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ORIGINAL ARTICLE

Threonine 74 of MOB1 is a putative key phosphorylation site by MST2 to form the scaffold to activate nuclear Dbf2-related kinase 1

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Mammalian nuclear Dbf2-related (NDR) kinases (LATS1 and 2, NDR1 and 2) play a role in cell proliferation, apoptosis and morphological changes. These kinases are regulated by mammalian sterile 20-like kinases (MSTs) and Mps one binder (MOB) 1. Okadaic acid (OA), which activates MST2, facilitates the complex formation of MOB1, MST2 and NDR1 in HEK293FT cells. The *in vitro* biochemical study demonstrates the phosphorylation of MOB1 by MST2. The phosphorylated MOB1 alone is capable to partially activate NDR1 *in vitro*, but MST2 is also required for the full activation. The knockdown of MOB1 or MST2 abolishes the OA-induced NDR1 activation in HEK293FT cells. Among MOB1 mutants, in which each serine or threonine residue is replaced with alanine, MOB1 T74A and T181A mutants fail to activate NDR1. Thr74, but not Thr181, is phosphorylated by MST2 *in vitro*, although MOB1 is also phosphorylated by MST2 at other site(s). The interaction of MOB1 T74A with NDR1 is barely enhanced by OA treatment. These findings indicate that the phosphorylation of MOB1 at Thr74 by MST2 is essential to make a complex of MOB1, MST2 and NDR1, and to fully activate NDR1.
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Introduction

Nuclear Dbf2-related (NDR) kinases are members of a subfamily of serine/threonine kinases that are conserved from yeast to mammals. In fly, a tumor suppressor, Warts, and its related protein, Tricornered, belong to this family. Genetic screens in *Drosophila* have revealed a signaling pathway that regulates Warts (Edgar, 2006; Hariharan, 2006). This pathway is called Hippo pathway and comprises two serine/threonine kinases (Hippo and Warts), a WW domain-containing adaptor (Salvador (Sav)/Shar-pei) and Mps one binder (MOB) superfamily proteins (Mats). Hippo and Sav proteins physically interact with each other through the C-terminal Sav/RASSF/Hippo domain (Harvey *et al*., 2003; Jia *et al*., 2003; Pantalacci *et al*., 2003; Udan *et al*., 2003; Wu *et al*., 2003). Mats binds Warts and stimulates its kinase activity (He *et al*., 2005; Lai *et al*., 2005). Sav interacts through its WW-domains with the PPXY motif of Warts and functions as a scaffold to form the tripartite complex of Hippo, Sav and Warts (Tapon *et al*., 2002; Harvey *et al*., 2003). These studies lead to the hypothesis that Hippo/Sav complex activates Warts/Mats complex. The molecular mechanism to regulate tricornered is less studied. Mats activates Tricornered, but how Hippo and Sav are involved in the regulation is unclear (He *et al*., 2005).

Hippo, Salvador and Mats have mammalian homologs, mammalian sterile 20-like kinases (MSTs), hSav/hWW45 and MOB1, respectively (Creasy and Chernoff, 1995; Luca and Winey, 1998; Valverde, 2000). There exist four NDR kinases in the mammalian genome (LATS1 and 2, NDR1 and 2) (Hergovich *et al*., 2006a, b). LATS1 and 2 are tumor suppressors and regulated by MSTs and MOB1 (Stavridi *et al*., 2003; Bothos *et al*., 2005; Chan *et al*., 2005; Hergovich *et al*., 2006a, b). MOB1 associates with LATS1 and activates it. MST2 phosphorylates LATS1 and 2 and activates them. MOB1 also binds NDR1 and 2 and induces the autophosphorylation (Bichsel *et al*., 2004; Hergovich *et al*., 2005). MST3 phosphorylates and activates NDR2 but fails to activate the MOB1-binding deficient NDR2 mutant (Stegert *et al*., 2005). A report has been published during our study that in fly, Hippo phosphorylates Mats to enhance the affinity of Mats to Warts, and that Mats is critical for Hippo-mediated Warts activation (Wei *et al*., 2007). These studies imply that MST kinases and MOB1 co-operatively regulate NDR kinase activity. However, it has not been established whether and how the phosphorylation of MOB1 by MST kinases is involved in the activation of NDR kinases. We have started this study to address this

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question. Because of the plethora of isoforms of each component, multiple combinations are possible. Among them, we have focused on MOB1, MST2 and NDR1, because they functionally rescue their respective *Drosophila* mutants, Mats, Hippo and Tricornered *in vivo*.

Results

MOB1, MST2 and NDR1 form a tripartite complex, and the okadaic acid treatment enhances this formation

MOB1 binds NDR1 and 2 and activates them (Bichsel *et al*., 2004). MOB1 and MST3 co-operatively activate NDR2 (Hergovich *et al*., 2005). We here examined the interaction of NDR1 with MST2 and the effect of MOB1 on this interaction. Myc-MOB1 was coimmunoprecipitated with FLAG-NDR1 from HEK293FT cells (Figure 1a, lane 3, a white arrow). Okadaic acid (OA) treatment augmented the coimmunoprecipitation of Myc-MOB1 with FLAG-NDR1, and endogenous MOB1 was also detected in the immunoprecipitates (Figure 1a, lane 4, a white arrow and a white arrowhead). Myc-MST2 was not detected in the immunoprecipitates with FLAG-NDR1 under the basal condition (Figure 1b, lane 1). Even when Myc-MOB1 was exogenously expressed, Myc-MST2 was not coimmunoprecipitated with FLAG-NDR1 (Figure 1b, lane 3). However, Myc-MST2 was coimmunoprecipitated with FLAG-NDR1 from OA-treated cells (Figure 1b, lane 2, an arrow). It is to note that endogenous MOB1 was coimmunoprecipitated (Figure 1b, lane 2, an arrowhead). Exogenous expression of Myc-MOB1 enhanced OA-induced coimmunoprecipitation of Myc-MST2 with FLAG-NDR1 (Figure 1b, lane 4, an arrow). Conversely, when MOB1 was knocked down, OA treatment did not enhance the coimmunoprecipitation of Myc-MST2 with FLAG-NDR1 (Figure 1c, lane 4, an arrow). OA treatment also enhanced the interaction of FLAG-His6 (FH)-MST2 with Myc-MOB1 and endogenous MOB1 (Figure 1a, lane 6, an arrow and an arrowhead). These findings suggest that MOB1 functions as a scaffold to link MST2 to NDR1, and that OA treatment enhances both the interactions between MOB1 and NDR1 and between MOB1 and MST2 to facilitate the tripartite complex formation.

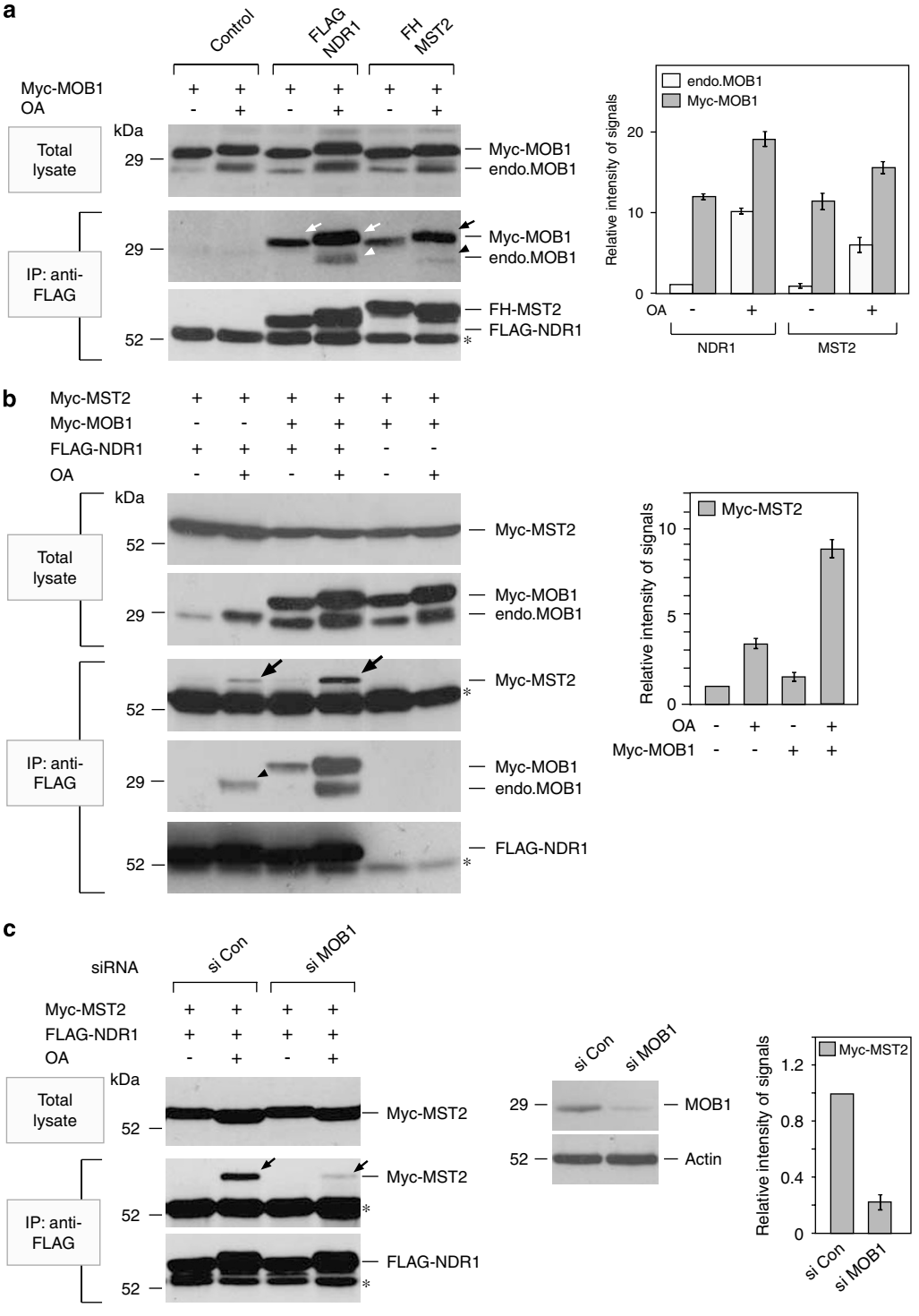
MST2 phosphorylates MOB1

We next examined the phosphorylation of MOB1 by MST2. FH-MST2 or FH-MST2-kinase negative (KN) was expressed in HEK293FT cells, immunoprecipitated with anti-FLAG agarose gel, and eluted with FLAG peptide. FH-MST2, but not FH-MST2-KN, phosphorylated glutathione S-transferase (GST)-MOB1 (Figure 2a). A unit of 0.8 pmol of phosphate was incorporated into 1 pmol of MOB1. As MST2 is a serine/threonine kinase, we tested whether serine and threonine residues of MOB1 are phosphorylated by MST2 (Creasy and Chernoff, 1995). GST-MOB1 was incubated with FH-MST2 or FH-MST2-KN in the kinase buffer with or without ATP and then immunoblotted with anti-phosphoserine or anti-phosphothreonine antibody (Figure 2b). GST-MOB1 exhibited the mobility shift when incubated with FH-MST2 in the ATP-containing kinase buffer (Figure 2b, lane 2, arrows). The signal detected by anti-phosphothreonine antibody increased simultaneously. The signal detected by anti-phosphoserine antibody did not show significant change. GST-MOB1 was detected with both antibodies even when incubated in the kinase buffer without ATP (Figure 2b, lanes 1 and 3). This implies that GST-MOB1 is phosphorylated in *Escherichia coli*. Indeed, when GST-MOB1 treated with protein phosphatase 1 was used as a substrate, the *in vitro* phosphorylation increased by about 20 % (data not shown).

Figure 1 MOB1, MST2 and NDR1 form a tripartite complex and OA treatment enhances this complex formation. (**a**) MOB1 binds both NDR1 and MST2. Myc-MOB1 was expressed alone (lanes 1 and 2) or coexpressed with either FLAG-NDR1 (lanes 3 and 4) or FH-MST2 (lanes 5 and 6) in HEK293FT cells. The cells were treated with either the mock (ethanol) (lanes 1, 3 and 5) or 1 μM of OA for 1 h (lanes 2, 4 and 6). Immunoprecipitation was performed with anti-FLAG antibody. Immunoprecipitates were blotted with anti-MOB1 antibody. The upper panel (Total lysate) demonstrates Myc-MOB1 and endogenous MOB1 in the inputs. The lower panel (IP: anti-FLAG) shows Myc-MOB1, endogenous MOB1, FH-MST2 and FLAG-NDR1 in the immunoprecipitates. Asterisk indicates the immunoglobulin heavy chain. Myc-MOB1 was coimmunoprecipitated with FLAG-NDR1 (lane 3, a white arrow) and FH-MST2 (lane 5) from the mock-treated cells. The association of Myc-MOB1 with FLAG-NDR1 and FH-MST2 was enhanced in the OA-treated cells (lane 4, a white arrow; lane 6, a black arrow). Endogenous MOB1 was also coimmunoprecipitated (lane 4, a white arrowhead; lane 6, a black arrowhead). Protein standards are indicated on the left. The signals of immunoprecipitated Myc-MOB1 and endogenous MOB1 were quantified (the right panel). Error bars indicate s.d. of three independent experiments. (**b**) MST2 interacts with NDR1 in the presence of MOB1. Myc-MST2 was expressed with FLAG-NDR1 (lanes 1 and 2), Myc-MOB1 and FLAG-NDR1 (lanes 3 and 4), or Myc-MOB1 (lanes 5 and 6). OA treatment, immunoprecipitation, immunoblotting and the quantitative analysis were performed as for (**a**). Myc-MST2 was not coimmunoprecipitated with FLAG-NDR1 from the mock-treated cells (lane 1). It was coimmunoprecipitated with FLAG-NDR1 from the OA-treated cells (lane 2, an arrow) with endogenous MOB1 (lane 2, an arrowhead). Exogenous expression of Myc-MOB1 increased the coimmunoprecipitated Myc-MST2 (lane 4, an arrow). In the last two lanes, Myc-MST2 and Myc-MOB1 were expressed without FLAG-NDR1 as negative control samples. (**c**) The knockdown of endogenous MOB1 inhibited the OA-induced coimmunoprecipitation of Myc-MST2 with FLAG-NDR1. HEK293FT cells were transfected with either control (si Con) or MOB1-specific (si MOB1) double stranded (ds) RNA. The middle panel shows the suppression of endogenous MOB1. The immunoblotting for actin is shown as a control. Myc-MST2 was subsequently coexpressed with FLAG-NDR1, and the immunoprecipitation was performed with anti-FLAG antibody. In the control ds RNA-transfected cells, the coimmunoprecipitation of Myc-MST2 with FLAG-NDR1 was enhanced with OA treatment (lane 2, an arrow). The OA-induced enhancement was attenuated in the MOB1-specific ds RNA-transfected cells (lane 4, an arrow). The quantitative data are shown (the right panel). ds, double stranded; FH, FLAG-His6; MOB1, Mps one binder; MST2, mammalian sterile 20-like; NDR1, nuclear Dbf2-related; OA, okadaic acid.

MOB1 and MST2 co-operatively activate NDR1

To test how MOB1 and MST2 influence NDR1 activity *in vitro*, Myc-NDR1 was immunoprecipitated from HEK293FT cells and used to phosphorylate a synthetic peptide. The basal activity of Myc-NDR1 was very low (Figure 3a, an open circle). The addition of GST-MOB1 alone or FH-MST2 alone barely affected the kinase activity (Figure 3a, a closed circle and an open triangle). When FH-MST2 and GST-MOB1 were added together, the NDR1 kinase activity was enhanced (Figure 3a, closed triangles). Thus, as reported for MOB1, MST3 and NDR2, both of MST2 and MOB1 are necessary for the full activation of NDR1. This effect of GST-MOB1 was dose dependent (Figure 3b). We subsequently tested whether the phosphorylated MOB1 is sufficient for the activation of NDR1. A unit of 2 pmol of GST-MOB1



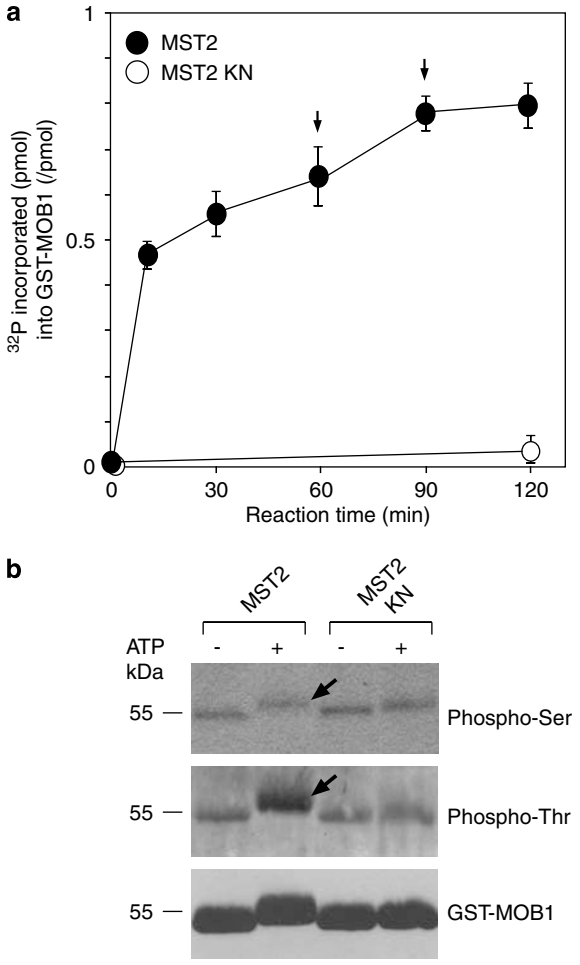


Figure 2 MST2 phosphorylates MOB1. (**a**) Time course of the phosphorylation of GST-MOB1. FH-MST2 and FH-MST2-KN were expressed in HEK293FT cells. The cell lysates were incubated with anti-FLAG agarose gel. FH-MST2 and FH-MST2-KN were eluted with FLAG peptide. A unit of 10 pmol of GST-MOB1 was incubated with 1 pmol of FH-MST2 (closed circles) or FH-MST2-KN (open circles) at 30 °C. The reactions were resolved on a 10% SDS-PAGE, and the incorporated ^{32}P was counted using the liquid scintillation counter. New aliquots of the enzyme (0.5 pmol) were added at 60 and 90 min (arrows). Error bars indicate s.d. of three independent experiments. (**b**) Immunoblottings of the phosphorylated GST-MOB1 with anti-phosphoserine and anti-phosphothreonine antibodies. 4 pmol of GST-MOB1 was incubated with 0.2 pmol of FH-MST2 or FH-MST2-KN in the kinase buffer with or without ATP for 60 min at 30 °C and analyzed. GST-MOB1 showed a mobility shift when it was incubated with FH-MST2 in the ATP-containing kinase buffer and more remarkably detected with anti-phosphothreonine antibody (an arrow). It is to note that GST-MOB1 was detected with anti-phosphoserine and anti-phosphothreonine antibodies even when incubated in the kinase buffer without ATP. Protein standards are indicated on the left. FH, FLAG-His6; GST, glutathione S-transferase; KN, kinase negative; MST, mammalian sterile 20-like; MOB1, Mps one binder; SDS-PAGE, SDS-polyacrylamide gel electrophoresis.

was preincubated with FH-MST2 fixed on anti-FLAG agarose gel for 90 min. After the incubation, MST2 was removed by a centrifugation. The amount of the phosphorylated MOB1 in the supernatant was calculated as approximately 1.6 pmol. We confirmed by the immunoblotting that MST2 was not carried over in the supernatant (data not shown). This supernatant was added to the NDR1 assay mixture. The reaction was stopped at 90 min. The supernatant itself (the phosphorylated MOB1 alone) activated the NDR1 (Figure 3c). The addition of FH-MST2 at the second stage further increased the NDR1 activity. We confirmed that MOB1 was not additionally phosphorylated during the second stage (Figure 3c, the bottom panel). FH-MST2-KN did not cause an additional increase above the phosphorylated MOB1. Therefore, the phosphorylated MOB1 is not sufficient for the activation of NDR1, and the active MST2 is also required. We confirmed that Myc-NDR1 could be phosphorylated by GST-MST2, but not GST-MST2-KN (Figure 3d and data not shown). The addition of GST-MOB1 enhanced the phosphorylation of NDR1.

MOB1 and MST2 are involved in the activation of NDR1 in vivo

We next examined whether MOB1 and MST2 are involved in the activation of NDR1 *in vivo*. FLAG-NDR1 was immunoprecipitated with anti-FLAG antibody from the mock-treated or the OA-treated HEK293FT cells. OA treatment drastically activated FLAG-NDR1 (Figure 4a). The amount of immunoprecipitated NDR1 slightly increased in OA-treated cells, suggesting that OA treatment affects the amount of NDR1 in the detergent soluble fraction (data not shown). We quantified the immunoprecipitated NDR1 by the protein staining and normalized the kinase activity. The specific activity increased by OA treatment. To investigate the involvement of MOB1 and MST2 in the OA-induced activation of NDR1, we utilized double-stranded RNA (ds RNA) to knockdown human MOB1 and MST2. The ds RNA for MST2 suppressed endogenous MST2 in HEK293FT cells (Figure 4b, the right panel). The suppression of MOB1 or MST2 partially but significantly decreased the OA-induced activation (Figure 4b, the left panel). As reverse experiments, we exogenously expressed MOB1 and MST2. The expression of MOB1 and/or MST2 had no significant influence on the NDR1 activity from the mock-treated HEK293FT cells (Figure 4c, asterisks). The expression of MOB1 alone, but not of MST2 alone, significantly enhanced OA-induced NDR1 activation (Figure 4c, columns 2, 4 and 6). The additional expression of MST2 did not augment the effect of MOB1 (Figure 4c, columns 4 and 8). These findings suggest that MOB1 and MST2 are involved in OA-induced NDR1 activation in HEK293FT cells and that the amount of MOB1 is a limiting factor.

Thr74 and Thr181 of MOB1 are involved in the activation of NDR1

As shown in Figure 2b, MST2 phosphorylates threonine of MOB1. It is not clear whether MST2 phosphorylates serine of MOB1. We attempted to determine which serine and threonine residues are phosphorylated by MST2 and involved in the activation of NDR1. We prepared a series of MOB1 mutants, in which each

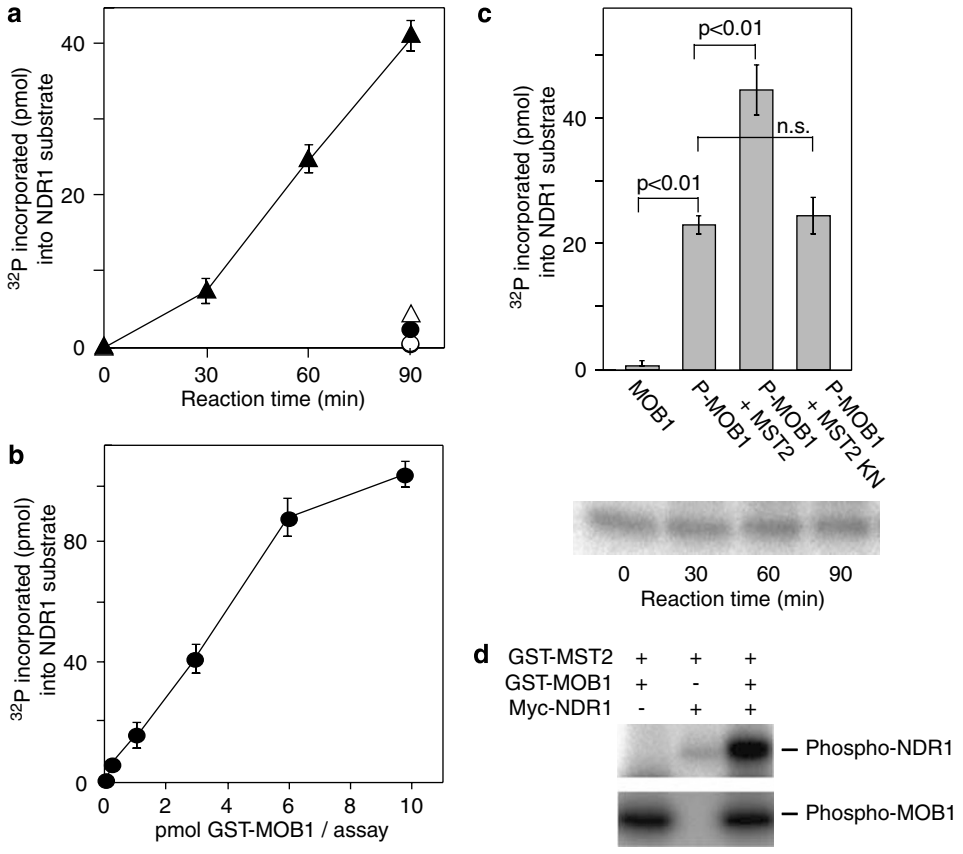


Figure 3 Activation of NDR1 by MST2 and MOB1. (**a**) NDR1 assay was performed using the synthetic peptide as a substrate. Open circles, 1 pmol of Myc-NDR1 alone; closed circles, with 2 pmol of GST-MOB1; open triangles, with 0.2 pmol of FH-MST2; and closed triangles, with 2 pmol of GST-MOB1 and 0.2 pmol of FH-MST2. Error bars indicate s.d. of three independent experiments. (**b**) Dose effect of MOB1 on the stimulation of NDR1 activity. NDR1 assay was performed with indicated amounts of GST-MOB1 and immunoprecipitated FH-MST2 for 60 min. (**c**) The effect of MST2 on NDR1 activity in the presence of the phosphorylated MOB1 (P-MOB1). GST-MOB1 was phosphorylated by immunoprecipitated FH-MST2 fixed on anti-FLAG agarose gel for 90 min. GST-MOB1 was separated from FH-MST2 by the centrifugation. Some GST-MOB1 was trapped by FH-MST2. GST-MOB1 recovered in the supernatant was quantified and used for the second incubation. To confirm that GST-MOB1 was not further phosphorylated for the second incubation with FH-MST2, aliquots from the reaction mixture at 0, 30, 60, and 90 min in the second incubation were resolved on a 10% SDS-PAGE, and visualized with a PhosphorImager (the bottom panel). The additional phosphorylation of GST-MOB1 did not take place during the second incubation. $P<0.01$ and NS, not significant; *t*-test. (**d**) A unit of 5 pmol of the immunoprecipitated Myc-NDR1 was phosphorylated by 0.4 pmol of GST-MST2 with or without 2 pmol of GST-MOB1. The phosphorylation of Myc-NDR1 was enhanced by MOB1. FH, FLAG-His6; GST, glutathione S-transferase; MST, mammalian sterile 20-like; MOB1, Mps one binder; NDR1, nuclear Dbf2-related; SDS-PAGE, SDS-polyacrylamide gel electrophoresis.

serine or threonine residue is replaced with alanine. We could not detect remarkable reduction of phosphorylation in any mutant, although phosphorylation was slightly lower in some mutants such as MOB1 S9, 10A, T35A and T74A (Figure 5a). The capacity to activate NDR1 *in vitro* was significantly impaired in T74A and T181A mutants (Figure 5b, asterisks). The expression of MOB1 T74A or T181A did not enhance NDR1 activity in the OA-treated HEK293FT cells as much as the wild type of MOB1 (MOB1 WT) did (Figure 5c). GST-fused peptides covering 24 amino acids around Thr74 (GST-MOB1; 62–85) and Thr181 (GST-MOB1; 170–193) were used as substrates for the *in vitro* phosphorylation by MST2. MST2 phosphorylated GST-MOB1 (62–85) but not GST-MOB1 (62–85) mutant with alanine instead of threonine at 74 (GST-MOB1; 62–85; T74A) (Figure 5d). Under the same condition, MST2 did not phosphorylate GST-MOB1 (170–193). Although the efficiency of the phosphorylation is lower compared with the full-length MOB1, this result indicates that MST2 can phosphorylate Thr74 of MOB1. However, GST-MOB1 T74A was phosphorylated by MST2 at almost the same level as MOB1 WT *in vitro* (Figure 5a). The initial velocity of the phosphorylation was not significantly different (Figure 5e). These results suggest that MOB1 has additional phosphorylation site(s) besides Thr74 and that these site(s) may be phosphorylated more efficiently in MOB1 T74A.

MOB1 T74A does not function as a scaffold to link MST2 and NDR1 in vivo

MOB1 plays a role as a scaffold to facilitate the complex formation including MST2 and NDR1 in OA-treated HEK293FT cells (Figure 1). When Myc-MOB1 T74A was expressed, this mutant was not immunoprecipitated

with NDR1 (Figure 6a, the fourth panel, lane 7). Even after OA treatment, Myc-MOB1 T74A was hardly detected in the immunoprecipitates (Figure 6a, the fourth panel, lane 8, an arrowhead). This makes a sharp contrast to Myc-MOB1 WT (Figure 6a, the fourth panel, lanes 5 and 6). The capability of Myc-MOB1 T74A to facilitate the coimmunoprecipitation of Myc-MST2 with FLAG-NDR1 from OA-treated cells was significantly impaired (Figure 6a, the third panel, lanes 6 and 8, arrows). Under the expression of Myc-MOB1 T74A, the immunoprecipitates contained endogenous MOB1 besides FLAG-NDR1 and Myc-MST2, indicating that Myc-MOB1 T74A does not interfere with the OA-dependent complex formation including endogenous MOB1 (Figure 6a, the fourth panel, lane 8). For the further confirmation, we performed the *in vitro* interaction experiments. GST-MOB1 WT or GST-MOB1 T74A was incubated with FH-MST2 in the kinase buffer with or without ATP. After the centrifugation to remove FH-MST2, the supernatant was mixed with FLAG-NDR1 immobilized on anti-FLAG agarose gel. The incubation in the complete kinase buffer resulted in the mobility shift for both of GST-MOB1 and GST-MOB1 T74A (Figure 6b, Input, lanes 2 and 4). GST-MOB1 was only slightly captured with FLAG-NDR1 when it was incubated in the kinase buffer without ATP (Figure 6b, lane 1). When incubated in the complete kinase buffer, GST-MOB1 remarkably bound to FLAG-NDR1, while GST-MOB1 T74A did not (Figure 6b, lanes 2 and 4). As the mobility shift suggests, GST-MOB1 T74A appears to be phosphorylated by MST2 *in vitro*. This observation also supports that MOB1 has additional phosphorylation site(s) but that the phosphorylation at that site is not sufficient to enhance the interaction of MOB1 with NDR1. In contrast, GST-MOB1 T74A bound MST2, as well as GST-MOB1 did (data not shown). In the immunofluorescence, NDR1 was distributed in the cytosol and the nucleus, when expressed alone (Figure 6c). Both of

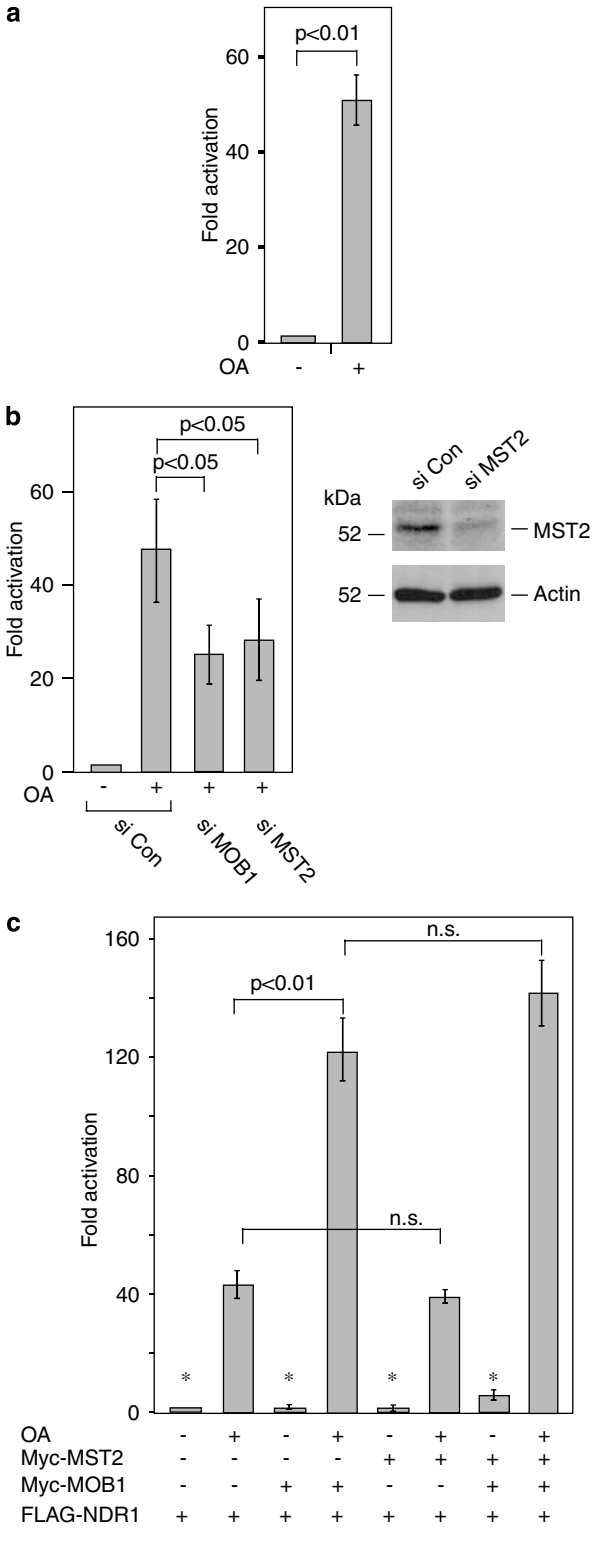


Figure 4 Involvement of MOB1 and MST2 in the OA-induced activation of NDR1. (**a**) NDR1 is activated by OA treatment in HEK293FT cells. HEK293FT cells were transfected with pCIneoFLAG NDR1 and treated with either the mock or OA. FLAG-NDR1 was immunoprecipitated with anti-FLAG antibody from the mock-treated or the OA-treated cells. The amount of the immunoprecipitated NDR1 was evaluated by the protein staining, and the activity of NDR1 was normalized. Error bars indicate s.d. of three independent experiments. $P<0.01$; *t*-test. (**b**) The effect of the knockdown of MOB1 and MST2 on the OA-induced activation of NDR1. HEK293FT cells were transfected with control ds RNA (si Con) or ds RNA specific for MOB1 (si MOB1) or MST2 (si MST2). The right panel shows the suppression of endogenous MST2. The validity for the knockdown of MOB1 is shown in Figure 1c. FLAG-NDR1 was expressed in these HEK293FT cells and immunoprecipitated after the OA treatment. The suppression of MOB1 or MST2 attenuated the OA-induced activation of NDR1. $P<0.05$; *t*-test. (**c**) The effect of exogenous expression of MOB1 and MST2 on the OA-induced activation of NDR1. FLAG-NDR1 was expressed with various combinations of Myc-MOB1 and Myc-MST2 in HEK293FT cells. FLAG-NDR1 was immunoprecipitated from the mock-treated or the OA-treated cells. The *in vitro* kinase assays were performed by using the equal amount of FLAG-NDR1. The exogenous expression of Myc-MOB1 and Myc-MST2 did not affect NDR1 activity in the mock-treated cells (asterisks). The expression of Myc-MOB1 enhanced the OA-induced activation (columns 2 and 4). The expression of Myc-MST2 had no effect (columns 2 and 6). It did not augment the effect of Myc-MOB1 either (columns 4 and 8). $P<0.01$ and NS, not significant; *t*-test. ds, double stranded; MST, mammalian sterile 20-like; MOB1, Mps one binder; NDR1, nuclear Dbf2-related; OA, okadaic acid.

MOB1 WT and MOB1 T74A were diffusely detected outside the nucleus, although some signals were detected in the nucleus. The subcellular fractionation also indicates that the distributions of both MOB1 proteins are not different. When NDR1 and MOB1 WT were coexpressed, NDR1 was recruited outside the nucleus. However, when MOB1 T74A was expressed, NDR1 remained in the nucleus. These findings support that MOB1 T74A does not interact with NDR1 as well as MOB1 WT in cells.

MOB1 is phosphorylated in HEK293FT cells

Finally, we expressed FH-MOB1 in HEK293FT cells, labeled the cells with ^{32}P, and then performed the immunoprecipitation from the mock-treated and the OA-treated cells. FH-MOB1 was slightly phosphorylated in the mock-treated cells, but the OA treatment significantly enhanced the phosphorylation (Figure 7a). The knockdown of MST2 attenuated the interaction between MOB1 and NDR1 in not only in the OA-treated cells but also in the non-treated cells (Figure 7b, an arrowhead and an arrow). This finding implies that the overexpressed MOB1 is phosphorylated by MST2 even in the non-treated cells and is consistent with the result of the labeling experiment.

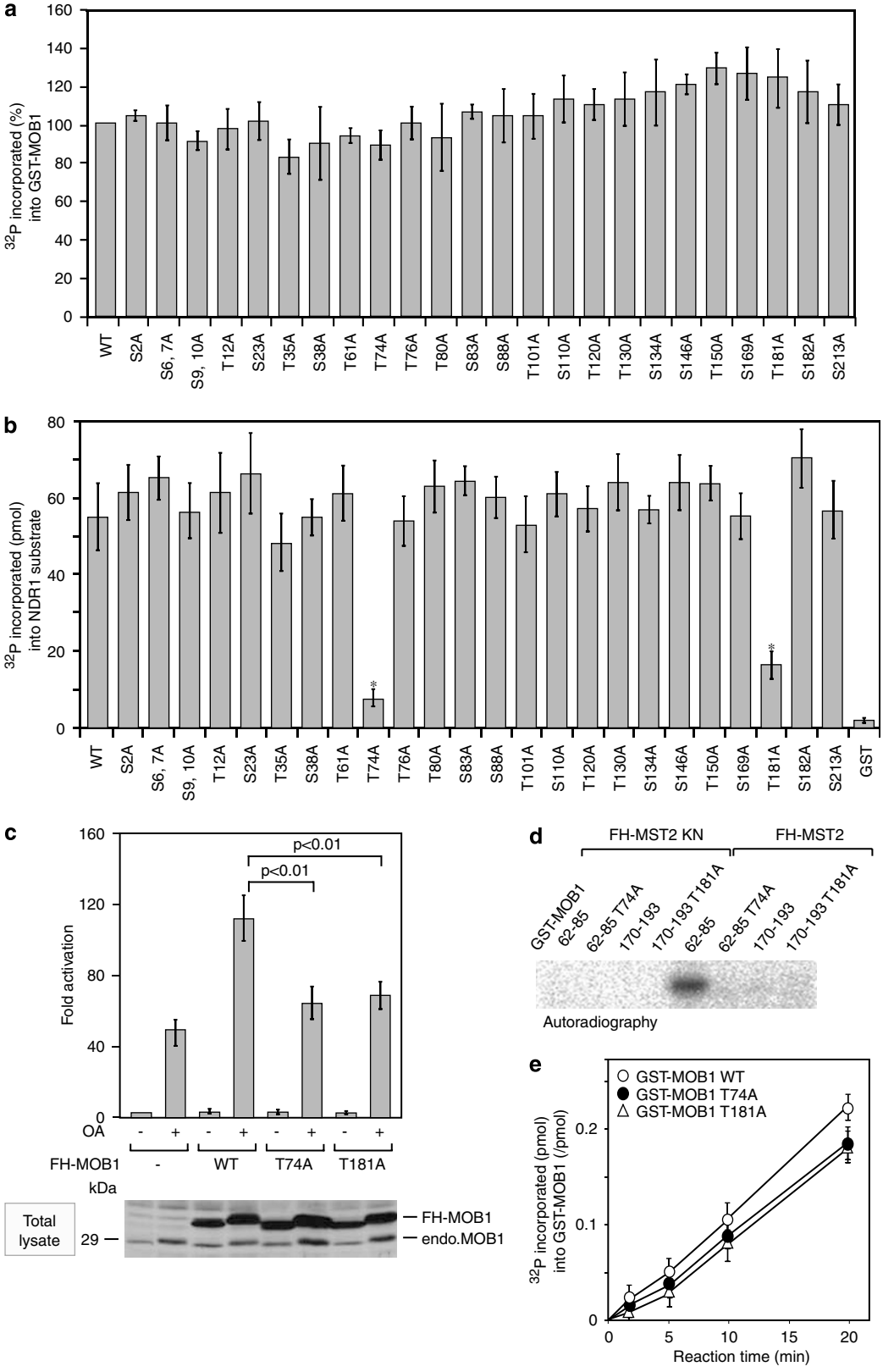
Discussion

NDR kinases play a role in cell proliferation, morphological changes and apoptosis (Hergovich *et al*., 2006a, b). Preceding studies have suggested the involvement of MST kinases and MOB family proteins in this regulation. We have here focused on MST2 and MOB1 and directly revealed the significance of the MST2-dependent phosphorylation of MOB1 in the activation of NDR1. First, we have shown that NDR1 forms a complex with MOB1 and MST2 in the OA-treated HEK293FT cells. MOB1 is coimmunoprecipitated with NDR1 from the mock-treated cells. The OA treatment significantly augments the interaction between MOB1 and NDR1 and the knockdown of MST2 suppresses this OA-induced interaction. The coimmunoprecipitation of MST2 with NDR1 is detected only from the OA-treated cells and depends on MOB1. OA induces the autophosphorylation of MST2 and activates it (Lee and Yonehara, 2002). Thereby, we speculated that MST2 is activated by OA and phosphorylates MOB1, and that the phosphorylated MOB1 functions as a scaffold to link MST2 to NDR1. We next analyzed the phosphorylation of MOB1 by MST2 *in vitro*. To minimize the contamination of other kinases, we immunoprecipitated FH-MST2 from HEK293FT cells, vigorously washed it using a high salt buffer, and eluted FH-MST2 with FLAG peptide. MST2 phosphorylates MOB1 *in vitro*, but the kinase negative mutant does not. In the course of this study, the interaction between *Drosophila* Hippo and Mats has been reported (Wei *et al*., 2007). The authors also reported the phosphorylation of Mats by Hippo and of MOB1 by MST1. Their results with ours indicate that the phosphorylation of Mats/MOB1 by MSTs is conserved in human and fly.

The molecular activation mechanism of NDR kinases is well studied *in vitro* and *in vivo* (Bichsel *et al*., 2004; Hergovich *et al*., 2005; Stegert *et al*., 2005, 2006a, b). However, because of the presence of many isoforms, there is no report to directly discuss the involvement of MST2 and MOB1 in the activation of NDR1. As human genes encoding these proteins substitute for their fly homologs, we consider that it is important to study the activation mechanism using these proteins. Our results are consistent with the previous reports. Neither MST2 nor MOB1 alone significantly activates NDR1, but when MST2 and MOB1 are together added to NDR1, they remarkably stimulate the kinase activity. To analyze the underlying molecular mechanism more closely, we performed a two-step experiment. In the first step, we phosphorylated MOB1 with MST2 and then removed MST2. In the second step, we tested various combinations of MOB1 and MST2 for the activation of NDR1. The phosphorylated MOB1 alone significantly but only partially activates NDR1. MST2 alone does not induce the full activation of NDR1. The combination of the phosphorylated MOB1 and MST2 activates NDR1 to the maximum. The phosphorylation state of MOB1 does not change at the second step, suggesting that the newly supplemented MST2 works not with MOB1 but with NDR1. Indeed, MST2 phosphorylates NDR1 and this phosphorylation is enhanced by MOB1. The kinase-negative MST2 fails to further activate NDR1 even in the presence of the phosphorylated MOB1. Thereby, the kinase activity of MST2 is required for the phosphorylation of both MOB1 and NDR1. The knockdown of either MOB1 or MST2 inhibits the OA-induced activation of NDR1 in HEK293FT cells, indicating that both of MOB1 and MST2 are involved in the activation of NDR1 *in vivo*.

The subsequent key question is which residues of MOB1 are phosphorylated by MST2. MOB1 has total 26 serine and threonine residues (15 of serine and 11 of threonine), among which 21 residues are conserved in Mats. We replaced these residues with alanine and tested whether each mutant is phosphorylated by MST2 *in vitro* and activates NDR1. We could not detect a significant reduction of phosphorylation in any mutant. This observation suggests that MOB1 has more than one phosphorylation sites. Almost 0.8 pmol of phosphate is incorporated to 1 pmol of GST-MOB1. The immunoblottings with anti-phosphoserine and antiphosphothreonine antibodies suggest that GST-MOB1 is phosphorylated in *E. coli*, which may cause incomplete phosphorylation *in vitro*, but the treatment with protein phosphatase 1 only slightly improves the stoichiometry. Thereby, it is likely that all potential phosphorylation sites of MOB1 are not phosphorylated simultaneously. There may be some mechanism to regulate which site is phosphorylated in response to various cellular contexts. This is an intriguing question of future studies. The capacity to activate NDR1 *in vitro* is significantly impaired in two mutants, T74A and T181A. These mutants fail to activate NDR1 *in vivo*,

too. The *in vitro* phosphorylation indicates that Thr74 can be phosphorylated by MST2. Thr181 is not phosphorylated under the same condition. It is not yet concluded whether Thr181 is phosphorylated by MST2 under the different condition or by some other kinase. The exogenous expression of MOB1 T74A still facil-



itates to some extent the complex formation of NDR1 and MST2, which is consistent with that MOB1 T74A slightly activates NDR1 *in vitro*. However, *in vitro* binding assay and the immunofluorescence study indicate that the interaction of MOB1 T74A with NDR1 is weak. Thereby, it can be concluded that Thr74 of MOB1 is important for the activation of NDR1. As NDR1 is suggested to function as a proto-oncogene, it is also interesting to examine whether the enhanced phosphorylation of Thr74 is implicated in oncogenesis.

Crystal structure of MOB1 demonstrated that the core is a long bundle of four α-helices, which is stabilized by Zn (Stavridi *et al*., 2003). Thr74 is at the C-terminal end of H2, one of long helices. This region includes the Zn-binding site and is well conserved. Thr181 belongs to H7, which faces to H2 and also forms a bundle. Thereby, the phosphorylations at Thr74 and Thr181 may induce a large conformational change, which affects the interaction with NDR1. Although we have focused on Thr74 in this study, the additional phosphorylation at Thr181 might be also necessary to activate NDR1.

Materials and methods

Construction of expression vectors and recombinant proteins
cDNA of human MST2 (BC010640) was purchased from Open Biosystem. cDNAs of human MOB1 and NDR1 were obtained from human lung cDNA library (BioChain Institute Inc., Hayward, CA, USA) by PCR using the primer sets (5′-acgcgtatgagcttcctcttcagcag-3′ and 5′-gtcgactatctgtcttttgatc caag-3′ for MOB1; and 5′-acgcgtgccatggcaatgacaggctcaac-3′ and 5′-gtcgactattttgctgctttcatgta-3′ for NDR1). pCIneoMyc, pCIneoFH and pGex4T-1 (GE Healthcare Bio-sciences, UK) vectors were used to generate expression constructs (Ikeda *et al*., 2007). MST2, MOB1 and NDR1 expression constructs cover the following regions; pCIneoMyc and pGex4T-1 MOB1 2–216 (AAH03398); pCIneoMyc, pCIneoFH and pGex4T-1 MST2, 2–491 (AAH10640); pCIneoMyc and pCIneoFLAG NDR1, 1–465 (AAH12085). The kinase-negative mutant of MST2, MST2-KN, was generated by PCR, in which lysine at 56 is replaced by arginine. All mutants of MOB1 were produced by PCR and the sequences were confirmed.

Antibodies
Rabbit anti-MOB1 and anti-MST2 antibodies were raised against GST-MOB1 and GST-MST2-KN. Other antibodies were obtained from commercial sources; anti-Myc (9E10), (the American Type Culture Collection); anti-FLAG, anti-phosphoserine, anti-phosphothreonine and antiactin (Sigma-Aldrich, St Louis, MO, USA); fluorescein-isothiocyanate-conjugated and rhodamine-conjugated secondary (Millipore Corp., Billerica, MA, USA).

Cell culture and immunocytochemistry
HEK293FT and HeLa cells were grown in Dulbecco's Modified Eagle Medium containing 10% fetal bovine serum, 100 U/ml of penicillin, and 100 μg/ml of streptomycin under 5% CO_2 at 37 °C. Immunocytochemistry was performed as described previously (Ikeda *et al*., 2007).

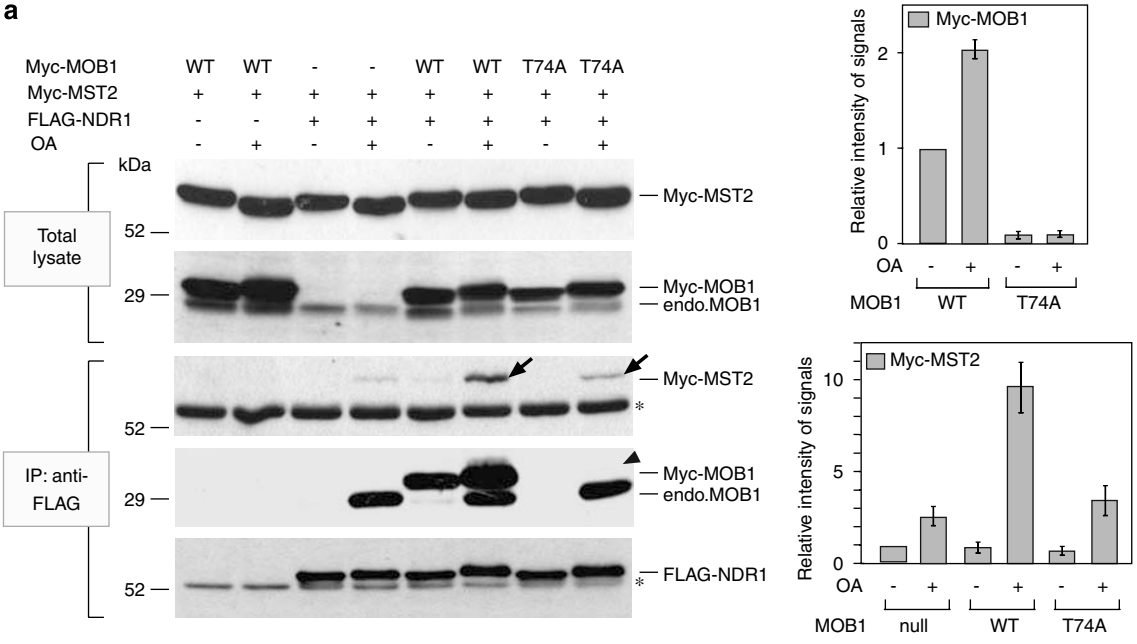
Immunoprecipitation
HEK293FT cells were transfected with expression vectors using Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA). After 48 h, cells were treated with either the mock (ethanol) or 1 μM of OA for 1 h. Cells from 12-well plate were homogenized in 300 μl of the lysis buffer containing 25 mM Tris-HCl (pH 8.0), 100 mM NaCl and 1% (w/v) Triton X-100 and centrifuged at 100 000 × *g* for 15 min at 4 °C. The supernatant was incubated with 0.2 μl of anti-FLAG antibody fixed on 10 μl of protein G-Sepharose 4 fast flow beads (GE Healthcare Bio-science). After the beads were washed, the precipitates were analyzed on SDS-polyacrylamide gel electrophoresis (SDS-PAGE) and immunoblotted with anti-Myc, anti-FLAG or anti-MOB1 antibody.

RNA interference
HEK293FT cells were transfected with 21-nucleotide oligomers (MOB1-specific ds RNA (Silencer predesigned siRNA no. 26356, Applied Biosystems, Foster City, CA, USA); and MST2-specific ds RNA (STK3-HSS110314, Invitrogen)) using Lipofectamine 2000.

Protein kinase assays
HEK293FT cells transfected with pCIneoFH MST2, pCIneoFH MST2-KN, pCIneoMyc NDR1 or pCIneoFLAG NDR1 were harvested in ice-cold phosphate-buffered saline (PBS) containing 1 mM Na_3VO_4 and 20 mM β-glycerophosphate and homogenized in 500 μl of buffer A (25 mM Tris-HCl (pH 8.0), 100 mM NaCl, 1% (w/v) Triton X-100, 1 mM NaF, 1 mM Na_3VO_4, 1 mM β-glycerophosphate, 100 μM 4-amidinophenylmethanesulfonyl fluoride, 1 mg/l leupeptin, and 2 mg/l aprotinin). After centrifugation at 100 000 × *g* for 15 min, the

Figure 5 Analysis of MOB1 mutants. (**a**) *In vitro* phosphorylation of MOB1 mutants by FH-MST2. A series of MOB1 mutants, in which each serine and threonine residue is replaced with alanine, were prepared by PCR. Ser6 and 7 and Ser9 and 10 are simultaneously replaced with alanine to generate MOB1 S6, 7A and S9,10A. 10 pmol of GST-MOB1 proteins were incubated with FH-MST2 *in vitro*. The phosphorylation of each protein is demonstrated as the ratio against that of GST-MOB1 WT. The significant reduction of phosphorylation was not detected in any mutant. Error bars indicate s.d. of three independent experiments. (**b**) The effect of MOB1 mutants on NDR1 activity. Myc-NDR1 was isolated from HEK293FT cells and used for the *in vitro* kinase assay with FH-MST2 and each GST-MOB1 mutant. The last column shows the effect of control GST. The effects of MOB1 T74A and T181A were significantly low, although they still exhibited some activation (asterisks). (**c**) Myc-NDR1 was expressed alone or with indicated FH-MOB1 proteins in HEK293FT cells. Cells were treated with the mock or OA. The activities of the immunoprecipitated Myc-NDR1 were evaluated. FH-MOB1 T74A and T181A were expressed similarly as FH-MOB1 WT (the bottom panel). The effects of MOB1 T74A and T181A on the OA-induced activation of NDR1 were significantly smaller. (**d**) *In vitro* phosphorylation of GST-MOB1 (62–85) and (170–193). GST-fusion proteins covering 24 amino acids around Thr74 and Thr181 were prepared and incubated with FH-MST2-KN or FH-MST2. GST-MOB1 (62–85) was phosphorylated by FH-MST2 but GST-MOB1 (170–193) was not. GST-MOB1 (62–85) T74A was not phosphorylated. (**e**) Various GST-MOB1 proteins were phosphorylated by MST2 *in vitro*. To evaluate the initial velocity, the incubation was performed at 25 °C instead of at 30 °C. ds, double stranded; FH, FLAG-His6; GST, glutathione S-transferase; MST, mammalian sterile 20-like; MOB1, Mps one binder; NDR1, nuclear Dbf2-related; OA, okadaic acid; WT, wild type.

supernatant was incubated for 3 h at 4 °C with anti-Myc or anti-FLAG antibody prebound to protein G Sepharose 4 fast flow beads, or anti-FLAG agarose gel (Sigma-Aldrich). For the MST2 kinase assay, the beads were washed three times with buffer A containing 500 mM NaCl, and twice with buffer B (20 mM Tris-HCl (pH 7.4), 1 mM Na_3VO_4, and 20 mM β-glycerophosphate). FH-MST2 was eluted with 0.2 g/l FLAG peptide (Sigma-Aldrich) in buffer B. Various amounts of GST-MOB1 were phosphorylated by FH-MST2 kinase in 20 µl of 40 mM Hepes-NaOH (pH 7.4) containing 10 mM $MgCl_2$, 1 mM dithiothreitol (DTT) 1 mM NaF, 1 mM Na_3VO_4, 1 mM β-glycerophosphate, and 10 µM γ^{32}P-ATP (5000 c.p.m./pmol) at 30 °C. For the phosphorylation of Myc-NDR1, GST-MST2 was used, because Myc-NDR1 and FH-MST2 are not completely separated on SDS-PAGE. The reaction was stopped by adding 10 µl of SDS-PAGE loading buffer. The samples were analyzed by SDS-PAGE and the gel was stained with Sypro Orange (Invitrogen). Protein staining and autoradiography was analyzed by FLA-3000 Image Analyzer (Fujifilm, Tokyo, Japan). The incorporated ^{32}P was counted by liquid scintillation counter LSC-5100 (Aloka). For the NDR1 assay, Myc-NDR1 was immunoprecipitated, washed three times with buffer A containing 500 mM NaCl and twice with buffer B, and incubated with various



combinations of GST-MOB1 proteins and FH-MST2 in 30 µl of buffer B containing 10 mM $MgCl_2$, 1 mM DTT, 20 µM γ^{32}P-ATP (2100 c.p.m./pmol), and 0.25 mM NDR1 substrate peptide (KKRNRRLSVA, single letters stand for amino-acid residues) at 30 °C. The amount of immunoprecipitated Myc-NDR1 was evaluated by the protein staining to normalize the kinase activity. To analyze the effect of MST2 on MOB1 and on NDR1 separately, 2 pmol of GST-MOB1 was incubated with FH-MST2 immobilized on anti-FLAG agarose gel in 30 µl of the reaction mixture containing 100 µM γ^{32}P-ATP (1500 c.p.m./pmol) for 90 min. The reaction mixtures were centrifuged at 10 000 × *g* for 5 min, and 20 µl of the supernatant was collected. The amount of the phosphorylated GST-MOB1 in the supernatant was calculated based on the incorporated ^{32}P. The supernatant was mixed with 10 µl of buffer B containing 10 mM $MgCl_2$, 1 mM DTT, and 0.75 mM NDR1 substrate peptide for the second incubation. Reactions were stopped by adding 3 µl of 0.5 M ethylenediaminetetraacetic acid (EDTA). The reaction mixtures were centrifuged at 10 000 × *g* for 1 min and 15 µl of the supernatant was spotted onto P-81 phosphocellulose paper (Whatmann). The paper was washed four times for 5 min each in 1% phosphoric acid.

In vitro *protein interaction assay*

20 pmol of GST-MOB1 was incubated with 1 pmol of immobilized FH-MST2 in 50 µl of buffer B containing 10 mM $MgCl_2$ and 1 mM DTT with or without 20 µM ATP for 1 h at 30 °C. After the incubation, the mixture was centrifuged at 15 000 × *g* for 1 min at 4 °C to remove FH-MST2. The supernatant was collected and the recovered GST-MOB1 was quantified. 4 pmol of the treated MOB1 was added to 4 pmol of immobilized FLAG-NDR1, and incubated in 300 µl of buffer

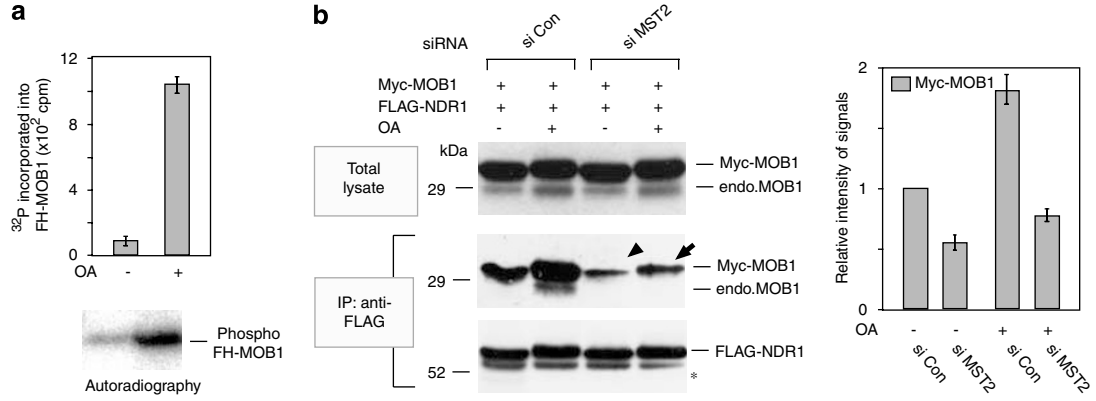


Figure 7 MOB1 is phosphorylated in the non-treated HEK293FT cells and MST2 facilitates the interaction between MOB1 and NDR1. (**a**) FH-MOB1 was expressed in HEK293FT cells. Cells were labeled with $^{32}P_i$ at 0.5 mCi/ml for 3 h and were treated with the mock or 1 µM OA for 1 h. Cells were collected and FH-MOB1 was immunoprecipitated. The precipitates were analyzed on SDS-PAGE and the incorporated ^{32}P into FH-MOB1 proteins were counted. FH-MOB1 was slightly phosphorylated even in the non-treated cells. (**b**) Myc-MOB1 and FLAG-NDR1 were expressed in HeLa cells that were transfected with the control ds RNA or MST2-specific ds RNA, and FLAG-NDR1 was immunoprecipitated. The knockdown of MST2 attenuated the coimmunoprecipitation of MOB1 in both the non-treated and the OA-treated cells (an arrowhead and an arrow). Asterisk indicates the immunoglobulin heavy chain. The quantitative data are shown (the right panel). ds, double stranded; FH, FLAG-His6; GST, glutathione S-transferase; MST, mammalian sterile 20-like; MOB1, Mps one binder; NDR1, nuclear Dbf2-related; OA, okadaic acid; WT, wild type.

Figure 6 MOB1 T74A does not function as a scaffold to link MST2 to NDR1. (**a**) FLAG-NDR1 and Myc-MST2 were expressed in HEK293FT cells. Cells were treated with the mock or OA, and the immunoprecipitation was performed with anti-FLAG antibody. Immunoprecipitates were blotted with anti-Myc (the first and the third panels), anti-MOB1 (the second and the fourth panels) and anti-FLAG (the fifth panel) antibodies. Asterisks indicate the immunoglobulin heavy chain. In the first two lanes, Myc-MST2 and Myc-MOB1 were expressed without FLAG-NDR1 as negative control samples. Myc-MST2 was coimmunoprecipitated with FLAG-NDR1 from OA-treated cells and the endogenous MOB1 was detected in the immunoprecipitates (lane 4). The expression of Myc-MOB1 WT enhanced the interaction of Myc-MST2 and FLAG-NDR1, especially in OA-treated cells (lane 6, an arrow). The interaction of Myc-MOB1 and endogenous MOB1 with FLAG-NDR1 was also augmented by OA treatment. Under the expression of Myc-MOB1 T74A, Myc-MST2 and endogenous MOB1 were coimmunoprecipitated with FLAG-NDR1 from OA-treated cells, but Myc-MOB1 T74A was not detected in this exposure (lane 8, an arrowhead). The signals of Myc-MOB1 WT, Myc-MOB1 T74A, and Myc-MST2 in the immunoprecipitates from mock-treated or OA-treated cells were quantified and the data are shown (the right panels). Error bars indicate s.d. of three independent experiments. Protein standards are indicated on the left. (**b**) GST-MOB1 WT and GST-MOB1 T74A were incubated with FH-MST2 immobilized on anti-FLAG agarose gel in the kinase buffer with or without ATP, and centrifuged to remove FH-MST2. GST-MOB1 WT and GST-MOB1 T74A were then incubated with FLAG-NDR1 immobilized on anti-FLAG agarose gel and the attached proteins were detected with anti-MOB1 antibody. GST-MOB1 WT was efficiently captured by FLAG-NDR1 when it was incubated in the complete kinase buffer and phosphorylated (lane 2). GST-MOB1 T74A did not bind to FLAG-NDR1, even when incubated in the complete kinase buffer. It is to note that GST-MOB1 T74A shows the mobility shift in the input, because this mutant has other phosphorylation site(s) than Thr74. (**c**) The subcellular distribution of MOB1 WT and MOB1 T74A in HeLa cells and the recruitment of NDR1 outside the nucleus. FLAG-NDR1, Myc-MOB1 WT and MOB1 T74A were expressed in HeLa cells (the top panels). FLAG-NDR1 was coexpressed with either Myc-MOB1 WT or Myc-MOB1 T74A. FLAG-NDR1 was recruited by Myc-MOB1 WT, but not by Myc-MOB1 T74A, from the nucleus (the middle and the bottom panels, arrowheads). Bars, 10 µm. In the subcellular fractionation, Myc-MOB1 WT and Myc-MOB1 T74A were recovered in both of the cytosolic and the membrane fractions (the right panels). ds, double stranded; FH, FLAG-His6; GST, glutathione S-transferase; MST, mammalian sterile 20-like; MOB1, Mps one binder; NDR1, nuclear Dbf2-related; OA, okadaic acid; WT, wild type.

A for 3 h at 4 °C. The mixture was centrifuged at 15 000 × *g* for 1 min and the gel was washed three times with buffer A containing 500 mM NaCl, and once with PBS. GST-MOB1 was detected by immunoblotting with anti-MOB1 antibody.

Subcellular fractionation
HeLa cells were collected by scraping and homogenized in the hypotonic buffer (10 mM Hepes-NaOH (pH 7.4), 10 mM NaCl, 1.5 mM $MgCl_2$, 4 μM leupeptin, 0.5 mM phenylmethane sulfonylfluoride and 1 mM DTT). The samples were centrifuged for 90 min at 150 000 × *g* at 4 °C. The comparable amount of the total lysates, the cytosolic fraction and the membrane fraction were analyzed.

Metabolic labeling
HEK293FT cells were transfected with pCIneoFH MOB1 or the empty vector. After 48 h, cells were washed twice with the phosphate-free medium (Invitrogen) and labeled with $^{32}P_i$ at 0.5 mCi/ml. After 3 h labeling, cells were treated with 1 μM OA for 1 h. FH-MOB1 was immunoprecipitated and the incorporated ^{32}P was measured.

Protein quantification
Purified recombinant proteins and various amounts of bovine serum albumin were separated by SDS-PAGE, stained with Sypro Orange, and detected with FLA-3000 Image Analyzer. The quantification was performed with Fujifilm Image Gauge software.

Abbreviations

Ds, double stranded; DTT, dithiothreitol; FH, FLAG-His6; GST, glutathione S-transferase; KN, kinase negative; MOB, Mps one binder; MOB1 WT, the wild type of MOB1; MST, mammalian sterile 20-like; NDR, nuclear Dbf2-related; OA, okadaic acid; PBS, phosphate-buffered saline; Sav, Salvador; SDS–PAGE, SDS–polyacryalmide gel electrophoresis.

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