

**Supplemental Fig. 1.** Binding analysis of MOB1B to peptide epitopes in the inter domain linker region of MST1. A, Sequence of (*top*) a Nud1 native phosphopeptide found to interact with yeast Mob1 or (*bottom*) a Nud1-like phosphopeptide optimized for yeast Mob1 binding that was co-crystalized with human MOB1A by Rock *et al.* (2013; *Science*, **340**:871–5). B, Schematic showing the relative positions of T353, T367, T380 and the SARAH domain in the C-terminal portion of MST1. C, 15-residue peptide spot array of MST1 probed with GST-MOB1B. Peptides containing pT353 and pT367 are indicated, as well as a non-phospho-dependent binding sequence shown previously to contribute to bipartite recognition (Ni *et al.*, 2015, *Genes Dev.*, **29**:1416–31).

## T353 peptide -2 to +2 Alanine walk



Supplemental Fig. 2. Binding analysis of MOB1B to single site variants of a MST1 T353 phosphopeptide. Positions P+1, P+2 P-1, and P-2 relative to the P0 pT353 site were substituted individually for each of the 20 natural occurring amino acids as indicated. Peptide spot arrays were tested for binding with GST-MOB1B in a Far-Western assay.

20 amino acid scan at +2 position

## T367 peptide +1 to +4 Alanine walk

20 amino aciu scan al 11 position	20 amino acid scan at +2 position
T L P S Q L G pT A V I N A E D   T L P S Q L G pT A V I N A E D   T L P S Q L G pT R V I N A E D   T L P S Q L G pT N V I N A E D   T L P S Q L G pT C V I N A E D   T L P S Q L G pT E V I N A E D   T L P S Q L G pT H V I N A E D   T L P S Q L <td>T L P S Q L G PT M A I N A E D   T L P S Q L G PT M R I N A E D   T L P S Q L G PT M N I N A E D   T L P S Q L G PT M D I N A E D   T L P S Q L G PT M D I N A E D   T L P S Q L G PT M Q I N A E D   T L P S Q L G PT M Q I N A E D   T L P S Q L</td>	T L P S Q L G PT M A I N A E D   T L P S Q L G PT M R I N A E D   T L P S Q L G PT M N I N A E D   T L P S Q L G PT M D I N A E D   T L P S Q L G PT M D I N A E D   T L P S Q L G PT M Q I N A E D   T L P S Q L G PT M Q I N A E D   T L P S Q L
T L P S Q L G pT T V I N A E D T L P S Q L G pT W V I N A E D T L P S Q L G pT V V I N A E D T L P S Q L G pT V V I N A E D T L P S Q L G pT V V I N A E D	$ \begin{array}{cccccccccccccccccccccccccccccccccccc$
20 amino acid scan at +3 position	20 amino acid scan at +4 position
20 amino acid scan at +3 position T L P S Q L G pT M V A N A E D T L P S Q L G pT M V R N A E D T L P S Q L G pT M V N N A E D T L P S Q L G pT M V D N A E D T L P S Q L G pT M V C N A E D T L P S Q L G pT M V C N A E D T L P S Q L G pT M V Q N A E D T L P S Q L G pT M V Q N A E D T L P S Q L G pT M V G N A E D T L P S Q L G pT M V E N A E D T L P S Q L G pT M V E N A E D T L P S Q L G pT M V G N A E D T L P S Q L G pT M V I N A E D	$\begin{array}{cccccccccccccccccccccccccccccccccccc$

Supplemental Fig. 3. Binding analysis of MOB1B to single site variants of a MST1 T367 phosphopeptide. Positions P+1, P+2, P+3, and P+4 relative to the P0 pT367 site were substituted individually for each of the 20 natural occurring amino acids as indicated. Peptide spot arrays were tested for binding with GST-MOB1B in a Far-Western assay.



**Supplemental Fig. 4.** Binding analysis of human and yeast MOB proteins to MST1 phosphopeptides and to an optimized Nud1-like phosphopeptide. A, Fluorescence polarization analysis of MOB1A binding to differentially phosphorylated MST1 15-mer peptides containing T329 and T340. B. Fluorescence polarization analysis of MOB1A binding to differentially phosphorylated MST1 15-mer peptides containing T380 and T387. C, Fluorescence polarization analysis of human MOB1A (HsMOB1A) and yeast Mob1 (ScMob1) binding to the MST1 phosphopeptide pT367. D, Fluorescence polarization analysis of human MOB1A (HsMOB1A) and yeast MOB1A) and yeast MOB1 (ScMob1) binding to the optimized Nud1-like phosphoserine containing peptide. Data is plotted as the mean ± SEM (n=3).

HsMOB1A HsMOB1B HsMOB2 HsMOB3A HsMOB3A HsMOB3C HsMOB4 DmMATS ScMOB1	GAMASFLFSSRSSKT F.KPKKNI   GAMASFLFGSRSSKT F.KPKKNI   GAMASFLFGSRSSKT F.KPKKNI   GAMASFLFGSRSSKT F.KPKKNI   GAMASFLFGSKSKA KPNGKK   GAMASSPFLKQVFNKDKT KPNGKK   GAMASSPFLKQVFNKDKT F.RPKRKF   GAMASIA GAMASSPFLKQVFNKDKT   GAMASSPFLKQVFNKDKT F.RPKRKF   GAMASIA GAMASIA   GAMASPFLKQVFNKDKT F.RPKRKF   GAMASIA GAMASIA   GAMASIA F.KPKKNI	22 22 25 24 24 33 22 41
HsMOB1A HsMOB1B HsMOB2 HsMOB3A HsMOB3C HsMOB4 DmMATS ScMOB1	a1 c0 a1   PEGSHQYELLKHAEATLGSGNLRQAVMLPEGEDLNEWIAVNTVDFF   PEGSHQYELLKHAEATLGSGNLRMAVMLPEGEDLNEWVAVNTVDFF   PAAEERKAYLEPEHTKARITDFQFKELVVLPREIDLNEWLASNTTTFF   EPGTQRFELHKKAQASLNAG.LDLRLAVQLPPGEDLNDWVAVHVVDFF   EPGTQRFELHKKAQASLNSG.VDLKAAVQLPSGEDQNDWVAVHVVDFF   EPGTQRFELYKKAQASLNSG.UDLRSVVRLPPGENIDDWIAVHVVDFF   DEMDSTLAVQQYIQQNIRADCSNIDKILEPPEGQDEGVWKYEHLRQFC   PEGTHQYDLMKHAAATLGSGNLRNAVALPDGEDLNEWVAVNTVDFF   TVTTHQ.DIKQIVEMTLGSE.GVLNQAVKLPRGEDENEWLAVHCVDFY   70 80	68 67 72 71 71 81 68 87
HsMOB1A HsMOB1B HsMOB2 HsMOB3A HsMOB3C HsMOB4 DmMATS ScMOB1	NQINMLYGTITEFCTEETCGIMSAGPRYEYHWADGLTVKKPIKCSAPK NQINMLYGTISEFCTGETCQTMAVCNTQ.YYWYDERGKKVKCSAPK HHINLQYSTISEFCTGETCQTMAVCNTQ.YYWYDERGKKVKCTAPQ NRVNLIYGTISDGCTEQSCPVMSGGPKYEYRWQDEHKFRKPTALSAPR NRINLIYGTICEFCTERTCPVMSGGPKYEYRWQDEHKFRKPTALSAPR NRINLIYGTMAERCSETSCPVMAGGPRYEYRWQDERQYRRPAKLSAPR LELNGLAVKLQSECHPDTCTQMTATEQWIFLCAAHKTPKECPAID NQINMLYGTITEFCTEETCGIMSAGPKYEYHWADGLTVKKPIKCSAPK 120 130 140 150 160	116 112 120 119 126 116 134
HsMOB1A HsMOB1B HsMOB2 HsMOB3A HsMOB3B HsMOB3C HsMOB4 DmMATS ScMOB1	C3 C4   YIDYLMTWVQDQLDDETLFPSKIGVPFPKNFMS.VAKTILKRLFRVYA   YIDYLMTWVQDQLDDETLFPSKIGVPFPKNFMS.VAKTILKRLFRVYA   YVDFVMSSVQKLVTDEDVFPTKYGREFPSSFES.LVRKICRHLFHVLA   YMDLLMDWIEAQINNEDLFPTNVGTPFPKNFLQ.TVRKILSRLFRVFV   YMNLLMDWIEQUNNEEIFPTCVGVPFPKNFLQ.ICMKILCRLFRVFV   YMALLMDWIEGLINDEEVFPTRVGVPFPKNFLQ.VCTKILTRLFRVFV   YMALLMDWIEGLINDEEVFPTRVGVPFPKNFLQ.VCTKILTRLFRVFV   YMALLMDWIEGLINDEEVFPTRVGVPFPKNFLQ.VCTKILTRLFRVFV   YMALLMDWIEGLINDEEVFPTRVGVPFPKNFLQ.VCTKILTRLFRVFV   YMALLMDWIEGLINDEEVFPTRVGVPFPKNFLQ.VCTKILTRLFRVFV   YMALLMDWIEGLINDEEVFPTRVGVPFPKNFV   YMALLMDWIEGLINDEEVFPTRVGVPFPKNFV   YMALLMDWIEGLINDEEVFPTRVGVPFPKNFV   YMALLMDWIEGLINDEEVFPTRVGVPFPKNFV   YMALLMDWIEGLINDEEVFPTRVGVPFPKNFV   YMALLMDWIEGLINDEEVFPTRVGVPFPKNFV   YMALLMDWIEGLINDEVFPTRVGVPFPKNFV   YMALLMDWIEGLINSNKYFPSRVSIK.   YVECLMRWCQDQLDDETLFPSKVTGTFPEGFIQRVIQPILRRLFVYA   YVECLMRWCQDQFDDESLFPSKVTGTFPEGFIQRVIQPILRRLFVYA   170 180	163 159 167 166 166 171 163 182
HsMOB1A HsMOB1B HsMOB2 HsMOB3A HsMOB3B HsMOB3C HsMOB4 DmMATS ScMOB1	a5 a6 a7   H I Y HQ H F D S V MQ L Q E E A H L N T S F K H F I F F V Q E F N L I D R R E L AP L Q E AP L Q E   H I Y HQ H F D P V I Q L Q E E A H L N T S F K H F I F F V Q E F N L I D R R E L AP L Q E AP L Q E   H I Y HQ H F D P V I Q L Q E E A H L N T S F K H F I F F V Q E F N L I D R R E L AP L Q E AP L Q E   H I Y WA H F K E T L A L E L H G H L N T L Y V H F I L F A R E F N L L D P K E T A T M D D AT M D D   H V Y I H H F D R I A Q M G S E A H V N T C Y K H F Y Y F V K E F G L I D T K E L E P L K E E P L K E   H V Y I H H F D R V I V M G A E A H V N T C Y K H F Y Y F V T E M N L I D R K E L E P L K E E P L K E   H V Y I H H F D S I L S M G A E A H V N T C Y K H F Y Y F V T E M N L I D R K E L E P L K E E P L R E   H Y Y I H H F D S I L S M G A E A H V N T C Y K H F Y Y F I R E F S L V D Q R E L E P L R E E P L R E   H A Y F H H R Q I F D E Y E N E T F L C H R . F T K F V M K Y N L M S K D N L I V P T L E E A P L Q E   H I Y H Q H F T E V V T L G E E A H L N T S F R H F C L F A Q E F N L I E R R E L AP L Q E A P L Q E   H I Y C H H F N E I L E L N L Q T V L N T S F R H F C L F A Q E F E L L R P A D F G P L L E AP L Q E   210 A I M S K I N L I V N T S F R H F C L F A Q E F E L L R P A D F G P L L E AP L Q E	209 209 213 212 212 212 216 209 228
HsMOB1A HsMOB1B HsMOB2 HsMOB3A HsMOB3A HsMOB3C HsMOB4 DmMATS ScMOB1	LIEKLGSKDR. 219   LIEKLTSKDR. 219   LTEVLCSGAGGVHSGGSGDGAGSGGPGAQNHVKER 240   MTARMCH. 220   MTSRMCH. 219   LTEVLOSGAGGVHSGGSGDGAGSGGPGAQNHVKER 240   MTSRMCH. 219   MTSRMCH. 219   MTERICH. 219   LIDKLTAKDERQI. 219   ZZ22 220	

1

10

20

★ P0 phosphate coordinating residues

• P+1, P+2 and P+3 contact residues

• secondary contact residues for a hydrophobic motif of bipartite interacting phosphopeptides

**Supplemental Fig. 5.** Structure-based sequence alignment of MOB proteins. *Homo sapiens* (*H.s.*) MOB1A (Q9H8S9-1), *H.s.* MOB1B (Q7L9L4-1), *H.s.* MOB2 (Q70IA6-1), *H.s.* MOB3A (Q96BX8-1), *H.s.* MOB3B (Q86TA1-1), *H.s.* MOB3C (Q70IA8-1), *H.s.* MOB4 (Q9Y3A3-1), Drosophila melanogaster (*D.m.*) MATS (Q95RA8-1) and *Saccharomyces cerevisiae* (*S.c.*) MOB1A (P40484-1) are shown. Contact residues are indicated as per inset.



**Supplemental Fig. 6.** Structural analysis of MOB1A binding to high-affinity phosphopeptide ligands from MST1. A, Ribbons diagram of the overall structure of MOB1A bound to a pT353-containing peptide from MST1. Inset: Zoom in surface representation of the molecular interactions governing the specificity of MOB1A phosphopeptide recognition. B, Superposition of complexes of MOB1A bound to a high-affinity pT353 peptide (*right*) and a high-affinity pT367 peptide (*left*) of MST1 with a complex of MOB1A bound to a sub-optimal phosphopeptide derived from yeast Nud1 (PDB: 4JIZ; Rock *et al.*, 2013; *Science*, **340**:871–5). C, Superposition of complexes of MOB1B bound to high-affinity pT353 peptide (*right*) and a high affinity pT367 peptide (*left*) of MST1 with a complex of MOB1B bound to high-affinity phosphopeptide of MST2 (orange; PDB: 5BRK; Ni *et al.*, 2015, *Genes Dev.*, **29**:1416–31). Note that the 31-residue MST2-MBM phosphopeptide is a suboptimal match to the MOB1B phosphopeptide binding consensus but compensates by forming additional bipartite contacts with the core domain of MOB1A through the short highlighted helical element.