

A

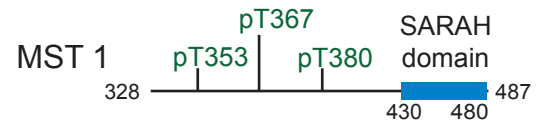
Nud1 native sequence:

T V L N N Y S pT<sup>78</sup> V H Q K V P S

Optimized sequence for yeast Mob1:

T V A R I Y H pS V V R Y A P S

B



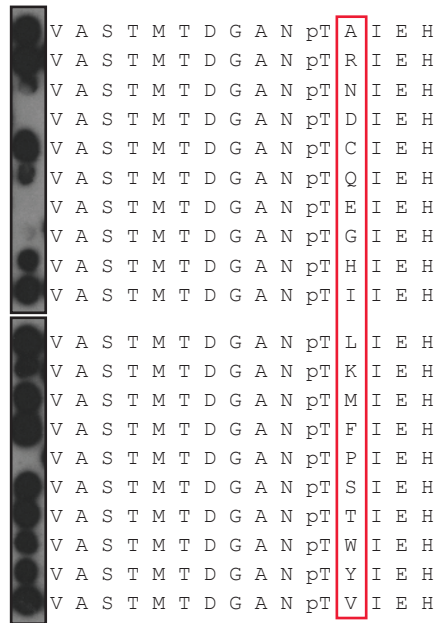
C

TVARIYHSVVRVAPS		
TVARIYHpSVVRVAPS	Nud1 control	
SEEDEMDSGMTVRVAV		
EDEMDSGMTVRVAVGD		
AVGDEMGTVRVASTM		
GTVRVASTMTDGANT		
TMIEHDDTLPSQLGT		
TLPSQLGTMVINAED		
EDEEEEGTMKRREDET		
TMKRREDETMQPAKPS		
AK <u>PSFLEY</u> FQEKEKE	non-phospho-dependent binding peptide	
EKENQINSFGKSVPG		
QINSFGKSVPGPLKN		
VPGPLKNSSDWKIPQ		
PGPLKNSSDWKIPQD		
KIPQDGDYEFKLSWT		
GDYEFKLSWTVEDLQ		
YEFKLSWTVEDLQKR		
AKPSFLEpYFEQKEKE		
EKENQINpSFGKSVPG		
QINSFGKpSVPGLKN		
VPGPLKNpSSDWKIPQ		
PGPLKNpSDWKIPQD		
KIPQDGDpYEFKLSWT		
GDYEFKpSWTVEDLQ		
YEFKLSWpTVEDLQKR		
GpTMVRAVGDEMGTVR		
GTMVRAVGDEMGpTVR		
GpTMVRAVGDEMGpTVR		
VApSTMTDGANTMIEH		
VASpTMTDGANTMIEH	Unphosphorylated T353	
VASTMpTDGANTMIEH		
VASTMTDGANpTMIEH	*pT353	
VApSpTMpTDGANpTMIEH		
DDpTLPSQLGTMVINA	Unphosphorylated T367	
DDTLpPpSQLGTMVINA		
KSVPGPLKNSSDWKI		
KSVPGPLKNSSDWKI		
KSVPGPLKNSSDWKI		
PQDGDYEFKLSWTVE		
PQDGDYEFKLSWTVE		
PQDGDYEFKLSWTVE		
PQDGDYEFKLSWTVE		
DLQKRLALDPMMEQ		
EIEEIRQKYQSKRQP		
ILDAIEAKRRQQNF		
space (empty)		
SEEDEMDpSGpTMVRAV		
EDEMDSGpTMVRAVGD		
AVGDEMGPpTVRVASTM		
GTVRVASpTMTDGANT		
TMIEHDDpTLPSQLGT		
TLPSQLGpTMVINAED	*pT367	
EDEEEEGpTMKRREDET		
TMKRREDEpTMQPAKPS	*pT387	
GTMVRAVGDEMGTVR		
GTMVRAVGDEMGTVR		
GTMVRAVGDEMGTVR		
VASTMTDGANTMIEH		
VASTMTDGANTMIEH		
VASTMTDGANTMIEH	Unphosphorylated T353	
VASTMTDGANTMIEH		
DDTLPSQLGTMVINA		
DDTLPSQLGTMVINA	Unphosphorylated T367	
DDTLPSQLGTMVINA		
EDEEEEGTMK		
RRDETMQPAK <u>PSFLE</u>	non-phospho-dependent bipartite binding peptide (partial)	
RRDETMQPAK <u>PpSFLE</u>		
RRDETMQPAK <u>PpSFLE</u>		
YFEQKEKENQINSFG		
KSVPGPLKNSSDWKI		
DDTLPSQLGpTMVINA	*pT367	
DDpTLpPpSQLGpTMVINA		
EDEEEEGpTMK		
RRDEpTMQPAK <u>PSFLE</u>	non-phospho-dependent bipartite binding peptide (partial)	
RRDETMQPAK <u>PpSFLE</u>		
RRDEpTMQPAK <u>PpSFLE</u>		
YFEQKEKENQINpSFG		
KpSVPGPLKNSSDWKI		
KSVPGPLKNpSSDWKI		
KSVPGPLKNpSSDWKI		
KpSVPGPLKNpSpSDWKI		
PQDGDYEFKpSWTVE		
PQDGDYEFKLSWpTVE		
PQDGDYEFKpSWpTVE		
DLQKRLALDPMMEQ		
EIEEIRQKYQpSKRQP		
ILDAIEAKRRQQNF		
space (empty)		

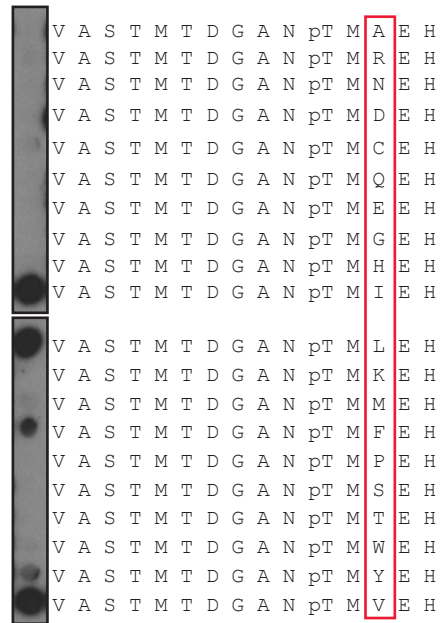
**Supplemental Fig. 1.** Binding analysis of MOB1B to peptide epitopes in the inter domain linker region of MST1. A, Sequence of (*top*) a Nud1 native phosphopeptide found to interact with yeast Mob1 or (*bottom*) a Nud1-like phosphopeptide optimized for yeast Mob1 binding that was co-crystallized with 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

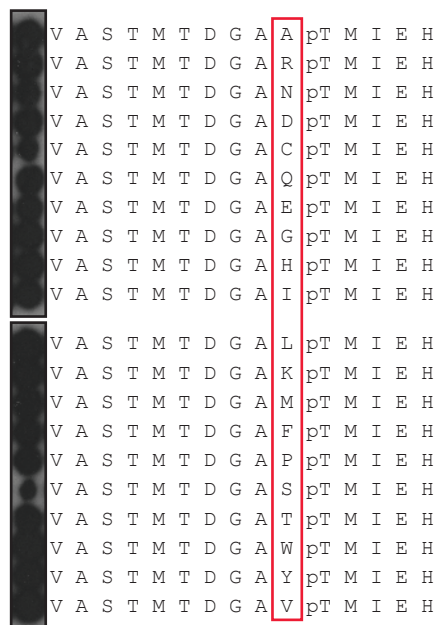
20 amino acid scan at +1 position



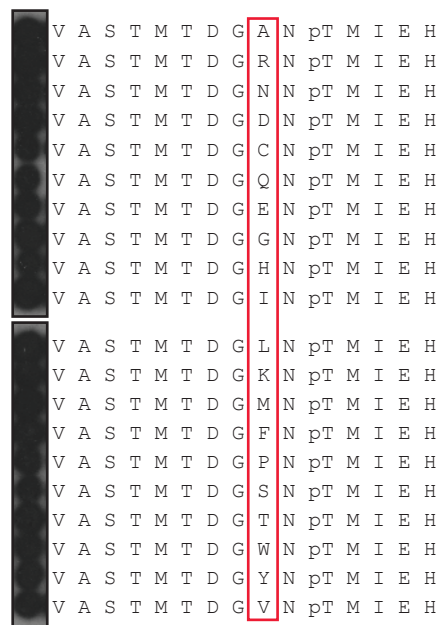
20 amino acid scan at +2 position



20 amino acid scan at -1 position



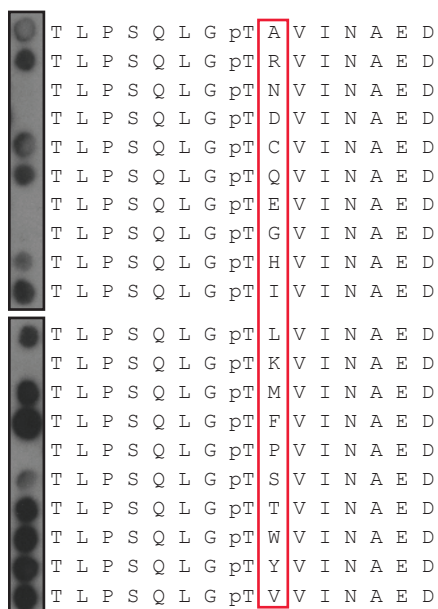
20 amino acid scan at -2 position



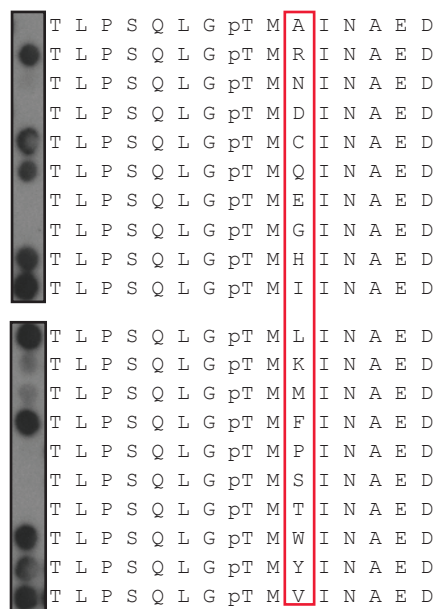
**Supplemental Fig. 2.** Binding analysis of MOB1B to single site variants of a MST1 T353 phosphopeptide. Positions P+1, P+2 P-1, and P-2 relative to the P0 pT353 site were substituted individually for each of the 20 natural occurring amino acids as indicated. Peptide spot arrays were tested for binding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

### T367 peptide +1 to +4 Alanine walk

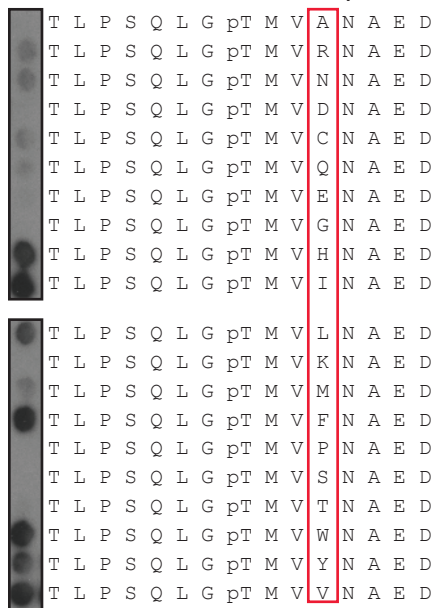
20 amino acid scan at +1 position



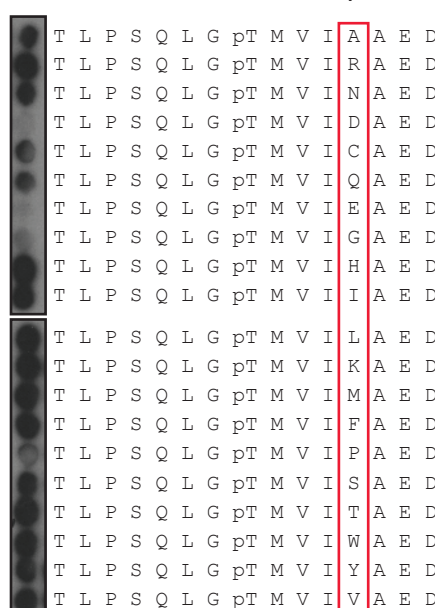
20 amino acid scan at +2 position



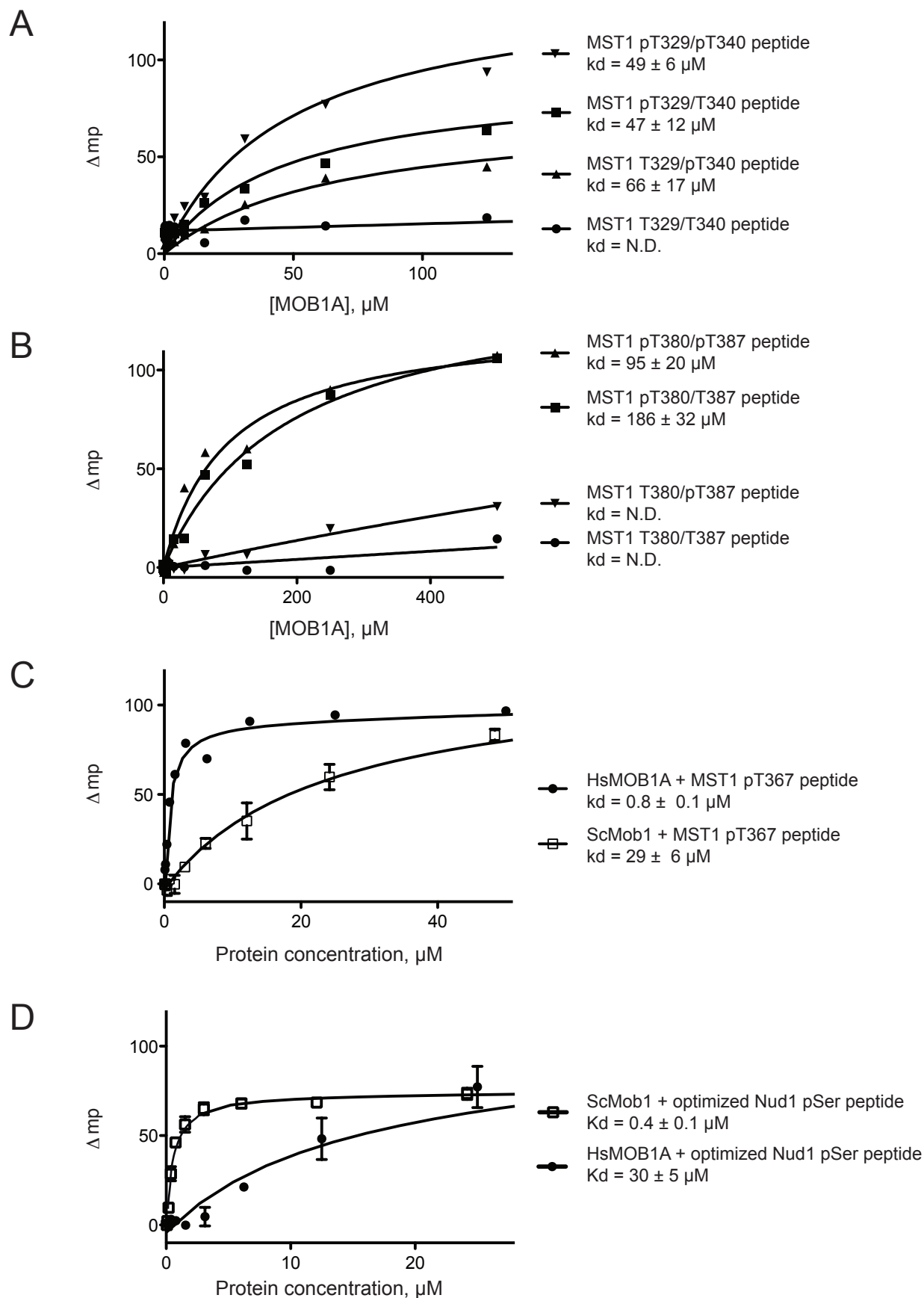
20 amino acid scan at +3 position



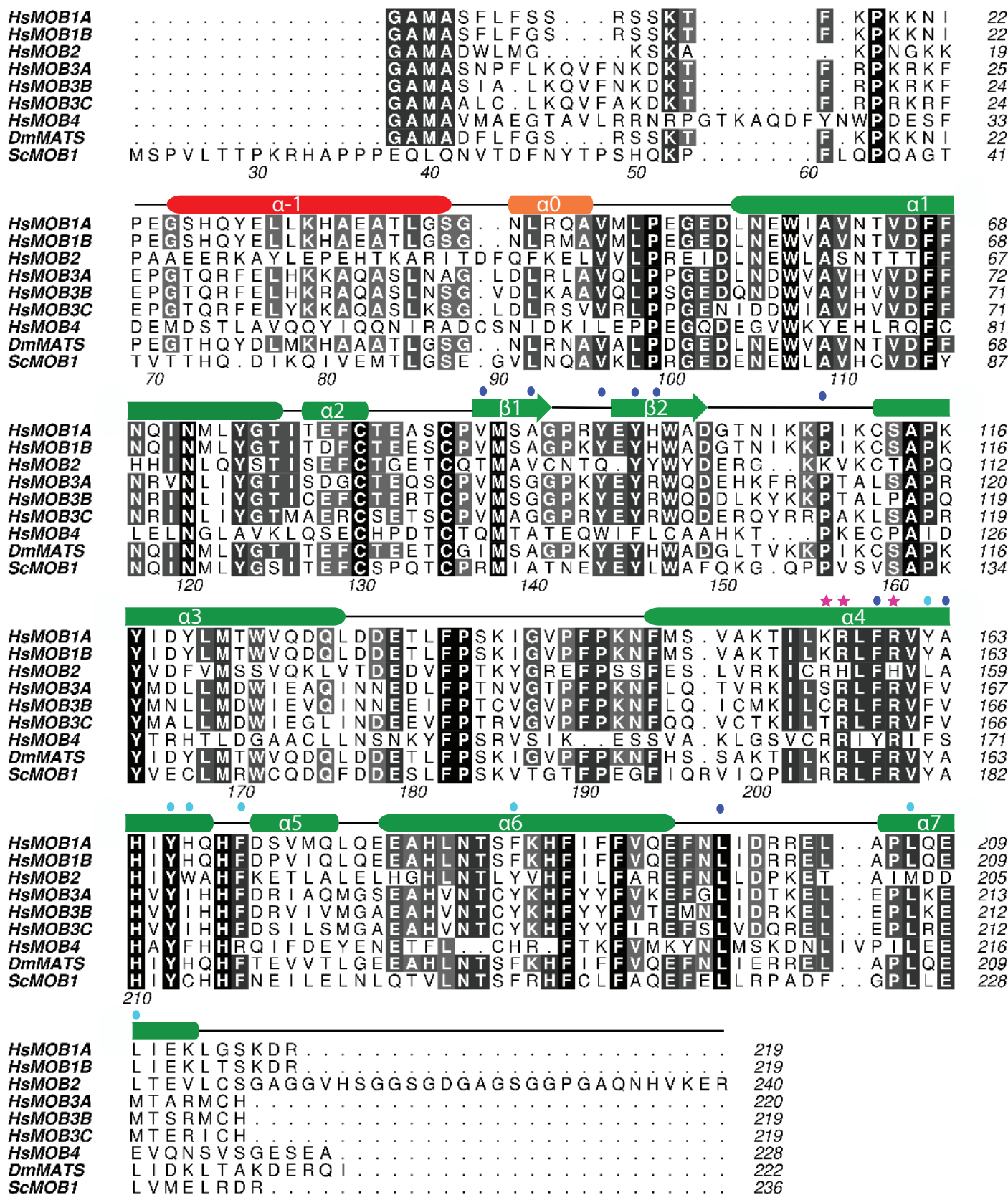
20 amino acid scan at +4 position



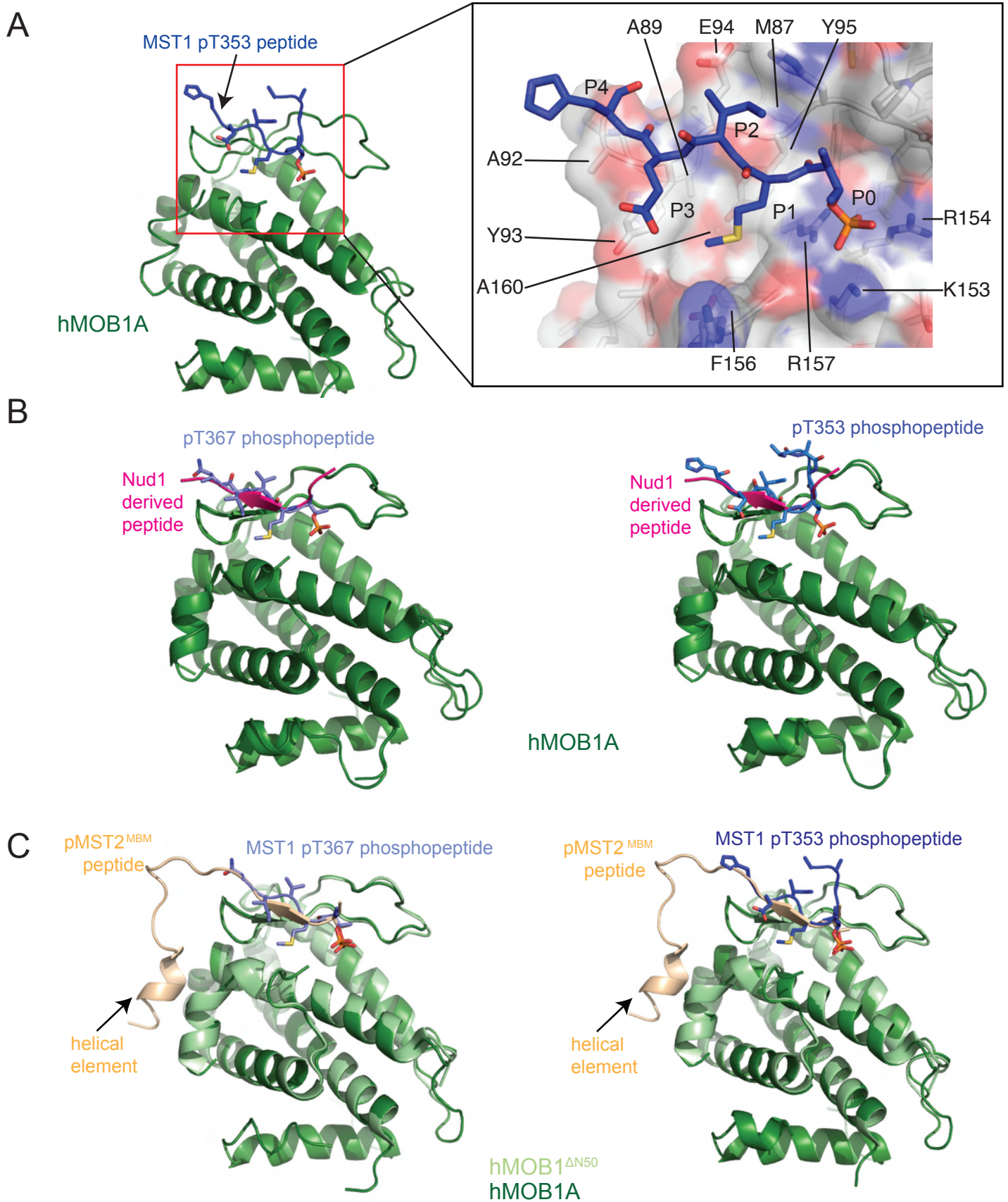
**Supplemental Fig. 3.** Binding analysis of MOB1B to single site variants of a MST1 T367 phosphopeptide. Positions P+1, P+2, P+3, and P+4 relative to the P0 pT367 site were substituted individually for each of the 20 natural occurring amino acids as indicated. Peptide spot arrays were tested for binding 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 MOB1 (ScMob1) binding to the optimized Nud1-like phosphoserine containing peptide. Data is plotted as the mean  $\pm$  SEM (n=3).



**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 MOB1A bound to a 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.