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The human tumour suppressor LATS1 is activated by human MOB1 at the membrane

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Abstract

Downregulation of the LATS1 tumour suppressor protein kinase contributes to tumour formation in mammals and flies. Strikingly, the tumour suppressor activity depends on the interaction with Dmob (*Drosphila* Mps1-One binder) in *Drosophila melanogaster*. Recently, human LATS1 was reported to interact with human MOB1 (hMOB1), but the activation of LATS1 was not addressed. Here, we identified a highly conserved hMOB1-binding motif within LATS1's primary structure. While co-expression of LATS1 with hMOB1 did not elevate LATS1 kinase activity in mammalian cells, membrane-targeting of hMOB1 resulted in a significant increase of LATS1 activity. This stimulation was dependent on intact activation segment and hydrophobic motif phosphorylation sites, and was further found to occur a few minutes after membrane association. Therefore, we suggest a potential in vivo mechanism of LATS1 activation through rapid recruitment to the plasma membrane by hMOB1 followed by multi-site phosphorylation, thereby providing insight into the molecular regulation of the LATS tumour suppressor.

Keywords: Signal transduction; LATS; Kinase; Tumour suppressor; MOB; Membrane; Activation mechanism

The conserved NDR (nuclear Dbf2-related) family represents a subclass of the AGC serine/threonine protein kinases and consists of mammalian NDR1, NDR2, LATS1 (large tumour suppressor 1) and LATS2, *Drosophila melanogaster* TRC and LATS, *Caenorhabditis elegans* SAX-1 and LATS, and a number of fungi and plant kinases [1]. Human, fly, and yeast NDR kinases have been reported to require phosphorylation on both the activation segment and the hydrophobic motif for activation [2–9]. All family members have also a conserved N-terminal regulatory (NTR) domain of different lengths, best characterized in mammalian NDR1/2 [8–12]. Human MOB1A (hMOB1A) binds to this domain, thereby probably releasing autoinhibition of activation segment autophosphorylation [10]. However, although this activation mechanism is readily recapitulated in vitro, co-expression of the various components in tissue culture cells proved inefficient to activate NDR1/2 kinases [10,11], but when NDR1/2 itself or its co-activator hMOB1A/B/2 (hMOBs) is targeted to the membrane, an increase in NDR1/2 kinase activity was readily achieved [11,13].

While the biological functions of mammalian NDR1/2 remain the subject of intense research, mammalian LATS kinases are implicated in regulating cell cycle progression and apoptosis [14–23]. Moreover, LATS1 deficient mice developed ovarian stromal cell tumours and soft tissue sarcomas [24]. Promoter inactivation and missense mutations affecting LATS1 have further been reported in human sarcomas, ovarian carcinomas, and breast cancer [25–27]. These data suggest that dysregulated LATS1 expression contributes to tumour formation in mammals. Strikingly, the *warts*/*lats* gene confers also tumour suppressor activity in invertebrates [28–30].

The signalling pathway involving Lats/Warts, Hippo, Salvador, and Yorkie proteins was previously shown to be essential for proliferation control in *D. melanogaster*

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[31–38]. However, recently it was reported that the catalytic activity of the *Drosophila* Lats protein kinase can be regulated by co-activator binding [39,40]. The Mats protein (MOB as tumour suppressor; also termed Dmob1), a member of the superfamily of MOB proteins, interacts genetically and physically with Lats. The human ortholog of Mats, hMOB1A, is able to rescue the lethality of Mats loss-of-function in flies, suggesting that the regulation of LATS by MOBs is conserved from *Drosophila* to human. In support of this notion, human LATS1 was recently found to bind to hMOB1A [14], however, this study did not address the activation of LATS1 by hMOB1.

Here, we explored further the interaction of human LATS1 with hMOB proteins. Furthermore, considering that the biochemical analysis of Lats activation by Mats was carried out solely in the presence of okadaic acid (OA) [39], a potent PP2A inhibitor proven to also activate human LATS1 kinase [2], we further investigated the activation of human LATS1 by hMOBs in a cellular context without any drug treatment. Moreover, given that human LATS1 and NDR1/2 kinases appear to be regulated in a similar manner, we also tested the role of altered subcellular localization of hMOBs on human LATS1 activation, since we reported previously that in vivo activation of human NDR1/2 kinases can be achieved by translocation to the plasma membrane [11,13].

Materials and methods

Cell culture, chemicals, drug treatments, transfections, and antibodies. COS-7 cells were cultured and transfected as described [11]. In some experiments, cells were treated for 60 min with 1 μM okadaic acid (OA; Alexis Corp.). In other experiments, cells were serum-starved for 2 h prior to transfection. The transfection mixture was removed after 4 h and cells were serum-starved overnight before stimulation with 12-*O*-tetradecanoylphorbol 13-acetate (TPA; 100 ng/ml; Amersham Biosciences). Anti-HA 12CA5 and anti-myc 9E10 antibodies were used as hybridoma supernatants. Anti-HA Y11 antibodies were from Santa Cruz.

Construction of plasmids. All human hMOB1A, hMOB1B, and hMOB2 constructs have been described previously [11]. Human LATS1 cDNAs were subcloned into pcDNA3-derivatives containing a haemagglutination (HA) eptitope. Except for LATS1 (D846A), all mutants of LATS1 were generated by using the QuikChange procedure with pcDNA3-HA-LATS1 as template. All constructs were confirmed by sequence analysis. Experimental details of the generation of constructs are available upon request.

Fractionation of cells and immunofluorescence microscopy. Cytosolic and membrane-associated proteins were separated as described recently [11]. Processing of cells for immunofluorescence has also been described elsewhere [41].

Immunoblotting, immunoprecipitation, and HA-LATS1 kinase assay. To detect LATS1 or hMOB proteins, samples were resolved by 6% or 12% SDS–PAGE and processed as reported [11]. Harvesting and processing of samples for immunoprecipitation was also carried out as described [11]. Analysis of immunoprecipitated HA-LATS1 kinase activity was performed as reported for HA-NDR using the same buffer conditions and substrate peptide [11].

HA-LATS1 autophosphorylation assay. Cells were processed for immunoprecipitation as described elsewhere [11]. After the final wash with IP buffer, immunocomplexes were washed twice with kinase buffer (25 mM Hepes, pH 7.4, 50 mM NaCl, 5 mM $MgCl_2$, 5 mM β-glycerophosphate, 1 mM benzamidine, 4 μM leupeptin, and 1 mM DTT). Beads were resuspended in 30 μl kinase buffer containing 1 μM microcystin, 1 μM cAMP-dependent protein kinase inhibitor peptide, and 100 μM [γ-P^{32}]-ATP (~1000 cpm/pmol). After 30 min incubation at 30 °C, reactions were stopped by addition of Laemmli buffer, and samples were analysed by SDS–PAGE, followed by autoradiography.

Results

Human LATS1 kinase activity can be measured using the substrate peptide of human NDR1/2

Since LATS kinase activity has previously only been monitored by autophosphorylation [2,39], we established a kinase assay to monitor human LATS1 transphosphorylation activity. By applying the same conditions as reported for human NDR1/2 kinases [11], we could readily measure human LATS1 kinase activity towards the standard NDR1/2 substrate peptide (Fig. 1). As predicted, HA-LATS1 immunoprecipitates displayed elevated kinase activity once HA-LATS1 wild-type (wt) expressing cells had been treated with OA (Fig. 1B). The kinase activity measured by peptide phosphorylation was mirrored by increased autophosphorylation activity of LATS1 (Fig. 1A). HA-LATS1 kinase dead (D846A), and phosphorylation site mutants (S909A), (T1079A) or

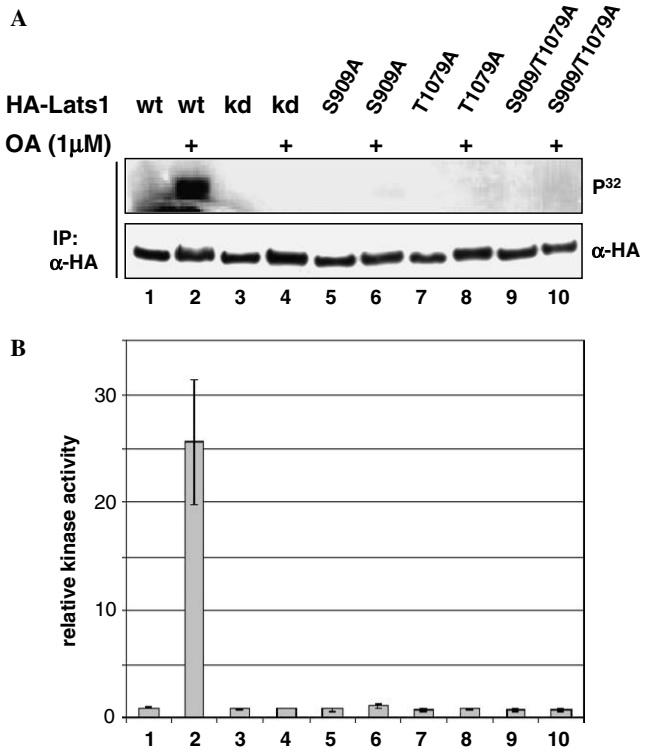

Fig. 1. Activation of LATS1 requires Ser909 and Thr1079. (A) Cells expressing HA-LATS1 (wt), (kd), (S909A), (T1079A) or (S909/T1079A) were incubated in the absence (−) or presence (+) of OA, before processing for immunoprecipitation using anti-HA 12CA5 antibody. Complexes were analysed by immunoblotting using anti-HA (bottom panel) or subjected to an autophosphorylation assay (top panel). (B) In parallel, immunocomplexes were subjected to peptide kinase assays.

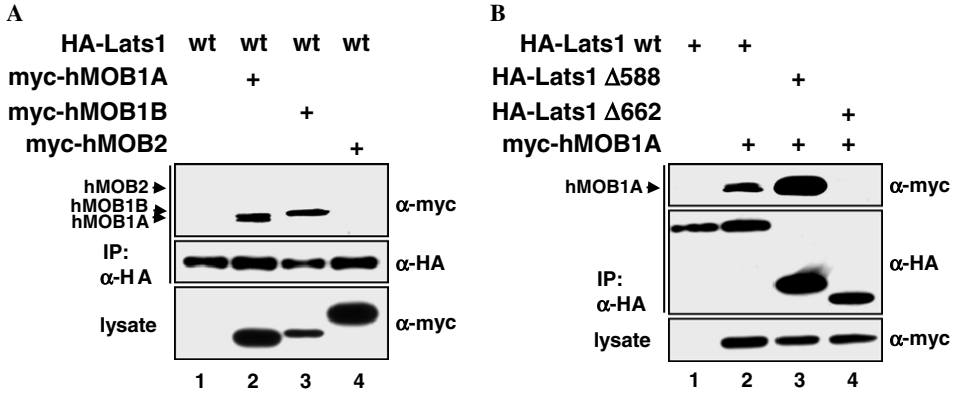

Fig. 2. Human LATS1 binds to hMOB1A/B, but not hMOB2. (A) Lysates of cells co-expressing HA-LATS1 (wt) with the indicated myc-tagged hMOB species were analysed by immunoprecipitation using anti-HA antibody. Complexes were assayed by Western blotting using anti-myc (top panel) or anti-HA antibody (middle panel). Input lysates were analysed by anti-myc (bottom panel). (B) Lysates containing indicated HA-LATS1 and myc-hMOB1A forms were analysed by immunoprecipitation. Complexes were assayed by immunoblotting using anti-myc (top panel) or anti-HA antibody (middle panel). Input lysates were analysed by anti-myc (bottom panel).

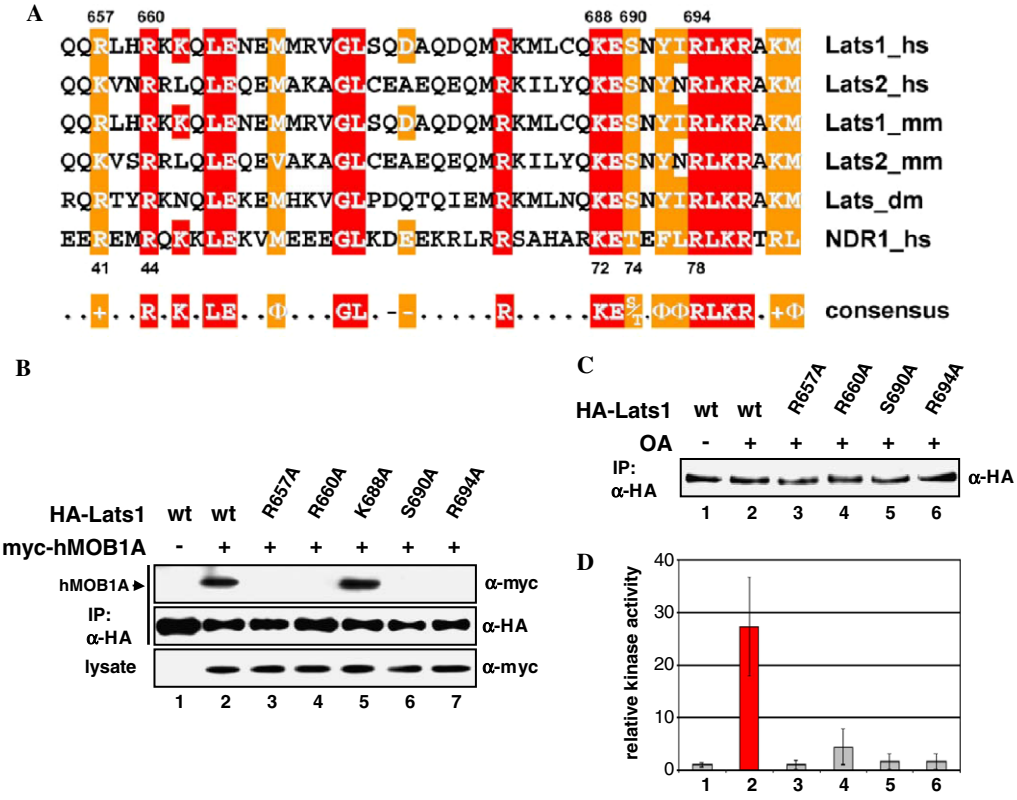

Fig. 3. LATS1 interacts with hMOB1 through a conserved domain. (A) Alignment of human (hs), mouse (mm), and drosophila (dm) LATS protein sequences with amino acids 39–84 of human NDR1. Identical residues are marked in red, similar residues in orange. The consensus sequence is indicated below. Hydrophobic residues (Φ). Positive charge (+). Negative charge (−). (B) Cells expressing HA-LATS1 (wt), (R657A), (R660A), (K688A), (S690A) or (R694A), and myc-hMOB1A were analysed by immunoprecipitation. Complexes were assayed by Western blotting using anti-myc (top panel) or anti-HA (middle panel). Lysates were analysed by anti-myc (bottom panel). (C) Cells expressing indicated HA-LATS1 species were processed for immunoprecipitation using anti-HA antibody, before analysis by immunoblotting with anti-HA. Before harvesting, cells were incubated with OA (+). (D) In parallel, immunocomplexes were tested by a peptide kinase assay. Significantly increased kinase activity is highlighted in red.

(S909/T1079A) were not activated by OA, further demonstrating the specificity of the assay. These data are in full agreement with recent observations [2] showing that phosphorylation in the activation segment (Ser909 of LATS1) and the hydrophobic motif (Thr1079 of LATS1) is essential for LATS1 kinase activity upon OA treatment.

LATS1 binds to hMOB1 through a conserved motif

To address the interaction of human LATS1 with both hMOBs, HA-LATS1 was co-expressed with myc-tagged hMOB1A/B and hMOB2, prior to processing for immunoprecipitation and subsequent immunoblotting (Fig. 2). In full support of recent findings [14], hMOB1A and hMOB1B (hMOB1) bound to HA-LATS1, whereas hMOB2 did not (Fig. 2A). Considering that a LATS1 deletion mutant lacking the first 588 amino acids (Δ588) was still activated by OA while a mutant deleted of the first 662 residues (Δ662) was not [2], we speculated that this difference in activation might represent the capability of LATS1 to associate with hMOB1. Consequently, cells co-expressing HA-LATS1 wt, (Δ588) or (Δ662) with myc-hMOB1A were subjected to a co-immunoprecipitation experiment (Fig. 2B). As predicted, HA-LATS1 (Δ588) interacted with hMOB1A, but HA-LATS1 (Δ662) did not (Fig. 2B), illustrating that the first 588 amino acids of LATS1 are dispensable for hMOB1-binding. Subsequently, by aligning the putative MOB1 association residues of LATS1 with human NDR1/2 in silico, we were able to determine a region conserved between human LATS1/2 and NDR1 (Fig. 3A), which has already been demonstrated to be crucial for hMOB1A binding by human NDR1/2 kinases [10]. To test the role of the conserved residues, five potential key residues of LATS1 were mutated and tested for their ability to associate with hMOB1 (Fig. 3B). Four out of the five LATS1 mutants [(R657A), (R660A), (S690A), and (R694A)] did not co-immunoprecipitate hMOB1 (Fig. 3B), and were not significantly activated by OA treatment (Fig. 3D). Similar to results obtained with HA-NDR1 (K72A) [10], HA-LATS1 (K688A) bound to hMOB1A comparable to wild-type LATS1 (Fig. 3B, lane 5), but was not activated by OA treatment (data not shown). Overall, this analysis demonstrates that LATS1 and NDR1/2 share a common motif required for hMOB1-binding and kinase activation.

LATS1 is activated by membrane-bound hMOB1

Although LATS1 and hMOB1 readily interacted (Figs. 2 and 3), we did not detect any LATS1 activity changes when co-expressed with hMOBs (Figs. 4A and B),

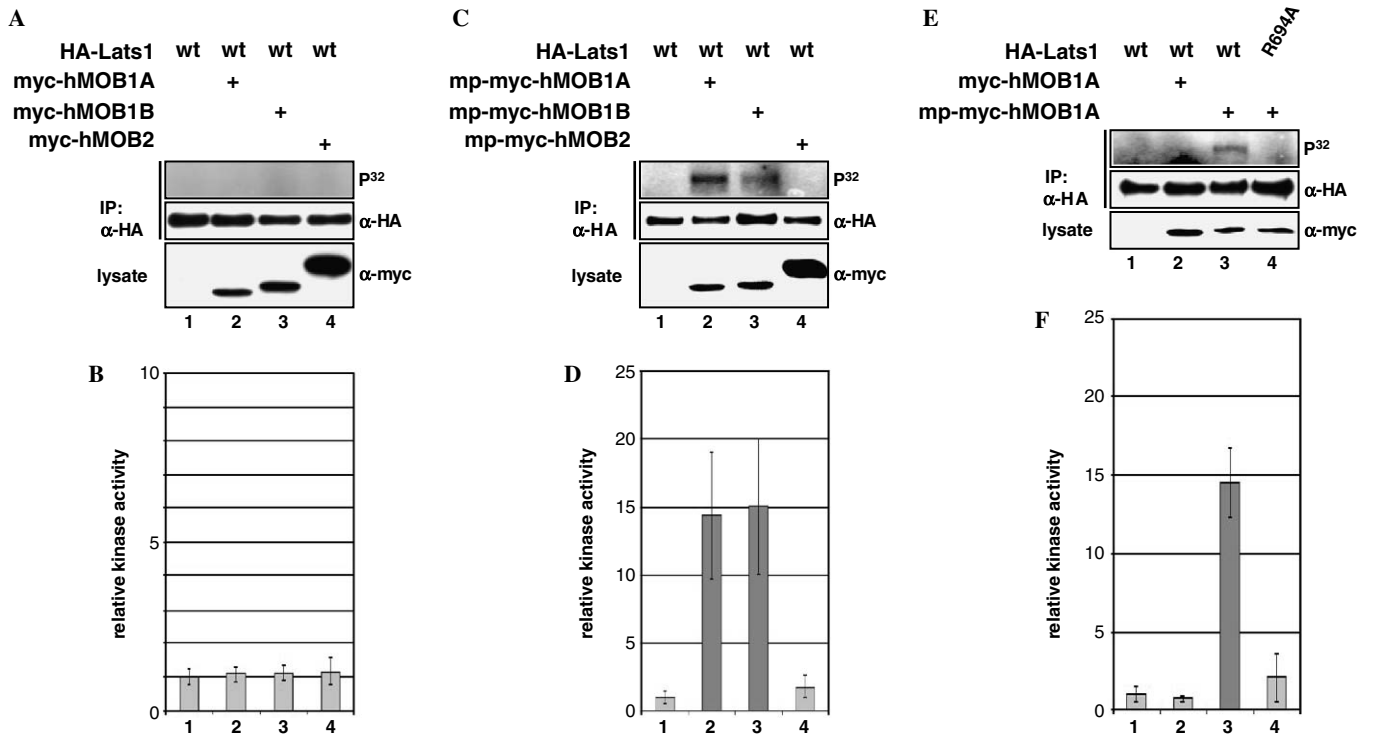

Fig. 4. Membrane-targeted, but not non-targeted hMOB1, stimulates LATS1 kinase activity. (A) Lysates of cells expressing the indicated combinations of HA-LATS1 and myc-hMOBs species were analysed by immunoprecipitation using anti-HA, before assaying by Western blotting using anti-HA (middle panel) or an autophosphorylation assay (top panel). Lysates were immunoblotted with anti-myc antibody (bottom panel). (B) In parallel, immunocomplexes were subjected to peptide kinase assays. (C) Lysates containing the indicated combinations of HA-LATS1 and membrane-targeted hMOBs (mp-myc-hMOB) were analysed by immunoprecipitation. Complexes were tested by immunoblotting with anti-HA (middle panel) or an autophosphorylation assay (top panel). Lysates were immunoblotted with anti-myc antibody (bottom panel). (D) In parallel, immunocomplexes were subjected to peptide kinase assays. Increased kinase activity is marked in dark gray. (E) Cells were transfected with indicated cDNAs and subjected to immunoprecipitation using anti-HA, before assaying by immunoblotting with anti-HA (middle panel) or an autophosphorylation assay (top panel). Lysates were immunoblotted with anti-myc antibody (bottom panel). (F) Immunocomplexes were also subjected to peptide kinase assays. Of note, kinase activity of LATS1 was only increased by mp-myc-hMOB1 (dark gray bar).

mirroring recent findings for human NDR1/2 [10,11]. Thus, we sought to alter the subcellular localization of hMOB1, as such manipulations lead to increased NDR1/2 kinase activity in mammalian cells [11,13]. Significantly, when the myristoylation/palmitoylation (mp) motif from the Lck tyrosine kinase was fused to the N-terminus of hMOB1, the resulting membrane-targeted hMOB1 (mp-hMOB1) activated LATS1 upon co-expression in the absence of OA, whereas mp-hMOB2 did not (Figs. 4C and D). Furthermore, the hMOB1-binding deficient mutants, HA-LATS1 (R694A) and (R660A), were not stimulated by mp-hMOB1 (Figs. 4E and F; data not shown), suggesting that LATS1 activation by mp-hMOB1 is dependent on their interaction.

Activation of LATS1 at the membrane

To address whether this activation occurred indeed at the membrane, we separated transfected cells into cytoplasmic and membranous fractions (Fig. 5A). While most LATS1 accumulated in the cytoplasm when expressed alone, LATS1 was significantly enriched in the membrane fraction upon co-expression of mp-hMOB1A (Fig. 5A, lanes 1–4). HA-LATS1 (R694A) and (R660A) were not recruited to membranous structures by mp-hMOB1A (Fig. 5A, lanes 5 and 6; data not shown), demonstrating that LATS1 is recruited to the membrane by binding to mp-hMOB1A. Moreover, increased LATS1 kinase activity was solely observed in the membrane fraction when LATS1 (wt) and mp-hMOB1A were co-expressed (Figs. 5B and C), hence, showing that only membrane-recruited LATS1 species are active. However, this analysis did not reveal the intracellular membrane structures where LATS1 activation occurs. Therefore, we examined cells by immunofluorescence microscopy (Fig. 5D). LATS1 was mostly cytoplasmic regardless whether untargeted hMOB1A was co-expressed or not (Fig. 5D, top and top middle panels). In contrast, when mp-hMOB1A was co-expressed, LATS1 decorated the plasma membrane (Fig. 5D, bottom middle panels). Of note, HA-LATS1 (R694A) was not recruited to the plasma membrane (Fig. 5D, bottom panels), indicating that association of LATS1 with the plasma membrane depends on binding to mp-hMOB1A.

Next, considering that phosphorylation of LATS1 on the activation segment (S909) and hydrophobic motif (T1079A) is essential for kinase activation following OA treatment (see Fig. 1), we tested the stimulation of HA-LATS1 (S909A) and (T1079A) by mp-hMOB1A (Figs. 6A and B). This analysis revealed that HA-LATS1 (S909A) and (T1079A) displayed only slightly increased kinase activity when co-expressed with mp-hMOB1A (Figs. 6A and B). Thus, residues S909 and T1079 of LATS1 are crucial for

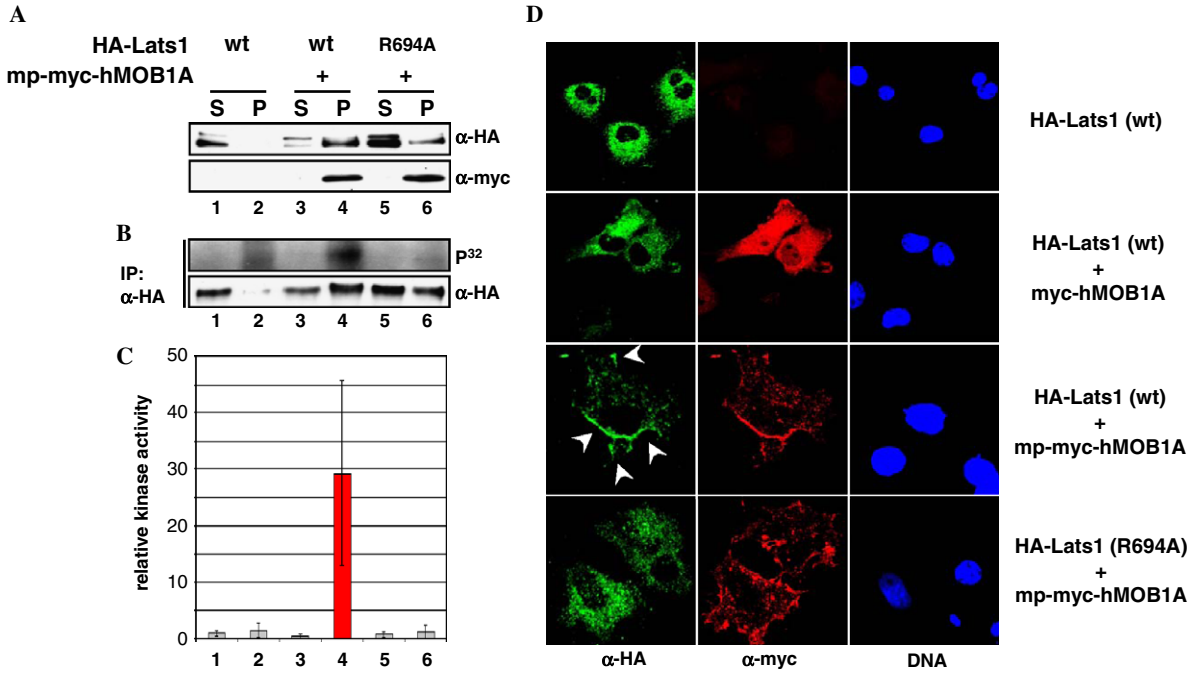

Fig. 5. LATS1 is stimulated by membrane-bound hMOB1 at the plasma membrane. (A) Cells transfected with the indicated HA-LATS1 and myc-hMOB1A constructs were subjected to S100/P100 fractionation (S, cytoplasm; P, membrane), before immunoblotting with anti-HA (top panel) and anti-myc antibody (bottom panel). (B) Equal amounts of S100 and P100 fractions were submitted to immunoprecipitation with anti-HA antibody, before analysis by immunoblotting using anti-HA (bottom panel) or an autophosphorylation assay (top panel). (C) In parallel, immunocomplexes were subjected to peptide kinase assays. Of note, kinase activity of LATS1 was only increased in the membrane fraction (red bar). (D) Cells co-expressing HA-LATS1 (wt)/empty vector (top panels), HA-LATS1 (wt)/myc-hMOB1A (middle top panels), HA-LATS1 (wt)/mp-myc-hMOB1A (middle bottom panels), or HA-LATS1 (R694A)/mp-myc-hMOB1A (bottom panels) were processed for immunofluorescence analysis with anti-HA Y11 (green) and anti-myc 9E10 antibody (red). Nuclei are shown in blue.

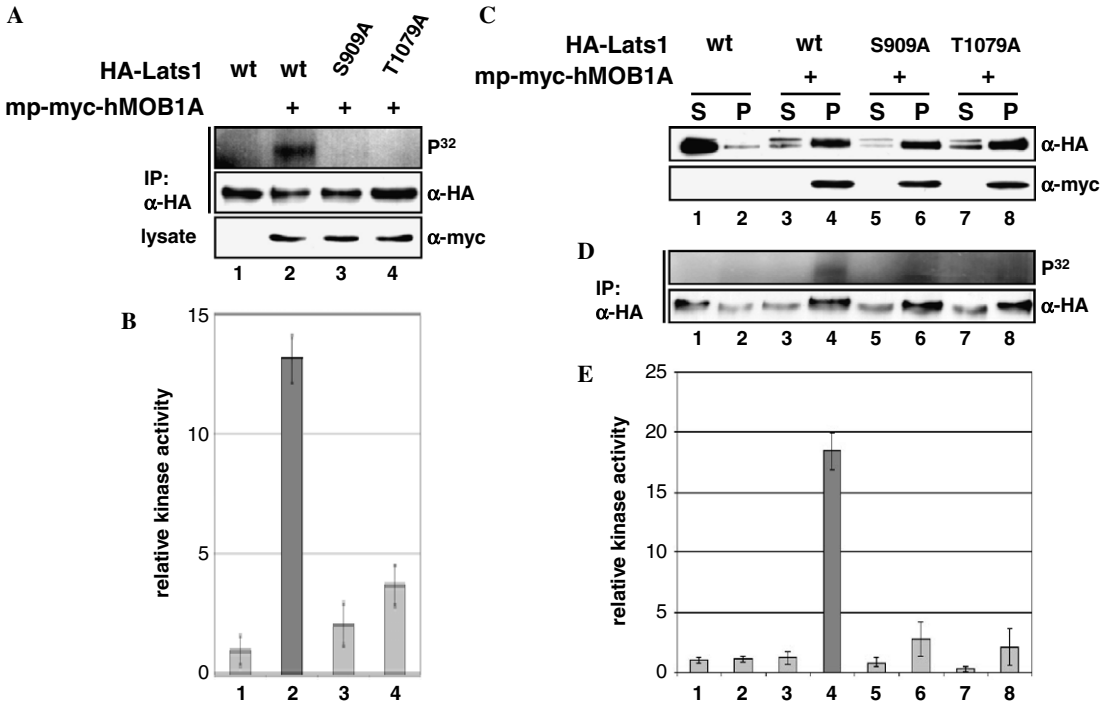

Fig. 6. Residues S909 and T1079 of LATS1 are required for activation at the membrane. (A) Lysates of cells expressing the indicated combinations of HA-LATS1 and mp-myc-hMOB1A were analysed by immunoprecipitation using anti-HA, before assaying by Western blotting using anti-HA (middle panel) or an autophosphorylation assay (top panel). Lysates were immunoblotted with anti-myc antibody (bottom panel). (B) In parallel, immunocomplexes were subjected to peptide kinase assays. Strongly increased kinase activity is shown in dark gray. (C) Cells transfected with the indicated HA-LATS1 and mp-myc-hMOB1A constructs were subjected to S100/P100 fractionation (S, cytoplasm; P, membrane), before immunoblotting with anti-HA (top panel) and anti-myc antibody (bottom panel). (D) Equal amounts of S100 and P100 fractions were submitted to immunoprecipitation with anti-HA antibody, before analysis by immunoblotting using anti-HA (bottom panel) or an autophosphorylation assay (top panel). (E) In parallel, immunocomplexes were subjected to peptide kinase assays. Of importance, kinase activity of LATS1 was only strongly elevated in the membrane fraction of cells co-expressing HA-LATS1 (wt) and mp-myc-hMOB1A (dark gray bar).

both, activation by PP2A inhibition and by membrane-targeting. Significantly, both LATS1 mutants were recruited to the membrane to the same extent as wild-type species (Fig. 6C), suggesting that their inability to be activated was not due to a lack of enrichment at the membrane. This notion was further supported by the fact that membrane fractions of HA-LATS1 (S909A) and (T1079A) expressing cells displayed only weakly elevated kinase activity in comparison to wild-type protein (Fig. 6E), although equal amounts of LATS1 kinase were immunoprecipitated (Fig. 6D). Overall, these data strongly suggests that membrane recruitment of LATS1 through hMOB1A is not sufficient to activate LATS1. In addition, the activation segment and hydrophobic motif of LATS1 have to be intact in order to allow stimulation at the membrane.

Induced membrane association of hMOB1A leads to rapid activation of LATS1

To finally analyse the kinetics of LATS1 activation by membrane-associated hMOB1, we made use of a construct that allows rapid and inducible translocation of hMOB1A to the membrane [11]. The C1 domain of PKCα was fused to the N-terminus of hMOB1A (C1-hMOB1A), leading to membrane binding after a few minutes of TPA stimulation (Fig. 7A, bottom panel). While HA-LATS1 (wt) was readily enriched at membranous structures 10 min after TPA addition (Fig. 7A, lanes 1–6), co-expression of HA-LATS1 (R694A) with C1-hMOB1A did not lead to recruitment of LATS1 species to the membrane (Fig. 7A, lane 10). LATS1 (wt) expressed alone was not enriched at the membrane after TPA treatment (data not shown). Overall, these findings suggest that LATS1 is rapidly recruited to the membrane by hMOB1A in an interaction dependent manner. To address whether this recruitment promoted activation of LATS1, we immunoprecipitated HA-LATS1 species and analysed kinase activities (Figs. 7B and C). Here, we could show that LATS1 was only potently activated when the co-expressed C1-hMOB1A was induced by TPA treatment (Figs. 7B and C, lane 4). In stark contrast, LATS1 (wt) expressed alone, or LATS1 (R694A) co-expressed with C1-hMOB1A, displayed no change in kinase activity (Figs. 7B and C, lanes 1 and 2, 5 and 6), excluding the possibility that the observed activation of LATS1 after TPA induction was simply due to direct activation of LATS1 by TPA. Taken together, these data show that LATS1 species are rapidly activated following recruitment to the membrane by hMOB1A.

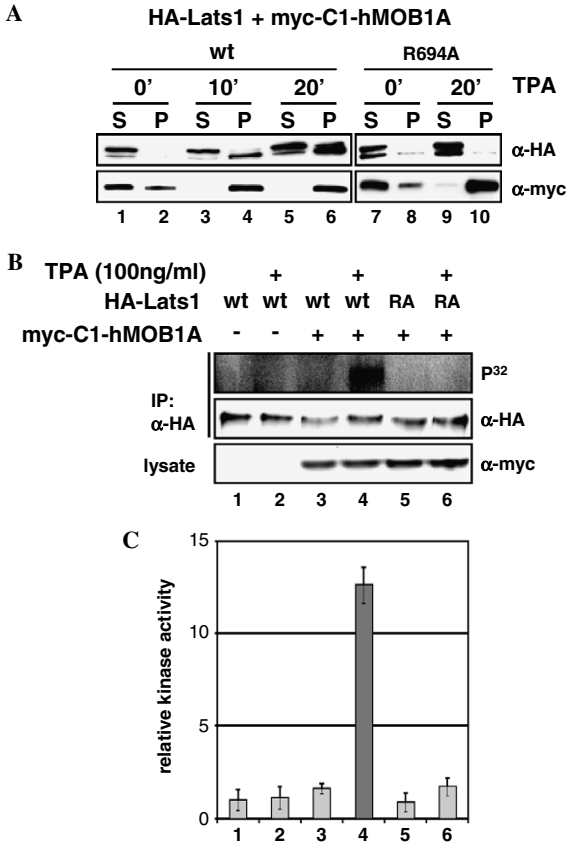

Fig. 7. Induced membrane association of hMOB1A results in rapid activation of LATS1. (A) Cells expressing myc-tagged hMOB1A fused to the C1 domain of PKCα (myc-C1-hMOB1A) and HA-LATS1 (wt) or a MOB1-binding deficient mutant (RA, R694A) were serum-starved overnight, then incubated with TPA for the indicated times and subsequently S100/P100 fractionated (S, cytoplasm; P, membrane), before immunoblotting with anti-HA (top panel) and anti-myc antibody (bottom panel). (B) Indicated cell lysates were analysed by immunoprecipitation, before assaying by Western blotting using anti-HA (middle panel) or an autophosphorylation assay (top panel). Lysates were immunoblotted with anti-myc antibody (bottom panel). Prior to lysis cells were incubated for 20 min with (+) or without (−) TPA. (C) In parallel, immunocomplexes were subjected to kinase assays. The elevated LATS1 activity is highlighted in dark gray.

Discussion

In summary, our interaction data are in full agreement with recent observations [14], by showing that LATS1 associates with hMOB1A and hMOB1B, but not hMOB2. Further, we show that human LATS1 and NDR1/2 share a very similar hMOB1-binding motif through which kinase activity is regulated. The hMOB1-binding motif also exists in human LATS2 and even in Lats from flies (see Fig. 3A), predicting that mutations at key residues defined in our study will also affect their interaction with MOBs. By having defined a hMOB1-binding consensus sequence, it is also of high interest to address which other kinases/proteins contain the same motif, and to test these putative MOB-regulated molecules experimentally.

All experiments carried out so far suggest that mammalian LATS1 and NDR1/2 are not activated by simple overexpression of their hMOB co-activators in spite of strong physical interaction [10,11]. However, data reported here, and elsewhere [11,13], show that hMOBs can fully activate human NDR/LATS kinases once targeted to the membrane. Interestingly, human NDR1/2 and LATS1/2 are also activated by phosphorylation through mammalian Ste20-like (MST) kinases [2,13]. MST1/2 can phosphorylate LATS1/2 in vitro [2], while MST3 targets NDR1/2 [13]. Considering that MST3 is responsible for phosphorylation of human NDR1/2 at the membrane, it is very tempting to speculate that membrane recruitment of LATS1 by hMOB1 facilitates LATS1 phosphorylation by MST1/2 in vivo. Furthermore, LATS1 derivatives carrying mutations at the activation segment or hydrophobic motif phosphorylation sites are not stimulated by mp-hMOB1, showing that phosphorylation on both sites is essential for activation. However, whether MST1/2 specifically phosphorylate LATS1 on the hydrophobic motif and not the activation segment is yet to be established.

Overall, these findings illustrate the conservation of the regulation mechanism of human LATS1 and NDR1/2. Given the detailed biochemical characterization of human NDR1/2, it is very tempting to speculate that like NDR1/2 itself [10], human LATS1 is also regulated by autophosphorylation of S909, while phosphorylation on T1079 is performed by a Ste20-like kinase such as MST1/2. Considering the conservation of the hMOB1-binding site, it is very likely that hMOB1-binding also leads to release of inhibition of LATS1 autophosphorylation activity, as already proposed for human NDR1/2 [10]. Moreover, since replacing the hydrophobic motif of NDR2 by the phosphorylation mimicking hydrophobic motif of PRK-2 has been shown to result in a constitutively active kinase [8], it will also be of interest to test the consequence of such manipulations on LATS1, in particular its tumour suppressor function. Overall, we believe that knowledge gained from studies of human NDR1/2 kinases can be used to predict the regulatory mechanism(s) of the LATS tumour suppressor in mammals and invertebrates. A more detailed study of the NDR/LATS protein kinase regulation at the molecular level will be essential to fully understand the role of this family in cancer, especially the regulation of LATS1 as tumour suppressor.

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