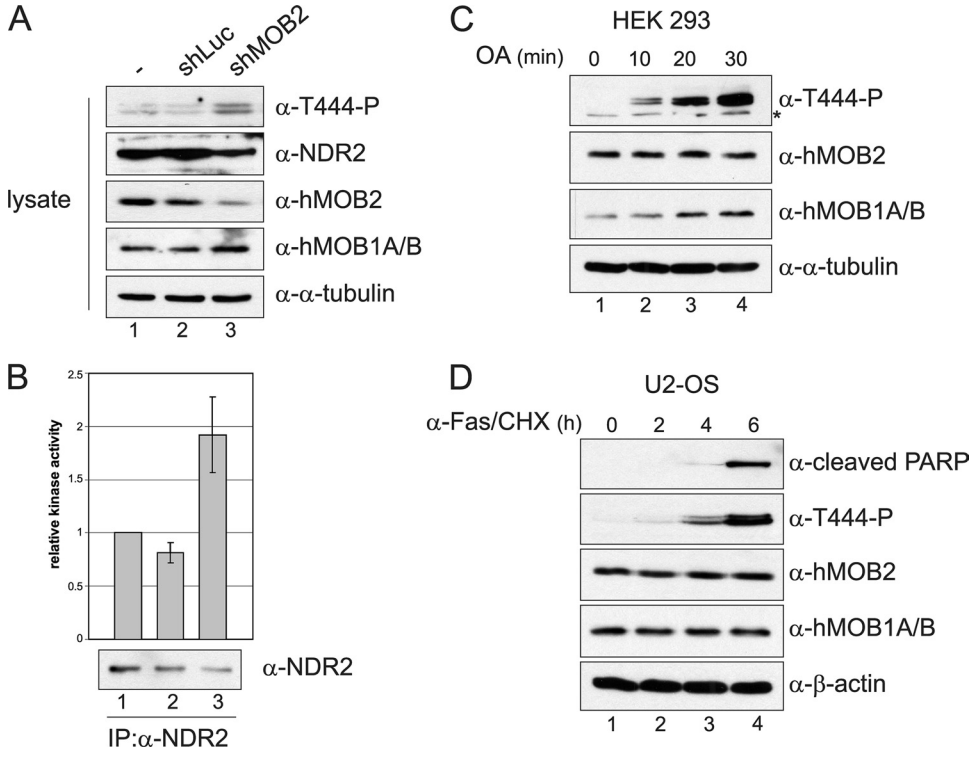

FIG. 8. Reduction of hMOB2 protein results in increased NDR1/2 activity. (A) HEK 293 cells were transfected with plasmids encoding shLuc or shMOB2 and were processed 72 h later for immunoblotting with the indicated antibodies. (B) In parallel, samples were subjected to IP using rabbit IgG or anti-NDR2 antibody before peptide kinase assays were performed. Data from at least two independent experiments with two replicates per experiment are shown. The error bars represent standard deviations. (C) HEK 293 cells were treated with 1 μM OA for the indicated times before being processed for immunoblotting with the indicated antibodies. A background band is marked by an asterisk. (D) Apoptosis was induced in U2-OS cells by adding Fas antibody in combination with CHX for the indicated time and analyzed as for panel C.

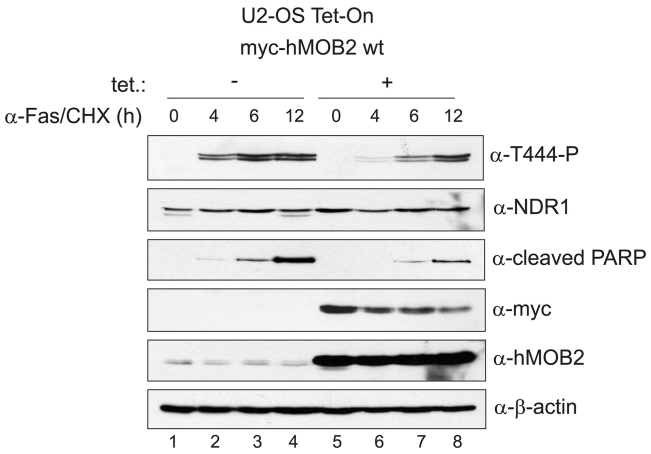

FIG. 9. Overexpression of hMOB2(wt) impairs death receptor-induced activation of NDR kinases and interferes with apoptosis signaling. U2-OS cells expressing myc-hMOB2(wt) in a tetracycline-inducible manner were incubated without (lanes 1 to 4) or with (lanes 5 to 8) tetracycline for 24 h before apoptosis was induced by the addition of Fas antibody in combination with CHX. Cells were harvested after 0, 4, 6, and 12 h and processed for immunoblotting using the indicated antibodies.

lines 2 and 3 to 6 and 7). Concurrently, we investigated whether this decrease in NDR activation was matched by a reduction in apoptotic markers. Indeed, the signal for cleaved PARP was reduced in cells overexpressing hMOB2(wt) compared with control cells (Fig. 9, compare lanes 3 and 4 to 7 and 8). These results indicate that hMOB2(wt) can interfere with the physiological activation of NDR kinases and consequently also interfere with NDR kinase apoptotic function.

We then sought to determine whether hMOB2 can also affect NDR functions in centrosome duplication. As previously reported (1), centrosomes overduplicate in U2-OS cells upon S-phase arrest. Therefore, U2-OS cells transiently expressing empty vector, myc-tagged hMOB2(wt), or hMOB2(H157A) were arrested in S phase for 72 h and then analyzed by immunoblotting and immunofluorescence (Fig. 10A and B). As expected centrosome overduplication was observed in control cells (Fig. 10C) but was reduced by overexpression of hMOB2(wt) (Fig. 10C, lane 3). Overexpression of the NDR binding-deficient mutant hMOB2(H157A) had no effect (Fig. 10C, lane 4), despite expression and localization patterns similar to those of hMOB2(wt) (Fig. 10A and B). Overall, these results suggest that wild-type hMOB2 also negatively affects centrosome overduplication during S phase in an NDR binding-dependent manner. Therefore, two biological functions of human NDR kinases can be negatively regulated by increased hMOB2 expression.

DISCUSSION

MOB proteins are critical regulators of kinases of the NDR family and are conserved from yeast to humans (16). In budding yeast, two distinct complexes of MOB-NDR modules exist, Mob1p-Dbf2p and Mob2p-Cbk1p. Moreover, MOB proteins are essential activating subunits of the respective NDR kinases (19, 20, 22, 38). In multicellular organisms, such as *Drosophila*, dMOB1/Mats is required for the function of both warts and trc kinase (10, 24), indicating that MOB1 proteins do not specifically bind to a single NDR kinase, as in yeast. Also, in human cells, hMOB1A/B bind to and activate all four NDR kinases (2, 4, 13, 15, 29) and are essential for the function of NDR1/2 kinases in apoptosis and centrosome duplication (13, 36).

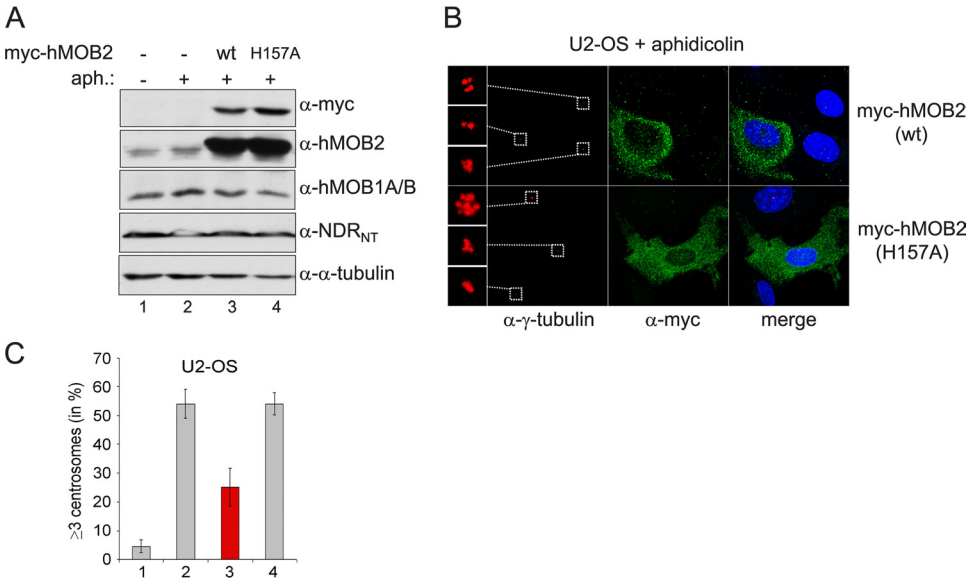

FIG. 10. Ectopic expression of hMOB2(wt) impairs centrosome overduplication. (A and B) U2-OS cells transfected with myc-tagged hMOB2(wt) or hMOB2(H157A) were treated with aphidicolin (2 μg/ml) for 72 h before being processed for immunoblotting (A) or immunofluorescence assay (B) with the indicated antibodies. The insets show enlargements of centrosomes in red. Myc-hMOB2 variants are in green. DNA is stained blue. (C) Histograms showing percentages of cells with excess centrosomes (≥3, more than three per mononucleated cell). Shown are cumulative data from at least three independent experiments with at least two replicates of 100 cells counted per experiment. The error bars indicate standard deviations.

However, human cells express six MOB proteins (Table 1) and four NDR kinases. We show here that hMOB3A, -B, and -C do not physically interact with or activate any of the four NDR/LATS kinases (Fig. 1 and 2). Despite their significant homology to hMOB1 proteins compared with hMOB2 (Table 1), hMOB3 proteins display significant sequence variation in or around amino acids previously shown to be important in conditional mutants in budding yeast MOB1p (32). Such variation might explain why hMOB3 proteins did not associate with NDR/LATS kinases. In support of this, it was shown recently that overexpression of hMOB3 proteins did not significantly affect centrosome duplication, a known function of NDR1/2 kinases (13). Therefore, the physiological binding partners and functions of hMOB3 proteins remain undefined.

Our data demonstrated that hMOB2 is a specific interaction partner of human NDR1/2 and not LATS1/2 (Fig. 1 and 2). Accordingly, we focused our investigation on hMOB2 and NDR1/2. Our findings demonstrate that hMOB2 binds to the N-terminal regulatory domain of NDR1/2 kinases (Fig. 3), the same region reported earlier for hMOB1A/B (2). Interestingly, mutational analysis of the N-terminal region of NDR1/2 revealed that the mode of binding of hMOB2 differs significantly from that of hMOB1A/B, because mutations in the NDR1/2 protein that interfere with hMOB1 binding (2) do not affect hMOB2 association (Fig. 3 and Table 2). hMOB2 binds NDR1/2, most likely through multiple contact points, since the interaction could not be ablated by single or combined point mutations (Fig. 3 and Table 2). Therefore, structural analysis of NDR1/2 kinases in complex with hMOB1 and hMOB2 proteins will be required in the future to examine differences in the two modes of interaction and also the mechanistic differences in activation/inhibition of these two complexes.

Additionally, we described for the first time competitive binding of hMOB2 and hMOB1 proteins to the N terminus of human NDR1/2 kinases (Fig. 4). Moreover, hMOB2 impaired okadaic acid-induced activation of endogenous NDR species (Fig. 4B), indicating distinct functions for different human MOB proteins in the regulation of NDR1/2 kinases, since hMOB1A was shown to potentiate NDR activity in a similar experiment (2). In addition, these data are strengthened by the concurrent use of a phosphospecific antibody to the hydrophobic motif phosphorylation (anti-T444-P) and by our biological experiments. However, in the literature, conflicting reports on the effects of hMOB2 overexpression on NDR activity describe overexpressed hMOB2 activating NDR1/2 kinases upon OA stimulation (5, 7). This could be due to the assays used to measure NDR kinase activity. In both studies, the nonspecific kinase substrates myelin basic protein and histone H1 were used. Therefore, the presence of an associated kinase may have contributed to the increase in phosphorylation of these substrates, whereas in our assays, an established NDR kinase substrate peptide was used (11, 13, 33, 34).

hMOB2 interferes with the activation of ectopic and endogenous NDR1/2 kinases by membrane-targeted hMOB1A in an NDR binding-dependent manner (Fig. 5), since an hMOB2 variant incapable of binding to NDR1/2 did not affect activation of NDR (Fig. 5). The expression of hMOB2(wt) retained NDR in the cytoplasm, and also, a fraction of mp-hMOB1A was observed in the cytoplasmic fraction (Fig. 5D). Therefore, it is possible that hMOB2 inhibits activation of NDR by mp-hMOB1A by retention of the NDR-MOB1 complex, or even by retaining mp-hMOB1A itself in the cytoplasm. However, the analysis of this observation requires further investigation. Moreover, we analyzed endogenous complexes of NDR1/2 and hMOB1A/B or hMOB2 (Fig. 6 and 7). In full agreement with previous work (36), we showed that phosphorylated endogenous NDR species associate with hMOB1A/B. Interestingly, unphosphorylated NDR proteins coimmunoprecipitated with hMOB2, in contrast to active NDR species, which were found to be associated mostly with hMOB1A/B (Fig. 7). This finding uncovers a novel and distinct role of hMOB2 in the regulation of NDR1/2 kinases.

Strikingly, by RNAi depletion of hMOB2 in HEK293 cells, we found evidence that the endogenous role of hMOB2 is to inhibit NDR kinases, since knockdown of hMOB2 increased phosphorylation and kinase activity of endogenous NDR species (Fig. 8A and B). We did not observe an effect on hMOB1A/B protein, but we detected a decrease in total NDR protein. Therefore, it is tempting to speculate that hMOB2 might also play a role in NDR protein stability. Nevertheless, we describe for the first time an endogenous inhibitory function of a human MOB protein. We tried to address the mechanism through which hMOB1A/B and hMOB2 regulate NDR activation and inhibition by analyzing the abundance of hMOB1A/B and hMOB2 during the activation of NDR kinases (Fig. 8C and D and 10A). Whereas the hMOB2 protein level did not change during both treatments, hMOB1A/B protein increased during okadaic acid stimulation (Fig. 8), despite activation of NDR in both treatments (Fig. 8). Therefore, the endogenous mechanism through which hMOB1A/B and hMOB2 regulate activation/inhibition of NDR kinases remains unknown, since the total protein level might not represent the composition of NDR-MOB complexes during the course of activation. Future research in this direction is warranted.

We subsequently addressed the putative inhibitory function of hMOB2 in the context of two biological functions of NDR1/2 kinases, the proapoptotic role of NDR and the contribution of NDR to centrosome duplication (13, 36). Importantly, both functions depend on the interaction of hMOB1A/B proteins and NDR1/2 kinases.

First, inducible expression of hMOB2 interfered with the activation of NDR1 in U2-OS cells after anti-Fas treatment and in turn delayed apoptotic progression, as assessed by cleaved PARP (Fig. 9). Since cleaved PARP is a marker for apoptotic cells, this indicates that hMOB2 expression delayed the onset of apoptosis and most likely reduced the total apoptotic cell population in our settings. Furthermore, ectopic hMOB2 impaired centrosome overduplication in an NDR binding-dependent manner (Fig. 10). Significantly, the expression of kinase-dead NDR1 had a comparable effect on centrosome overduplication in a similar assay (13). This is indicative of an inhibitory effect of hMOB2 on NDR1 activity, which in turn was necessary for centrosome duplication in our experimental settings.

Interestingly, the role of the MOB2 protein in flies, dMOB2, appears to also differ from that of dMOB1/Mats, because mutations in the dMOB2 gene do not significantly enhance a phenotype of trc mutants or overexpression of a dominant-negative trc kinase (10). More precisely, overexpression of a truncated form of dMOB2 (amino acids 148 to 354) leads to a

phenotype similar to the trc mutant in fly wings (10), suggesting a dominant-negative role of dMOB2 in NDR kinase regulation in flies. Intriguingly, the truncated variant of dMOB2 shares high similarity with the full-length human MOB2 protein (data not shown). Therefore, it is tempting to speculate that dMOB2 has competitive properties similar to those of hMOB2 shown in our study. Determining whether dMOB2 negatively regulates trc kinase by competing with dMOB1/Mats is a question for future studies.

Our data show for the first time that hMOB2 has inhibitory effects on NDR1/2 functions. hMOB2 is found in unphosphorylated NDR complexes, and when overexpressed, hMOB2 can compete with hMOB1A/B, possibly physically displacing endogenous hMOB1A/B from NDR. hMOB2-NDR1/2 complexes that accumulate also appear to be inactive/quiescent. As a result, the activation of NDR1/2 by hMOB1A/B and possibly also by upstream kinases, such as MST1, could be impaired. Future challenges will be to address whether hMOB2 hinders NDR activation by mechanisms other than competition and steric restriction of the access of hMOB1 to the N terminus of NDR1/2, which in turn will have to be addressed by highly defined quantitative biochemical and biological assays. Moreover, the role of dMOB2 in flies has yet to be clarified. In light of our findings, the investigation by *Drosophila* geneticists of a negative function of dMOB2 on tricornered, warts, or even hippo kinase, will be of considerable interest.

In conclusion, our data indicate a novel role for hMOB2 in the regulation of NDR1/2 kinases. In contrast to hMOB1, hMOB2 is present in unphosphorylated NDR complexes. RNAi-mediated reduction of hMOB2 resulted in increased NDR activity. Overexpression negatively affects biological functions of NDR kinases, such as apoptotic progression and centrosome duplication. Altogether, our data indicate that hMOB2 plays an inhibitory role in the regulation of human NDR1/2 kinases.

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3.1.3. Supplemental Material (data not shown)

Figure S1. Kohler *et al.*

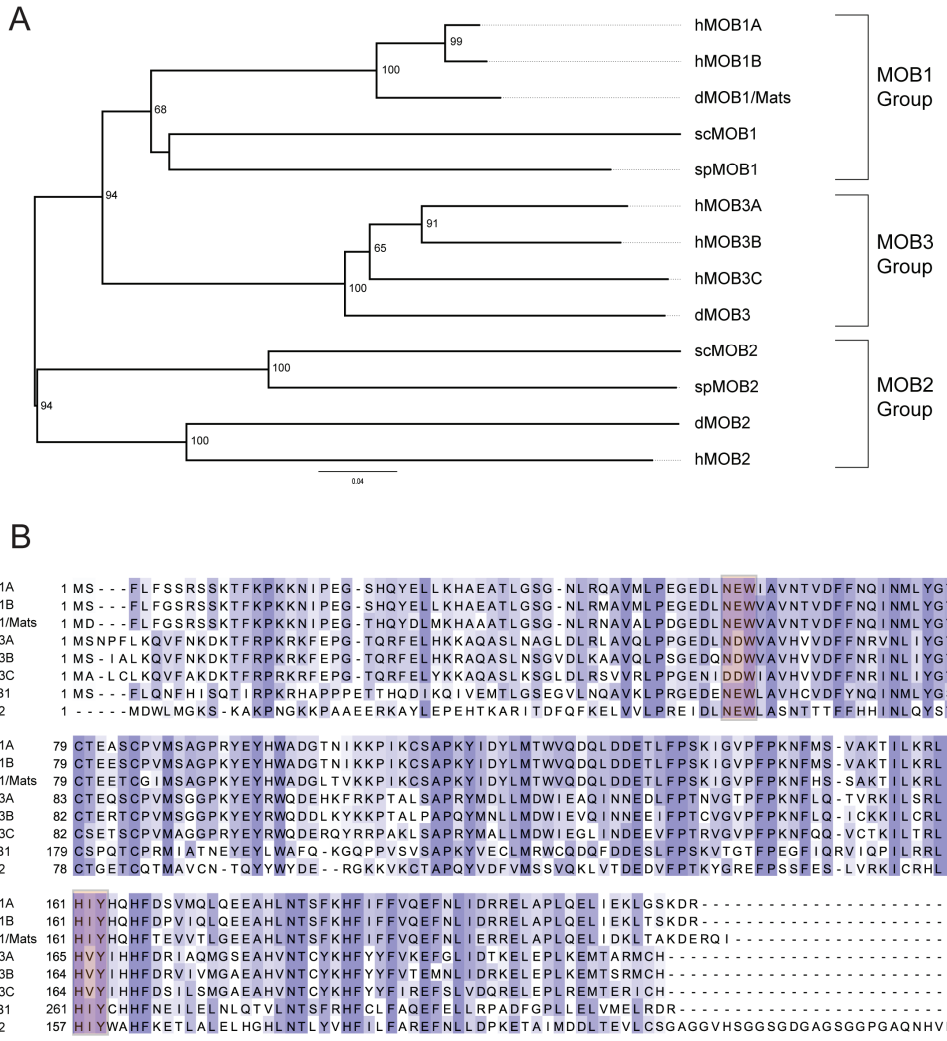

Figure S1. Alignment of human hMOB proteins and phylogenetic analysis of the MOB protein family. (A) Phylogenetic relationships between proteins of the MOB family from human, fruit fly and yeast species. Only the residues of the conserved MOB-phocein domain (pFam accession number PF03637) corresponding to residues 29-205 of hMOB1A were aligned and used for the phylogenetic analysis. The tree was constructed with quicktree using the neighbor-joining method. The numbers for interior branches represent bootstrap values. Only values higher than 50 are shown. Protein accession numbers form top to bottom: human MOB1A (*Homo sapiens*), Q9H8S9; human MOB1B, NP_775739; fruit fly dMOB1/Mats (*Drosophila melanogaster*), NP_651041; human MOB3A, CAE45267; human MOB3B, CAE45268; human MOB3C, CAE45269; fruit fly MOB3 (dMOB3), AAF52892; budding yeast MOB1 (*Saccharomyces cerevisiae*, scMOB1), P40484, and MOB2 (scMOB2), P43563; fission yeast MOB1 and 2 (*Schizosaccharomyces pombe*, spMOB1, NP_595191; spMOB2, NP_587851) human MOB2, NP_443731; fruit fly MOB2 (dMOB2), NP_729715. (B) Alignment of amino acid sequences of full-length MOB proteins. Alignment was performed using default parameters of ClustalW2 and illustrated using Jalview. Residues 17-85 and 95-121 of scMOB1 are not shown in the alignment.

Figure S2. Kohler *et al.*

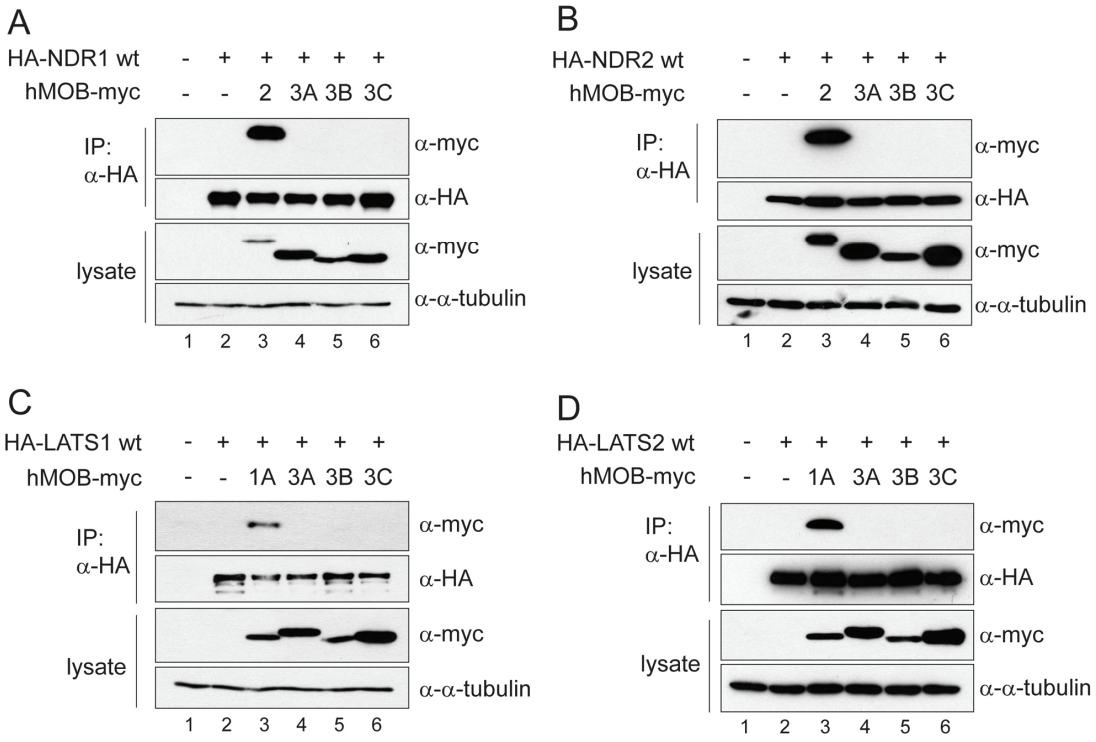

Figure S2. Human NDR and LATS kinases do not interact with C-terminally tagged hMOB3A/B/C proteins. (A) and (B) Lysates of HEK 293 cells co-expressing the indicated combinations of HA-tagged NDR1 wild-type (wt), HA-tagged NDR2(wt) and hMOB species containing a C-terminal myc-tag were analyzed by immunoprecipitation (IP) using anti-HA 12CA5 antibody. Complexes were assayed by immmunoblotting using anti-myc antibody (top panel) or anti-HA antibody (middle panel). Input lysates were analyzed by immunoblotting using anti-myc antibody and a-α-tubulin antibody. hMOB2 served as positive control. (C) and (D) Lysates of HEK 293 cells co-expressing the indicated HA-tagged LATS1(wt) or LATS2(wt) and hMOB species containing a C-terminal myc-tag were analyzed as described in A and B, except that hMOB1A served as positive control.

Figure S3. Kohler *et al.*

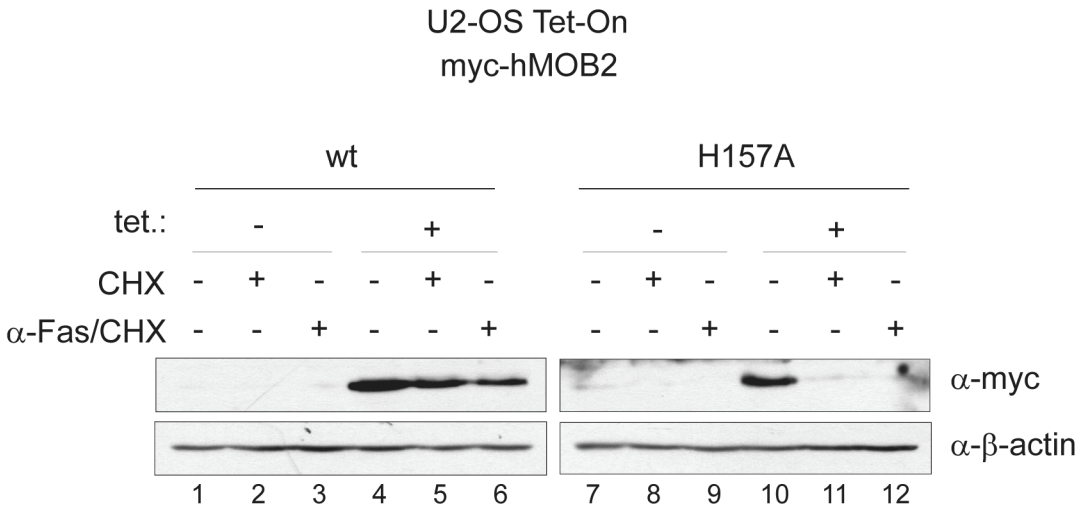

Figure S3. The hMOB2(H157A) mutant displays reduced protein stability. U2-OS cells expressing hMOB2(wt) or hMOB2(H157A) in a tetracycline inducible manner were incubated without or with tetracycline for 24 h before treatment with DMSO (lanes 1, 4, 7 and 10), cycloheximide (CHX) (lanes 2, 5, 8 and 11) or CHX in combination with activating anti-Fas antibody (lanes 3, 6, 9 and 12). Cells were harvested 4 h and processed for immunoblotting using anti-myc and anti-actin antibodies.

Figure S4. Kohler *et al.*

Figure S4. Alignment of the amino acid sequence of human MOB2 and fly dMOB2(dC). The truncated dMOB2(dC) protein (aa148-354) as described in (He et al. 2005) shares 47% identity and 61% similarity with full-length human MOB2.

3.2. Discovery of Novel Human NDR Kinases Substrates:

A Chemical Genetic Approach

3.2.1. Introduction

The identification of kinase-substrate pairs has so far been the bottleneck of protein kinase research mainly because the highly conserved structural similarity of the kinase domain prevents prediction of substrate specificity for a single kinase.

Shokat and colleagues developed a method by which with the help of a mutation a subtle structural distinction between wild-type and mutant kinases is created therefore facilitating the identification of novel substrates [1]. The method is based on the mutation of a highly conserved bulky hydrophobic residue in the ATP-binding pocket present in most of all protein kinases [2]. Changing this so-called gatekeeper residue in the active site of the kinase (usually a methionine, leucine, phenylalanine or threonine) to alanine or glycine grants access to a hydrophobic groove within the ATP-binding pocket, therein allows binding of bulky ATP analogs with substituent groups appended to the N^6-amine such as N^6-(benzyl)-ATP. Since wild-type kinases are not able to utilize ATP analogs, the addition of a single analog-sensitive kinase to cell extracts in the presence of a radiolabeled ATP analog ([γ–^{32}P] N^6-(benzyl)-ATP) should ultimately result in specific labeling of direct substrates which can subsequently be identified by mass spectrometry [3-6].

Additionally, the analog-sensitive kinase is exclusively sensitized to the orthogonal inhibitors 1Na-PP1, 1NM-PP1 or 3-MB-PP1. Since wild-type kinases remain insensitive to the addition of these orthogonal inhibitors, the chemical genetic analysis offers single-kinase inhibition, and unlike genetic disruption of protein kinase function, cell-permeable inhibitors act quickly, reversibly and do not allow the cell to

compensate for the missing kinase activity. This allows for direct investigation of the cellular kinase activity-dependent functions of the modified protein kinase [7-9].

Although NDR1/2 kinases are well characterized in terms of regulation and functions, downstream signaling events remain elusive. Indeed, the first NDR substrate was only described recently (Cornils et al., see Appendix). Therefore, we aimed to employ the chemical genetic method to NDR kinases in order to identify novel substrates. Hence, we created analog-sensitive variants of NDR1/2 kinases by the mutation of the gate-keeper residues Met166 and Met167, respectively. Subsequently, we have tried to identify direct substrates of analog-sensitive NDR1/2 variants by performing assays on cell extracts and immunocomplexes in the presence of a radiolabeled ATP analog.

3.2.2. Results

Generation of an active analog-sensitive NDR1-hMOB complex to screen for novel substrates in cell extracts. We have characterized a recombinant analog-sensitive NDR1 kinase, MBP-NDR1 PIF M166G (Kohler R Masterthesis 2006). However, this recombinant kinase diplayed reduced basal activity when compared to wild-type NDR1 and therefore the discovery of novel substrates in cell lysates was likely to be hindered by the limited kinase activity of recombinant NDR1 M166G from bacteria.

For the discovery of novel substrates of Cdk1 and Cdk7, analog-sensitive kinases were expressed and purified from insect cells using the baculovirus system [3, 4, 10]. We thus changed to the baculoviral system and purified polyhistidine-tagged

NDR1(M166G) in dimeric complexes with either hMOB1A or hMOB2 or NDR1(M166G) without hMOB proteins instead containing the PIFtide (6xHis-NDR1(M166G) PIF) rendering it constitutively active (Fig. 1A). The recombinant proteins were essentially pure and we could demonstrate the distinct NDR/hMOB complexes by Coomassie stain or immunoblotting (Fig. 1A). The activity of these NDR1(M166G) variants was then compared to the previously used MBP-NDR1(M166G) PIF from *E. coli* using a peptide kinase assay and radiolabeled ATP-analog [γ–^{32}P] N^6-(benzyl)-ATP (Fig. 1B). As apparent from this panel, the analog-sensitive NDR1 kinases from Sf9 cells displayed 6-16 fold increase in kinase activity compared to the MBP-NDR1(M166G) PIF (Fig. 1B, lanes 2-4 vs lane 1). Interestingly, the NDR1(M166G)/hMOB1A complex showed comparable kinase activity to the constitutively active NDR1(M166G) PIF (Fig. 1B, lanes 2 and 3). Therefore, the NDR1/hMOB1A complex was used for further experiments since it possibly reflected a physiological NDR1 kinase better than the artificial PIFtide containing chimeric kinase. Furthermore, the complex of NDR1(M166G)/hMOB2 displayed reduced activity when compared to NDR1(M166G)/hMOB1A (Fig. 1B, compare lanes 2 and 4), which is in full agreement with our recently published manuscript [11]. Significantly, we could further increase the activity of the NDR1(M166G)/hMOB1A complex by adding the upstream kinase MST3 (Fig. 1C). The incubation of NDR1(M166G)/hMOB1A together with GST-MST3 purified from okadaic acid (OA)-treated HEK 293 cells before assaying kinase activity resulted in a robust increase in phosphorylation of the hydrophobic motif T444 as well as kinase activity (~100-fold) measured by peptide kinase assay (Fig. 1C, lane 2). To demonstrate that this increase in activity was specific to the analog-sensitive NDR1 kinase, the orthogonal inhibitor 1NM-PP1 was added to the kinase reaction after

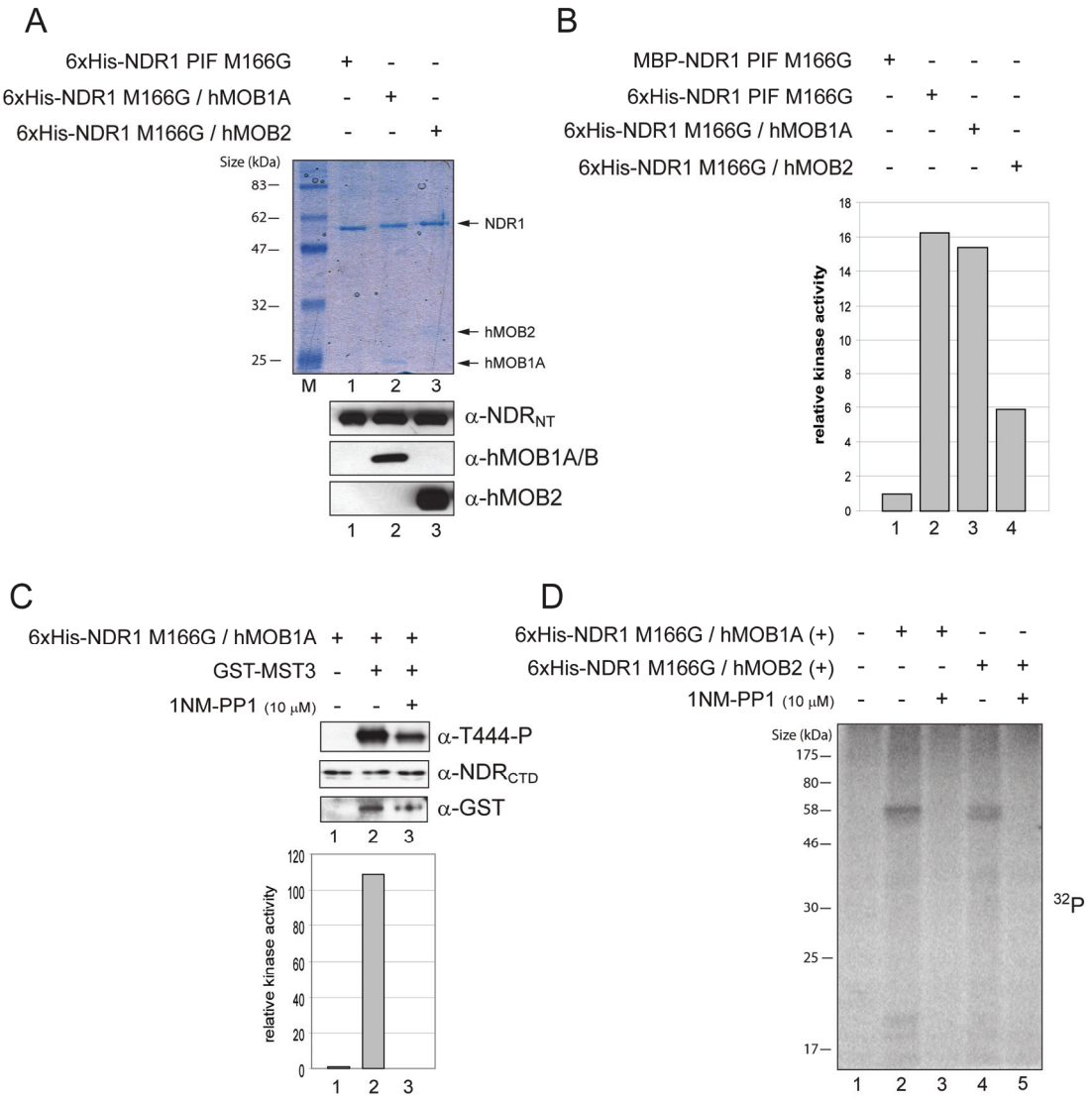

Figure 1. Generation and characterization of active recombinant NDR1(M166G)/ hMOB complexes. **(A)** Purification of NDR1(M166G)/hMOB complexes. Purified polyhistidine-tagged NDR1(M166G) variants from Sf9 cells were analyzed by SDS-PAGE followed by Coomassie staining and in parallel by immunoblotting using anti-NDR, anti-hMOB1A/B and anti-hMOB2 antibodies. **(B)** Recombinant NDR1(M166G)/hMOB1A complex displays high activity. 500 ng of purified proteins and complexes from (A) were assayed for kinase activity in a peptide kinase assay using [γ-^{32}P] N^6-(benzyl)-ATP. The results in duplicates are shown relatively to the activity of MBP-NDR1(M166G) PIF purified from *E. Coli*. **(C)** Activation of NDR1(M166G)/hMOB1A by MST3 *in vitro*. NDR1(M166G) in complex with hMOB1A was incubated with or without GST-MST3 purified from okadaic acid (OA)-activated HEK 293 cells. Aliquots of the reactions were analysed by immunoblotting using anti-T444P, anti-NDR and anti-GST antibodies. In parallel, kinase assays on NDR substrate peptide containing [γ-^{32}P] N^6-(benzyl)-ATP were performed in duplicates with or without the orthogonal inhibitor 1NM-PP1. **(D)** Screening membrane extracts for direct substrates. Activated NDR1(M166G)/hMOB complexes (+) treated as in (C) were mixed with 100 µg membrane extracts in the presence of [γ-^{32}P] N^6-(benzyl)-ATP, an ATP regenerating system and with or without the orthogonal inhibitor 1NM-PP1. Labeled bands were analysed by SDS-PAGE and exposed to a Phosphor screen ad visualized with a Phosphorimager.

incubation with GST-MST3, which abolished NDR1 kinase activity in the peptide kinase assay (Fig. 1C, lane 3).

We generated a highly active analog-sensitive NDR1/hMOB1A complex, and the usage of an ATP regeneration system as previously described [3] greatly reduced the background in our assays (Fig 1D, Lane 1). However, when we screened for direct substrates in cell lysates or subcellular fractions such as membranous, nuclear or cytosolic fractions, we did not observe a reproducible pattern of labeled bands. As a representative experiment we show here membranous fraction of HEK 293 cells assayed with activated dimeric NDR1(M166G)/hMOB complexes (Fig. 1D). We did not observe strong background staining (Fig. 1D, lane 1), however, the only bands in the reaction that responded to the orthogonal inhibitor 1NM-PP1 treatment were NDR1(M166G) at ~58 kDa and a faint band at ~20 kDa (Fig 1. lanes 2 and 3). In conclusion, although we successfully optimized the activity of our analog-sensitive NDR1 kinase, we did not observe a reproducible pattern of phosphorylated bands that could possibly lead to the discovery of novel substrates.

Kinase assays on HA-NDR1(M166G) immunocomplexes reveal a distinct pattern of labeled bands specific for the activity of the analog-sensitive NDR kinase. The labeling reactions performed with activated NDR1(M166G)/hMOB1A and cell extracts did not lead to the discovery of directly phosphorylated proteins. (Fig. 1). Therefore, we sought to leverage another approach which did not involve production of recombinant analog-sensitive kinases and assays in cell extracts. We adapted the method described by Eblen et al., which consists of a kinase assay on immunocomplexes of the respective analog-sensitive kinase, in order to identify novel Erk2 substrates [5]. Briefly, HEK 293 cells overexpressing HA-NDR1(M166G) are treated with OA for 45 min before harvesting in order to accumulate active NDR (Fig. 2). Then the HA-tagged protein is immunoprecipitated using covalently coupled anti-

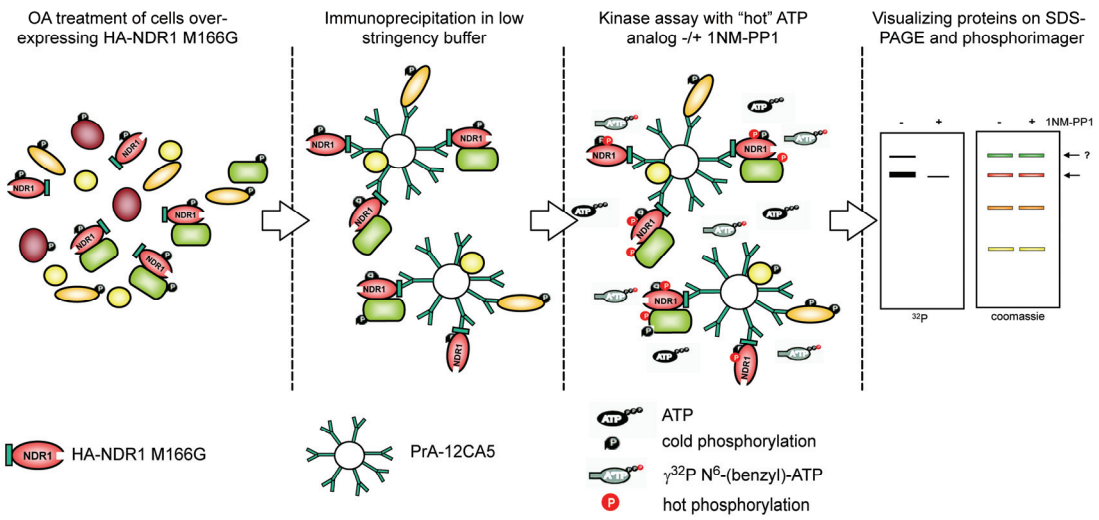

Figure 2. Experimental outline for kinase assays on HA-NDR1(M166G) immunocomplexes. HEK 293 cells expressing HA-NDR1(M166G) are treated with OA before lysis in low stringency buffer. Subsequently, NDR1 associated proteins are immunoprecipitated using anti-HA antibody covalently coupled to beads. After several washes in low-stringency buffer, kinase assays using radiolabeled [γ-^{32}P] N^6-(benzyl)-ATP are performed on beads in the presence or absence of the orthogonal inhibitor 1NM-PP1. Labeled proteins are then visualized by SDS-PAGE and exposure to a Phosphorimager screen.

HA antibodies in low-stringency buffer where we would expect many NDR-binding proteins to remain associated. The HA-immunocomplexes are then directly assayed on the PrA-beads in reaction buffer containing [γ–^{32}P] N^6-(benzyl)-ATP in combination with or without the orthogonal inhibitor 1NM-PP1. Other co-immunoprecipitated kinases would be able to use "cold" ATP to phosphorylate substrates. However, only the analog-sensitive HA-NDR1(M166G) should be able to use the radiolabeled ATP analog, therefore directly labeling substrates. The proteins are separated by SDS-PAGE and the labeled bands are visualized by exposure to a phosphorimager screen, which will show bands responsive to the inhibition of NDR1(M166G) by 1NM-PP1 (Fig. 2).

Congruently, HA-tagged NDR1(M166G) and NDR2(M167G) from unstimulated or OA-treated HEK 293 cells were immunoprecipitated and associated proteins were labeled in an *in vitro* kinase assay using [γ–^{32}P] N^6-(benzyl)-ATP with or without the orthogonal inhibitor 1NM-PP1 (Fig. 3A). The kinase assay on immunoprecipitates

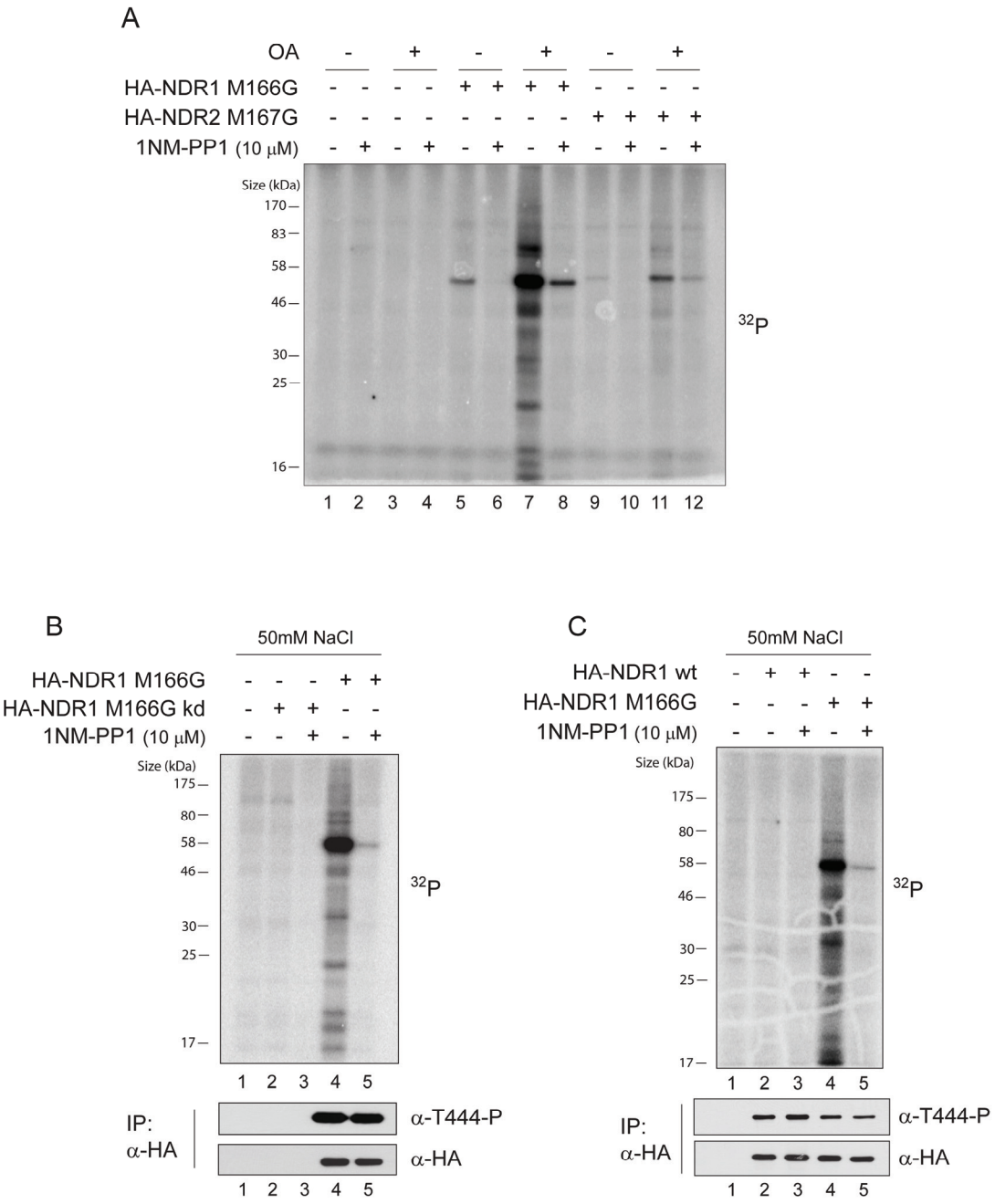

Figure 3. Kinase assays on NDR1(M166G) immunocomplexes. **(A)** A distinct pattern of labeled bands is observed after immunocomplex kinase assays. After immunoprecipitation in IP buffer (without NaCl) with anti-HA antibody of HEK 293 cells overexpressing HA-NDR1 or HA-NDR2 treated or untreated with OA for 45 min kinase assay reactions were performed in the presence of [γ-^{32}P] N^6-(benzyl)-ATP and with or without 1NM-PP1. Labeled bands were visualized using a Phosphorimager screen. **(B)** The pattern of the specifically labeled band is reproducible in more stringent IP buffer. Immunoprecipitation of HA-NDR1(M166G) was performed in IP buffer containing 50 mM NaCl before kinase assays using [γ-^{32}P] N^6-(benzyl)-ATP. In parallel complexes were assayed using anti-HA and anti-T444-P antibodies. **(C)** The labeled bands are specific for HA-NDR1(M166G) activity. Lysates of HEK 293 cells overexpressing HA-NDR1(wt) or HA-NDR1(M166G) were subjected to immunoprecipitation in IP buffer containing 50 mM NaCl before kinase assays on immunocomplexes using [γ-^{32}P] N^6-(benzyl)-ATP were performed. Labeled bands were visualized using a Phosphorimager screen. In parallel complexes were analyzed using anti-HA and anti-T444-P antibodies.

from transfected control cells showed negligible background phosphorylation (Fig. 3A, lanes 1-4). Interestingly, the kinase assays of HA-NDR1(M166G) immunocomplexes from OA-stimulated cells generated a distinct pattern of labeled bands at high and low molecular masses (Fig. 3A, lane 7). Significantly, the signals were responsive to the inhibition by 1NM-PP1, indicating that these proteins were directly phosphorylated by HA-NDR1(M166G) (Fig. 3A, lane 8). We observed similar results when kinase assays of HA-NDR2(M167G) immunoprecipitates were analyzed (Fig. 3A, lanes 11 and 12). However, the signals were significantly weaker as in the case of NDR1 probably due to differences in expression levels. Therefore, we used HA-NDR1(M166G) for further experiments.

We then increased the stringency of our immunoprecipitation and washing protocol by using IP buffer containing 50 or 150 mM NaCl. The obtained pattern of labeled bands was highly reproducible in buffer containing 50 mM NaCl (Fig. 3B, lanes 4 and 5). However, the signals were greatly diminished using buffer containing 150 mM NaCl (data not shown). Therefore, we used buffer with 50 mM NaCl also to decrease background by proteins binding unspecifically to the beads.

In order to show that the labeling of these bands is derived from NDR1(M166G) activity, we performed kinase assays on immunocomplexes of HA-NDR1(wt) from OA-stimulated cells in parallel (Fig. 3C). Whereas we observed again a similar pattern of bands in the kinase assay using HA-NDR1(M166G) immunocomplexes (Fig. 3C, lanes 4 and 5), the reaction with HA-NDR1(wt) immunoprecipitates generated only signals resembling background phosphorylation of transfected control cells (Fig. 3C, lanes 1-3) despite similar amounts of active HA-tagged NDR species were being immunoprecipitated (Fig. 3C, bottom panels).

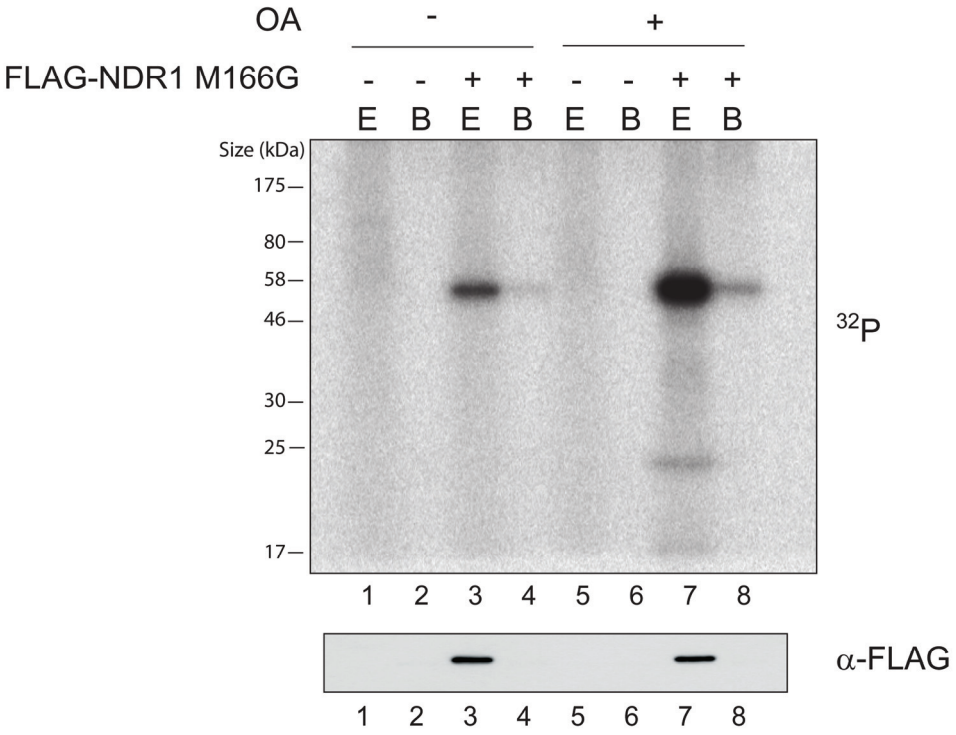

Figure 4. Kinase assays on FLAG-NDR1(M166G) immunocomplexes do not result on a distinct pattern of bands. Complexes from HEK 293 cells overexpressing FLAG-NDR1(M166G) treated with or without OA, were immunoprecipitated using anti-FLAG antibody in IP buffer containing 50 mM NaCl. Kinase assay reactions were performed in the presence of [γ-^{32}P] N^6-(benzyl)-ATP, with or without 1NM-PP1. Then, proteins were eluted using 3xFLAG peptide and analyzed by SDS-PAGE. Elution efficiency was assessed by immunoblotting using anti-FLAG antibody. Labeled bands were visualized using a Phosphorimager screen. E, eluate, B, beads.

In order to identify ERK2 substrates, FLAG-tagged ERK2 was used which facilitated the elution of immunocomplexes and subsequent mass spectrometrical analysis [5]. Thus, we repeated our experiments with FLAG-tagged NDR1(M166G) followed by elution using 3xFLAG peptide. Despite efficient elution from FLAG-NDR1(M166G) of the beads, we did not observe a distinct pattern of bands except for the autophosphorylation of NDR1(M166G) (Figure 4).

In conclusion, after switching from recombinant analog-sensitive NDR kinase to assays using HA-NDR immunocomplexes from OA treated cells we were actually able to reproducibly obtain a distinct pattern of bands directly phosphorylated by HA-NDR1(M166G). Unfortunately, we were not able to use FLAG-NDR1(M166G) which would have facilitated elution and further analysis by mass spectrometry.

3.2.3. Discussion

The molecular regulation and the functions of NDR1/2 kinases in apoptosis and centrosome duplication [11-21] are well described. However, downstream mechanisms of NDR functions remained elusive and only recently the first substrate of human NDR kinases was identified, the cyclin-CDK inhibitor p21 (Cornils and Kohler. see Appendix).

Hence, we aimed to identify novel NDR substrates using a chemical genetic approach originally developed by Shokat and colleagues [1]. We established recombinant sources of analog-sensitive NDR1(M166G) kinase (Fig. 1). Comparing the activity of NDR1(M166G) variants either purified from *E.Coli* or Sf9 cells, we could show that recombinant NDR1(M166G) purified using the baculovirus system displayed significantly higher basal activity (Fig. 1B). Recombinant analog-sensitive kinases such as CDK1, CDK2 or CDK7 need to be activated by pretreatment with upstream kinases prior to use for labeling direct substrates [3, 4, 10].Accordingly, we were able to increase the activity of the polyhistidine-tagged dimeric NDR1/hMOB complexes by 100-fold following preincubation with GST-MST3 (Fig. 3C). This highly active analog-sensitive NDR/hMOB1A complex was subsequently used for screening cell extracts and subcellular fractions for direct NDR substrates (Fig. 1D). Unfortunately,

the addition of activated NDR1(M166G)/hMOB complexes to whole cell lysates or subcellular fractions such as membranous fraction did not result in labeling of proteins except for the observed autophosphorylation of NDR itself (Fig. 1D, lane 2). The labeling of substrates using recombinant analog-sensitive kinases and cell lysates or cellular fractions worked exceptionally well with CDKs such as CDK1, CDK2 and CDK7 [3, 4, 10]. However, with mammalian kinases of other classes such as PKA, JNK or RAF-1, the clear-cut substrate labeling in lysates was more difficult to achieve [22-24].

Several hypothetical explanations can be thought of to justify the ineffective labeling of substrates in cell lysates *in vitro*: saturation of phosphosites by endogenous protein kinases; association of substrates with endogenous kinases, therefore blocking access for phosphorylation; reduced activity and substrate specificity of the recombinant analog-sensitive kinase; missing co-factors in the dimeric NDR1(M166G)/hMOB1 complex; limited abundance of direct substrates in cell lysates; complexity of the lysates/fractions.

Binding partners of a kinase are potential substrates, therefore immunoprecipitation of the overexpressed analog-sensitive kinase under low-stringency conditions should leave binding partners associated and thus make them available for labeling [5]. We adapted this method and observed for the first time a reproducible pattern of labeled bands when immunocomplexes of HA-tagged NDR1(M166G) from OA-treated HEK 293 cells were assayed in the presence of radiolabeled ATP analog (Fig. 3). The bands are likely to be direct targets of NDR1(M166G) since the signals were abolished when the orthogonal inhibitor 1NM-PP1 was added. Furthermore, the signals were not visible in HA-tagged NDR1(wt) complexes (Fig. 3C). However, assays performed on FLAG-NDR1(M166G)

immunocomplexes did not result in the previously labeled bands (Fig. 4). Therefore, the identification of the nature of those bands was largely impaired.

In order to validate the result obtained with HA-tagged NDR1(M166G) the reactions should be tested for the abundance of phosphorylated p21, the recently described first substrate of NDR kinases (Cornils and Kohler, see Appendix). The assays could be performed as described but after labeling, instead of analysis by SDS-PAGE, the reaction are boiled in SDS and immunoprecipitation of p21 is performed followed by analysis by western blot and autoradiography. Moreover, a combined approach of immunocomplex kinase assays and covalent capture of phosphoproteins as described in [10] could help to identify substrates of NDR1(M166G).

In conclusion, we have established a recombinant source of active analog-sensitive NDR1/hMOB1A complex yet only the immunoprecipitated HA-NDR1(M166G) yielded a specific and reproducible pattern of bands. However, these results should open avenues for the discovery of novel direct substrates of human NDR1/2 kinases and for further chemical genetic analysis of NDR kinase function.

3.2.4. Materials and Methods

Construction of Plasmids and recombinant Baculoviruses. Human NDR1 and NDR2 cDNAs were subcloned into pcDNA3-HA or pcDNA3-FLAG using BamHI and XhoI restriction sites. The generation of a constitutively active NDR1 kinase by exchanging the C-terminus of NDR1 by the hydrophobic motif of PRK2 has been described earlier ([12], Kohler R. Masterthesis). The gatekeeper mutations Met166 or Met167 to glycine were introduced by site-directed mutagenesis according to the

manufacturer's instructions (Stratagene). The NDR1 PIF M166G cDNA was subcloned into pMAL-2c or pFastBac HTb using BamHI and XhoI sites. Human hMOB1A and hMOB2 cDNAs were amplified by PCR, digested by XhoI and SphI and inserted into pFastBac Dual or digested by BamHI and XhoI and ligated into pFastBac HTb. To generate pFastBac Dual coding for NDR1(M166G) and hMOB1A or hMOB2, NDR1(M166G) cDNA was subcloned into pFastBac HTb using BamHI and XhoI restriction sites. Then NDR1 M166G cDNA containing an N-terminal 6x His tag followed by an rTEV cleavage site was subcloned from pFastBac HTb using RsrII and HindIII restriction sites and ligated into pFastBac Dual constructs. To generate pFastBac1-GST, the coding sequence of Glutathione-S-transferase (GST) of pGEX-6P-3 including multiple cloning site was amplified by PCR and digested with BglII and NotI and inserted into pFastBac1 vector. Human NDR1(wt) or NDR1(kd) and NDR2(wt) or NDR2(kd) were subcloned into pFastBac1-GST using BamHI and XhoI restriction sites. All constructs were confirmed by sequence analysis.

Recombinant baculoviruses were generated using the Bac-to-Bac baculovirus system (Invitrogen). Briefly, pFastBac plasmids were transformed into DH10Bac to generate bacmid DNA. Correct insertion of coding sequences in bacmid DNA was verified by PCR. To generate recombinant baculovirus, individual bacmids were transfected into Sf9 cells and high titer viruses were harvested after several rounds of amplification.

Expression and Purification of Recombinant Proteins. To produce recombinant protein using the Bac-to-Bac system, Sf9 cells (20 x 10^6 cells/15-cm dish) were infected with the respective high-titer baculovirus stock and cells were harvested 48 or 72 h after infection. Recombinant proteins containing a polyhistidine tag were purified according to manufacturer's instructions (Invitrogen). Briefly, Sf9 cells were

lysed in binding buffer (50 mM NaH_2PO_4, 500 mM NaCl, 1% NP-40, 1 mM Na_3VO_4, 1 mM benzamidine, 4 μM leupeptin, 0.5 mM phenylmethylsulfonyl fluoride (PMSF) and 1 μM microcystine at pH 8.0) containing 10 mM imidazole and 6x His tagged proteins were bound to Ni-NTA agarose. After extensive washes with binding buffer containing 20 mM imidazole, proteins were eluted using 250 mM imidazole followed by dialysis vs. 20 mM Tris at pH 7.5 containing 20 % glycerol. Purified recombinant proteins were stored at – 80°C.

Active recombinant NDR kinases containing N-terminal Glutathione-S-Transferase (GST) tags in complex with hMOB1A were purified on GS-4B beads according to the manufacturer's instructions (GE Lifesciences). Briefly, Sf9 were co-infected with baculoviruses encoding GST-NDR1/2 variants and 6xHis-hMOB1A and harvested 72 hours after infection. To activate NDR kinases, cells were treated with 0.1 μM okadaic acid (OA) 4 hours before harvest. Complexes were purified on GS-4B beads.

To make bacterially synthesized NDR1 PIF M166G, transformed BL21/DE3 *E. Coli* were induced with IPTG for 3 hours and MBP-tagged proteins were purified using amylose resin according to the manufacturer's instruction (New England Biolabs).

The purification of GST-MST3 from OA-treated HEK293 cells was described previously [12].

Cell Culture, Transfections and Chemicals. Sf9 cells were maintained in Grace's insect medium containing penicillin and streptomycin and 10% fetal calf serum in a humidified incubator at 25°C. HEK 293 cells were maintained in Dulbecco's modified eagle medium (DMEM) supplemented with 10% fetal calf serum. Exponentially growing HEK 293 cells were transfected in solution at

consistent confluence (5 x 10^6 cells/10-cm dish) using jetPEI (Polyplus Transfections) according to the manufacturer's instructions. The orthogonal inhibitor 4-amino-1-(*tert*-butyl)-3-(1′-naphthylmethyl) pyrazolo [3,4-*d*] pyrimidine (1NM-PP1) was purchased from Calbiochem and okadaic acid (OA) was from Alexis Biochemicals (Enzo Life Sciences).

Generation of [γ-^{32}P] N^6-(benzyl)-ATP. 100 units of Nucleoside diphosphate kinase (NDPK, *S. Cerevisiae*, Sigma) and 400 μCi of [γ–^{32}P] ATP (Hartmann Analytic) were added in a 100-μl reaction containing HBS (150 mM NaCl, 20 mM HEPES, pH 7.4, and 5 mM $MgCl_2$). The NDPK was allowed to autophosphorylate at 30°C for 5 min. ^{32}P-labeled NDPK was purified from free [γ–^{32}P] ATP by centrifugation through a Probequant G50 micro column (Amersham Bioscience) at 735 x g for 2 min. 1 nmole of N^6-(benzyl)-ADP (Biolog) in HBS was added to the ^{32}P-labeled NDPK, and the reaction was incubated for 20 min at 30°C. After the reaction, [γ–^{32}P] N^6-(benzyl)-ATP was purified from NDPK using a Microcon-YM10 (Millipore) at 13000 rpm for 15 min. This procedure usually resulted in production of ~200 μCi of purified [γ–^{32}P] N^6-(benzyl)-ATP.

Antibodies. The generation and purification of anti-T444-P, anti-NDR_{NT}, anti-NDR_{CTD}, anti-hMOB1A/B and anti-hMOB2 antibodies has been described previously [17, 19-21]. Anti-GST antibody was purchased from Santa Cruz. Anti-HA 12CA5 and 42F13 were used as hybridoma supernatants. Anti-FLAG M2 and 3xFLAG peptide were purchased from Sigma.

Immunoblotting. Immunoblotting experiments were performed as described [13].

Fractionation of Cells. Cytosolic, nuclear and membrane-associated proteins were separated by fractionation as described [13].

Peptide Kinase Assays. Purified recombinant NDR1(M166G) kinase variants (500 ng) were incubated in reaction buffer (20 mM Tris/HCL pH 7.5, 10 mM $MgCl_2$, 1 mM benzamidine, 4 μM leupeptin, 1 μM microcystin, 1 mM DTT, 1 μM cyclic AMP-dependent protein kinase inhibitor peptide) containing 500 μM ATP for 90 min at 30°C before 1mM NDR substrate peptide (KKRNRRLSVA) and 2.5 μCi [γ–^{32}P] N^6-(benzyl)-ATP were added. After 30 min at 30°C reaction was stopped by adding 50 mM EDTA. 20 μl of the reaction mix was then spotted onto squares of P-81 phosphocellulose paper (Whatman) and washed four times for 10 min each in 1% phosphoric acid and once in acetone before counting in a liquid scintillation counter was performed. Experiments were performed in duplicates.

In vitro **Activation of NDR1(M166G) Kinases by MST3.** 500 ng of purified 6xHis-tagged NDR1(M166G) in complex with hMOB1A or hMOB2 was incubated with or without ~1 μg GST-MST3 for 90 min at 30°C in 60 μl reaction buffer containing 1mM ATP.

Protein Phosphorylation in Cell Extracts. 100 μg cytosolic, nuclear or membrane extracts in 20 mM Tris/HCl pH 7.5 supplemented with protease inhibitors, phosphatase inhibitors (80 mM sodium-glycerophosphate, 50 mM NaF, 1 mM Na3VO4) and an ATP regenerating system (40 mM creatine phosphate, 0.2 mg/ml creatine phosphokinase) were mixed with 0.5 – 1 μg of activated NDR1(M166G)/ hMOB complexes and 5 μCi [γ–^{32}P] N^6-(benzyl)-ATP in a final volume of 70 μl. After 30 min at 30°C reactions were stopped by the addition of 20 μl 5x SDS-PAGE loading buffer and boiling at 95°C for 5 min. Reactions were analyzed on a 12% SDS-PAGE gel and labeled bands were visualized by exposure to film or Phosphorimager screen.

Immunoprecipitation and Protein Phosphorylation on Immunocomplexes. For immunoprecipitation and phosphorylation, cells were treated with 1 μM OA for 45 min, harvested, pelleted at 1000 *g* for 3 min, and washed with cold PBS before lysis in low-stringency immunoprecipitation buffer (IP buffer) (20 mM Tris, 10% glycerol, 1% NP-40, 5 mM EDTA, 0.5 mM EGTA, 20 mM β-glycerophosphate, 50 mM NaF, 1 mM Na_3VO_4, 1 mM benzamidine, 4 μM leupeptin, 0.5 mM phenylmethylsulfonyl fluoride (PMSF), 1 μM microcystine and 1mM dithiothreitol (DTT) at pH 8.0) containing no or 50 mM NaCl. Lysates were centrifuged for 10 min at 16000 *g* at 4°C and split in two before preclearing with protein A (PrA)-Sepharose followed by immunoprecipitation with anti-HA 12CA5 antibody covalently coupled to PrA-sepharose. Beads were washed four times with IP buffer and two times in 20 mM Tris/HCl containing 1 mM benzamidine, 4 μM leupeptin, 0.5 mM PMSF and 1mM DTT at pH 7.5. Immunocomplexes were assayed by resuspention in 30 μl reaction buffer containing 1mM ATP and 5 μCi [γ–^{32}P] N^6-(benzyl)-ATP supplemented with or without 10 μM 1NM-PP1. After 30 min incubation at 30°C reactions were stopped by adding 5x SDS-PAGE loading buffer and boiling at 95°C for 5 min. Labeled bands were visualized by SDS-PAGE and exposure to a Phosphorimager screen.

3.2.5. References

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4. General Discussion

The aim of the thesis was to investigate the functional role of all human MOB proteins in the regulation of human NDR kinases. During the course of this study we were able to identify a novel role for hMOB2. In contrast to hMOB1A/B proteins, hMOB2 has endogenous inhibitory properties towards NDR1/2 kinases.

Furthermore, the basis for the identification of novel substrates of human NDR1/2 kinases was established. Applying a chemical genetic strategy to NDR1, we observed proteins specifically phosphorylated by NDR1(M166G) in immunocomplex kinase assays. In addition, while investigating the role of NDR in cell cycle regulation, we identified the first *in vivo* substrate of NDR1/2 kinases, the cyclin-dependent kinase inhibitor p21.

Differential regulation of NDR kinases by MOB proteins

MOB proteins are essential components of the NDR-MOB signaling modules across species [6]. Going from unicellular to multicellular species, there is a progressive expansion of the MOB family from two MOB proteins in yeast (Mob1p and Mob2p) to six MOB proteins in human (hMOB1A/B, hMOB2 and hMOB3A/B/C). We observed differential interactions with the human NDR kinase family NDR1/2 and LATS1/2. Three out of the six MOB proteins analyzed did not bind to or activate NDR1/2 or LATS1/2 kinases. Therefore, the binding partners of hMOB3 proteins remain to be identified. Furthermore, we could show that hMOB2 is a NDR1/2-specific binder of human NDR kinase family members. Interestingly, hMOB2 binds to the same region as shown for hMOB1A/B, the NTR, and we could for the first time show competitive binding of two MOB proteins. Moreover, hMOB2 was observed in an inactive complex with NDR1/2 and reduction of hMOB2 by

RNAi resulted in increased NDR kinase activity. In agreement with this finding, overexpression of hMOB2 impaired NDR function and activation in the context of FAS-mediated apoptosis and centrosome duplication. In summary, this data indicate a negative role for hMOB2 in NDR1/2 kinase regulation (Figure 5).

In yeast, the MOB proteins are essential for activity and function of NDR kinases and, in *Drosophila,* Mats/dMOB1 is essential for Wts activity and is required for Trc function [45-47]. Interestingly, one study already indicated that dMOB2 might act as an inhibitor of Trc since overxpression of a dMOB2 truncation mutant sharing high sequence similarity with hMOB2 resulted in a weak phenotype as observed in Trc-DN mutant flies [47]. In mammals, hMOB1A/B are established activators of human NDR1/2 kinases [8, 35, 36, 39, 113] and hMOB2 was previously shown to activate NDR1/2 *in vitro* [37, 49]. However, these studies lack a specific substrate for NDR and none of the effects were confirmed *in vivo* while our study included analysis using specific substrate, a phospho-specific antibody and investigated cellular functions of NDR1/2 [50]. Nevertheless, our study demonstrates that hMOB2 can act as a negative regulator. We speculate that in an overexpression setting hMOB2 out-competes endogenous hMOB1A/B leading to the accumulation of hMOB2-NDR1/2 complexes. These inactive complexes cannot be activated by upstream effectors such as MST1 since the activation by MST1 of NDR requires hMOB1A/B binding. Ultimately, the downstream

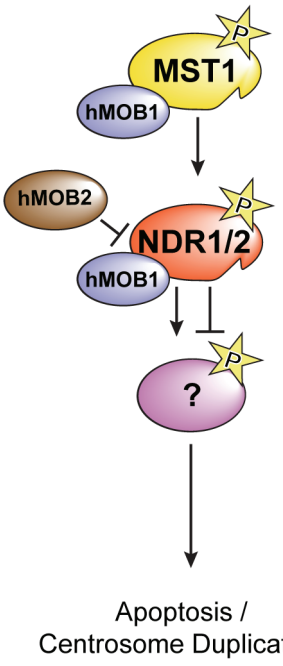

Figure 5. Model summarizing the role of hMOB2 in regulation of human NDR1/2 kinases. hMOB2 directly competes with hMOB1 proteins for NDR/1/2 binding and impairs hMOB1 and MST1-dependent activation and functions of NDR1/2 kinases in apoptosis and centrosome duplication. Arrowed or blunted ends indicate activation or inhibition, respectively.

signaling is inhibited resulting in impaired functions of NDR1/2 when hMOB2 is overexpressed (Figure 6A). Besides inhibition of NDR1/2 kinases via competition with hMOB1 proteins, it is also likely that binding of hMOB2 to the NTR of NDR kinases leads to a distinct conformational change in the kinase domain. Residues in the NTR essential for interaction with hMOB1 are known and binding the NTR stimulates autophosphorylation of NDR1/2 probably by release from autoinhibiton [8]. Since hMOB2 binds the NTR distinctly from hMOB1A/B, it is likely that hMOB2 induces an inactive conformation of the kinase domain of NDR1/2 kinases or induces autoinhibition by the AIS. Therefore, structural analysis of active hMOB1-NDR in comparison to inactive hMOB2-NDR complexes is highly recommended.

Based on our data and previously published work on NDR activation by hMOB1A/B, we propose the following model of subunit exchange during NDR activation (Figure 7B). We found that in cycling cells NDR1/2 is present in an inactive complex with hMOB2 and active complexes with hMOB1 proteins. Initially, hMOB1A/B, but not hMOB2, is phosphorylated by MST1/2 which increases its affinity towards NDR1/2 [51, 113]. Phosphorylated hMOB1A/B is then capable to replace hMOB2 from the inactive hMOB2-NDR1/2 complex thereby facilitating binding and phosphorylation of the hydrophobic motif by MST1 resulting in an active hMOB1A/B-NDR1/2 complex (Figure 6B). The large scaffold protein Furry might also be involved in governing the subunit exchange during NDR activation. In *Drosophila*, Furry is essential for Trc activity and functions [56, 61] and it was recently reported that hMOB2 and NDR1/2 interact with a mammalian Furry protein [37]. However, further work is needed to establish Furry as an essential contributor of NDR activation in the context of apoptosis and centrosome duplication.

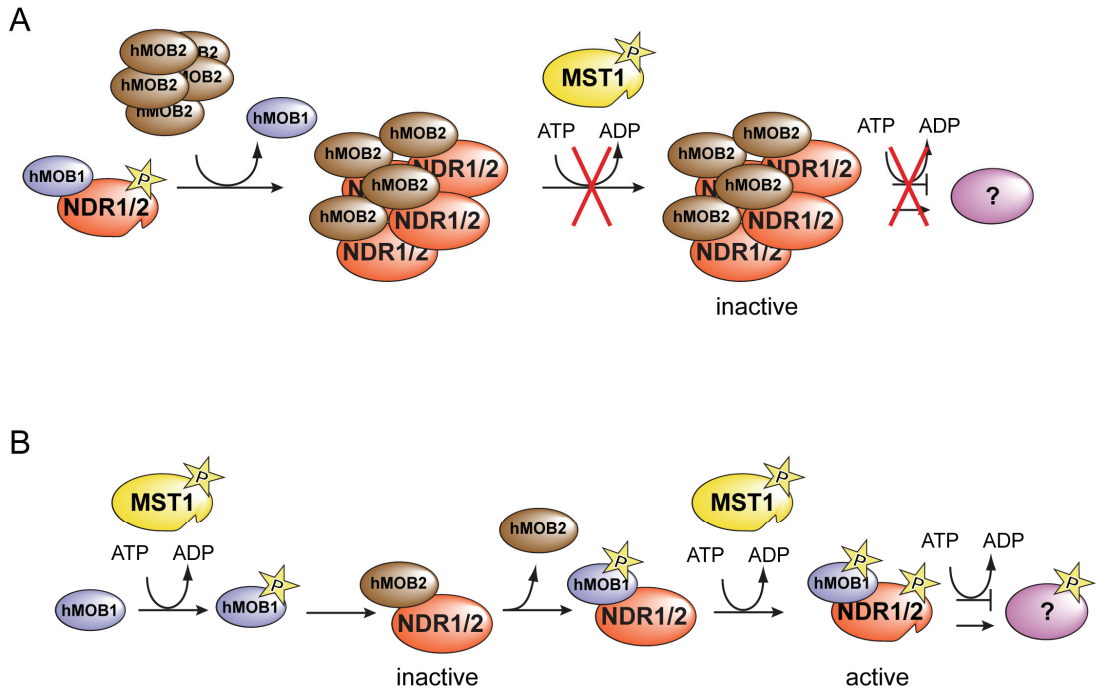

Figure 6. Model of the effect of overexpression of hMOB2 on NDR-hMOB1 signaling and of the role of hMOB2 in physiological NDR activation. (A) Overexpressed hMOB2 displaces hMOB1 from NDR1/2 kinases leading to the accumulation of inactive hMOB2-NDR complexes which cannot be activated by upstream kinases such as MST1. The signaling downstream of NDR and therefore the functions in apoptosis and centrosome duplication are impaired. (B) MST1 phosphorylates hMOB1 proteins thereby increasing affinity for interaction with human NDR1/2 and replacing hMOB2 from the inactive hMOB2-NDR complex. hMOB1-NDR complexes can then be activated by upstream kinases such as MST1 which leads to the phosphorylation of downstream targets and apoptotic progression and centrosome duplication.

Active kinases are usually targeted by the ubiquitin-proteasome system for degradation [114]. Therefore, hMOB2 might protect NDR kinases from degradation by competing with hMOB1A/B proteins for binding to NDR1/2 thereby sequestering NDR1/2 into an inactive complex. Indeed, we observed that hMOB2 depletion by RNAi leads to a decrease in total NDR protein and hMOB2 overexpression drastically stabilizes NDR1/2 (D. Schmitz, R. Kohler, unpublished observation). Hence, it is tempting to speculate that hMOB2 functions in controlling NDR protein levels. However, this observation requires further investigation. It is also quite peculiar that human NDR1/2 kinases are the only NDR family members that were shown to exist in functionally distinct complexes with different MOB proteins. Nevertheless, the reason why human NDR1/2 acquired this additional level of regulation in contrast to

LATS1/2 which restrictively bind to hMOB1A/B has yet to be investigated. Perhaps the hMOB2-NDR1/2 complex serves additional functions such as an adaptor-like or scaffold-like protein. We could also speculate that, under specific conditions, even this complex can be activated and is required for a yet unknown function of human NDR kinases especially since the related MOB2p proteins are essential for the activity and functions of NDR kinases in yeast. Therefore, genetically modified mice harboring targeted deletion or overexpression of mammalian MOB2 would complement ongoing research in tissue cultured cells in order to decipher the physiological roles of MOB2 proteins in mammals.

The future of human NDR kinase substrates identification

Recently, substrates for almost all NDR kinase family members from yeast to men were discovered (Table 2). However, most substrates, if not all, were identified by a candidate substrate approach via which a protein implicated in the same process is tested as a direct substrate *in vitro* and then verified *in vivo* [112]. To date, no unbiased approach to discover novel substrates was reported to be applied on NDR kinases. Although analog-sensitive alleles of yeast NDR kinases (Cbk1p-as and Orb6p-as) have been established, these strains have not yet been used to discover novel substrates [43, 70] as reported for other analog-sensitive yeast kinases [115-117]. Therefore, we aimed to identify targets of human NDR1/2 kinases by creation of an analog-sensitive NDR based on the chemical genetic approach developed by Shokat and colleagues [118].

Analogous to how novel Cdk7 substrates were identified [119] we established a recombinant source of highly active analog-sensitive NDR1(M166G)-hMOB1A complexes. However, when we performed kinase assays on cellular fractions in the

presence of radiolabeled analog-ATP we did not observe any specifically labeled bands. Hence, we adopted another method using analog-sensitive NDR1(M166G) previously used to identify ERK2 substrates [120]. By using immunoprecipitated NDR1(M166G) and subsequent *in vitro* kinase assays on immunocomplexes we observed for the first time a reproducible pattern of labeled bands responsive to the addition of the ortholog inhibitor 1NM-PP1. Therefore, we established a basis for the identification of novel substrates of human NDR kinases using a chemical genetic approach which should be followed up by mass spectrometric analysis of phosphoproteins.

More recently, we identified p21 as the first *in vivo* substrate of human NDR1/2 kinases (see Appendix and Table 2). NDR1/2 phosphorylate p21 on Ser146, a residue previously shown to have a destabilizing effect on p21 [121], resulting in NDR-mediated degradation of p21. As hypothesized earlier and similar to the peptide substrate of NDR1/2 [21], the phosphorylation site Ser 146 is preceded by four positively charged residues: $RKRRxxS^{146}$. Does the identification of the first downstream target of human NDR1/2 impede the unbiased search for novel substrates? The discovery of p21 as an *in vivo* substrate might prove immensely valuable for further development and improvement of a chemical genetic approach. Significantly, known substrates serve as essential positive controls when novel methods are applied to protein kinases [115, 119, 122]. Thus, having p21 as a direct target, we are now able to validate the functionality of the analog-sensitive NDR1(M166G) in our assays. Furthermore, one should try to find phosphorylated p21 in the established immunocomplex assays by a second immunoprecipitation step or by direct immunoblotting with antibodies directed against p21 and p21-pSer146. Furthermore, the knowledge of a phosphorylation motif for human NDR1/2,

RKRRxxS in p21, can complement the search for substrates via a chemical genetic approach. Putative interactors identified by the immunocomplex assay can be scanned for a similar motif and tested *in vitro*. Therefore, the discovery of p21 as a first substrate might actually accelerate the identification of novel targets.

In addition, the availability of MEFs lacking both NDR1 and NDR2 can also facilitate substrate identification. By complementation of NDR1/2 null MEFs with an analog-sensitive NDR variant, one will be able to apply total cell phosphoproteomics to cells treated with or without the orthogonal inhibitor 1NM-PP1 for instance upon FAS-mediated apoptosis, therefore identifying putative downstream effectors of NDR in its role in apoptotic progression.

Overall, with the identification of the first *in vivo* substrate and the development of an unbiased approach for substrate identification, we have now the basis to dissect the downstream signaling of mammalian NDR kinases. Importantly, uncovering the targets of NDR1/2 signaling will help to gain more detailed understanding of the functions of NDR in tumor suppression, apoptosis and centrosome duplication.

5. References

(This section contains the references cited in the introduction and the general discussion. References that are important for the results part can be found in the respective results section or manuscript.)

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6. Appendix

This section contains two additional manuscripts which I have contributed to during the course of the thesis. The manuscripts contain work on NDR kinases in the context of cell cycle regulation and centrosome duplication which is discussed in the main text and put in relation to the work obtained during this thesis.

The first manuscript describes a novel role of human NDR1/2 in regulation of G1/S cell cycle progression. Work on this manuscript lead to the identification of the first *in vivo* substrate of human NDR kinases, the cyclin-dependent kinase inhibitor p21. This result is intensively discussed in the main text of this thesis. Further, this study was a close collaboration between Hauke Cornils and me which resulted in co-first authorship.

The second manuscript describes the essential role of hMOB1 and MST1 proteins regulating NDR kinase phosphorylation in the context of centrosome duplication. This study showed that binding to hMOB1 is crucial for activation of NDR kinases and subsequent centrosome duplication. This result was incorporated and discussed in the manuscript in which we describe a negative role for hMOB2 in NDR kinase regulation.

6.1. Human NDR Kinases Control G1/S Cell Cycle Transition by Directly Regulating p21 Stability

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Hauke Cornils*, Reto S. Kohler*, Alexander Hergovich and Brian A. Hemmings

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Human NDR Kinases Control G_1/S Cell Cycle Transition by Directly Regulating p21 Stability▽

Hauke Cornils,†* Reto S. Kohler,† Alexander Hergovich,‡ and Brian A. Hemmings*

Friedrich Miescher Institute for Biomedical Research, Maulbeerstrasse 66, 4058 Basel, Switzerland

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The G_1 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. Multiple protein kinases, among them the cyclin-dependent kinases (Cdks), control G_1-phase progression and S-phase entry. With the regulation of apoptosis, centrosome duplication, and mitotic chromosome alignment downstream of the HIPPO pathway components MST1 and MST2, mammalian NDR kinases have been implicated to function in cell cycle-dependent processes. Although they are well characterized in terms of biochemical regulation and upstream signaling pathways, signaling mechanisms downstream of mammalian NDR kinases remain largely unknown. We identify here a role for human NDR in regulating the G_1/S transition. In G_1 phase, NDR kinases are activated by a third MST kinase (MST3). Significantly, interfering with NDR and MST3 kinase expression results in G_1 arrest and subsequent proliferation defects. Furthermore, we describe the first downstream signaling mechanisms by which NDR kinases regulate cell cycle progression. Our findings suggest that NDR kinases control protein stability of the cyclin-Cdk inhibitor protein p21 by direct phosphorylation. These findings establish a novel MST3-NDR-p21 axis as an important regulator of G_1/S progression of mammalian cells.

The G_1 phase of the cell cycle is a crucial integrator of internal and external cues, allowing cells to grow, process outside information, or repair damage before entering S phase (32). Entry into S phase is mediated by the action of cyclin-dependent kinases (Cdk) complexed with their respective cyclin subunits. Initially cyclin D-Cdk4/6 and later cyclin E-Cdk2 complexes phosphorylate the retinoblastoma (Rb) tumor suppressor protein, allowing dissociation of Rb from E2F transcription factors and subsequent transcription of genes required for S phase entry (17). The activity of Cdks is controlled on multiple levels (44). The association of Cdks with cyclin subunits is a prerequisite for Cdk activation. This process is initially controlled by the availability of the cyclin subunit, whose abundance is regulated by both transcriptional and post-transcriptional processes (44). Furthermore, cyclin-Cdk inhibitor (CKI) proteins of the Cip/Kip (e.g., p21 and p27) and INK4 (e.g., p16) families control cyclin-Cdk activity by different mechanisms. Cip/Kip proteins associate with and inhibit cyclin E-Cdk2 complexes, and INK4 proteins inhibit cyclin D-dependent Cdks by sequestering Cdk4/6 into binary Cdk-INK4 complexes, thereby blocking assembly of active cyclin D-Cdk4/6 complexes. Multiple signaling pathways have been shown to directly or indirectly affect the activity of cyclin-Cdk complexes, thereby controlling the G_1/S transition. Since the correct regulation of the G_1/S transition is essential for mammalian cells, much research has been invested in understanding this process. However, investigations of the complex regulation of Cdk activity are still needed.

Members of the nuclear-Dbf2-related (NDR) family of Ser/Thr kinases are highly conserved from yeast to human and have been implicated in the regulation of a variety of biological processes (24). NDR family kinases in *Saccharomyces cerevisiae* have distinct roles in the regulation of mitotic exit by Dbf2p (38, 51) and the control of polarized cell growth by Cbk1p (3, 53). Similarly, in *Schizosaccharomyces pombe*, Sid2p has a role in cytokinesis (16) and Orb6p functions in cell polarity and morphogenesis (11, 25). In *Drosophila melanogaster* the roles of NDR kinases also differ substantially. Warts regulates cell proliferation and apoptosis (26), and tricornered regulates cell morphogenesis and dendritic tiling (13, 18). These findings indicate that two distinct branches of NDR signaling exist across species. Nevertheless, in a subset of those functions NDR kinases in yeast and flies can function cooperatively (14, 40). With the regulation of mitotic exit, cell growth, proliferation, centrosome duplication, and morphogenesis, NDR family kinases across species have been shown to function in processes tightly linked to the cell cycle (24). The human genome encodes for four different NDR kinase family members, NDR1/2 and LATS1/2 (20). The kinases LATS1/2 function as part of the HIPPO pathway controlling the localization and function of the YAP oncogene (56). Furthermore, roles for LATS1 and LATS2 in controlling mitotic exit and genomic stability have been described (4, 34). Although they are well characterized in terms of biochemical regulation, functions for the other two NDR family kinases in the human genome, NDR1 and NDR2, have only recently started to be unraveled. In cellular systems, NDR kinases have been implicated in the regulation of centrosome duplication, apoptosis, and the alignment of mitotic chromosomes (7, 23, 50). Furthermore, a recent study indicated a tumor-suppressive function, by controlling proper apoptotic responses, for NDR1/2 in

* Corresponding author. Mailing address: Friedrich Miescher Institute for Biomedical Research, Maulbeerstrasse 66, CH-4058 Basel, Switzerland. Phone: 41 61 6974872. Fax: 41 61 6973976. E-mail for Hauke Cornils: hauke.cornils@fmi.ch. E-mail for Brian A. Hemmings: brian.hemmings@fmi.ch.

† These authors contributed equally to this work.

‡ Present address: UCL Cancer Institute, University College London, London WC1E 6BT, United Kingdom.

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mice (10). NDR1/2 activity is regulated by phosphorylation of the hydrophobic motif (HM) through the mammalian Ste20-like kinases MST1, MST2, and MST3 (7, 22, 47, 50). Whereas NDR kinase activation during apoptosis and centrosome duplication is mediated by MST1 (22, 50), MST2 regulates NDR in the context of mitotic chromosome alignment (7). However, the functional context of NDR kinase activation by MST3 has not been reported so far. Furthermore, although functions and regulators of NDR1/2 were defined recently, downstream signaling remained elusive. Here we addressed NDR1/2 activation throughout the cell cycle. We show that NDR1/2 are selectively activated in G_1 phase by MST3, establishing the first functional context for NDR kinase regulation by MST3. More importantly, with the direct regulation of p21 stability by phosphorylation on Ser 146, we define here the first downstream signaling mechanisms by which NDR kinases can control G_1/S progression.

MATERIALS AND METHODS

Construction of plasmids. The construction of plasmids encoding cDNAs and retroviral constructs for tagged variants of NDR1, NDR2, MST1, MST2, and MST3 has been described elsewhere (19, 22, 50). RNA interference (RNAi) rescue constructs for NDR2 were obtained by introducing silent mutations into the short hairpin RNA (shRNA) target sites using PCR mutagenesis. For constructs expressing cDNAs fused to an internal ribosome entry site-green fluorescent protein (IRES-GFP), the IRES-GFP cassette was excised from the pMIG-vector 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, France) 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, Switzerland), and hemagglutinin (HA)-tagged c-myc was obtained similarly 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 PCR mutagenesis. Primer sequences are available upon request. Vectors encoding cDNA for FBW7 or ubiquitin (Ub) were kind gifts from B. Clurman (Fred Hutchinson Cancer Research Center, Seattle, WA) and W. Filipowicz (Friedrich Miescher Institute, Basel, Switzerland). For production of recombinant protein, kinase-dead NDR1 (NDR1kd) (K118R) cDNA was cloned in the pMal-C2 vector.

Cell culture, transfections, and treatments. All cell lines used in this study were maintained in Dulbecco modified Eagle medium (DMEM) supplemented with 10% fetal calf serum (FCS). Cells were transfected using Fugene 6 (Roche), Lipofectamine 2000 (Invitrogen), or jetPEI (Polyplus Transfection) as described by the manufacturer. For small interfering RNA (siRNA)-mediated knockdown of MST1, MST2, MST3, or p21, cells were transfected with predesigned siRNA (Qiagen) using Lipofectamine 2000. For si_p21-mediated rescue experiments, cells were transfected twice at 24-h intervals. HeLa cells expressing tetracycline (TET)-inducible shRNA against NDR1 and NDR2 and U2OS cells stably expressing shRNA against NDR1 together with a wild-type NDR1 (NDR1wt) rescue construct have been described elsewhere (23, 50). HeLa and U2OS cells stably expressing shRNA against NDR1 or NDR2 alone were generated as described previously (23, 50). To determine protein stability, cells were treated with 50 μg/ml cycloheximide (CHX) or 10 μM MG132.

Reagents and antibodies. The generation of antibodies against T444-P, NDR1, NDR2, and NDR1/2 has been described previously (50). 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, MST1, MST2, MST3, and myc-tagged proteins (71D10) were from Cell Signaling. Antibodies against HA tag (12CA5 and 42F13), tubulin (YL1/2), and c-myc (9E10) were used as hybridoma supernatants. Additional antibodies used included anti-p21-pS146 (Abgent), anti-P-MST4-T178/-MST3-T190/-STK25-T174 (referred to as P-MST3) (Epitomics), anti-MST3 (BD Biosciences), and anti-FLAG (M2) (Sigma). Nocodazole, thymidine, propidium iodide (PI), and cycloheximide were from Sigma. Okadaic acid (OA), SB203580, and SB202190 were from Alexis (Enzo Life Sciences). MG132 was from Calbiotech. Bromodeoxyuridine (BrdU) and anti-BrdU antibody were from BD Biosciences.

Protein extraction, immunoprecipitation, immunoblotting, and ubiquitination analysis. Protein extraction from cultured cells, immunoprecipitation, and immunoblotting were done as described previously (19). The following antibodies were used for immunoprecipitation: anti-HA (12CA5), anti-c-myc (9E10 and N262), anti-p21, anti-MST3, and a mixture of NDR1- and NDR2-specific antibodies to assess endogenous NDR species. For quantification using the Li-Cor Odyssey system, Western blots were incubated with secondary antibodies conjugated with fluorescent dyes. Quantifications were carried out using the Li-Cor Odyssey software. Analysis of c-myc ubiquitination was performed as described previously (41).

Cell cycle analysis. HeLa and HeLa S3 cells were synchronized using either a double thymidine block with subsequent nocodazole arrest and mitotic shake-off (48) or a single treatment with 100 ng/ml nocodazole for 14 h. Cells were washed free of nocodazole with ice-cold phosphate-buffered saline (PBS) and released into fresh medium for the indicated times before harvesting. Cell cycle distribution was assessed using either BrdU labeling as described by the manufacturer or PI staining as described previously (23). To detect cells blocked in G_1, a method described by Mikule et al. was used (35). In short, cells were seeded at defined densities into 10-cm dishes, and 24 h later 2.5 μg/ml nocodazole was added and left for 14 to 16 h to terminally arrest cells at the G_2/M border. Cells were harvested by trypsinization and processed for fluorescence-activated cell sorter (FACS) analysis.

Kinase assays. Methods to determine the activity of endogenous NDR kinases have been described earlier (50). To assay p21 phosphorylation by NDR1/2 *in vitro*, HEK293 cells were transfected with cDNAs encoding HA-tagged NDR kinase isoforms and mutants. Cells were stimulated with 1 μM okadaic acid for 60 h prior to lysis and immunoprecipitation. *In vitro* kinase assays using purified glutathione *S*-transferase (GST)-tagged p21 isoforms were performed as described previously (19) with minor modifications. Before addition of [γ-^{32}P]ATP and GST-p21, the immunoprecipitated kinases were preincubated for 90 min at 30°C in reaction buffer without ^{32}P-labeled ATP. The labeling reaction was stopped after 60 min by boiling the samples in sample buffer for 5 min at 95°C. Samples were resolved on SDS-PAGE, stained with Coomassie blue, and exposed to a phosphorimager (Amersham Biosciences). Kinase activity of endogenous MST3 species was assessed by immunoprecipitation of endogenous MST3 and by using recombinant maltose binding protein (MBP)-tagged NDR1 K118R as a substrate in an *in vitro* kinase assay as described earlier (47).

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 times, cells were harvested by trypsinization and counted using a ViCell-XR automated cell counter (Beckman Coulter). The decrease in proliferation of HeLa shNDR1/2 cells with or without TET was calculated using the formula $y = 100 \times$ (cell count on day 6 with TET)/(cell count on day 6 without TET). The result represents the mean of triplicates for three different clones.

RNA isolation, quantitative real-time PCR, and luciferase assays. Total RNA from cells was isolated with TRIzol reagent (Invitrogen) and further purified using an RNeasy kit (Qiagen). cDNA was generated from 2 μg of total RNA using Moloney murine leukemia virus (M-MuLV) reverse transcriptase (NEB) and oligo(dT) primers. Quantitative RT-PCR to detect p21, p27, and c-myc (primer sequences are available upon request) was carried out using SYBR green technology in an ABI Prism 7000 detection system (Applied Biosystems). Luciferase assays using the wild-type (LDH-WT) or E-box-mutated (LDH-MT) version of the LDA-H promoter (a kind gift from C. V. Dang, Johns Hopkins University, Baltimore, MD) (45) were performed using the dual-luciferase reporter assay from Promega.

Statistical analysis. Statistical analyses were performed with Student's *t* test for the comparison between two samples.

RESULTS

NDR kinases are activated in G_1 by MST3. Mammalian NDR kinases are implicated in the regulation of cell cycle-dependent processes such as centrosome duplication and the alignment of mitotic chromosomes (7, 22). To better define the cell cycle function(s) of NDR kinases, we analyzed NDR kinase activity during cell cycle progression. Hydrophobic motif (HM) phosphorylation of NDR1 and NDR2, as an indicator of NDR kinase activity (47), was nearly absent in M phase, in-

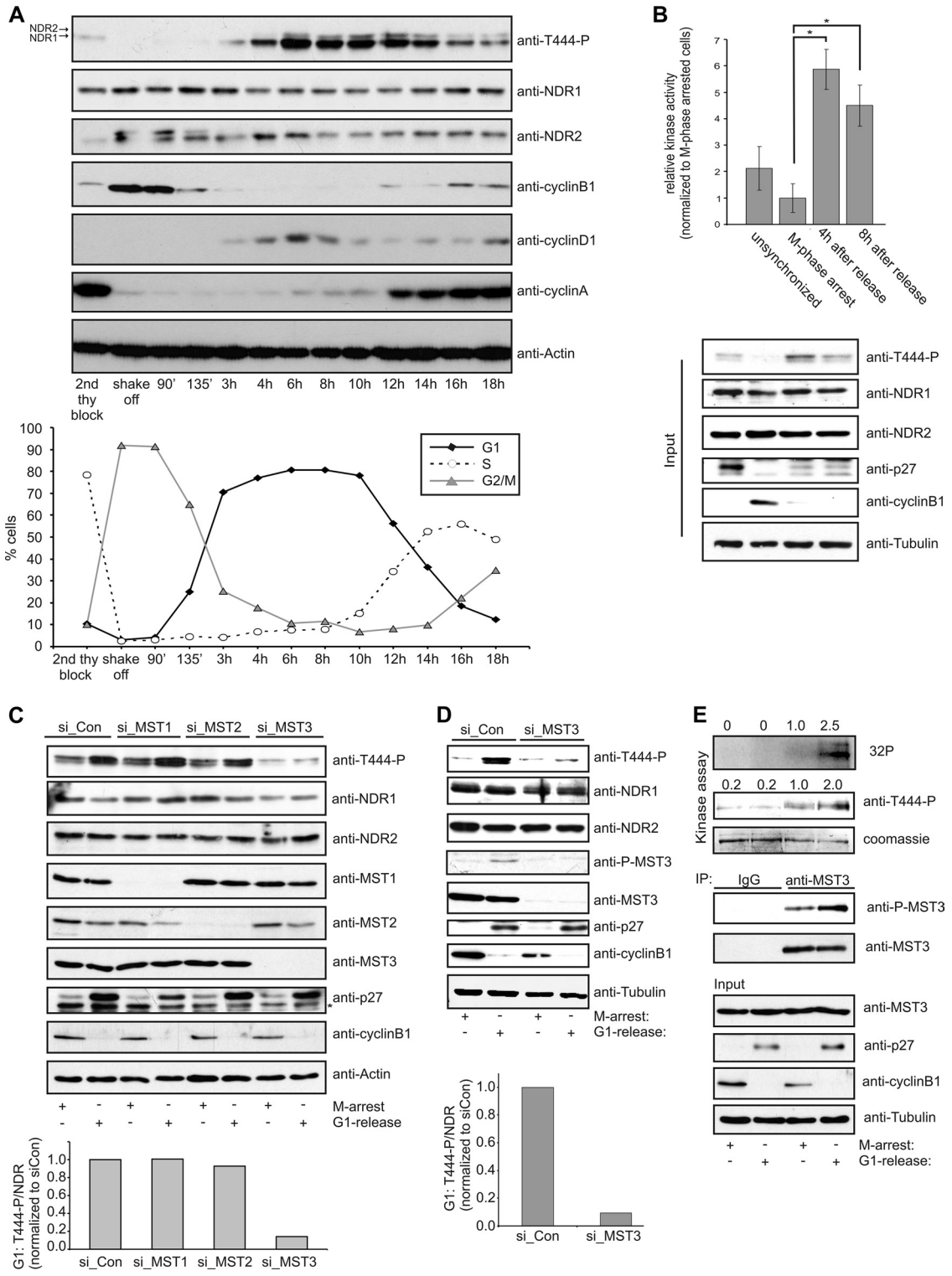

FIG. 1. NDR kinases are activated by MST3 in the G_1 phase of the cell cycle. (A) NDR kinases are activated in a cell cycle-dependent manner. Synchronized HeLa S3 cells were harvested after mitotic shake-off and replated in fresh medium for the indicated times. Activation of NDR1/2 was assessed using anti-T444-P, -NDR1, and -NDR2 antibodies. Cell cycle distribution was assessed using propidium iodide (PI) staining and FACS analysis. (B) Endogenous NDR kinase activity is increased in G_1 phase. HeLa cells were arrested at the G_2/M border using nocodazole treatment for 14 h and released for the indicated times before harvesting. Lysates were subjected to immunoblotting and immunoprecipitation of endogenous NDR species using a mixture of isoform-specific antibodies. NDR kinase activity was assessed using peptide kinase assays ($n = 3$; $P < 0.002$). (C) Depletion of MST1/2/3 kinases using isoform-specific siRNAs. HeLa cells were transfected with control siRNA (si_Con) or siRNAs targeting MST1/2/3 kinases (si_MST1, si_MST2, and si_MST3) and 48 h later were arrested with nocodazole for 14 h. Arrested cells were harvested or released into G_1 for 8 h before harvesting. NDR activation was assessed using T444-P antibody. Cell cycle phases were confirmed by analyzing

creased 3 h after mitotic shake-off upon entry into G_1 phase, and peaked at around 6 to 8 h in G_1 phase. Activation of NDR persisted into S phase (12 to 14 h) and started to decrease 14 h after shake-off (Fig. 1A). Analysis of cell cycle markers by immunoblotting and FACS staining of the cell population (visualized by a plotted graph) confirmed that NDR activation peaked in G_1 phase with the activation persisting into S phase. G_1 activation of NDR1/2 was confirmed by analyzing endogenous NDR1/2 activity using a peptide kinase assay (Fig. 1B). Since three members of the mammalian Ste20-like kinases (MST1/2/3) can regulate NDR kinases by phosphorylation of the HM of NDR (7, 22, 47, 50), we tested which MST kinase was important for NDR1/2 activation in G_1. To this end, we analyzed NDR phosphorylation in G_1 upon siRNA-mediated knockdown of MST1 to -3 (Fig. 1C). Although depletion of MST1 and MST2 hardly affected NDR activation, knockdown of MST3 expression significantly reduced NDR phosphorylation in this setting. This finding was confirmed using overexpression of dominant negative (DN) variants of MST1 to -3 (data not shown), suggesting that MST3 is the main upstream kinase in this cell cycle phase. Interestingly, we observed an increase in phosphorylated MST3 in G_1-phase cells versus M-phase-arrested cells, indicating that MST3 activity is increased in G_1 phase of the cell cycle (Fig. 1D). We then addressed whether increased MST3 phosphorylation reflected an increase in kinase activity. Endogenous MST3 kinase was immunoprecipitated in cells arrested in M phase or released into G_1 phase. The increase in phosphorylation of MST3 in G_1 was paralleled by approximately 2-fold-increased kinase activity when an *in vitro* kinase assay on kinase-dead NDR1 (MBP-NDR1 K118R) was performed (Fig. 1E), therefore demonstrating that kinase activity of MST3 is increased in G_1. Collectively, these results revealed that NDR kinases were activated in G_1 phase of the cell cycle, with the activation persisting into S phase. Furthermore, our experiments revealed MST3 as the responsible upstream kinase for NDR1/2 in this setting, thereby providing the first functional link between NDR1/2 and MST3.

Depletion of NDR kinases results in G_1 arrest and subsequent proliferation defects. To analyze whether the activation of NDR kinases contributed to cell cycle progression and proliferation, we generated HeLa cells expressing inducible shRNA against NDR1 and NDR2 (Fig. 2A). Both isoforms were simultaneously targeted to avoid any compensatory effects as described earlier for NDR1-deficient mice (10). Knockdown of NDR kinases consistently resulted in decreased proliferation of around 50%, which was not observed in control clones expressing shRNA against firefly luciferase (Fig. 2B and C). Single knockdown of NDR kinase isoforms also resulted in a significant decrease in proliferation (data not shown). Accordingly, depletion of MST3 by RNAi resulted in reduced proliferation similar to that after knockdown of NDR1/2 (Fig. 2D). Reduced proliferation in NDR-depleted cells was accompanied by an increase in cells in G_1 and a decrease in cells in S phase (Fig. 2E). G_1 phase arrest was confirmed by treating cells with nocodazole to accumulate cycling cells at the G_2/M border (Fig. 2F). Briefly, at 3 days after induction of shRNA expression, the cells were treated with nocodazole to depolymerize microtubules, activating the spindle assembly checkpoint and arrest cells at the G_2/M border. (35). Therefore, cells blocked in G_1 will not proceed to G_2/M border, and as was apparent, cells depleted of NDR1/2 were retained in the G_1 peak (Fig. 2F, +TET). Since depletion of NDR can result in centrosome defects (23), we asked next whether the G_1 arrest was due to activation of the p38-p53 centrosome integrity checkpoint (35, 46). NDR1/2-depleted cells were treated with p38 inhibitors (SB203580 and SB202190) prior to G_1 arrest assessment (Fig. 2G), revealing that inhibition of p38 had no detectable effect. These results suggest that p38-p53 signaling does not contribute to the cell cycle arrest upon NDR1/2 depletion. Therefore, we analyzed the mechanisms underlying the G_1 block by investigating expression levels of other known G_1/S regulators (Fig. 3A). Interestingly, the expression of p21 and p27 was elevated in NDR1/2 knockdown cells without a significant decrease in the expression of cyclins and Cdks (Fig. 3A). In addition, the expression of the c-myc proto-oncogene was reduced. These results were confirmed in HeLa cells expressing shRNA against NDR1 and NDR2 alone, as well as in transiently transfected HCT116 cells (data not shown). Furthermore, stable expression of shRNA-resistant NDR1wt in U2-OS cells counteracted p21 and p27 upregulation and restored cell proliferation (data not shown). This suggested that the observed G_1 block upon depletion of NDR1/2 might be due to the inhibition of cyclin-Cdk complexes by increased levels of p21 and p27. In addition, we analyzed the effects of MST3 depletion on p21, p27, and c-myc levels (Fig. 3B). As found upon NDR depletion, we also observed upregulation of p21 and p27, while c-myc levels were reduced in MST3-depleted cells (Fig. 3B). These experiments suggest the existence of an MST3-NDR axis as regulator of cell proliferation.

It has been shown that c-myc is able to repress p21 and p27 expression (9, 54). Therefore, we tested whether depletion of NDR1/2 would result in increased expression of p21 and p27 mRNAs. Strikingly, although p27 mRNA levels were clearly increased, we did not observe any elevation of p21 mRNA in our settings (Fig. 3C). In addition, NDR depletion did not affect c-myc mRNA expression, suggesting that NDR kinases

cyclin B1 and p27 expression (*, unspecific band). Phospho-T444 levels after G_1 release were compared to those in control samples and analyzed using the Li-Cor Odyssey system. (D) Reduction of MST3 impairs G_1 activation of NDR. HeLa cells were transfected with control siRNA (si_Con) or siRNA against MST3 (si_MST3) and treated and analyzed as described for panel C. MST3 activation was assessed 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. (E) Kinase activity of MST3 is increased in the G_1 phase of the cell cycle. HeLa cells were arrested at the G_2/M border using nocodazole treatment for 14 h and released for 8 h before harvesting. Lysates were subjected to immunoblotting and in parallel to immunoprecipitation (IP) of endogenous MST3 species using anti-MST3 or control antibodies. Endogenous MST3 kinase activity was assessed by an *in vitro* kinase assay using recombinant MBP-NDR1 K118R as a substrate. Reactions were analyzed by SDS-PAGE followed by immunoblotting against phosphorylation of Thr 444 in NDR1 and in parallel by exposure to a phosphorimager screen. NDR phosphorylation was quantified and normalized to the activity of MST3 in M phase.

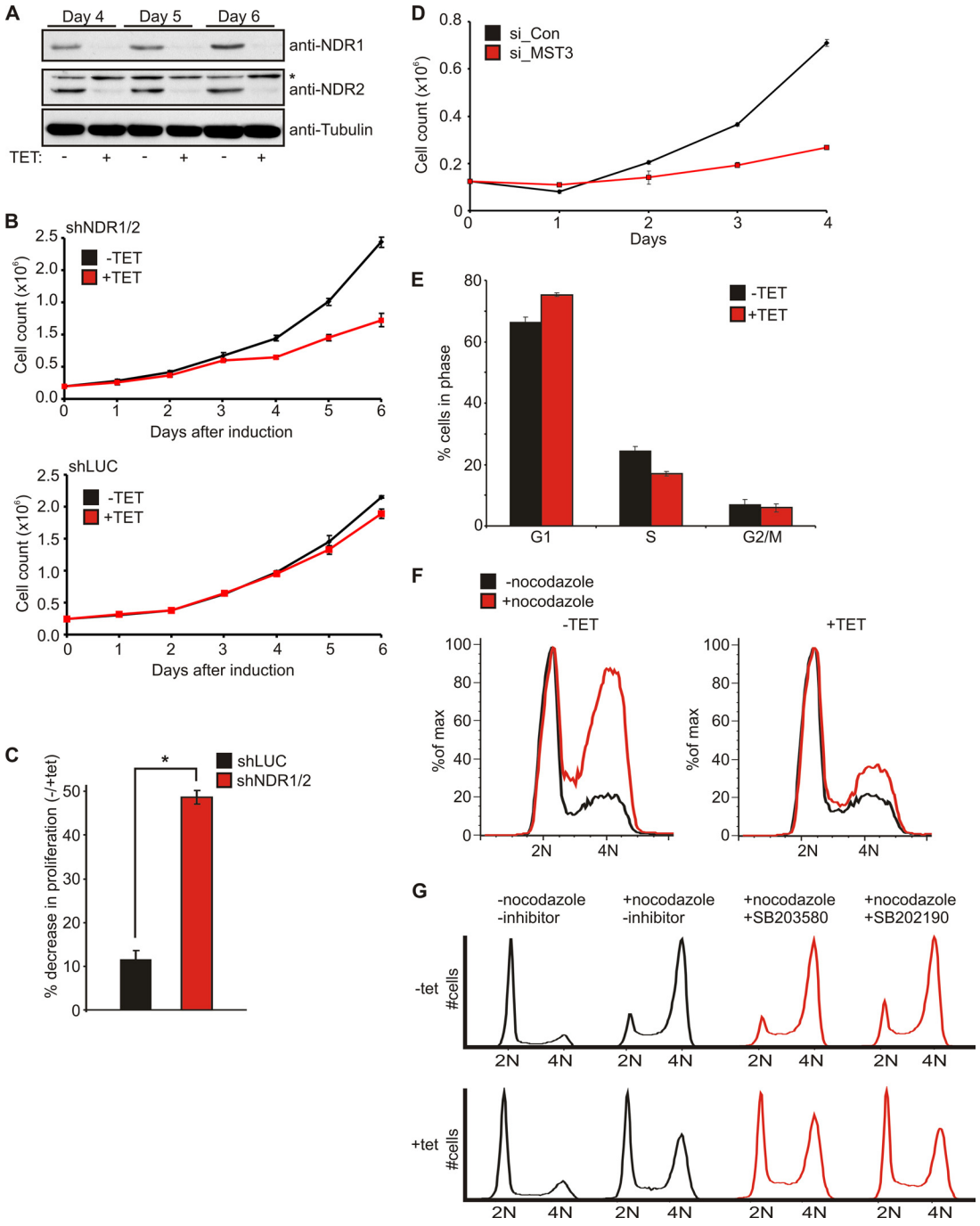

FIG. 2. shRNA-mediated knockdown of NDR1/2 results in cellular proliferation defects due to a G_1 block. (A) Characterization of T-Rex-HeLa cells stably expressing shRNA against NDR1 and NDR2. Cells were seeded in 10-cm dishes, and shRNA expression was induced by the addition of tetracycline (TET) for the indicated times. Lysates from harvested cells were analyzed for NDR1 and NDR2 expression using isoform-specific antibodies (*, unspecific band). (B) NDR1/2 depletion results in proliferation defects. 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 times, cells were harvested by trypsinization and counted using a ViCell automated cell counter. (C) Validation of proliferation defects in different clones stably expressing shNDR1/2 or shLUC ($n = 3$; $P < 0.001$). Experiments were performed as for panel B, and differences in proliferation were calculated as the percentage of cells without tetracycline to cells with tetracycline counted on day 6 after induction of shRNA expression. (D) Depletion of MST3 results in proliferation defects similar to those observed in NDR-depleted cells. HeLa cells were transfected with control siRNA (si_Con) or siRNA against MST3 (si_MST3); 24 h later, cells were seeded at defined densities in triplicates and cell counts were analyzed as for panel B. (E) Depletion of NDR1 and NDR2 results in an increase in G_1 phase cells accompanied by a decrease in S phase cells. HeLa cells expressing shRNA against NDR1/2 were induced for 4 days with tetracycline. BrdU was added directly to the cell medium and left for 30 min before harvesting and processing for FACS analysis ($n = 3$). (F) Depletion of NDR1/2 results in G_1 arrest. Knockdown of NDR1/2 was induced for 4 days using tetracycline. At 14 h before harvesting and processing for FACS analysis, cells were treated with 2.5 μg/ml nocodazole to induce G_2/M accumulation. Fixed cells were stained with PI and analyzed by FACS. Histograms were overlaid to allow better comparison of cells in a given cell cycle phase. (G) Treatment of NDR1/2-depleted cells with inhibitors against p38 does not suppress G_1 arrest. HeLa cells expressing shRNA against NDR1/2 were induced for 4 days with tetracycline. At 24 h before analysis, SB203580 or SB202190 (10 μM final concentration) was added to the cells. G_1 arrest analysis was performed as for panel F.

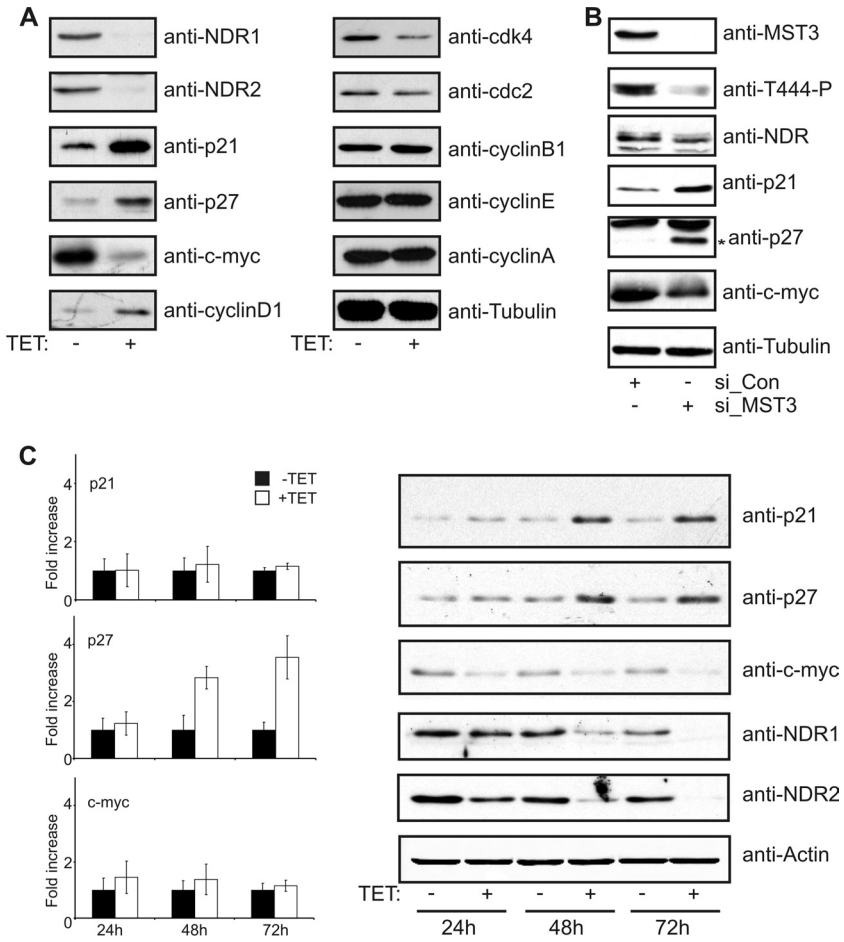

FIG. 3. Depletion of NDR1/2 kinases results in an increase in p21 and p27 protein levels and a decrease in c-myc protein level. (A) Reduced NDR kinase levels increase p21 and p27 and decrease c-myc. Knockdown of NDR1/2 was induced for 4 days, and cell lysates were analyzed for the expression of the indicated cell cycle regulators using Western blotting. (B) Depletion of MST3 increases p21 and p27 and decreases c-myc similarly to knockdown of NDR kinases. HeLa cells were transfected with control siRNA (si_Con) or siRNA against MST3 (si_MST3). After 3 days, cell lysates were analyzed by Western blotting (*, p27). (C) NDR1/2 depletion results in increased p27 mRNA levels, whereas changes in c-myc and p21 are observed only at the protein level. Knockdown of NDR1/2 was induced for the indicated times, and RNA extracts were prepared to analyze p21, p27, and c-myc mRNAs by quantitative RT-PCR. Values for p21, p27, and c-myc mRNAs are given as fold change relative to untreated samples ($n = 3$). In parallel, the samples were analyzed for the levels of the indicated proteins by Western blotting.

regulated p21 and c-myc protein levels posttranscriptionally (Fig. 3C).

NDR kinases regulate c-myc protein levels by direct binding and interfering with FBW7-mediated ubiquitination. A recent report analyzing posttranscriptional modifiers of c-myc in human B cells proposed that NDR1 could regulate c-myc protein stability (52). In full agreement with this previous report, c-myc protein levels were rescued by the addition of the proteasomal inhibitor MG132 in NDR1/2-depleted HeLa cells (data not shown). In addition, we could confirm that NDR1 and c-myc interacted on both overexpressed and endogenous levels (data not shown). Furthermore, NDR2 bound to c-myc with similar affinity as NDR1 (data not shown). Next, we analyzed the currently unknown determinants for NDR binding to c-myc. Using coimmunoprecipitation experiments, we found that c-myc interacted mainly with the N-terminal region (NTR) (residues 1 to 82) of NDR1 (Fig. 4A). In addition, interaction was shown to be modulated by HM phosphorylation (Thr 444) (Fig. 4B). Both the NTR and the HM phosphorylation site have been shown to be essential for full kinase activity of NDR (2, 36, 47), suggesting that NDR might bind to c-myc in an active conformation. Nevertheless, kinase-dead NDR1 (NDR1kd) associated with c-myc similarly to NDR1wt (Fig. 4B). Both NDR1wt and NDR1kd significantly stabilized c-myc levels (Fig. 4C). NDR1 mutants defective in or with reduced c-myc interaction (NDR1-ΔNTR and NDR1-TA) had minor effects on c-myc stability, indicating that binding of NDR1 to c-myc is required to stabilize c-myc levels. Furthermore, increasing HM phosphorylation of overexpressed NDR by coexpression of MST3 increased complex formation and c-myc stability (Fig. 4D and data not shown). The effects of overexpression and HM phosphorylation of NDR1 on endogenous c-myc levels were also tested. Strikingly, overexpression of NDR1wt and NDR1kd increased endogenous c-myc levels, which could be further increased by stimulating HM phosphorylation through coexpression of MST3 (data not shown). Besides stabilizing c-myc protein levels, NDR1wt but not NDR1TA (T444A) overexpression stimulated c-myc-mediated transcrip-

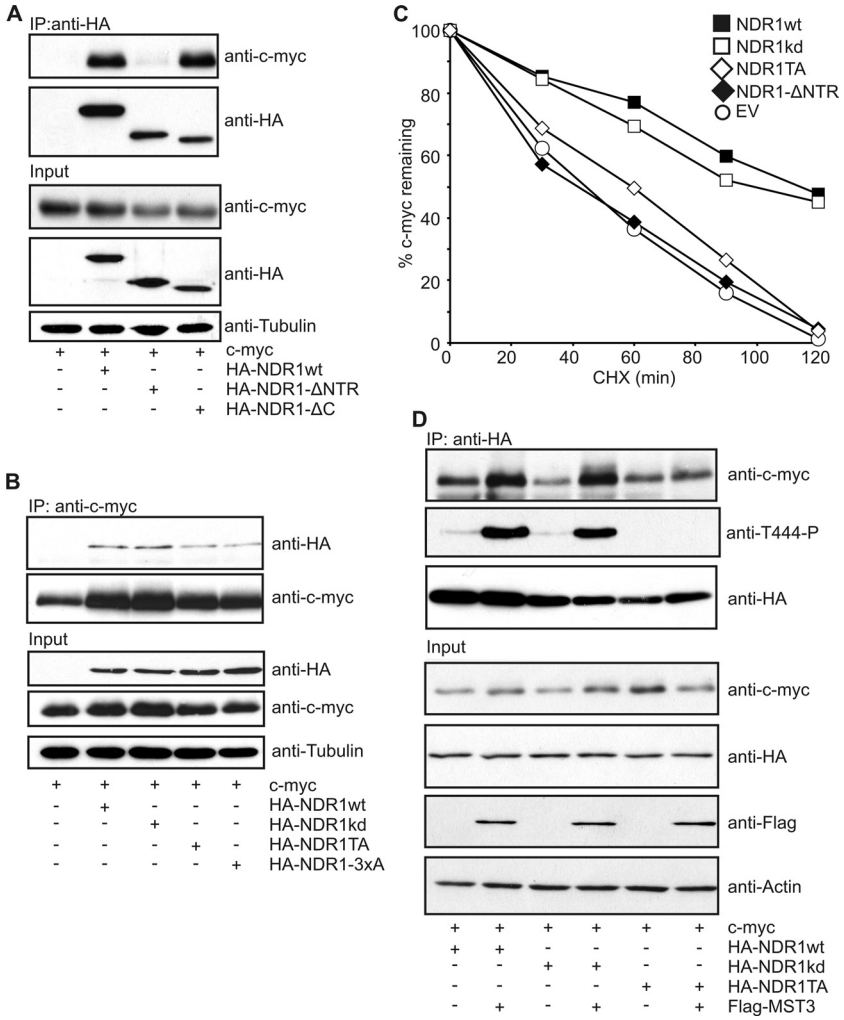

FIG. 4. NDR1/2 in an active conformation stabilizes c-myc. (A) c-myc binds to the N-terminal region of NDR1. HEK293 cells were transfected with c-myc together with the indicated HA-tagged NDR1 constructs. NDR1 species were immunoprecipitated, and c-myc binding was analyzed by SDS-PAGE. (B) Binding of NDR1 to c-myc is modulated by hydrophobic motif phosphorylation (T444). HEK293 cells were transfected with the indicated NDR1 constructs (NDR1TA, T444A; NDR1-3xA, T74, S281, and T444A). c-myc was immunoprecipitated, and bound NDR1 species were analyzed by immunoblotting. (C) Overexpression of NDR1wt and NDR1kd stabilizes c-myc. HEK293 cells were transfected with c-myc and the indicated NDR1 cDNA; 24 h later, cells were treated with cycloheximide (CHX) for the indicated times and c-myc levels were analyzed using the Li-Cor Odyssey system. (D) Hydrophobic motif phosphorylation of NDR increases binding of NDR to c-myc. HEK293 cells were transfected with c-myc and the indicated NDR1 cDNAs together with a vector encoding FLAG-MST3, and complex formation was analyzed.

tion (reference 45 and data not shown). This suggests that NDR expression and phosphorylation can positively affect c-myc protein levels and activity.

Degradation of c-myc is tightly regulated by the ubiquitin-proteasome system (49), and hence we tested whether NDR expression affected c-myc ubiquitination. Indeed, we observed that the stabilizing effect of NDR1 overexpression on c-myc was due to impaired c-myc ubiquitination (Fig. 5A). The FBW7 E3 ubiquitin ligase complex has been shown to mainly regulate c-myc ubiquitination and degradation (49). We tested the effect of NDR overexpression on FBW7-mediated ubiquitination (Fig. 5B). Ubiquitination of c-myc by FBW7 was impaired by NDR1wt overexpression, although NDR1 did not compete with FBW7 for c-myc interaction (Fig. 5C). Collectively, our analysis suggests a role for NDR kinases in the regulation of c-myc protein stability by interfering with FBW7-mediated ubiquitination. Interestingly, NDR kinases interacted with c-myc supported by HM phosphorylation, but independent of NDR kinase activity.

G_1 arrest upon depletion of NDR is not dependent on c-myc. Depletion of NDR1/2 results in G_1 arrest accompanied by an increase in the p21 level and a decrease in c-myc. To test the potential contribution of a reduced c-myc level to the G_1 arrest observed in NDR-depleted cells, we restored the c-myc level by exogenous expression (Fig. 6). Although restoring c-myc prevented, as expected, accumulation of p27, it failed to rescue cells from G_1 arrest in this setting. These experiments therefore indicated that the increased levels of p21 might mediate the observed G_1 arrest in NDR kinase-depleted cells.

NDR kinases regulate p21 stability by phosphorylation of S146 on p21. Since restoration of c-myc level was not sufficient

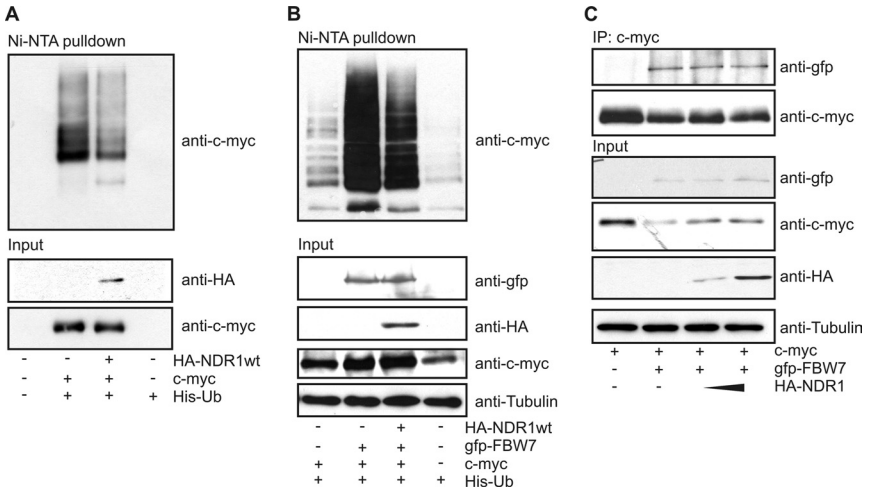

FIG. 5. NDR overexpression impairs FBW7-mediated c-myc ubiquitination. (A) HEK293 cells were transfected with c-myc and His-tagged ubiquitin (His-Ub) together with HA-NDR1wt where indicated. Ubiquitinated proteins were pulled down from cell lysates using Ni-nitrilotriacetic acid (NTA)–Sepharose and analyzed by SDS-PAGE. (B) NDR decreases FBW7-mediated ubiquitination of c-myc. The experiment was performed as for panel A, but where indicated, GFP-FBW7 was coexpressed. (C) NDR1 does not compete with FBW7 for c-myc binding. HEK 293 cells were transfected with c-myc, FBW7, and increasing amounts of NDR1wt. c-myc was immunoprecipitated and analyzed for bound FBW7.

to compensate for NDR depletion and release of the subsequent G_1 arrest, we addressed the well-known cell cycle regulator p21 in our setting. NDR depletion results in increased p21 protein levels, without accompanying upregulation of p21 mRNA (Fig. 3). This indicates that NDR kinases could affect p21 protein stability. Indeed, knockdown of NDR1/2 significantly increased p21 protein stability (Fig. 7A). No binding of NDR1/2 to p21 could be detected (data not shown), suggesting a mechanism distinct from the regulation of c-myc by NDR. Earlier studies have implicated several phosphorylation sites on p21 in regulating p21 stability (8), which suggested p21 as a potential NDR kinase substrate. Recombinant NDR2 kinase was able to phosphorylate GST-p21 in an *in vitro* kinase assay (Fig. 7B). Strikingly, purified NDR1 and NDR2wt phosphorylated p21 *in vitro*, while NDR1/2kd did not (Fig. 7C and data not shown). Although no substrates of NDR1/2 have been described, one study reported that NDR1 prefers a stretch of positively charged basic amino acids in the vicinity of the phosphorylation site (36). Intriguingly, the p21 primary sequence contains a stretch of four basic amino acids upstream of the known phosphorylation sites T145 and S146 (43), GRKRRQT^{145}S^{146}MT (G, glycine; R, arginine; K, lysine; Q, glutamine; T, threonine; S, serine; and M, methionine). Therefore, T145 or S146 alanine mutants were subjected to kinase assays (Fig. 7D and E). Phosphorylation of p21 by NDR1/2 was abolished when Ser 146 was mutated to alanine but not when Thr145 was mutated (Fig. 7D and data no shown). These findings were confirmed using a phospho-specific antibody for phosphorylation on Ser 146 (anti-p21-pS146) (Fig. 7E). Therefore, our analysis showed that NDR1/2 phosphorylates p21 mainly on S146.

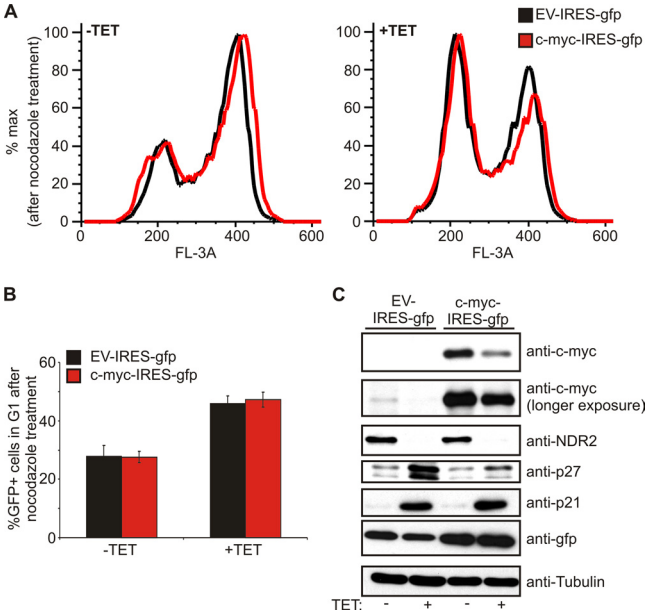

FIG. 6. G_1 arrest in NDR-depleted cells is not dependent on c-myc. (A) HeLa cells expressing shRNA against NDR1/2 were induced for 72 h with tetracycline and transfected with vectors expressing c-myc together with an IRES-GFP as a transfection marker. G_1 arrest analysis was performed as described in the legend to Fig. 2F. Cell cycle profiles of GFP-positive cells were overlaid to allow for better comparison. (B) Analysis of GFP-positive cells in G_1 after nocodazole arrest upon depletion of NDR1/2 and overexpression of c-myc ($n = 3$). (C) Analysis of c-myc, p21, and p27 in NDR1/2-depleted cells upon overexpression of c-myc. Cells were treated as described for panel A, but lysates were prepared before G_1 arrest analysis.

NDR kinases are efficiently activated by okadaic acid (OA) treatment. Indeed, overexpression of NDR1wt but not kinase-dead NDR1 or an HM mutant increased phosphorylation of p21 upon OA treatment (Fig. 8A). Similar results were obtained when NDR2wt or kinase-dead NDR2 was used (Fig. 8B). Furthermore, reduction of NDR kinases in cells by shRNA reduced OA-mediated as well as steady-state phosphorylation of p21 on S146 (Fig. 8C). Significantly, steady-state phosphorylation of endogenous p21 on S146 was reduced upon

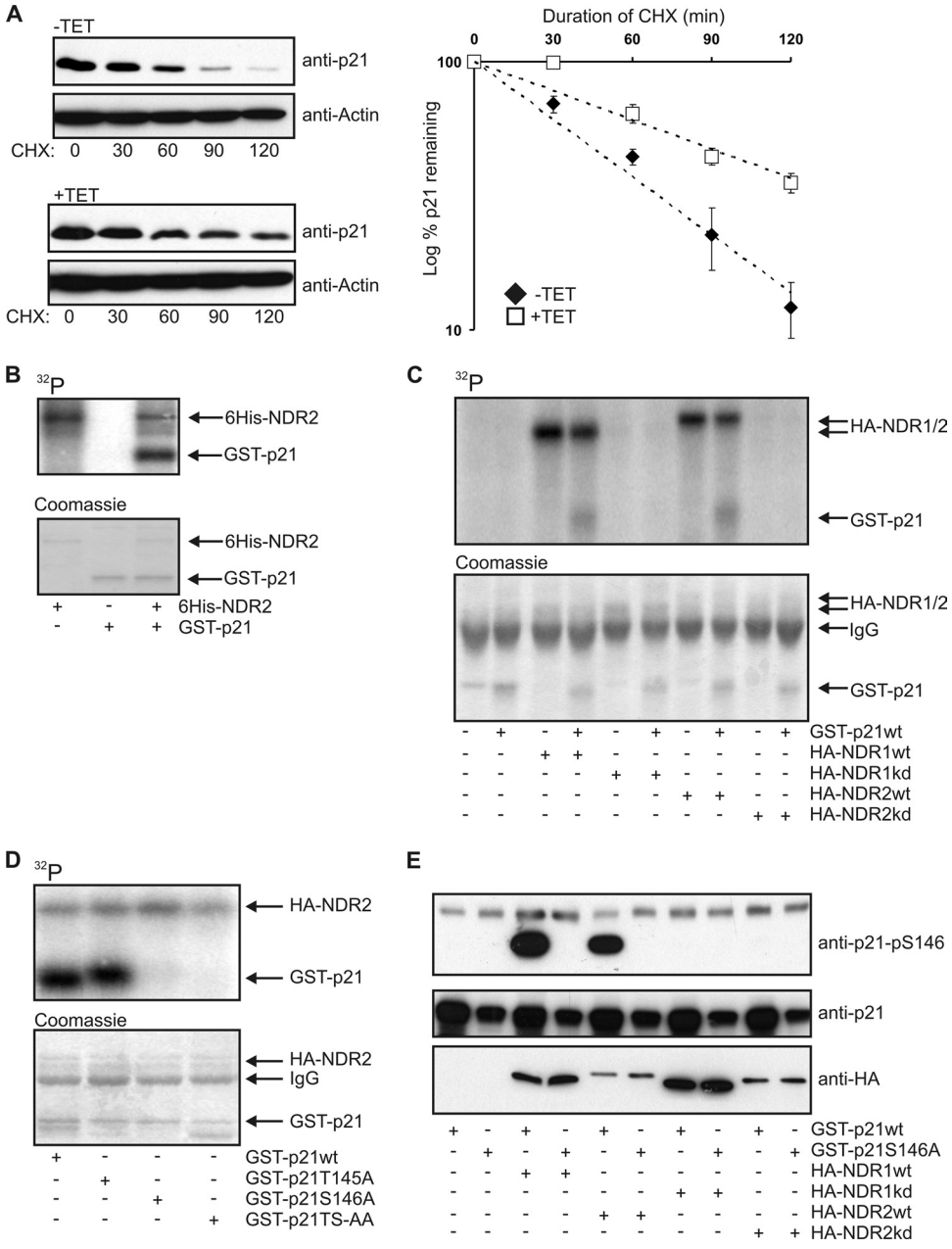

FIG. 7. NDR kinases phosphorylate p21 on S146 *in vitro*. (A) Depletion of NDR1/2 increases p21 stability. HeLa-shNDR1/2 cells were treated with tetracycline for 72 h. Prior to harvesting, cells were treated for the indicated times with CHX. p21 levels were analyzed using the Li-Cor Odyssey system ($n = 3$). p21 levels are depicted as the percentage remaining relative to the level at time zero, and trend lines were added to the data. r^2 values for the trend lines are 0.98 (−TET) and 0.92 (+TET). Equation for the trend lines are $y = 100e^{-0.0166x}$ (−TET) and $y = 100e^{-0.0083x}$ (+TET). (B) Recombinant NDR2 phosphorylates recombinant p21. GST-p21 was used in an *in vitro* kinase assay with polyhistidine-tagged NDR2 from Sf9 cells in the presence of [γ-^{32}P]ATP. After 30 min, reactions were analyzed by SDS-PAGE and exposure to a phosphorimager. (C) NDR1 and 2 phosphorylate p21 *in vitro*. HA-tagged NDR1/2wt or HA-NDR1/2kd was immunoprecipitated from okadaic acid-stimulated HEK293 cells and used for *in vitro* kinase assays with GST-p21 as a substrate. Reactions were analyzed as for panel B. (D) Ser 146 is the major site of p21 phosphorylated by NDR kinases. GST-p21 with mutated T145, S146, or T145/S146 phospho-acceptor sites was used as a substrate for *in vitro* kinase assays as described for panel B. (E) NDR1 and -2 phosphorylate p21 on Ser 146. GST-p21wt or GST-p21 S146A was used as a substrate in *in vitro* kinase assays using HA-tagged NDR1/2wt or HA-NDR1/2kd from okadaic acid-stimulated HEK 293 cells. Assays were analyzed by Western blotting using a phospho-specific antibody of p21 recognizing only if Ser 146 of p21 is phosphorylated.

knockdown of NDR1/2 in HeLa cells without OA treatment, despite an increase in the total level of p21 (Fig. 8D). Since it has been shown that phosphorylation of S146 destabilizes p21 (42), we tested the effect of overexpressing NDR1 together with MST3 on the stability of p21 (Fig. 8E). Although overexpression of NDR1wt or NDR1kd did not significantly affect p21 steady-state level, treatment of cells with cycloheximide (CHX) to inhibit translation revealed that overexpression of

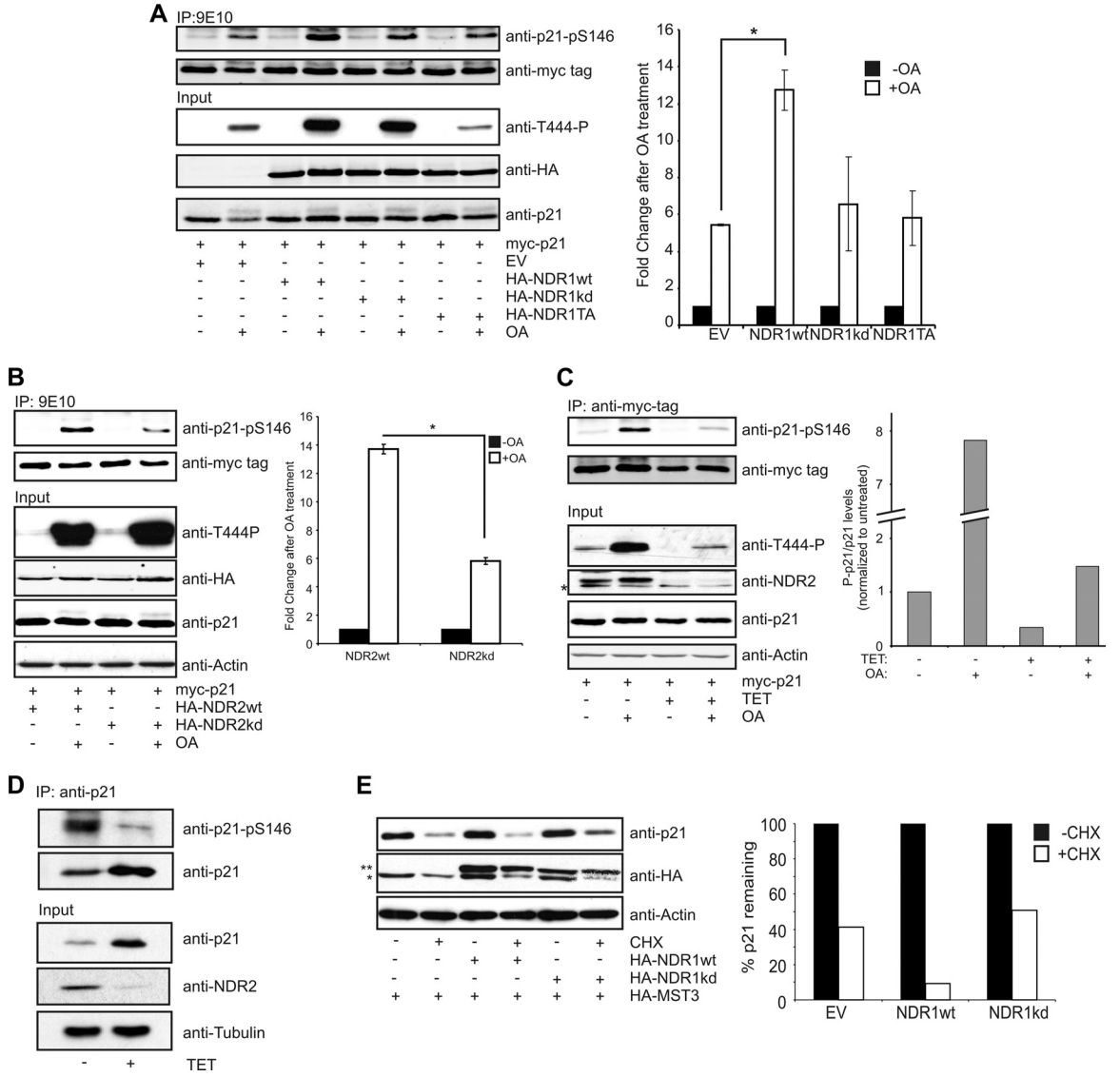

FIG. 8. NDR phosphorylates p21 *in vivo* and regulates p21 stability. (A) Overexpression of NDR1wt increases okadaic acid (OA)-induced phosphorylation of p21 on S146. HeLa cells were transfected with the indicated HA-tagged NDR1 species together with myc-tagged p21. Samples were stimulated with OA for 1 h before lysis where indicated. myc-tagged p21 was immunoprecipitated from lysates, and p21-pS146 levels were analyzed and quantified using the Li-Cor Odyssey system ($n = 3$; $P < 0.002$). p21-pS146 levels were normalized to controls without OA. (B) NDR2 phosphorylates p21 on S146 *in vivo*. HeLa cells were transfected with the HA-tagged NDR2wt or NDR2kd together with myc-tagged p21. Samples were stimulated with OA for 1 h before lysis where indicated. myc-tagged p21 was immunoprecipitated from lysates, and p21-pS146 levels were analyzed and quantified using the Li-Cor Odyssey system ($n = 3$; $P < 0.0001$). p21-pS146 levels were normalized to controls without OA. (C) Depletion of NDR1/2 decreases phosphorylation of p21 on S146. HeLa-shNDR1/2 cells were induced for 72 h with tetracycline and transfected with cDNA encoding myc-tagged p21. At 24 h after transfection, cells were stimulated with OA for 1 h and lysed. p21-pS146 levels were analyzed as for panel B (*, unspecific band). p21-pS146 levels were normalized to the untreated control sample. (D) Depletion of NDR results in a reduction of phosphorylated p21. HeLa-shNDR1/2 cells were induced for 72 h with tetracycline before lysis and immunoprecipitation of endogenous p21 were performed. p21-pS146 levels were analyzed by Western blotting. (E) Overexpression of NDR decreases p21 stability. HeLa cells were transfected with empty vector (EV) or with cDNAs encoding HA-NDR1wt or HA-NDR1kd in the presence of HA-MST3. Prior to lysis, cells were treated with CHX for 60 min where indicated (*, HA-MST3; **, HA-NDR).

NDR1wt but not NDR1kd decreased p21 protein stability in this setting (Fig. 8E). Taken together, these results show that NDR kinases directly phosphorylated p21 on S146 *in vitro* and *in vivo*, establishing p21 as the first substrate for mammalian NDR kinases. Importantly, since phosphorylation on S146 can negatively affect the protein stability of p21, our results suggest that loss of NDR activity results in an increase in total p21 level and that increased activity of NDR leads to destabilization of p21.

G_1 arrest upon depletion of NDR kinases is dependent on increased p21 stability. To confirm the effects of NDR on p21, we performed experiments to rescue the effects of depletion of NDR by transient overexpression of NDR mutants refractory to shRNA (Fig. 9A). Indeed, whereas overexpres-

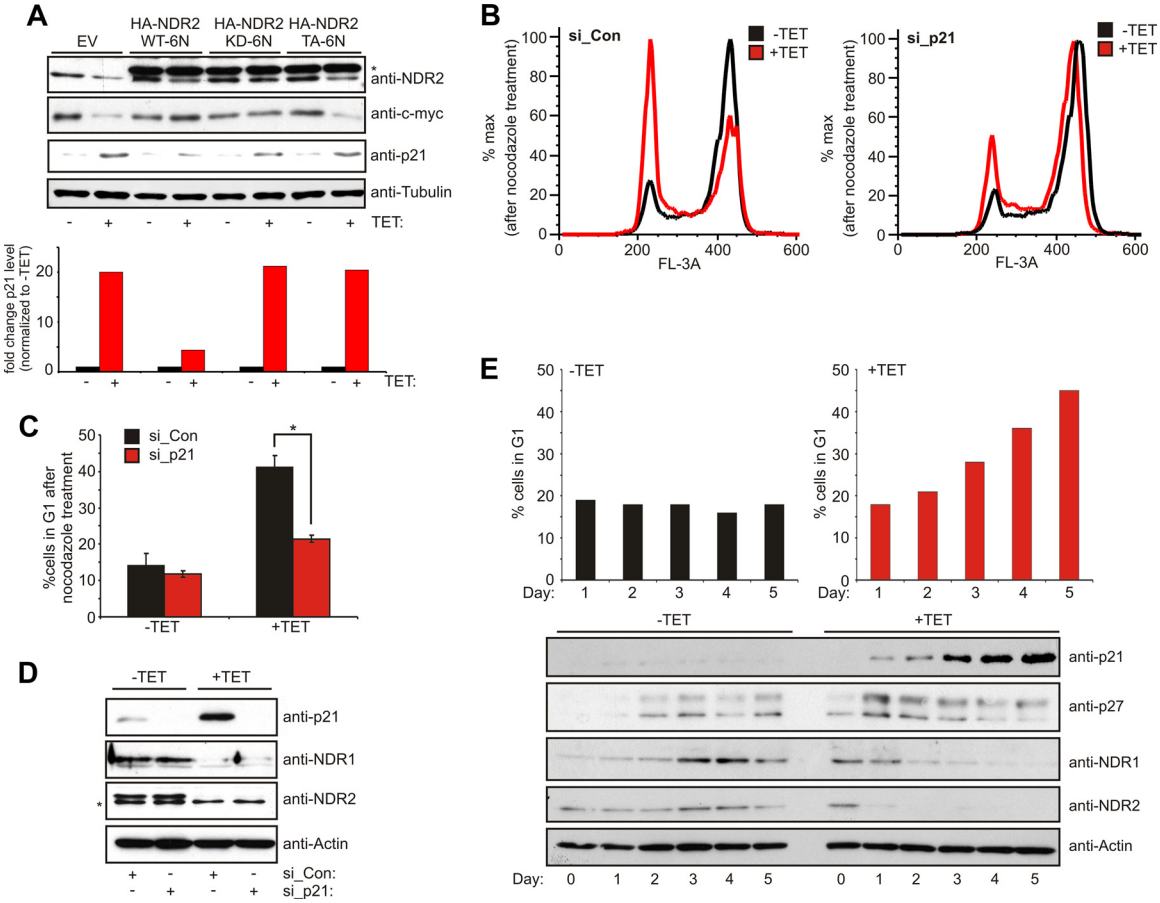

FIG. 9. G_1 arrest after NDR kinase knockdown is rescued by reducing p21 levels. (A) The effects of NDR kinase depletion can be rescued depending on the NDR mutant. HeLa cells expressing shRNA against NDR2 were treated for 48 h with tetracycline and subsequently transfected with the indicated NDR2 mutants refractory to shNDR2 (*, HA-tagged NDR2). p21 levels were quantified using the Li-Cor Odyssey system. (B) HeLa cells expressing shRNA against NDR1/2 were treated with tetracycline for 48 h before being transfected with siRNA against p21 on two consecutive days. The cells were treated for G_1 arrest analysis as described in the legend for Fig. 2F at 24 h after the second transfection. (C) Quantification of cells in G_1 after nocodazole treatment ($n = 3$; $P < 0.002$). (D) Cells obtained from the experiment for panel B were analyzed for the expression of p21 and NDR1/2 (*, unspecific band). (E) p21 levels correlate with G_1 arrest in NDR1/2-depleted cells. HeLa cells expressing shRNA against NDR1/2 were induced with tetracycline for the indicated times before harvest and lysis. Cells used for cell cycle analysis were treated with 2.5 μg/ml nocodazole for 14 h before harvest.

sion of NDR2wt in this setting rescued both the effects on c-myc and p21 levels, overexpression of NDR2kd only restored c-myc levels. Importantly, NDR2TA failed to rescue either of the effects. Additionally, reexpression of refractory NDR1wt in U2OS shNDR1 cells rescued the effect on p21 levels upon NDR1 knockdown (data not shown). These observations fully confirm our previous findings, namely, that NDR can regulate c-myc stability irrespective of kinase activity, while the effect of NDR kinases on p21 levels is dependent on NDR kinase activity.

Next we analyzed whether altered p21 levels mediated the G_1 arrest upon depletion of NDR. Indeed, siRNA-mediated depletion of p21 was fully sufficient to result in a significant restoration of NDR1/2-depleted cells from G_1 blockade (Fig. 9B to D). Furthermore, we have confirmed this finding by the use of a second, independent siRNA against p21 (data not shown). In full agreement with this finding, p21 levels were shown to correlate with G_1 arrest in shNDR1/2 cells. Prolonged depletion of NDR1/2 resulted in an increasing amount of cells in G_1 and correspondingly in an accumulation of p21 protein, whereas p27 levels did not correlate (Fig. 9E). Collectively, these experiments revealed that p21 is a key mediator of the G_1 arrest observed in NDR kinase-depleted cells.

DISCUSSION

In eukaryotes NDR kinases have been shown to function in processes tightly linked to the cell cycle, such as mitotic exit, cell growth, proliferation, centrosome duplication, and morphogenesis (24). Here we identify human NDR kinases as novel regulators of cell cycle progression. NDR kinases are activated in G_1, with the activation persisting into S phase (Fig. 1), in full accordance with NDR kinase function in centrosome duplication (23). Conversely, knockdown of NDR1/2 results in G_1 arrest (Fig. 2). Importantly, although depletion of NDR kinases can result in centrosome duplication defects (22, 23), our experiments show that the G_1 arrest is completely inde-

pendent of the reported p38-p53 centrosome integrity checkpoint (35, 46).

Although they are well described in terms of upstream regulation and biochemical activation, signaling mechanisms downstream of mammalian NDR kinases remained elusive. Here we describe a novel function for NDR kinases in regulating the G_1/S transition, identify c-myc and p21 as downstream signaling targets for mammalian NDR kinases, and provide detailed mechanistic insight into this regulation: whereas NDR kinases regulate c-myc protein stability by direct interaction independent of kinase activity (Fig. 4 and 5), p21 is directly phosphorylated by NDR kinases on Ser 146, thereby regulating p21 stability (Fig. 7 and 8). Furthermore, knockdown of NDR1/2 resulted in G_1 arrest and was dependent on increased p21 stability but not on c-myc (Fig. 9).

NDR1 has been shown previously to bind to c-myc and increase its stability; however, mechanistic insight into this regulation has not been reported so far (52). Here we confirm the binding of NDR kinases to c-myc (Fig. 4 and data not shown). Strikingly, we find that both binding and stabilization of c-myc by NDR are independent of NDR kinase activity (Fig. 4), indicating a novel adaptor-like function for NDR kinases. We observed distinct effects on NDR–c-myc interaction upon point mutation of the hydrophobic motif phosphorylation site T444 or deletion of the C terminus. The C-terminal deletion mutant still interacted with c-myc, whereas point mutants (T444A, T444D, or T444E) displayed reduced or no interaction (Fig. 3 and data not shown). Since we found the N terminus of NDR to be responsible for the interaction with c-myc, deletions or mutations of the C terminus might have distinct conformational consequences for the structure of the N-terminal region of NDR. Therefore, future structural analysis of NDR kinases bearing mutations or deletions will be required to put these findings into the context of how NDR–c-myc interaction is modulated and hence how exactly NDR stabilizes c-myc.

c-myc protein levels are tightly regulated in cells. Apart from a pronounced regulation at the transcriptional level, protein levels of c-myc are controlled by ubiquitin ligases such as FBW7 (49). Here we show that NDR kinases do not compete with FBW7 for c-myc binding. Nevertheless, NDR kinases can inhibit c-myc ubiquitination by FBW7 (Fig. 5), indicating a novel mechanism by which NDR kinases interfere with c-myc degradation. Potentially, NDR kinases bound to c-myc inhibit ubiquitination directly or facilitate recruitment of deubiquitinating enzymes. Future studies to further define the effects of NDR kinases on c-myc stability are warranted.

Various mechanisms control expression of the Cdk inhibitor p21. The transcriptional regulation of p21 is well described and includes p53-dependent and p53-independent mechanisms (15). Here we show that the deregulation of p21 levels upon knockdown of NDR is not based on changes in transcription of the p21 gene (Fig. 3), indicating a novel aspect of p21 regulation on a posttranscriptional level by NDR kinases. Degradation of p21 is mediated by both ubiquitin-dependent and -independent mechanisms (1). In addition, phosphorylation of p21 has been shown to modulate a variety of p21 functions by affecting p21 localization, complex formation, and degradation (8). We show here that NDR1 and NDR2 phosphorylate p21 on S146 *in vitro* and *in vivo*, identifying p21 as the first *in vivo* substrate for human NDR1/2 (Fig. 7 and 8). Direct substrates of NDR/LATS orthologs in yeast and flies and also of mammalian LATS1/2 kinases have been discovered (6, 12, 28, 29, 33, 37). Interestingly, the aforementioned reports also defined consensus phosphorylation motifs for NDR/LATS kinases in yeast, flies, and mammals: for human LATS1/2 and fly warts, HXRXXS; for yeast Dbf2p and Sid2p, RXXS; and for yeast Cbk1p, $HX^R/_KX^R/_KXS$ (H, histidine; R, arginine; K, lysine; S, serine; and X, any amino acid). Interestingly, NDR1/2 kinases seem to have a less stringent consensus, since they prefer a stretch of positively charged basic amino acids N terminal of the phosphorylation site (36). Nevertheless, the p21 phosphorylation site of NDR, GRKRRQTS^{146}M, matches a minimal consensus for NDR kinases across species, RxxS. However, further research in this area in order to define a precise consensus phosphorylation motif of mammalian NDR1/2 kinases is warranted.

Interestingly, endogenous p21 phosphorylation is reduced upon knockdown of NDR kinases despite an increase in total levels of p21 (Fig. 8). Phosphorylation of p21 on S146 has been reported to both increase and decrease p21 protein stability, depending on the cellular context and whether endogenous or overexpressed p21 was analyzed (30, 39, 42, 55). In our setting we could confirm the destabilizing effect of phosphorylated S146 on p21 protein turnover (Fig. 8). Even more importantly, our results show that increased p21 stability and subsequent accumulation of p21 correlated with the increase of cells in G_1 (Fig. 9). Furthermore, in rescue experiments using siRNAs targeting p21 in cells depleted of NDR kinases, we observed a significant release from G_1 arrest compared to that in control cells (Fig. 9) Therefore, p21 indeed is the main regulator of the G_1 arrest observed in NDR kinase-depleted cells.

Previous reports revealed a role for NDR kinases in regulating mitotic chromosome alignment, centrosome duplication, and apoptosis (7, 22, 50). In these contexts, however, NDR kinases have been shown to function downstream of MST1 and MST2 kinases (7, 22, 50), which have been established as tumor suppressors as part of the HIPPO pathway (21, 56). A third member of the MST kinase family, MST3, has also been reported to act as an upstream kinase for NDR kinases (47), but the biological significance of this MST3-NDR axis has remained elusive. Here we identified MST3 as the main upstream kinase responsible for NDR activation in G_1 (Fig. 1). In addition, we observed an increase of active MST3 in G_1 compared to M phase, showing that MST3 activity can change in a cell cycle-dependent manner. Furthermore, our experiments confirmed a role of MST3 signaling in cell cycle regulation, as depletion of MST3 resulted in effects on proliferation similar to those observed for NDR kinases (Fig. 2D and 3B). MST3 signaling has been implicated in the regulation of axon outgrowth, cellular migration, and stress-induced apoptosis (5, 27, 31); however, any involvement of MST3 in cell cycle progression has not been described so far. Now, given our findings regarding this novel role of MST3 in the control of cell cycle progression, future research addressing the molecular activation mechanisms of MST3 is warranted.

Together, the findings described in this report lay a new foundation for research addressing functions of NDR kinases in cell cycle control. In combination with previous reports regarding apoptotic signaling (10, 50), our results actually indicate a potential dual role for NDR kinases in regulating cell

proliferation and apoptosis. Intriguingly, the different and partially opposing functions of mammalian NDR kinases seem to depend on the MST kinase input: whereas NDR kinase function in apoptosis, tumor suppression, and centrosome duplication is dependent on MST1 (10, 22, 50), NDR kinases regulate mitotic chromosome alignment downstream of MST2 (7). In line with these findings, the regulation of cell proliferation by NDR kinases described here depends not on MST1 or MST2 but on MST3. In conclusion, the MST3-NDR axis reported here can promote cell proliferation via direct phosphorylation of p21, thereby restricting p21 levels. Therefore, our work might provide a platform for establishing a dual and most likely cell context-dependent role for NDR kinases in normal and cancer cell biology.

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Supplemental Information

Human NDR Kinases Control G1/S Cell Cycle Transition by Directly Regulating p21 Stability

Hauke Cornils, Reto S. Kohler, Alexander Hergovich and Brian A. Hemmings

Figure S1; Cornils, et al.

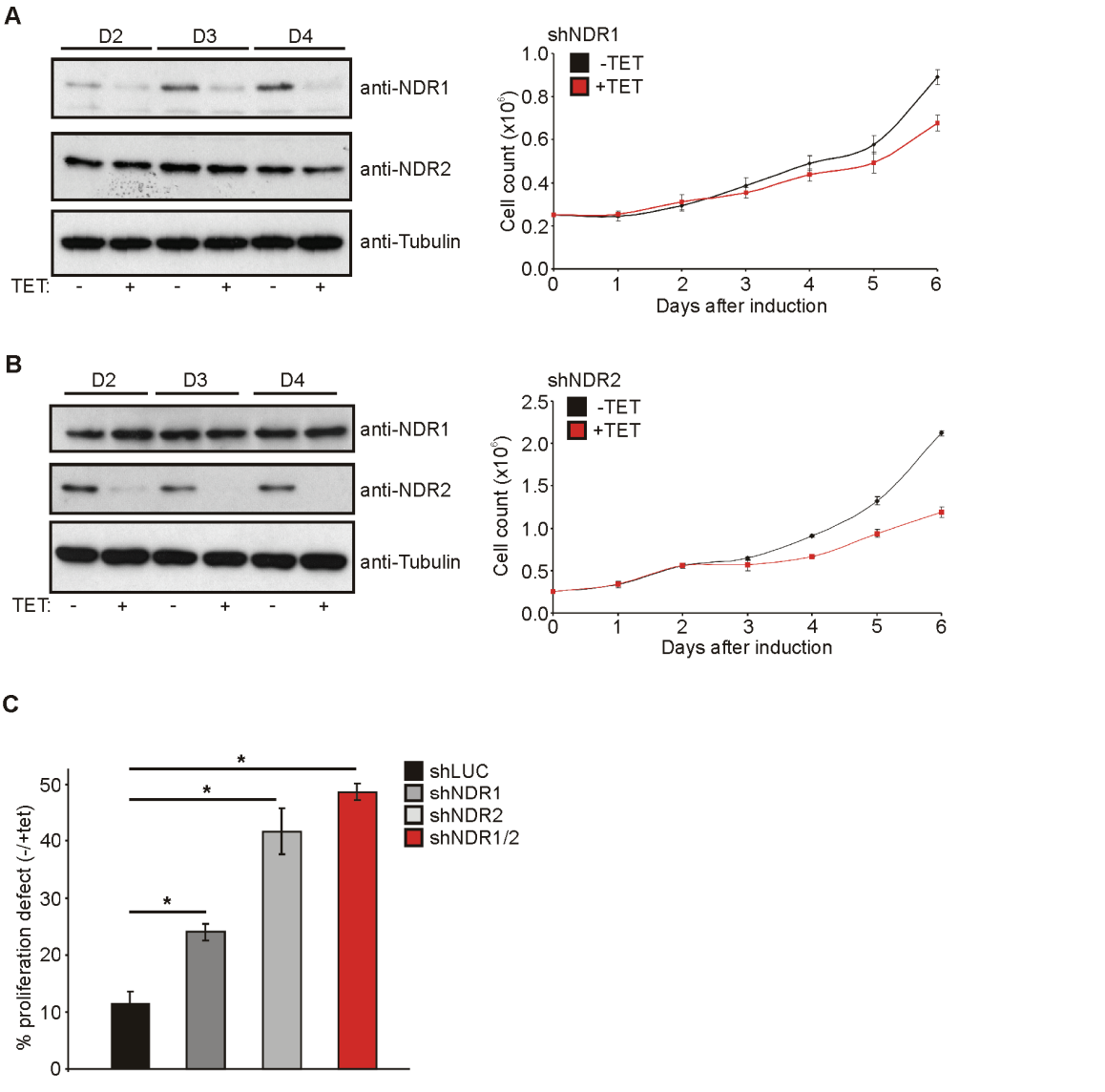

Figure S1. Single depletion of NDR kinase isoforms results in proliferation defects and G1-arrest independent of the centrosome checkpoint. (A) Knock-down of NDR1 alone results in proliferation defects. HeLa cells expressing shRNA against NDR1 alone were seeded in triplicates and treated with tetracycline for the indicated time. Cells were either processed for immuno-blotting (left panel) or counted using a Vicell-automated cell counter. (B) Knock-down of NDR2 alone results in proliferation defects. 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; *, $P < 0.005$). Experiments were performed as in (A) and differences in proliferation were calculated as percentage of cells without tetracycline to cells with tetracycline counted on day 6 after induction of shRNA expression.

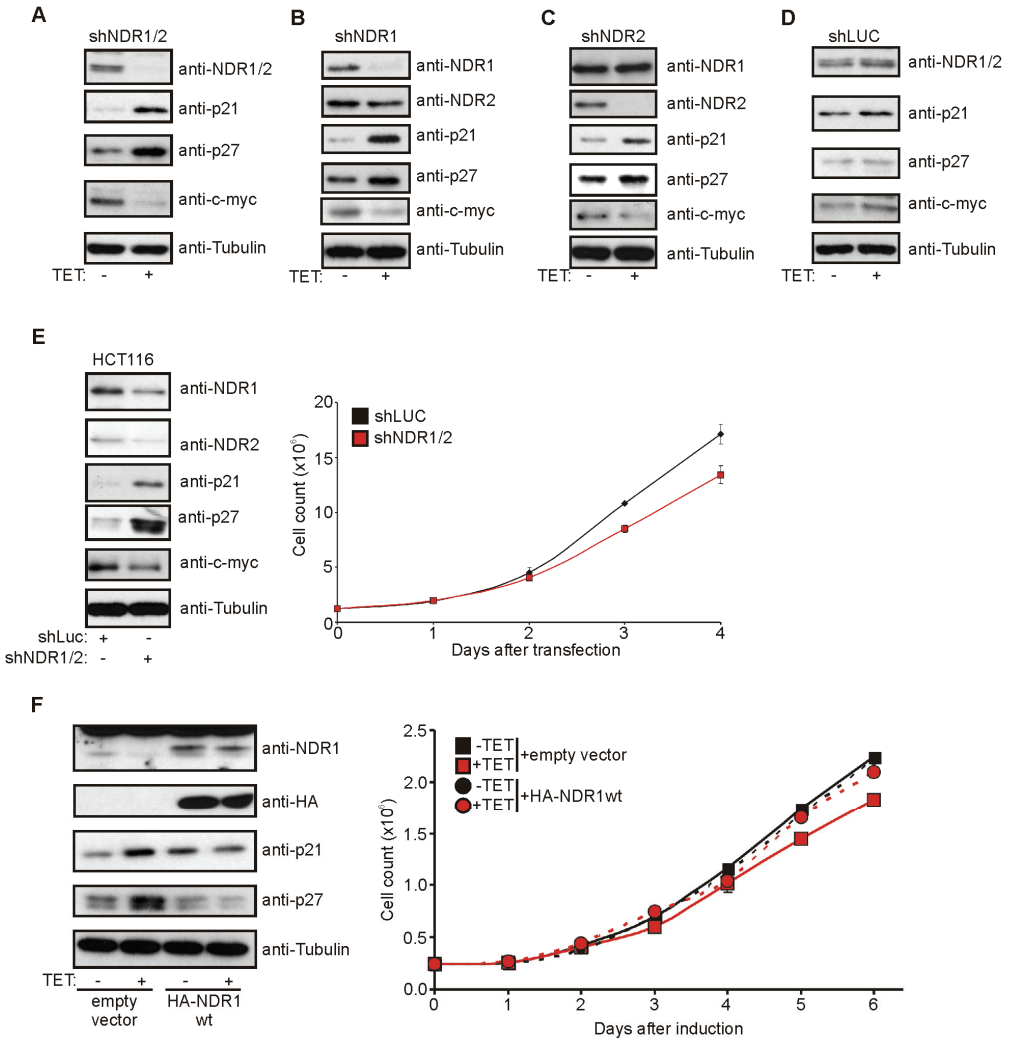
A
shNDR1/2
anti-NDR1/2
anti-p21
anti-p27
anti-c-myc
anti-Tubulin
TET: - +
B
shNDR1
anti-NDR1
anti-NDR2
anti-p21
anti-p27
anti-c-myc
anti-Tubulin
TET: - +
C
shNDR2
anti-NDR1
anti-NDR2
anti-p21
anti-p27
anti-c-myc
anti-Tubulin
TET: - +
D
shLUC
anti-NDR1/2
anti-p21
anti-p27
anti-c-myc
anti-Tubulin
TET: - +
E
HCT116
anti-NDR1
anti-NDR2
anti-p21
anti-p27
anti-c-myc
anti-Tubulin
shLuc: + -
shNDR1/2: - +
shLUC
shNDR1/2
Cell count (x10⁶)
Days after transfection
F
anti-NDR1
anti-HA
anti-p21
anti-p27
anti-Tubulin
TET: - + - +
empty vector
HA-NDR1 wt
-TET
+TET
+empty vector
-TET
+TET
+HA-NDR1wt
Cell count (x10⁶)
Days after induction

Figure S2. Validation of the effects of depletion of NDR kinases on proliferation, p21, p27 and c-myc levels. (A) Analysis of p21, p27 and c-myc levels 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) Effects of NDR1 depletion on p21 and p27 level and on proliferation are rescued by re-expression of an NDR1wt rescue construct. U2OS cells expressing shNDR1 and rescue constructs were described and validated in Hergovich et al., 2007. Protein lysates were obtained 72h after tetracycline induction and cell counts were obtained as described in (E).

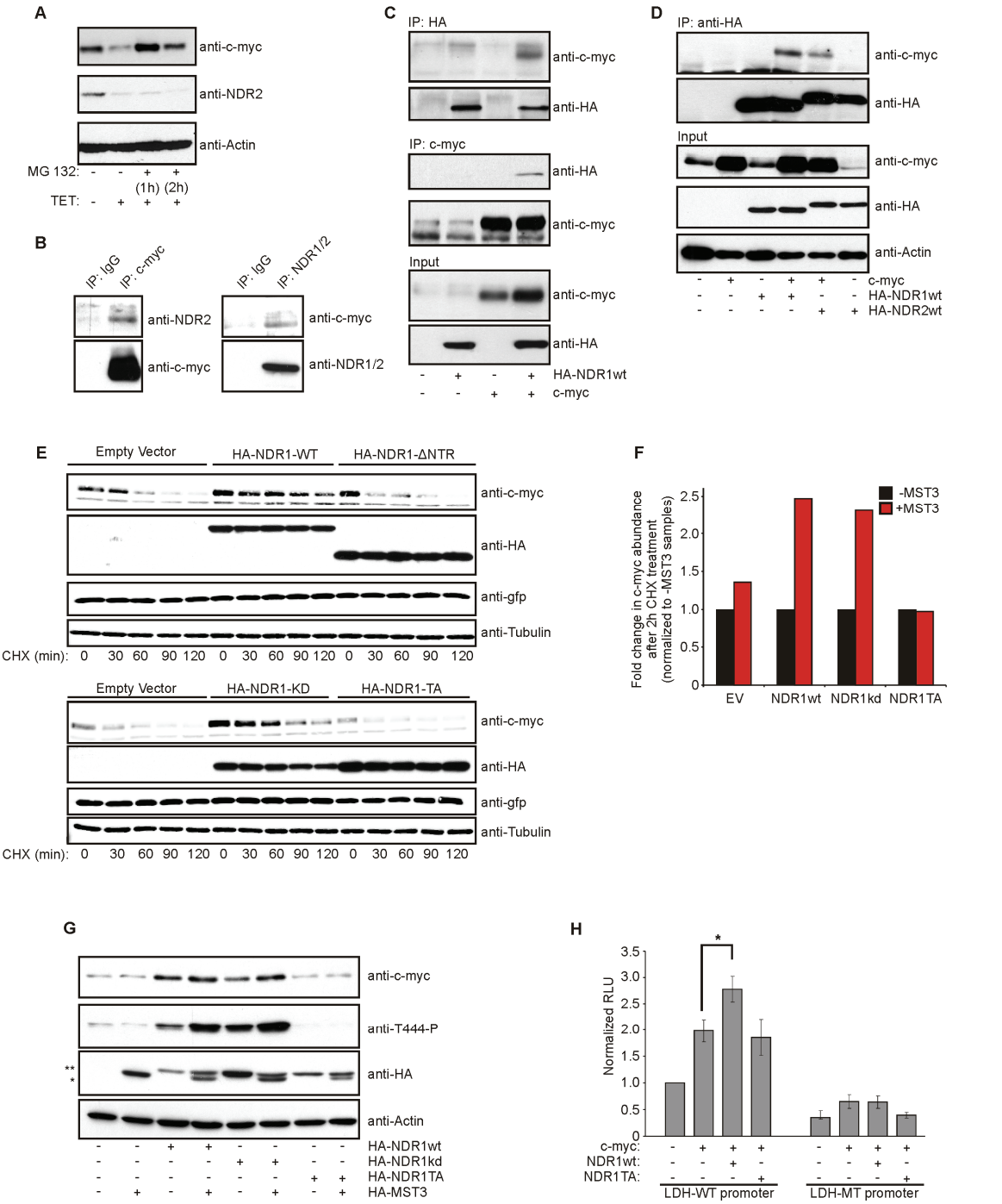
A
anti-c-myc
anti-NDR2
anti-Actin
MG 132: - - + +
(1h) (2h)
TET: - + + +
B
IP: IgG
IP: c-myc
IP: NDR1/2
anti-NDR2
anti-c-myc
anti-NDR1/2
C
IP: HA
anti-c-myc
anti-HA
IP: c-myc
Input
HA-NDR1wt
c-myc
D
IP: anti-HA
Input
c-myc
HA-NDR1wt
HA-NDR2wt
E
Empty Vector
HA-NDR1-WT
HA-NDR1-ΔNTR
anti-gfp
anti-Tubulin
CHX (min): 0 30 60 90 120 0 30 60 90 120 0 30 60 90 120
HA-NDR1-KD
HA-NDR1-TA
F
Fold change in c-myc abundance after 2h CHX treatment (normalized to -MST3 samples)
-MST3
+MST3
EV
NDR1wt
NDR1kd
NDR1TA
G
anti-T444-P
HA-NDR1wt
HA-NDR1kd
HA-NDR1TA
HA-MST3
H
Normalized RLU
c-myc:
NDR1wt:
NDR1TA:
LDH-WT promoter
LDH-MT promoter

Figure S3. NDR1 and NDR2 interact with and stabilize c-myc. (A) Effects of NDR depletion on c-myc levels can be rescued by proteasome inhibition. 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) Endogenous NDR kinases and c-myc interact. NDR species or c-myc were immunoprecipitated from HeLa cell lysates using the indicated antibodies and complex formation was analyzed using SDS-PAGE. (C) Overexpressed NDR1 interacts with c-myc. HEK cells were transfected with cDNAs for c-myc and HA-NDR1wt and HA or myc precipitates were analyzed for interaction. (D) Both NDR1 and NDR2 interact with c-myc. HEK cells were transfected with HA-NDR1, HA-NDR2 and c-myc and complexes were analyzed as in (C). (E) Overexpression of NDR1wt and NDR1kd stabilizes c-myc. HEK cells were transfected with c-myc and the indicated NDR1 cDNAs. 24h later cells were treated with CHX for the indicated time and c-myc levels were assessed by SDS-PAGE and LICOR Odyssey system. (F) Hydrophobic-motif phosphorylation of NDR increases c-myc stability. HEK293 cells transfected with c-myc and NDR1 constructs in the presence of absence of FLAG-MST3 were treated with CHX for 120min and lysates were analyzed for the expression of c-myc, as compared to untreated samples, using the LI-COR Odyssey system. (G) NDR overexpression stabilizes endogenous c-myc levels. HEK293 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 (*, HA-MST3; **, HA-NDR). (H) NDR overexpression promotes c-myc dependent transcription. HEK293 cells were transfected with the indicated constructs and c-myc dependent transcription of the LDH-A promoter was analyzed (n = 3; $P < 0.001$).

Figure S4; Cornils, et al.

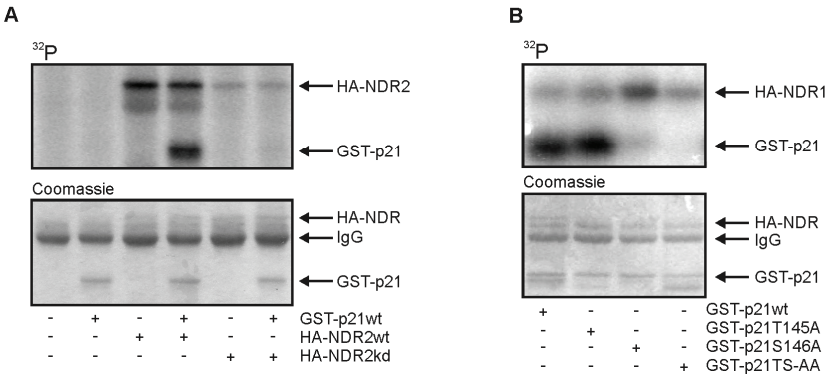

Figure S4. Validation of p21-Ser146 phosphorylation for both NDR1 and NDR2 *in vitro*. (A) Both NDR1 and NDR2 phosphorylate p21 *in vitro*. HA-tagged NDR2wt or HA-NDR2kd was immunoprecipitated from okadaic acid-stimulated HEK293 cells and after pre-incubation with cold ATP used for *in vitro* kinase assays with GST-p21 as substrate. (B) NDR1 phosphorylates p21 on S146. *In vitro* kinase assay to determine NDR phosphorylation 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 (A). Here NDR1 was immunoprecipitated from cells.

Figure S5; Cornils, et al.

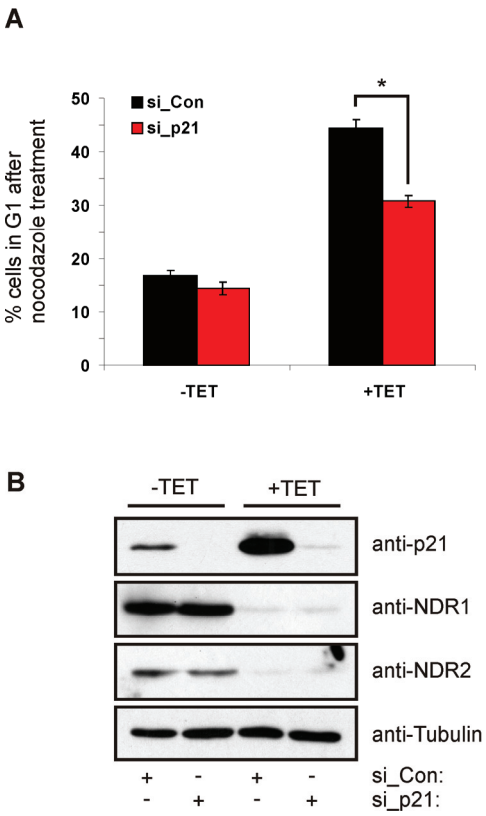

Figure S5. G1-arrest after NDR kinase knock-down is rescued by reducing p21 levels using an additional independent siRNA targeting p21. (A) HeLa cells expressing shRNA against NDR1/2 were treated with tetracycline for 48h before being transfected with siRNA against p21 on two consecutive days. The cells were treated for G1-arrest analysis as described 24h after the second transfection. Quantification of cells in G1 after nocodazole treatment (n=3; $P < 0.0025$). (B) Cells obtained in (A) before nocodazole treatment were analyzed for the expression of p21 and NDR1/2.

Figure S6; Cornils, et al.

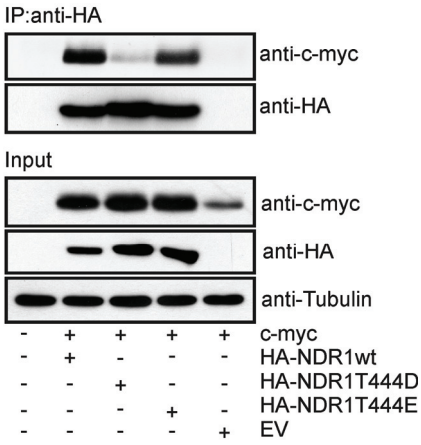

Figure S6. NDR1 T444D and T444E display loss or reduced binding to c-myc. HEK 293 cells were transfected with c-myc together with the indicated HA-tagged NDR1 constructs. HA-tagged NDR1 species were immunoprecipitated and c-myc binding was analyzed by SDS-PAGE.

Figure S7; Cornils, et al.

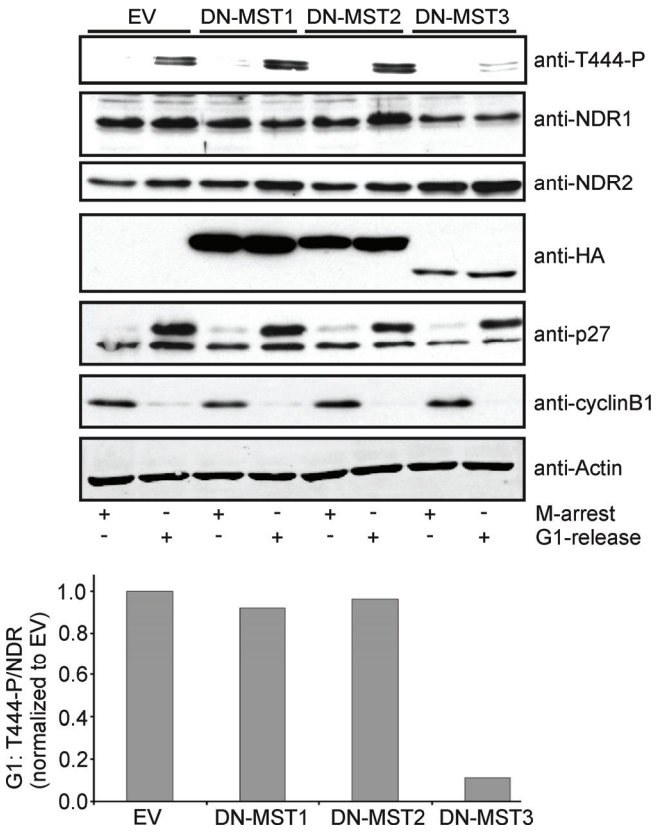

Figure S7. Expression of DN-MST3 reduces G1-activation of NDR. HeLa cells were transfected with HA-tagged dominant-negative (DN) variants of MST1, MST2 or MST3 and 24h later arrested with nocodazole for 14h. Arrested cells were harvested or released into G1 for 8h before harvesting. NDR activation was assessed using T-444-P antibody. Cell cycle phases were confirmed by analyzing Cyclin B1 and p27 expression. Phospho-T444 levels after G1 release were compared to control samples and analyzed using the LI-COR Odyssey system.

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

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Article

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

Alexander Hergovich,[1,*] Reto S. Kohler,[1] Debora Schmitz,[1] Anton Vichalkovski,[1] Hauke Cornils,[1] and Brian A. Hemmings[1]
[1]Friedrich Miescher Institute for Biomedical Research, Maulbeerstrasse 66, CH-4058 Basel, Switzerland

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

*Correspondence: hergo@fmi.ch

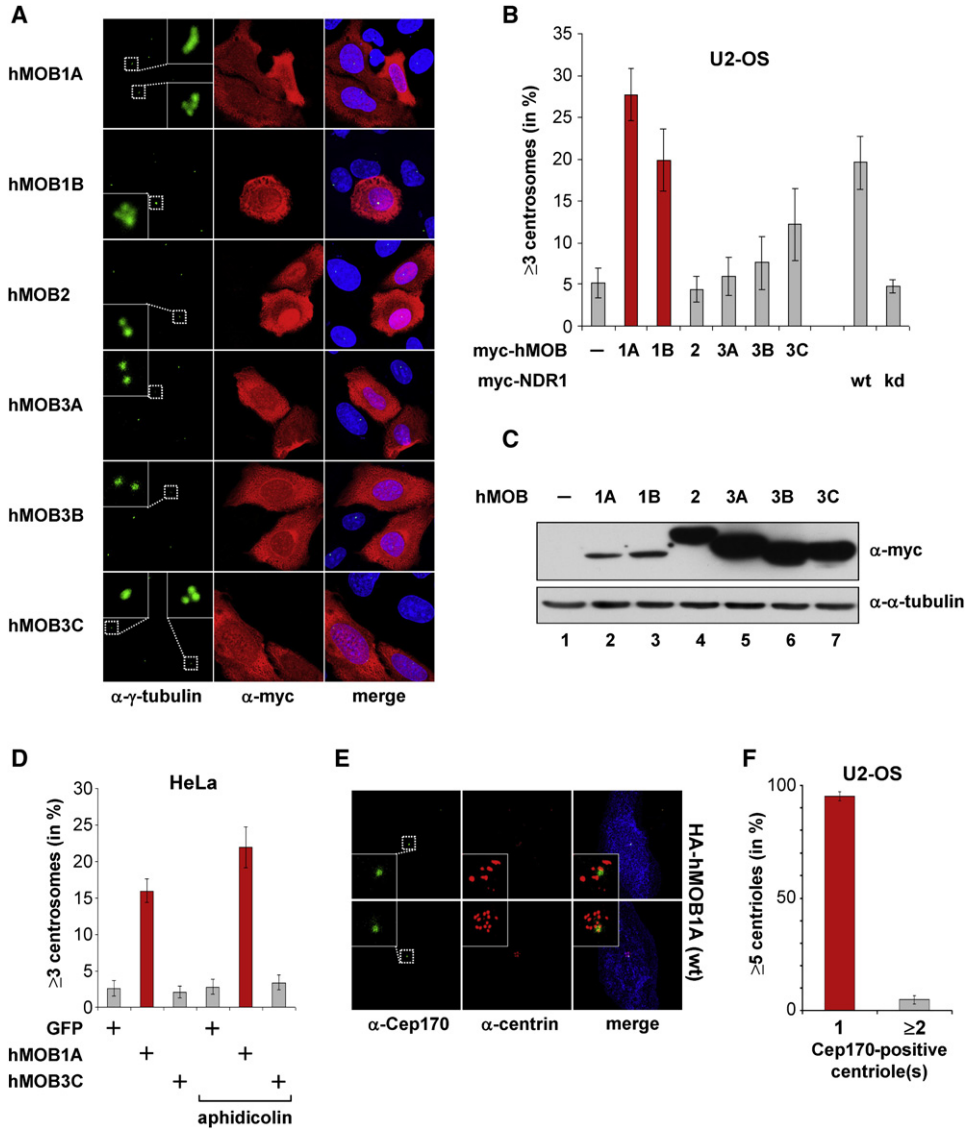

Figure 1. Overexpression of hMOB1A/B Leads to Centrosome Overduplication

(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

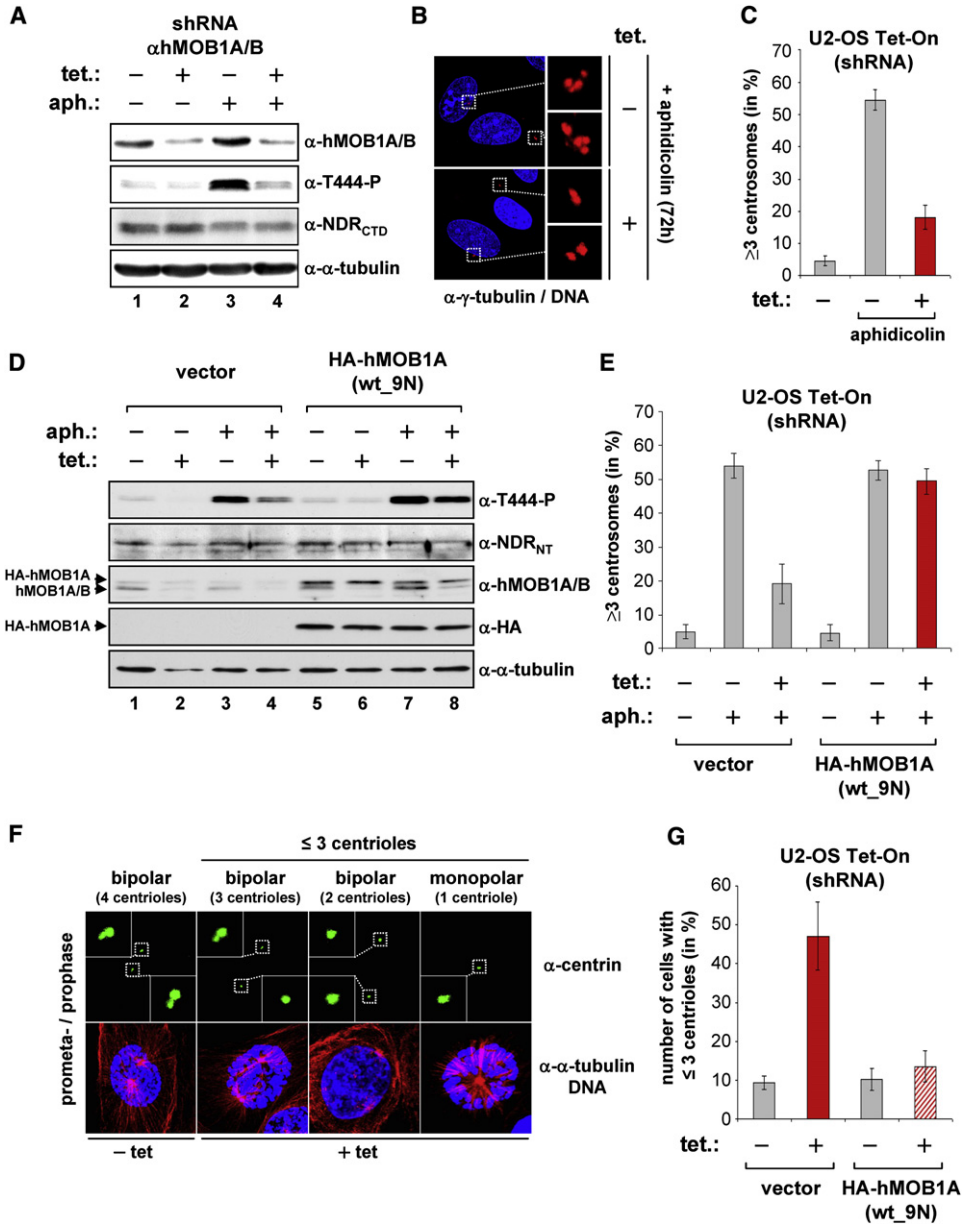

Figure 2. Endogenous hMOB1A/B Is Required for Centrosome Duplication

(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.

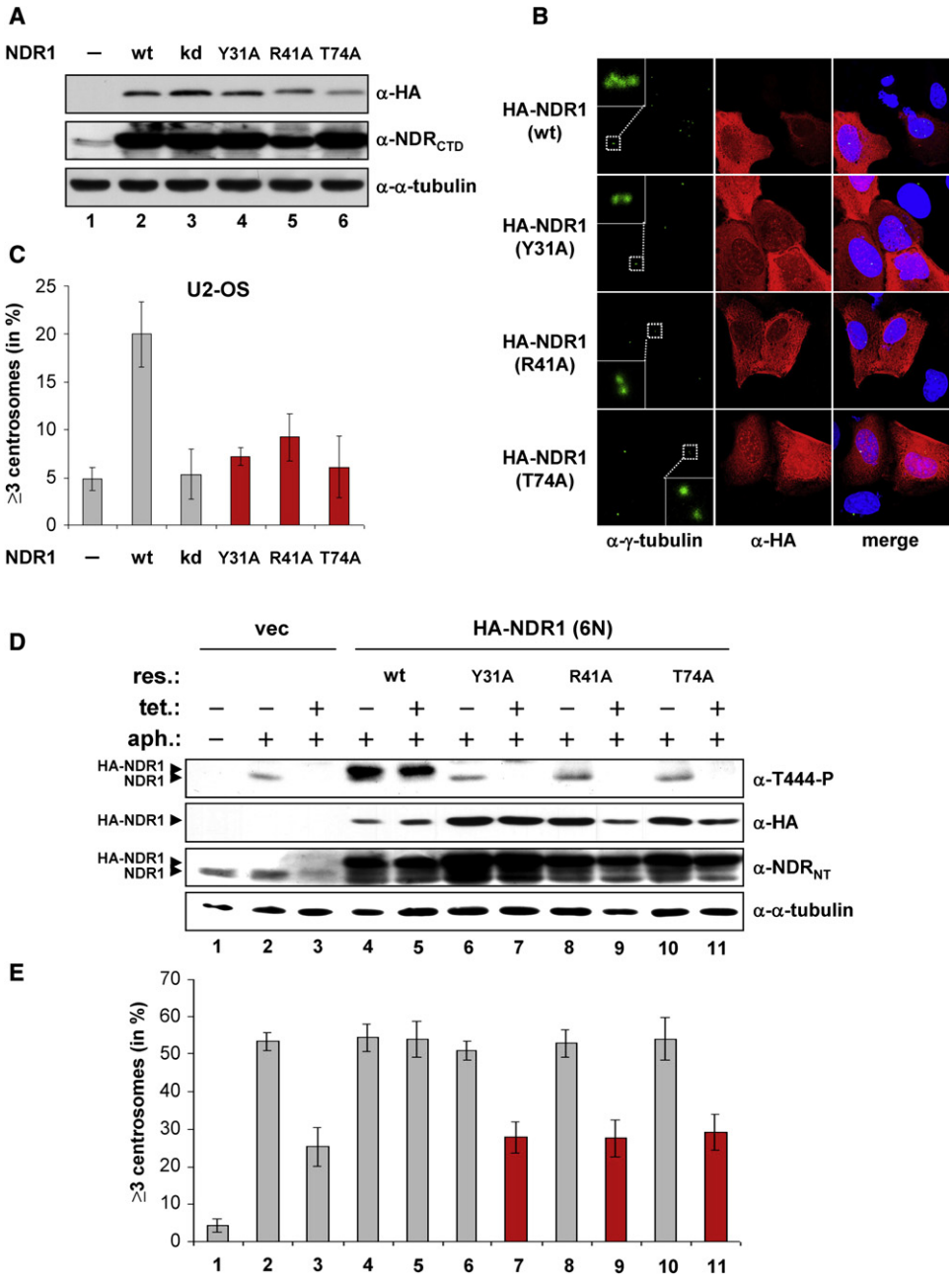

Figure 3. NDR1 Kinase Deficient in hMOB1A/B Binding Does Not Support Centrosome Amplification

(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

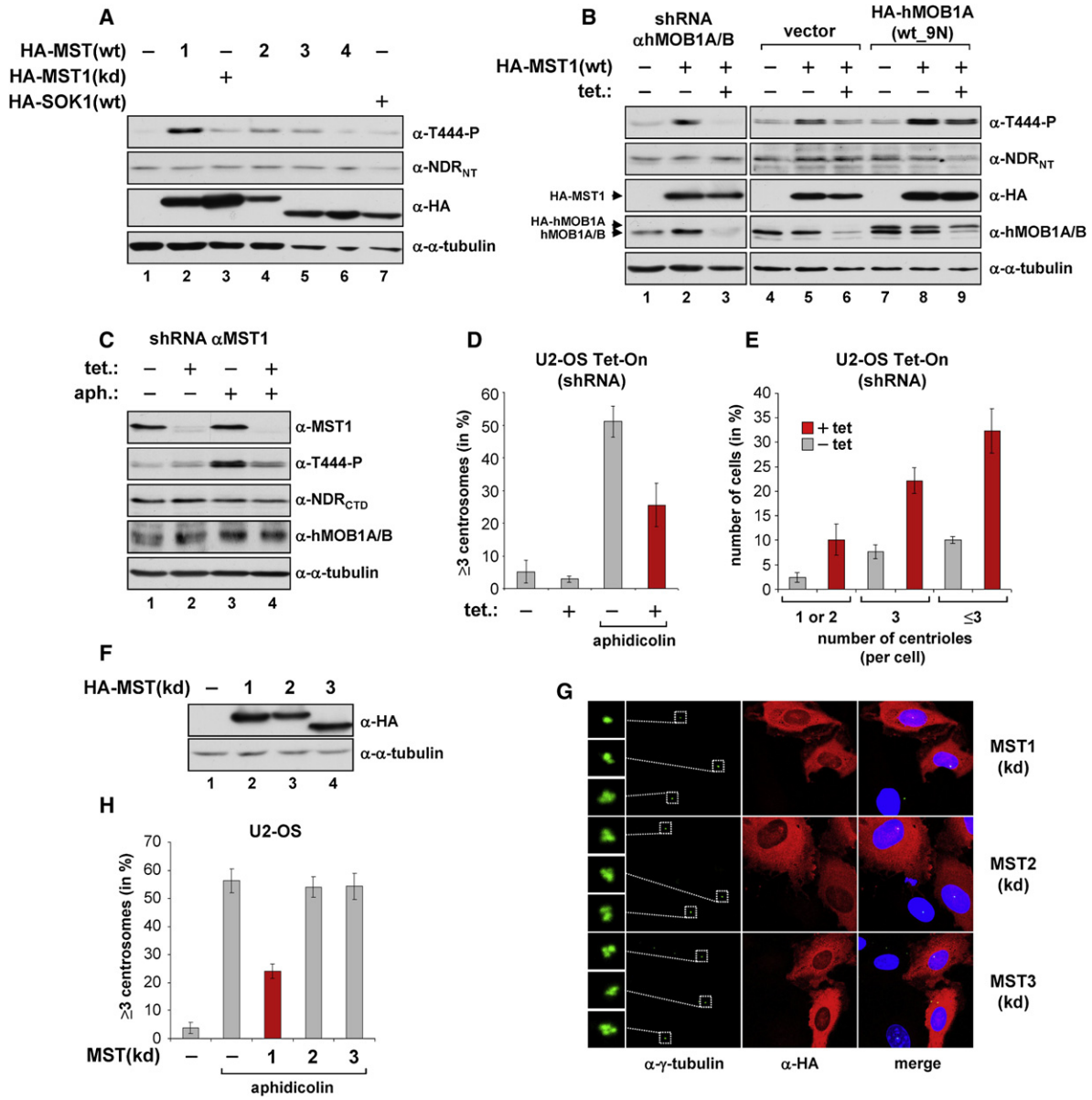

Figure 4. Human MST1 Kinase Is Crucial for Centrosome (Over)duplication

(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

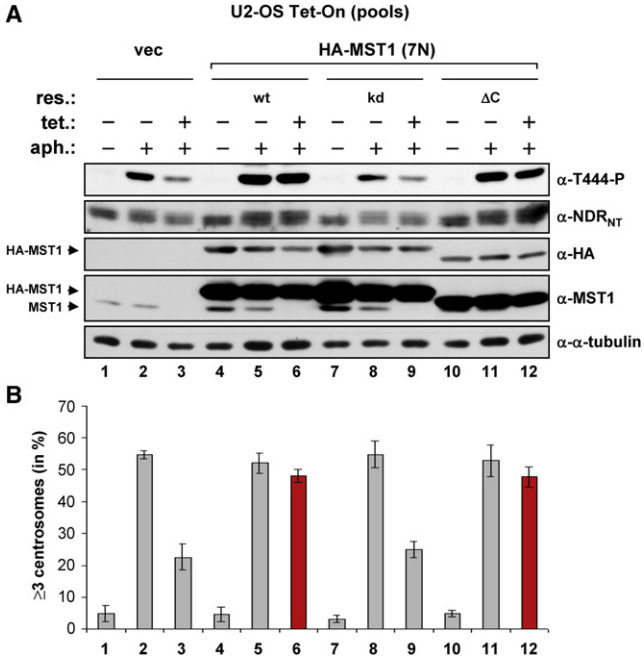

Figure 5. MST1 Kinase Activity, Not the SARAH Domain, Is Required for Centrosome Overduplication in Human Cells

(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

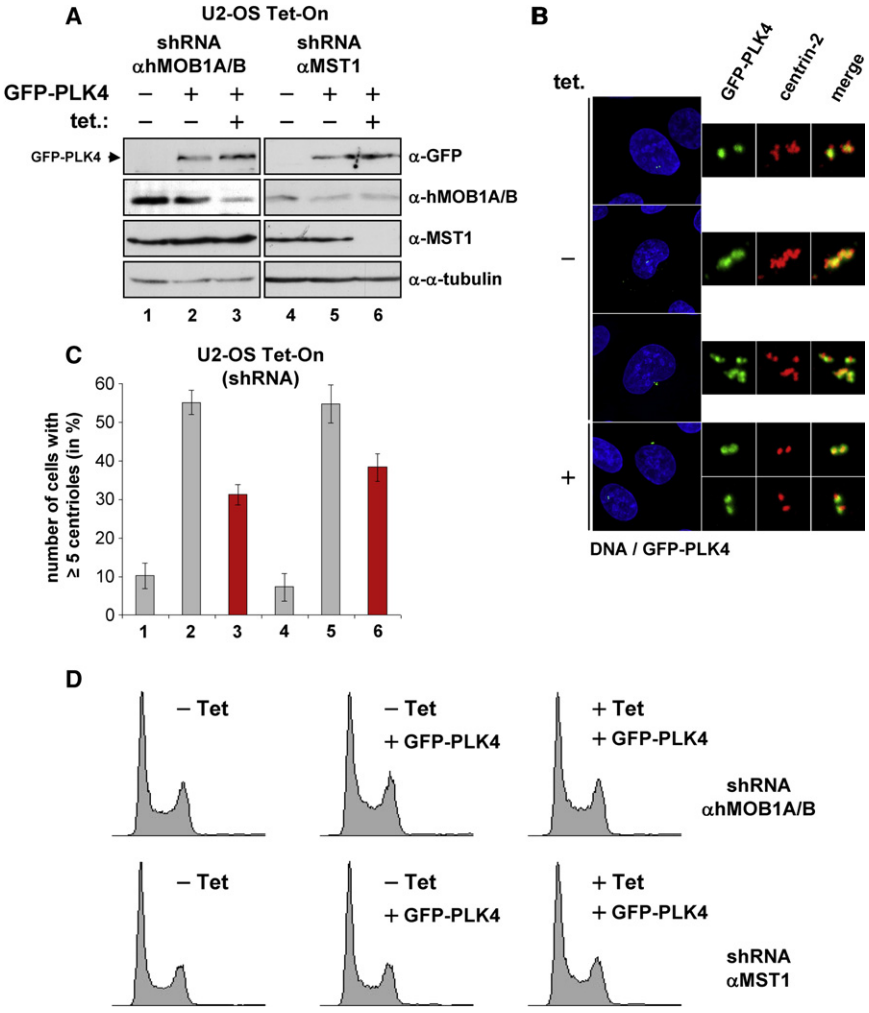

Figure 6. PLK4-driven centriole biogenesis is impaired upon hMOB1A/B or MST1 depletion

(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

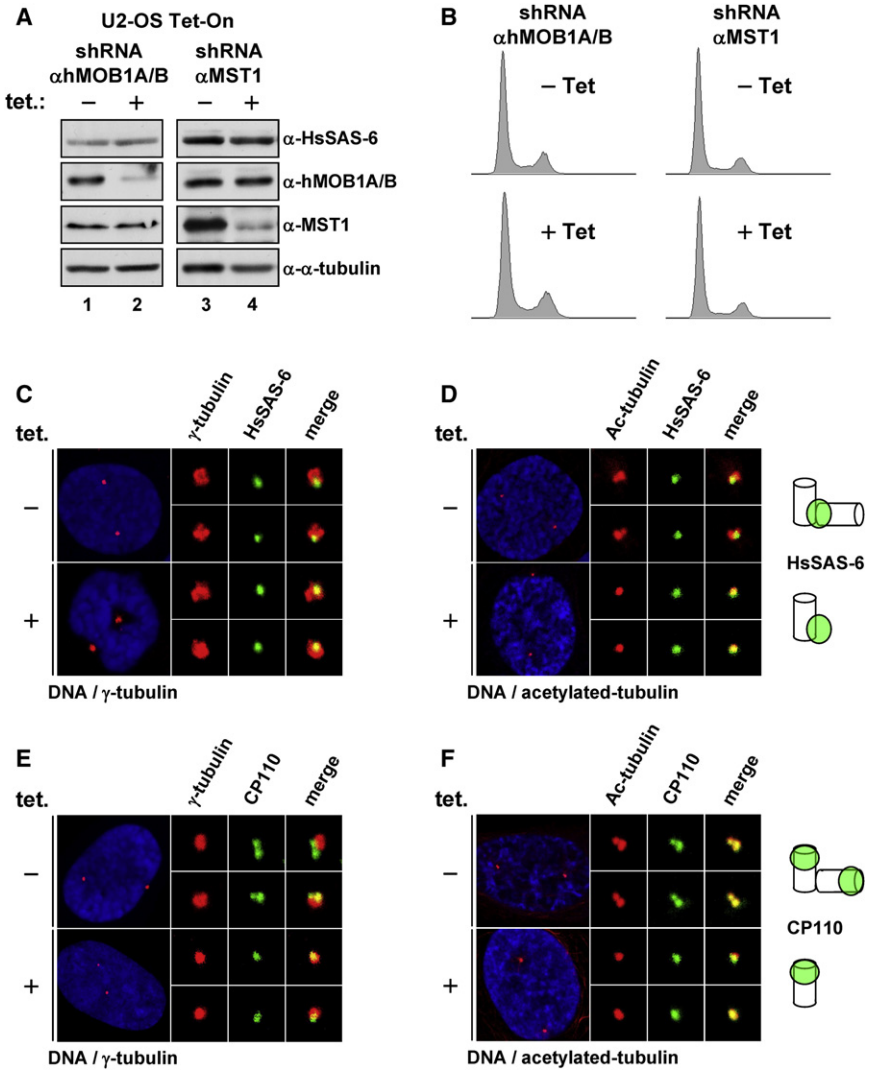

Figure 7. Centriole Localization of CP110, but Not of HsSAS-6, Depends on MST1/hMOB1 Signaling

(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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8. Curriculum Vitae

Personal Information

Reto Kohler
Winkelfeldstrasse 6,
4566 Halten, Switzerland
Date of Birth: 15 February 1982
Nationality: Swiss
reto.kohler@fmi.ch

Education and Professional Training

Since 10/2006	Friedrich Miescher Institute for Biomedical Research, Basel, Switzerland
	Phd studies in biochemistry in the laboratory of Brian A. Hemmings
09/2006	Friedrich Miescher Institute for Biomedical Research, Basel, Biozentrum, University of Basel, Switzerland
	MSc thesis in the laboratory of Brian A. Hemmings: "The Quest for Substrates of Human NDR Kinases"
10/2001 – 09/2006	MSc studies in Molecular Biology, Major in Biochemistry Biozentrum, University of Basel, Switzerland
08/2004-11/2004	Institute of applied microbiology, University of Natural resources and Applied Sciences, Vienna, Austria
	Practical Internship

Publications

Cornils H*, **Kohler RS***, Hergovich A and Hemmings BA (2011) Human NDR Kinases Control G1/S Cell Cycle Transition by Directly Regulating p21 Stability. *Mol Cell Biol 31*, 1382-1395. *equal contribution.

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Attended Conferences and Presentations

11/2010	The 2nd Workshop on the Hippo Tumor Suppressor Pathway, Rome, Italy Oral presentation (in place of Brian Hemmings)
09/2010	FMI 40th Anniversary Symposium, Basel, Switzerland Poster presentation
12/2006	Targeting the Kinome, Basel, Switzerland
2005-2009	Internal FMI annual meeting Poster presentation