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

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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, tricorned (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 relocation 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-

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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 tri-cornered 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 myristoylation/palmitoylation 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'-GATCCCGCTGGTGACGGATGAGGA CTTC AAGAGAGTCCTCATCCGTCACCGCTTTTGGAAA-3' (targeting sequences of the hMOB2 coding sequence are underlined) and 5'-AGC TTTTCAAAAAGCTGGTGACGGATGAGGACTCTCTTGAAGTCCT CATCCGTCACCGCTGG-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 μ g/ml) in combination with cycloheximide (CHX) (10 μ g/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 μ g/ml hygromycin B (InvivoGen). Stable transformants were maintained in DMEM supplemented with 0.5 mg/ml G418 and 50 μ g/ml hygromycin B. Expression of myc-hMOB2 variants was induced by the addition of 2 μ g/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 benzamide, 4 μ M leupeptin, 0.5 mM phenylmethylsulfonyl fluoride [PMSF], 1 μ M 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	MOB1 α , MOBKL1B, MOBKL1B, MOB4B, hMats1
hMOB1B	95	216	MOB1 β , 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(Δ NTR), 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(Δ 26)] still interacted with hMOB2 (Fig. 3F, top, lane 3), whereas an NDR1 mutant lacking the first 33 residues [NDR1(Δ 33)] 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 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 anti-

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,

bodies. 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.

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 μ M 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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