Stable MOB1 interaction with Hippo/MST is not essential for development and tissue growth control

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The Hippo tumor suppressor pathway is essential for development and tissue growth control, encompassing a core cassette consisting of the Hippo (MST1/2), Warts (LATS1/2), and Tricornered (NDR1/2) kinases together with MOB1 as an important signaling adaptor. However, it remains unclear which regulatory interactions between MOB1 and the different Hippo core kinases coordinate development, tissue growth, and tumor suppression. Here, we report the crystal structure of the MOB1/NDR2 complex and define key MOB1 residues mediating MOB1’s differential binding to Hippo core kinases, thereby establishing MOB1 variants with selective loss-of-interaction. By studying these variants in human cancer cells and Drosophila, we uncovered that MOB1/Warts binding is essential for tumor suppression, tissue growth control, and development, while stable MOB1/Hippo binding is dispensable and MOB1/Trc binding alone is insufficient. Collectively, we decrypt molecularly, cell biologically, and genetically the importance of the diverse interactions of Hippo core kinases with the pivotal MOB1 signal transducer.

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The Hippo pathway is vital for organ growth control and tissue homeostasis, and its dysregulation has been linked to various human cancers. Therefore, it is imperative to understand how major core components of the Hippo pathway are regulated in different cellular contexts in health and disease. The Hippo tumor suppressor pathway signals through three main levels: (i) a central core cassette of transcriptional programs, (ii) downstream regulators of transcriptional programs, and (iii) a diverse series of regulators. The Drosophila core cassette comprises the Warts (Wts) and Hippo (Hpo) kinases supported by the adaptor protein Mats. The Hpo-Wts-Mats casette acts together to inhibit Yorkie (Yki), which when uncontrolled promotes tissue overgrowth. The Hippo core cassette is conserved from flies to humans, mammalian MST2, LATS1, and MOB1A by compensating for Hpo, Wts, and Mats loss-of-function, respectively. Thus, Drosophila is well suited for in vivo studies of essential molecular mechanisms in Hippo core signaling.

The mammalian Hippo core cassette contains MST1/2, LATS1/2, and MOB1 which together regulate the transcriptional co-activators YAP/TAZ, MST1/2, LATS1/2, MOB1, and YAP/TAZ. Upon activation, the core cassette is activated by MST1/2, LATS1/2, and MOB1 which together regulate the transcriptional activity of the core cassette. The Hippo pathway is vital for organ growth control and tissue homeostasis, with human MST2, LATS1, and MOB1A conserved across species. The overall structure of the MOB1/NDR2 complex is similar to the reported MOB1/LATS1 complex, as an alternative branch.

MOB1 proteins are highly conserved among eukaryotes, constituting signal transducers in essential processes via their regulatory interactions with NDR/LATS. In Drosophila, Mats (aka dMOB1) can function together with Wts and Hpo in Hippo signaling. However, Mats also interacts genetically with Terc, the fly counterpart of human NDR1/2. Mammals express at least six MOBs, with MOB1A and MOB1B sharing 95% sequence identity. MOB1A/B (aka MOB1) can function redundantly as regulators of LATS1/2 signaling, but are also required for NDR1/2 activation, as an alternative branch.

The Hippo core cassette. Biochemical evidence suggests that MOB1 can associate with the highly conserved N-terminal regulatory domain (NTR) of NDR/LATS kinases to promote their activities. MST1/2 phosphorylation of MOB1 can influence MOB1 binding to the NTR, as an alternative branch.

Although it is yet to be determined whether this phosphorylation is required for NDR/LATS kinase activity in vivo. Nevertheless, MOB1 (Mats) is very likely acting as a central molecular switch in Hippo signaling. However, we still lack structural and molecular insights on how the regulatory binding composed of MOB1 to MST1/2, LATS1/2, or NDR1/2 are mediated and how MOB1 differentiates between these interactions. Two crystal structures of MOB1 bound to LATS1 were reported, but the crystal structure of the MOB1/NDR complex has yet to be documented. The importance of stable MOB1 binding to MST1/2 (Hpo) is currently debated based on recent published biochemical data and the analysis of a chimeric conformation sensor.

In general, the biological significance of MOB1 interactions with Hippo core kinases is not defined for tumor suppression, development, and tissue growth control. However, by comparing these crystal structures we could identify Asp63 as a key MOB1 residue that specifically mediates binding to LATS1. Thus, we characterized the interactions of Hippo core kinases with full-length MOB1 variants carrying specific point mutations, resulting in the discovery of MOB1 variants that are selectively impaired in their binding to MST1/2 (Hpo) or LATS1/2 (Wts) in human and fly cells. Using these MOB1 variants with selective loss-of-interaction, we found that a stable interaction of MOB1 with LATS1/2, but not with MST1/2, is essential for tumor suppressive properties of MOB1 in human cancer cells. By employing fly genetics, we discovered that the MOB1/Wts interaction is essential for development and tissue growth control, while stable MOB1 binding to Hpo is dispensable. Taken together, our study decrypts the nature and functional importance of the diverse interactions of Hippo core kinases with the central MOB1 signaling adaptor.

**Results**

**Crystal structure of MOB1 bound to the NTR of human NDR2.** To delineate the interaction of MOB1 with NDR2 on the atomic level, we determined the crystal structure of the MOB1/NDR2 complex at 2.1 Å using purified MOB1 (residues 33–216) and the NTR of NDR2 (residues 25–88) (Fig. 1a–c and Table 1). The structure of MOB1 adopts a globular shape consisting of nine α-helices (α1–α9) and two β-strands (Fig. 1a), as reported for human MST2. MOB1 is a large protein that consists of nine domains, each domain

MOB1 binds differently to the NTRs of NDR2 and LATS1 kinases. To define possible differences between MOB1/NDR2 and MOB1/LATS1 complexes, we compared available MOB1/LATS1 structures, with our MOB1/NDR2 structure (Fig. 1). This revealed fully conserved core interactions, but also dissimilarities (Fig. 2 and Supplementary Fig. 2). Most significantly, we discovered that His646 of LATS1 bonds with Asp63 of MOB1 (Fig. 2b) supported by a cluster of surrounding residues involving Phe642, Met643, Gln645, Val647, and Val650. While Phe31 of NDR2 does not interact with Asp63 of MOB1, this could be a key MOB1 residue that specifically mediates binding to LATS1. Thus, we characterized the interactions of Hippo core kinases with full-length MOB1 variants carrying specific point mutations, resulting in the discovery of MOB1 variants that are selectively impaired in their binding to MST1/2 (Hpo) or LATS1/2 (Wts) in human and fly cells. Using these MOB1 variants with selective loss-of-interaction, we found that a stable interaction of MOB1 with LATS1/2, but not with MST1/2, is essential for tumor suppressive properties of MOB1 in human cancer cells. By employing fly genetics, we discovered that the MOB1/Wts interaction is essential for development and tissue growth control, while stable MOB1 binding to Hpo is dispensable. Taken together, our study decrypts the nature and functional importance of the diverse interactions of Hippo core kinases with the central MOB1 signaling adaptor.
b). Consequently, our structural comparison suggests that Asp63 of MOB1 specifically bonds with LATS kinases through His646 (Fig. 2b), which is conserved in human LATS1/2 and fly Wts, but replaced by a bulky Phe/Tyr residue in human NDR1/2 and fly Trc (Fig. 2a). Thus, our evidence indicates that MOB1 binds differently to NDR vs. LATS kinases.

To investigate whether the interaction thermodynamics differ, we performed isothermal titration calorimetry (ITC) assays to determine the dissociation constant ($K_d$) of full-length MOB1 with the NTRs of NDR1, NDR2, LATS1, or LATS2 (Fig. 2c–f and Supplementary Fig. 3). Significantly, unphosphorylated full-length MOB1 bound to NDR1 and NDR2 (Fig. 2c, left panel and Supplementary Fig. 3a), while an interaction with LATS1 or LATS2 was undetectable (Fig. 2d, left panel, and Supplementary Fig. 3b). MOB1(Q67A) and MOB1(H185A) mutants did not interact with NDR2 (Supplementary Fig. 4), illustrating that the

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**Diagram Captions**

- **a)** Human MOB1
- **b)** Human NDR2
- **c)** MOB1 % Conservation
- **d)** Interaction with NDR2
- **e)** 1st interaction interface of MOB1/NDR2
- **f)** 2nd interaction interface of MOB1/NDR2
observed MOB1/NDR2 interaction is specific. Considering that MST1/2 phosphorylation of MOB1 can influence in vitro MOB1 binding to the NTR of LATS1, we measured the Kd of MST1/2-phosphorylated full-length MOB1 (phospho-MOB1) with NDR1, NDR2, LATS1, or LATS2 (Fig. 2c, d, right panels, and Supplementary Fig. 3a, b). This revealed that MOB1 phosphorylation is essential for MOB1 binding to LATS1 or LATS2 in our experimental settings using protein fragments representing the NTR domains (Fig. 2d and Supplementary Fig. 3b). We further uncovered that NDR1 and NDR2 bound with enhanced affinity to phospho-MOB1 than to non-phosphorylated MOB1 (Fig. 2c and Supplementary Fig. 3a). Phospho-MOBO1 also bound with an at least 15-fold (or higher) increased affinity to NDR1 or NDR2 compared to LATS1 or LATS2 (Fig. 2c, d, right panels and Supplementary Fig. 3a, b).

Since we discovered Asp63 of MOB1 as a potential key determinant in the differential binding of MOB1 to NDR2 vs. LATS1 (Fig. 2b), we tested the contribution of residues surrounding His646 of LATS1 and Phe31 of NDR2 to MOB1 binding. Specifically, considering that Tyr32 of NDR2 (and Tyr31 of NDR1, respectively) is essential for MOB1 binding and kinase activation15, 17, 25 but not conserved in LATS1/215, 17, 25 we hypothesized that by switching Val647 of LATS1 (or Val610 of LATS2) to a tyrosine we may change the binding affinities of LATS1 and LATS2 into the ones observed for NDR1 and NDR2. Thus, we studied NDR1(Y31V), NDR2(Y32V), LATS1(V647Y), and LATS2(V610Y) mutants. As expected based on the central role of Tyr32 in MOB1/NDR2 complex formation (Figs. 1e and 2a), NDR2(Y32V) and NDR1(Y31V) did not associate with non-phosphorylated MOB1 (Fig. 2e, left panel, and Supplementary Fig. 3c). However, NDR2(Y32V) and NDR1(Y31V) bound to phospho-MOBO1 (Fig. 2e, right panel, and Supplementary Fig. 3c), although with a 20-fold decreased binding affinity compared to wild-type NDR2 or NDR1, respectively (compare Fig. 2c, e and Supplementary Fig. 3a, c). Significantly, LATS1(V647Y) and LATS2(V610Y) interacted with non-phosphorylated MOB1 with a similar Kd as observed between wild-type LATS1 or LATS2 and phospho-MOBO1 (compare Fig. 2d, f and Supplementary Fig. 3b, d). LATS1(V647Y) and LATS2(V610Y) even displayed a 5-fold increased binding affinity for phospho-MOB1 compared to wild-type LATS1 or LATS2, respectively (compare Fig. 2d, f, and Supplementary Fig. 3b, d).

Taken together, we demonstrate in Figs. 1 and 2 that MOB1 relies on different residues to bind to NDR2 and LATS1. MST1/2 phosphorylation of MOB1 can play a significant role in modulating in vitro the diverse binding affinities of MOB1 to the NTRs of NDR/LATS kinases. MOB1/NDR2 complex formation is dramatically increased by prior MST1/2 phosphorylation of MOB1, while the MOB1/LATS1 interaction appears to be fully dependent on MST1/2 phosphorylation of MOB1. In this regard, a single substitution of Val647 to Tyr of LATS1 (or Val610 of LATS2) is sufficient to support MOB1/LATS complex formation independent of MOB1 phosphorylation. More specifically, LATS1(V647Y) and LATS2(V610Y) bound to non-phospho and phospho-MOBO1 with much increased affinities as observed for LATS1 and LATS2 wild-type, hence indicating that a single substitution in LATS1 or LATS2 can switch the binding mode of LATS kinases. Noteworthy, these conclusions are solely based on our ITC assays (Fig. 2 and Supplementary Fig. 3), hence the in vivo relevance has yet to be determined. In this regard, it has been documented that unphosphorylated MOB1 can bind to a LATS1 fragment in vitro19, 20. Therefore, more research is needed to decipher the reason(s) for this discrepancy. Possibly our ITC assay requires higher concentrations to detect lower affinity interactions, or ITC assays with higher affinity kinase fragments are necessary. Certainly, identical kinase fragments will need to be tested to allow a proper comparison of our findings (Fig. 2 and Supplementary Fig. 3) with previous studies19, 20, but most importantly, the in vivo implications of MST1/2 (Hpo) phosphorylation of MOB1 need to be delineated in future studies.

Table 1 Data collection and refinement statistics

| Data collection | Space group | P212121 |
|-----------------|-------------|---------|
| Unit-cell parameters | a = 57.5 Å, b = 94.4 Å, c = 102.2 Å; α = β = γ = 90° |
| Number of molecules/asymmetric unit | 2 |
| Resolution range (Å) | 50-2.10 (2.18-2.10) |
| Completeness (%) | 99.6 (100.0) |
| Redundancy | 6.9 (6.4) |
| Total observations | 226,859 |
| Unique reflections | 32,723 |
| Rmerge (%) | 10.0 (61.3) |
| I/σ(I) | 16.3 (3.1) |
| Refinement | |
| Rwork (%) | 15.3 |
| Rfree (%) | 24.4 |
| Overall B factor | 42.3 |
| RMSD bond lengths (Å) | 0.013 |
| RMSD bond angles (°) | 1.507 |
| Ramachandran plot (favored, allowed, disallowed, %) | 99.0, 0.6, 0.4 |
| Final model (number of protein/solvent atoms) | 4,149/267 |

RMSD root-mean-square deviations from ideal geometry: Rmerge = Σ|Fl –Fc|/Σ|Fc|, for the intensity (I) of observation i of reflection h. R factor = Σ|Fl –Fc|/Σ|Fl|, where Fl and Fc are the observed and calculated structure factors, respectively. Rfree = R factor calculated using 5% of the reflection data chosen randomly and omitted from the start of refinement. Data for the highest resolution shell are shown in parentheses.
Defining MOB1 variants with selective loss-of-interactions. To empower the translation of our structural and biochemical findings into studies of human cells and flies, we studied the interactions of full-length human MOB1 variants with human fly Hippo core kinases (Fig. 3 and Supplementary Figs. 5–7). In human HEK293 cells myc-tagged MOB1 mutants were co-expressed with HA-tagged wild-type kinases, followed by co-immunoprecipitation experiments in low-stringency conditions (Supplementary Figs. 5–7). Moreover, myc-tagged MOB1 binding to fly HA-tagged Wts, Trc, and Hpo was examined in Drosophila S2R+ cells (Supplementary Figs. 5e, 6e, f, and 7e). These experiments revealed that MOB1(D63V) does not form stable complexes with LATS1/2 or Wts, while stably associating with NDR1/2, Trc, MST1/2, and Hpo (Supplementary Figs. 5–7).
Thus, together with our structural data (Figs. 1 and 2) these findings indicate that Asp63 of MOB1 plays a specific role in MOB1 binding to fly and human LATS kinases.

To study the entire spectrum of MOB1 interactions with Hippo core kinases (Supplementary Fig. 1a), we next determined key residues mediating MOB1 binding to MST1/2 and Hpo. As observed for the MOB1 interactions with NDR/LATS kinases (Figs. 1 and 2 and refs. 19, 20), we speculated that charged residues of MOB1 are also centrally important for stable complex formation with human MST1/2 and fly Hpo. Consequently, we performed co-immunoprecipitation assays with MST1/2 and a panel of MOB1 variants. Significantly, a MOB1(K104E/K105E) version failed to stably bind to MST1/2 and Hpo (Fig. 3a, b). MOB1(K104E/K105E) displayed selective loss-of-interaction with MST1/2 and Hpo since it was still proficient in binding to LATS1/2, Wts, NDR1/2 or Trc (Fig. 3a, b and Supplementary Figs. 5–7). Moreover, unlike wild-type MOB1, MOB1(K104E/K105E) did not associate with human MST2 in three additional co-immunoprecipitation conditions (Supplementary Fig. 8). Collectively, these data suggest that Lys104 and Lys105 of MOB1 play specific roles in MOB1 binding to Hpo and MST1/2.

In the hope to generate an alternative MOB1 variant displaying selective loss-of-interaction with MST1/2 and Hpo, we considered modifications of the MOB1 residues Lys153 and Arg154 as promising candidates due to their central roles in the phosphothreonine binding interface supporting MOB1/MST1 and MOB1/MST2 interactions20, 28. However, MOB1(K153A/R154A) and MOB1(K153E/R154E) displayed defective interactions with full-length wild-type MST2 and LATS2, while MST1 binding was intact (Supplementary Fig. 9). MOB1(K153A/R154A) and MOB1(K153E/R154E) were also defective in Wts and Hpo binding, while they bound to Trc in insect cells (Supplementary Fig. 9). Thus, as suggested previously37, as central P0 phosphate coordinating residues28, 37, Lys153 and Arg154 are likely to represent the core of a more general phospho-serine/threonine binding domain of MOB1. This conclusion is reinforced by the finding that the region surrounding Lys153 and Arg154 of MOB1 also supports Praja2 binding38. As a result, we concluded that Lys153 and Arg154 modifications of MOB1 are not suitable to develop MOB1 variants with selective loss-of-interaction with MST1/2 and Hippo.

Since MOB1(E51K) is deficient in NDR1/2 binding39, we also profiled MOB1(E51K) (Supplementary Figs. 5–7). However, as summarized in Fig. 3c, MOB1(E51K) associated inconsistently with Hippo core kinases and was thus excluded from further cellular studies. Alternatively, we engineered a MOB1(D63V/K104E/K105E) mutant to establish a MOB1 version that only associates with NDR1/2, but not with LATS1/2 and MST1/2 (Fig. 3c and Supplementary Figs. 5–7).

To complete the biochemical characterization, we performed additional experiments. First, we investigated the importance of Asp63 and Lys104/Lys105 of MOB1 for in vitro binding to Hippo core kinases using gel filtration chromatography, revealing that recombinant full-length MOB1(D63V) and MOB1(K104E/K105E) displayed the same binding patterns as observed for full-length proteins expressed in human and fly cells (Fig. 3c and Supplementary Fig. 10). Second, we performed ITC assays to determine the dissociation constant of full-length MOB1(K104E/K105E) with the NTRs of NDR1, NDR2, LATS1, and LATS2 (Supplementary Fig. 11). Unphosphorylated MOB1(K104E/K105E) bound to all four NTRs comparable to unphosphorylated wild-type MOB1 (compare Fig. 2 and Supplementary Figs. 3 and 11). Likewise, phospho-MOB1(K104E/K105E) displayed similar affinities to all four NTRs as observed for wild-type MOB1 (compare Fig. 2 and Supplementary Figs. 3 and 11). Binding of MOB1(K104E/K105E) to NDR1(Y31V), NDR2(Y32V), LATS1(V647Y), and LATS2(V610Y) NTR mutants was also comparable to wild-type MOB1 (compare Fig. 2 and Supplementary Figs. 3 and 11). Third, we measured MST1/2 (Hpo) phosphorylation of our MOB1 variants (Supplementary Figs. 12 and 13), since MOB1 phosphorylation can influence the binding affinities of MOB1 to NDR/LATS (Fig. 2 and refs. 19, 22, 28, 29). MST1/2 (Hpo) phosphorylation of MOB1 on Thr12 and Thr35 was comparable for all MOB1 versions tested (Fig. 3c and Supplementary Figs. 12 and 13). Thus, the selective loss-of-interaction of MOB1(D63V) is not a consequence of altered Thr12/Thr35 phosphorylation, but rather caused by the substitution of a key residue that is essential for MOB1/LATS complex formation. Our data (Fig. 3 and Supplementary Figs. 7–13) further argue that a stable interaction of MST1/2 (Hpo) with MOB1 is not required for MOB1 phosphorylation by MST1/2 (Hpo).

Taken together, we discovered that distinct MOB1 residues mediate the interactions with the different mammalian and fly Hippo core kinases. Specifically, Asp63 and Lys104/Lys105 of MOB1 represent key residues mediating the differential binding properties of MOB1 with LATS1/2 (Wts) and MST1/2 (Hpo), respectively (Fig. 3d).

Testing of MOB1 variants in anchorage-independent growth. To define which MOB1 interactions with Hippo core kinases are necessary for tumor suppression, we engineered pools of MCF-7 human breast cancer cells stably expressing either empty vector (EV), HA-MOB1 wild-type (wt), HA-MOB1(D63V), HA-MOB1(K104E/K105E), or HA-MOB1(D63V/K104E/K105E) (Fig. 4a). Then, we determined proliferation and colony formation in two-dimensional (2D) culture conditions. Specifically, we measured proliferation using IncuCyte live cell analysis technology (Fig. 4b) and performed colony formation assays (Fig. 4c, d) to determine
cell survival based on the ability of single cells to grow into colonies. Expression of all MOB1 variants resulted in decreased proliferation in 2D compared to controls (Fig. 4b). Likewise, except for MOB1(D63V/K104E/K105E), our MOB1 variants reduced colony formation (Fig. 4c, d). Transient expression of our MOB1 variants in HCT116 colon cancer cells also diminished proliferation and colony formation in 2D (Supplementary Fig. 14). These data show that MOB1(D63V) and MOB1(K104E/K105E) suppress proliferation and colony formation similarly to MOB1(wt), suggesting that the interactions of MOB1 with LATS1/2 and MST1/2 are dispensable. Considering that MOB1(D63V) and MOB1(K104E/K105E) still bind to NDR1/2 (Fig. 3c and Supplementary Fig. 6) and that MOB1(K104E/K105E) is phosphorylated on Thr12 and Thr35 in MCF-7 cells comparable to wild-type MOB1 (Supplementary Fig. 15), we are therefore tempted to conclude that MOB1 binding to NDR1/2 can be...
sufficient to at least in part suppress cancer-related features in human cancer cells grown in 2D.

Next, we performed anchorage-independent growth assays (Fig. 4e, f), a more stringent method to determine malignant transformation in three-dimensional (3D) tissue culture. In contrast to our 2D observations (Fig. 4b–d), MOB1(wt) or MOB1 (K104E/K105E), but not MOB1(D63V) or MOB1(D63V/K104E/ K105E), significantly suppressed anchorage-independent growth in 3D (Fig. 4e, f and Supplementary Fig. 14). This suggests that MOB1 interactions with MST1/2 are dispensable, while MOB1 interactions with LATS1/2 are required to suppress anchorage-independent growth of human cancer cells.
binding to LATS1/2 is important and MOB1 binding to NDR1/2 alone is insufficient in this 3D setting. In general, our anchorage-independent growth data of human cancer cells (Fig. 4e, f) mirrored our fly genetics discoveries (see Fig. 5 and 6 and Supplementary Fig. 16), suggesting that tissue culture experiments performed under more physiological conditions can reflect in vivo tissue overgrowth experiments.

MOB1/Hpo in fly development and tissue growth control. To study our MOB1 variants in a complex multicellular organism, we generated and characterized transgenic flies that ubiquitously expressed our myc-tagged MOB1 versions in a mats mutant background (Figs. 5 and 6 and Supplementary Fig. 16). As human MOB1 expression can rescue mats mutants\(^{10,16}\), this allowed us to determine the functional significance of altering MOB1 binding in vivo. Using Phic31-mediated recombination we integrated our MOB1 variants at the same chromosomal location (89E11 on chromosome 3) under control of the ubiquitous ubiquitin-63E promoter (ubi > MOB1, Supplementary Fig. 16a). Western blotting of whole flies confirmed similar expression of myc-tagged MOB1 variants (Supplementary Fig. 16b).

We then tested which MOB1 transgene can rescue the larval lethality of mats deficient flies\(^{10}\). As expected, wild-type MOB1 expression rescued the lethality of a null mats trans-heteroallelic combination (mats\(^{roo}\)/mats\(^{23b}\), Supplementary Fig. 16c). Likewise, mats deficient animals expressing MOB1(K104E/K105E) were viable and fertile (Supplementary Fig. 16c), suggesting that stable MOB1/Hpo binding is dispensable for normal fly development. In contrast, neither MOB1(D63V) nor MOB1(D63V/K104E/K105E) rescued mats mutants (Supplementary Fig. 16c), showing that MOB1/Wts complex formation is essential for normal development, while MOB1 binding to Trc alone is insufficient to promote normal development.

To test the rescue effect of our MOB1 mutations on the mats tissue overgrowth phenotype, we generated mats mutant clones in the head using the eyFLP/FRT system\(^{42}\). Expression of wild-type MOB1 and MOB1(K104E/K105E) fully rescued the overgrown and misshapen head phenotype of eyFLP mats animals (compare Fig. 5b, d with Fig. 5a, f). In contrast, expression of MOB1(D63V) or MOB1(D63V/K104E/K105E) only partially suppressed the mats overgrowth phenotype (compare Fig. 5c, e with Fig. 5a, f). Thus, stable MOB1 binding to Hpo is dispensable for tissue growth control, while MOB1/Wts complex formation is necessary.

Finally, we tested the effect of our MOB1 transgenes on Yki transcriptional activity by examining the levels of Expanded (Ex), a well-characterized Yki transcriptional target\(^{42}\). We generated mutant clones for mats in wing imaginal disks (the larval precursors to the adult wing) using the FLP/FRT system under control of the heat shock promoter (Fig. 6). While mats clones displayed a robust increase in Ex expression (Fig. 6a–e) expression of either wild-type MOB1 or MOB1(K104E/K105E) restored Ex levels to control levels (Fig. 6f–j, p–t). In contrast, Ex levels (and therefore Yki activity) were still strongly upregulated when MOB1(D63V) or MOB1(D63V/K104E/K105E) were expressed in mats clones (Fig. 6k–o, u–y). Thus, in full agreement with the animal viability (Supplementary Fig. 16) and head overgrowth data (Fig. 5), the interaction of MOB1 with Wts is required to repress Yki activity, while the MOB1/Hpo interaction is dispensable and MOB1/Trc complex formation alone is insufficient for a complete rescue.

As previously observed\(^{10}\), mats clones were usually small (Fig. 6a). Mats mutant clones were rarely recovered in the wing.
pouch, likely because to their tendency to delaminate due to excessive overgrowth. Interestingly, although Ex levels were strongly upregulated in both MOB1(D63V) or MOB1(D63V/K104E/K105E) expressing clones, MOB1(D63V) expressing clones were larger and survived readily in the pouch (Fig. 6k), in contrast to MOB1(D63V/K104E/K105E) expressing clones, which were more similar to mats clones not expressing MOB1 (compare Fig. 6a, u). In this regard, we also noted that adult heads expressing MOB1(D63V/K104E/K105E) were more severely affected than adult heads expressing MOB1(D63V) in mats null tissue (compare the more rippled appearance of Fig. 5c, e). These findings collectively suggest that when MOB1 activity is weakened by loss of the MOB1/Wts interaction, MOB1 function is further compromised by loss of the Hpo/MOB1 interaction.

Fig. 6 Human MOB1(wt) or MOB1(K104/K105E), but not MOB1(D63V) or MOB1(D63V/K104E/K105E), expression suppresses elevated Yki activity in mats null mutant wing imaginal disk clones. Analysis of Ex expression in wing imaginal disks of the indicated genotypes (wt wild-type, DV D63V, KE K104E/K105E, D63V/K104E/K105E). All panels show third instar wing imaginal disks containing mats mutant clones marked by the absence of GFP expression (green in b, g, l, q, v). GFP-negative mats null clones are indicated by dashed lines. Maximum intensity projections of XY confocal stacks are shown in red to visualize Expanded (Ex) labeling (red in a, f, k, p, u). X-Z confocal sections through the same wing imaginal disks were acquired c, h, m, r, w. Nuclei are stained with DAPI in blue. Ex (d, i, n, s, x) and GFP (e, j, o, t, y) protein levels are indicated. All scale bars are 20 µm. As expected, mats null clones displayed increased Yki activity as judged by elevated Ex levels a–e. Expression of MOB1(wt) f–j or MOB1(K104E/K105E) p–t suppresses Yki activity to normal levels in mats mutant clones (compare i, s with d), while expression of MOB1(D63V) k–o or MOB1(D63V/K104E/K105E) u–y does not (compare n, x with d and i)
while disruption of the Hpo/MOB1 interaction alone does not affect MOB1 function.

**Discussion**

Despite extensive progress in elucidating Hippo growth control signaling, studies comparing the biological significance of the regulatory interactions of MOB1 with Hippo core kinases have remained elusive. This point is crucial, since loss-of-function of MOB1 in flies and mice causes the most severe phenotypes of Hippo core cassette components\(^1\), indicating that MOB1 represents a multipurpose hub in Hippo core signaling. By combining genetics, structure, molecular, and cell biology our present study addresses this pressing issue. We uncovered key mechanisms promoting selective binding of MOB1 to Hippo core kinases in mammalian and fly cells. Precisely, we discovered that Asp63 of MOB1 is indispensable for interacting with LAT51/2 and Wts, while Lys104/Lys105 of MOB1 are essential for stable complex formation with MTT1/2 and Hpo. Thus, MOB1 can differentiate between interactions in the Hippo core cassette, thereby most likely enabling MOB1 to regulate the specificity and amplitude of Hippo core kinase signaling.

While our biochemical and molecular data regarding Asp63 of MOB1 are further supported by a comparison of crystal structures of MOB1/NDR2 and MOB1/LATS2 complexes, we can currently only speculate on the structural level concerning Lys104/Lys105 of MOB1 and stable complex formation with MTT1/2 (Hpo). Based on available structural data\(^2\), one can draw the conclusion that Pro106 of MOB1 significantly contributes to the MTT1 binding surface. Thus, we are tempted to speculate that modifications of Lys104/Lys105 of MOB1 impact the neighboring Pro106 and thereby impair stable MOB1/MMT1 complex formation.

**MOB1/NDR (Trc) complex formation alone is insufficient to support development and normal tissue growth control. In this regard, His646 and Val647 of LAT51 (see this study), and possibly selective inhibitory MOB2 binding to the NTR of NDR1/2\(^3\), are promising candidates for the fine-tuning of MOB1-mediated signaling. Consequently, future studies are warranted to address these possibilities. In particular, crystal structures of full-length NDR kinases bound to full-length MOB1 in its non-phosphorylated vs. phosphorylated state will help to further our understanding with regard to the recently proposed auto-inhibition model for MOB1 binding\(^4\). Furthermore, the in vivo importance of MTT1/2 (Hpo) mediated phosphorylation of MOB1 (Mats) needs to be deciphered. In this regard, our conclusions regarding MOB1 phosphorylation are currently based on in vitro experiments, which might be cautiously interpreted regarding in vivo implications.

By discovering MOB1 variants displaying selective loss-of-interactions and decrypting the biological significance of regulatory interactions of MOB1 in Hippo core signaling, we believe that our study helps to settle the recent controversy\(^5\),\(^6\),\(^7\),\(^8\),\(^9\),\(^10\),\(^11\) regarding the importance of MOB1 binding to MTT1/2 (Hpo). Ni et al. previously concluded that stable MTT2 binding to MOB1 functions as an important step in activating the MTT1/2-LATS1/2 kinase cascade\(^12\). However, no biological functions were addressed\(^12\). In this regard, using a chimeric sensor to measure Wts conformation in fly tissues, Vrabioiu and Struhl\(^13\) found that MOB1 can act as a Hpo-independent activator of Wts, hence contrasting the model proposed by Ni et al.\(^12\). Manning and Harvey\(^14\) proposed a unifying model, wherein MOB1 acts before and after Hpo-mediated phosphorylation of Wts and MOB1. However, in support of Luo and colleagues\(^15\), the Sichler and Gingras laboratories recently showed that ternary MTT1/2-MOB1-LATS1/2 (NDR1/2) complex formation is important, at least when tested in vitro\(^16\),\(^17\). Conversely, our study rather supports the model drawn by Vrabioiu and Struhl\(^13\), namely that a ternary complex is not required for MOB1-LATS1 activation in vivo. Therefore, it is currently difficult to reconcile all published data into one general model. Nonetheless, we are proposing an updated four-step model (Supplementary Fig. 17) attempting to consolidate these models\(^18\),\(^19\),\(^20\),\(^21\) with our discoveries reported here and other important biochemical data\(^22\),\(^23\),\(^24\),\(^25\),\(^26\),\(^27\). First, activated Hpo (MTT1/2) phosphorylates MOB1 to release MOB1 from an auto-inhibitory conformation\(^19\),\(^20\),\(^22\). Significantly, this first step does not seem to require formation of a stable Hpo/MOB1 complex (see this study), suggesting that MTT1/2 phosphorylation of MOB1 only requires a brief transient kinase-substrate interaction. However, we currently do not understand how MOB1 (Mats) phosphorylation actually fits into the regulation of Hippo core kinase signaling in vivo. Second, MOB1 binds to Wts (LAT51/2) to “open up” Wts\(^28\). In this second step the formation of a stable MOB1/Wts complex is essential (see this study and refs. \(^21\), \(^22\), \(^23\), \(^25\), \(^26\)). Third, “open” LAT51/2 is phosphorylated by MTT1/2 in the C-terminal hydrophobic motif (HM)\(^20\),\(^21\). This third step can occur without formation of a stable ternary MTT1/2-MOB1-LATS1/2 complex, as proposed by the MTT1/2-binding deficient K104E/K105E mutant characterized in this study. Fourth, HM-phosphorylated LAT51/2 autophosphorylates on the activation loop\(^20\),\(^21\), a step that can occur independent of MOB1 binding to LAT51/2\(^21\). Intriguingly, the MTT1/2-MOB1-NDR1/2 signaling model is very similar, but different\(^17\),\(^45\) since MOB1 binding to NDR1/2 can occur independently of MOB1 phosphorylation (see this study). Even more importantly, it is crucial to note that our proposed model (Supplementary Fig. 17) is mainly supported by in vitro experiments.
Our study together with the report by Vrabioiu and Struhl would argue that the MOB1/Hippo interaction is dispensable for tissue growth control by Hippo signaling. But we strongly caution from drawing a broad and general conclusion from these studies, since we cannot rule out the possibility that the MOB1/Hippo interaction is only dispensable in selective aspects of tissue growth control. Thus, our model (Supplementary Fig. 17) may exemplify interaction is only dispensable in selective aspects of tissue growth since we cannot rule out the possibility that the MOB1/Hippo from drawing a broad and general conclusion from these studies, although it can be sufficient to normally support proliferation of human cancer cells. The MOB1/Trc interaction is essential for development, tissue growth control, and Yki regulation in Drosophila, but it can be dispensable for some tumor suppressive properties of MOB1 in human cells. Therefore, our study significantly advances our understanding of the biological importance of the regulatory interactions of MOB1 with Hippo core kinases, in addition to providing structural and molecular insights into the differential binding of MOB1 to Hippo core kinases.

In the course of our in vivo studies we noted further interesting aspects. While disruption of the stable MOB1/Hpo interaction alone did not have a detectable effect on MOB1 function (see K104E/K105E mutant), loss of the MOB1/Hpo interaction could affect MOB1 function in the context of disrupted MOB1/Wts interaction (see D63V/K104E/K105E mutant). This is illustrated by the observations that, in the eye overgrowth assay, D63V/K104E/K105E mutant tissues were noticeably more overgrown than in K104E/K105E mutants. In the wing clone experiments, only D63V/K104E/K105E mutants displayed a high frequency of clone delamination and loss in the wing pouch, indicative of a strong overgrowth phenotype and Ex upregulation, these phenotypes were still markedly weaker than the full overgrowth phenotype and Ex upregulation, these phenotypes...
LATS2 proteins in the sample cell. By varying the stoichiometry (n), the enthalpy for the reaction (ΔfH), and the association constant (K_a), data were fitted with the non-linear least-square method using a single-site binding model with Origin for ITC version 7.0 (MicroCal).

**Gel filtration chromatography.** Full-length MOB1A wild-type, D63V or K104E cDNAs were subcloned into the pET28a vector, expressed in bacteria and subsequently purified as described above for MOB1A (33–216). Purified full-length MOB1 proteins were incubated with NDR2 (25–88), LATS1 (618–697), or MST2 (2–392) protein at 4 °C for 20 min in assembly buffer, before the protein mixtures were loaded onto a 16/60 Superdex 200 gel filtration chromatography column (GE Healthcare) and 1.4 ml fractions were collected. Samples from selected Superdex 200 fractions were analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and Coomassie Blue staining. For the complex formation assay between MOB1A and LATS1, MOB1A protein was in vitro phosphorylated by MST2 (230) as described above prior to incubation with LATS1 (618–697).

**Antibodies for immunoblotting and immunoprecipitations.** For immunoblott- ing, samples were resolved by 8 or 12% SDS-PAGE, followed by transfer onto Immobilon-P membranes (Millipore). Membranes were blocked for at least one hour with TBST (50 mM Tris pH 7.5, 150 mM NaCl, 0.5% Tween 20) containing 5% skim milk powder and then probed overnight with primary antibody. Bound antibodies were visualized using IRDye680 (LI-COR Biosciences) or IRDye800 (LI-COR Biosciences) secondary antibodies and followed by enhanced chemiluminescence. For co-immunoprecipitations, cells were collected by centrifugation at 1000 x g for 3 min, followed by lysis in corresponding buffers (see below). After 30 min, cell lysates were centrifuged for 10 min at 16,000 x g at 4 °C before pre-clearing with protein A-Sepharose, followed by immunoprecipitation with 2 μg of 2C3A anti-phosphotyrosine antibodies. Beads were washed at least three times with the corresponding lysis buffer before samples were analyzed by SDS-PAGE and immunoblotting. The characterization of LATS1/2, NDR1/2, MST1/2, Warts, Trc, and Hpo binding to MOB1A variants was carried out in low-stringency buffer (50 mM HEPES pH 7.4, 20 mM beta-glycerophosphate, 20 mM KCl, 1% TX-100, 1 mM EDTA, 2 mM NaF, 1 mM Na_3VO_4, 1 mM benzamidine, 4 μM leupeptin, 0.5 mM pepstatin, 1 mM PMSF, 1 mM microcystin, and 1 mM DTT). Alternatively (Supplementary Fig. 8), co-immunoprecipitation experiments were performed in RIPA (8006, Cell Signalling; 20 mM Tris pH 7.5, 150 mM NaCl, 1 mM EDTA, 1 mM EGTA, 1% NP-40, 1% w/v sodium deoxycholate, 2.5 mM sodium sulphocholate, 1 mM beta-glycerophosphate, 1 mM Na_3VO_4, 1 mM NaF, 1 mM Na_2HPO_4, 1 mM leupeptin, 1 mM PMSF, 0.5 mM PMSF, 1 mM microcystin, and 1 mM DTT).

**Cell culture and transient transfections.** HEK293, HEK293T, MCF-7, and HCT116 cells were obtained from ATCC and grown at 37 °C in 5% CO_2 humidified chambers in DMEM (D6429, Sigma) supplemented with 10% fetal bovine serum (FBS; F7524, Sigma) and penicillin/streptomycin. Cells were authenticated through STR (Short Tandem Repeat) profiling and mycoplasma test performed by Microsynth (Switzerland). Exponentially growing HEK293 cells were plated at a consistent confluence and transfected with plasmids using Fugene 6 (E2692, Promega) according to the manufacturer’s instructions as described elsewhere. 1.2 x 10⁶ HCT116 cells were transiently transfected with 1.0 μg pcDNA3-based plasmids using the nucleofector kit V (VCA-1003, Lonza) as defined by the manufacturer. Drosophila S2R + cells were maintained at 24 °C in Schneider’s Drosophila Medium (DMEM, Invitrogen) supplemented with heat-inactivated 10% FBS (10082-16, Invitrogen) and penicillin/streptomycin. S2R + cells were transiently transfected with pcDNA3-based plasmids using Effectene (301425, Qiagen) according to the manufacturer’s instructions.

**Generation of stable cell line pools.** To generate stable lentiviral cell pools using pLEX_HA-MOB1A plasmids, 8 x 10⁶ of the HEK293T packaging cells were transduced with 2.4 μg of pMD.G, 0.8 μg of p8.91, and 4.8 μg of pLX305 plasmids using Lipofectamine 2000 (11668, Invitrogen) as recommended by the manufacturer. Tissue culture supernatants were harvested 24 h later, passed through a 0.45-μm filter and added to the target cell lines in the presence of 1 μg ml⁻¹ polybrene. Infected cells were selected by growth in the presence of 2.5 μg ml⁻¹ puromycin. Stable pools (uncloned mass culture) of cells were maintained in DMEM with 1% FBS, 0.1% puromycin. Retroviral (uncloned mass culture) were generated using pCMV-Retro based plasmids and maintained in the presence of G418 as described elsewhere.

**Proliferation and colony formation assays.** HCT116 transiently transfected with pcDNA3-based plasmids were seeded 24 h post-transfection, or stable MCF-7 cell line pools were analyzed. Media were replenished every 72 h during the duration of each experiment. For cell proliferation analysis, 10,000 cells were plated in 12-well plates, followed by non-invasive IncuCyte live cell imaging (Essen BioScience) to measure the kinetic of cell growth/proliferation based on area (confluence) metrics. Phase-contrast images were continuously collected on IncuCyte ZOOM (Essen BioScience) for at least one week. For each cell proliferation experiment, a specific definition (Phase-contrast processing module) was applied to count objects (cells) for the duration of the assay. The Phase object area was expressed as relative cell confluence for each well at each time point and

**Construction of plasmids.** Human MOB1A/R, NDR1/2, LATS1/2, and MST1/2 cDNAs cloned in pcDNA3-based vectors were described previously.21, 35, 39, 44, 45, 56 pcDNA3-myc-hMOB1A(wt) served as template for the generation of the following MOB1A mutants by PCR-based mutagenesis: E94R, E155K, E155V, D395E, K104E, K105E, K135E, H161Q, and K164Q, and D63E and E51K/K104E/K105E were synthesized. To subclone N-terminally HA-tagged MOB1 cDNAs into the pLEX vector, the tagged cDNAs were first inserted using KpnI and Xhol into the pENTR-3C plasmid (Invitrogen) and then recombined into the pLEX destination plasmid using Gateway technology (Invitrogen). To subclone HA-tagged MOB1 cDNAs into the pCMV-R-neo plasmid, the tagged cDNAs were inserted using Pmel and Xhol. Hpo and Warts cDNAs have been described.28 The plex vector, MaTS (LD47533) and Trc (LD37189) cDNAs were from the Drosophila Genomics Resource Center (Indiana University, USA). To subclone N-terminally tagged cDNAs into the pAW or pKC26w-pUbiq fly expression vectors, the tagged cDNAs were first inserted into the pENTR-3C plasmid (Invitrogen) and then recombined into these two destination plasmids using Gateway technology (Invitrogen). Myc-hMOB1A variants, myc-Hats, MaTS-HTrc, and HA-Hpo were inserted into pENTR-3C using KpnI and NotI. All constructs were confirmed by sequence analysis of the entire cDNAs at every cloning step.
subsequently exported into GraphPad Prism Software for final analysis. To evaluate colony formation, 1,000 cells were seeded per well (6-well format). After 8–12 days, colonies were fixed with methanol/acetic acid (3:1) for 5 min at room temperature, stained with 0.5% (w/v) crystal violet for 15 min at room temperature, washed with water and finally scanned using the G:BOX HR gel documentation system (Syngene). Colonies composed of at least 50 cells were scored as positive.

**Soft agar assays for anchorage-independent growth**. After trypanosnosis, cells were passed 4–5 times through a 21 G syringe, before 1 × 10⁴ E4 cells were resuspended in complete medium (DMEM containing 10% FBS and appropriate antibiotic) with 0.6% agarose (16200520, Thermo Fisher Scientific), and subsequently cultured in wells (6-well format) underlaid by a layer of 1% agarose in complete medium and overlaid with complete medium without agarose. Top layer media were replenished every 72 h. After three weeks, colonies were stained with 2.5 mg ml⁻¹ methylene blue tetrazolium blue (MTT, Sigma), scanned, and quantified. Cell clusters of at least 50 cells were scored as colonies.

**Western blotting and PCR genotyping of transgenic flies**. To analyze adult flies using immunoblotting, three flies per genotype were frozen at −80 °C overnight, followed by homogenization on ice, and 20 µl of Lamelli sample buffer perfly (1610737, Bio-Rad) using a Squisher manual homogenizer (Zymo Research). Following boiling samples at 95 °C for 10 min, samples were centrifuged and supernatants transferred to fresh tubes. Finally, DTT was added to a final concentration of 50 mM and equal volumes of samples were analyzed by SDS-PAGE, followed by western blotting. For PCR genotyping, flies were collected, frozen in liquid nitrogen, and subsequently crushed using a micropestle, followed by preparation of genomic DNA using a Qiagen kit (69506). Genomic DNA was stored at −20 °C, followed by PCR genotyping using the following primers: 5′-GGCCGCTCAAGA-TAGCGAGAT-3′ and 5′-GCACACTCTGGAACCCGTCGAT-3′ to detect the Bso transposon and 5′-AGAGGGCCCTCTGCATCCAGGCGAGCA-3′ and 5′-CCGCGACAGCGCATTCGAGTC-3′ to detect the FRT site.

**Statistical analysis**. Graphs and statistical analyses were carried out using the GraphPad Prism software. Data are presented as mean ± s.e.m., unless stated otherwise. The significance of differences between the means or the population distributions was determined using two-tailed unpaired Student t-test. Differences were considered statistically significant when p-values were below 0.05 (*), 0.01 (**, or 0.001 (***) p-values are listed in the corresponding figure legends where appropriate.

**Data availability**. The coordinates and structure factor files of the complex of human MOB1 (residues 33–216) bound to NDR2 (residues 25–88) have been deposited in the Protein Data Bank (PDB) with accession number 5XQZ. All relevant data are available from the corresponding authors upon reasonable request.

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**Author contributions**

Y.K. performed and interpreted most experiments in Figs. 3–6 and the Supplementary Figures. K.L. contributed to Z.G, performed and interpreted most experiments in Figs. 1 and 2 and Supplementary Figs. 2–4, 10, 11, and 18. M.H. and Y.K. supported the experiments and interpretation of Figs. 16–19. M.G., B.A.S., M.M., L.H., J.A.D.S. and E.S.I. assisted Y.K. with the experiments shown in Supplementary Figs. 5–15. I.B. and N.T. participated in the analyses and interpretation of all experiments, in particular the design, analyses, and interpretation of Figs. 5, 6 and 8.
Supplementary Fig. 16. G.W. and A.H. participated in the conception, planning, analyses, and interpretation of all experiments, and wrote the manuscript. All authors approved the submitted manuscript version.

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