Expanding the Hippo pathway:

hMOB3 modulates apoptotic MST1 signaling and supports tumor growth in glioblastoma

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List of abbreviations

AGC	Protein kinase A(PKA)/PKG/PKC-like
ATP	Adenosine triphosphate
Drosophila	*Drosophila melanogaster* (fruit fly)
LATS	Large Tumor Suppressor
STK	Serine/Threonine Kinase
MST	Mammalian sterile-20 like
NDR	Nuclear dbf2 related
NTR	N-terminal regulatory domain
AS	Activation segment
MOB	Mps one binder
YAP	Yes associated protein 65 (YAP65)
TAZ	WW domain containing transcription regulator 1 (WWTR1)
TEAD	Transcriptional enhancer factor TEF-1
WT	Wild type
KD	Kinase dead
PKB	Protein Kinase B
PI3K	Phosphoinositide 3 kinase
PDK1	Phosphoinositide-dependent kinase 1
TSC	Tuberous sclerosis
ABL	Abelson murine leukemia viral oncogene homolog 1
JNK	c-Jun N-terminal kinase
EGFR	Epidermal growth factor receptor

PDGF	Platelet-derived growth factor
PDGFR	Platelet-derived growth factor receptor
MAPK	Mitogen-activated protein kinase
S. cerevisiae	*Saccharomyces cerevisiae* (budding yeast)
S. pombe	*Schizosaccharomyces pombe* (fission yeast)

Summary

Protein kinases are critical players of signal transduction pathways involved in development, physiological and pathological processes. Deregulation of protein kinase signaling is found to be causal or related to varieties of human diseases, such as cancer, cardiovascular disease and diabetes. The human genome encodes 518 protein kinases. Approximately 60 out of them belong to the AGC group of Serine/Threonine protein kinases, including the ste20 like MST kinase family and NDR kinase family. Members of these families are highly conserved from yeast to men and regulate essential processes such as growth, proliferation and apoptosis. The Hippo pathway is a recently identified tumor suppressive network, where the MST-NDR family kinases form a kinase cascade regulating the downstream signaling through the effector YAP/TAZ.

In addition to signaling through the NDR family kinases, the Hippo/MST kinases also control cell apoptosis bypass these classical effectors YAP/TAZ. Despite the fact that JNK, FOXO3, H2B are well characterized downstream targets of apoptotic MST kinases, the regulatory mechanisms of apoptotic MST signaling are still largely unknown.

The human MOB family consists of six members encoded by six different genes (hMOB1A, -1B, -2, -3A, -3B and -3C). While as an activator for hMOB1A/B in MST-LATS/NDR kinase cascade, hMOB2 is a specific negative regulator of NDR kinase by competing the binding of hMOB1 to NDR kinase. Although hMOB3 family members share higher amino acid identity with hMOB1 than hMOB2, hMOB3 proteins do not interact or (de)activate NDR family kinases. Hence, the functions of hMOB3A/B/C are completely undefined.

A previous microarray study performed in the lab indicated that hMOB3 family members were deregulated in glioblastoma. In the present study, we first investigated the pathological roles of

human MOB3 proteins and found that hMOB3 is highly upregulated in glioblastoma. Moreover, mRNA expression levels of hMOB3 members correlate with survival, suggesting hMOB3 members as potential prognostic markers. We extended the biochemical analysis by looking for the interaction partners of hMOB3 and demonstrated that hMOB3 binds to MST1 and inhibits the apoptotic cleavage of MST1 kinase. We further verified that hMOB3 promotes tumorigenesis of gliobalstoma cells *in vivo* by a U87MG derived flank model. Taken together, our results suggest that manipulate hMOB3 might represent a therapeutic strategy in malignant gliomas.

1. Introduction

1.1 Protein kinases in organ size control

Protein kinases are essential components of intracellular signaling pathways and mediating most of the signal transduction in cells. Protein kinases are kinase enzymes that modify targeted proteins (substrates) by catalyzing the transfer of phosphate groups to substrates' hydroxyl group of serine, threonine or tyrosine amino acid side chains (1). The phosphorylation usually results in functional changes of substrates, such as enzyme activity, subcellular localization, binding affinities to other proteins and protein stability. Thus, protein kinases play critical roles by orchestrating signaling transductions involved in development, cell growth and differentiation. Therefore, deregulation of protein kinases by mutation, fusion with other kinases/proteins or altered expression is causal or associated with many human diseases such as cardiovascular diseases, cancer and metabolic diseases.

The protein kinase complement of the human genome, also known as the kinome, encodes 518 protein kinases and represents one of the biggest gene family of the human genome (2). To gain insight into kinase function and evolution, all 518 protein kinases were classified into a hierarchy of groups, families and subfamilies based on the sequence similarity of their catalytic domains, domain structure outside of the catalytic domains (2). The biggest kinase group is tyrosine kinases (90 kinases), followed by the CAMK (74 kinases), the AGC (63 kinases) and then the CMGC group of kinases (61 kinases) (Figure 1) (2). Since aberrant protein kinase signaling is causal or associated with the development of human diseases, protein kinases have emerged as a major class of drug targets for therapeutic intervention (3).

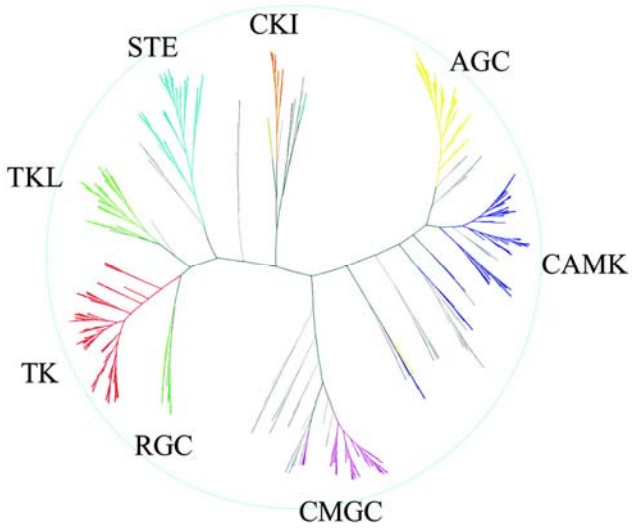


Figure 1. Dendrogram of 491 eukaryotic protein kinase domains from 478 genes. Major groups are labeled and colored. Taken from (2).

The development of a fully functional organ depends on the precise patterning and size-sensing signals. Precise control of organ size is a fundamental and critical process during animal development and tissue regeneration, which is a highly coordinated process involving variety of physiological signaling integrations. In general, the finale organ size is dependent on the cell size and cell number. While cell size is regulated by cell growth, the hemostasis of cell number is maintained by the balance between cell proliferation and cell death. The TOR and Hippo pathways, both of which are controlled by protein kinase signaling networks, are among the key signaling pathways involved in the regulation of organ size through their respective function in the controlling cell size and cell number (4) (Figure 2).

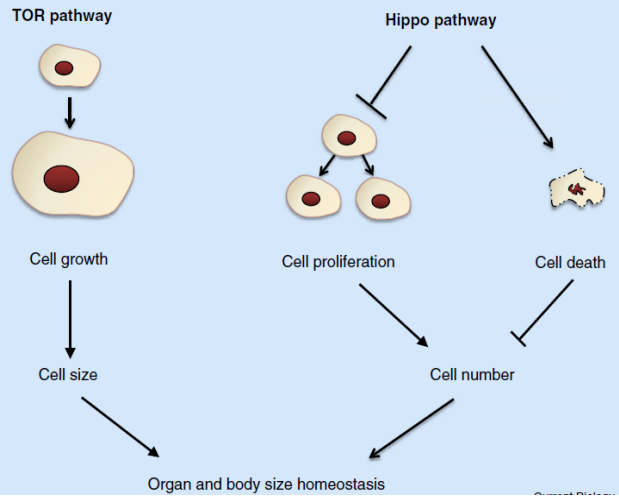


Figure 2. Organ size control by the TOR and Hippo pathways. TOR pathway stimulates cell growth and thereby increases the cell size. The Hippo pathway regulates organ size by promoting cell proliferation and inhibiting cell death. Images adapted from (4).

TOR was firstly identified in yeast as "Target Of the FKBP-Rapamycin complex" (short as "Target Of Rapamycin") by Hall's laboratory (5) and later on characterized as a master cell growth regulator (6, 7). The TOR kinase acts as a central signaling sensor by adjusting cellular metabolic output to match the energy status and growth factor availability. In a simple model, under nutrient rich condition, growth factors activates PI3K/PKD1/PKB (also known as AKT) signaling, activated PKB phosphorylates and inhibits TSC2 leading to the accumulation of Rheb-GTP, which activates TOR (8, 9). Activated TOR stimulates cell growth and thus increases cell mass by coordinating signaling such as protein synthesis, ribosome biogenesis and proper cell cycle entry (6, 10). In the starvation condition, TOR activity is inhibited. Inactivated TOR restricts the cell metabolic activity and results in cell cycle block. Thus, TOR signaling is appreciated as a temporal and spatial regulator of cell growth (7). Hyperactivated TOR signaling results in increased cell growth and sometimes promotes some cells into cell cycle progression (7, 10).

1.2 The Hippo pathway

The Hippo pathway is an emerging organ size control network by inhibiting cell proliferation and promoting cell death. The Hippo pathway was originally identified by *Drosophila* genetics with the discovery that the mutants represent overgrowth phenotype. The first Hippo component identified from the mosaic-based screens was named *Warts* (*Wts*, also called *Lats*) in 1995 (11, 12), which encodes a kinase of the nuclear dbf-2-related (ndr) family (Figure 3). *Wts* was considered as an orphan tumor suppressor until 2002, when *Salvador* (*Sav*, also called *Shar-pei*) mutant was identified (Figure 3) (13, 14). *Salvador* encodes a WW domain-containing protein, and its mutations result in a similar cell-autonomous overgrowth as *Wts* mutant clones. Importantly, Tapon et al. demonstrated that *Sav* and *Wts* genetically and physically interacted with each other, suggesting that these two protein function in the same pathway (13). The breakthrough came in 2003 when 5 independent groups reported the finding of the *Hippo* mutants, which encodes a mammalian homolog of Ste20 family kinase MST1/MST2 (Figure 3) (15-19). Remarkably, Hippo kinase formed a complex with Sav and Wts, thus putting the three tumor suppressors together for the first time as the Salvador-Warts-Hippo pathway (15).

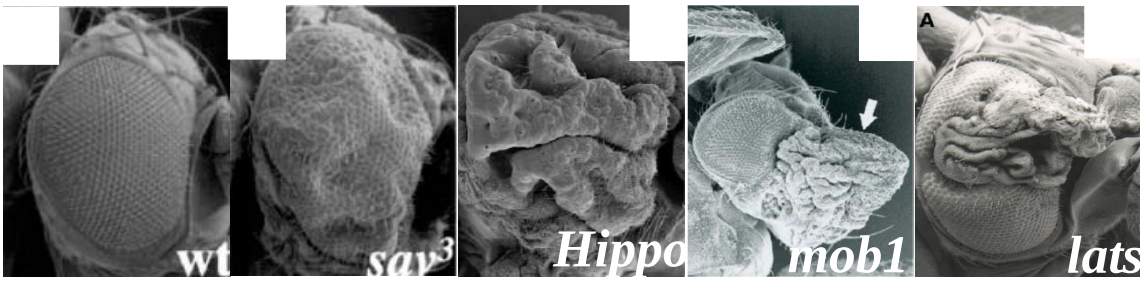


Figure 3. Overgrowth phenotype of the Hippo pathway mutants. Images adapted from (11, 13, 15, 20)

In addition to *Wts*, the nuclear dbf-2-related family kinases, such as ndr kinases, are reported to regulate cell cycle progression and cell morphogenesis from yeast to man (21). The ndr family kinases are identified to function in a complex with an adaptor protein MOB (Mps one binder) (22, 23), raising the possibility that Wts might also bind to a fly mob. Lai et al. demonstrated this hypothesis by nicely showing that mutant of *drosophila mats* (Mob as tumor suppressor, also known as mob1) leads to the similar overgrowth phenotype in *Hippo*, *Wts* and *Sav* mutants (Figure 3) (20). Biochemically, *Drosophila* mob1 binds to Wts and bridges it to the upstream Hippo (24). This finding demonstrates that Mob1 is a bona fide key component of the Hippo pathway.

Since *Cyclin E* and the cell death inhibitor *Diap1* was observed to be increased in *Wts* and *Sav* mutants (15-19), which suggested Hippo downstream might be the transcriptional program related, researcher focused on the transcription factor related protein which interacts with LATS, searching for Hippo effectors. To this end, Pan's Lab identified the *Yorkie*, a *drosophila* homolog of mammalian *YAP* and *TAZ*, from a yeast two-hybrid screening that binds to Lats kinase (25, 26). As yorkie is a transcriptional co-activator, a TEAD/TEF family transcription factor named Scalloped was found to form a complex with yorkie (27-30). Thus, yorki/Scalloped, YAP/TEAD and TAZ/TEAD in mammals, served as the downstream effector for the Hippo-LATS signaling.

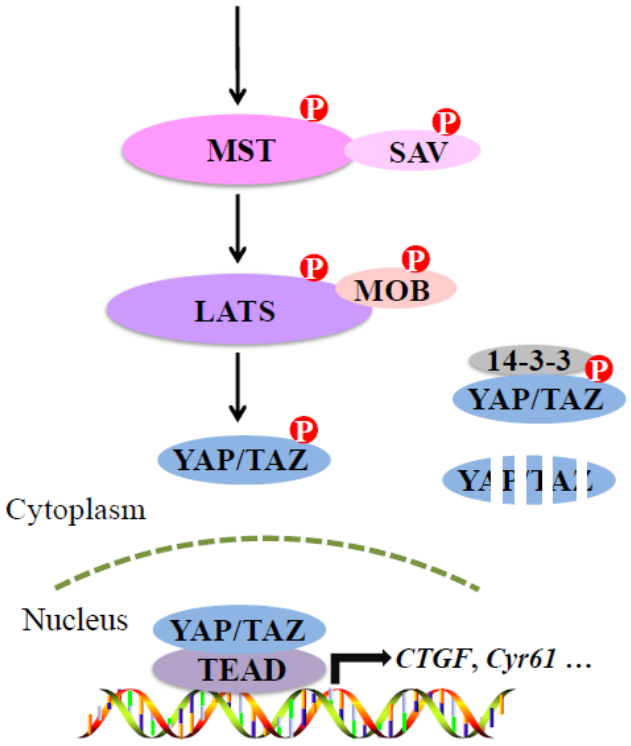


Figure 4. The core Hippo pathway. MST kinases in complex with SAV phosphorylates and activities MOB/LATS. LATS phosphorylates and deactivates YAP/TAZ. Intact YAP/TAZ forms transcriptional active complex with TEAD. Phosphorylated YAP/TAZ binds to 14-3-3 resulting cytoplasmic retention and degradation.

Taken together, these findings orchestrate a linear model for the canonical Hippo pathway (31). Mechanistically, the Hippo kinase (MST in mammals) forms a kinase cascade with the downstream Wts kinase (NDR/LATS in mammals), whereas Sav (SAV in mammals) and Mats (MOB in mammals) proteins function as positive co-activators. Classically, once activated by the Hippo/MST kinase, LATS kinase in turn phosphorylates the transcriptional co-activator YAP/TAZ and restrains its activity by preventing its nuclear translocation (32) and promoting its degradation (33) (Figure 4) .

The classical Hippo pathway control organ size by regulating the transcriptional program. As a transcription co-activator, YAP does not contain any DNA binding domain but functions through forming a complex with TEAD, whereby initiating the transcription of a subset of pro-survival genes. Recent findings further depicted the mechanism of YAP-TEAD interaction by showing that YAP competes the binding of TEAD to a default repressor VGLL4 (Tgi in *Drosopholia*)

(34-36). Once YAP/TAZ is phosphorylated by active Hippo signaling, they are restricted in cytoplasm and not able to enter nucleus to form the functional complex with TEAD.

1.3 Regulation of the Hippo pathway

Ever since the discovery of the Hippo pathway, one key issue in Hippo research is to identify its regulators. Using genetic models or biochemical approaches, many additional components have been identified to either modulate the core Hippo activity or interact with the Hippo effectors YAP/TAZ (Figure 5).

Inactivation of YAP by activated Hippo signal was first observed in cell-cell contact condition (32), but the upstream activator was not known. The initial work identified two adaptor proteins Ex (Expaned) and FERM (4.1, Ezrin, Radxin, Moesin) domain protein Mer (Merlin, also known as NF2 for neurofibromatosis 2) (37). Later on, Kibra (a WW domain and C2 domain containing protein) was found to form a ternary complex with Mer and Ex (38-40). This complex activates the Hippo pathway through recruiting the Hippo/Sav/Wts (MST/SAV/LATS in mammals) to the membrane for activation (Figure 5A) (37-43). Recent findings further deciphered the mechanism for this activation process by showing that NF2 complex does not enhance the intrinsic activity of MST, but rather NF2 interacts with the N-terminus of LATS and thus facilitate the activation of LATS by MST1/SAV through the hydrophobic motif phosphorylation (44).

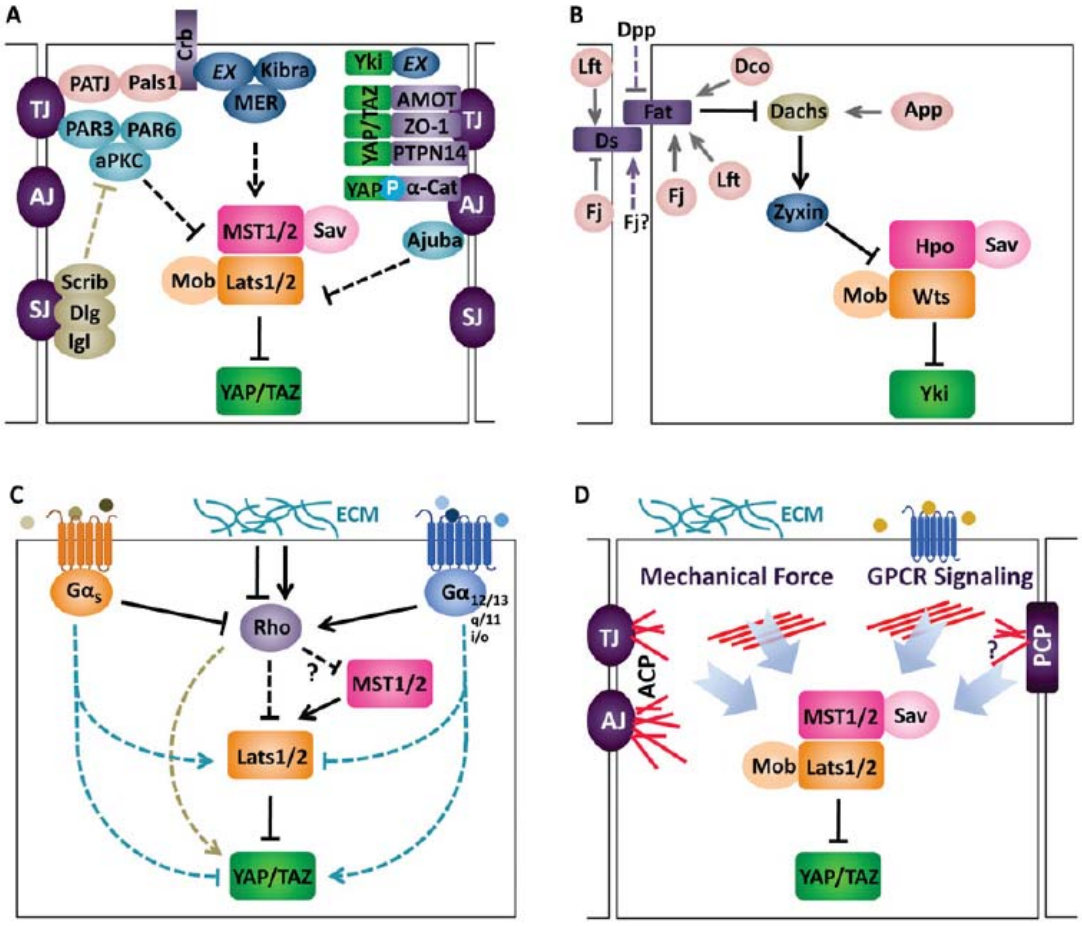


Figure 5. Regulatory inputs of the Hippo pathway. Regulation of the Hippo pathway by apical-basal polarity (A), PCP (B), mechanical cues and GPCR (C), and actin cytoskeleton (D). Arrowed or blunted ends indicate activation or inhibition, respectively. Dash lines indicate indirect or unknown mechanisms. Red lines in D represent actin filaments. Images taken from (45).

Apical-basal polarity components are revealed as another group of key players mediating the cell-cell contact induced Hippo signaling (Figure 5A). Apical-basal polarity network is orchestrated via the Crumbs (Crb) complex, the Par complex and the Scribble (Scrib) complex (Figure 5A). Crumbs (Crb) complex was delineated as an upstream regulator by interaction of

the intracellular FERM -binding motif (FBM) to Ex, thus altering the cellular location of Ex containing complex (46-50). The Par complex also regulates the Hippo pathway. Overexpression of atypical PKC (aPKC) induces activation of the Hippo effector Yorkie (49, 51). *Drosophila* epistatic interaction revealed that aPKC acts upstream of the Hippo pathway by changing the cellular location of RASSF and Hippo (49). However, the detail mechanism for this regulation is not fully understood yet. Recently, Par protein, MARK kinases in mammals, is also found to positively impact on the Hippo pathway through coordinating the Par/Scrib/MST/Sav complex (52-54). The Scrib complex was found to be a positive input for Hippo activity (55). Whilst Lgl acts antagonistically to aPKC to regulate Hippo and RASSF localization (49), the membrane located Scrib direct actives the Hippo pathway by scaffolding core kinase MST to the membrane for activation (55, 56). The *Drosophila* planar cell polarity complex also signals to the Hippo pathway (Figure 5B) (57-59) and this regulation is well reviewed in (60-62).

Recent finding suggest that G protein couple receptors (GPCRs) also regulate the Hippo-YAP pathway (Figure 5C) (63-65). Several chemokines in the serum, such as LPA and S1P, were found to activate YAP/TAZ activity through GPCRs-RhoA GTPase (63, 65) mediated deactivation of LATS kinase (63). In the same report, several other diffusible ligand/factors, such as glucagon, epinephrine and dopamine were also identified as YAP/TAZ regulator through their corresponding GPCRs (63). Meanwhile, Thrombin also stimulates the YAP/TAZ activity through protease activated receptors (PARs) (64). Nevertheless, the mechanism how GPCRs are linked to RhoA GTPase and how RhoA-GTPase regulates LATS are not known yet. GPCR represents the largest family of plasma membrane receptors, which are able to be activated or blocked by a variety of ligands or pharmaceutical agents. Thus, the YAP/TAZ activity might be a fine-tuned readout by multiple GPCR signaling integration in a specified condition.

In addition, extracellular matrix induced cytoskeleton changes is another contributor to the regulation of the Hippo pathway (Figure 5D). Several reports demonstrate that YAP/TAZ as a sensor of machanotransduction in response to cell geometry changes (66-68), attachment/detachment (69) and stress fibers (66, 70). In general, mechanotransduction leads to a cellular morphology and cytoskeleton change, suggesting that cytoskeleton might be a signal integrator to the mechanical clues, which transduces further down to the Hippo pathway (71). Indeed, YAP/TAZ is regulated by F-actins, F-actin-capping/serving proteins and microtubules (66, 68, 70). RhoA is the major downstream of actin cytoskeleton in response to mechanical stresses. But how RhoA transduces to Hippo effector YAP/TAZ and whether the core kinase cascade is involved in RhoA-YAP/TAZ are still under debate and merit further investigation.

1.4 MST/MOB/NDR core complex

1.4.1 MST kinases

MST kinases (sterile 20 like kinase, MST1/2) were firstly cloned as Ste20 like kinase and found to be activated under severe stress (72-74). Later on, MST1/2 kinases were identified as the mammalian Hippo kinase when Sav-Hippo-Wts pathway was discovered in *Drosophila* (15-19).

Regulation and regulators of MST kinases

MST1 contains an N-terminal kinase domain, followed by an auto-inhibitory domain and a C-terminal protein-protein interaction domain called SARAH (Salvador-RASSF-Hippo) (75). In addition to mediating the signal integration from RASSF/SAV(15, 76, 77), the SARAH domain is also essential for the activation of MST kinases themselves by inter-dimerization (MST1/MST2, MST1/MST1, MST2/MST2) induced trans-phosphorylation (78-80).

In addition to signaling through the classical Hippo downstream LATS kinase regulating proliferation and apoptosis (26), MST kinases are pro-apoptotic kinase by themselves (79, 81-83). Under apoptotic conditions, activated MST kinases cross-talk with caspases, resulting in a proteolytic N terminus (81, 84). Cleaved N terminal of MST translocates into the nucleus (83) and phosphorylates H2B (85), FOXO3 (86), JNK (87-90) and activates p53 family members (77, 91, 92) to execute the biological functions.

Several upstream regulators and kinases have been characterized for MST1 activation/deactivation. As described above, while the Mer/Kibra/NF2 complex and the Crb and Scrib polarity complex activate the MST-LATS cascade activity via membrane recruiting, aPKC complex negatively regulates MST-LATS activation. Besides, TAOK1 positively regulates MST kinases activity by direct phosphorylation (93, 94). Homeodomain-interacting protein kinase was also found to regulate Hippo-dependent tissue growth probably by promoting the nuclear accumulation of YAP (95, 96). In addition, C-ABL phosphorylates MST1 at Tyrosine 433, activates and stabilizes MST1 (97, 98). Activation of MST by GPCRs and cytoskeleton is still under debate as the results from difference groups were inconsistent (63, 65, 68, 99). Some other negative regulators include protein kinases such as PKB (100-105), JNK (87-90), Salt induced kinase (SIK) (106) and RAF-1(107-109), and phosphatase like PHLLP (110) and PP2A (111, 112). PKB and JNK phosphorylate MST1 at Threonine 120/387 (100-103) and Serine 82 (89), respectively, and inhibit the proteolytic activation of MST1 initiated apoptotic signaling. SIK phosphorylates Sav at Serine 413 and disrupts Sav mediated Hippo/Wts interaction (106). RAF-1 inhibits MST2 activity through SARAH domain mediated protein-protein interaction and further recruits phosphatase to deactivate MST1. The mechanism of phosphatase, like PHLLP (110) and

PP2A (111, 112), to deactivate MST activity is via the catalytic nature of phosphatase by removing the phosphate group of Threonine 183/180 from MST1 or MST2, respectively.

Tumor suppressive role of MST kinases

The physiological roles of MST kinases are unrevealed using knock-out mouse models. MST kinases are essential for early embryonic development as MST1/2 double knock-out mice were dead at embryonic day 8.5 due to the growth retardation, failed placental development, defect vascular patterning and hematopoietic development (113-115). MST1 and MST2 function redundantly for embryonic development as MST1 or MST2 single deletion mice were viable, fertile and development normally. Conditional knock out models in liver, intestine, pancreas and heart suggest that MST1/2 is a bona fide essential regulator controlling cell proliferation, apoptosis and differentiation. The phenotypes are summarized in table 1.

Table 1. Phenotypes of the Mst1/2 conditional knockout mice (downloaded from (116) and modified)

Tissues	Phenotypes	Reference
Liver	Dramatic hepatocyte proliferation and hepatomegaly; Development of hepatocellular carcinoma and cholangiocarcinoma within 2 months.	(26, 114, 115, 117-120)
Intestine	Intestinal hyperplasia; An expansion of stem-like undifferentiated cells; An almost complete absence of all secretory lineages; Development of the polypoid lesions and colonic adenomas within 3 months old.	(120-122)
Pancreas	A significantly decrease in pancreas mass; Acinar cell atrophy; Overabundance of ductal structures; Smaller islets with abnormal α/β cell ratios in pancreas	(123, 124)
Heart	Expansion of trabecular and subcompact ventricular myocardial layers; Thickened ventricular walls, and enlarged ventricular chambers without a change in myocardial cell size.	(125, 126)

Key role of MST1 in immune deficiency

The murine Mst kinases are most peaked in lymphoid tissues (127). Mst1 kinase plays critical roles in T cell adhesion (128), migration (99, 127, 129, 130), survival (127, 131-133). Depletion

of MST1 and MST2 does not have significant impact in the development of thymocyote. However, ablation of MST1 results in a dramatic decrease of peripheral $CD4^+$ and $CD8^+$ T cells and $B220^+$ B cells (99, 127, 129, 130). Interestingly, although the total numbers of peripheral $CD4^+$ and $CD8^+$ T cells and $CD62L^{hi}/CD44^{lo}$ naïve T cells are decreased, the ratio of $CD62L^{low}/CD44^{high}$ effector/memory T cells are increased in MST null mice (127). Clinically, patients bearing loss of function mutations of MST1 have been reported with a primary immunodeficiency syndrome characterized as T cell lymphopenia, neutropenia, infection and autoimmune dysfunction (132, 133).

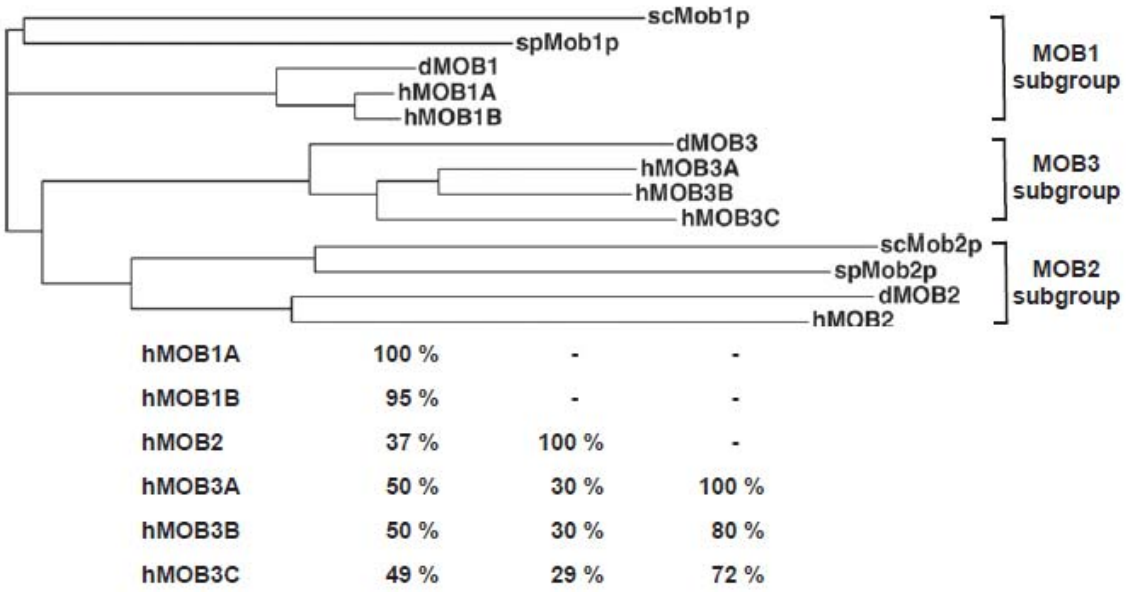
Deletion of Mst1, or both of Mst1 and Mst2, impairs the thymocyte egress and induces an accumulation of mature single positive thymocytes in thymus and a decreased number of peripheral lymphocytes (99, 134). Mst1-null mice show defects in T cell adhesion, trafficking, and intranodal migration in vivo (130). Thymocytes egress is controlled by gradient of S1P and requires the activation of RAC and RhoA (135-137). MST null mature thymocytes show impaired sensitivity of RAC and RhoA activation (99). Biochemical studies further demonstrate that phosphorylation of MOB1 by MST1 is essential to enable MOB1 interact with and activate DOCK8 (99). These studies suggest MST1/MOB1/DOCK8 axis might be a critical signal mediator from the S1P to cytoskeleton changes (99). Recently, Mst1 has also been shown to be involve in LFA-1/ICAM-1-dependent high-velocity medullary migration and is required for migrating thymocytes to associate with rare populations of $Aire^+$ ICAM-1^{hi} medullary thymic epithelial cells , suggesting MST1 might be a key factor in regulating thymocytes self-antigen scanning in the medulla (138).

Collectively, the mice phenotype and clinical observations have clearly suggested MST kinases are one of the critical genes essential for maintaining the immune homeostasis.

1.4.2 MOB proteins

MOB proteins are small adaptor proteins without any enzymatic activity and are conserved from yeast to human. In yeast, mob proteins are reported to be essential for mitotic exit and septation initiation networks by regulating ndr kinases (22, 23). In *Drosophila*, Mats (MOB as tumor suppressor)/dMOB1 physically interacts with Wts and is necessary for Wts activity (20, 24). Importantly, human Mob1 could functionally rescue the phenotype resulting from loss of Mats, indicating that the function of MOB protein is evolutionally conserved (20). Murine *Mob1* has been shown to be essential for embryogenesis. *Mob1* null embryos have a defect in primitive endoderm formation (139). Mice with a single allele of *mob1a* or *mob1b* developed spontaneous tumors at 70 weeks in a broad range of tissues, confirming the tumor suppressive role of mob1 in mammalian system (139). The tumor suppressive function of MOB1 has further been validated by a keratinocyte specific double knock out model (139).

The human genome encodes six MOB genes, namely *hMOB1A/B*, *hMOB2* and *hMOB3A/B/C* (Figure 6) (140). Another closely related gene (Phocein) was found to be part of PP2A/Striatin/MST3 complex (141). Interestingly, whereas hMOB1A/B physically interact and activate all four human NDR/LATS kinases (142-144), hMOB2 specifically negative regulates NDR kinase activity by completing the binding of NDR kinase to hMOB1 (Figure 7) (140). Although hMOB3A/B/C show higher sequence similarity to hMOB1 than hMOB2, hMOB3A/B/C proteins do not interact with or (de)activate all four NDR/LATS kinases (140, 145).



hMOB1A	100 %	-	-
hMOB1B	95 %	-	-
hMOB2	37 %	100 %	-
hMOB3A	50 %	30 %	100 %
hMOB3B	50 %	30 %	80 %
hMOB3C	49 %	29 %	72 %

Figure 6. phylogenetic analysis of the MOB protein family. Phylogenetic relationships within the MOB protein family. Top: phylogenetic tree using Clustal W phylogenetic calculation based on the neighbour-joining method. Budding and fission yeast scMob1p and spMob1p, respectively, group together with dMOB1 and hMOB1A/B (MOB1 subgroup), while scMob2p and spMob2p fall into a group together with dMOB2 and hMOB2 (MOB2 subgroup). dMOB3 together with hMOB3A/B/C forms a third group (MOB3 subgroup). Bottom: display of primary sequence identities within human MOB protein family. Adapted from (145).

The roles of hMOB1 have been extensively characterized in tissue cultured system. hMOB1A/B proteins are essential for the functions of human NDR1/2 in apoptosis and centrosome duplication in a NDR binding dependent manner (146, 147). In addition to the association with NDR kinases, hMOB1A/B proteins also interact with human MST1/2, bridging NDR/LATS to the upstream MST kinases (148). Moreover, human MST1/2 kinases and the *Drosophila* Hippo kinase phosphorylate hMOB1A/B and Mats, respectively, thereby increasing MOB1/Mats protein affinity towards NDR/LATS or Wts kinase (148). Furthermore, Binding of NDR kinases to hMOB1A/B is reported to release the kinase from the auto-inhibitory status by the auto-

inhibitory segment (AIS) (142). In summary, current findings proposed a model of NDR activation, in which hMOB1A/B is phosphorylated by MST1/2 which results in efficient ternary complex formation of hMOB1/MST/NDR, which in turn facilitates the phosphorylation of NDR1/2 by MST kinases (Figure 7).

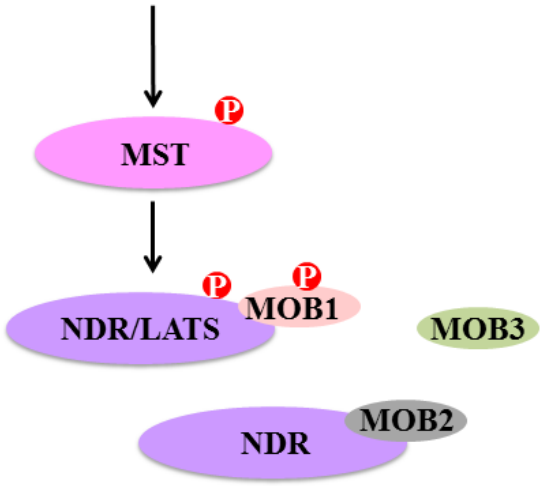


Figure 7. The MST/hMOB/NDR complex. hMOB1 binds to both LATS and NDR. hMOB1 is phosphorylated by MST1/2 which results in efficient ternary complex formation of hMOB1/MST/NDR. hMOB2 specifically interacts with un-phosphorylated NDR kinases, which competes the binding of hMOB1 to NDR. hMOB3 does not interact with or (de)activate NDR/LATS.

Moreover, spatial relocalization seems to be another critical aspect in NDR kinase activation. Artificial targeting of hMOB1 or Mats proteins to the plasma membrane leads to rapid and robust activation of NDR/LATS kinase or Wts, respectively (24, 144). Direct membrane targeting of Trc kinase itself could also rescues the effect of *trc* mutant flies (24, 144). These observations indicate cellular membrane might be a key place for activation of NDR/LATS kinases. However, the mechanisms of NDR kinases being recruited to and activated at the plasma membrane by MOB1 remain to be elucidated.

Recently, a correlation of loss of hMOB1 with pathological grade of human brain tumor has been explored (149). This finding suggest that proteolytic degradation of hMOB1 by the up-regulated

ubiquitin ligase praja2 is a pathological triggers in the gliomagenesis (149). Taken together, all these findings point that hMOB1 a critical tumor suppressive adaptor by regulating downstream NDR/LATS activity.

hMOB2 protein share approximately 37% sequence identity with hMOB1 (Figure 6) (140, 145). While hMOB1 proteins bind to both NDR and LATS kinases, hMOB2 only interacts with NDR kinases (Figure 7) (140). hMOB2 associates with NDR kinase through the N terminal region of hMOB1 binding motif (140). Therefore, it is reasonable to observe the competing binding between hMOB1 and hMOB2 towards NDR kinases. Interestingly, while hMOB1 was found to associate with activated NDR kinases, hMOB2 forms a complex with intact un-phosphorylated NDR kinases (140). This affinity preference indicates that hMOB2 restricts hMOB1 induced activation process of NDR kinases (Figure 7). However, the mechanism of interplay between hMOB2 and hMOB1 towards NDR activation remains to be depicted. One possible explanation is the subcellular location of hMOB2. hMOB1 proteins predominantly locate at cytoplasm, but hMOB2 is found to be accumulated in the nucleus. This alternation of subcellular localization of hMOB2 might block the membrane location and activation of NDR kinase by hMOB1. Nevertheless, the functions of hMOB2 merit further investigation.

hMOB3A/B/C proteins are three distinct protein products from three different genes. hMOB3 group proteins share about 50% amino acid identity with hMOB1 (Figure 6) (145). However, hMOB3A/B/C do not interact with or (de)activate NDR/LATS kinases. The biochemical roles and potential physiopathological roles of uncharacterized hMOB3 need to be deciphered.

Taken together, MOB proteins are essential regulators of NDR/LATS kinases. While MOB1 proteins function as activators, the role for human MOB2 protein is a specific negative regulator for human NDR kinases. The function of the hMOB3A/B/C proteins has to be defined.

1.4.3 NDR kinases

Activation of NDR kinases

The NDR kinases belong to the AGC group of serine/threonine kinases (150). Members of the NDR family are highly conserved throughout evolution and can be found in organisms such as *S. cerevisiae* (Dbf2p, Dbf20p and Cbk1p), *S. pombe* (Sid2p and Orb6p), *C. elegans* (SAX-1 and LATS) and *D. melanogaster* (Warts and Trc) as well as other fungi, plants and protozoans (21). The human genome encodes four NDR family kinases: NDR1 (STK38), NDR2 (STK38L), LATS1 and LATS2 (21). Genetic and biochemical studies showed that NDR kinases are crucial regulators of important functions such as mitosis, cytokinesis, cell polarity and morphogenesis, cell cycle progression, apoptosis, proliferation, centrosome duplication (21, 151, 152).

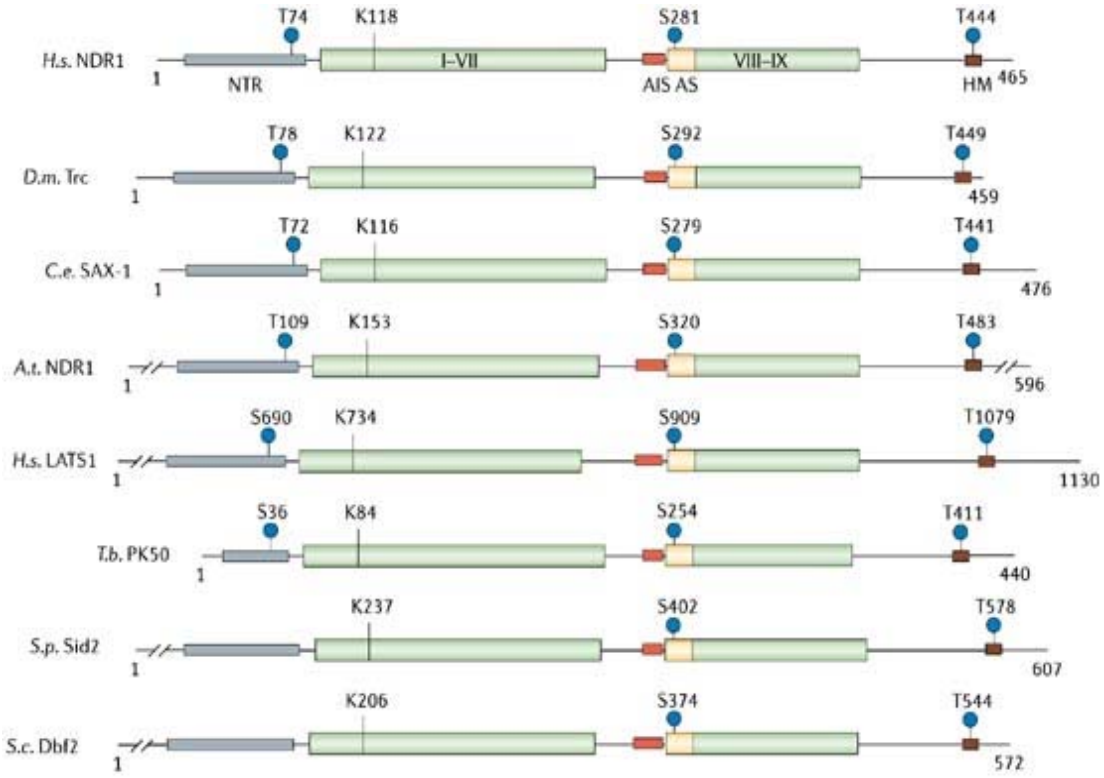


Figure 8. Primary structure of selected NDR kinases. Eight members of NDR family kinases from unicellular to multicellular organisms are shown (H.s. Homo sapiens, D.m. Drosophila melanogaster, C.e. Caenorhabditis elegans, A.t. Arabidopsis thaliana, T.b. Trypanosoma brucei). The N-terminal regulatory domain (NTR, grey), the kinase domain (green) with the activation segment (yellow) and the hydrophobic motif (brown) are shown. In addition, the auto-inhibitory sequence (red) and conserved phosphorylation sites (blue dots) are indicated. Taken from (21).

The primary structure of NDR kinases is conserved from yeast to men (Figure 8) (21). All NDR kinases contain both typical characteristics of AGC kinases required for activation but are unique among the AGC group because they exhibit two distinct features only present in the NDR family: an N-terminal regulatory domain (NTR) also known as the S100B/hMOB1 association domain (SMA) and an insert of about 30-60 amino acids between subdomains VII and VIII of the kinase domain (Figure 8) (21). The NTR is responsible for the interaction with S100B and hMOB

proteins (21). The NDR/LATS NTR contains a number of basic and hydrophobic residues which were shown to be critical for the binding to hMOB1A. Strikingly, mutating the positive charged area in NDR or negative charged residues in MOB1 abolished their interaction, suggesting that the NDR/MOB complex formation is based on electrostatic interactions (145).

The 30-60 residues insert between kinase subdomains VII and VIII contains a stretch of positively charged residues. This basic residue containing motif precedes the activation segment and seems to inhibit NDR kinase activity as mutation of these residues to alanine leads to a significant increase in NDR1/2 kinase activity. Therefore, this motif is also referred to as an auto-inhibitory sequence (AIS) (21).

All NDR kinases contain two regulatory phosphorylation sites: the hydrophobic motif (HM) phosphorylation site (Threonine 444 in human NDR1) and the activation segment (AS) (Serine 281 in human NDR1). While the hydrophobic motif is phosphorylated by upstream MST kinases (146, 147, 153, 154), the activation segment (AS) phosphorylation is not targeted any upstream kinase but is regulated via autophosphorylation (152). Interestingly, a third phosphorylation site located at the NTR of NDR (Threonine 74 in NDR1) kinases was found to be important for fully activation of NDR kinase since mutating of this site to alanine reduces kinase activity and abolishes the binding to hMOB1 (155). However, whether this phosphorylation site has intrinsic impacts on kinase activity or whether the reduced activity is from loss of hMOB1 binding still need to be defined.

As discussed before, MST kinases are upstream kinases responsible for the HM phosphorylation. However, MST kinases seem not to function in a redundant manner towards NDR phosphorylation in different cellular processes. For instance, MST1 kinase is the predominant

kinase responsible for the activation of NDR1/2 during centrosome duplication and Fas ligand induced apoptosis (146, 147), while for chromosome alignment during mitosis, the major player shifts to MST2 (156). However, during cell cycle progression, MST3 kinases, but not MST1 or MST2, is essential for induction of NDR activation (154). In addition to MST kinases, the activation process of NDR kinases is also regulated by hMOB proteins as describe above and might require a subcellular localization change, such as membrane targeting. Furthermore, phosphatase such as PP2A could also deactivate NDR kinases (21). Both of HM and AS phosphorylation sites are targets of PP2A. Treatment with okadaic acid, a potent PP2A inhibitor, dramatically activates NDR kinases (153, 157). Furthermore, recombinant PP2A completely deactivates human NDR kinases (157). Nevertheless, how PP2A regulates NDR kinases underlying physiological processes still needs to be addressed. Another level of regulation of NDR kinase activity by scaffold protein Furry is still largely unknown (156).

Biological functions of NDR kinases

The function of NDR kinases was initially identified to be involved in cell division in yeast (21, 151, 152). C.elegant NDR kinase *SAX1* and *Drosophila Wts* and *Trc* was reported to regulates neurite outgrowth and dendritic tiling (151, 158, 159). The NDR family kinase *Wts* was identified as a tumor suppressors in *Drosophila* and later *Wts* homolog *Lats* was shown to act as a tumor suppressor in mice (11, 12, 160). The other NDR family kinase *Trc* in *Drosopholia* was identified as a critical regulator of epidermal outgrowth and dendritic tiling and branching (158, 159). Since our work mainly related to NDR kinases but not LATS kinases, we are going to focus on the role of mammalian NDR kinases during the following discussion.

The first biological role of NDR kinases was identified by Hergovich and colleagues by showing that NDR kinases regulates centrosome duplication (161). Further investigation indicated that

MST1/hMOB1/NDR forms a functional ternary complex regulating this process (147). Meanwhile, another study carried out in the Hemmings laboratory show that NDR kinases are activated by RASSF1A and MST1 during Fas ligand induced apoptosis (146). Further, the same lab performed another investigation examining the role of NDR kinase during cell cycle progression and unraveled a functional MST3/NDR/p21 axis regulating G1/S cell cycle transition (154, 162). Interestingly, NDR kinases could also regulate c-Myc stability, however, the mechanism in this regulation is not fully known. NDR1/2 were also implicated in the alignment of chromosomes during mitosis (156). The first *in vivo* role of NDR kinases came from the NDR1 whole body knock out model. Aged Ndr1 knock-out mice developed spontaneous T cell lymphoma, suggesting a critical role of NDR kinases in T cell hemostasis (163). Taken together, all these cell biology and *in vivo* studies suggest that NDR kinases are bona fide downstream targets of Hippo/MST kinases. The function of mammalian NDR1/2 merits further investigation using *in vivo* models.

1.5 Gliomas

Gliomas, the most common type of brain tumor, are originated from mature glial cells or less differentiated glial progenitor cells (Figure 9) (164). Based on their aggressive nature, gliomas are classified into 4 grades according to the World Health Organization (WHO). Compared with lower grades (I and II), high-grade (III and IV) tumors have a worse prognosis and display histological features such as nuclear atypia, increased proliferation, microvascular proliferation and necrosis (164).

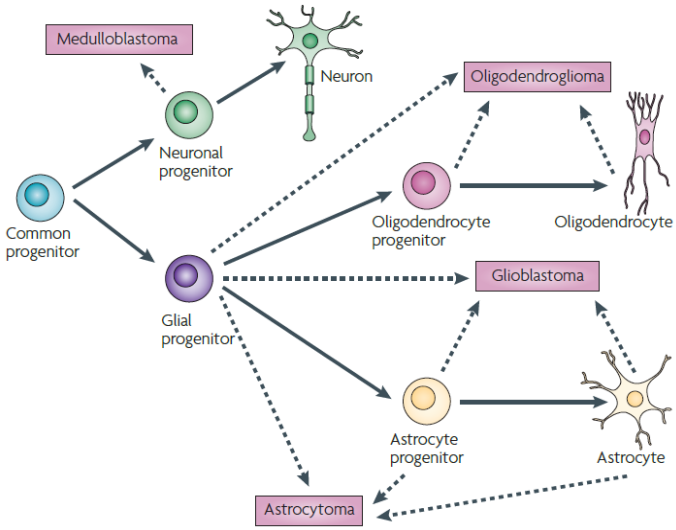


Figure 9. The neuroglial lineage tree. Self-renewing, common progenitors are thought to produce committed neuronal and glial progenitors that eventually differentiate into mature neurons, astrocytes and oligodendrocytes. Although the precise cells of origin for diffuse glioma variants and medulloblastoma remain largely unknown, a selection of likely candidates for each (dashed arrows) is indicated. Taken from (164)

Glioblastoma multiforme (GBM) is the grade IV gliomas with a median survival of approximately 14 months after diagnosis (165). Approximately 90% of GBM arise *de novo*, while 10% originate from lower grade astrocytoma and are known as "secondary" GBM (166).

1.5.1 Resistance of high grade gliomas

Despite the benefits of surgical resection and the use of adjuvant radiochemotherapies, patients almost invariably succumb to recurrent widespread tumor growth (Figure 10) (167-169). Thus,

defining the mechanism of resistance of GBM cells and discovering further effective therapeutic targets are crucial medical goals.

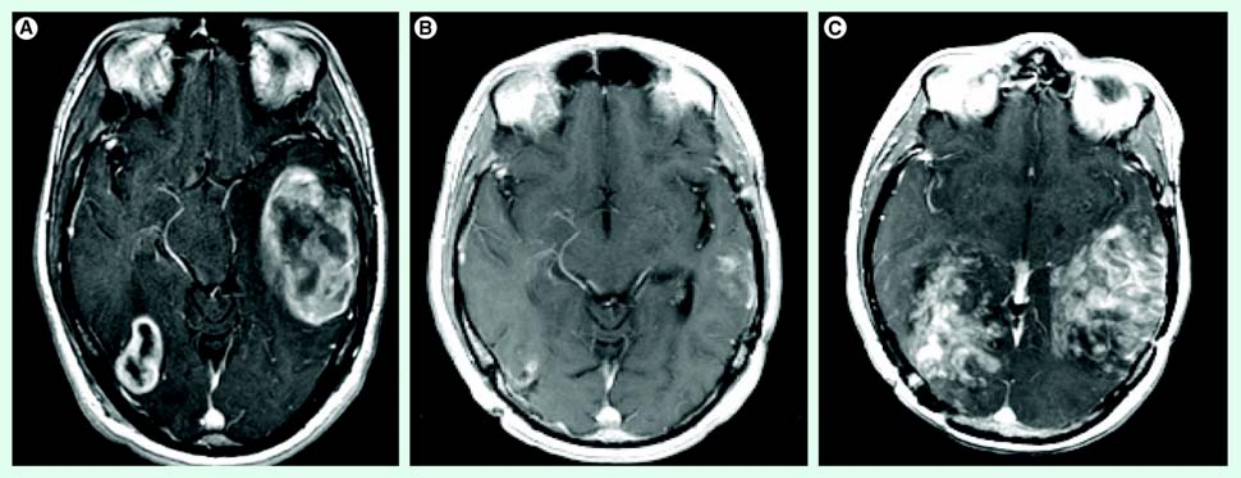


Figure 10. Axial T1-weighted post-contrast MRI sequences of a patient with glioblastoma. (A) At diagnosis an enhancing multifocal temporal and right parietoccipital tumor is seen. **(B)** The patient received combined chemo- and radiation-therapy and achieved a response. **(C)** The tumor recurred 2 years after diagnosis in a diffusely infiltrating fashion and the patient died. Taken from (169).

The key challenge with malignant gliomas is that tumor cells escaping surgical resection are able to survive and invade adjacent brain tissues, even under the stress of intensive radiochemotherapy. Indeed, DNA damage response was shown to enhance the capacity for therapy resistance and invasiveness of residual tumor cells (170, 171). However, the mechanisms underlying radio-resistance remain largely unknown.

Another recent emerging concept of resistance comes from the glioma stem cells. Glioma stem cells or glioma initiating cells have characteristics of stem cells with the property of long-term self-renewal and the capacity to differentiate (172). Although it has been demonstrate that $CD133^-$ can give rise to $CD133^+$ cells *in vivo*, CD133 is still the most frequently used marker to

identify glioma stem cells (173, 174). In addition to CD133, several other markers, such as SSEA-1, Nestin, Sox2 and Musashi-1 have been also used (170, 175, 176). The DNA damage checkpoint is preferentially activated in $CD133^{+}$ tumor cells and it has been demonstrated radiation induced DNA damage is more efficiently repaired in CD133 positive cells than in negative cells (170). Indeed, inhibition of the checkpoint kinases Chk1 and Chk2 sensitizes glioma stem cells to radiation induced cell death, indicating that targeting the DNA damage checkpoint may improve the efficacy of radiotherapy in GBM (170). Nevertheless, deeper investigation of the biological nature of glioma stem cells will shed light on the development of high effective therapy for high grade gliomas.

1.5.2 Current therapy for gliomas

After diagnosis, patients usually firstly get surgical removal of tumors as much as possible (177). Fractionated focal radiotherapy is the standard treatment after resection or biopsy. Exclusive chemotherapy (usually Temozolomide, TMZ) has been proposed for elderly patients (177). Concomitant and adjuvant TMZ chemotherapy significantly improved median, 2- and 5- year survival, and is the current standard of care for patients with glioblastoma up to age 70 (168). Selecting patients likely to benefit from TMZ therapy has been suggested basing on the basis of the methyl-guanine methyl transferase (*MGMT*) gene promoter methylation (178).

Several potential therapeutic targets have been proposed based on the deregulated signaling pathways, which has led to the first generation of drugs that inhibit these pathways in clinical trials. These agents are classified into growth factor receptor inhibitors, intracellular signal transducer inhibitors and angiogenesis blockers. Of note, EGFR appears to be the most attractive candidate, which is overexpressed in over 40% of primary GBM (179). The *EGFR* gene with a deletion of exon 2-7 (EGFRvIII) is ligand-independent constitutively active and promotes cell

proliferation and survival in many cancer types (180-182). The PDGFR subtypes and PDGF isoforms are also overexpressed and hyperactivated in malignant gliomas (183). In addition, key component of signaling pathways such as Ras, PI3K, PKB, MAPK, mTOR have also been proposed as attractive targets (164, 165). Furthermore, given the high vascularization of GBM tumors, anti-angiogenic drugs blocking interactions between secreted pro-angiogenic inducers and the correspondence receptors are considered as alternative strategies (184).

1.5.3 The Hippo pathway in malignant gliomas

The Hippo pathway has been characterized as tumor suppressive signaling networks by antagonizing the pro-oncogenic effectors YAP/TAZ. Deregulation of Hippo signaling components, such as NF2, MST and LATS/NDR kinases, MOB1 proteins, as well as the downstream effectors YAP/TAZ, has been reported in numerous animal tumor models and human malignancies (185). Of note, several critical and potential components of the Hippo pathway, such as Mer, hMOB1, CD44, YAP and TAZ, have been described to be relevant to gliomagensis and will be discussed below.

Mer (also known as NF2 for Neurofibromatosis 2) is a member of Band 4.1 superfamily of proteins, which links the transmembrane proteins to the actin cytoskeleton. Mutation of Mer has been found in several types of nervous system tumors. In addition to mutational inactivation of the *NF2* gene in NF2-related tumors, mutation and loss of Mer has been reported in other types of cancers, including high grade gliomas (186). Mer is found to be downregulated in human malignant gliomas . Re-expression of functional Mer, but not loss of function mutant, inhibits the growth of human glioma cells and promotes apoptosis *in vivo* (186). A link between Mer to MST kinases signaling provides the mechanism for this phenotype, indicating Mer activates the tumor suppressive Hippo signaling.

hMOB1 is an adaptor protein that orchestrates the MST-LATS/NDR kinase cascade. hMOB1 has been demonstrated as a tumor suppressor in both *Drosophila* and mammalian models (20, 139) . The first clinical relevance came from the study identifying the downregulating of hMOB1 in glioblastomas. Upregulated RING ligase praja 2 ubiquitylates and degrades hMOB1, leading to the decreased protein level of hMOB1(149). This study links the ubiquitin proteasome system to the deregulated Hippo signaling in the progression of gliomas.

CD44 is a major cell surface hyaluronan receptor that has been implicated in the progression of many types of cancers. In glioblastomas, CD44 is found to be highly upregulated with pro-oncogenic function by promoting GBM growth and survival *in vivo* (187). Interestingly, Merlin has been reported to mediate contact inhibition through CD44 (188). Therefore, it is tempting to speculate that CD44 signals upstream of the mammalian Hippo pathway via Mer and antagonizes the stress induced activation of MST-LATS kinases in gliomas.

As CD44 is well established cancer stem cell marker, it is tempting to speculate the roles of Hippo signaling in cancer stem cells. Indeed, TAZ, one of the hippo effectors, has been reported to confer cancer stem cell-related traits on breast cancer cells (56). Moreover, the hippo transducer TAZ has been found to be implicated in the differentiation of glioma stem cells as well (189, 190). Compared with mesenchymal GBMs, where *TAZ* is highly expressed and active, *TAZ* expression is lower in proneural GBMs and lowere grade gliomas. The expression pattern is associated with CpG island methylation status of the *TAZ* promoter. TAZ is functional essential for mesenchymal glioma stem cells as silencing of TAZ in mesenchymal glioma stem cells leads to decreased expression of mesenchymal markers, invasion, self-renewal and tumor formation. Interestingly, CD44 seems to be a transcriptional target of TAZ, suggesting a potential feedback loop between CD44 and TAZ (189). However, the parallel YAP is found to

be dispensable maintaining the mesenchymal glioma stem cells (189). Nevertheless, YAP is also highly upregulated in high grade gliomas and is required for cell proliferation in GBM tumor cells (191).

Several investigations suggest general epigenetic hypermethylation of the promoters of RASSF (192), MST (193) and LATS (194) kinases in many cancer types, which result in decreased protein levels in tumors. Therefore, it is not surprise to speculate the similar pattern would occur in malignant gliomas as well.

In general, the YAP/TAZ destructive Hippo signaling seems to be suppressed in gliomas, either by mutational loss of upstream positive input Mer, or by epigenetic silencing of core kinases, or by proteolytic degradation of key co-activator hMOB1, or by upregulation of upstream inhibitor CD44. Inhibition of Hippo signaling results with hyperactivation of the transcriptional co-activator YAP/TAZ. Therapeutically, one would think restoring of functional tumor suppressive Hippo signal by blocking the upstream negative regulators or reinforcing the positive inputs. Alternatively, targeting the binding of YAP/TAZ to its transcriptional coactivator TEAD would be another general and effective approach. One small molecular Verteporfin (VP) tested by Duo-Jia Pan's Lab represents one promising example to targeting YAP/TEAD interaction by small inhibitors (195). It has been tested recently that introducing a default repressive VGL4 functional fragment blocks the YAP/TEAD or TAZ/TEAD complex, pointing towards alternative therapeutic strategy with pharmaceutical peptide (35).

Taken together, current investigations strongly suggest that modulating the Hippo-YAP/TAZ pathway might represent a promising therapeutic strategy in malignant gliomas.

2. Scope of thesis

The human genome encodes six MOB family members (hMOB1A, -1B, -2, -3A, -3B and -3C). While hMOB1A/B functions as an activator for MST-LATS/NDR kinase cascade, hMOB2 was found as a specific negative regulator of NDR kinase by competing the binding of hMOB1 to NDR kinase. Although hMOB3 share higher sequence identity to hMOB1 than hMOB2, hMOB3 proteins do not interact or interfere the NDR kinases. Thus, the biochemical functions of hMOB3A/B/C are completely undefined. Additionally, hMOB3 mRNA levels seem to be deregulated in several pathological diseases, such as mantel cell lymphoma, colon cancer and amyotrophic lateral sclerosis. A previous microarray analysis carried out in the lab identified hMOB3A /B/C were deregulated in glioblastoma multiform (GBM) as well.

In this thesis, we investigated the role of hMOB3 in GBM and found that total hMOB3 proteins are upregulated in GBM. Importantly, we observed a correlation of hMOB3A/B/C mRNA expression with clinical survival, suggesting hMOB3A/B/C being a potential prognostic biomarker. Additionally, we explored the biochemical function by analyzing the interaction of hMOB3 with the upstream MST kinase and found hMOB3 specifically interact with MST kinases upon apoptotic stimulation and under cell-cell contact condition. Moreover, we demonstrated that binding of hMOB3 to MST1 inhibits the apoptotic cleavage of MST1 in GBM cells. Taken together, our results indicate hMOB3 negatively regulates apoptotic MST1 signaling, suggesting modulating hMOB3 might represent a potential therapeutic approach in GBM.

3. Results

The results obtained during course of my thesis are shown in the following manuscript. Parts of the text in the manuscripts were taken from the summary, introduction, scope of the thesis and general discussion of this thesis. The numbering of references and figures of the manuscript is separate to that from the introduction and general discussion meaning that the first reference and the first figure of each manuscript is numbered as “1”.

hMOB3 inhibits apoptotic cleavage of MST1 in glioblastoma multiform

This part of this result will be presented in the manuscript entitled “hMOB3 modulates apoptotic MST1 signaling and supports tumor growth in glioblastoma multiforme”. Currently it is published in Cancer Research.

hMOB3 modulates apoptotic MST1 signaling and supports tumor growth in glioblastoma multiforme

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Running Title: hMOB3 inhibits apoptotic MST1 cleavage and promotes tumor growth

Keywords: apoptosis, etoposide, proliferation, STK4, caspase cleavage

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Word count: 5263 words 6 Figures +3 Supplemental Figures

Abstract:

New therapeutic targets are needed that circumvent resistance in glioblastoma multiforme (GBM). Here we show that the uncharacterized adaptor protein human Msp One Binder 3 (hMOB3) is upregulated in GBM. In a search for its biochemical function, we find that hMOB3 specifically interacts with MST1 kinase in response to apoptotic stimuli and cell-cell contact. Moreover, hMOB3 negatively regulates apoptotic MST1 signaling in GBM cells by inhibiting the MST1 cleavage activation process. We also demonstrate that the physical interaction between hMOB3 and MST1 is essential for this regulation of MST1. Further, we show that hMOB3 sustains GBM cell growth at high cell density and promotes tumorigenesis *in vivo*. In summary, our results suggest hMOB3 as a potential therapeutic target for the treatment of malignant gliomas.

Introduction

Glioblastoma multiforme (GBM) is the most common and aggressive primary human brain tumor, with a median survival of approximately 14 months after diagnosis. Despite the benefits of surgical resection and the use of adjuvant radiochemotherapies, patients almost invariably succumb to recurrent widespread tumor growth (1, 2). Thus, defining the mechanism of resistance of GBM cells and discovering further effective therapeutic targets are crucial medical goals.

The Hippo pathway is an evolutionarily conserved tumor suppressive signal originally identified in *Drosophila* as a tumor suppressive signal (3-9). Deregulation of Hippo signaling components,

such as MST and LATS/NDR kinases, MOB1 proteins, as well as the downstream effector YAP, has been reported in numerous animal tumor models and human malignancies (10).

MST1 (Sterile 20-like kinase 1), the mammalian homolog of the Hippo kinase, plays a critical role in regulating cellular apoptosis and proliferation (11-15). MST1 contains an N-terminal kinase domain, followed by an auto-inhibitory domain and a C-terminal protein-protein interaction domain called SARAH (Salvador-RASSF-Hippo) (16). In response to apoptotic stimuli, MST1 is activated by dimerization-mediated trans-phosphorylation and caspase-mediated cleavage (17-20). Cleaved MST1 translocates from the cytoplasm into the nucleus and induces chromatin condensation by phosphorylation different targets (21-25). Although Akt and JNK have been reported to phosphorylate MST1 and modulate its cleavage (26-29), the regulation of apoptotic MST1 signaling has not been completely defined.

MOB1 (Mps One Binder 1) proteins were first characterized in yeast, where they are essential components of mitotic exit and septation initiation networks (30, 31). *Drosophila* mob1/mats functions as a tumor suppressor by regulating the activation of the Warts kinase (32, 33). The mammalian genome encodes 6 MOB proteins through 6 different genes, namely MOB1A/B, MOB2 and MOB3A/B/C (34, 35). Mammalian Mob1A and Mob1B are essential for embryonic development and prevent tumorigenesis in a broad range of tissues via a mechanism similar to that reported in flies (36, 37). The function of human MOB1 has been characterized as a co-activator of the MST-NDR/LATS kinase cascade (38, 39). Human MOB2 has been reported to restrict NDR kinase signaling (34). Although hMOB3 shares higher amino acid sequence identity (50%) with hMOB1 than hMOB2 (37%), it neither interacts with nor activates NDR/LATS kinases (34, 35). Its biochemical functions remain unknown. Therefore, the

molecular roles of hMOB3 in the context of the mammalian Hippo pathway merit further investigation.

In the present study we have found that the previously uncharacterized hMOB3 is overexpressed in GBM. Biochemically, hMOB3 directly interacts with MST1 kinase in response to apoptotic stimuli and at high cell density. Functionally, hMOB3 negatively regulates MST1 cleavage during etoposide-induced apoptosis and attenuates the apoptotic response. Moreover, hMOB3 is required to sustain tumor cell proliferation and growth *in vitro* and *in vivo*. Taken together, our study reveals that hMOB3 restricts the crosstalk between MST1 and caspases during apoptosis and supports tumorigenesis in GBM suggesting hMOB3 as a potential target for GBM therapy.

Materials and Methods

Patients. Tissue samples of primary GBM and adjacent non-neoplastic brain were processed in accordance with the guidelines of the Ethical Committee of the University Hospital of Basel. Tumors were diagnosed and graded according to the World Health Organization (WHO) Classification of Tumors of the Nervous System (40).

Cell culture, transfection, and stimulation. HEK293 cell line was obtained from American Type Culture Collection (ATCC). Glioma cell lines were described previously (41, 42). All the cell lines in this study were confirmed with absence of mycoplasma contamination (MycoAlert^{TM}, Lonza) and regularly authenticated by growth and morphological observations. HEK293 and glioma cell lines were maintained in Dulbecco's modified Eagle's medium supplemented with 10% fetal calf serum. Transfection of HEK293 and GBM cells were carried out using jetPEI (PolyPlus Transfections, Dietikon, Switzerland) and Lipofectamine 2000

(Invitrogen, CA, USA) according to the manufacturer's instructions, respectively. Apoptosis was induced as indicated in the figure legends. Okadaic acid was purchased from Alexis Biochemicals (Enzo Life Sciences, Lausen, Switzerland). Cyclohexylamine (CHX), actinomycin D and etoposide were obtained from Sigma (St Louis, MO, USA).

Annexin V assay. Annexin V staining was performed according to the manufacturer's instructions (BD Bioscience) and analyzed by FACSCalibur. The results were from three independent experiments and presented as mean ± standard deviation. Statistical analysis is performed in Excel with two tailed-paired-student t test.

Tumor Implantation: Aythymic Nude–*Foxn1*^{nu} mice (Harlan, France) were maintained in Specific and Opportunistic Pathogen Free (SOPF) facility with food and water *ad libitum*. U87MG cells ($8x10^5$ in 200µl DMEM:Matrigel(1:1 ratio)) were implanted into left flanks. Tumor diameters were regularly measured via caliper and tumor volumes calculated as follows: Volume = $d^2 \bullet D \bullet \pi/6$, where *d* is shorter tumor diameter and D is longer tumor diameter. All in-vivo experiments were performed under approved authorization within the Swiss Federal Animal Welfare Law.

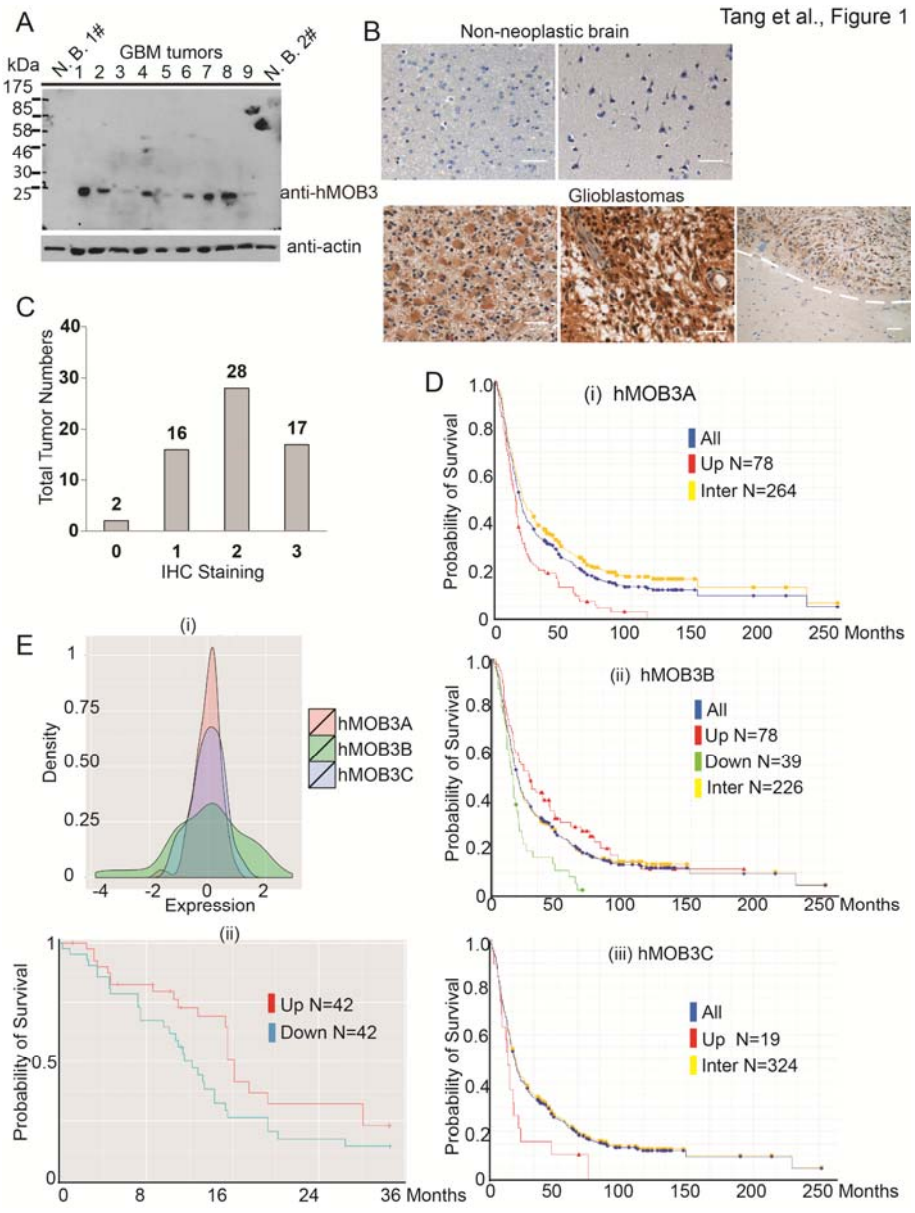
Results

hMOB3 is overexpressed in human GBM

In a previous study, we performed a microarray analysis of 30 human gliomas (41). Interestingly, the mRNA levels of uncharacterized hMOB3 family members were found to be deregulated in GBM. Of these, hMOB3A and hMOB3C expression were elevated while hMOB3B was

downregulated (Supplemental Figure 1A). hMOB3A/B/C are three unique genes located on different chromosomes. Given that the three hMOB3 isoforms hMO3A/B/C are about 80% identical (35), we set out to investigate the function of total hMOB3 protein in GBM, instead of analyzing each isoform separately. To this end, we generated a rabbit polyclonal antibody against total hMOB3 that recognizes hMOB3A/B/C proteins but not hMOB1 or hMOB2 (Supplemental Figure 1B-C and 1E).

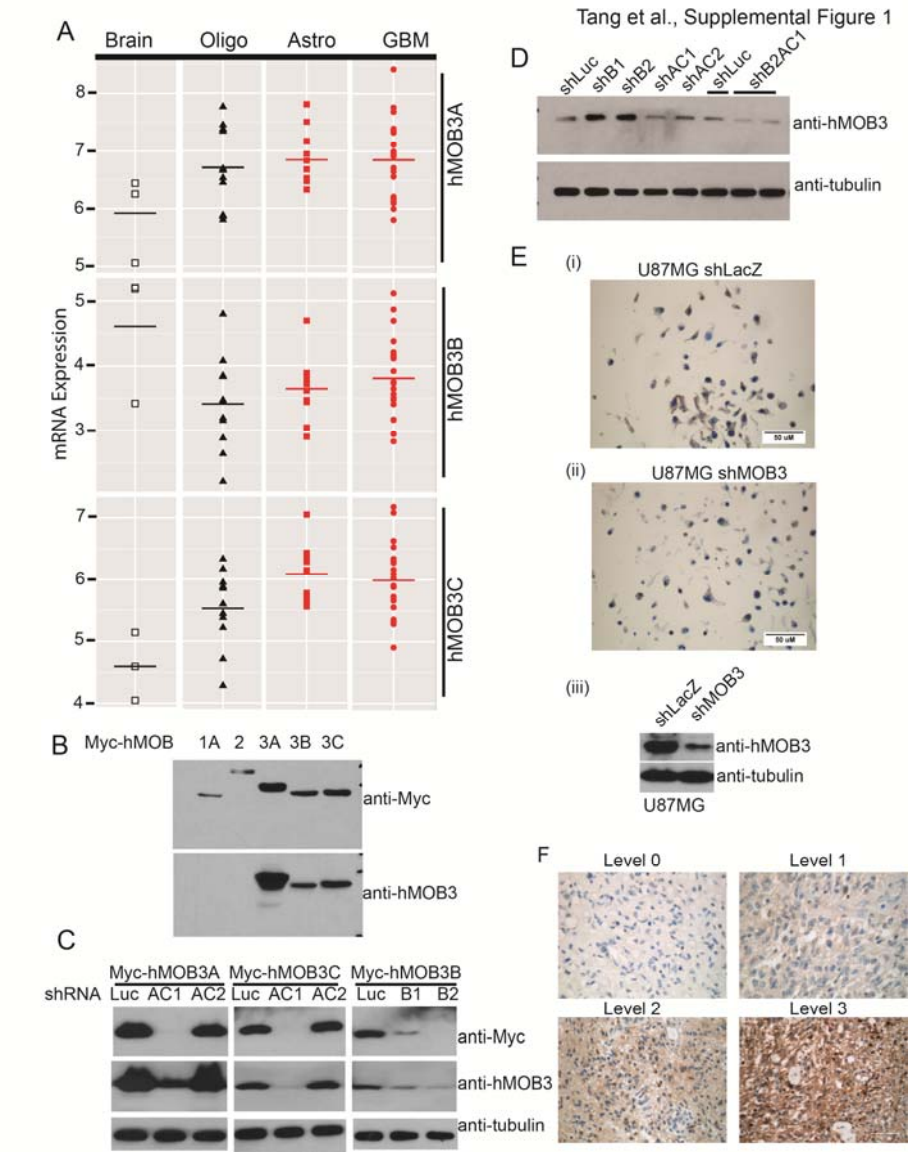
Using this novel antibody, we determined the total hMOB3 protein levels in human GBM samples by Western blotting and found it to be upregulated in the majority of solid GBM tumor samples compared with non-neoplastic human brain tissue (Figure 1A). Immunohistochemical staining confirmed total hMOB3 protein upregulation in human glioblastomas (Figure 1B). Moreover, scoring of hMOB3 protein expression in 63 clinical GBM samples revealed that 71.4% (45/63) of tumors displayed either medium or high hMOB3 expression levels (Figure 1C and Supplemental Figure 1F).



120

121 **Figure 1. hMOB3 is overexpressed in human GBM.** (A) Western blotting analysis of GBM
122 lysates and non-neoplastic brain tissues (N.B.) with an anti-hMOB3 antibody. Molecular weights
123 are expressed in kilo-Daltons (kDa). (B) Representative immunohistochemistry images of non-
124 neoplastic brain tissue using an anti-hMOB3 antibody (left: white matter; right: grey matter) and
125 various human GBM tumors (left: GBM with partly gemistocytic differentiation; middle: GBM
126 with focal spindle-shaped cytomorphology; right: GBM with specific staining in the tumor
127 (upper right) but not adjacent non-neoplastic tissue (lower left)). (C) Scoring of hMOB3
128 immunohistochemical staining in 63 human GBM samples (0: negative; 1: low; 2: medium; 3:
129 high). For representative images, see Supplemental Figure 1F. (D) Kaplan-Meier (KM) survival
130 curves for hMOB3 expression taken from the Rembrandt database. Cut-off is a twofold change.
131 *P* value is provided by the database using log-rank test. Curves represent all patients (blue),
132 patients with upregulation of hMOB3 (red), downregulation (green), and intermediate expression

(yellow). N=patient numbers. (i) KM curve for hMOB3A. $P_{up\text{- vs inter-}}$<0.01; (ii) KM curve for hMOB3B. $P_{up\text{- vs down-}}$<0.01, $P_{up\text{- vs inter-}}$=0.15, $P_{down\text{- vs inter-}}$<0.05; (iii) KM curve for hMOB3C. $P_{up\text{- vs inter-}}$<0.05. (E) (i) Density plot of hMOB3 isoforms expression levels. Displayed are the normalized expression values for hMOB3A/B/C where 0 represents the expression mean of all samples. (ii) Prognostic value of hMOB3B expression in the TCGA-Gene Expression (IlluminaHiSeq data subset) database. KM survival curves for 25% of patients with highest ("Up") versus lowest ("Down") of hMOB3B expression levels. $P_{up\text{- vs down-}}$=0.05. A detailed description of data extraction and processing as well as statistical analysis is provided in the "Statistical analysis and Bioinformatics " section in the supplementary Materials and Methods.



Supplemental Figure 1. Upregulation of hMOB3 in GBM and characterization of hMOB3 antibody and shRNAs. (A) mRNA expression of hMOB3A/B/C in normal human brain (Brain), oligodendroglioma (Oligo), astrocytoma (Astro), and glioblastoma multiforme (GBM). (B) Characterization of a purified home-made hMOB3 antibody. Lysates of HEK293 cells overexpressing Myc-targeted hMOB1A, 2, 3A, 3B, 3C were immunoblotted. (C) Characterization of hMOB3A/B/C shRNA constructs. Lysates of HEK293 cells overexpressing Myc-tagged hMOB3A, B or C in combinations of shLuc control, shMOB3AC1#, shMOB3AC2#, shMOB3B1#, or shMOB3B2# were analyzed by immunoblotting. (D) Characterization of the knockdown efficiency of hMOB3 shRNA constructs. Lysates of U87MG cells transiently transfected with the indicated plasmids were analyzed by immunoblotting. (E) Characterization of the hMOB3 antibody in immunohistochemical conditions. Immunohistochemical staining images of stable (i) U87MG_shLacZ and (ii) U87MG_shMOB3 cells. (iii) The knockdown efficiency was demonstrated by immunoblotting. (F) Representative images of the immunohistochemical staining of hMOB3 in GBM used for quantification in Figure 1C. Level 0: negative; Level 1: low; Level 2: moderate; Level 3: high.

To explore the potential prognostic value of hMOB3, we compared clinical outcome and hMOB3 gene expression using the Rembrandt database (43). In agreement with our finding of upregulation of hMOB3A and hMOB3C (Supplemental Figure 1A), we did not identify any sample with hMOB3A and hMOB3C downregulation in human GBM in Rembrandt dataset (data not shown). Because of limited GBM sample numbers, we extended our analysis from "GBM" to "all glioma". In this dataset, we found a statistically significant correlation between poor survival and high mRNA expression of hMOB3A and hMOB3C (Figure 1D (i) and (iii)); the opposite was found for hMOB3B where low expression correlates with poor survival (Figure 1D (ii)).

Next, we sought to validate these clinical correlations using the online TCGA-Gene Expression database (44). A total of 167 patient samples with available hMOB3A/B/C mRNA expression and survival data were extracted (denoted IlluminaHiSeq data subset). Within these samples, we again observed highly variable expression of the hMOB3B gene but relatively stable hMOB3A and hMOB3C expression levels (Figure 1E (i)), suggesting frequent genetic or epigenetic

alterations in the hMOB3B genomic locus. We further generated Kaplan-Meier curves for differential hMOB3B gene expression from the same dataset (Figure 1E (ii)). Since records for normal human brain control tissue were not available, we followed the common strategy to define the top 25% of the samples with highest expression as "Up" and the 25% with lowest hMOB3B expression as "Down". Based on these criteria, 50% survival of patients with low hMOB3B levels was reduced by 40% compared to patients with high hMOB3B levels (10 versus 16.6 months). These findings indicate that downregulation of hMOB3B predicts poor survival, fully consistent with the results from the Rembrandt dataset.

To investigate the discrepancy between upregulated total hMOB3 protein levels in human GBM and its variable prognostic values from the Rembrand and TCGA mRNA datasets, we studied the interplay within hMOB3 members by single knockdown of the most variable member, hMOB3B. Interestingly, upregulation of total hMOB3 protein in U373MG cells by specific knockdown of hMOB3B pointed towards compensatory mechanisms of hMOB3A/C and hMOB3B (Supplemental Figure 1D). Therefore, it appears that depletion of hMOB3B results in the upregulation of hMOB3A/C protein, which indicates that low levels of hMOB3B in GBM might result in high hMOB3A/C levels. This could potentially explain the observed association between poor survival and low hMOB3B expression (Figure 1D).

Collectively, our analysis indicates that total hMOB3 is up-regulated at the protein level in GBM and that expression of hMOB3A/B/C is associated with clinical outcomes. Based on these findings it is tempting to speculate that total hMOB3 protein has proto-oncogenic properties.

hMOB3 interacts with MST1 in response to apoptosis and high cell density

Unlike hMOB1 and hMOB2, hMOB3 does not bind to LATS and NDR kinases (34, 35). To investigate the involvement of hMOB3 in GBM, we asked whether hMOB3 plays a role in regulating the upstream Hippo kinase MST1. Since hMOB1 was reported to form a complex with MST1 and NDR1 upon apoptotic stimulation (38), we firstly analyzed the physical interaction between MST1 and hMOB3 under apoptotic conditions. To this end, we performed MBP pull-down assays using purified *E. coli*-expressed MBP-tagged hMOB3A as a bait to purify N terminal Flag-tagged MST1 from etoposide-treated HEK293 cells. Interestingly, hMOB3A interacted with MST1 in this experimental setting (Figure 2A). Consistently, endogenous MST1 could be co-immunoprecipitated by overexpressed hMOB3 in response to okadaic acid (Figure 2B, left panel) and *vice versa* (Figure 2B, right panel). However, although hMOB3 bound to MST1, hMOB3 did not interact with endogenous NDR/LATS (Supplemental Figure 2A). We next analyzed the interactions of MST1 with all hMOB3 members using purified MBP-tagged hMOB3 to pull down purified untagged human MST1 expressed in Sf9 insect cells. Consistently, untagged MST1 purified from Sf9 cells could be pulled down with MBP-hMOB3A/B/C, excluding tag-mediated unspecific binding and illustrating that MST1 and hMOB3 interact directly (Figure 2C and Supplemental Figure E). However, to our surprise, the interaction between MST1 and hMOB3 in HEK293 cells was lost at low cell density (50%) without any stimulation (Figure 2B and 2D lane 1 and Supplemental Figure 2B).

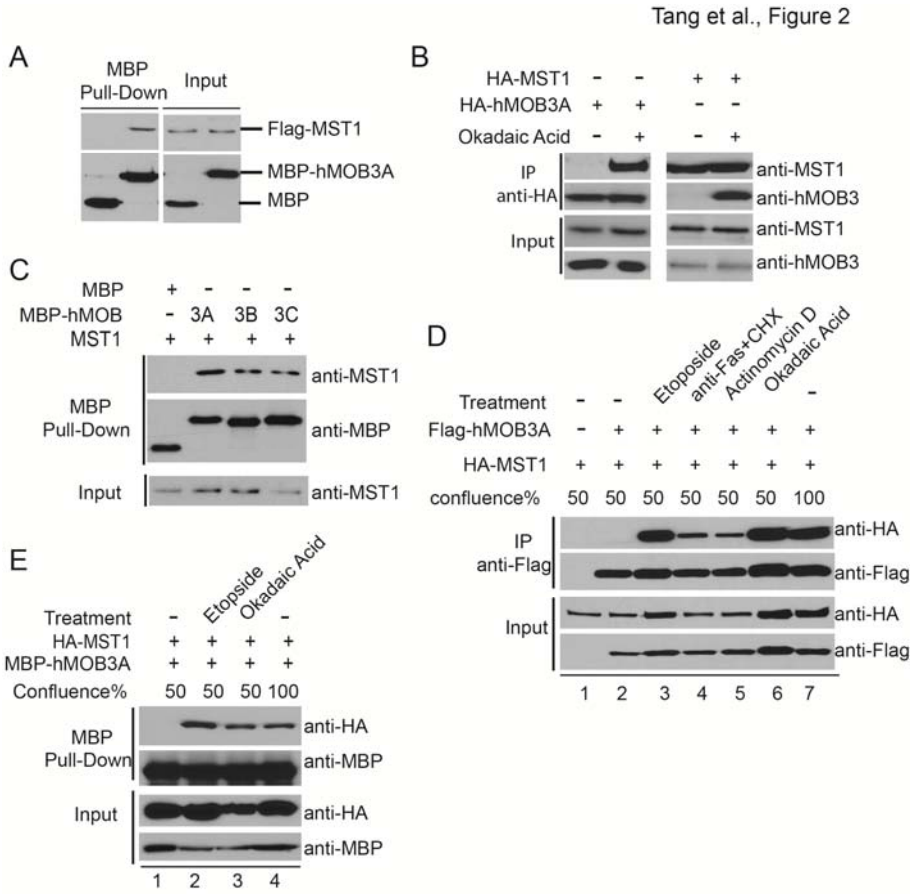


Figure 2. hMOB3 interacts with MST1. (A) hMOB3A interacts with Flag-tagged MST1. After incubation of purified *E.coli*-expressed MBP or MBP-hMOB3A with etoposide-pretreated Flag-tagged MST1 overexpression HEK293 cell lysates, complexes were analyzed via MBP pull-down assay followed by immunoblotting. (B) Overexpressed hMOB3 interacts with endogenous MST1 upon okadaic acid (0.5nM) treatment (left panel) and *vice versa* (right panel). U87MG cell lysates overexpressing HA-tagged hMOB3 (left panel) or HA-tagged MST1 (right panel) with or without treatment were analyzed by immunoprecipitation (IP). Complexes and input lysates were assayed by immunoblotting. (C) hMOB3A/B/C interacts with MST1 directly. After incubation of purified *E.coli*-expressed MBP or MBP-hMOB3A/B/C with purified SF9-expressed untagged MST1, the complexes were analyzed via MBP pull-down assay followed by immunoblotting. (D) hMOB3 interacts with MST1 upon stimulation of cell death and at high cell density. Lysates of HEK293 cells co-expressing HA-tagged MST1 and Flag-tagged hMOB3A were treated with the indicated reagents (Etoposide, 100μM; anti-Fas, 0.5 μg/ml, CHX, 15ug/ml; Actinomycin D 2μM; Okadaic Acid, 0.5nM) at around 50% confluence or cultured to 100% confluence, and were analyzed by immunoprecipitation (IP). Complexes and input lysates were assayed by immunoblotting. (E) hMOB3 interacts with active MST1. After incubation of purified *E.coli*-expressed MBP-hMOB3A with untreated or okadaic acid (0.5nM) pretreated, etoposide (100μM) -pretreated or cell-cell contact-conditioned Flag-tagged MST1 overexpressing HEK293 cell lysates, complexes were analyzed via MBP pull-down assay followed by immunoblotting.

We next asked whether the interaction of MST1 with hMOB3 occurs under general apoptotic stress. To address this question, we co-expressed hMOB3A with MST1 in HEK293 cells. Prior to immunoprecipitation, the cells were treated with various apoptotic stimuli or grown to 100% confluence. Strikingly, in contrast to low cell density without any treatment, hMOB3/MST1 complex formation was induced by a broad range of apoptotic stimuli as well as by increased cell-cell contact due to high cell density (Figure 2D). To avoid artificial binding of two overexpressed proteins inside cells, we confirmed the interaction by *in vitro* pull-down using purified *E.coli*-expressed MBP-tagged hMOB3A as bait. Consistently, MST1 was only pulled down from stressed HEK293 cells (Figure 2E). In the reverse approach, hMOB3A was also immunoprecipitated by MST1 in HEK293 cells (Supplemental Figure 2D).

To gain insight into the domains of MST1 responsible for binding to hMOB3, we generated a series of MST1 truncation mutants illustrated in Figure 3A. We tested the interaction between these mutants and wild-type hMOB3A by co-immunoprecipitation and found that the minimal fragment of MST1 required for binding of hMOB3 comprises the kinase domain and the auto-inhibitory domain (Figure 3B and 3C). However, neither the kinase domain nor the auto-inhibitory domain alone was sufficient for the association with hMOB3 (Figure 3B lane 7 and 3C lane 3). Notably, the SARAH domain was found to be dispensable for the interaction as SARAH domain deletion mutant showed similar affinity as the wild-type full-length MST1 (Figure 3B). A C-terminal MST1 fragment containing the auto-inhibitory domain and the SARAH domain also did not form a complex with hMOB3, further confirming that the SARAH domain is not involved in this binding (Figure 3C lane 2).

Next, we asked whether the kinase activity of MST1 is required for its interaction with hMOB3. . As illustrated in Figure 3A, the regulation of MST1 activity requires the ATP-binding site Lys_{59}

and the phosphorylation of Thr_{183} (20). Therefore, we investigated the interaction using two inactive MST1 mutants. Remarkably, the interaction between MST1 and hMOB3 was abolished when MST1 was mutated into an inactive form either by conversion of the ATP-binding site Lys_{59} to Arg or the phosphorylation site Thr_{183} to Ala (Figure 3B lane 5 and 3D lane 4). As expected, binding was restored when Thr_{183} was mutated to glutamic acid (Figure 3D lane 5). We also observed the binding of MST1T_{183}E and hMOB3 at 50% cell density (Supplemental Figure 2F), further indicating that phosphorylation of threonine 183 is critical for the interaction. To further confirm the observation of activity of MST1 dependent interaction with hMOB3, we treated untagged MST1 purified from Sf9 cells with lambda-phosphatase to dephosphorylate MST1 prior to pull down experiments. Significantly, phosphatase treatment decreased binding of MST1 to hMOB3A (Figure 3E lane 3).

Taken together, our analysis indicates that the interaction between MST1 and hMOB3 is induced by apoptosis and cell-cell contact stress and depends on MST1 phosphorylation and kinase activity, while the SARAH domain is dispensable.

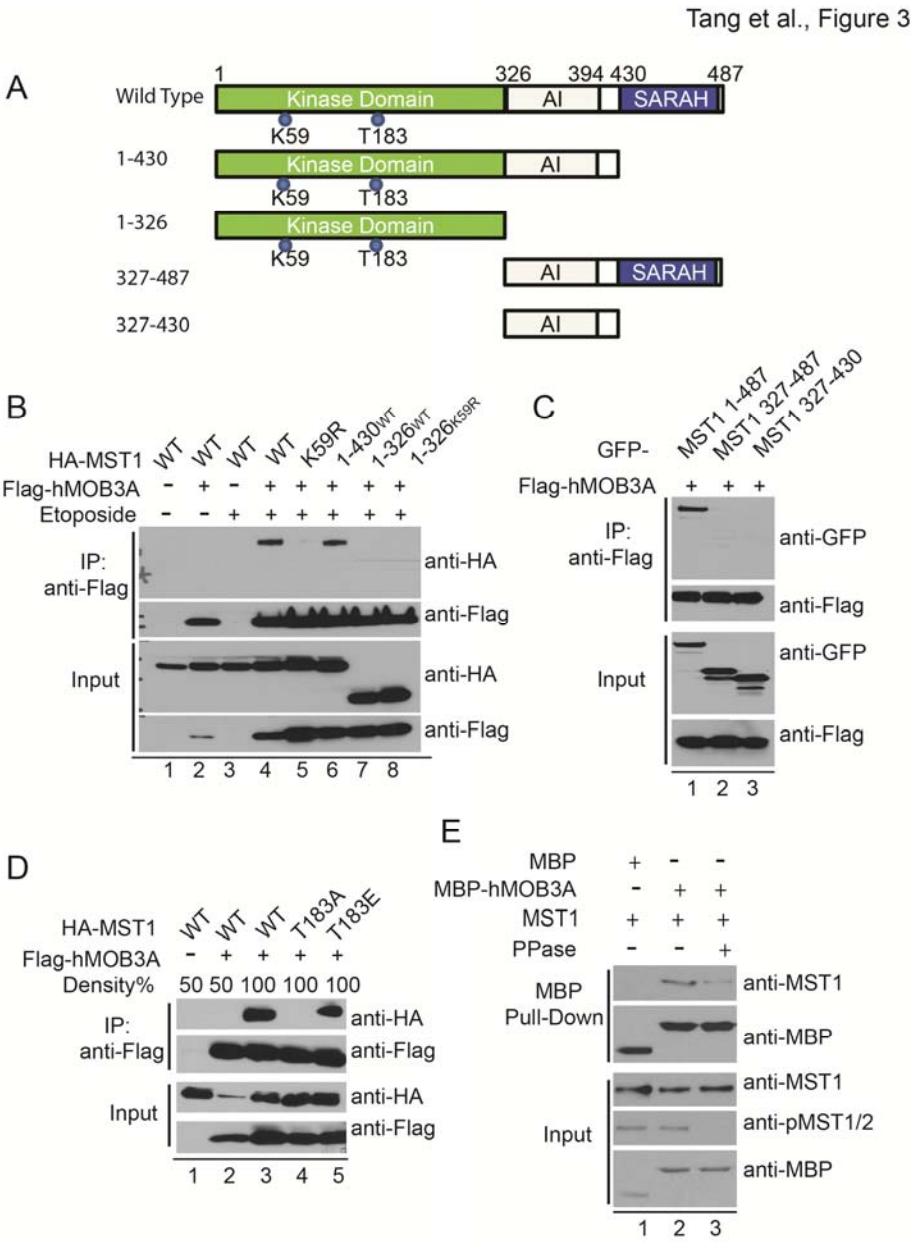
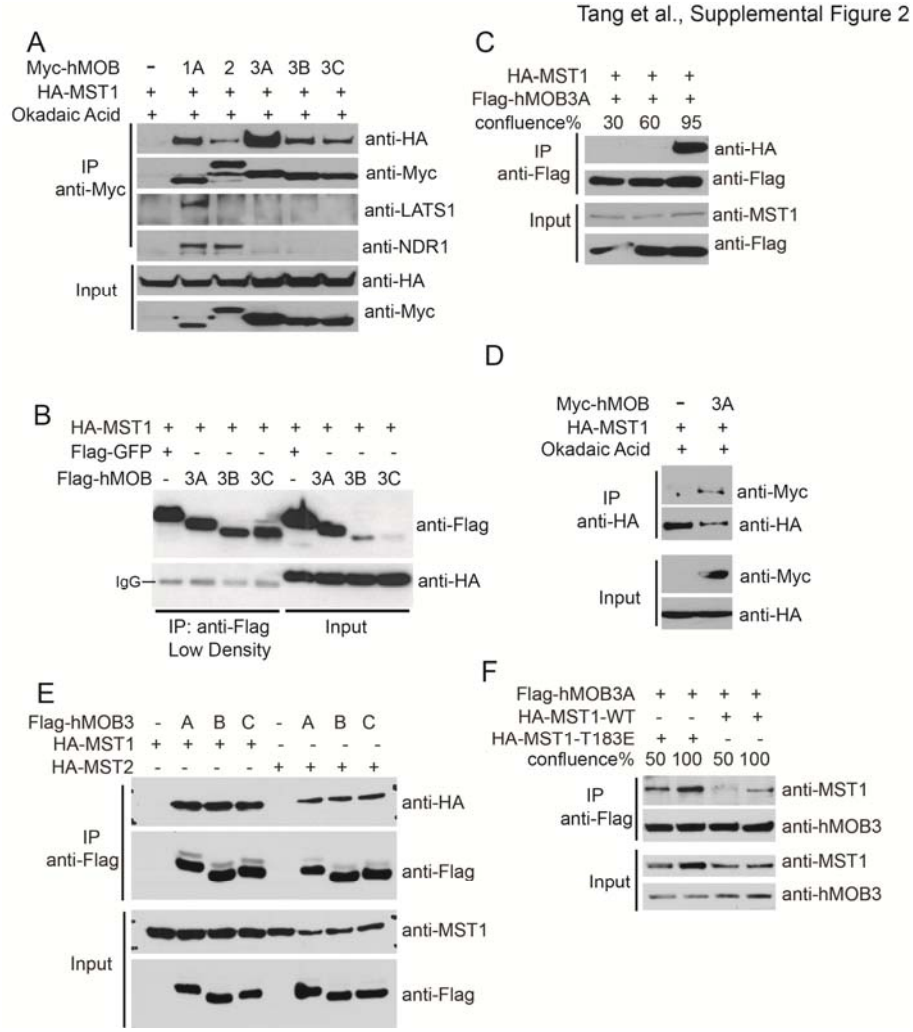


Figure 3. hMOB3 binds to the active MST1 N-terminus kinase domain and auto-inhibitory domain. (A) Primary structure of wild-type MST1 and an overview of HA- or GFP-tagged mutant derivates. Amino acids Arg_{59} and Thr_{183} are the ATP-binding and auto-phosphorylation sites, respectively. Arg_{59} and Thr_{183} were mutated to Lys and Ala, respectively. K59R and T183A mutants are kinase dead. (B) Etoposide (100 μM) -pretreated or untreated lysates of HEK293 cells containing the indicated combinations of HA-tagged MST1 forms and Flag-tagged hMOB3A (wild type) were analyzed by immunoprecipitation (IP). Complexes and input lysates were analyzed by immunoblotting. (C) Etoposide (100 μM) -pretreated HEK293 cell lysates co-overexpressing the indicated combinations of GFP-tagged MST1 forms and Flag-tagged hMOB3A (wild type) were analyzed by immunoprecipitation (IP). Complexes and input lysates were analyzed by immunoblotting. (D) HEK293 cells overexpressing HA-tagged MST1 alone or

co-overexpressing the indicated combinations of HA-tagged MST1 forms and Flag-tagged hMOB3A (wt) were harvested and lysed at different cell confluences and the lysates analyzed by immunoprecipitation (IP). Complexes and input lysates were analyzed by immunoblotting. The T183E mutant is not constitutively active but functions similar to the wild type (20). (E) Purified SF9-expressed, untagged active wild-type MST1 was first treated with lambda phosphatase (PPase). Untreated or treated MST1 was then subjected to pull-down assay with purified *E.coli*-expressed MBP or MBP-tagged hMOB3A. Complexes and input lysates were analyzed by immunoblotting.



Supplemental Figure 2. hMOB3 specifically interacts with MST1. (A) hMOB3 and MST1 form a complex without NDR or LATS kinases upon okadaic acid (0.5nM) treatment. Okadaic acid-treated lysates of HEK293 cells expressing HA-tagged MST1 alone or co-expressing HA-tagged MST1 and Myc-tagged hMOB1A/2/3A/3B/3C were analyzed by immunoprecipitation (IP). Complexes and input lysates were assayed by immunoblotting. (B) hMOB3 does not bind to MST1 at low cell density without stimulation. HEK293 cells co-expressing HA-tagged MST1

and Flag-tagged GFP or hMOB3A/B/C were harvested at 50% confluence without any pre-treatment and the lysates analyzed by immunoprecipitation (IP). Complexes and input lysates were assayed by immunoblotting. (C) High cell density induces the interaction between MST1 and hMOB3. HEK293 cells co-expressing HA-tagged MST1 and Flag-tagged hMOB3A were harvested at the indicated confluences without pre-treatment. Lysates were analyzed by immunoprecipitation (IP). Complexes and input lysates were assayed by immunoblotting. (D) Binding of MST1 and hMOB3. Okadaic acid (0.5nM) -treated lysates of HEK293 cells expressing HA-tagged MST1 alone or co-expressing HA-tagged MST1 and Myc-tagged hMOB3A were analyzed by immunoprecipitation (IP) using an anti-HA antibody. Complexes and input lysates were assayed by immunoblotting. (E) High cell density induces the interaction between MST1/2 and hMOB3A/B/C. HEK293 cells co-expressing HA-tagged MST1/2 and Flag-tagged hMOB3A/B/C were harvested at cell-cell contact condition. Lysates were analyzed by immunoprecipitation (IP). Complexes and input lysates were assayed by immunoblotting. (F) Interaction between MST1 mutant and hMOB3. HEK293 cells co-expressing HA-tagged MST1-T183E or wild type with Flag-tagged hMOB3A were harvested indicated culture confluence. Lysates were analyzed by immunoprecipitation (IP). Complexes and input lysates were assayed by immunoblotting.

hMOB3 negatively regulates cleavage of MST1 in GBM cells

Having demonstrated that hMOB3 is highly upregulated in GBM and that hMOB3 interacts with MST1, we next addressed the biological effect of hMOB3 on the activity of MST1 in GBM (Figure 4). The kinase domain and auto-inhibitory domain of active MST1 also interacts with caspases during apoptosis (17-21). Therefore we focused on the interplay of hMOB3, MST1 cleavage and caspases in the cellular apoptotic response of GBM cells. To this end, we generated hMOB3-overexpressing U373MG cells and evaluated the apoptotic response to the standard chemotherapy drug etoposide. Notably, the cleavage of endogenous MST1 was reduced in hMOB3-overexpressing cells compared to control cells (Figure 4A). We next determined the biological consequences of hMOB3 overexpression by analyzing the apoptotic response. In agreement with decreased levels of cleaved MST1, overexpressed hMOB3 attenuated apoptosis, as reflected by decreased levels of cleaved poly (ADP-ribose) polymerase and caspase3 proteins

(Figure 4A). Furthermore, Annexin V staining revealed a decrease in apoptotic cells of approximately 40% in hMOB3A/B/C overexpressing cells upon etoposide treatment (Figure 4B).

As cleaved MST1 translocates into nucleus (21), we next analyzed signaling downstream of MST1 cleavage. In the absence of a suitable immunofluorescence antibody to analyze the subcellular distribution of endogenous MST1, we performed subcellular fractionation assays to examine nuclear translocation of cleaved MST1 in etoposide treated cells. Consistent with a decrease in total levels of cleaved MST1, nuclear cleaved MST1 was also significantly reduced in hMOB3-overexpressing U373MG cells (Figure 4C). Histone H2B has been reported to be a key substrate mediating apoptotic MST1 signaling (24). We further analyzed H2B Ser_{14} phosphorylation in response to etoposide and found a decrease in Ser_{14} phosphorylation in hMOB3B-overexpressing U373MG cells (Figure 4D).

To further test our conclusions on the effect of endogenous hMOB3 on MST1 cleavage and apoptosis, and given the functional redundancy of hMOB3A/B/C (Figure 4A-B), we generated a construct containing two independent short hairpin RNAs targeting all hMOB3 isoforms (shRNA-MOB3 B2#AC1#, described in Supplementary Methods and Materials; Supplemental Figure 1C and 1D, hereafter referred shMOB3). We subsequently used this construct to generate tetracycline-inducible hMOB3 knockdown U373MG cells. Strikingly, etoposide induced MST1 cleavage was higher after tetracycline-induced hMOB3 knockdown than in control cells without tetracycline treatment (Figure 4E). We observed a similar phenotype in three additional clones (Supplemental Figure 3A). To examine further whether hMOB3 attenuates apoptotic cleavage of MST1, we also generated stable knockdown cells in the U87MG and LN229 lines. Similar enhanced cleavage of MST1 and apoptosis was observed in stable hMOB3 knockdown U87MG and LN229 cells compared with U87MG_shLacZ or LN229_shLuc control cells, respectively

(Figure 4G and Supplemental Figure 3B). Moreover, Annexin V staining revealed an increase in apoptosis of approximately 1.8 fold and 1.6 fold in hMOB3 depleted U373MG and U87MG cells, respectively (Figure 4F and Figure 4H). Collectively, these data further support the notion that hMOB3 negatively regulates apoptotic cleavage of MST1 in GBM cells.

To examine whether there is any correlation between hMOB3 protein levels and the apoptotic response in GBM cell lines, we treated ten glioma derived cell lines with etoposide for 24 hours before measuring MST1 cleavage and apoptosis markers (Supplemental Figure 3C). Based on their sensitivity to etoposide, we performed a Pearson correlation test and found a correlation coefficient of -0.6 between hMOB3 protein and cleaved MST1, indicative of a moderate negative correlation. Taken together, our data suggest that hMOB3 significantly contributes to modulate the sensitivity of GBM cells to the chemotherapeutic agent etoposide.

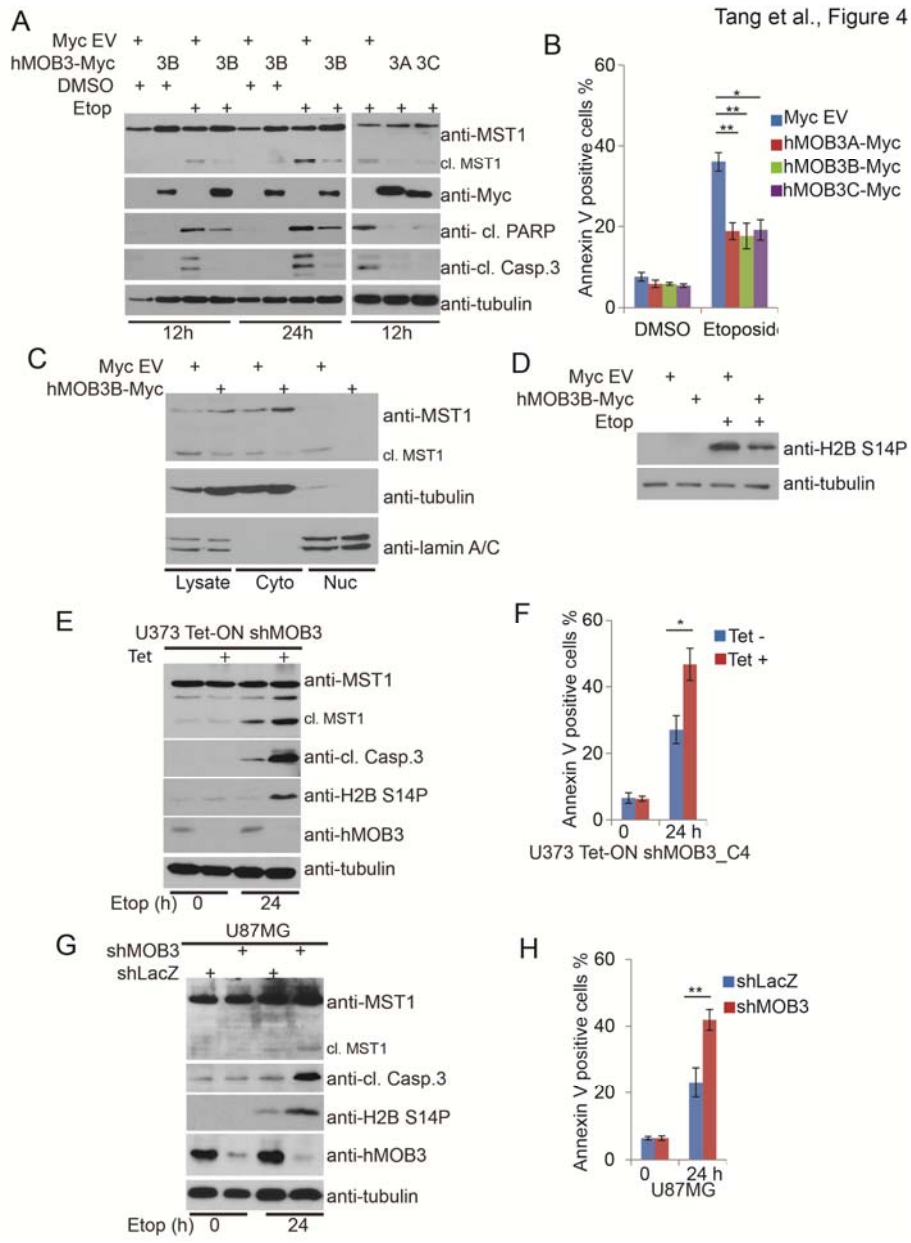
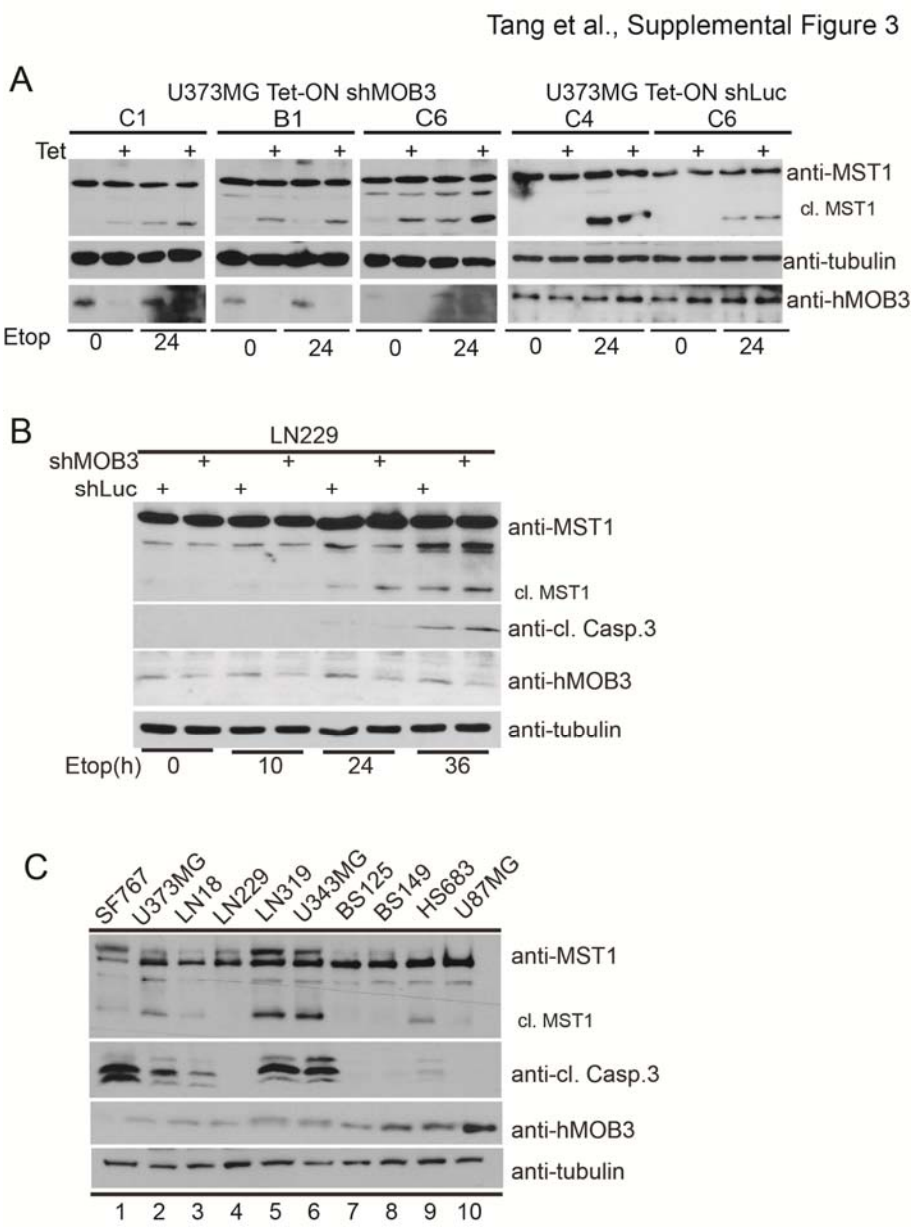


Figure 4. hMOB3 inhibits apoptotic MST1 cleavage. (A) U373MG cells stably expressing Myc-empty-vector (Myc EV) or Myc-tagged hMOB3A/B/C were treated with DMSO or etoposide (Etop, 100 μM) for 12 or 24 hours. Treated cells were harvested for immunoblotting. (B) In parallel, apoptosis after a 24 hours treatment was analyzed by FACS-based Annexin V staining. (C) U373MG cells overexpressing either empty vector (EV) or Myc-tagged hMOB3 (hMOB3-Myc) were treated for 24 hours with etoposide (Etop, 100 μM), fractionated (lysate: total lysate; Cyto: cytoplasma; Nuc: nucleus), and processed for immunoblotting. (D) In parallel, pretreated and treated U373MG cell total lysates were analyzed by immunoblotting. (E) U373MG clone C4 cells stably expressing shRNA targeting all three hMOB3 isoforms in a tetracycline-inducible manner (U373MG Tet-ON shMOB3 C4) were treated with etoposide (Etop,100 μM) in the presence or absence of tetracycline (left panel). The untreated and 24 hours

treated cells were harvested for immunoblotting. (F) In parallel, apoptosis before and after 24 hours etoposide treatment was analyzed by FACS-based Annexin V staining. (G) Pooled U87MG cells constitutively and stably expressing shRNA targeting *E.coli* LacZ or all three hMOB3 isoforms were treated with etoposide (Etop, 200 μM) (right panel). The untreated and 24 hours treated cells were harvested for immunoblotting. (H) In parallel, apoptosis before and after 24 hours etoposide treatment was analyzed by FACS based Annexin V staining.



Supplemental Figure 3. hMOB3 negatively regulates apoptotic MST1 cleavage. (A) Western blotting analysis of the effect of knockdown of hMOB3 on MST1 cleavage. Different U373MG Tet-ON shMOB3 or shLuc control clones were treated with 100μM etoposide (Etop) for 24

hours. Pretreated and treated lysates were analyzed by immunoblotting. (B) Effect of hMOB3 knockdown in LN229 cells. LN229 shMOB3 or shLuc control cells were treated with 100μM etoposide (Etop) for the indicated times. The cells were harvested and lysed for immunoblotting. (C) Analysis of the response of in-house glioma cell lines to etoposide. The indicated cell lines were treated with 100μM etoposide for 24 hours, harvested, and lysed for immunoblotting.

hMOB3 binding to MST1 is essential for hMOB3-mediated MST1 regulation

We next examined the activity of hMOB3 on MST1 using MST1 binding-deficient hMOB3 mutants. Since hMOB1 phosphorylation on Thr_{12} and Thr_{35} by MST1 is needed for MST1/hMOB1/LATS complex formation (11), we mutated Thr12 and Ser38 to alanines and tested the interaction with MST1 (Figure 5A). Surprisingly, we found that these two residues, which are conserved between hMOB3 and hMOB1 (34, 35), are dispensable for the hMOB3/MST1 interaction (Figure 5A). Therefore, based on the structure of hMOB1A (45), we generated a series of mutations in residues potentially critical for this interaction. Significantly, we found that Arg_{108}/Lys_{109} in hMOB3A, which are conserved among hMOB3A/B/C (Lys_{107}/Lys_{108} and Arg_{107}/Arg_{108} for hMOB3B and hMOB3C, respectively), are critical residues, since hMOB3/MST1 complex formation was completely abolished by mutating both residues to glutamic acid (Figure 5A). We used the $R_{108}K_{109}$EE mutant to generate U373MG cells stably expressing binding-deficient mutant hMOB3A (Figure 5B). Cleavage of MST1 and apoptotic responses were analyzed in control, hMOB3A wild-type and hMOB3A binding deficient cells upon etoposide treatment. Significantly, MST1 cleavage and caspase3 activation were reduced in wild-type hMOB3A overexpressing cells but not in cells overexpressing the MST1 binding-deficient mutant (Figure 5B). Quantification of Annexin V positive cells confirmed a decrease in apoptosis of 48% in U373MG cells overexpressing wild-type hMOB3, while cells expressing the

MST1 binding deficient mutant were affected by etoposide to a similar level as controls (Figure 5C).

Furthermore, we rescued the U87MG hMOB3 knock-down cells with wild type or MST1 binding mutant hMOB3A and analyzed their apoptotic response to etoposide (Figure 5D-E). Indeed, apoptotic response (Figure 5D-E) was only rescued by wild type hMOB3A but not the binding deficient mutant.

Taken together, our results strongly suggest that hMOB3 restricts MST1 apoptotic signaling via a direct physical interaction.

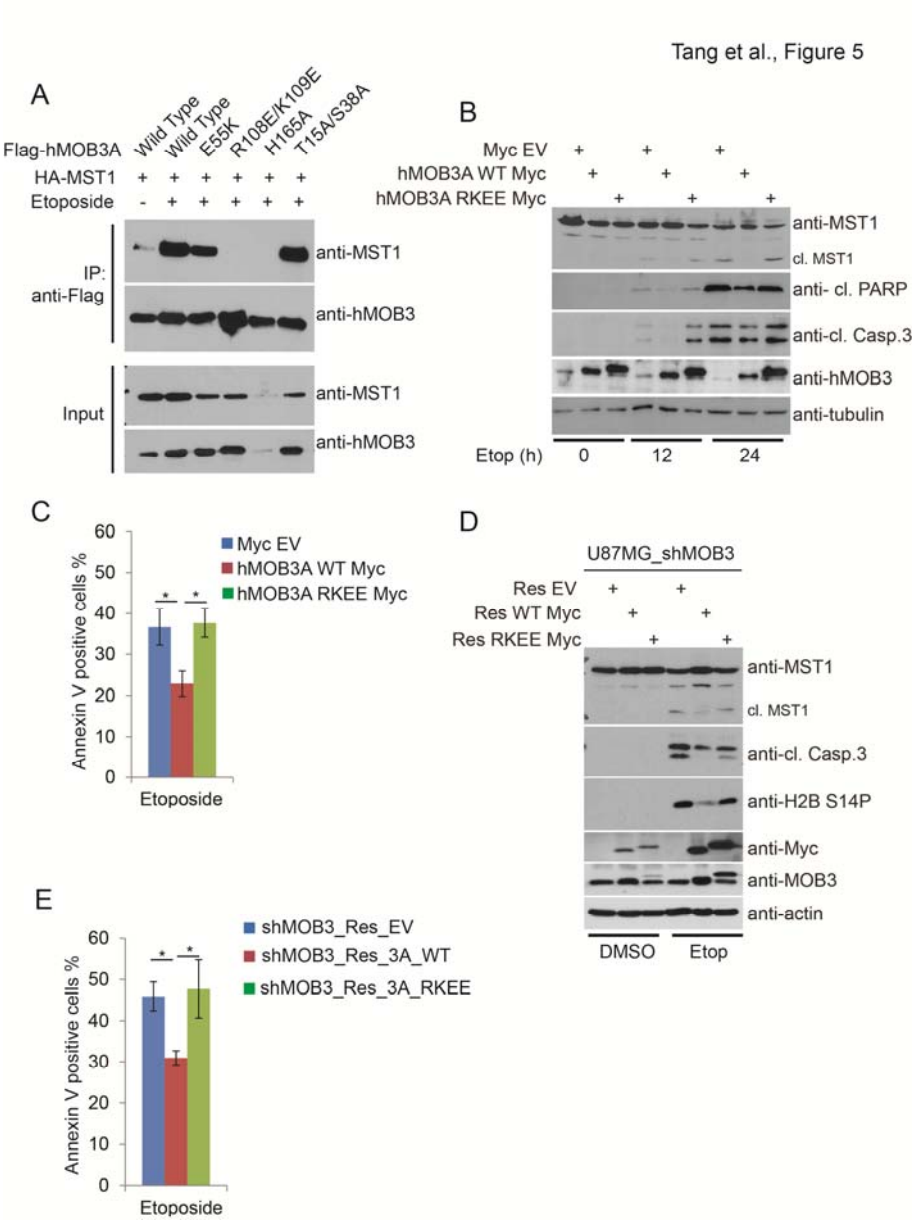


Figure 5. Binding capacity of hMOB3 is essential for its inhibitory effect on apoptotic MST1 cleavage. (A) Generation and characterization of an MST1 binding-deficient mutant. Glu_{55}, $Arg_{108}Lys_{109}$, His_{165} and Thr_{15}/Ser_{38} were mutated to Lys_{55}, $Glu_{108}Glu_{109}$, Ala_{165} and Ala_{15}/Ala_{38}, respectively. Lysates of etoposide-treated or untreated HEK293 cells containing the indicated combinations of HA-tagged full-length wild-type MST1 and Flag-tagged hMOB3A forms were analyzed by immunoprecipitation (IP). Complexes and input lysates were analyzed by immunoblotting. (B) U373MG cells stably expressing the Myc-empty-vector (Myc EV) or Myc-tagged wild-type hMOB3A or the Myc-tagged $R_{108}E/K_{109}E$ (RKEE) mutant were treated with etoposide (Etop, 100μM) for 12 or 24 hours. Treated cells were harvested for immunoblotting. (C) In parallel, apoptosis after 24 hours treatment was analyzed by FACS-based Annexin V staining.(D) U87MG_shMOB3 cells stably expressing the Myc-empty-vector (Myc EV) or Myc-tagged wild-type rescue hMOB3A or the Myc-tagged rescue $R_{108}E/K_{109}E$ (RKEE)

mutant were treated with etoposide (Etop, 200μM) for 24 hours. Treated cells were harvested for immunoblotting. (E) In parallel, apoptosis after 24 hours treatment was analyzed by FACS-based Annexin V staining.

hMOB3 sustains cancer cell proliferation and tumor growth

We found that hMOB3 protein levels are increased in human GBM samples (Figure 1A and 1B) and hMOB3 interacts with MST1 under high cell density (Figure 2D and 2E). To investigate whether hMOB3 contributes to cancer cell proliferation *in vitro* and tumorigenesis *in vivo*, we analyzed cell proliferation upon tetracycline-inducible hMOB3 knockdown in U373MG cells. Knockdown of hMOB3 did not alter cell proliferation at low density up to day 2. However, from day 4 onwards, when cells started to form cell-cell contacts, knockdown of hMOB3 progressively led to a drop in cell proliferation, with a maximum of 32% reduction at day 8 (Figure 6A). Cell viability measurements indicated that the reduction in cell number was not due to apoptosis (data not shown). End-point MTT assays indicated a decline in cell growth of approximately 40% (Figure 6B). We next investigated the involvement of hMOB3 in tumorigenesis *in vivo* using a mouse xenograft model. As our standard U373MG flank tumor model did not yield tumors after 6 months (data not shown), we established a flank tumor model using constitutively hMOB3 depleted U87MG cells as defined in Figure 4G. While the shLacZ control cells started to form palpable tumors after approximately 8 weeks, MOB3 knockdown cells showed negligible growth *in vivo* up to 14 weeks (Figure 6C). Tumor weight analysis at week 15 further confirmed that hMOB3 is essential for tumor growth (Figure 6D). Collectively, our U87MG cell based flank tumor model suggests that hMOB3 is required for GBM cell growth *in vivo*. In summary, our findings suggest that hMOB3 supports tumor cell growth *in vitro* and *in vivo*.

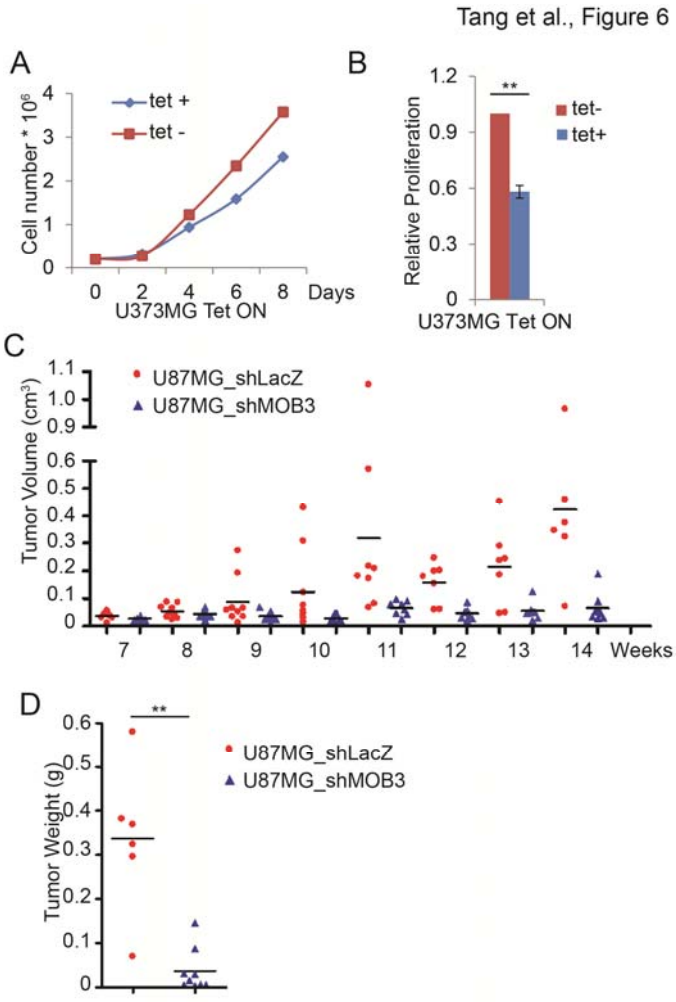


Figure 6. hMOB3 sustains tumor cell growth *in vitro* and *in vivo*. (A) Growth curve of pooled U373MG Tet_ON shMOB3 cells (C1/C4/C6) in the absence or presence of tetracycline. Depletion of hMOB3 was achieved by addition of 2 μg/ml tetracycline for 96 h. (B) End-point MTT analysis of U373MG Tet_ON shMOB3 cells in the absence or presence of tetracycline. (C) Growth curves of flank tumors derived from constitutively hMOB3-depleted U87MG cells (U87MG_shMOB3) versus U87MG_shLacZ cells *in vivo*. Tumor volumes (cm^3) were measured and plotted. (D) Tumors weight (g) derived from U87MG_shLacZ and U87MG_shMOB3 cells were measured and plotted.

Discussion

Genetic profiling has greatly advanced our understanding of molecular mechanisms underlying tumorigenesis and drug resistance. Based on microarray analysis of malignant gliomas, we

recently identified Mnk1 and MerTK as potential therapeutic targets for the treatment of GBM (41, 42). In the present study, we have discovered that the previously uncharacterized adaptor protein hMOB3A/B/C is deregulated in GBM (Figure 1). Of note, we found that hMOB3A and hMOB3C were increased at the mRNA level (Supplemental figure 1A). Consistent with our findings, data obtained from the Rembrandt database also showed upregulation of hMOB3A and hMOB3C in human GBM samples (Figure 1D); conversely, hMOB3B appears to be downregulated. The chromosome locus 9p21, on which the hMOB3B and IFNK genes are located, is frequently deleted (52%) or epigenetically inactivated in GBM (46-48), which might explain the varying mRNA levels of hMOB3B in our array and the publicly available Rembrandt and TCGA datasets. Importantly, we found that total hMOB3 protein levels were prominently upregulated in human GBM samples (Figure 1A, 1B and 1C), suggesting possible oncogenic properties of hMOB3 protein in GBM. Consistently, hMOB3A/C upregulation correlated with unfavorable clinical outcomes in the Rembrandt database, also pointing towards a potential proto-oncogenic function of hMOB3. In an attempt to reconcile the converse de-regulation of the respective hMOB3 isoforms in GBM, we found that knockdown of hMOB3B resulted in an upregulation of total hMOB3 protein levels (Supplemental Figure 1D). This suggests that, in response to inactivation of hMOB3B, compensatory upregulation of hMOB3A and C might provide GBM cells with increased oncogenic potential. However, the basis of the compensation mechanisms is currently unknown and requires further analysis.

While the role of hMOB1 protein has already been studied in tissue cultured cells and recently confirmed in a knock-out mouse model (36, 38, 39), the function of hMOB3 protein remained unknown. In a previous study we found that hMOB3 does not interact with NDR or LATS (34), but did not address MST1 as a potential interaction partner. As hMOB1 interacts with hMOB1,

493 we asked whether hMOB3 might do so as well. Indeed, we observed that all three hMOB3
494 isoforms directly interact with mammalian Hippo, MST1 (Figure 2). Importantly, the
495 hMOB3/MST1 interaction is induced by apoptosis and high cell density. Moreover, MST1
496 kinase activity is required for the interaction (Figure 3B, 3D and 3E). MST1 kinase activity is
497 regulated by various mechanisms (16), importantly by binding to SAV, RASSAF or to itself via
498 the SARAH domain . Interestingly, the SARAH domain of MST1 is dispensable for hMOB3
499 binding (Figure 3B and 3C), suggesting a different mode of their interaction rather than classical
500 SARAH mediated signaling integration.

501 MST1 is a well-characterized pro-apoptotic kinase that potentiates apoptosis by cross-talking
502 with caspases and regulating downstream substrates (18, 19). Significantly, our observations
503 suggest that hMOB3 regulates the cleavage process of MST1, which potentiates the apoptotic
504 activity of the kinase (19). Overexpression of hMOB3 restricts MST1 cleavage and protects
505 against the induction of apoptosis. Conversely, depletion of hMOB3 in GBM cell lines reduces
506 MST1 cleavage and sensitizes cells to apoptosis induction (Figure 4). Importantly, we
507 demonstrate that the protective effect of hMOB3 critically depends on its direct binding to MST1,
508 since MST1-binding deficient mutant did not protect against apoptosis induction (Figure 5). This
509 anti-apoptotic property of hMOB3 is likely to confer a considerable survival benefit to brain
510 tumor cells, particularly if exposed to chemotherapeutic agents such as etoposide. A
511 comprehensive recent meta-analysis revealed that etoposide treatment significantly improves
512 overall survival in high grade gliomas (49). Our data suggest that concomitantly blocking the
513 interaction between hMOB3 and MST1 might further increase the therapeutic benefit of
514 etoposide.

Tumor cells tend to have a proliferative advantage over the surrounding non-neoplastic cells. We also find that hMOB3 significantly contributes to the proliferation rate of GBM cells (Figure 6A and 6B), further supporting its potential role as an onco-protein. Importantly, we confirm this observation in a xenograft model *in vivo* where depletion of hMOB3 suppresses tumor growth (Figure 6C and 6D).

Taken together, our results characterize hMOB3 as a potential biomarker with clinical prognostic value for GBM. Negative regulation of the apoptotic cleavage of MST1 signaling by hMOB3 improved cellular survival in response to the standard chemotherapeutic agent etoposide. In our study, targeting hMOB3 sensitized GBM cells to etoposide and blocked tumor cell growth. Thus, hMOB3 manipulation warrants further in depth analysis as it may represent a promising therapeutic strategy to target GBM.

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662

663 **Supplementary Information**

664 **Supplementary Materials and Methods**

665 **Antibodies.** Anti-myc 9E10, anti-HA 12CA5 and 42F13, and anti-α-tubulin YL1/2 were used as
666 hybridoma supernatants. Anti-HA antibody (Y-11) and anti-β-actin were purchased from Santa

Cruz (CA, USA), and anti-Fas (CH-11) from Millipore (Zug, Switzerland). Anti-MST1, anti-phospho-MST1/2, anti-cleaved caspase3, and anti-phospho-H2B Ser14 antibodies were purchased from Cell Signaling (Beverly, MA, USA) and anti-cleaved poly(ADP-ribose) polymerase (PARP) from BD Bioscience (San Jose, CA, USA). Anti-Flag antibody (M2) was obtained from Sigma. Anti-hMOB3 antibody was raised against full length MBP-tagged hMOB3A and affinity purified.

Construction of plasmids. hMOB1, hMOB2, hMOB3A/BC, MST1 constructs have been described previously (34, 39). Mutants of MST1 and hMOB3 were generated by site-directed mutagenesis according to the manufacturer's instructions (Stratagene, La Jolla, CA, USA). Truncation mutants of MST1 were cloned via PCR with BamH1 and XhoI sites. To generate an shRNA vector that simultaneously targets all three hMOB3A/B/C isoforms, termed pTERshMOB3, two independent oligonucleotide pairs, one targeting hMOB3A/C and the other hMOB3B, were cloned stepwise into the pTER vector as described before (38). The targeting sequences used were: 5′- GCTACAAGCACTTCTACTA -3′ for hMOB3A/C, 5′-CAGGATGATCTCAAGTATA -3′, 5′- GAGGTTTGAGCTGCACAAA -3′ for hMOB3B, and 5′- GACTACACAAATCAGCGATT -3′ for LacZ. Generation of the pTERshLuc control vector has been described previously (39). All constructs were confirmed by sequence analysis.

Generation of stable cell lines. To generate inducible U373MG cell lines, U373MG were transfected with pCDNA6/TR (Invitrogen) and pTERshMOB3. Cells were selected in the presence of 100 ug/ml blasticidine (Invivogen, Toulouse, France) and 400 μg/ml Zeocin (Invivogen). Induction of shMOB3 was achieved by addition of 2 μg/ml tetracycline for 96 h. To generate stable knockdown cell lines, LN229 and U87MG cells were transfected with the

pTERshMOB3 construct. Cells were selected in the presence of 400 µg/ml zeocin (LN229_shMOB3 and U87MG_shMOB3).

Immunoprecipitation, pull-down and fractionation of cells. Immunoprecipitation experiments were performed as described previously (34). Pull-down assays were performed in pull-down buffer (20 mM HEPES, 50 mM β-glycerophosphate, 100 mM NaCl, 10 mM NaF, 1 mM Na3VO4, 0.5% TX-100 at pH 7.4) supplemented with Roche protease inhibitor cocktail (Basel, Switzerland). Cytosolic and nuclear proteins were separated as described previously (34).

Immunohistochemistry. Staining was performed with the Discovery XT machine (Ventana Medical Systems Inc., Tucson, Egg/ZH, Switzerland, AZ, USA). Haematoxylin-counterstained sections were photographed (Nikon, YTHM) and analyzed using ImageAccess Enterprise7 software (Glattbrugg, Switzerland).

Statistical analysis and Bioinformatics. ***Figure 1E***-Survival analysis and differences in expression values were analyzed with help of the R/Bioconductor Survival analysis is based on the preprocessed GBM Deep-Sequencing datasets from TCGA (TCGA GBM HiSeqV2 version:2013-04-22). Correction for batch effects could be excluded, following the suggestions stated in the documentation of the 'TCGA Batch Effects Tool' (http://bioinformatics.mdanderson.org/main/TCGABatchEffects:Overview). Samples with missing information were excluded from the datasets. The datasets do not include 'normal tissue controls'. Hence, the provided preprocessed expression values of the hMOB3-genes were each normalized to the overall median of the cognate's isoforms expression values in all samples. Samples with expression values greater than or equal to the upper quartile were considered upregulated, such with expression values lesser than or equal to the lower quartile were

711 considered downregulated. Kaplan-Meier estimates were calculated based on the samples
712 displaying up- or downregulation. Curves were compared applying a Peto and Peto modified
713 Gehan-Wilcoxon test (http://CRAN.R-project.org/package=survival).

714 ***mRNA expression analysis(supplemental figure 1A).*** Preprocessed expression values where
715 derived as described in (41). Statistical significance of the differences in expression values
716 between samples was tested by Welch Two Sample t-tests.

717

4. General discussion

Ever since the discovery of the Hippo pathway, enormous effort in Hippo research is to identify its regulators. Human MOB proteins are small essential adaptor protein bridging NDR/LATS kinases to the upstream MST kinases. Previous studies from the lab demonstrated hMOB1 recruits NDR kinases to the membrane for activation and plays vital roles in the MST/hMOB1/NDR signaling complex (145). The hMOB2 was characterized as a NDR kinase specific negative regulator by competing the activation of NDR kinase by hMOB1 (140). Although hMOB3 shares higher amino acid identity (50%) with hMOB1 than hMOB2 (37%), hMOB3 was found to not interact with or (de)activate NDR/LATS. Thus the biochemical function of hMOB3 remains unknown (145).

This thesis was aiming to investigate the function of uncharacterized human MOB3 proteins in the context of the Hippo pathway. To investigate the involvement of hMOB3 in the Hippo pathway, in the present study, we analyzed the protein-protein interaction of hMOB3 to the upstream MST kinases. Interestingly, we did not observe any interaction between hMOB3 with MST1 under normal low cell density tissue culture condition. But we found an induced binding of hMOB3 and MST1 upon apoptotic stimulations or under cell-cell contact stress. We next investigated the functional interplay between hMOB3 and MST1 focusing on the proteolytic process of MST1 based on the domains of MST1 essential for binding to hMOB3 and found that hMOB3 negatively regulates caspase mediated MST1 cleavage.

Furthermore, we extended our investigation to analyze the potential pathological roles of hMOB3. A previous glioblastoma transcriptome screen carried out in the lab indicated all three hMOB3 isoforms were deregulated. Therefore, we focused on examining the pathological role of

hMOB3 in GBM. Indeed, we observed a correlation of worse survival and hMOB3A/C high expression group from public available Rembrandt database. Interestingly, the opposite correlation, meaning unfavorable survival was explored with hMOB3B low expression, from both Rembrandt and TCGA databases. A further analysis of hMOB3 isoforms suggested a compensatory mechanism between all three isoforms at the post-transcriptional level. Single knock-down of the most variable hMOB3B resulted in an up-regulation of total hMOB3 at protein level. Thus, our observations indicated that hMOB3 protein has certain pro-oncogenic functions in GBM. Indeed, from the GBM tumor lysates we observed a highly up-regulation of hMOB3 total protein compared with non-neoplastic brain. Immunohistochemistry staining further confirmed approximately 70% GBM tumor with moderate or high hMOB3 expression. The oncogenic function of hMOB3 was further validated by a U87MG derived flank tumor by showing that depletion of hMOB3 delays the flank tumor onset.

Alternations in the expression profile of hMOB3 members have also been reported in colon cancer (196), mantle cell lymphoma (197, 198) and amyotrophic lateral sclerosis (ALS) (199, 200). Importantly, hMOB3B is found to be down-regulated via chromosome locus deletion in all four diseases. However, whether deletion of hMOB3B locus is instructive or permissive to the disease onset is not fully known yet. Additionally, because of the general pattern of down-regulation of hMOB3B and decreased hMOB3B correlated with inferior survival in colon cancer, mantle cell lymphoma and GBM, one would speculate specific function of hMOB3B out of hMOB3A/B/C family members. However, single knock down hMOB3B in HEK and glioblastoma cell lines resulted in an increased of total hMOB3 protein, suggesting compensatory mechanisms of hMOB3A/C and hMOB3B. Therefore, it appears that depletion of hMOB3B results in the upregulation of hMOB3A/C protein, which indicates that low levels of

hMOB3B might result in high hMOB3A/C protein levels. This might explain the observed upregulation of total hMOB3 protein in GBM. Given an approximately 80% sequence identity across the whole protein but not specific domains, one would speculate that hMOB3A/B/C function redundantly at the protein levels. Indeed, our result demonstrated that hMOB3A/B/C protein functions redundantly and all three isoforms could impair the apoptotic cleavage of MST1 in glioblastomas. Hence, it is critical to evaluate the total hMOB3 at the protein level in the reported human tumors with hMOB3B downregulation to further clarify the expression of total hMOB3 at protein level.

hMOB3 is found to regulate the cleavage of MST1 by direct protein- protein interaction under apoptosis in glioblastomas. Importantly, hMOB3 could also bind to MST1 at high cell density. We have investigated the expression pattern of mMOB3 in different mouse tissues. Significantly, mMOB3 proteins are highly abundant in spleen, thymus, lymph node and colon, but expressed at very low level in liver and small intestine (data unpublished). Interestingly, the cleavage of MST1 in liver and small intestine is significantly higher than other organs. The inverse correlation in mice tissues fits our model in glioblastomas that MOB3 modulates the cleavage of MST1.

MST kinases have been shown to play a key role in hepatocyte hemostasis in mouse. Ablation of MST kinases in mouse liver results in hyperproliferation of hepatocytes and the progression of healthy liver to Hepatocellular Carcinoma (HCC) within 14 weeks. Interestingly, the majority of MST1 is cleaved in normal hepatocytes, proposing that cleaved MST1 might be a critical regulator of the quiescence of hepatocytes (114). Indeed, about 70% HCC samples show intact full-length MST1, suggesting that cleavage of MST1 is blocked in majority of HCC (114). These results indicate that de-regulation of MST1 cleavage might be a causer for the progression of

HCC (114, 201). Our results suggest hMOB3 might be a critical regulator of MST1 by inhibiting the cleavage of MST1 by direct protein-protein interaction. Therefore, it would be interesting to investigate the role of hMOB3 in liver cancer progression focusing on the cleavage process of MST1. A straightforward analysis would be to test the hMOB3 protein levels in paired adjacent non-neoplastic liver and HCC samples.

Recently, MST1 has been identified as a critical regulator of apoptotic beta cell death and function, which is strongly activated in beta cells under diabetogenic conditions and induces apoptosis by upregulation of BCL-2 homolog-3 (BH3)-only protein BIM (202). Moreover, active MST1 phosphorylates PDX1 at Threonine 11 and promotes the ubiquitination mediated degradation of PDX1, which results in impaired insulin secretion (202). This study suggests that manipulation of MST1 may serve as potential therapeutic approach for diabetes (202). Our hMOB3 study indicates hMOB3 interacts with MST1 and inhibits the apoptotic cleavage of MST1. Therefore, it would be interesting to characterize the role(s) of hMOB3 in the beta cells under diabetogenic stress.

Taken together, study of hMOB3 in the present thesis clearly suggests its biochemical role in regulating MST1 signaling and the pro-oncogenic roles of total hMOB3 proteins in glioblastomas. Manipulating hMOB3 sensitizes the glioma cells to chemotherapy drug induced apoptosis and blocks the tumorigenesis *in vivo*, pointing towards that hMOB3 might be a potential therapeutic target in GBM. Moreover, it is worth to extent the characterization the pathological roles of total hMOB3 proteins in colon cancer, liver cancer, mantle cell lymphomas, neurodegenerative diseases and metabolic disorders.

5. Reference

This section contains the references cited in the Introduction and the General Discussion parts.

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7. Curriculum vitae

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