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Article

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

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Summary

Background: Human MST/hSAV/LATS/hMOB tumor suppressor cascades are regulators of cell death and proliferation; however, little is known about other functions of MST/hMOB signaling. Mob1p, one of two MOB proteins in yeast, appears to play a role in spindle pole body duplication (the equivalent of mammalian centrosome duplication). We therefore investigated the role of human MOB proteins in centrosome duplication. We also addressed the regulation of human centrosome duplication by mammalian serine/threonine Ste20-like (MST) kinases, considering that MOB proteins can function together with Ste20-like kinases in eukaryotes.
Results: By studying the six human MOB proteins and five MST kinases, we found that MST1/hMOB1 signaling controls centrosome duplication. Overexpression of hMOB1 caused centrosome overduplication, whereas RNAi depletion of hMOB1 or MST1 impaired centriole duplication. Significantly, we delineated an hMOB1/MST1/NDR1 signaling pathway regulating centrosome duplication. More specifically, analysis of shRNA-resistant hMOB1 and NDR1 mutants revealed that a functional NDR/hMOB1 complex is critical for MST1 to phosphorylate NDR on the hydrophobic motif that in turn is required for human centrosome duplication. Furthermore, shRNA-resistant MST1 variants revealed that MST1 kinase activity is crucial for centrosome duplication whereas MST1 binding to the hSAV and RASSF1A tumor suppressor proteins is dispensable. Finally, by studying the PLK4/HsSAS-6/CP110 centriole assembly machinery, we also observed that normal daughter centriole formation depends on intact MST1/hMOB1/NDR signaling, although HsSAS-6 centriolar localization is not affected.
Conclusions: Our observations propose a novel pathway in control of human centriole duplication after recruitment of HsSAS-6 to centrioles.

Introduction

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

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

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

Results

Overexpression of hMOB1A/B Results in Centrosome Overduplication

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

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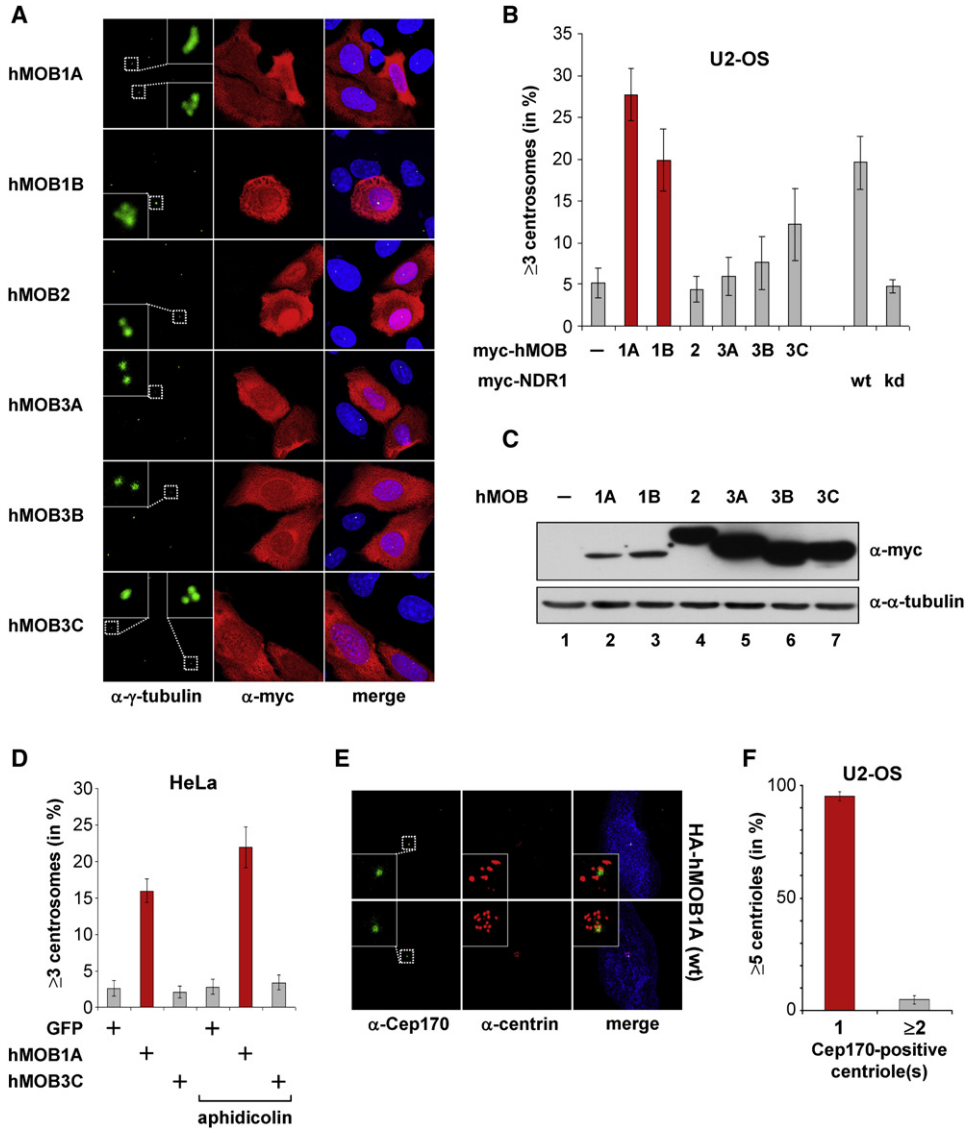

Figure 1. Overexpression of hMOB1A/B Leads to Centrosome Overduplication

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

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

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

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

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

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

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

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

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

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

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

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

hMOB1A/B Regulates Hydrophobic Motif Phosphorylation of NDR Kinase

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

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

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

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

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

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

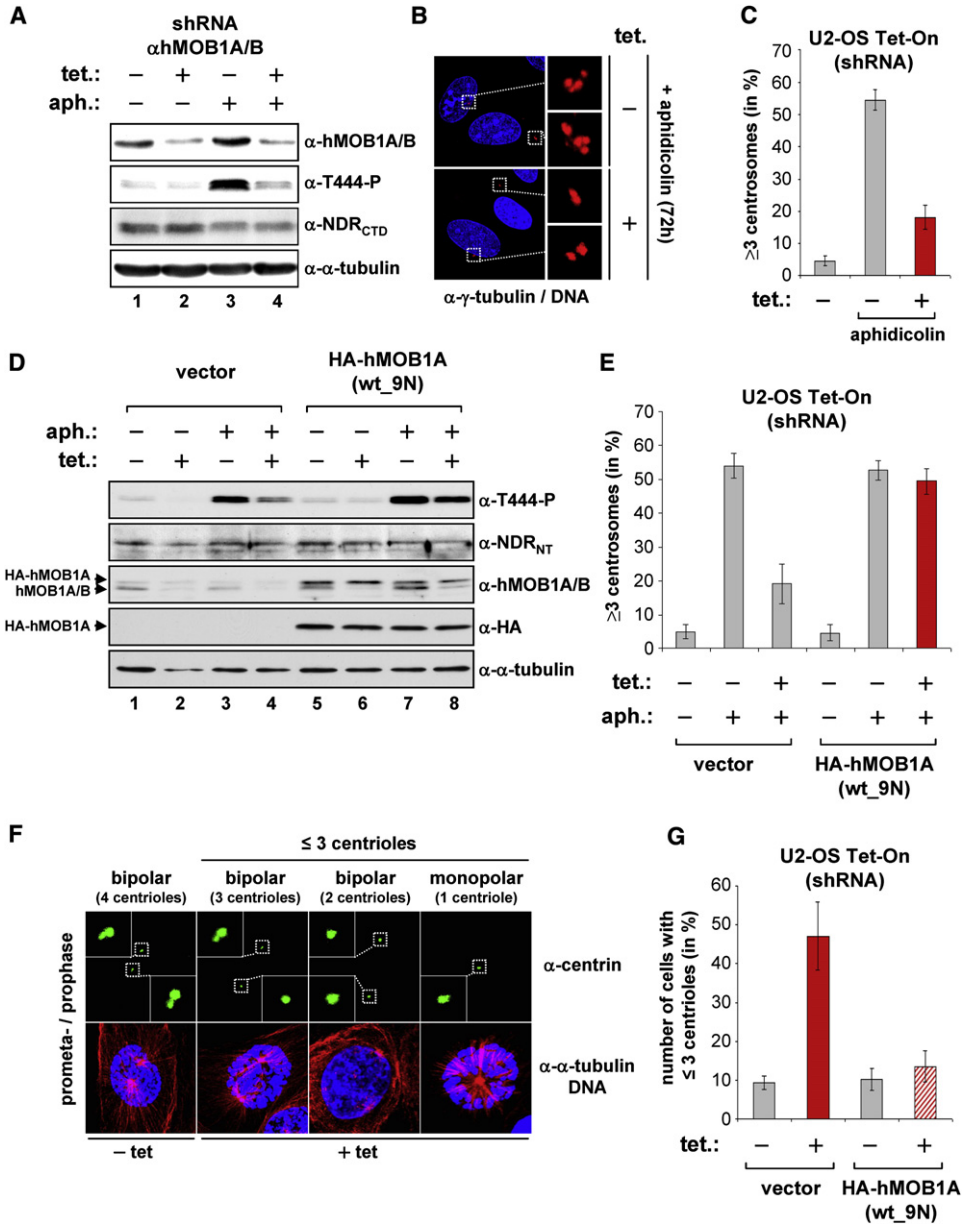

Figure 2. Endogenous hMOB1A/B Is Required for Centrosome Duplication

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

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

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

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

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

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

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

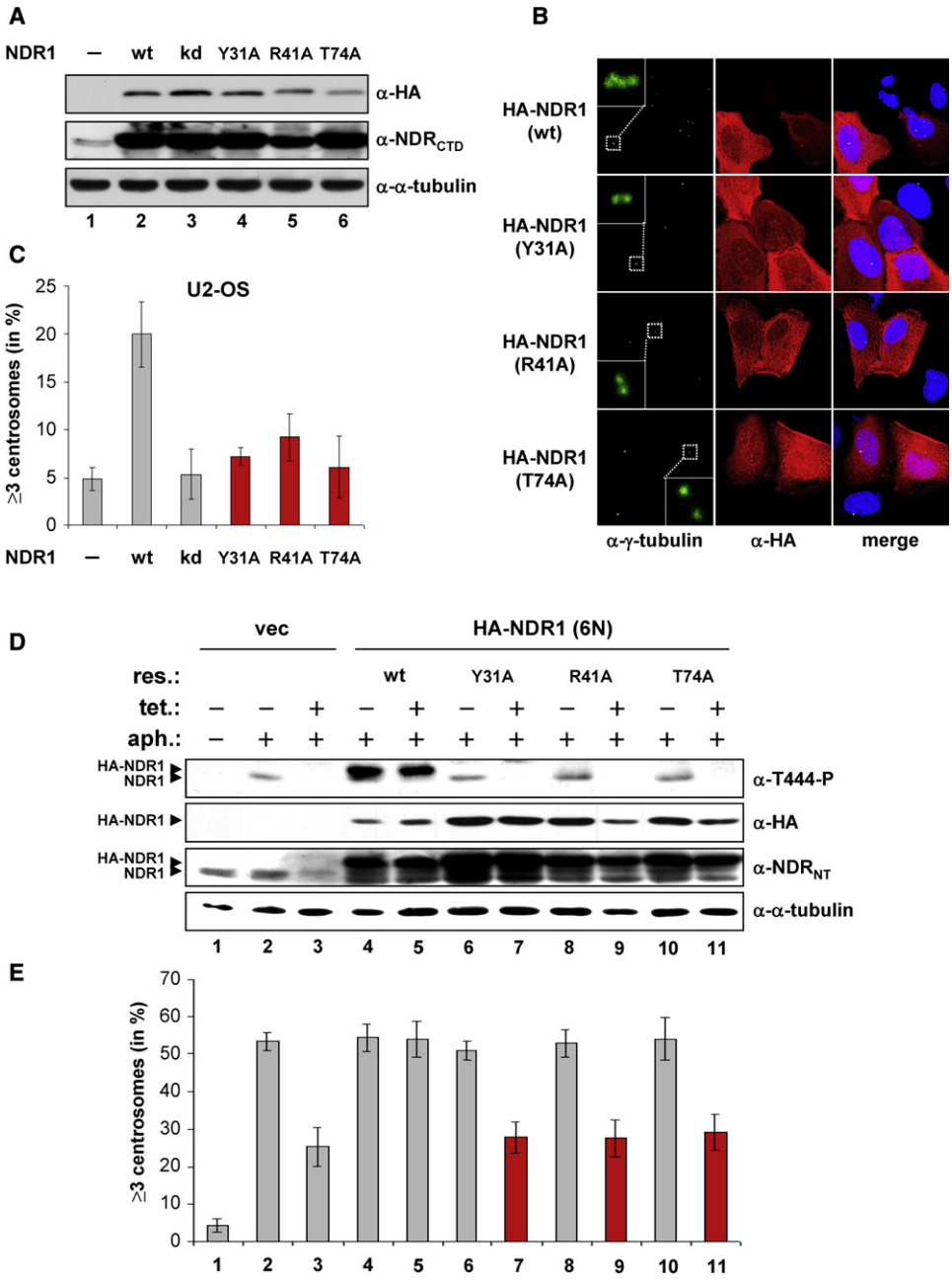

Figure 3. NDR1 Kinase Deficient in hMOB1A/B Binding Does Not Support Centrosome Amplification

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

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

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

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

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

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

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

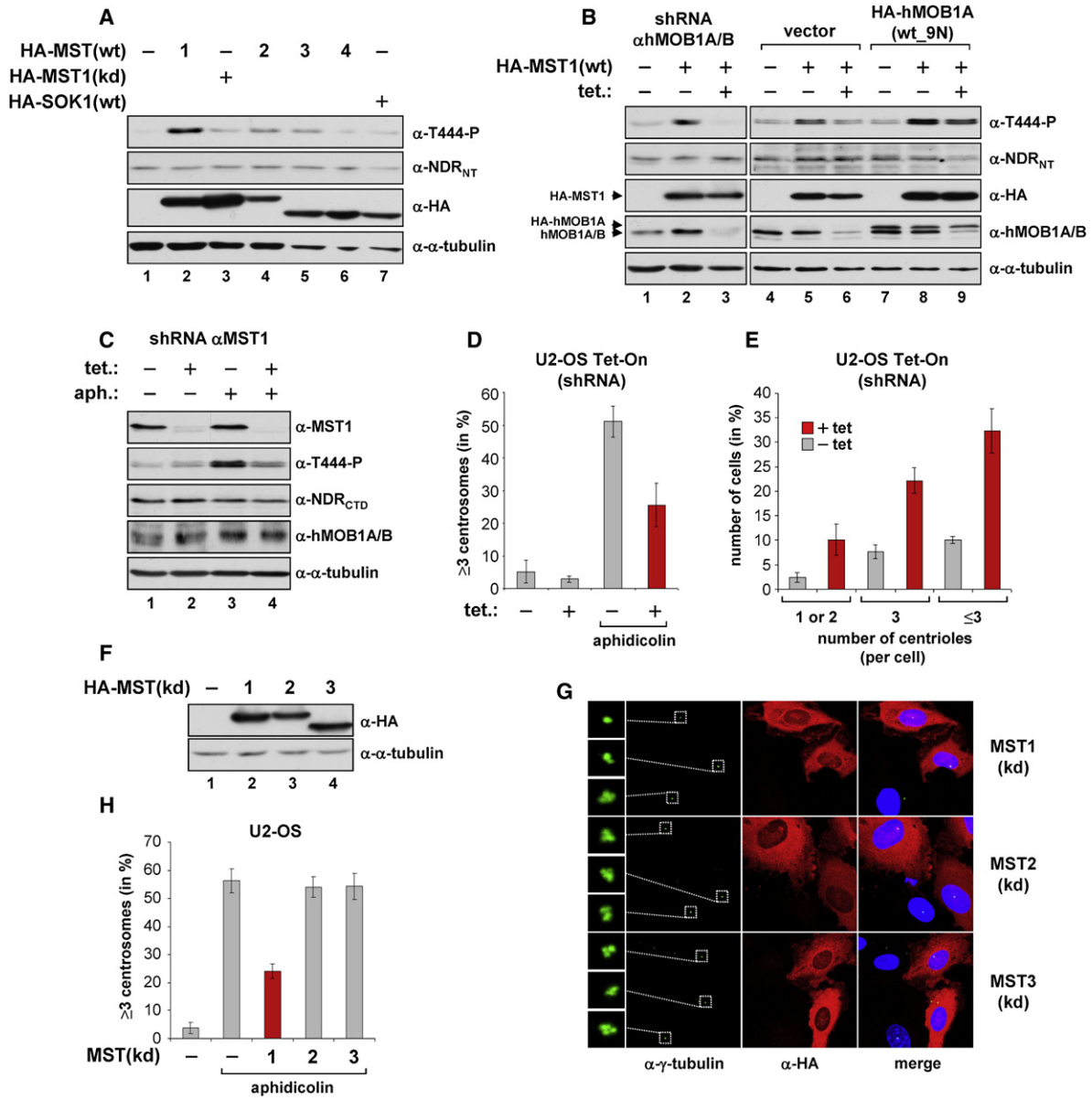

Figure 4. Human MST1 Kinase Is Crucial for Centrosome (Over)duplication

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

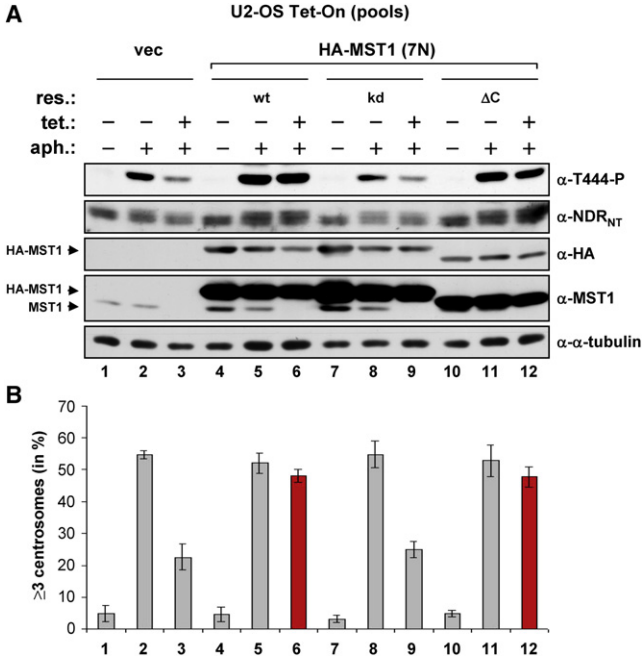

Figure 5. MST1 Kinase Activity, Not the SARAH Domain, Is Required for Centrosome Overduplication in Human Cells

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

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

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

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

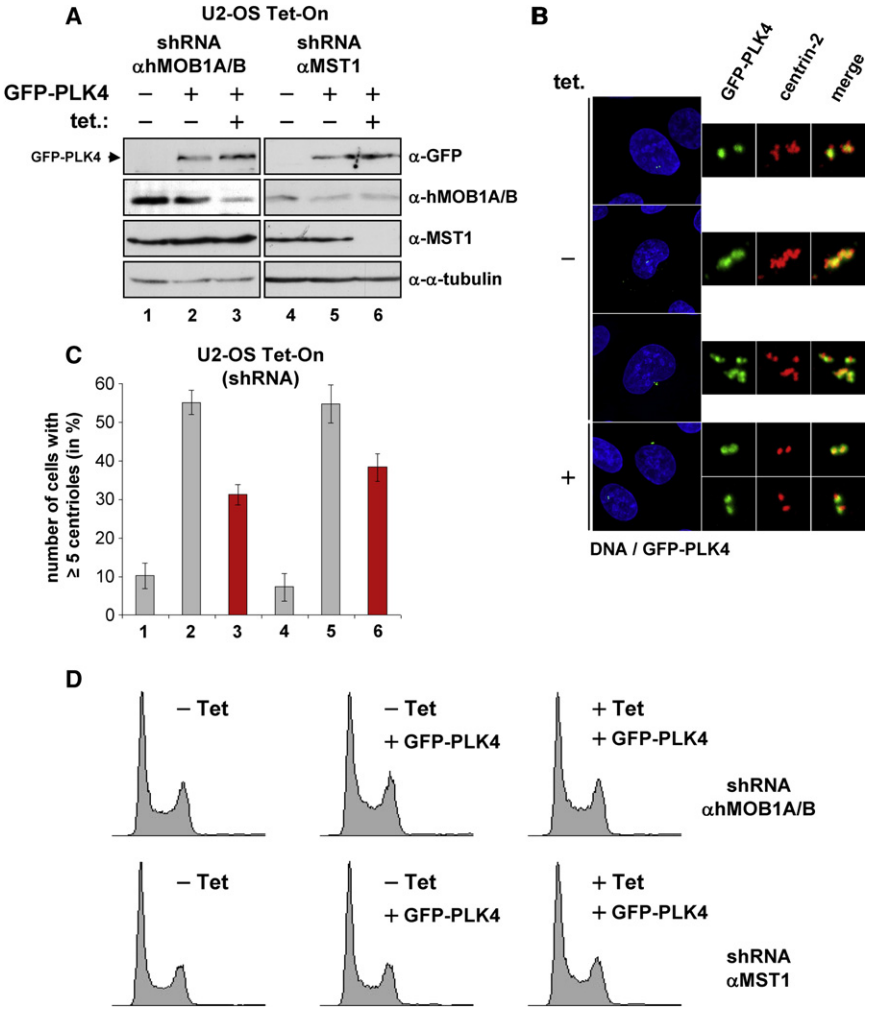

Figure 6. PLK4-driven centriole biogenesis is impaired upon hMOB1A/B or MST1 depletion

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

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

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

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

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

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

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

Discussion

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

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

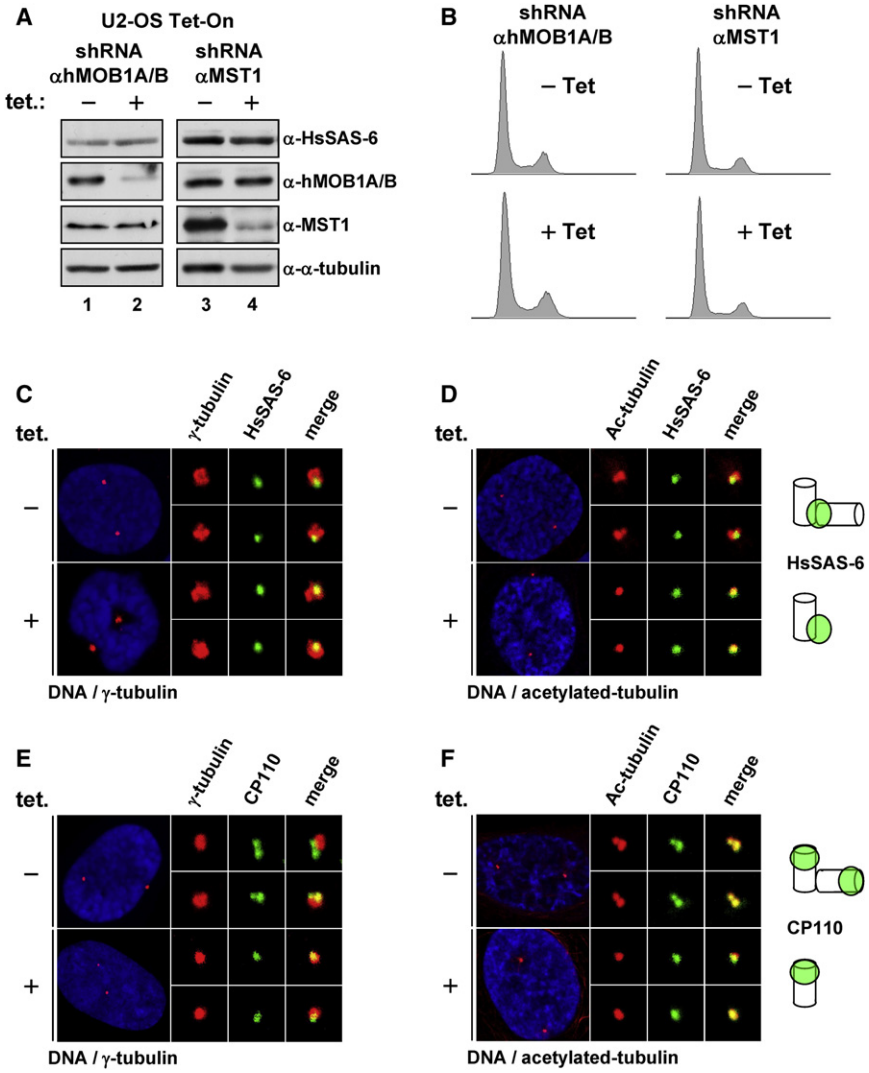

Figure 7. Centriole Localization of CP110, but Not of HsSAS-6, Depends on MST1/hMOB1 Signaling

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

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

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

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

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

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

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

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

Experimental Procedures

Cell Culture, Transfections, and Chemicals

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

Generation of Stable Cell Lines

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

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

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

Supplemental Data

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

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