Supplementary Information Borek et al.



		Total		
		amino	Alignment	Alignment
	Accession	acid	starts at	ends at
Organism	number	residues	residue	residue
Schizosaccharomyces_pombe	NP_595204	397	1	397
Schizosaccharomyces_cryophilus	EPY50518	397	1	397
Schizosaccharomyces_octosporus	EPX71557	396	1	396
Schizosaccharomyces_japonicus	XP_002174423	463	1	437
Magnaporthe_grisea	XP_359559	1153	479	1074
Neurospora_crassa	XP_962857	1096	426	995
Gibberella_zeae	XP_387151	1170	503	1088
Penicillium_marneffei	XP_002144160	976	349	877
Coccidioides_immitis	XP_001248197	965	350	897
Paracoccidioides_brasiliensis	XP_002791015	975	317	875
Ajellomyces_capsulatus	XP_001538699	682	32	595
Penicillium chrysogenum	XP 002562312	789	134	689
Aspergillus nidulans	XP 662149	950	319	854
Aspergillus_clavatus	XP_001272930	1013	369	915
Aspergillus fumigatus	XP 749412	1008	353	908
Aspergillus terreus	XP 001218235	964	307	866
Aspergillus flavus	XP 002379344	964	321	871

	S pombe	S cryophilus	S octosporus	S japonicus	M grisea	N crassa	G zeae	P marneffei	C immitis	P brasiliensis	A capsulatus	P chrysogenum	A nidulans	A clavatus	A fumigatus	A terreus	A flavus
S pombe	100	40.76	38.21	27.93	17.99	20.18	21.24	20.6	22.77	21.74	20.28	20.85	20.66	22.25	23.93	23.36	21.5
S cryophilus	40.76	100	71.07	28.82	17.68	17.83	19.51	20.55	21.66	19.83	19.65	21.28	21.23	20.18	21.83	22.87	21.8
S octosporus	38.21	71.07	100	25.88	18.65	17.14	19.88	22.15	23.15	21.35	20.87	21.35	22.22	21.45	24.26	22.35	21.9
S japonicus	27.93	28.82	25.88	100	18.33	19.61	18.97	19.37	18.36	20.38	20.91	22.04	21.59	22.07	23.69	21.1	21.0
M grisea	17.99	17.68	18.65	18.33	100	44.93	46.35	28.95	28.23	32.74	29.55	28.92	29.07	29.04	30.18	29.88	31.0
N crassa	20.18	17.83	17.14	19.61	44.93	100	47.23	30.6	31.12	33.54	31.21	32.69	32.4	33.55	34.67	33.12	32.6
G zeae	21.24	19.51	19.88	18.97	46.35	47.23	100	31.33	32.26	32.39	32.14	32.71	32.78	32.51	33.47	32.12	31.8
P marneffei	20.6	20.55	22.15	19.37	28.95	30.6	31.33	100	43.11	43.43	41.44	44	43.56	44.82	45.21	42.19	42.2
C immitis	22.77	21.66	23.15	18.36	28.23	31.12	32.26	43.11	100	50.38	47.96	41.67	40.24	45.22	41.95	43.7	40.7
P_brasiliensis	21.74	19.83	21.35	20.38	32.74	33.54	32.39	43.43	50.38	100	71.72	42.52	41.22	45.03	43.87	44.47	44.0
A_capsulatus	20.28	19.65	20.87	20.91	29.55	31.21	32.14	41.44	47.96	71.72	100	42.53	41.65	45.92	44.03	44.05	43.4
P chrysogenum	20.85	21.28	21.35	22.04	28.92	32.69	32.71	44	41.67	42.52	42.53	100	52.53	51.87	52.41	53.9	52.8
A nidulans	20.66	21.23	22.22	21.59	29.07	32.4	32.78	43.56	40.24	41.22	41.65	52.53	100	55.21	58.06	61.25	61.
A clavatus	22.25	20.18	21.45	22.07	29.04	33.55	32.51	44.82	45.22	45.03	45.92	51.87	55.21	100	70.33	60.66	63.0
A fumigatus	23.93	21.83	24.26	23.69	30.18	34.67	33.47	45.21	41.95	43.87	44.03	52.41	58.06	70.33	100	62.03	62.3
A terreus	23.36	22.87	22.35	21.1	29.88	33.12	32.12	42.19	43.7	44.47	44.05	53.9	61.25	60.66	62.03	100	66.6
A flavus	21.51	21.86	21.92	21.01	31.03	32.62	31.89	42.26	40.78	44.08	43.48	52.83	61.3	63.06	62.2	66.67	10

b

Supplementary Figure 1. Identification of Mto2 homologs.

(a) GlobPlot (Supplementary Ref. 1) of Mto2 amino-acid sequence (397 residues), showing regions predicted to be disordered (upward slope) or ordered (downward slope). Regions conserved in alignment of sequence homologs (Supplementary Fig. 2) are highlighted in green. Hatched region indicates a short predicted coiled-coil.
(b) Species names and GenBank accession numbers of representative Mto2 sequence homologs identified by PSI-BLAST (Supplementary Ref. 2). Because most Mto2 homologs contain N- or C-terminal extensions (e.g. *M. grisea, N. crassa,* etc.), the specific amino-acid residues used to generate the alignment are also indicated.
(c) Percentage identity between individual Mto2 homologs (Clustal Omega alignment; Supplementary Ref. 3). Black indicates comparisons among *Schizosaccharomyces* species, green indicates comparisons among filamentous fungi, and red indicates comparison between *Schizosaccharomyces* species and filamentous fungi. Note that conservation between *Schizosaccharomyces* species and filamentous fungi is low (18-24%) compared to conservation within filamentous fungi (typically 30-60%).

	[T35 S39] S47 S49 T52 S58 S61
S_pombe/1-397 S_cryophilus/1-397	1 M S E H NY Q S D <mark>R</mark> E V A E D P F L NY E A S A NQ L S S N S R E S D P R G G P WR A GMR G A G L MD E P - L E D G M Y G D N NY L D 67 1 M S R N D L S F D K G V A E D P F L S Y E A S V K S R S N V S P K T R E S Y A Q N A K HQ H A L P S M K A G T D L - D S R D NY A 64
S_octosporus/1-396	1 · · · · · · · · · · · · · · · SNDF SFDK GVT EDP FLSY EA SVK SR PN FSL · · · · · · NP R E SY IQNANHR DDLP · · · · RLNH SP DP · A S · · · · · · R ENY G · · · · · · · · · · · · · · · · · ·
M_grisea/479-1074	479 A DA QMTP P D 5 A V A T P DA DN TA DL FMK (A SE BA P P A P E D G D G Q H E P SAV SR VAR SF H R P L ST V I P P TH T P T S P P - Q T R R L S DQ E T S H F Q R A S S E Q P G Q 378 16 DM S M T P P D S A T A S D N E M T A L F M N A B D T P T E D G S A S A S A S A S A S A S A S A S A
G_zeae/503-1088	503 EALR SAP V S S R R G A F DN E DT G DV F LK I A R E E T SQ R Q A D E - Q P S D D N R S I V S R A T R F T H R R P L S S V V - P I H S P N S P P - R V R R R L S D Q R E T S R S R S R G Y E D D R A 601
C_immitis/350-897	350 A S SK D S D D S R Q K N E D V F LN I A K S S S S · R R D S V - R G S E R Q R S K F G L T G · L S S R L R T · · K · · · E D T · P P P · D R N I Y D S Q D V S P L N · · · · · · · · · · · · · · · · · ·
A_capsulatus/32-595	32 ····································
P_cnrysog./134-689 A_nidulans/319-854	134
A_clavatus/369-915 A_fumigatus/353-908	369 · · · · · · · D G ARI P LAE DPRSK NEDI FLNI AR SDSK · RR DSL - GR SELRR SR LGLSG · SHLR SPISR FN · · · EQI - P SP · DHQ · QLNI YEI P LHSH · · HI SP SNP · · 454 353 · · · · · · · D G ART <mark>P</mark> V A ED S <mark>K</mark> SK NEDI FLNI AR SD SK · RR DSL - GR SEHRR SR LGLSN · GHLR SP NSR LN · · · EQI - P SP · DHQ · QLNI YEI P LHSH · · HI SP SNP · · 454
A_terreus/307-866 A_flavus/321-871	307 DGSRTPVADDSRSKNEDIFLNIARSDST- RRESF- GRSDLRRSRFRMSG- SGLRSPASRVN - SEQT- ASP- DQL- RLNTYDNPLHSN - NGSPSTP - 393 321 DGNRT <mark>P</mark> VADDA <mark>R</mark> S <mark>DIFLNIARSDS</mark> S- RR <mark>ESL- GRSELRRSRMRMSG- SGFRSSTSRAN EHT- SSP-</mark> DQL- RLNTYESPLHSN - NGSPSQP - 403
	S71 T73 S81
S_pombe/1-397 S_cryophilus/1-397	68
S_octosporus/1-396 S_japonicus/1-437	65
M_grisea/479-1074 N_crassa/426-995	579 Q T T R DWAQR A V G G R E R S V Q · I D D R A R S R V L V · · A P P A L K S S P V T P R S P P T H D T P D G F F · · · · · · · · · · · · · · · · S H S R R T S I T E N S M · L P S R T A S L K S · S A Y · · · · · · · 660 504 V R · · · E P N L R G N P R D R L I T N I A Q E D L G R S Q S · · V R T S L R Q A P P T P R Q I A F Q D A Y E A G A · · · · · · · · G L R R · S S I A D I S S V G · · R N S Q Y R N S N L A · · · · · · 582
G_zeae/503-1088 P_marneffei/349-877	602 T E V S RM ST Y RT T P R DK A A S A H P G E D L G R S R A - S T S T M R P S P V T P R S I V F Q E P S S E N S N Y S R R P S I T D N N A T G Q S R S S A Y <mark>K</mark> S - I H H 685 427 N S N - Y S T P G P A S A H P L D E G S R L R H H S T A - G S R S V V G V P R S R Y N Q E T P P - E L P H I D F K A S L A D A R L R - Y <u>S Q</u> M S N Q 496
C_immitis/350-897 P_brasiliensis/317-875	424 • • • • • • YTT• G I L R S A A S S H P L D D S S R L K Y • • L G S T A R S T I G L P R S R F G R S T A R E L S P E P P Q Q • • • • • • • Q R P S E R R G S • A Q D T • • F Q L R A • • H R Q S N I P • • • • • • 502 412 • • • • • • R E S S S I R S P T S T Q H P M D E N S R L R Y • • F G V S T R S S I G L P G S R L T R T T R E • T S P E T L S N • • • • • • Y • T S E R R G S I A Q D Q V Q G Q L R T • Y R Q S N L S • • • • • • 492
A_capsulatus/32-595 P_chrysog./134-689	118 • • • • • • • • • • • • • • • • • •
A_nidulans/319-854 A_clavatus/369-915	409 - · · · · H S · L P Y S Y S A S A H P L D E S <mark>G R S P</mark> Q S N F A S S S H S T V <mark>G L P R S R L S R T N P E · D E P I S · · · · · · · · · E R R A S L H D S R · · · · · S · · · F R H S G L S · · · · · · · 478 455 - · · · · · Y S A · L P Y S Y S A S A H P L D D H S R S R H S V I G S S R S T I G L P R S R F N R T S P D · D S P Q S P E I · · · · · · · · P I E R R G S L Q D P R · · · · A · · · Y R H S A L A · · · · · · 529</mark>
A_fumigatus/353-908 A_terreus/307-866	439 • • • • • • Y S A • L P Y S Y S A S A H P L D D H P R S R H S V V G S S S R S T L G I S R S R L G Q A S P D • S S P Y S A E L • • • • • • • H I E R R G S L Q D P R • • • • • • • Y R H S A L A • • • • • 5 13 394 • • • • • • Y S S • L S Y S Y A A S A H P L D D H S R S H H S G Y V S S R S T I G L P R S R F S R V S P D • A S P R A S E T • • • • • • • N A E R R A S L Q D P R • • • • • • • Y R H S T L S • • • • • • • • • • • • • • • • • •
	404 • • • • • • R S S • LAY SY SA SA HP L D D H S <mark>R</mark> S L H S G F I S S <mark>K S T I G</mark> V P R S R L SQ I S P D • D S P E K • • T • • • • • S I E R R G S L P D P R • • • • T • • • Y R H S T L S • • • • • • 476
S_pombe/1-397	S159" 99 NA IQ - EHKAAK F V S EK S - L EK V STADNNLVLQ ELENLR ER LNQ V ELQL S ERP S SYLGYHNNL (D P Y S P N S Y P S L L 171
S_cryophilus/1-397 S_octosporus/1-396	94 · · · L · · · · NHP S · MRNA I GT · · · · · · · LA ET S · L · · · · ER A SP S DN STVLR ELD ELX F R L S N I ELH L S · · · · · · · HT S PT L S TT S Q P Y S P Y GH L G ST S S Y · 166 94 · · · I · · · · S H S S · MRNA V GT · · · · · · · LA ET S · L · · · · ER E S P S D S S T V LR ELD ELK F R L S N I ELH L S · · · · · DT S RT L P F S S Q S Y S P Y G N Y R NP A S H · 166
S_japonicus/1-437 M_grisea/479-1074	131 DT A L ER AT Q Q L S - L T S P Q P Q - · · · · · · V R S R E - L E - · · H T V P N P AT N E F I F R E L R S L R E R L G Q V E A E L A - · · · · · · · · R T R M E AT T T · · · · · · · · · · · · · · · ·
N_crassa/426-995 G_zeae/503-1088	583 V G G R I Y N S <mark>S P L V P R A P E P Q R P E T S Q N A E T N Q G A E G T - D S S T S T</mark> A D <mark>P S T V WD E L DD L K S R I H R L E R T G K K P P - A G A G N S R S S E E R P P T A T T N A T T M S A S P <mark>K R G S G G T T V 688</mark> 686 <mark>G H N K T Y N S S</mark> P L V R S F D F Q V P S N I D T G N G A E G T - E S T L S N T A P S T V WD E L DD L K S R I H R L E L T G K L P P - A G A G N S R S S E E R P P T A T T N A T T M S A S P <mark>K R G S G G T T V 688</mark> 686 G H N K T Y N S S P L V R S F D F Q V P S N I D T G N G A E G T - E S T L S N T A P S T V WD E L DD L K S R I H R L E L T G K L P S T S G A V S R L S D D R P P T A T T T M S A S P K R V E N G H R Q 788</mark>
P_marneffei/349-877 C immitis/350-897	497 - • - S S R T V R Q S <mark>S - M - S D A A E R A R L D - • - • - G E K A R H D G T E S T L S T T A P S T V WD E L D D L K S R I K K L E L T G K F P S S S A A A M S S V S G E P R T A A T T A T L S S S P K Q K H V R K S S 597 503 - • • G G R G Y R A L S - N - S D G T D P A K L D - • • • • V E R S R Y D G T - E S T L S T T A P S T V WD E L D D L K S R I R K L E L T G K L P T S S A A A M S S V S G E P R T A T T T T L S S S P K Q K H V R K S S 597</mark>
P_brasiliensis/317-875 A capsulatus/32-595	493 - • - T M R T T Q N S S - S V D A A A E R S R L D - • • • A E K S R L D G T - E S T L <mark>S T T A P S T V WD E L D D L K S R I R K L E L T G K L P P S</mark> S A A A M S S V S G E P R T A T T T A T T L S S S P K H G R K M T P P 593 199 - • • T M R S T L N S S • V A D A V A E O S R L E • • • • • A E K S R L D G T • E S T L S T T A P S T V WD E L D D L K S R I R K L E L T G K L P P S S A A A M S S V S G E P R T A T T T A T T L S S S P K H G R K M T P P 593
P_chrysog./134-689	292 TIR SSRQPS-A-SEVTGRPRAD TER SRADGT-EST LSTTAPST WUDELDDLKDRIKKLELTGRLPPSSSAAMYTPTNERPRTANTAITTLSSSPKQRRKASV-390
A_clavatus/369-915 A_fumigatus/353-908	530 SIR S SRQP S- G- S D VT ER AR V E P DR SRQ DGT - E ST L ST T AP ST VWD EL ED LK SR I K K LELT GK LP P S SQ AA I S SV S G ER PRT AT TT VT T V S S S P K HN HK T SN - 628
A_terreus/307-866	469 TIRGSRHTS-T-SEATERAQQE HDRTRHDGT-ESTLSTTAPSTWDELEDLKSRIRKLELTGKLPPSSQEAISSASNDRPRTATTTVTTVSSSPRHGRKTSG-567
	<u>\$176 \$179</u> \$220
S_pombe/1-397 S_cryophilus/1-397	172 · · · P ST H SP H SP A P L S · T M Q T A L M R L R T Y H P SP I I L K P V E Q A V N H A I T L V N T · · · · SP S · · · · S V · · · · · · · V D A L C R S L A E L C L G L V Q E A I D A S I L S 250 167 · · · · A N N T SY SP A P L N · T M Q T A L S R L Q T Y H P SP V V L E P V Q Q A V Q H A I T L T Q T · · · · SP S · · · · A V · · · · · · · · V D G L C R S L A E L C L G L V Q A I D A S L S 244
S_octosporus/1-396 S_japonicus/1-437	167 · · · · INAHSFSPAPLN-TMQTALSQLQSYH <mark>P</mark> SPAVLEPIQQAVQHAITLIHT· · · · TPS· · · · · TV· · · · · · · · · · · · · ·
M_grisea/479-1074 N_crassa/426-995	762 A A D A S ST - T S SQ R E A Q P I L L S A V SK A - R P HMNNDA F G A L E S A A S E V L S L A Q M L G S V G Q P G P I S S G A ST I G G - G S N V T D R Q L R K A D S I C R S L T EMC L S L A D E A A Q R K N S A 868 689 N H G E T A S N A S S R E T Q P I L L S A L L K T - K G L I S A E V F NA I E S A A NDA L A L T S M I G A A G Q P G P I S S G A S V V G G Y G S G V T D R Q L R K A D S I C R S L T E L C I A L T D E A N Q S K P A Q 796
G_zeae/503-1088 P_marneffei/349-877	789 T A D A V ST T S SHHQ R E SHN I LQ SA LA K S' K V L LD T D I Y Q A LE SA AN D A I S LA SMMG <mark>T P G Q P G P</mark> I S S S A ST I G S' NVT V T <mark>D R Q L R K A E SV C R S L T E LC L A LG E E A T Q T R M P R 897 598 I S' P E V LA A T A SA N S I Q T L LQ SA LA K A' K T T V G P E V Y NA LE A T A T D A LT LT NM LA T T T T I V - S G N S ST V T G - T G L S E R Q A K K K A D S L C R G LT E LC LA LT E E Q A I V Q P N S 702</mark>
C_immitis/350-897 P_brasiliensis/317-875	603 P N - N S G D A A T T A D A Q M Q T L L R T G L T K V - K P A V S P E I Y S A L E A T A N D A L T L S SM F G S N A Q Q L - A S T M S V V G T - A T G S D R Q F K R K V D SM C R S L T E L C I A L A D Q K L I A A S K N 707 594 A P E P D A A V V A D Q I H P L L H T A L A K A - K P T L N A D V Y R A L E T T A A D A L T L A T I L S SN G P Q H G S G M S V V N G A G I S D R Q M R K A D S L C R G L T E L C L A L S D E Q L A S S S N N 696
A_capsulatus/32-595 P_chrysog./134-689	300 P L GQ E S ST V T DQ I H P L L H T A L A K A - K P I L N A D V P T L E A T T T D A L T L A A I L S N A P Q H R S GM P - V N G A ST S D R Q M R K A D S L C R G L T E L C L V L S D Q Q R E S I S K T 401 391 - S - T A D T E R S P V H P I L Q S A L A K A - K A V L S G D V T S L E A T I T D A L N L S T A L G V N T A P S G S V S V V N G G Y T S P E R H A R R K A D S V C R S L T E L C L A L T D E Q L K S A R P A 490
A_nidulans/319-854 A_clavatus/369-915	572 - V - S G E S DT I T A P N P V H P L L Q S A L V K V - R S V V N K D V Y T A L E A A A T D A MA L S Q I L G'A G K T P S G N V S I V N - G Y G S A E R Q S R K K A D S V C R S L T E L C L A L S D E H H T K QQ S S 674 629 - L - S G D S E N V A N P V H P L L H S A L L K A - K T V L S N E V F K A L D A V T D A L A L S N T L G T NQ A P S G G V S C V N - G Y S P S D R Q N R K A D S V C R S L T E L C L A L S D V Q P P Q Q Q A 729
A_fumigatus/353-908 A_terreus/307-866	613 - S - GA ET EA ST AT N <mark>P V H</mark> P LLQ SA LL KA - R DV LNN EVYK SLEAAVT DALALS SLLGTNKAP S - · GGV SVVN · G - · Y SS SDRQ SR KA D SV C R SLT ELC LALSD EQ L SKQQ - M 714 568 - P - SN E SDT I T I H SQ I H P LLQ SAMAKA - K SV L SK EVY A A L EVT VT DALALSA LLN SGKT P S - · G SV SV LN · G - · Y SS SER Q SR KA D SV C R SLT ELC LALSD EQ L R R Q A S 670
A_flavus/321-871	576 - S- SPGSDSIPPTNPVHPLLQSALSKA-KTVLNKDVFTALEATATDAISLSTTLSTNKAPSGGISVVN-GYGPTORLSRKADSVCRGLTELCLALSDEQLRRHQAS 678
S_pombe/1-397	251 QQ ESSNSLDLVRHQ
S_cryophilus/1-39/ S_octosporus/1-396	245 QQ AGQ I SMDLIPHS IP - LDLAT SIT - NSSPSK S. ASSFATNSP DKSLF
S_Japonicus/1-437 M_grisea/479-1074	200 DE SQT PREP GROEE - EEM SGT ATTIL, AASTO, ESGKN ST SFY PTTAFT 869 GP ET PR DK E- AVT T SP TR V F SV LNGA SQ R T V AT S EV GL SR IN - T T I SP RT LA RP DE RR AT FLGLGVA S SP R L AM SP AT P NA EP ST P GT G R R S SL L - 963
N_crassa/426-995 G_zeae/503-1088	/9/ PA APNRELERVII PII AAKFIGTIGKRKSSTMVETALPQP- SVT SPRAPTIMEQRKTSLLAASALPSPRISAVPALPV DFG PGRKSSLL 886 898 Q SI EVPTPTQNEAPIT PT INKT FSG FSQ RRQ SI GRPDK NVPKE- AVT SPRTMSK FEERRLSILNG SSLPT SRA
C_immitis/350-897	708 RP CS SSQ EA SUG VIGAD TET NER SA SHEP EVIGUED SAV REPSIREANITIL USAGR RER SHD SASSF REPSILAP SK LA 800 708 RP CS SSQ EA SUG VIGAD TET NER SA SHEP EDV GRQ AGS LGRT LES SR LGRR SK LL ST SG VR SSQ EA SUG VIG
A_capsulatus/32-595	09/ R*GS RD4ASSQTQTDSMMDRESTIPNLATKRSASHEPEEPQRQQAPSTRLAAGSRLESKRASLESLIGTTSTR NTQENTN-PESTPINSTPPSRLN
A_nidulans/319-854	491 S SHET
A_fumigatus/353-908	715 PA S DD DT TTQ LHT SIN BEAF TALFF AND LO FOR OF A CONSTRUCT AND A C
A_flavus/321-871	679 SKPDEDT ITQQP I GADDET LTPTTPY R STTQEP EGL SRRQ- STRAASRR SSFANP SGNTP SENNK EV NWGNDTT FDAKQTQ SPGS SLPT SR LS771
S_pombe/1-397	T314 T331* S341 314 ••••••••••••••••••••••••••••••••••••
S_cryophilus/1-397 S_octosporus/1-396	316 · · · · · P P R S S M S T S S T V HQ T S P K H L E N S Q G L · 365 316 · · · · · · · · · · · · · · · · · · ·
S_japonicus/1-437 M_grisea/479-1074	352 P R S Q <mark>R I I P</mark> - P T S S P L S D - · · · R V K T P V L R D R D G D Q M P A S S R F Q S K I S L S P N R - · · · · · · · · · · · · · · · · S P V S P R - S P P F H R K R - · F S K T A S S N V L V T P · · · 429 964 S R T R R A G T E E P E D T S G G R K P S L L M R T R R A G T E E · · · · P D E G R K T S L L L R S Q R P A Y A E D E D D S P R V R A P S R A A T E L G A F R R D Y V G - · · · · · · · · · · · · · · · · · ·
N_crassa/426-995 G_zeae/503-1088	887 A R N R R V A V E EP E E Q V N G R R S S L L L R S R R V G Q E E Q E EMP A E G R K T S L L L R S R K V F · · · N E E D E D R Y R T P S R A I T E V N G L R G T P R E · · · · P O F G R T S L S R R R A G T E E P D D · · · G R T S S L L L R T R R A G T E E · · · · P D E G R R T S L F V R N R R N T V G E D S E D E S R F R G P S R I R T D L N T I R V V P Q E · · · · · · · · · · · · · · · · · ·
P_marneffei/349-877 C_immitis/350-897	801 · · · R L S G · · · · · S C R I K R E D D S E · · · · E R G S · · · V F S R T I · · · · · · · · · · · · · · · · · ·
P_brasiliensis/317-875 A_capsulatus/32-595	793 · · · R A ST · · · · · · I L R N R R L Q D E D · · · · D L D D K · · · T L R P I · · · · · · · · · · · · · · · · · ·
P_chrysog./134-689 A_nidulans/319-854	577 · · · R M S S · · · · · · · S L R S R R L T I G E · · · · E S G E T E S P H S R S V · · · · · · · · · · · · · · · · · ·
A_clavatus/369-915 A_fumigatus/353-908	819 · · · R L ST · · · · · · T L R T K R L Q P D E · · · · D N G D E P N P H S R S F · · · · · · · · · · · · · · · · · ·
A_terreus/307-866 A_flavus/321-871	767 R L ST
5 pombe/1-307	S366 [*] S382 [*] [T394 S396] 362 SI HP(SPT SI R VA H
S_cryophilus/1-397	366 SFGP SF LDN SFHQ
S_japonicus/1-437	$\frac{1}{430}$ $\frac{1}{430}$ $\frac{1}{430}$ $\frac{1}{430}$ $\frac{1}{437}$ $\frac{1}$
N_crassa/426-995	968 LA SQ A S S P D NTP LG S A LP R R MV P T S 995 Mto2[177] all rod otcos Mto2[177] all rod otcos
P_marneffei/349-877 C immitis/350-897	853 LSQ HQP KV SP SI S SI SQ RR SY ATP MID 877 867 Mto2[6A] = all sites with green asterisk.
P_brasiliensis/317-875 A capsulatus/32-505	855 SQQLPLQQQQT GQPR STTQ GQ 565 OL SPORHADQAHP LSQVQ SN IP V R NY GTP. 595 N.B. S382A is unique to Mto2[6A]
P_chrysog./134-689 A nidulans/319-854	646 SVAYSPR SPQYQ SSQVPQPQVQ SSQPRTPTL - SSSL SFRR SY
A_clavatus/369-915 A_fumigatus/353-908	886 SR L
A_terreus/307-866 A_flavus/321-871	833 P R F

Supplementary Figure 2. Alignment of Mto2 homologs and position of phosphosite mutations.

Clustal Omega alignment (Supplementary Ref. 3) of fission yeast *S. pombe* Mto2 with homologs from other *Schizosaccharomyces* species and from filamentous fungi. Circled amino-acid residues indicate residues mutated to alanine in various *mto2-phosphomutant* strains; the labeling scheme used to distinguish different mutants is shown in figure. Boxed regions indicate cases in which one or more [S/T]-[P] sites present in *S. pombe* Mto2 are also present in the same general neighborhood in homologs; as is often the case for unstructured proteins (Supplementary Ref. 4), positioning of these sites is not highly conserved in the alignment. Boundaries of boxes indicate the range of candidate-similar [S/T]-[P] sites in the full collection of homologs. Box with dashed line shows an [S/T]-[P] site conserved in *Schizosaccharomyces* species but not in most other organisms.







Supplementary Figure 3. Identification of Mto2 phosphorylation sites by mass spectrometry.

(a) Coomassie-blue stain of purified Mto2 (purification of Mto2-HTB) after TEV cleavage. The Mto2 band was excised prior to digestion and MS analysis.

(b) Representative spectra of Mto2 peptides containing high-confidence phosphorylation sites (see also Supplementary Data 1). Phosphorylated Ser159 was identified in the peptide

LNQVELQLSERPSSYLGYHNNLSPYR. Phosphorylated Thr264 and Ser276 were identified separately in two different forms of the peptide HTPPLNYTSSVDSSPQR. Precursor ions are shown in blue, and fragment ions in red. • - indicates loss of phosphate; 9 - indicates loss of water. MD, Mascot Delta Score.



Supplementary Figure 4. Identification of Mto2[17A] phosphorylation sites by mass spectrometry.

(a) Coomassie-blue stain of purified Mto2-GFP (anti-GFP purification). Interphase and mitotic Mto2[17A]-GFP bands were excised prior to digestion and MS analysis.

(b) Example spectra of an Mto2[17A] peptide, ASPASQSFPSLQDAPAPR, in which three distinct phosphorylation sites (Ser382, Ser385 and Ser387) were identified with high confidence, each in a separate monophosphopeptide. Note that Pro397 is the last amino acid of Mto2[17A] itself; Arg398 is the first amino acid of a linker (RIPGLIN) between Mto2[17A] and GFP. See also Supplementary Data 1. PSD, Phospho (STY) Score Difference for the indicated residue (Ser382, Ser385 and Ser387, respectively).



mto1



$mto1\Lambda$

Supplementary Figure 5. Mto2[24A]-GFP and Mto2[17A]-GFP have similar functional properties during interphase.

(a) Schematic showing distribution of serine and threonine residues in wild-type Mto2 (blue, total 92 residues; 14 tyrosines residues are not shown), together with sites mutated in phosphomutant Mto2 proteins (red). Mutated sites that were experimentally identified as phosphorylated are indicated in bold (these represent a subset of total identified phosphosites; see Supplementary Table 1). Mutated residues are also shown in sequence alignment in Supplementary Fig. 2.

(b) Quantification of microtubule bundle number per cell, in the strains indicated, expressing GFP-tubulin (nmt81:GFP-Atb2). For each strain, 70 cells were scored. Images and cartoons at left indicate method of guantification. P values (Wilcoxon rank-sum test): mto2+ vs mto2-phosphomutant, p<1e-3; mto2/24A] vs mto2[13A] and mto2[6A], p<0.02. Gray box shows data reproduced from Fig. 3c, for comparison.

(c) Quantification of microtubule nucleation events per cell during a 10 min interval, in the strains indicated. For each strain, 50 cells were scored. Median with interguartile range is shown in red. ** p<1e-3, *** p<1e-4 (Wilcoxon rank-sum test). Gray box shows data reproduced from Fig. 3d, for comparison.

(d) Images showing localization of wild type and phosphomutant Mto2-GFP in $mto1^+$ and $mto1\Delta$ cells. Z-projections. Gray box shows data reproduced from Fig. 4b, for comparison. Scale bar, 5 µm.

Mto2-GFP	Mto2[6A]-GFP	Mto2[13A]-GFP	Mto2[17A]-GFP	Mto2[24A]-GFP
		• • * *		. 3 P.
and the		2.4	19 C	
		an in the second		N. C. C. S.

b



Supplementary Figure 6. Mitotic Mto2[24A]-GFP forms puncta that localize to the nuclear envelope.

(a) Localization of wild-type and phosphomutant Mto2-GFP, as indicated, in mitotic cells. Z-projections. Note absence of nuclear envelope (NE)-associated puncta of wild-type Mto2-GFP and Mto2[6A]-GFP, weak puncta of Mto2[13A]-GFP and Mto2[17A]-GFP, and more robust puncta of Mto2[24A]-GFP. All proteins localize to spindle pole bodies (SPBs).

(b) Z-projection (top row) and individual Z-sections (other rows) of the *mto1[NE]* mitotic cell shown in Fig. 6c, expressing Mto2[24A]-GFP together with SPB marker Cut12 fused to tandem-dimer Tomato (Cut12-tdT). Individual Z-sections demonstrate that Mto2[24A]-GFP localizes to the nuclear envelope and not in the nuclear interior. Contrast settings are different between the Z-projection and individual Z-sections. Scale bars, 5 μm.



Supplementary Figure 7. Impaired nucleocytoplasmic transport due to *pim1* mutation leads to increased cytoplasmic microtubules and defects in spindle pole body separation in mitotic *mto2-phosphomutant* cells.

Quantification of cytoplasmic MTs (mCherry-tubulin) in mitotic wild-type (*mto2*+) and phosphomutant *mto2[17A]* and *mto2[24A]* cells, in *pim1-F201S* mutant genetic background at 37°C (n=182, 177 and 186 cells, respectively). Bright Plo1-GFP signal at spindle pole bodies (SPBs) was used to identify mitotic cells independent of spindle assembly state. Note that "minimum spindle length" is 0 μ m (i.e. unseparated SPBs), but to avoid overcrowding of datapoints, some datapoints are displayed slightly above and below the zero position on the vertical axis. Tables show percent of mitotic cells containing one or more cytoplasmic MTs, and percent of cells in which bright Plo1-GFP signal was observed as a single spot, indicating unseparated SPBs. *** p<1e-4 (Fisher's exact test), **** p<1e-9 (Wilcoxon rank-sum test, one-tailed).



Supplementary Figure 8. Mutation of Mto2 phosphorylation sites to phosphomimetic glutamate or aspartate leads to loss of Mto1-Mto2 and Mto1/2- γ -TuC interactions, decreased Mto2-GFP puncta, and decreased numbers of cytoplasmic microtubules during interphase.

(a) Anti-Mto1, anti-Mto2 and Anti-Gtb1 (*S. pombe* γ -tubulin) Western blots of interphase cell extracts, and corresponding IgG pulldowns, from wild-type, *mto2-phosphomutant*, and *mto2-phosphomimetic* cells expressing Mto1-SZZ (Protein A tag). Mto2 expression levels are relative to the value for *mto1+ mto2+* cells (first lane, set to 100). Graphs show quantification of Mto2 (or phosphomutant/phosphomimetic Mto2) and Gtb1 co-purifying with Mto1-SZZ in the pulldowns above, normalized to the values obtained for wild-type (WT) cells (second column). (b) Localization of GFP-tagged wild-type and phosphomimetic Mto2 as indicated, together with microtubule distribution (shown by mCherry-tubulin; mCh-Atb2). *mto2* Δ cells are also shown for comparison. Scale bar, 5 µm. (c) Quantification of microtubule bundle number per cell, in the strains indicated, from images of the type shown in (b). For each strain, 70 cells were scored. Images and cartoons at left indicate method of quantification. P-values (Wilcoxon rank-sum test): *mto2-GFP(WT)* vs *mto2-phosphomimetic* and *mto2* Δ , p<1e-14; *mto2* Δ vs *mto2-phosphomimetic*, p<1e-5.

					Net Phos	[S/T]P in homol	mto2+ MS2	mto2 [17A] MS2 phos	mut'd in	mut'd in	mut'd in	mut'd in	mut'd in	mut'd in	mut'd in	mut'd in
aa	aa #	Sequence	Comment	Conserv'n	predic'n	region	prob.	prob.	mto2[6A]	mto2[13A]	mto2[17A]	mto2[NT1]	mto2[24A]	mto2[FCC]	mto2[CT1]	mto2[CT2]
S Y	2	MSEHNY SEHNYQSDR		Sz only -	0.00 0.91			1.00				yes				
s	8	HNYQSDREV			0.79			0.50				yes				
Y	20 23	PFLNYEASA NYEASANOI	nlo	Sz only	0.31		, i	0.09				Ves				
s	28	ANQLSSNSR	pio	-	0.05			0.22				yes				
S	29	NQLSSNSRE	pbd	+	1.00			0.22				yes				
s	31	NSRESTPRG		Sz only	1.00			0.22				yes				
Т	35	SRESTPRGS	TP PBD	-	1.00	++		N/A			yes	yes	yes	yes	yes	yes
S	39 47	TPRGSPWRA	CDK	+	0.99	++		N/A			yes	yes	yes	yes	yes	yes
s	49	MRSASLMTE		+	0.89								yes			
T	52	ASLMTEPLE	DL O	+	0.04								yes			
Y	60	EDSMYSDNN	PLO	-/+	0.15								yes			
s	61	DSMYSDNNY		-/+	0.07								yes			
Y S	65 71	SDNNYLDNG DNGVSETKD		-/+ +	0.99								Ves			
T	73	GVSFTKDEN		-/+	0.29								yes			
Y	80 81		SP	/+	0.80	++	0.65	N/A		VAS	Ves	Ves	Ves	VAS	VAS	VAS
s	83	LYSPSWPSL	01	-/+	0.02		0.00	19/4		yes	yes	yes	yes	yc3	yc3	yes
S	86	PSWPSLADA		+++	0.39		0.06									
S	94 97	NSMKSNNAI		-/+ +	0.46			1.00								
s	111	AKFV <mark>S</mark> EKSL		-	0.97			0.06								
S	114	VSEKSLEKV	PLO	++	0.90			1.00						yes ves		
T	120	EKVSTADNN	pbd	+++	0.50			0.46						yes		
S	145	ELQLSERPS		+	0.87	j	0.42	0.33						yes		
S	149	SERPSSYLG ERPSSYLGY	pbd	++++	0.94		0.43	0.33						yes		
Y	151	RPSSYLGYH		-	0.09		0.08	0.22								
Y	154	SYLGYHNNL	СDК	-/+ +++	0.77	++	0.97	N/A	ves	ves	ves	Ves	ves	ves	ves	ves
Ŷ	161	NLSPYRSPN	obit	Sz only	0.24		0.22		,	,	,	,	,	,	,	,
S	163	SPYRSPNSY	SP	+	0.73	++	0.12	0.17								
Y	167	SPNSYPSLL		-	0.60		0.13	0.11								
S	169	NSYP <mark>S</mark> LLPS		+	0.31	1	0.35	0.11								
S T	173	SLLPSTHSP	nbd	-/+ -/+	0.33		0.37	0.11								
s	176	PSTHSPHSP	SP	+	0.93	+	0.58	N/A		yes	yes	yes	yes	yes	yes	yes
S	179	HSPHSPAPL	SP	+	0.99	+	0.97	N/A		yes	yes	yes	yes	yes	yes	yes
T	185	APLSTMQTA	pbd	-	0.80		0.14									
Т	188	STMQTALMR		+++	0.12											
Y	195 196	RIRTYHPS		-/+ Sz only	0.28											
s	199	TYHPSPIIL	SP	Sz only	0.09	-										
T	215	NHAITLVNT		++	0.36											
s	219	LVNTSPSSV	SP	+	0.06	-/+		N/A		yes	yes	yes	yes	yes	yes	yes
s	222	NTSPSSVVD		++	0.09											
s	223 231	ALCRSLAFI	pbd	++	1.00											
s	247	AIDASILSQ	PLO	-/+	0.01											
S	250	ASILSQQES		-/+ /+	0.88											
s	255	QQESSNSLD	plo pbd	-/+	0.10											
S	257	ESSNSLDLV	TD	-/+	0.03		1.00	NI/A	VOC	VOC	VOC	VOC	VOC	VOC	VOC	VOC
Ý	269	PPLNYTSSV	IF	-	0.17	**	0.89	0.44	yes	yes	yes	yes	yes	yes	yes	yes
Т	270	PLNYTSSVD	PLO	-/+	0.06		0.32	0.53							yes	
S	271	NYTSSVDS	pbd	+ -/+	0.96		0.52	0.34		yes	yes	yes	yes	yes	ves	yes
S	275	SSVDSSPQR		-/+	0.64		0.49								yes	
S	276 282	SVDSSPQRM ORMASDSVC	CDK PBD	++ ++	0.99 n ao	++	0.94	N/A	yes	yes	yes	yes	yes	yes	yes	yes
S	284	MASDSYGRP		-	0.98		0.56	19/2		,	,	,	,	,	,	,
Y	285	ASDSYGRPS		Sz only	0.90		0.32									
S	∠89 298	DPFPSVDLQ	ARK	++++	0.98		0.44	0.13								
S	303	VDLQSNELS		+	0.22			0.13								
S T	307 313	SNELSHHNV HNVRTTLES	plo	+++ -/+	0.16			0.95								
Ť	314	NVRTTLFSD	ARK	+	0.89			N/A		yes	yes	yes	yes	yes	yes	yes
S	317		nlo	/+	0.34											
S	320	SRFHSKIHT	μu	-/+	0.27											
T	328	SKIHTHSTP		-	0.03		0.27	0.62								
S T	330 331	HTHSTPPS	TP PBD	-/+ -/+	0.38	++	0.75	0.87 N/A	yes	ves	yes	yes	yes	yes	yes	yes
s	334	STPPSQMYS		+	0.10		0.84									
Ŷ	337	PSQMYSAAS		- _/+	0.08		0.06									
S	341	YSAASHFRY		-/+	0.03			N/A		yes	yes	yes	yes	yes	yes	yes
Y	345	SHFRYRSDP		-/+	0.26			0.10								
S	347 350	RSDPSTRHV	plo	-/+ -/+	0.85			0.42								
Т	351	SDPSTRHVS	pbd	-	0.11			0.29								
S	355			- Sa onlu	0.92		0.05	0.56								
S	357 358	USNSTNKS	plo pbd	Sz only -	0.80			0.75								
S	361	STNKSSLHP	plo	-/+	0.05		0.75	0.47								
S	362 366	INKS <mark>S</mark> LHPS SLHP <mark>S</mark> PTSI	ARK pbd SP	-/+ +	0.78	++	0.94	0.10 N/A	ves	ves	ves	ves	ves	ves	ves	ves
Т	368	HPSPTSLRV	0.	-	0.26		0.33		,	,	,	,	,	,	,	,
S	369	PSPTSLRVA	QD	+	0.48	++	0.33	0.00	VAS							VAS
S	385	ASPASQSFP	or.	-/+	0.99			1.00	903							yes
S	387	PASQSFPSL		-/+	0.01			1.00								yes
S T	390 394	USEPSLQDT SLQDTPSP	TP	-/+ +	0.99	++		0.10 N/A			ves	ves	ves	ves	yes	ves
s	396	QDTPSP	SP	+	0.33	++		N/A			yes	yes	yes	yes	yes	yes

Comment: SP, TP: [S]-[P] or [T]-[P] (i.e. when not in strong CDK site). **CDK**: strong CDK site = [pS/pT]-P-X-[K/R]. **ARK**: strong AURORA site = [K/R]-X-[pS/pT]-[I/L/M/V] (Supplementary Refs. 5,6). PLO: strong Polo site = [D/E/N]-X-[pS/pT]-[F/I/L/M/M], plo: weak Polo site = [D/E/N]-X-[pS/pT]-[no P] (Supplementary Refs. 7-9). PBD: strong Polo Box Domain binding site = S-[pS/pT]-P. **pdb**: weak Polo Box Domain binding site = S-[pS/pT] (Supplementary Ref. 10). **Conservation**: Qualitative assessment based on alignment shown in Supplementary Fig. 2, considering both extent of conservation among homologs and quality of alignment in the neighborhood. "Sz only" = conserved in *Schizoaaccharomyces* species. **NetPhos prediction**: Probability score from NetPhos algorithm (Supplementary Ref. 11). [*ST*/**T**] *in homologous region*: Indicates if similar site is present nearby in homologs; see Supplementary Fig. 2 for details. **MS2 Phosphorylation probabilities**: Summary of MS/MS analysis of Mto2 and Mto2[17A]; see Supplementary Data 1 for details. Red shading = high probabilities. Blue shading = low probabilities. NA = not applicable (because mutated in Mto2[17A]). For residues grouped in double-lined boxes, at least one residue in box is phosphorylated, but MS2 data cannot make unambiguous assignment. In some cases, it is possible that some molecules of the same peptide are phosphorylated on a different residue. For residues in thick-lined box (Ser355, Ser357, Thr358), peptide is doubly-phosphorylated, and thus at least two of the residues within the box are phosphorylated. Red and blue colors indicate high and low probabilities, respectively. **Mut'd in:** indicates which residues are mutated in the mutants listed.

Supplementary Table 2: Yeast strains used in this work

Strain	Genotype	Source
KS516	b- ade6-M210 /eu1-32 ura4-D18	Lab stock
KS1235	h+ kanX1.mt81.GEP-atb2 ade6-M210 leu1-32 ura4-D18	Lab stock
KS1459	h+ mto2-GFP:kanMX mto1∆::kanMX ade6-M216 leu1-32 ura4-D18	Lab stock
KS1740	h+ nda3-KM311 ade6-M216 leu1-32 ura4-D18	Lab stock
KS1890	h- mto2-GFP:kanMX ade6-M216 leu1-32 ura4-D18	Lab stock
KS3575	h+ mto1-SZZ:kanMX nda3-KM311 ade6-M216 leu1-32 ura4-D18	Lab stock
KS3953	h- mto2-HTB:kanMX ade6-M210 leu1-32 ura4-D18	This study
KS4323	h- mto1-SZZ:kanMX mto2∆::kanMX ade6-M216 leu1-32 ura4-D18	Lab stock
KS5285	h- mto2-GFP:kanMX nda3-KM311 ade6-M216 leu1-32 ura4-D18	Lab stock
KS6404	h- mto2[13A]:hphMX mto1-SZZ:kanMX nda3-KM311 ade6 leu1-32 ura4-D18	This study
KS6407	h- mto2[13A]:hphMX kanMX:nmt81:GFP-atb2 ade6-M216 leu1-32 ura4-D18	This study
KS6412	h+ mto2[6A]:hphMX mto1-SZZ:kanMX nda3-KM311 ade6-M216 leu1-32 ura4-D18	This study
KS6415	n-mto2[174]:npnMX nda3-KM311 ade6-M210 leU1-32 ura4-D18	This study
KS6512	n+ mto2[17A]:npnMX kanMX:nmt81:GFP-atb2 ade6-M216 leu1-32 ura4-D18	This study
KS6744	n+ mto1[0A].npniMX kaniMX.nimto1.GFP-al02 adeo-m210 leu1-32 ura4-016	This study
KS6760	h+ mto1-SZZ.kanimX mto2[17A].npmixX mta3-Kiv311 ade6-w210 ieu1-S2 uta4-D16	This study
KS6775	h+ alp4-GEP·hphMX natMX ^{·7·} ADH15·mCherry-atb2 ade6-M210 leu1-32 ura4-D18	Lab stock
KS7055	h+ mto2-GFP:kanMX natMX:Z:ADH15:mCherry-atb2 ade6-M216 leu1-32 ura4-D18	Lab stock
KS7083	h- mto2[17A]-GFP:natMX nda3-KM311 ade6-M210 leu1-32 ura4-D18	This study
KS7093	h+ mto2[17A]-GFP:natMX car2∆:kanMX arg1-230 lys3-37 nda3-KM311	This study
KS7099	h+ mto2[17A]-GFP:natMX ade6 leu1-32 ura4-D18	This study
KS7158	h+ mto2[17A]-GFP:natMX mto1∆::kanMX ade6 leu1-32 ura4-D18	This study
KS7221	h+ mto2[FCC]:hphMX nda3-KM311 ade6-M210 leu1-32 ura4-D18	This study
KS7223	h+ mto2[NT1]:hphMX nda3-KM311 ade6-M210 leu1-32 ura4-D18	This study
KS7225	h+ mto2[CT1]:hphMX nda3-KM311 ade6-M210 leu1-32 ura4-D18	This study
KS7227	h+ mto2[24A]:hphMX nda3-KM311 ade6-M210 leu1-32 ura4-D18	This study
KS7229	h+ mto2[CT2]:hphMX nda3-KM311 ade6-M210 leu1-32 ura4-D18	This study
KS7413	h+ mto2[24A]-GFP:natMX:hphMX mto1∆::kanMX ade6-M210 leu1-32 ura4-D18	This study
KS/41/	n+ mto2/24AJ-GFP:natMX:npnMX ade6-M210 leu1-32 ura4-D18	This study
KS7504	h- mto2[24A]-GFF.IId(MA.IIpIIMA IIud3-KM311 due0-M210 leu I-32 uld4-D10	This study
KS7504	h+ mto2[17A]-GEP:natMX.natMX7:ADH15:mCherry-atb2 ade6 leu1-32 ura4-D18	This study
KS7510	h+ mto2_GEP:kanMX mto1/NEI:kanMX cut12-tdT:kanMX ade6 leu1-32 ura4-D18	This study
KS7518	h+ natMX:nmt81:mto1INE1:kanMX natMX:nmt81:mto2-GEP:kanMX ade6-M216 leu1-32 ura4-D18	This study
KS7525	h+ mto2[CT1]-GFP:natMX:hphMX ade6-M210 leu1-32 ura4-D18	This study
KS7534	h- mto2[FCC]-GFP:natMX:hphMX ade6-M210 leu1-32 ura4-D18	This study
KS7536	h- mto2[CT2]-GFP:natMX:hphMX ade6-M210 leu1-32 ura4-D18	This study
KS7541	h+ mto2[NT1]-GFP:natMX:hphMX ade6-M210 leu1-32 ura4-D18	This study
KS7542	h- mto2[24A]-GFP:natMX:hphMX mto1[NE]:kanMX cut12-tdT:kanMX ade6-M210 leu1-32 ura4-D18	This study
KS7545	h+ mto2[17A]-GFP:natMX mto1[NE]:kanMX cut12-tdT:kanMX ade6 leu1-32 ura4-D18	This study
KS7591	h- natMX:nmt81:mto1[NE]:kanMX natMX:nmt81:mto2-GFP:kanMX natMX:Z:ADH15:mCherry-atb2 ade6 leu1-32 ura4-D18	This study
KS7649	h+ mto2[24A]:hphMX kanMX:nmt81:GFP-atb2 ade6 leu1-32 ura4-D18	This study
KS7653	h+ mto1-SZZ:kanMX mto2[24A] nda3-KM311 ade6 leu1-32 ura4-D18	This study
KS/6/3	h+ natMX:nmt81:mto1[NE]:kanMX natMX:nmt81:mto2-GFP:kanMX alp4-td1:natMX ade6-M216 leu1-32 ura4-D18	This study
K97676	h+ mto2[17A];hphMX alp4-GFP.hphMX hatMX:2.ADH15:mCherry-atb2 ade6-M210 leu1-32 ura4-D18	This study
KS7681	h-mto2[17A].CEP:notMY:hnhMY.mto14::konMY.ade6.leu1-32.uro4-D18	This study
KS7683	h+ mto2[13A]-GFP:natMX:hphMX ade6 leu1-32 ura4-D18	This study
KS7725	h+ mto2[6A]-GEP:hphMX ade6 leu1-32 ura4-D18	This study
KS7726	h+ mto2[6A]-GFP:hphMX mto1\.:kanMX ade6 leu1-32 ura4-D18	This study
KS7736	h- mto1[bonsai]:kanMX natMX:nmt81:mto2-GFP:kanMX natMX:Z:ADH15:mCherry-atb2 ade6 leu1-32 ura4-D18	This study
KS7894	h+ mto1-SZZ:kanMX mto2[24E]:hphMX nda3-KM311 ade6-M216 leu1-32 ura4-D18	This study
KS7931	h- mto1-SZZ:kanMX mto2[24D]:hphMX nda3-KM311 ade6 leu1-32 ura4-D18	This study
KS7955	h+ plo1-mEGFP:kanMX pim1-F201S natMX:Z:ADH15:mCherry-Atb2 ade6 leu1-32 ura4-D18	This study
KS8002	h- mto2[24A]:hphMX plo1-mEGFP:kanMX pim1-F201S natMX:Z:ADH15:mCherry-atb2 ade6 leu1-32 ura4-D18	This study
KS8005	h- mto2[17A]:hphMX plo1-mEGFP:kanMX pim1-F201S natMX:Z:ADH15:mCherry-atb2 ade6-M210 leu1-32 ura4-D18	This study
KS8083	h+ mto2∆::kanMX natMX:Z:ADH15:mCherry-atb2 ade6 leu1-32 ura4-D18	This study
KS8085	h+ mto2[24D]-GFP:natMX:hphMX natMX:Z:ADH15:mCherry-atb2 ade6 leu1-32 ura4-D18	This study
KS8091	h+ mto2[24E]-GFP:natMX:hphMX natMX:Z:ADH15:mCherry-atb2 ade6 leu1-32 ura4-D18	This study

Supplementary Methods

Considerations underlying SILAC quantification of nonphosphopeptides

Peptides that are phosphorylated on multiple residues (here referred to as multiphosphopeptides) can be difficult to detect and/or identify experimentally, even after protein purification ^{12, 13}. For example, for the Mto2[17A] peptide sequence 381-A**S**PA**S**Q**S**FPSLQDAPAPR-398, we identified three distinct, high-confidence, singly-phosphorylated peptides (monophosphopeptides), each of which was phosphorylated on a different residue--one on S382, one on S385, and one on S387 (Supplementary Fig. 4, Supplementary Data 1)--but for the same sequence, we did not identify any di- or triphosphopeptides. These and similar results (e.g. Supplementary Fig. 3, Supplementary Data 1) illustrate the difficulty in detecting and identifying multiphosphopeptides with confidence, even when there is a good likelihood that they are present (although in principle our findings could also be due to mutually-exclusive monophosphorylation).

This may be particularly problematic for a protein such as Mto2[17A] (and more so for wild-type Mto2), because of the very high number of potential phosphorylation sites, and because a large proportion of Mto2 is predicted to be disordered. After excluding peptides containing five or fewer residues (which in general are too small to be uniquely identified) and taking account of incomplete cleavages, we determined that approximately 80% of Mto2[17A] tryptic peptides contain three or more serine or threonine residues, 70% contain four or more, and 50% contain five or more; percentages are marginally higher when tyrosine residues are also considered. Moreover, because of the high density of phosphorylation sites within Mto2, this situation is unlikely to be significantly improved by digestion with alternative proteases.

This raised the possibility that many Mto2[17A] peptides may be present as multiphosphopeptides *in vivo* but at the same time not easily detectable by mass spectrometry. We therefore sought to employ a method for SILAC-based relative quantification of phosphorylation that could overcome this obstacle. In the following example, we describe how two different approaches to quantifying the same experimental data can potentially lead to different conclusions, and we provide justification for why the second approach should be favored for analysis of Mto2[17A].

First, we consider that a given peptide of interest with multiple potential phosphorylation sites can exist in one of three states: nonphosphopeptide (denoted as "n"); monophosphopeptide ("p"); or multiphosphopeptide ("pp"). We further

consider that only the nonphosphopeptide and the monophosphopeptide can be detected; the multiphosphopeptide cannot be detected. We denote the true *in vivo* mole fractions of nonphosphopeptide, monophosphopeptide, and multiphosphopeptide in a "light" culture as " L_n ", " L_p ", and " L_{pp} ", respectively. Similarly, mole fractions of nonphosphopeptide, monophosphopeptide, and multiphosphopeptide in a "heavy" culture are denoted as " H_n ", " H_p ", and " H_{pp} ". By definition, $L_n + L_p + L_{pp} = 1.00$, and $H_n + H_p + H_{pp} = 1.00$. In the table below, we present hypothetical example values for these mole fractions for a hypothetical peptide that experiences a substantial increase in phosphorylation in the "heavy" culture relative to the "light" culture (e.g. in mitosis relative to interphase), accompanied by a shift towards multiphosphopeptides in the "heavy" culture:

State of peptide	Mole fraction in "light" culture (e.g. interphase)	Mole fraction in "heavy" culture (e.g. mitosis)
Nonphosphopeptide	$L_n = 0.90$	<i>H_n</i> = 0.20
Monophosphopeptide	$L_{p} = 0.10$	<i>H</i> _p = 0.05
Multiphosphopeptide	$L_{pp} = 0.00$	<i>H_{pp}</i> = 0.75
(Total peptide)	$(L_n + L_p + L_{pp} = 1.00)$	$(H_n + H_p + H_{pp} = 1.00)$

A first approach to quantification would involve simply determining the monophosphopeptide ratio in the "heavy" sample relative to the "light" sample:

monophosphopeptide ratio =
$$\frac{H_p}{L_p}$$

While this is straightforward to determine, for the data in the example one obtains:

monophosphopeptide ratio =
$$\frac{H_p}{L_p} = \frac{0.05}{0.10} = 0.5$$

By itself, this ratio would suggest that phosphorylation of the peptide is decreased in the "heavy" sample; however, this would be the opposite of the true trend of phosphorylation, because this approach does not take into account the (nondetectable) multiphosphopeptide. It is clear that this "false-negative" result is a direct consequence of the high mole fraction of multiphosphopeptide in the "heavy" sample; if this mole fraction were very small, then the "monophosphopeptide ratio" approach would in fact be suitable. However, *a priori* one can be sure of a low

multiphosphopeptide mole fraction only if the peptide of interest contains a single phosphorylatable residue, which is very rare within Mto2[17A]. More broadly, this approach also fails to give any indication of the stoichiometry of phosphorylation. For example, if $L_p = 0.005$ and $H_p = 0.05$, then the monophosphopeptide ratio $H_p / L_p = 10$, but the absolute level in phosphorylation may still be too low to be of biological significance. Finally, it is worth noting that if a given peptide were present as a multiphosphopeptide but not as a monophosphopeptide, then it would not even be possible to determine a monophosphopeptide ratio.

As an alternative to calculating monophosphopeptide ratio, one can follow a second approach, namely to quantify the nonphosphopeptide ratio in the "light" sample relative to the "heavy" sample ¹⁴:

nonphosphopeptide ratio =
$$\frac{L_n}{H_n}$$

For the data in the example (i.e., in which the combined mole fraction of monophosphopeptide and multiphosphopeptide is high), one obtains:

nonphosphopeptide ratio =
$$\frac{L_n}{H_n} = \frac{0.90}{0.20} = 4.5$$

With this approach, one identifies a substantial difference in nonphosphopeptide abundance that is consistent with the true trend of phosphorylation. The main disadvantage of this method in screening for phosphorylation sites is that it presumes that a decrease in the mole fraction of nonphosphopeptide in the "heavy" sample is specifically due to phosphorylation, when in fact such a decrease could be due to any modification (or, alternatively, proteolytic processing in vivo, resulting in the loss of an N- or C-terminal region--although in our case, purified Mto2[17A]-GFP showed similar migration on SDS-PAGE for "heavy" and "light" samples). As a result, this approach is likely to result in the greatest number of "false-positive" results. However, if the approach is further validated by direct demonstration of phosphorylation of the relevant peptide or, as shown in this work, demonstrated changes in protein phosphorylation (i.e. by Phos-tag Western blot) after mutation of the relevant residues, then it can be useful as a "first-pass" screening tool to identify protein regions subject to post-translational modification. In addition, a key advantage of quantifying nonphosphopeptides is that the ratios obtained should be at least approximately related to the stoichiometry of modification (again, with the presumption that any measured difference is due to only one type of modification).

Thus, when a modification of interest is expected to be present at relatively high stoichiometry (as is suggested, in our case, from Western blot data comparing Mto2[17A] migration in interphase vs. mitosis), this second approach will likely result in the smallest number of "false-negative" results. Finally, it is worth noting more generally that when the only two forms of a given peptide are nonsphosphopeptide and monophosphopeptide (i.e. multiphosphopeptide mole fraction is very low or zero), then SILAC quantification of nonphosphopeptide and monophosphopeptide to determine phosphorylation site occupancy much more accurately ¹⁵.

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