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Constitutively active NDR1-PIF kinase functions independent of MST1 and hMOB1 signalling

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Abstract

The human MST1/hMOB1/NDR1 tumour suppressor cascade regulates important cellular processes, such as centrosome duplication. hMOB1/NDR1 complex formation appears to be essential for NDR1 activation by autophosphorylation on Ser281 and hydrophobic motif (HM) phosphorylation at Thr444 by MST1. To dissect these mechanistic relationships in MST1/hMOB1/NDR signalling, we designed NDR1 variants carrying modifications that mimic HM phosphorylation and/or abolish hMOB1/NDR1 interactions. Significantly, the analyses of these variants revealed that NDR1-PIF, an NDR1 variant containing the PRK2 hydrophobic motif, remains hyperactive independent of hMOB1/NDR1-PIF complex formation. In contrast, as reported for the T444A phospho-acceptor mutant, NDR1 versions carrying single phospho-mimicking mutations at the HM phosphorylation site, namely T444D or T444E, do not display increased kinase activities. Collectively, these observations suggest that in cells Thr444 phosphorylation by MST1 depends on the hMOB1/NDR1 association, while Ser281 autophosphorylation of NDR1 can occur independently. By testing centrosome-targeted NDR1 variants in NDR1- or MST1-depleted cells, we further observed that centrosome-enriched NDR1-PIF neither requires hMOB1 binding nor MST1 signalling to function in centrosome overduplication. Taken together, our biochemical and cell biological characterisation of NDR1 versions provides novel unexpected insights into the regulatory mechanisms of NDR1 and NDR1's role in centrosome duplication.

Keywords

intracellular kinase signalling; MST1/STK4; NDR1/STK38; MOB proteins; hydrophobic motif phosphorylation; centrosome duplication

1. Introduction

Most signal transduction cascades transmit signals through protein kinases, which consequently represent one of the largest superfamilies found in the human genome [1]. In particular members of the AGC (protein kinase A (PKA)/PKG/PKC-like) subfamily of

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protein kinases have crucial cellular functions in cell growth, metabolism, proliferation and survival [2]. All AGC kinases share structural similarities, and many AGC kinases require phosphorylation of two conserved regulatory sites for activation: one conserved Ser/Thr residue within the activation segment (also termed T-loop) and one within the C-terminal hydrophobic motif (HM). As exemplified by the biochemical and structural characterization of the AGC kinase, Akt/PKB [3, 4], both regulatory sites must be phosphorylated simultaneously to achieve full kinase activation.

The NDR(nuclear Dbf2-related)/LATS(large tumour suppressor) kinase family is a subgroup of AGC kinases and consists of four related serine/threonine protein kinases (NDR1/STK38, NDR2/STK38L, LATS1, and LATS2) in the human genome [1, 5]. In mammals, LATS1/2 kinases are central to the Hippo tumour suppressor pathway [6-9], and NDR kinases regulate essential processes, such as centrosome duplication [10, 11], cell cycle/mitotic progression [12-15], ciliogenesis [16], neuronal dendrite/synapse formation [17], and apoptotic signalling [18, 19], where the latter function appears to be important for suppression of tumour growth [20]. In spite of the rapid progress in deciphering functions of mammalian LATS1/2, the mechanism of NDR regulation by phosphorylation must serve as a model for LATS regulation [8]. Therefore, studies addressing the regulatory mechanisms of NDR kinases are also very relevant for understanding the regulation of mammalian LATS-Hippo signalling.

Previously, it was reported that human NDR1 is dramatically activated upon inhibition of protein phosphatase 2A by okadaic acid (OA) [21, 22], which is not surprising since NDR1, like many other AGC kinases, must be phosphorylated on Ser281 (T-loop phosphorylation) and Thr444 (HM phosphorylation) for full activation [21-25]. However, NDR kinases are unique among AGC kinases because they have a distinctive activation mechanism. Competitive binding of the co-activator hMOB1 or the inhibitor hMOB2 to a conserved N-terminal regulatory (NTR) domain of NDR1 regulates kinase activity [23, 24, 26]. Initially, it was thought that binding of hMOB1 to the NTR only stimulates Ser281 autophosphorylation, but recent evidence suggests that hMOB1/NDR complex formation plays also a role in HM phosphorylation of NDR1, since Thr444 phosphorylation by MST1 is hMOB1 dependent in human cells [10, 27]. Therefore, NDR1 is regulated by a multistep process involving HM phosphorylation on Thr444, autophosphorylation of Ser281, and NTR binding of hMOB1. However, the order and dependency of these regulatory events are currently unknown [8].

Here, we dissected these three signalling steps by examining the basal and OA-stimulated activities of NDR1 variants carrying modifications that mimic HM phosphorylation and/or abolish hMOB1/NDR1 complex formation. These efforts revealed that NDR1-PIF, an NDR1 variant containing the PRK2 hydrophobic motif, is hyperactive and maintains high levels of Ser281 autophosphorylation despite loss of hMOB1 binding. Moreover, by studying NDR1-PIF in context of the previously reported MST1/hMOB1/NDR1 centrosome duplication signalling cascade [10], we demonstrate that expression of centrosome targeted and hMOB1-binding deficient NDR1-PIF is sufficient to restore centrosome amplification in MST1-depleted cells, thereby showing that NDR1-PIF can function independent of hMOB1 and MST1 signalling in human cells.

2. Materials and methods

2.1 Cell culture and transfections

Cos-7, U2-OS and PT67 cells were maintained in DMEM supplemented with 10% foetal calf serum. Exponentially growing COS-7 cells were plated at a consistent confluence (1×10^6 cells/10-cm dish) and transfected the next day using Fugene 6 (Roche) or Metafectene Pro (Biontex) as described by the manufacturer. U2-OS cells were transfected with Lipofectamine 2000 (Invitrogen) as described by the manufacturer.

2.2 Cell treatments

In OA experiments, cells were treated for 60 minutes with 1 μM okadaic acid (OA; Enzo Life Sciences). Aphidicolin and tetracycline were purchased from Sigma.

2.3 Generation of stable cell lines

U2-OS Tet-on cells expressing tetracycline-regulated shRNA against NDR1, hMOB1A/B, or MST1 have been described previously [10, 11]. Retroviral pools of rescue cell lines were generated and maintained as described elsewhere [11].

2.4 Construction of plasmids

Human NDR1, hMOB1A, hMOB1B, and hMOB2 pcDNA3-based vectors have been described previously [10, 11, 24]. New NDR1 mutants described herein were generated by PCR-based mutagenesis. NDR1 cDNAs refractory to siRNA were generated by PCR mutagenesis introducing silent mutations into residues 128 to 134 of human NDR1 (termed 6N for changing 6 nucleotides in the siRNA targeting sequence) [11]. To obtain centrosome-targeted NDR1 constructs, human NDR1 cDNAs were subcloned into a pcDNA3-HA(haemagglution)-AKAP derivative using *Bam*HI and *Xho*I [11]. For rescue experiments NDR1 cDNAs and their corresponding N-terminal tags were subcloned into the retroviral vector pCMV (neo-retro) [28]. All constructs were confirmed by sequence analysis. Further details of the generation of constructs are available upon request.

2.5 Antibodies

The generation and purification of anti-Ser281-P, anti-NDR_{CTD}, and anti-hMOB1A/B antibodies have been described previously [10, 11, 22, 24]. Mouse monoclonal antibodies against human STK38/NDR1 were purchased from AbD Serotec and Abnova, respectively. Rat monoclonal anti-α-tubulin (YL1/2) and mouse monoclonal anti-HA 12CA5 and anti-myc 9E10 antibodies were used as hybridoma supernatants. Additional anti-HA antibodies were from Cell Signaling (C29F4) and Roche (3F10). Anti-myc (71D10) and anti-MST1 were from Cell Signaling. Anti-γ-tubulin (GTU-88) was from Sigma. Secondary antibodies were purchased from GE Healthcare.

2.6 Immunoblotting and immunoprecipitation

Immunoblotting and co-immunoprecipitations (co-IPs) were performed as described [24]. The characterisation of NDR1 binding to hMOB1/2 was performed in low-stringency buffer

(30 mM HEPES pH 7.4, 20 mM beta-glycerophosphate, 20 mM KCl, 1 mM EGTA, 2 mM NaF, 1 mM Na3VO4, 1% TX-100) supplemented with protease inhibitors.

2.7 HA-NDR kinase assays

Analysis of immunoprecipitated HA-NDR species was performed as described previously [24]. [γ-P^{32}]-ATP was from Hartmann Analytic GmbH. The NDR substrate peptide (KKRNRRLSVA) was purchased from Biomatik.

2.8 Immunofluorescence microscopy

Cells were processed for immunofluorescence as defined elsewhere [11, 24]. In brief, cells were fixed in ice-cold methanol for 5 min at −20°C, then rehydrated in PBS for 5 min at room temperature and incubated with anti-γ-tubulin (GTU-88; Sigma) and anti-HA (C29F4; Cell Signaling) antibodies. Secondary antibodies were from Jackson Immunoresearch. DNA was counterstained with TO-PRO-3 iodide (Invitrogen) or DAPI (Sigma). Coverslips were mounted in Vectashield medium (Vector Lab.). Images were obtained with a LSM510 meta confocal laser scanning microscope (Carl Zeiss Ltd.). Photographic images were processed using Imaris 4.0 (Bitplane AG) and Photoshop CS4 (Adobe Systems Inc).

3. Results

3.1 PIFtide based hydrophobic motif (HM) modifications in human NDR1 result in hyperactive NDR1 kinases, while phospho-mimetic T444D and T444E mutations do not

To develop a research tool allowing the examination of the importance of HM phosphorylation of human NDR1, we characterised NDR1 variants carrying different HM modifications (Figs. 1 and 2). Like the majority of AGC kinases, NDR1 is stimulated by C-terminal HM phosphorylation on Thr444 phosphorylation [2, 5]. To test whether Asp/Glu substitutions at Thr444 can mimic Thr444 phosphorylation, thereby possibly generating constitutively active NDR1, we generated NDR1 mutants carrying Thr444 to Asp (T444D) or Glu (T444E) substitutions (Fig. 1A). Next, immunoprecipitated NDR1 wild-type (wt), kinase-dead (kd; K118A), (T444D) and (T444E) were subjected to kinase assays (Figs. 1B and 1C, lanes 1 to 4). Confirming the specificity of our peptide kinase assay [24], NDR1(kd) did not display any activity (Fig. 1C). NDR1(T444D) and NDR1(T444E) did not show increased activities when compared to NDR1(wt) (Fig. 1C). Given that these Asp/Glu modifications are known to elevate the basal kinase activities of other AGC kinases (for example Ser473D/Ser474D in Akt/PKB kinases dramatically increases basal Akt/PKB activities [3, 29]), this observation suggest that single phospho-mimetic mutations at the HM of NDR1, namely T444D or T444E, are not sufficient to trigger NDR1 activation.

As alternative approach to mimic HM phosphorylation of human NDR1, we introduced additional changes to the HM of NDR1. Based on the observation that the HM of PRK2 (PKC related kinase 2) functions as a potent mimic of HM phosphorylation in some AGC kinases, such as Akt/PKB [3, 4], the C-terminus of NDR1 was replaced by the HM of PRK2 to obtain NDR1-PIF (Fig. 1A). Furthermore, by introducing six changes in addition to the T444D modification we generated NDR1(FIDFDY), which represents a hybrid of the NDR1(T444D) and NDR1-PIF versions (Fig. 1A). Next, the kinase activities of

NDR1(FIDFDY) and NDR1-PIF were determined (Figs. 1B and 1C), revealing that NDR1(FIDFDY) was 6-fold and NDR1-PIF 20-fold more active than wild-type (Fig. 1C). Kinase activities were dependent on intact catalytic domains, since alterations of the ATP binding pocket by introducing a K118A mutation abolished kinase activities (Fig. 1C). Noteworthy, this activity increase of human NDR1-PIF is in full agreement with the reported elevation of human NDR2 and mouse NDR activities when carrying similar PIF modifications [17, 30]. However, considering that human NDR1-PIF has not yet been described and that the reported NDR-PIF versions [17, 30] were not examined with respect to autophosphorylation and hMOB1 binding, we expanded our characterisation of NDR1-PIF.

Considering that NDR1 is maximal activated by OA treatment of cells [5, 21], we compared OA activation of NDR1(T444D), NDR1(T444E), NDR1(FIDFDY) and NDR1-PIF (Fig. 2). The essential Ser281 autophosphorylation of NDR1 was examined (Fig. 2A), in addition to monitoring kinase activities by peptide kinase assays (Fig. 2B), allowing us to compare changes in auto- and trans-phosphorylation activities. As reported [22, 24], NDR1(wt) displayed dramatically increased Ser281 phosphorylation and was activated about 60-fold by OA treatment (Figs. 2A and 2B). In stark contrast, NDR1(T444A), NDR1(T444D) and NDR1(T444E) showed decreased Ser281 phosphorylation and more than 75% reduction in kinase activities when compared to OA-stimulated NDR1(wt) (Figs. 2A and 2B). NDR1(FIDFDY) and NDR1-PIF were activated by OA similarly to NDR1(wt) (Figs. 2A and 2B). Intriguingly, Ser281 phosphorylation of NDR1(FIDFDY) and NDR1-PIF was already elevated under basal conditions (Fig. 2A), indicating that NDR1(FIDFDY) and NDR1-PIF have increased basal auto- and trans phosphorylation activities. As expected, the kinase-dead NDR1(FIDFDY/kd) and NDR1(PIF/kd) versions displayed no kinase activities (Figs. 2A and 2B).

Taken together, the findings described in Figs. 1 and 2 strongly suggest that T444D and T444E substitutions at the regulatory Thr444 phosphorylation sites are not sufficient to transform NDR1 into a constitutively active kinase. The phospho-acceptor mutant NDR1(T444A) also had dramatically diminished auto- and trans-phosphorylation activities as judged by Ser281 and peptide phosphorylation events. In contrast, NDR1(FIDFDY) and NDR1-PIF displayed elevated basal activities, which were further amplified upon OA treatment, indicating that PIFtide based HM modifications of NDR1 function as Thr444 phospho-mimetic mutations of NDR1.

3.2 NDR1-PIF compensates for loss of NDR1 function in centrosome overduplication

To functionally characterise our panel of NDR1 HM variants, we chose the cell biological context of the previously reported role of NDR1 in centrosome overduplication [10, 11]. To do so, HA-tagged versions of short-hairpin RNA (shRNA)-resistant NDR1(wt), (T444D), NDR1(T444E), NDR1(FIDFDY) and NDR1-PIF were introduced into U2-OS cells stably expressing inducible shRNAs directed against human NDR1 (Fig. 3A). As reported previously for NDR1(wt), NDR1(kd), and NDR1(T444A) [10, 11], this approach allowed us to determine which NDR1 variant can compensate for the loss of function of endogenous NDR1 in centrosome overduplication assays. U2-OS cells expressing these selected NDR1

variants (Fig. 3A) were arrested in S-phase for a prolonged time and then analyzed by immunofluorescence microscopy for the number of centrosomes per mononucleated cell (Figs. 3B and 3C). In full agreement with previous reports [10, 11], centrosome overduplication was significantly decreased in NDR1-depleted cells and expression of shRNA-resistant NDR1(wt) allowed centrosome amplification in NDR1-depleted cells (Figs. 3A and 3C, lanes 2 to 5). In contrast, U2-OS cells expressing shRNA-resistant NDR1(T444D) or NDR1(T444E) did not compensate for NDR1 silencing (Figs. 3A and 3C, lanes 6 to 9), while expression of shRNA-resistant NDR1(FIDFDY) or NDR1-PIF restored centrosome overduplication (Figs. 3A and 3C, lanes 10 to 13). Noteworthy, the expression and subcellular localizations of the tested NDR1 variants were not obviously affected in our experimental settings (Figs. 3A and 3B; data not shown). Therefore, these data indicate that only NDR1 versions displaying normal OA-inducible activities (see Fig. 2) can compensate for the loss of NDR1 in our settings. While NDR1(T444D) and NDR1(T444E) failed to compensate for NDR1 depletion, as already reported for NDR1(T444A) [10], NDR1(FIDFDY) or NDR1-PIF were functional (Fig. 3). In summary, these findings indicate that NDR1(FIDFDY) and NDR1-PIF are suitable tools to examine the role and regulation of NDR1 in centrosome duplication, while NDR1(T444D) and NDR1(T444E) are not.

3.3 hMOB1A/B and hMOB2 binding to NDR1-PIF does not correlate with increased NDR1-PIF kinase activity

Given that the C-terminus of NDR1 is dispensable for all reported subcellular distribution patterns of NDR1 to the nucleus, cytoplasm, membrane and centrosome [11, 24], it was not surprising that the subcellular localisations of NDR1(T444D), NDR1(T444E), NDR1(FIDFDY) and NDR1-PIF were not obviously altered (Fig. 3; data not shown), and hence could not explain the observed differences with respect to kinase activities (Figs. 1 and 2) and cellular function (Fig. 3). Therefore, since NDR1(FIDFDY) and NDR1-PIF displayed increased Ser281 autophosphorylation activities (Fig. 2), and Ser281 phosphorylation depends on NDR1/hMOB1 complex formation according to previous reports [10, 23, 27], we speculated that increased binding of the co-activator hMOB1 to NDR1(FIDFDY) and NDR1-PIF might explain the observed changes in activities. In addition, we addressed hMOB2 binding to NDR1 [26], since reduced hMOB2/NDR1 complex formation might also help to explain the differences in kinase activities of our NDR1 HM variants. Consequently, the hMOB1 and hMOB2 binding properties of NDR1(wt), NDR1(T444D), NDR1(T444E), NDR1(FIDFDY) and NDR1-PIF were compared by co-immunoprecipitation assays (Fig. 4). To obtain a complete picture, NDR1 binding to both hMOB1 isoforms, hMOB1A and hMOB1B [27], was examined, revealing that NDR1(T444E) and NDR1(FIDFDY) bound normally to hMOB1A and displayed slightly reduced binding to hMOB1B (Figs. 4A and 4B). Unexpectedly, NDR1(T444D) and NDR1-PIF associated with hMOB1A and hMOB1B only very weakly when compared to NDR1(wt) (Figs. 4A and 4B). All NDR1 variants associated normally with hMOB2 (Fig. 4C), thereby ruling out the possibility that loss of binding of NDR1 to hMOB2 is triggering increased activities. Taking into account that hMOB1 and hMOB2 bind to the same NTR domain of NDR1 [23, 24, 26], these findings further suggest that the dramatic reduction of NDR1-PIF binding to hMOB1 cannot be attributed to a general loss of function of the NTR domain, since this would cause a loss of hMOB1 and hMOB2 binding, which is not the case.

In summary, these observations indicate that changes in hMOB1 or hMOB2 binding cannot explain the increased kinase activities of NDR1(FIDFDY) and NDR1-PIF. Rather these findings suggest that hMOB1 binding to NDR1-PIF might not play a central role in NDR1-PIF activation.

Therefore, we examined next the importance of endogenous hMOB1 for the activation of NDR1-PIF (Fig. 5). HA-tagged NDR1(wt) and NDR1-PIF were expressed in U2-OS cells depleted of both hMOB1 isoforms (hMOB1A/B), and subsequently cell lysates were processed for immunoprecipitation using anti-HA antibody (Fig. 5A). In full agreement with previous reports studying exogenous proteins [23, 24], this experiment revealed that the auto- and peptide-phosphorylation activities of NDR1(wt) are dependent on endogenous hMOB1 (Figs. 5A and 5B). In contrast, neither Ser281 nor peptide phosphorylation activities of NDR1-PIF were significantly affected upon hMOB1 depletion (Figs. 5A and 5B). Collectively, the data presented in Figs. 4 and 5 indicate that NDR1-PIF is hyperactive irrespective of efficient hMOB1 binding, possibly because hMOB1/NDR1-PIF complex formation is dispensable for NDR1-PIF activity.

3.4 NDR1-PIF displays auto- and trans-phosphorylation activities completely independent of hMOB1A/B binding

Before addressing the importance of hMOB1/NDR1-PIF complex formation, we confirmed the importance of Ser281 phosphorylation for NDR1-PIF activity (Supplementary Fig. S1). To do so, the phospho-acceptor residue Ser281 of NDR1-PIF was mutated to Ala, and then the activity of NDR1(S281A/PIF) was compared to NDR1-PIF. As already reported for NDR1(wt) [21, 22, 24], this revealed that Ser281 phosphorylation of NDR1-PIF is essential for kinase activity (Supplementary Fig. S1).

Next, we studied the importance of hMOB1/NDR1-PIF complex formation using NDR1 mutants deficient in hMOB1 binding (Fig. 6). In addition to HM modifications, Tyr31 or Arg41 of NDR1 were mutated to Ala (Y31A or R41A), since these mutations within the NTR domain of NDR1 are known to abolish hMOB1 binding without affection hMOB2/NDR1 interactions [10, 23, 24, 26]. As positive control, NDR1(wt) was fully activated upon OA treatment (Figs. 6A and 6B). In full support of our previous observations with NDR1(T444D) and NDR1(T444E) (see Fig. 2), NDR1(Y31A/T444D) and NDR1(Y31A/T444E) were not activated by OA stimulation (Figs. 6A and 6B). Similar results were obtained with NDR1(R41A/T444D) and NDR1(R41A/T444E) (data not shown). NDR1(Y31A/FIDFDY) and NDR1(R41A/FIDFDY) also did not display any significant activation upon OA stimulation (Figs. 6A and 6B). Cumulatively, these results suggest that hMOB1 binding is essential for basal and OA-stimulated activities of NDR1(T444D), NDR1(T444E), and NDR1(FIDFDY).

In contrast, NDR1(Y31A/PIF) and NDR1(R41A/PIF) maintained elevated basal activities and could be partially activated by OA stimulation (Fig. 6B). To our surprise, basal and OA-stimulated Ser281 autophosphorylation of NDR1(Y31A/PIF) and NDR1(R41A/PIF) was not affected when compared to OA-treated NDR1(wt), while trans-phosphorylation activities were reduced in comparison to fully activated wild-type NDR1 (Figs. 6A and 6B). Significantly, this indicates that Ser281 phosphorylation can occur completely independent

of hMOB1 binding once the HM has been modified to mimic phosphorylation. Furthermore, these data suggest that single analysis of Ser281 autophosphorylation (Fig. 6A) or peptide trans-phosphorylation (Fig. 6B) could lead to a misinterpretation of NDR1 kinase activities. Taken together, the findings shown in Fig. 6 demonstrate that Ser281 autophosphorylation of NDR1-PIF can take place without any hMOB1 binding to NDR1-PIF and further suggest that NDR1(Y31A/PIF) and NDR1(R41A/PIF) might represent suitable tools to dissect the functional (in-)dependency of NDR1-PIF with respect to upstream hMOB1 and MST1 signalling.

3.5 NDR1-PIF drives centrosome amplification independent of hMOB1A/B and MST1 signalling

To study NDR1(Y31A/PIF) and NDR1(R41A/PIF) in a functional setting, centrosome overduplication assays were performed in U2-OS cells expressing these NDR1 variants upon MST1 manipulation (Fig. 7 and Supplementary Fig. S2). This approach allowed us to examine simultaneously whether NDR1(Y31A/PIF) and NDR1(R41A/PIF) can function independent of hMOB1 binding (due to the Y31A and R41A mutations) and whether the PIF modification of the HM of NDR1 can mimic the Thr444 phosphorylation of NDR1 by MST1, and thereby compensate for the loss of endogenous MST1. Tagged cDNAs of NDR1(wt), NDR1(Y31A/PIF) and NDR1(R41A/PIF) were stably introduced into U2-OS cells expressing tetracycline-inducible shRNA directed against human MST1 (Fig. 7A), and after prolonged arrest in S-phase the number of centrosomes per cell was determined (Fig. 7B). In full agreement with a previous report [10], centrosome overduplication was reduced in MST1-depleted cells upon arrest in S-phase (Figs. 7A and 7B). Expression of NDR1(wt) did not restore centrosome amplification upon MST1 depletion (Figs. 7A and 7B), indicating that elevated basal activity of NDR1 is not sufficient to compensate for MST1 loss of function. Significantly, expression of NDR1(Y31A/PIF) or NDR1(R41A/PIF) restored normal centrosome overduplication in MST1-depleted cells (Figs. 7 and S2). NDR1(Y31A/PIF/kd) and NDR1(R41A/PIF/kd) carrying a K118A mutation in the catalytic domain were also tested (Figs. 7 and S2), revealing that the compensation by NDR1(Y31A/PIF) or NDR1(R41A/PIF) was kinase activity dependent. The expression and subcellular localizations of NDR1(Y31A/PIF) and NDR1(R41A/PIF) were not obviously affected in comparison to NDR1(wt) (Figs. 7 and S2; data not shown). Taken together, these findings indicate that active NDR1(Y31A/PIF) and NDR1(R41A/PIF) kinases can compensate for diminished MST1 signalling in spite of carrying mutations that abolish hMOB1 binding.

3.6 Centrosome-targeted NDR1 compensates for NDR1- or MST1-depletion in centrosome overduplication assays

Given that only a subpopulation of NDR1 associates with centrosomes, while the majority of NDR is cytoplasmic [10, 11, 24], we tested the importance of centrosome-enriched NDR1 in our settings. More specifically, to examine whether the centrosomal pool of NDR1 is sufficient to function in the regulation of centrosome duplication, we fused NDR1(wt), NDR1(Y31A/PIF) and NDR1(R41A/PIF) to the centrosome targeting domain of AKAP450, a mainly pericentriolar matrix protein [11, 31]. AKAP-NDR1 fusions of shRNA-resistant NDR1(wt), NDR1(Y31A/PIF) and NDR1(R41A/PIF) were introduced into U2-OS cells stably expressing tetracycline-inducible shRNAs directed against human NDR1 (Fig. 8A),

and the number of centrosomes per cell was determined by immunofluorescence microscopy (Fig. 8B). In full agreement with our previous observations [see Fig. 3; and [10, 11]] centrosome overduplication was reduced in NDR1-depleted cells (Figs. 8A and 8C). Expression of AKAP-NDR1(wt) at endogenous level restored centrosome amplification upon NDR1 depletion (Figs. 8A and 8C). Expression of AKAPNDR1(Y31A/PIF) or AKAP-NDR1(R41A/PIF) also compensated for loss of NDR1 function (Figs. 8A and 8C), suggesting that binding of hMOB1 to NDR1 is dispensable for centrosome overduplication once active NDR1 is enriched at centrosomes.

To study whether centrosome-enriched NDR1(wt), NDR1(Y31A/PIF) or NDR1(R41A/PIF) also restore centrosome amplification upon diminished MST1 signalling, AKAP-NDR1 fusions were introduced into U2-OS cells stably expressing tetracycline-inducible shRNAs directed against human MST1 (Fig. 9A). As already reported [10], MST1 depletion resulted in reduced centrosome overduplication (Figs. 9A and 9B). Expression of AKAP-NDR1(wt) only partially restored this defect (Figs. 9A and 9B), suggesting that increased basal NDR1 activity at centrosomes is not sufficient to drive efficient centrosome amplification upon MST1 depletion. In contrast, AKAP-NDR1(Y31A/PIF) or AKAP-NDR1(R41A/PIF) restored centrosome overduplication in MST1-depleted cells (Figs. 9A and 9B), indicating that NDR1 can function in centrosome duplication without hMOB1 binding and independent of MST1 signalling once NDR1 is enriched on centrosomes and the C-terminus of NDR1 has been modified to mimic Thr444 phosphorylation by MST1, the upstream HM kinase of NDR1 in this specific setting.

4. Discussion

Taken together, our study provides novel insights into the regulatory mechanisms of human NDR1 kinase and the role of NDR1 in centrosome duplication. PIFtide based HM modifications of NDR1 result in increased kinase activities (Figs. 1 and 2), which cannot be explained by altered binding of NDR1 to the co-activator hMOB1 or the inhibitor hMOB2 (Figs. 4 and 5). The analysis of hMOB1 binding deficient NDR1-PIF further demonstrated that NDR1-PIF remains active independent of hMOB1/NDR1-PIF complex formation (Figs. 6). Significantly, this analysis revealed further that Ser281 autophosphorylation of NDR1-PIF occurs independent of hMOB1 binding. Moreover, by studying selected NDR1 variants in NDR1- or MST1-depleted cells (Figs. 3, 7, 8 and 9), we further show that centrosome-enriched NDR1-PIF can compensate for the loss of hMOB1 and MST1 signalling in centrosome overduplication.

The current model of NDR1 activation involves at least three different steps: (1) binding of hMOB1 to the NTR domain, (2) autophosphorylation of Ser281 at the T-loop, and (3) HM phosphorylation on Thr444 by members of the MST kinase family [8]. Previously, Millward et al. established that Ser281- and Thr444-phosphorylations of NDR1 are essential for its activation by OA [21]. Significantly, the same study also showed that replacing Ser281 and/or Thr444 with negatively charged amino acids (Glu or Asp) was not sufficient to elevate basal NDR1 kinase activity [21]. Therefore, Hemmings and colleagues speculated that steric constraints in the T-loop and HM of NDR1 are too tight to allow effective mimicry of a phosphate group by a negatively charged amino acid side chain [21]. However,

this study neither examined the NDR1(T444D) and NDR1(T444E) mutants in a biological setting nor their activation by OA. Our work described here confirms that NDR1(T444D) and NDR1(T444E) do not display significantly elevated basal activities (Fig. 1) and demonstrates further for the first time that T444D and T444E mutants are only very modestly activated by OA treatment, very similar to T444A (Fig. 2). In addition, we observed that T444D and T444E mutants do not rescue centrosome overduplication upon NDR1 depletion (Fig. 3), suggesting that T444D and T444E mutants do not mimic Thr444 phosphorylation in these settings.

Consequently, we created constitutively active NDR1 by replacing the C-terminus of NDR1 by the HM domain of PRK2 (Fig. 1). As judged by dramatically increased auto- and trans-phosphorylation activities of NDR1-PIF (Figs. 1, 2, 5, 6 and S1), this approach successfully mimicked Thr444 phosphorylation and generated a constitutively active form of NDR1. NDR1-PIF further restored centrosome amplification upon NDR1 depletion (Fig. 3) indicating that NDR1-PIF is fully functional in mammalian cells. As already proposed for NDR2-PIF [30] it is very likely that NDR1-PIF functions in a manner analogous to the reported disorder-to-order conformational transition for Akt/PKB-PIF [3, 4]. Most likely, the C-terminal PIF sequence of NDR1-PIF allows the stabilisation of an intra-molecular interaction with the catalytic domain of NDR1, thereby facilitating a disorder-to-order transition of the kinase domain. Consequently, the activation segment surrounding the Ser281 site and other catalytic sites would be restructured, supporting efficient Ser281 autophosphorylation and substrate trans-phosphorylation. However, since crystal structures of member of the NDR kinase family [5, 8] have not been documented so far, the conformational changes of NDR1 as a consequence of Thr444 phosphorylation (or PIF-like alterations) are yet to be addressed experimentally.

Previous studies by the Hemmings laboratory also suggested that Ser281 is not phosphorylated by PDK1 like many other AGC kinases [2], but rather a result of hMOB1 binding to the NTR domain of NDR1, which stimulates the autophosphorylation of NDR1 on Ser281 [21-23]. Most likely, the explanation for this specific Ser281 autophosphorylation is an unusual structural feature of NDR1, namely an insert between the catalytic subdomains VII and VIII of NDR1, which is absent from all other known AGC kinases [5]. More specifically, this insert interrupts the canonical catalytic domain of AGC kinases, beginning C-terminally to the conserved magnesium-binding DFG motif and ending directly N-terminal to Ser281. Very likely, the close proximity of this insert to Ser281 favours the recognition of Ser281 by NDR1 itself, which is facilitated by hMOB1 binding to the NTR domain through a yet to be defined mechanism [8]. However, although these efforts established that NDR1 is regulated by a multistep process involving Ser281-, Thr444-phosphorylation and NTR binding of hMOB1, the order and co-dependency of regulatory events was not examined. Therefore, we particularly focused on understanding the relationship of hMOB1/NDR1 complex formation and regulatory phosphorylation events in mammalian cells. Here, we show that T444D and T444E mutants display severely decreased Ser281 autophosphorylation and that NDR1(T444A) is completely defective in Ser281 phosphorylation (Fig. 2). Moreover, NDR1-PIF showed strongly elevated basal Ser281 phosphorylation (Fig. 2), which is essential for NDR1-PIF activity but independent of complex formation with hMOB1 (Figs. 4, 5, 6 and S1). These unexpected observations

suggest that in mammalian cells Thr444 phosphorylation is instrumental for Ser281 phosphorylation.

These findings imply that once Thr444 phosphorylation has taken place, the subsequent conformational change of the C-terminal HM region is sufficient to support Ser281 phosphorylation due to the achieved proximity of the C-terminal HM region and the central T-loop of NDR1, as already described by the crystal structures of inactive and active Akt/PKB kinases [3, 4]. However, these structural interpretations of NDR1 based on reported crystal structures of other AGC kinases are complicated and remain speculative. All NDR kinases have unique features among all AGC kinases, in particular the insert in the catalytic domain that is not present in any other AGC kinase [5]. These specific features currently limit structural predictions and modelling of NDR1. Nevertheless, our comparative analyses of NDR1(wt), NDR1(FIDFDY) and NDR1-PIF (Figs. 1, 2 and 6) suggest that the flexibility (or the lack thereof) of the C-terminal HM domain of NDR1 is a key determinant of Ser281 phosphorylation and consequently NDR1 activity. NDR1-PIF displays about 20-fold elevated basal activity which is increased by about 2-fold upon OA treatment, while the NDR1(FIDFDY) basal activity is about 6-fold elevated and amplified nearly 10-fold upon OA treatment. Moreover, the NDR1(FIDFDY) activity is fully dependent on hMOB1 binding, while NDR1-PIF does not need to form a complex with hMOB1 to display auto- and trans-phosphorylation activities. These data suggest that on the structural level the FIDFDY modification results in a conformational change that supports the stabilisation of an intra-molecular interaction with the catalytic domain, while still allowing enough flexibility of the C-terminal HM domain to keep NDR1 activation dependent on the additional stabilisation by hMOB1 binding to the NTR domain. In contrast, the PIF modification appears to lock NDR1 in an active conformation with limited flexibility, which consequently renders NDR1-PIF independent of hMOB1 binding. To confirm, expand and challenge these structural predictions, the NDR/LATS signalling field now requires high-resolution crystal structures of NDR kinases, with particular emphasis on hMOB1 binding to NDR, Ser281 and Thr444 phosphorylation events and their conformational consequences.

As a whole, our characterization of NDR1 variants carrying HM modifications and hMOB1-binding abolishing mutations together with previously summarized work [8] proposes the following mode of NDR1 activation in mammalian cells (Supplementary Fig. S3): (1) hMOB1 binding to the NTR domain of NDR1 facilitates NDR1 phosphorylation on Thr444 by MST kinases, (2) followed by a conformational change due to Thr444 phosphorylation, (3) resulting in facilitated autophosphorylation of NDR1 on Ser281 (which is further supported by hMOB1 binding to the NTR domain of NDR1), finally (4) leading to full NDR1 activation due to additional conformational adjustments. This 4-step model proposes that hMOB1 binding to NDR1 could serve as rate-limiting step at the initial Thr444 phosphorylation of NDR1, which is in full agreement with our previous work, showing that hMOB1-binding deficient NDR1 is not phosphorylated on Thr444 in human cells [10]. Another prediction of this model is that activated NDR1 does not require hMOB1 binding to function in cells, which is supported by our findings described in Figs. 7, 8 and 9. Nevertheless, despite this advancement in our understanding of NDR1 activation, more future research is required. Besides taking on the challenge to solve the crystal structures of NDR kinases, we must understand how the complex MST and hMOB families [6, 8, 27] can

function upstream of NDR1. Perhaps other MST kinases can activate NDR1 by Thr444 phosphorylation independent of hMOB1. The competition between hMOB1 and hMOB2 for NDR1 binding [26] is also yet to be examined in our model. Possibly, given that MST-phosphorylated hMOB1 displays a higher affinity for NDR1 [32], the phosphorylation of hMOB1 by MST kinases could represent the initial trigger for the subsequent activation of NDR1, and hence warrants future investigations.

Importantly, since currently the mechanism of NDR regulation by phosphorylation and hMOB1 binding must serve as a model for LATS regulation [8], our study also expands our understanding of the regulation of mammalian LATS1/2 kinases. Very likely LATS1/2 kinases and other members of the NDR family [5] are regulated by a similar activation mechanism. Therefore, our research into the mechanism of NDR1 activation should be instrumental for a broad range of model organisms and cell systems, such as yeast, protozoan, invertebrate and perhaps even plants, besides serving as platform for mechanistic studies of LATS signalling in the Hippo pathway.

Besides further dissecting the activation mechanism of NDR1, we also investigated MST1/hMOB1/NDR1 signalling in centrosome overduplication, since NDR1 plays a role in this essential process [10, 11]. While these previous studies [10, 11] suggested that hMOB1 and MST1 are required for NDR1 to function in centrosome duplication, the importance of hMOB1/NDR1 complexes and HM phosphorylation of NDR1 by MST1 were not fully addressed. Moreover, the function of centrosome-associated NDR1 was not studied in these contexts. Given these open questions, we analysed a selection of NDR1 variants in NDR1- or MST1-depleted cells (Figs. 3, 7, 8 and 9), revealing that active centrosome-targeted NDR1 kinase is sufficient to drive centrosome duplication. Centrosome-targeted NDR1-PIF functioned in centrosome amplification without hMOB1 binding and independent of MST1 signalling, indicating that NDR1-PIF is suitable to dissect the downstream effects of MST1/hMOB1/NDR1 signalling in human centrosome duplication. Therefore, future research is warranted to identify the relevant NDR1 substrates with centrosome-targeted NDR1-PIF using a similar chemical genetics approach as recently applied for mouse NDR-PIF in neurons [17].

NDR1 signalling is also involved in the regulation of other essential cellular processes, such as cell cycle/mitotic progression, ciliogenesis, apoptosis, and neuronal dendrite/synapse formation [8]. Therefore, the activation mechanism and research tools defined here will also support the dissection of MST1/hMOB1/NDR1 signalling in these biological settings. Taking into account that centrosome-associated functions have started to gain more attention in neurobiology [33, 34], it will be especially interesting to study the role of the centrosome-enriched pool of NDR1 in neurons.

In summary, by defining different activation steps of NDR1 in mammalian cells, our report opens novel research avenues in the pursuit of MST1/hMOB1/NDR1 signalling in essential cell biological processes such as centrosome duplication, apoptosis signalling, and neuronal development. Possibly NDR1-PIF and other NDR1 variants described herein might even help to better our understanding of the recently reported link between NDR1 and c-myc [12, 13, 35, 36]. Our study also provides a framework for future studies aiming to dissect the

roles of the tumour suppressive LATS1/2 kinases downstream of the essential tumour suppressor proteins hMOB1 and MST1/2 in mammalian Hippo signalling.

5. Conclusion

In summary, our data presented in this study provide, for the first time, evidence suggesting that, once NDR1 has been modified to mimic Thr444 phosphorylation on the HM, Ser281 autophosphorylation of NDR1 can occur independent of complex formation with hMOB1. NDR1-PIF efficiently mimics Thr444 phosphorylation, remaining active even in the absence of hMOB1/NDR1-PIF complex formation. In human cells, centrosome-enriched and hMOB1-binding deficient NDR1-PIF compensates for NDR1- or MST1- depletion in centrosome overduplication assays. Taken together, these findings suggest that once NDR1 has been activated by Thr444 phosphorylation, the activities of hMOB1 and MST1 are not required anymore (1) to maintain elevated NDR1 kinase activity and (2) for NDR1 to support centrosome overduplication. Consequently, we provide novel insights into the regulatory mechanism of NDR1.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Abbreviations

NDR1	(Nuclear Dbf2-related 1)
MST1	(mammalian serine/threonine Ste20-like kinase 1)
STK	(serine/threonine kinase)
MOB	(Mps one binder)
HM	(hydrophobic motif)
PRK2	(PKC related kinase 2)
LATS	(large tumour suppressor)
PIF	(PDK1-interacting fragment)

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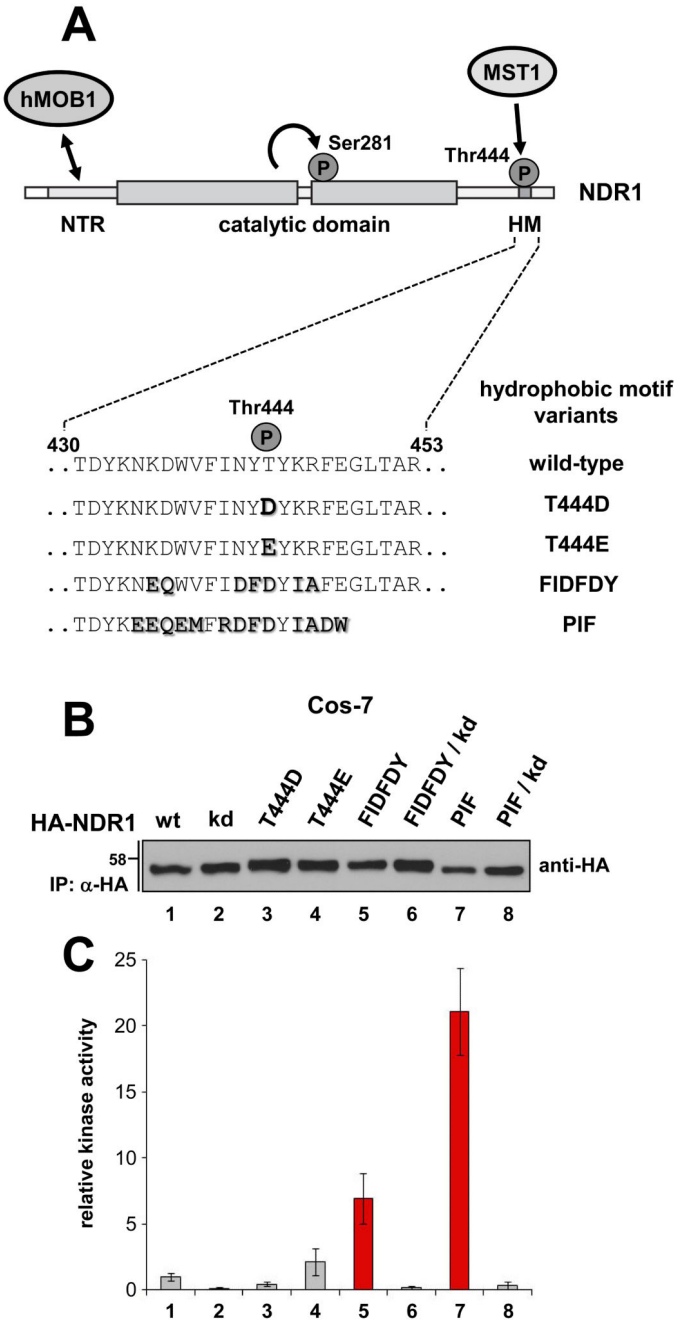

Figure 1. Characterisation of hydrophobic motif mutants of human NDR1 kinase

(A) Schematic representation of human NDR1 illustrating hMOB1 binding in the N-terminal regulatory region (NTR), Ser281 autophosphorylation in the catalytic domain, and hydrophobic motif (HM) phosphorylation on Thr444 by MST1 kinase. Alignment of the wild-type amino acid sequence of the region surrounding Thr444 and HM mutants tested herein. **(B)** Lysates of Cos-7 cells expressing indicated HA-tagged NDR1 variants (wild-type, wt; kinase-dead, kd) were subjected to immunoprecipitation (IP) using anti-HA 12CA5 antibody. Complexes were analysed by Western blotting using anti-HA antibody. Molecular

weight is shown. **(C)** In parallel, immunocomplexes were subjected to kinase assays. Cumulative data from three independent experiments with two replicates per experiment are shown. Error bars indicate standard deviations.

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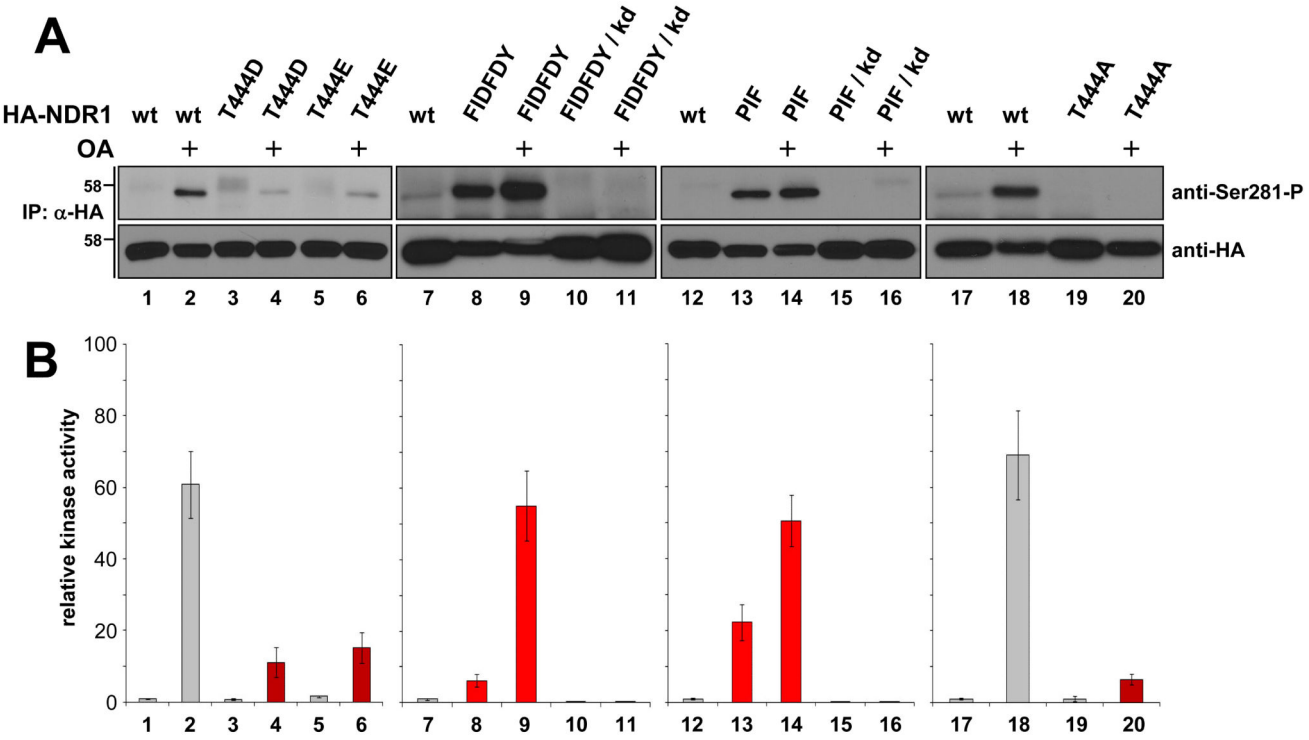

Figure 2. The NDR1(T444D) and NDR1(T444E) versions display impaired auto- and trans-phosphorylation activities

(A) Lysates of Cos-7 cells expressing indicated HA-tagged NDR1 versions (wild-type, wt) were subjected to immunoprecipitation (IP) using anti-HA 12CA5 antibody. Complexes were analysed by Western blotting using anti-Ser281-P (top panel) and anti-HA antibody (bottom panel). Prior to lysis, the marked cells (+) were incubated with okadaic acid (OA). Molecular weights are shown. **(B)** In parallel, immunocomplexes were subjected to kinase assays. Cumulative data from three independent experiments with two replicates per experiment are shown. Error bars indicate standard deviations. The wild-type, FIDFDY and PIF versions of NDR1 displayed a 60-fold increase in kinase activities upon OA treatment. In contrast, NDR1(T444D), NDR1(T444E) and NDR1(T444A) were only activated 5- to 15-fold (about 75-90% decrease in activation when compared to NDR1(wt)).

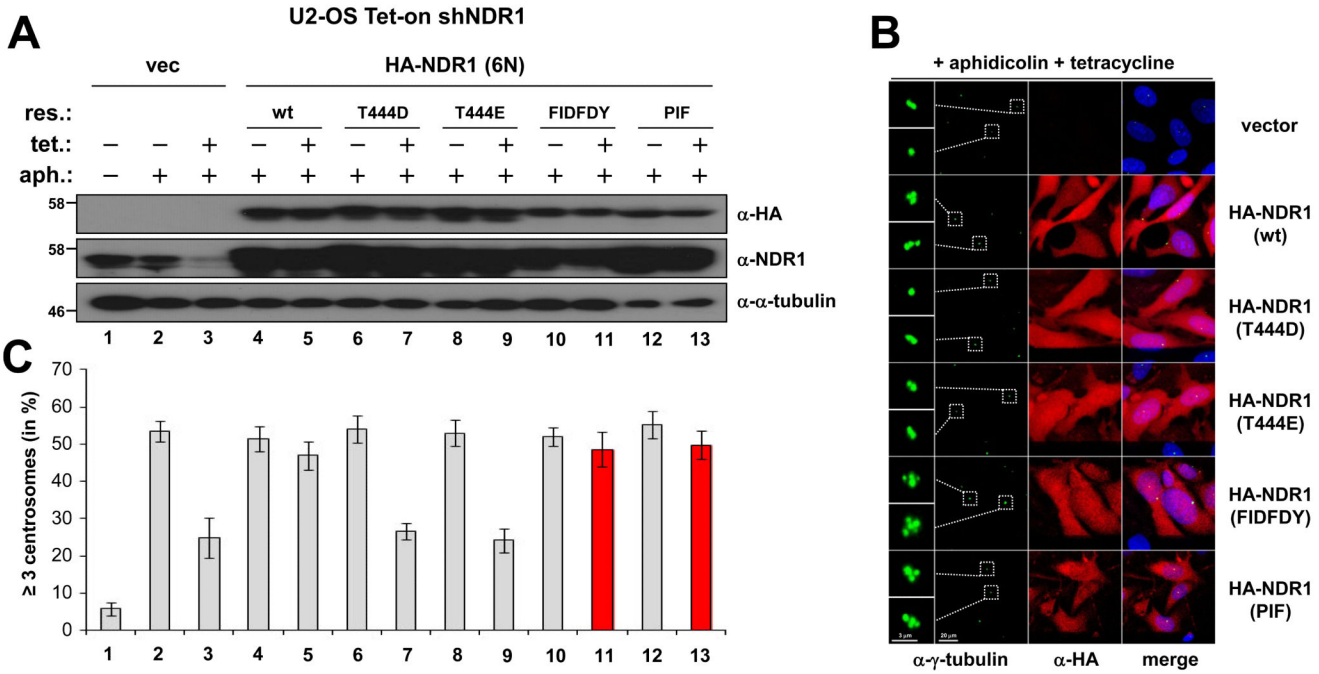

Figure 3. Active NDR1 versions drive centrosome overduplication, while NDR1(T444D) and NDR1(T444E) do not support centrosome amplification

(A) U2-OS cells stably expressing tetracycline-regulated short-hairpin RNA (shRNA) directed against human NDR1 were infected with empty vector (lanes 1 to 3), HA-NDR1 wild-type (lanes 4 and 5), or hydrophobic motif mutants (lanes 6 to 13) that are refractory to shRNA [HA-NDR1 (6N)]. After incubation for 72 hours without (-) or with (+) tetracycline (2 μg/ml) and for a further 72 hours with aphidicolin (2 μg/ml), cells were analysed by immunoblotting with indicated antibodies. Molecular weights are shown. **(B)** In parallel, cells were processed for immunofluorescence with anti-γ-tubulin (green) and anti-HA (red) antibodies. DNA is stained blue. Insets show enlargements of centrosomes in green. **(C)** Histograms showing percentages of cells with excess centrosomes (≥ 3). Cumulative data from three independent experiments with at least two replicates of 100 cells counted per experiment. Error bars indicate standard deviations.

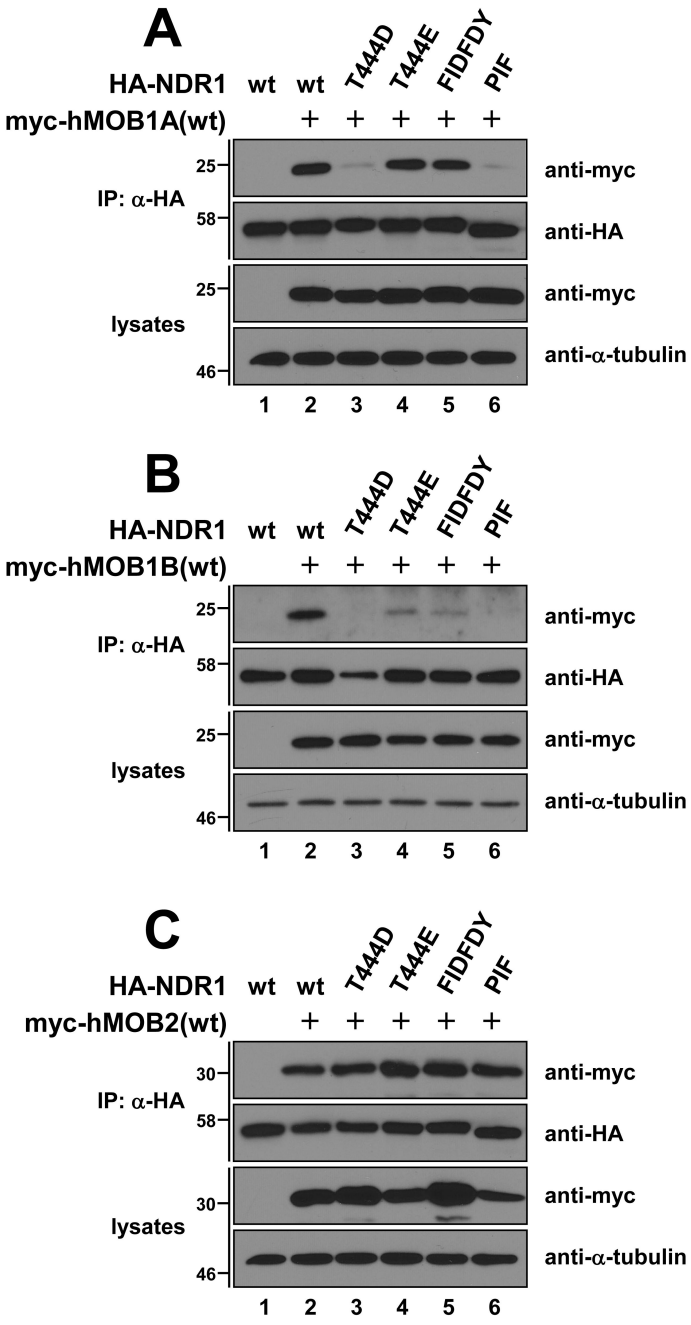

Figure 4. Increased kinase activities of NDR1 variants do not correlate with changes in hMOB1 and hMOB2 binding

(A-C) Lysates of Cos-7 cells expressing the indicated combinations of HA-tagged NDR1 forms and wild-type myc-tagged hMOB variants were analysed by immunoprecipitation (IP) using anti-HA 12CA5 antibody. Immunocomplexes were analysed by Western blotting using anti-myc 71D10 (top panel) and anti-HA 42F13 antibody (top middle panel). Input lysates were analysed with anti-myc 9E10 (bottom middle panel) and anti-α-tubulin YL1/2 antibody (bottom panel). Molecular weights are shown. Noteworthy, hyperactive NDR1-PIF

displays decreased binding to hMOB1A/B, while complex formation with hMOB2 is not impaired.

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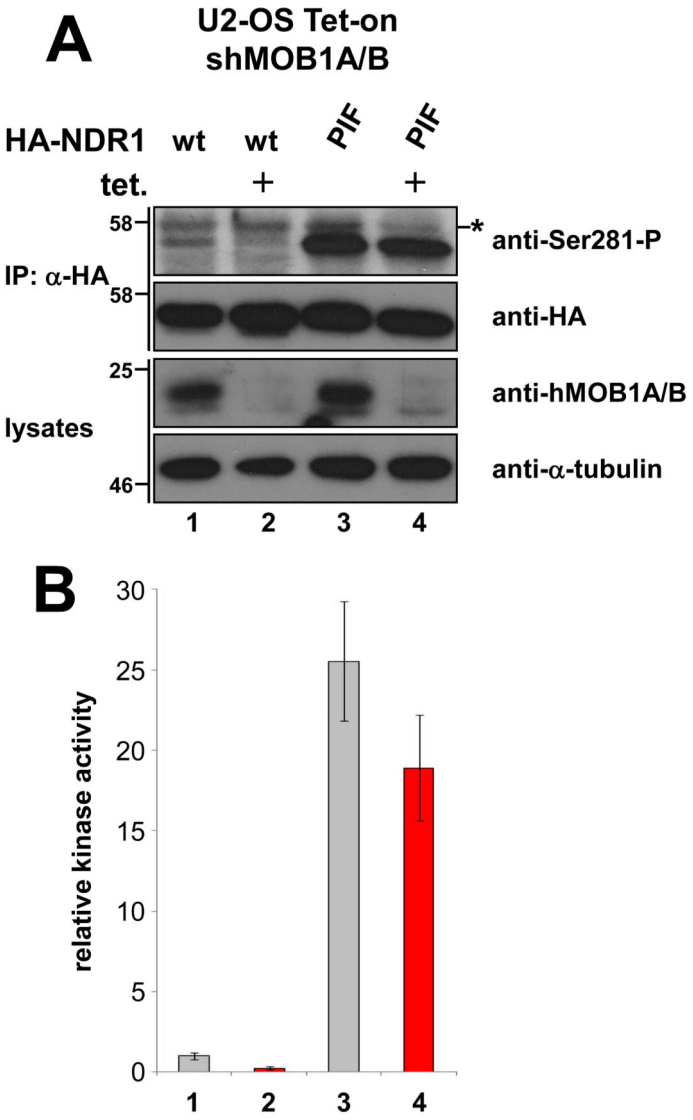

Figure 5. In contrast to NDR1-PIF, wild-type NDR1 requires endogenous hMOB1A/B for auto- and trans-phosphorylation activities

(A) U2-OS cells stably expressing tetracycline-regulated shRNA directed against hMOB1A/B were incubated for 72 hours without (-) or with (+) tetracycline before being transfected with indicated HA-NDR1 forms overnight. Subsequently, cells were subjected to immunoprecipitation (IP) using anti-HA 12CA5 antibody. Complexes and input lysates were analysed by Western blotting using indicated antibodies. Molecular weights are shown. An unspecific signal is indicated by an asterisk. **(B)** In parallel, immunocomplexes were subjected to kinase assays. Cumulative data from three independent experiments with two replicates per experiment are shown. Error bars indicate standard deviations.

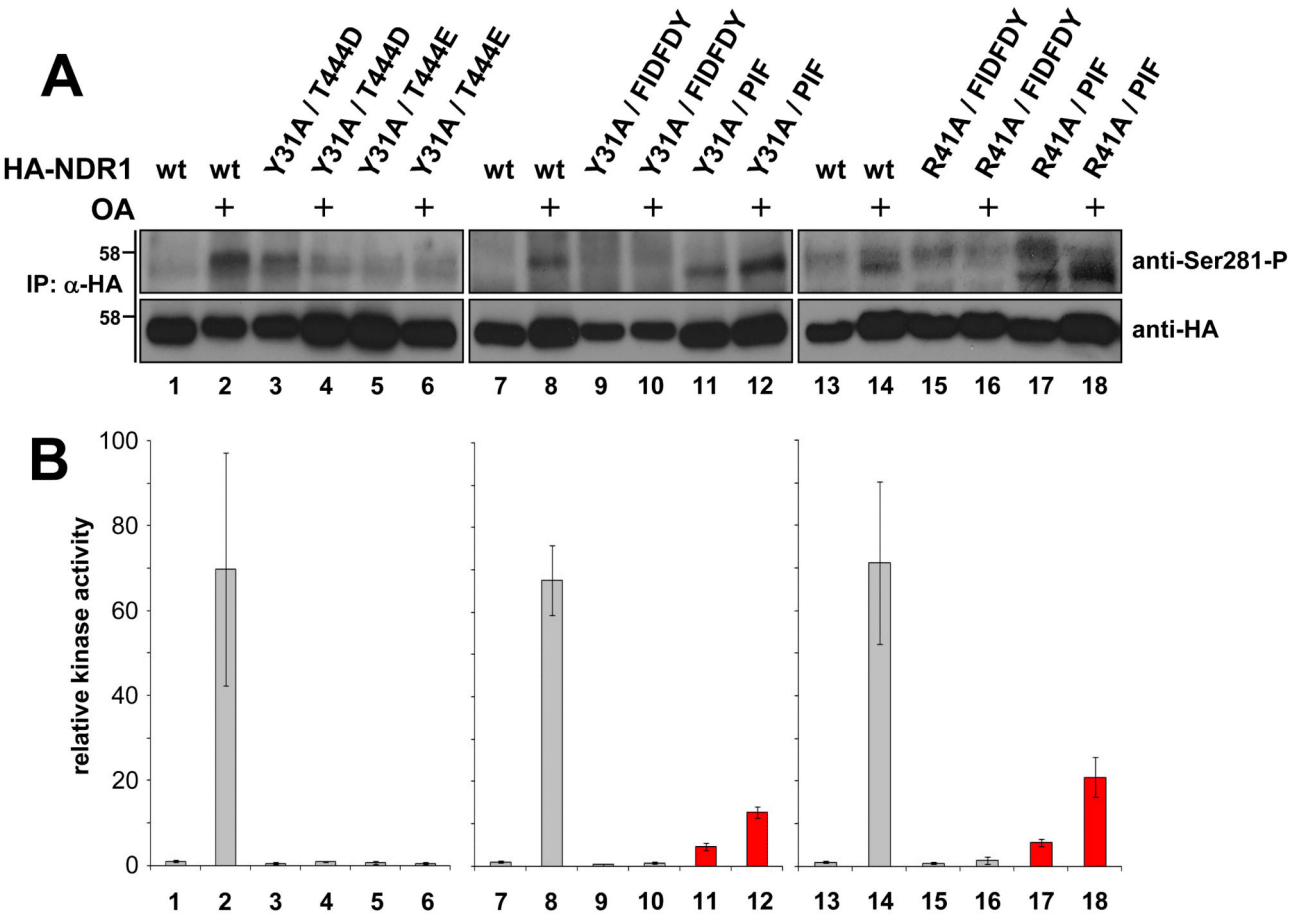

Figure 6. NDR1-PIF displays kinase activities despite abolished hMOB1A/B binding

(A) Lysates of Cos-7 cells expressing indicated HA-tagged NDR1 versions (wild-type, wt) were subjected to immunoprecipitation (IP) using anti-HA 12CA5 antibody. Complexes were analysed by Western blotting using indicated antibodies. Prior to lysis, the marked cells were incubated with OA (+). Molecular weights are shown. **(B)** In parallel, immunocomplexes were subjected to kinase assays. Cumulative data from three independent experiments with two replicates per experiment are shown. Error bars indicate standard deviations. Noteworthy, loss of hMOB1A/B binding (due to the Y31A or R41A mutations) did not block auto- and trans-phosphorylation activities of NDR1-PIF.

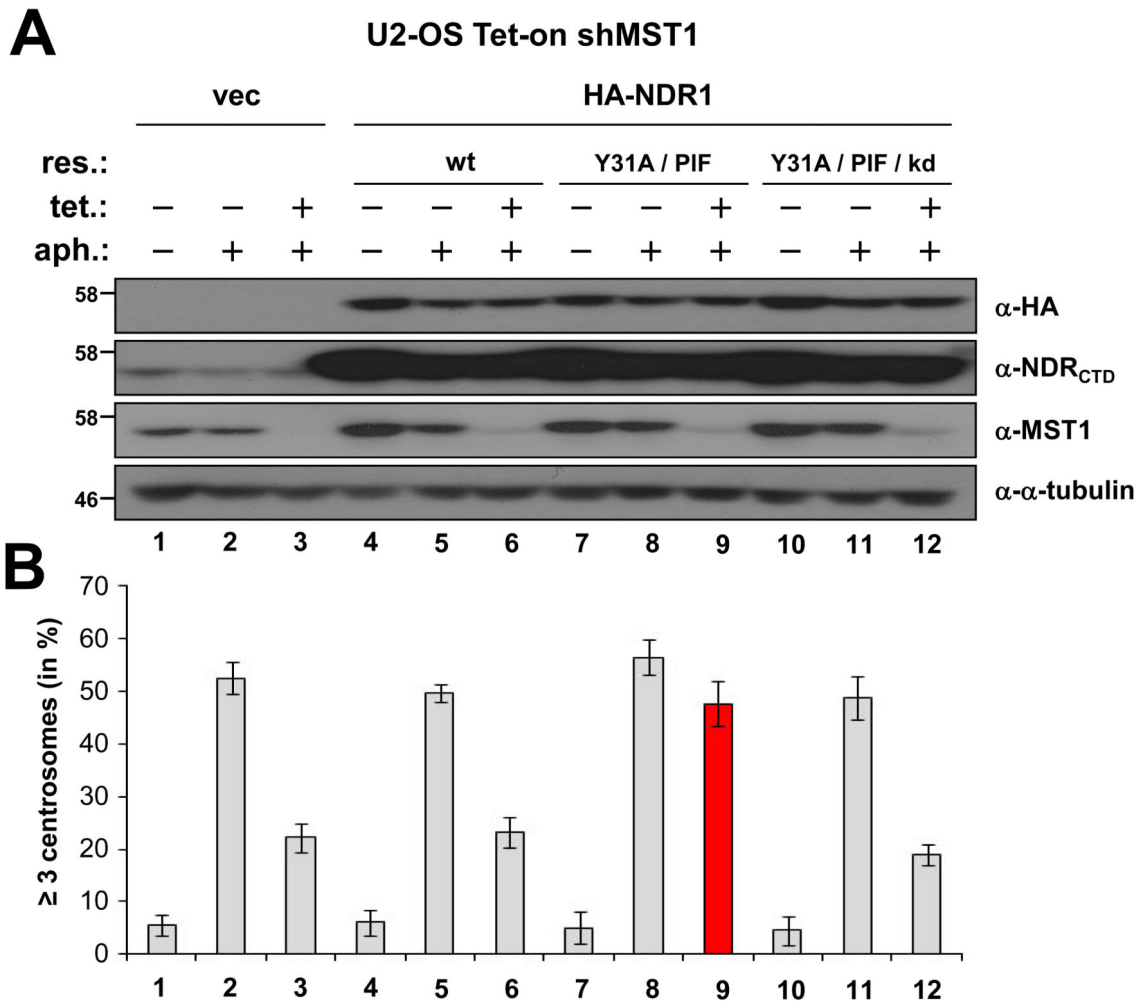

Figure 7. NDR1(Y31A/PIF) supports centrosome overduplication independent of hMOB1A/B binding and endogenous MST1 kinase

(A) U2-OS cells stably expressing tetracycline-regulated short-hairpin RNA (shRNA) directed against human MST1 were infected with empty vector (lanes 1 to 3), HA-NDR1 wild-type (lanes 4 to 6), NDR1(Y31A/PIF) deficient in hMOB1A/B binding (lanes 7 to 9), or kinase-dead (kd) NDR1(Y31A/PIF/kd) deficient in hMOB1A/B binding (lanes 10 to 12). After incubation for 72 hours without (-) or with (+) tetracycline and for a further 72 hours with aphidicolin, cells were analysed by immunoblotting with indicated antibodies. Molecular weights are shown. **(B)** In parallel, cells were processed for immunofluorescence to determine the number of centrosomes per cell (as shown in Figure 3B). 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.

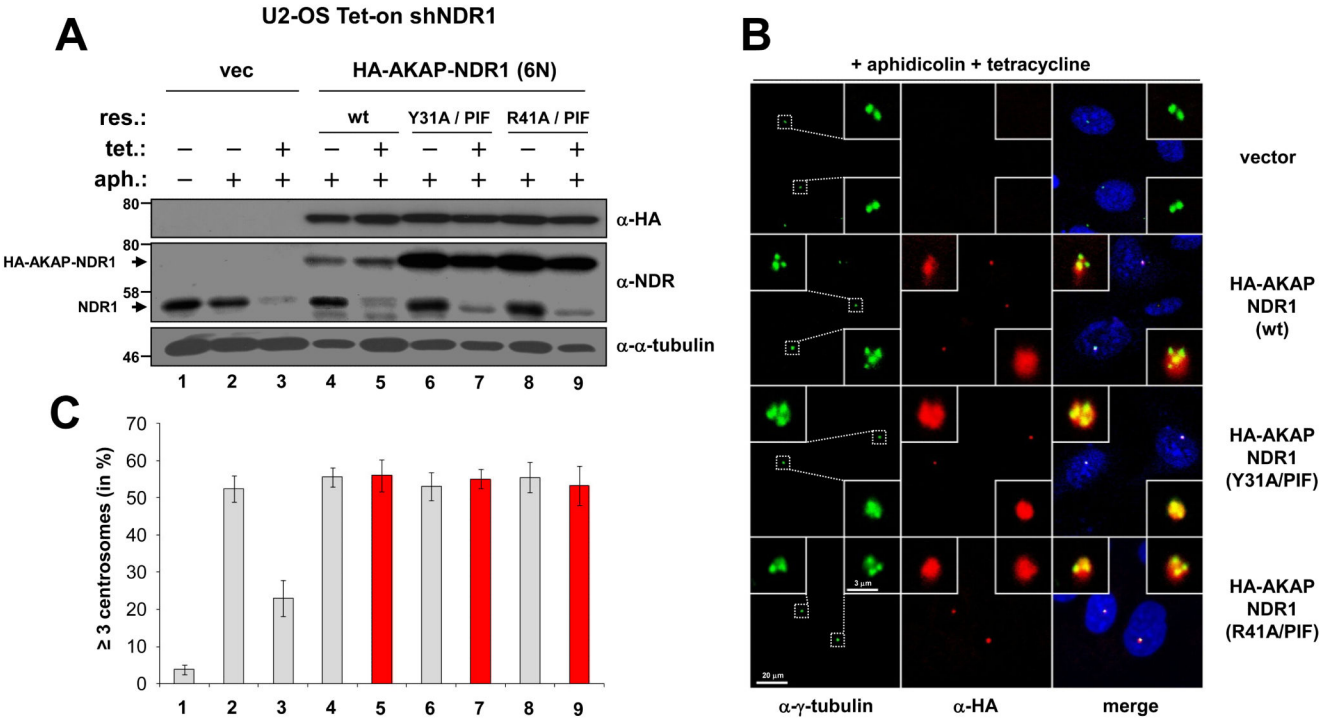

Figure 8. Centrosome-targeting of NDR1 is sufficient to restore centrosome amplification in NDR1-depleted cells

(A) U2-OS cells stably expressing tetracycline-regulated shRNA directed against human NDR1 were infected with empty vector (lanes 1 to 3), HA-AKAP-NDR1 wild-type (lanes 4 and 5), or HA-AKAP-NDR1-PIF deficient in hMOB1A/B binding (lanes 6 to 9) that are refractory to shRNA [HA-AKAP-NDR1 (6N)]. After incubation for 72 hours without (-) or with (+) tetracycline and for a further 72 hours with aphidicolin, cells were analysed by immunoblotting with indicated antibodies. The positions of HA-AKAP-NDR1 variants and endogenous NDR1 are highlighted by arrows. Molecular weights are shown. **(B)** In parallel, cells were processed for immunofluorescence with anti-γ-tubulin (green) and anti-HA (red) antibodies. DNA is stained blue. Insets show enlargements of centrosomes. **(C)** 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.

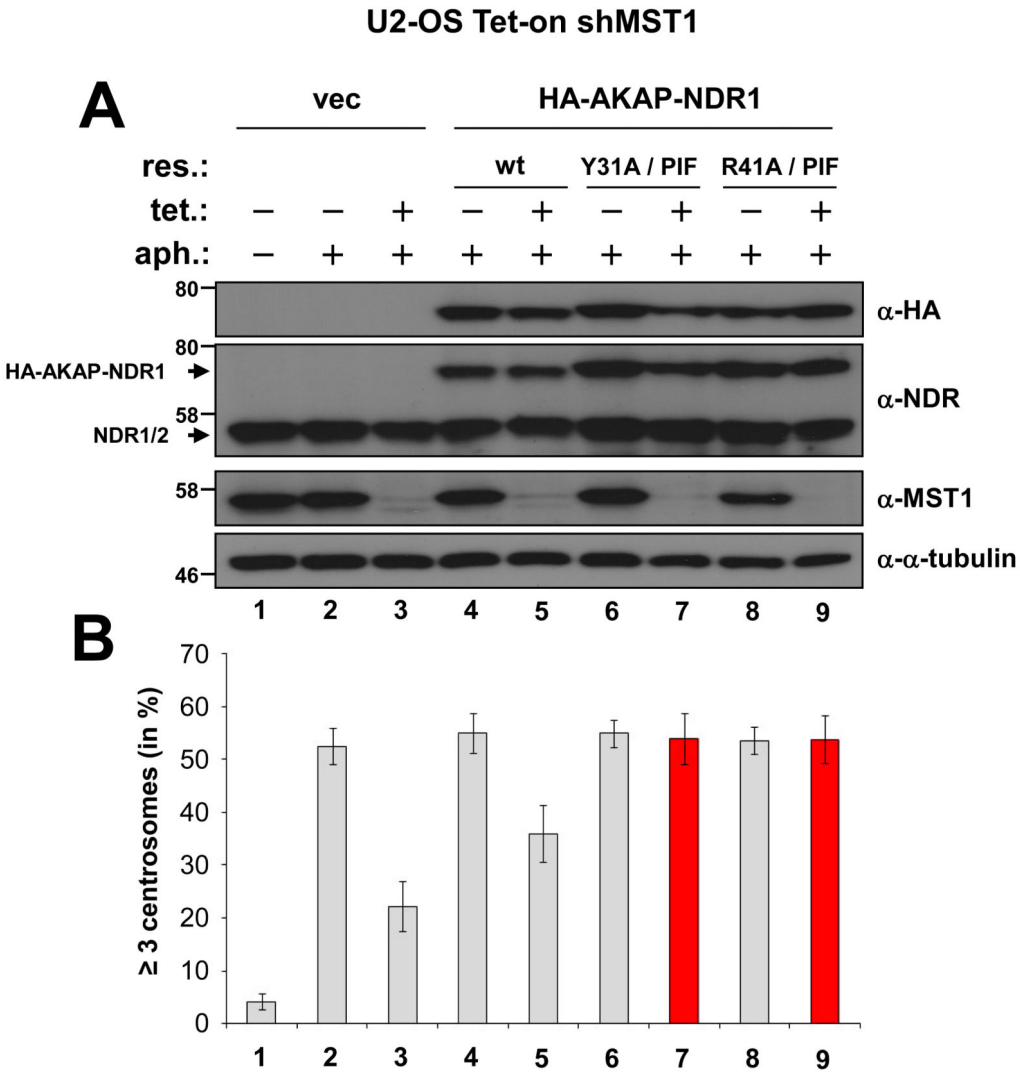

Figure 9. Centrosome-targeting of hMOB1A/B binding deficient NDR1-PIF restores centrosome overduplication upon MST1 depletion

(A) U2-OS cells stably expressing tetracycline-regulated shRNA directed against human MST1 were infected with empty vector (lanes 1 to 3), HA-AKAP-NDR1 wild-type (lanes 4 and 5), or HA-AKAP-NDR1-PIF versions deficient in hMOB1A/B binding (lanes 6 to 9). After incubation for 72 hours without (-) or with (+) tetracycline and for a further 72 hours with aphidicolin, cells were analysed by immunoblotting with indicated antibodies. The positions of HA-AKAP-NDR1 and endogenous NDR1 are highlighted by arrows. Molecular weights are shown. **(B)** In parallel, cells were processed for immunofluorescence to determine the number of centrosomes per cell (as shown in Figure 8B). 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.