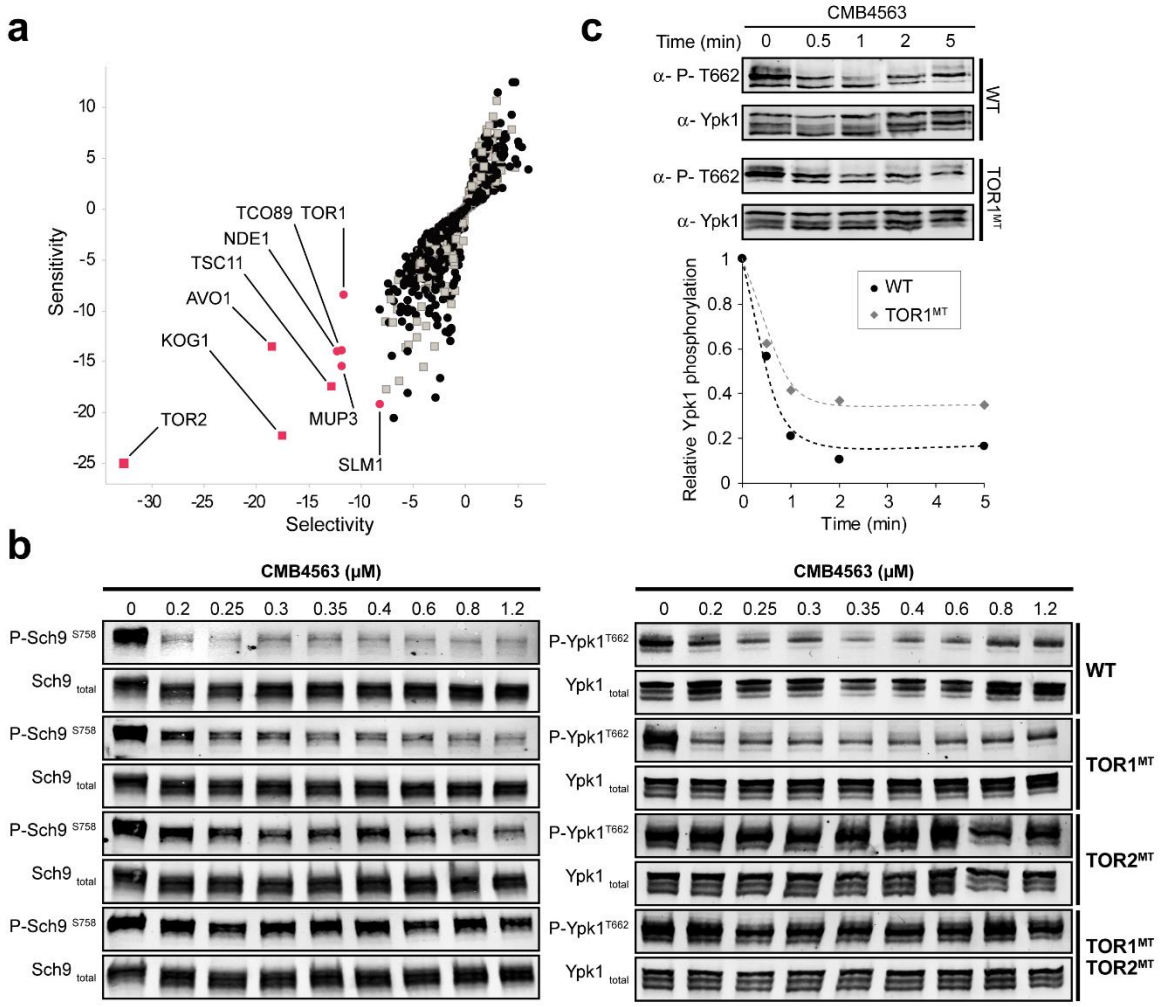
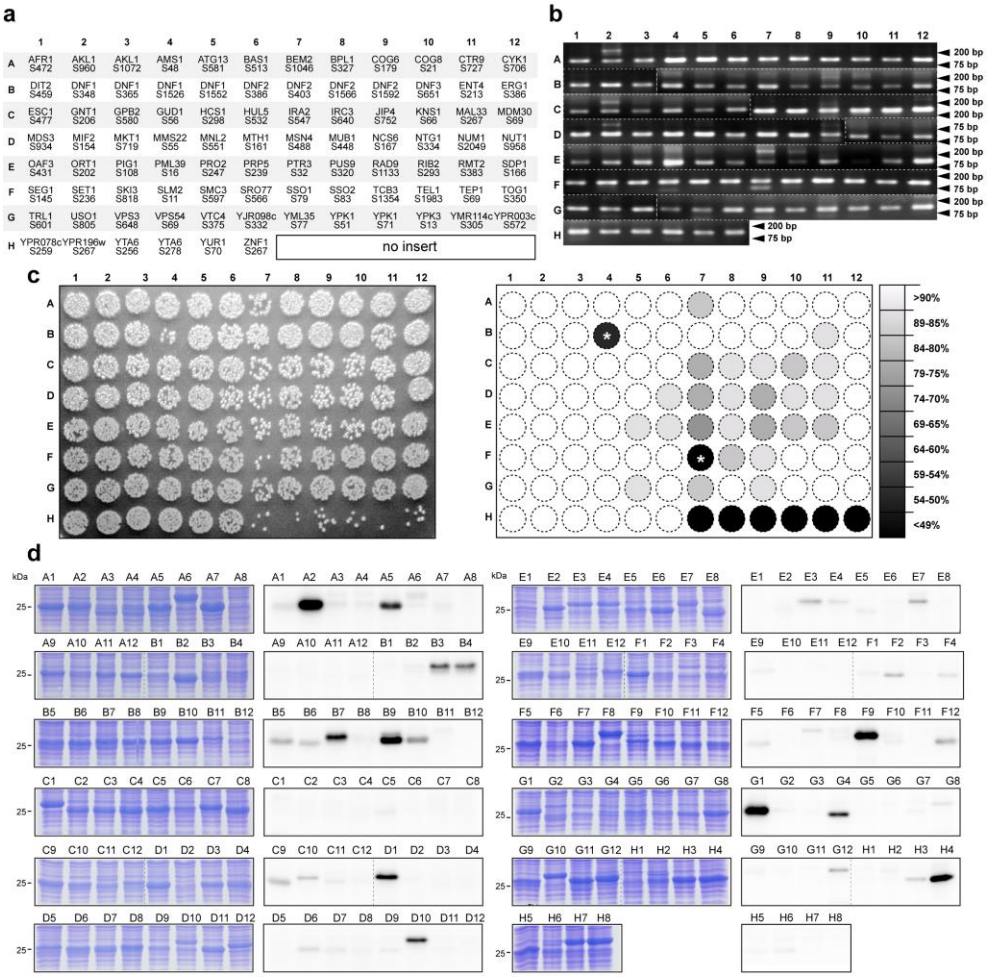
SUPPORTING INFORMATION

Supplemental figure 1



Supplemental figure 1: CMB4563 is a potent and fast inhibitor of TOR1 and TORC2. a, Heterozygous single deletion mutant strains covering 6200 genes (Open Biosystems) were grown as a pool in the presence or absence of 0.5 µM CMB4563 for 20 generations. The relative abundances of the individual strains were then compared. Sensitivity of individual deletion mutants are observed on the vertical axis and associated with a selectivity on the horizontal axis which was determined by statistical analysis to correct for how frequently particular mutants scored in multiple assays. The most selective and sensitive strains are found in the bottom left corner (1). **b,** Western blots assessing the respective activity of TORC1 and TORC2 after 12-minute CMB4563 treatments to cells of the indicated genotypes. The corresponding merge channels version is in Fig. 1d. TORC1 activity is followed by Sch9$^{P\text{-}S758}$ and TORC2 by Ypk1$^{P\text{-}T662}$ phosphorylation. **c,** Western blot and corresponding quantification assessing the kinetics of Ypk1^{T662} dephosphorylation in *TOR1*^{WT} and *TOR1*^{MT} strains with 0.8 µM CMB4563 treatment at indicated time points (above, minutes).

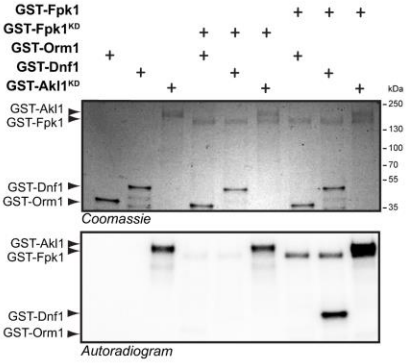
Supplemental figure 2



e

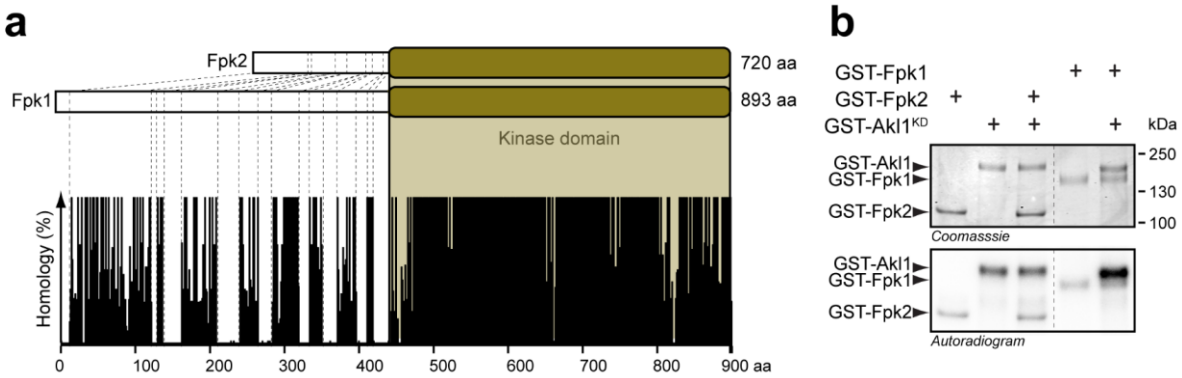
Protein	Function	Size (aa)	Motif	Putative Phospho-S Position (aa)	Detected S-Phospho
AKL1	Endocytosis	1108	ssvstrqpRqSLDlnfqevnl	960	YES
			mrksfaraRqSLDlervrrea	1072	YES
ATG13	Vesicles trafficking/Autophagy/TOR pathway factor	738	stsafnsrRnSLDkssnkqgm	581	YES
DNF1	Lipid transport	1571	*ktvgtsgpRsSLDsidsyrvs*	*348*	*NO*
			yrvsadygRpSLDydnleqga	365	YES
			grgpstnvRsSLDrtreqmia	1526	YES
			nrysveraRtSLDlpgvtnaa	1552	YES
DNF2	Lipid transport	1612	rnsrsfgpRgSLDsigsyrms	386	YES
			yrmsadfgRpSLDyenlnqtm	403	YES
			asargnklRtSLDrtreemla	1566	YES
DNF3	Lipid transport	1656	dapiekghRpSLDnfpksrns	651	YES
HCS1	DNA synthesis	683	*arlldsnkRhSLDilskknti*	*298*	*NO*
KNS1	TOR pathway factor	737	*nsfltdnlRnSLDlnqadnpl*	*66*	*NO*
MAL33	Maltose metabolism	468	*qpefvtdpRlSLDsffemirv*	*267*	*NO*
MDS3	TOR pathway factor	1487	enafsatpRaSLDgqmlgksl	934	YES
MTH1	Glucose sensing	433	*fpnhklitRvSLDealpktfy*	*161*	*NO*
MSN4	Transcription regulation	630	itptpafqRrSYDismnssfk	488	YES
NCS6	tRNA modification	359	*ctycgvfrRqSLDrgaaklgi*	*167*	*NO*
NTG1	DNA repair	399	*qflppddpRsSLDwdlqsqly*	*334*	*NO*
PTR3	Amino acids sensing	678	*ehdlylpqRlSLDqatqtdes*	*32*	*NO*
RAD9	DNA repair	1309	*llpsqesfRlSLDspskqgii*	*1133*	*NO*
SEG1	Eisosome	960	*rqkskthqRiSYDeaqrtfkd*	*145*	*NO*
SET1	Chromatin	1080	*rnslillpRiSYDryslgppp*	*236*	*NO*
SLM2	Membrane stress	656	msyqrnsaRaSLDlrsqyqql	11	YES
SSO1	Vesicles trafficking	290	neeqashlRhSLDnfvaqatd	79	YES
SSO2	Vesicles trafficking	295	*seeqemelRrSLDdyisqatd*	*83*	*NO*
TCB3	PI4P regulation	1545	lnstsvtpRaSLDydpsvpnt	1354	YES
TOG1	Fatty acid metabolism	794	*griistmsRrSLDaglnrwey*	*350*	*NO*
			snssvvpvRvSYDassststv	71	YES
YPR003c	Unkown function	754	*ddymmnmkRnSLDvegteeie*	*572*	*NO*
YTA6	Assymetric protein	754	erktnyksRaSLDnfssssks	255	YES
			kidnslikRrSLDvvrtsger	278	YES

f



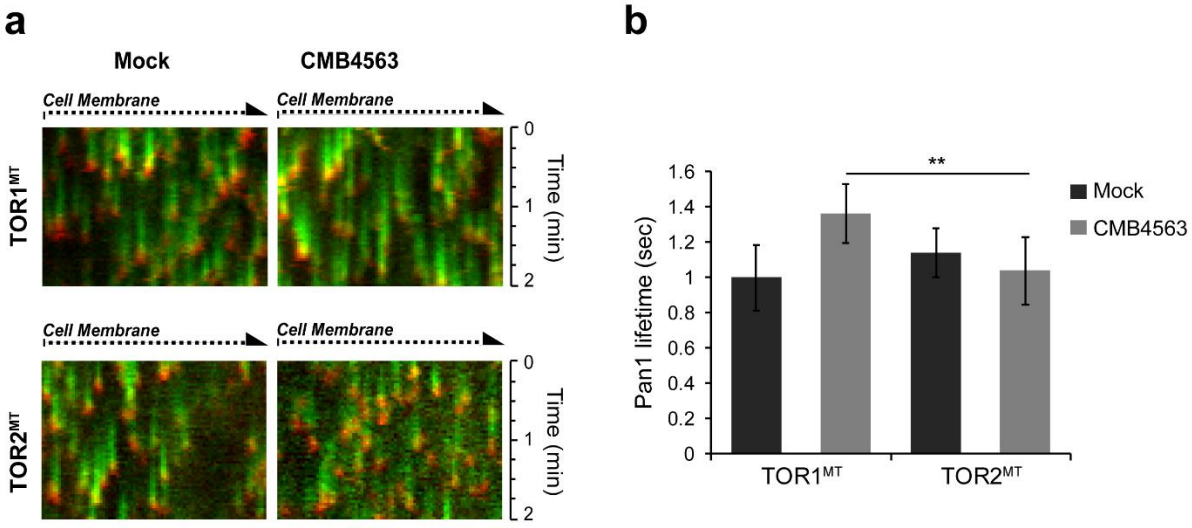
Supplemental figure 2: **Fpk1 substrate screen. a**, Organization of the 96-well-plate containing negative controls and the primer dimers corresponding to the 90 candidate peptide substrates. **b**, PCR products of the primer dimers analysed on 2% agarose gels **c,** Left Panel: Gibson assembly reactions were transformed into bacteria. 1/10 of the transformed cells were spotted onto LB plates containing kanamycin, including multiple negative controls corresponding to linearized vector without insert. Right Panel: Heat map reporting the cloning efficiency assessed with respect to the negative controls. The (*) indicated failed wells in which the cloning/transformation initially failed (these were subsequently repeated, successfully). **d**, Results of the initial *in vitro* kinase assays screen. Dashed lines define splicing of the gels. Bacterial lysates containing one of the 90 candidate peptides were individually incubated with GST-Fpk1 and ^{32}P-ATP for 30 min at 30°C. Reactants were subsequently resolved by SDS-PAGE, analysed by Coomassie stain (left panel) and autoradiography (right panel). **e**, Table recapitulating the peptides identified in both the initial and secondary screens (left) **f**, *In vitro* kinase assay assessing the ability of GST-Fpk1$^{WT/KD}$ to phosphorylate GST-Akl1^{KD}, GST-Orm1 (aa 1-85; negative control) or GST-Dnf1 (aa 1404-1571; positive control).

Supplemental figure 3:



Supplemental figure 3: Akl1 is not a target of Fpk2 *in vitro*. a, ClustalX alignments of Fpk1 and Fpk2 protein sequences. The green box represents the conserved kinase domain and the dashed lines delimit sequence insertions. **b,** Coomassie blue and autoradiogram SDS-PAGE images after *in vitro* kinase assay assessing the ability of Fpk1 and Fpk2 to phosphorylate Akl1. Dashed lines define splicing of the gel.

Supplemental figure 4



Supplemental figure 4: The inhibition of TORC1 does not cause delay the endocytosis process. a, Pan1-GFP and Abp1-mCherry kymographs were generated from *TOR1*^{MT} or *TOR2*^{MT} strains treated or not with CMB4563 for 30 min. **b**, Quantification of Pan1-GFP and Abp1-mCherry patch lifetimes determined from *TOR1*^{MT} or *TOR2*^{MT} cells as assessed in a. Bar graphs of Pan1-GFP lifetimes means, +/- standard deviation. Statistical parameters were extracted (Student's test: $P < 0.005$(**)) based on five patches/kymograph. Five kymographs were analysed.

Supplemental table 1

a

Forward primers

	1	2	3	4	5	6	7	8	9	10	11	12
A	TTTATTTTCAGGGC TCCATGCAGCCGCC GCCGCGCAGCAGC AAACGCCCGAGCC TGGATG	TTTATTTTCAGGGC TCCATGAGCAGCGT GAGCACCCGGCAG CCGCGCCAGAGCC TGGATCTGA	TTTATTTTCAGGGC TCCATGATGCGCAA AAGCTTCGCGCGC GCGCGCCAGAGCC TGGATCTGGA	TTTATTTTCAGGGC TCCATGCTGCCGAA ATTCTACGATAAAA AACGCATTAGCCTG GATCATG	TTTATTTTCAGGGC TCCATGAGCACCAG CGCGTTCAACAGCC GCCGCAACAGCCT GGATA	TTTATTTTCAGGGC TCCATGAGCGATAC CTTCAACCCGGAAT ACCGCACCAGCCT GGATA	TTTATTTTCAGGGC TCCATGCCGGCGA GTTCTACCGCGGCT GCGAGCTACGAGC CTGGATA	TTTATTTTCAGGGC TCCATGAAGTGCT GGAAGAAAACAAA ACCGCGCGAGCTA CGATG	TTTATTTTCAGGGC TCCATGAACCCGCTA CAGCGTGGAACGC GCGCGCGCGAGCC TGGATC	TTTATTTTCAGGGC TCCATGGATGCGCC GATTGAAAAAGGC CATGCGCCGAGCCT GGATA	TTTATTTTCAGGGC TCCATGGCGCTGG AAATTCTGCGCAAA GTGCGCGATAGCC TGGATA	TTTATTTTCAGGGC TCCATGGCGTTCAG CCGCGGCGCGCTG GTGCGCGAAAGCC TGGATC
B	TTTATTTTCAGGGC TCCATGCTGGCGG AAATGCTGAAACAG TTCCGCTGGAGCCT GGATC	TTTATTTTCAGGGC TCCATGAAAACCGT GGGCACCAGCGGC CCGCGCAGCAGCC TGGATAGCA	TTTATTTTCAGGGC TCCATGTACCGCGT GAGCGCGGATTAC GGCCGCCCGAGCC TGGATTACGA	TTTATTTTCAGGGC TCCATGGGCCGCG GCCGCGAGCACCAA CGTGCGCAGCA	TTTATTTTCAGGGC TCCATGAACGGCTA CAGCGTGGAACGC GCGCGCACCAGCC TGGATCTGCCGGG CGTGAC	TTTATTTTCAGGGC TCCATGCGCAACAG CCGCAGCTTCGGC CCGCGCGGCAGCC TGGATAGCAT	TTTATTTTCAGGGC TCCATGTACCGCAT GAGCGCGGATTTC GGCCGCCCGAGCC TGGATTACGA	TTTATTTTCAGGGC TCCATGACCACGAC ACTGCGCACCAGC CTGGAT	TTTATTTTCAGGGC TCCATGACCCGCTA CAGCGTGGAACGC GCGCGCGCGAGCC TGGATCTGCCGGG CATT	TTTATTTTCAGGGC TCCATGGATGCGCC GATT	TTTATTTTCAGGGC TCCATGACCCGCAA CACCCATGAATACG CGCGCTTCAGCCTG	TTTATTTTCAGGGC TCCATTGGAACTGCT GGATTACCATTTCG AACGCAAAAGCTAC GATAGCG
C	TTTATTTTCAGGGC TCCATGGAACCGG AAAAAGATGATATT ATTCGCAGCAGCCT GGATA	TTTATTTTCAGGGC TCCATGTACCTGGA TAACGATGCGATTC TGCGCAGCAGCCT GGAT	TTTATTTTCAGGGC TCCATGTGCATGG CACCAGCCTGATG GATCGCAGCAGCC TGGATA	TTTATTTTCAGGGC TCCATGAACGGCAT TATTCGCTTCGTGA ACCGCAACAGCCT GGATC	TTTATTTTCAGGGC TCCATGAACGGCCT GCTGGATAGCAAC AAACGCCATAGCCT GGAT	TTTATTTTCAGGGC TCCATGATGTTCAT TGCGCTGGATAAAA AGCCGCTGAGCCT GGATG	TTTATTTTCAGGGC TCCATGGCTACCAC CGCGGAACAGCAG AGCCGCGCGAGCT ACGATGCGCAT	TTTATTTTCAGGGC TCCATGGGCAAAA GCAGCAAAGATTTC ATTCGCCTGAGCCT GGATG	TTTATTTTCAGGGC TCCATGAGCGAGCG CGCGCGGCAACAA ACTGCGCACCAGC CTGGAT	TTTATTTTCAGGGC TCCATGACCCGCTA CAGCGTGGAACGC GCGCGCGCGAGCC TGGATCTGCCGGG CTGGATA	TTTATTTTCAGGGC TCCATGCAGCCGG TGGGCGCGATTAA AAGCCGCCATAGC CTGGATA	TTTATTTTCAGGGC TCCATGGCGCTAC CGATCGCTGGATTA ACCGCGAAAGCCT GGATC	TTTATTTTCAGGGC TCCATGGGCGTGC CGGGCAGCGAAAA CAAACGCGGCAGC CTGGATA
D	TTTATTTTCAGGGC TCCATGGAAACGC GTTCAGCGCGACC CCGCGCGCGAGCC TGGATGGCCAGAT	TTTATTTTCAGGGC TCCATGACCTACAA ACGCAAATACAGCA CCCGCTACAGCCTG GATA	TTTATTTTCAGGGC TCCATGAGCCTGAC CAGCGGCGAATTC AACCGCCTGAGCCT GGATA	TTTATTTTCAGGGC TCCATGCGCAGCCA GAAAATTGTGACCG AACGCCTGAGCCT GGATA	TTTATTTTCAGGGC TCCATGACCAACGA ACTGGATGTGACCA TTCGCAAAAGCTAC GATGTGAGCCTG	TTTATTTTCAGGGC TCCATGTTCCCGAA CCATAAACTGATTA CCCGCGTGAGCCT GGAT	TTTATTTTCAGGGC TCCATGATTACCCC GACCCCGGCGTTC CAGCGCCGCAGCT ACGATA	TTTATTTTCAGGGC TCCATGAAAATTAG CGATAACTTCGAAC GCCGCTGGAGCTA CGATA	TTTATTTTCAGGGC TTCCATGTGCACCTA CTGCGGCGTGTTCC GCCGCCAGAGCCT GGATCGCGGCGCG GCGAAACT	TTTATTTTCAGGGC TCCATGCAGTTCCT GCCGCCGGATGAT CCGCGCAGCAGCC TGGATTGGGA	TTTATTTTCAGGGC TCCATGAGCGAACT GATCGCCCGAGCC TGGAT	TTTATTTTCAGGGC TCCATGGGCGTGC CAAACGCGGCAGC CTGGATA
E	TTTATTTTCAGGGC TCCATGAGCTTCAA AATTAACCGCCATA CCCGCCCGAGCTA CGATA	TTTATTTTCAGGGC TCCATGATTGTGAA AAAAAGCCTGAAA GATCGCCATAGCCT GGATG	TTTATTTTCAGGGC TCCATGCCGTTCAT TGGCGGCGAAGAT CATCGCTACAGCCT GGATA	TTTATTTTCAGGGC TCCATGCTGGAAGT GCGCCTGAAAGC ATTCGCCATAGCCT GGAT	TTTATTTTCAGGGC TCCATGGATGCGG ATCTGATTAAAGCG AAACGCATTAGCCT GGATGC	TTTATTTTCAGGGC TCCATGAGCGAAAT CTGTACCTGCCGC AGCGCCTGAGCCT GGATA	TTTATTTTCAGGGC TCCATGGAACATGA TCTGTACCTGCCGC AGCGCCTGAGCCT GGATGGCGCGACC GGCACCG	TTTATTTTCAGGGC TCCATGAAACATGC GAGCGGCGAAAGC CACCAGCATTAGCTAC GATGG	TTTATTTTCAGGGC TCCATGCTGCTGCC GAAAACCGTGTTCA TTCCGCCTGAGCCT GGATA	TTTATTTTCAGGGC TCCATGAAACATGC GAAAACCGTGTTCC AGCGCGTGAGCTA CGATG	TTTATTTTCAGGGC TCCATGACGGCAT CCCGCTACAGCCTG GATG	TTTATTTTCAGGGC TCCATGATGAAATA CCATAACCTGGACC TGCGCCATAGCTAC GATC	TTTATTTTCAGGGC TCCATGATGAAATA CCATAACCTGGACC TGCGCCATAGCTAC GATC
F	TTTATTTTCAGGGC TCCATGCGCCAGAA AAGCAAAACCCATC AGCGCATTAGCTAC GATG	TTTATTTTCAGGGC TCCATGCGCAACAG CCTGATTCTGCTGC CGCGCATTAGCTAC GATC	TTTATTTTCAGGGC TCCATGATTGGCCT GGTGGAAGTGCTG ATGCGCTGCAGCCT GGATC	TTTATTTTCAGGGC TCCATGATGGACTA CCAGCGCAACAGC GCGCGCGCGAGCC TGGATCTGCGCAG CCAGT	TTTATTTTCAGGGC TTCCATTCCGCTGA ACCGCCTGAGCCT GGATA	TTTATTTTCAGGGC TCCATGGATGCGCT GCAGCTGAAATCA GCCGCTTCAGCCTG GATG	TTTATTTTCAGGGC TCCATGAACGAAGA ACAGGCGAGCCAT CTGCGCCATAGCCT GGATA	TTTATTTTCAGGGC TCCATGAGCGAAG AACAGGAAATGGA ACTGCGCCGCAGC CTGGATGATT	TTTATTTTCAGGGC TCCATGACTGAAG CACCAGCGTGACC CCGGCGGCGAGCC TGGATTACGA	TTTATTTTCAGGGC TCCATGCATACCCT GATGAACGTGCTG AGCCGCAACAGCC TGGATG	TTTATTTTCAGGGC TCCATGAACACCTA CCCGAAACTGCTGT ACCGCAACAGCCT GGATG	TTTATTTTCAGGGC TCCATGGGCCGCAT TATTAGCCATGAC GCCGCCGCAGCCT GGATGCGGGCCTG AACCG
G	TTTATTTTCAGGGC TCCATGGATGATGA AATTGAAACCGCGT TCCGCCGCAGCCT GGATT	TTTATTTTCAGGGC TCCATGCTGGAAAC CGAACTGAAAAAC GTGCGCGATAGTC TGGATG	TTTATTTTCAGGGC TCCATGACCATTAC CGTGGATATGTTCG AACGCGAAAGCTA CGATG	TTTATTTTCAGGGC TCCATGACCCCGCG GCGCGCGCCGCAGC AGCCGCCTGAGCC TGGATA	TTTATTTTCAGGGC TCCATGCAGCTGCC GGGCGATGCGCGC GTGCGCATTAGCCT GGATA	TTTATTTTCAGGGC TCCATGGTGCAGCA TATTGGCTACGATT GGCGCCTGAGCCT GGATA	TTTATTTTCAGGGC TCCATGCCGGAACA TATTGATGAAATTT CCGCATGAGCTAC GATTTC	TTTATTTTCAGGGC TCCATGGGCGAAC ATGATGCGAAGCTT ACCCGCAGCAGCC TGGAT	TTTATTTTCAGGGC TCCATGAGCAACAG CAGCGTGGTGCCG GTGCGCGTGAGCT ACGATG	TTTATTTTCAGGGC TCCATGTTCAGCCT GGATGAAGAACTG CATCGCGTGAGCCT GGATG	TTTATTTTCAGGGC TCCATGTTCAACCA CATGATGAACATGA AACGCAACAGCTA CGATGG	TTTATTTTCAGGGC TCCATGGATGATTA CATGATGAACATGC AACGCAACAGCCT GGATG
H	TTTATTTTCAGGGC TCCATGACCCTGCT GAAATACCGCTACG TGCGCACCAGCCT GGATA	TTTATTTTCAGGGC TCCATGCAGCCGG AAATTGTGACCGAT AGCCGCCTGAGCC TGGATA	TTTATTTTCAGGGC TCCATGGAACGCAA AACCAACTACAAAA GCCGCGCGAGCCT GGATAACT	TTTATTTTCAGGGC TCCATGAAAATTGA TAACAGCCTGATTA AACGCCGCAGCCT GGATG	TTTATTTTCAGGGC TCCATGTACTTCCC GAAAGTGAAACTG AGCCGCAACAGCT ACGATG	TTTATTTTCAGGGC TCCATGCAGCCGG AATTCGTGACCGAT CCGCGCCTGAGCC TGGATAGCT						

b

Reverse primers

	1	2	3	4	5	6	7	8	9	10	11	12
A	AATTCGGATCCCCG ATATCCTTAGAAGT AGCGCGCGGCTTTC GTTATCATCCAGGC TCGGGCGTTT	AATTCGGATCCCCG ATATCCTTACAGGT TCACTTCCTGGAAG TTCAGATCCAGGCT CTGGCG	AATTCGGATCCCCG ATATCCTTACGCTT CGCGGCGCACGCG TTCCAGATCCAGGC TCTGGC	AATTCGGATCCCCG ATATCCTTACCACC ACACTTTCAACATGA TCATGATCCAGGCT AATGCG	AATTCGGATCCCCG ATATCCTTACATGC CCTGTTTGTTGCTG CTTTTATCCAGGCT GTTGCGGCG	AATTCGGATCCCCG ATATCCTTAGCTCA GGAAATCGCTATCC ATGTTATCCAGGCT GGTGCGGTA	AATTCGGATCCCCG ATATCCTTAGGTTTT GCTCAGGCGGCCG GTGGATCCAGGCT ATCGCGCAT	AATTCGGATCCCCG ATATCCTTAATGCA GCAGGCTGCTGCT CGCCGCATCGTAG CTGCGCGCGGTA	AATTCGGATCCCCG ATATCCTTATTCCG GGTTCATCAGTTTC TGTTCATCCAGGCT CAGGCGTTC	AATTCGGATCCCCG ATATCCTTAGCTCT GCAGAATATCCTGC AGGAAATCCAGGC TCAGGCGTTT	AATTCGGATCCCCG ATATCCTTACAGGT TCAGCTGCACATCT TCGTTATCCAGGCT ATCGCGCAC	AATTCGGATCCCCG ATATCCTTAGCGTT TTCTGATCCAGGCT TTCGCGCAC
B	AATTCGGATCCCCG ATATCCTTAGGTCA GTTTTTCTTCCCATT CCGGATCCAGGCT CCAGCGGAA	AATTCGGATCCCCG ATATCCTTAGCTCA CGCGGTAGCTATCA ATGCTATCCAGGCT GCTGCG	AATTCGGATCCCCG ATATCCTTACGCGC CCTGTTCCAGGTTA TCGTAATCCAGGCT CGGGC	AATTCGGATCCCCG ATATCCTTACGCAA TCATCTGTTCGCGG GTGCGATCCAGGC TGCTGCGCACGTTG GTGCT	AATTCGGATCCCCG ATATCCTTACGCCG CGTTGGTCACGCCC GGCAGATCCA	AATTCGGATCCCCG ATATCCTTAGCTCA TGCGGTAGCTGCC AATGCTATCCAGGC TGCCGC	AATTCGGATCCCCG ATATCCTTACATGT TCTGTTCAGGTTT TCGTAATCCAGGCT CGGGC	AATTCGGATCCCCG ATATCCTTACGCCA GCATTTCTTCGCGG CATGGTTAATGCCC GTGGGATCCAGGC TGGTGCGCAGT	AATTCGGATCCCCG ATATCCTTATTCCG TGCGGCTTTTCGGG AAGTTATCCAGGCT GCGGATCCAGGCT GGGCAGATCCAGG	AATTCGGATCCCCG ATATCCTTAGCTGT TGCGGCTTTTCGGG AAGCTATCCAGGCT GCGGATCCAGGCT GAAGCGCGCGTAT	AATTCGGATCCCCG ATATCCTTATTCGCT ATATCCTTACATGC TCAGCACGTTAATC ACGCTATCGTAGCT TTTGCG	AATTCGGATCCCCG ATATCCTTACATGC TCAGCACGTTAATC ACGCTATCGTAGCT TTTGCG
C	AATTCGGATCCCCG ATATCCTTAGTTGTT GTTGCCATGGAAGT TTTTATCCAGGCTG CTGCGAAT	AATTCGGATCCCCG ATATCCTTAGTAGT TCGGCAGGAAGAA CAGTTCATCCAGGC TGCTGCGCAGA	AATTCGGATCCCCG ATATCCTTATTTCAG GTTGTTCAGCAGGC GCATATCCAGGCTG CTGCGATC	AATTCGGATCCCCG ATATCCTTAATGAT CCAGGCAATCTTTC GAATATCCAGGCTA TGTTGCGGTT	AATTCGGATCCCCG ATATCCTTAATGG TGTTTTTTTTGCTCA GAATATCCAGGCTA TGGCGTTTG	AATTCGGATCCCCG ATATCCTTACATGT CATCATCCAGGCTC AGGCGTTT	AATTCGGATCCCCG ATATCCTTAGTAGA AGGCTGTTCATCGC CGCG	AATTCGGATCCCCG ATATCCTTATTTGAA GCTGCTGTTCATGC TAATATCGTAGCTC GCGCCATC	AATTCGGATCCCCG ATATCCTTACTGCT TCACGTAGGTGGTC GCTGCTGATCGCCC GGGCTATCCAGGC CAGGCGAAT	AATTCGGATCCCCG ATATCCTTACAGCG GCTGCTGATCGCCC GGGCTATCCAGGC TATGGCGGCT	AATTCGGATCCCCG ATATCCTTAGTAGA GCTGCGCTGTCGCAG GCACATCCAGGCTC GTTGCGCAG	AATTCGGATCCCCG ATATCCTTAATCG GCACATCCAGGCTC AAGCTATCCAGGCT TCGCTATCCAGGCT AGAATATCCAGGCT TTCGCGGTT	AATTCGGATCCCCG ATATCCTTAATCG GCACATCCAGGCTC AAGCTATCCAGGCT TCGCTATCCAGGCT AGAATATCCAGGCT TTCGCGGTT
D	AATTCGGATCCCCG ATATCCTTAGCAGC TTTTGCCCAGCATC TGGCCATCCAGGCT CG	AATTCGGATCCCCG ATATCCTTAGCGCA CGCTCGGGCTTTCG CTGGTATCCAGGCT GTAGCGGGT	AATTCGGATCCCCG ATATCCTTAAATTT GCGCGCCCAGCCG GTGCTATCCAGGCT AAGTTATCCAGGCT CAGGCGGTT	AATTCGGATCCCCG ATATCCTTAGGTGC GCTCACATCGTAGC CAGGCGTTC	AATTCGGATCCCCG ATATCCTTACAGGC GGCAGCTGTACAG GCTTCATCCAGGCT TT	AATTCGGATCCCCG ATATCCTTAGTAGA AGGCTTGTTCATGC CACGCGGGGTA	AATTCGGATCCCCG ATATCCTTATTTGAA GCTGCTGTTCATGC TAATATCGTAGCTC CGGCGCTG	AATTCGGATCCCCG ATATCCTTAGTTG CAGCGGCG	AATTCGGATCCCCG ATATCCTTAATAT ATCCAGTTTCGCCGCG CCGCGAT	AATTCGGATCCCCG ATATCCTTAGTAA TGCTGC	AATTCGGATCCCCG ATATCCTTAATCG GCACATCCAGGCTC ATCCCAATCCAGGC GGGCGATCA	AATTCGGATCCCCG ATATCCTTAATCG GCACATCCAGGCTC TCGCTATCCAGGCT GCCGCGTTT	AATTCGGATCCCCG ATATCCTTAATCG GCACATCCAGGCTC TCGCTATCCAGGCT GCCGCGTTT
E	AATTCGGATCCCCG ATATCCTTAAATGT AATCGCTCGGTTTC ATAATATCGTAGCT CGGGCGGGT	AATTCGGATCCCCG ATATCCTTATTTGCT TTCATCGCGTTTCG GATCATCCAGGCTA TGGCGATC	AATTCGGATCCCCG ATATCCTTATTCCA AGAATATCCAGGCT GTAGCGATG	AATTCGGATCCCCG ATATCCTTAGCCCG TATGGCGAATG	AATTCGGATCCCCG ATATCCTTAGCCCG CCGGGTAGTTGGTT TTCGCATCCAGGCT AATGCGTT	AATTCGGATCCCCG ATATCCTTAGCCCG TGCCTTAATTTTAA TGTTATCCAGGCTC AGGCGCAG	AATTCGGATCCCCG ATATCCTTAGCCCG GAAGTTATCCAGGC TATGGCGCAG	AATTCGGATCCCCG ATATCCTTAGCATTT CACAATGCTGGTTT TGCCATCGTAGCTA ATGCGGT	AATTCGGATCCCCG ATATCCTTAAATAAT GCCGCCTTGCTCG GGCTATCCAGGCTC ATGCGGT	AATTCGGATCCCCG ATATCCTTAGCATTT CACAATGCTGGTTT GGCCATCGTAGCTG CGTTCATCCAGGCTG TAGCGGGT	AATTCGGATCCCCG ATATCCTTAATCGTT CGCGCGGCTTTTCA GCACATCCAGGCTC GTTCATCCAGGCTG TGCGTTCA	AATTCGGATCCCCG ATATCCTTATTTATC CGCGCGGCTTTTCA GCAGATCGTAGCTA TGCGCATCA
F	AATTCGGATCCCCG ATATCCTTAATCTTT GAAGGTGCGCTGC GCTTCATCGTAGCT AATGCGCTG	AATTCGGATCCCCG ATATCCTTACAGCA GCGGGCCCAGGCT GTAGCGATCGTAG CTAATGCGCGG	AATTCGGATCCCCG ATATCCTTACAGCA GGAAGCCCTGGCT GTACAGATCCAGG CTGCAGCGCAT	AATTCGGATCCCCG ATATCCTTAGTTGC GCTGGTACTGGCT GCGCAGATCCAG	AATTCGGATCCCCG ATATCCTTAGTTGC TCGGGAATTTCACA TCGCTATCCAGGCT AATGCGCAC	AATTCGGATCCCCG ATATCCTTAATCCA CCAGAATGGTTTTG CTATCATCCAGGCT CAGGCGGCTA	AATTCGGATCCCCG ATATCCTTAATCGG CTGCTCGCTGCCAC GAAGTTATCCAGGC TATGGCGCAG	AATTCGGATCCCCG ATATCCTTAATCGG TCGCCTGGCTAATG TAATCATCCAGGCT GCGGCG	AATTCGGATCCCCG ATATCCTTAGTAGT TCGGCAGCGTCGG CTCGCATCGTAGCT CACGCGCAC	AATTCGGATCCCCG ATATCCTTAGTATT TCGGCAGCTGTTC CTCGCATCGTAGCT TCGCGC	AATTCGGATCCCCG ATATCCTTACATGT GCTGCATTCGTTTT ATCATCCAGGCTA CGCGATG	AATTCGGATCCCCG ATATCCTTAGTATTC TCAGGAACAGAATC AGATCATCCAGGCT GTTGCGGTA	AATTCGGATCCCCG ATATCCTTAGTATTC CCAGCGGTTCAGG CCCGCATCCA TTGCGTTT
G	AATTCGGATCCCCG ATATCCTTAAATTTT GCGCACGGTCGGT TTGTAATCCAGGCT GCGGCGGAA	AATTCGGATCCCCG ATATCCTTACACAT CGCGCAGCTGGGT CATTTCATCCAGGC TATCGCGCAC	AATTCGGATCCCCG ATATCCTTACAGGT TAATTTCGCGAATA ATTTCATCGTAGCT TTCGCGTTC	AATTCGGATCCCCG ATATCCTTAGAAGC TGCGGCGCGGGGT AATGCTATCCAGGC TCAGGCGGCT	AATTCGGATCCCCG ATATCCTTATTCGC GCACCATGGTCAGT TCGGTATCCAGGCT GTTGCGGCT	AATTCGGATCCCCG ATATCCTTAGGTGG TCAGATGTTCGCG CTAATATCCAGGCT ATGCGG	AATTCGGATCCCCG ATATCCTTATTTGCT GCTGCGTGTTCCA TTGCGATCCAGGCT GCTGCGGGTA	AATTCGGATCCCCG ATATCCTTAGCTCG GGTTAATGGTGCCT TTGCGATCCAGGCT CACGCGCAC	AATTCGGATCCCCG ATATCCTTACACGG CTCGCATCGTAGCT TCGCGC	AATTCGGATCCCCG ATATCCTTACATTT TTCGTTTTTTTTCAG GCCATCGTAGCTGT CCACATCCAGGCTG TTGCGTTT	AATTCGGATCCCCG ATATCCTTATTAAT TTCTTCGGTGCCTT GCCATCGTAGCTGT CCACATCCAGGCTG TTGCGTTT
H	AATTCGGATCCCCG ATATCCTTAGCTGC TTTTTTCGCTGCCC AGCATATCCAGGCT GGTGCGCAC	AATTCGGATCCCCG ATATCCTTACACTTT GATCACTTCCAGGA AGCTATCCAGGCTC AGGCGGCT	AATTCGGATCCCCG ATATCCTTAGCTTTT GCTGCTGCTGCTGA AGTTATCCAGGCTC GCGCG	AATTCGGATCCCCG ATATCCTTAGCGTT CGCCGCTGGTGCG CACCACATCCAGGC TGCGGCGTTT	AATTCGGATCCCCG ATATCCTTAGCGGG TGTAGTTCAGGGTG TAATCATCGTAGCT GTTGCGGCT	AATTCGGATCCCCG ATATCCTTACACGC GAATCATTTCGAAG AAGCTATCCAGGCT CAGGCG						

Supplemental table 1: **List of primers used for the Fpk1 substrate library. a**, Forward primers organized following the order found in Supp. Fig1a. The consensus motif sequence is surrounded by 5 codons unique for each peptide. **b**, Corresponding reverse primers.

Supplemental table 2

Protein	Function	Size (aa)	Motif	Putative Phospho-S Position	Detected S-Phospho
AFR1	Shmoo formation	620	qpprsskRpSLDdnesaryf	472	YES
AKL1	Endocytosis	1108	ssvstrqpRqSLDlnfqevnl	960	YES
			mrksfaraRqSLDlervrrea	1072	YES
AMS1	Mannose metabolism	1083	lpkfydkkRiSLDhdhvkvww	48	NO
ATG13	Vesicles trafficking/Autophagy/TOR pathway factor	738	stsafnsrRnSLDkssnkqgm	581	YES
BAS1	Purine/histidine metabolism	811	sdtfnpeyRtSLDnmdsdfls	513	NO
BEM2	Cytokeleton organisation	2167	patpnqkmRdSLDttgrlskt	1046	YES
BPL1	Biotinylation	690	sfqfyrgyRaSYDaasssllh	327	NO
COG6	Vesicles trafficking	839	kvleenkeRlSLDeqklmnpe	179	NO
COG8	Vesicles trafficking	607	ddlteeqkRlSLDflqdilqs	21	NO
CTR9	Transcription	1077	aleilrkvRdSLDnedvqlnl	727	NO
CYK1	Budding	1495	afsrgalvReSLDqkssfykr	706	NO
DIT2	Sporulation	489	laemlkqfRwSLDpeweeklt	459	NO
DNF1	Lipid transport	1571	ktvgtsgpRsSLDsidsyrvs	348	NO
			yrvsadygRpSLDydnleqga	365	YES
			grgpstnvRsSLDrtreqmia	1526	YES
			nrysveraRtSLDlpgvtnaa	1552	YES
DNF2	Lipid transport	1612	rnsrsfgpRgSLDsigsyrms	386	YES
			yrmsadfgRpSLDyenlnqtm	403	YES
			asargnklRtSLDrtreemla	1566	YES
			trysveraRaSLDlpginhae	1592	YES
DNF3	Lipid transport	1656	dapiekghRpSLDnfpksrns	651	YES
ENT4	Trafficking	247	trntheyaRfSLDplaeedse	213	NO
ERG1	Ergosterol metabolism	496	elldyhfeRkSYDsvinvlsv	386	NO
ESC1	Telomeres	491	epekddiiRsSLDknfhgnnn	477	NO
GNT1	Glycans modifications	491	yldndailRsSLDeilfflpny	206	NO
GPB2	PKA inhibitor	880	vhgtslmdRsSLDmrllnnlk	580	NO
GUD1	Guanine metabolism	489	ngiirfvnRnSLDpvkdcldh	56	NO
HCS1	DNA synthesis	683	arlldsnkRhSLDilskknti	298	NO
HUL5	Ubiquitin/Proteasome	910	mfialdkkRlSLDddhnlinm	532	NO
IRA2	cAMP metabolism	3079	gytaeqqsRaSYDahktghtg	547	NO
IRC3	DNA synthesis	689	gesskdfiRlSLDdvttyvnt	640	NO
JIP4	Unkown function	876	aalgalksRhSLDspgdqqqq	752	NO
KNS1	TOR pathway factor	737	nsfltdniRnSLDinqadnpl	66	NO
MAL33	Maltose metabolism	468	qpefvtdpRlSLDsffemirv	267	NO
MDM30	Ubiquitin/Proteasome	598	rcydrwinReSLDiltgndyd	69	NO
MDS3	TOR pathway factor	1487	enafsatpRaSLDgqmlgks1	934	YES
MIF2	Kinetochore	549	tykrkystRySLDtsespsvr	154	YES
MKT1	Transcription regulation	830	sltsgefnRlSLDnfgwarki	719	NO
MMS22	Ubiquitin/Proteasome	1454	rsqkivteRlSLDstagesct	55	NO
MNL2	Mannose metabolism	849	tneldvtiRkSYDvslyscrl	551	NO

Protein	Function	Size (aa)	Motif	Putative Phospho-S Position	Detected S-Phospho
MTH1	Glucose sensing	433	fpnhklitRvSLDealpktfy	161	NO
MSN4	Transcription regulation	630	itptpafqRrSYDismnssfk	488	YES
MUB1	Ubiquitin/Proteasome	620	kisdnferRwSYDkmkkeltn	448	NO
NCS6	tRNA modification	359	ctycgvfrRqSLDrgaaklgi	167	NO
NTG1	DNA repair	399	qflppddpRsSLDwdlqsqly	334	NO
NUM1	Organel migration	2748	selrdqidRpSLDvlkekaai	2049	NO
NUT1	Transcription regulation	1132	gvpgsenkRgSLDsehvfdyf	958	NO
OAF3	Oleate metabolism	863	sfkinrhtRpSYDimkpsdyi	431	NO
ORT1	Arginine metabolism	292	ivkkslkdRhSLDdpkrdesk	202	NO
PIG1	Glycogen metabolism	648	pfiggedhRySLDildysdie	108	NO
PML39	RNA maturation	334	levrlksiRhSLDkntkllpg	16	NO
PRO2	Proline metabolism	456	dadlikakRiSLDaktnypag	247	NO
PRP5	RNA maturation	849	semeveelRlSLDnikikgtg	239	NO
PTR3	Amino acids sensing	678	ehdlylpqRlSLDgatgtdes	32	NO
PUS9	tRNA modification	462	khaktvfnRiSYDgktsivkc	320	NO
RAD9	DNA repair	1309	lipsgesfRlSLDspskggii	1133	NO
RIB2	tRNA modification	591	khaktvfqRvSYDqqtsivkc	293	NO
RMT2	Translation regulation	412	ygmkcdytRySLDeqlpdwnd	383	NO
SDP1	Slt2 phosphatase regulation	209	mkyhnlslRhSYDilksradk	166	NO
SEG1	Eisosome	960	rqkskthqRiSYDeaqrtfkd	145	NO
SET1	Chromatin	1080	rnsliilpRiSYDryslgppp	236	NO
SKI3	RNA degradation	1432	iglvevimRcSLDlysqgfil	818	NO
SLM2	Membrane stress	656	msyqrnsaRaSLDlrsqyqql	11	YES
SMC3	Mitosis	1230	rvtfiplnRlSLDsdvkfpsn	597	NO
SRO77	Vesicles trafficking	1010	dalglkfsRfSLDdsktilvd	566	NO
SSO1	Vesicles trafficking	290	neeqashlRhSLDnfvaqatd	79	YES
SSO2	Vesicles trafficking	295	seeqemelRrSLDdyisqatd	83	NO
TCB3	PI4P regulation	1545	lnstsvtpRaSLDydpsvpnt	1354	YES
TEL1	Telomeres	2787	htlmnvlsRnSLDenvkcsky	1983	NO
TEP1	PI3P Phosphatase	434	ntypkllyRnSLDdilfllv	69	NO
TOG1	Fatty acid metabolism	794	grilstmsRrSLDaglnrwey	350	NO
TRL1	tRNA modification	827	ddeietafRrSLDykptvrki	601	NO
USO1	Vesicles trafficking	1790	letelknvRdSLDemtqlrdv	805	NO
VPS3	Vesicles trafficking/Vacuole	1011	titvdmfeReSYDelireini	648	NO
VPS54	Protein recycling	889	tprarrssRlSLDsitprrsf	69	YES
VTC4	Vesicles trafficking/Vacuole	721	qlpqdarvRiSLDteltmvre	375	NO
YJR098c	Unkown function	656	vqqfgydwRlSLDlsakhltt	332	NO
YML35	Translation regulation	367	pehidelfRmSYDflegrssk	77	NO
YPK1	TOR pathway factor	680	gehdasitRsSLDrkgtinps	51	YES
			snssvvpvRvSYDassts tv	71	YES
YPK3	TOR pathway factor	525	fsldeelhRvSLDdkknd ikv	13	NO
YMR114C	Unkown function	368	fnqkkslkRnSYDglkkneeq	305	YES
YPR003c	Unkown function	754	ddymmnmkRnSLDvegteeie	572	NO
YPR078c	Unkown function	372	tllkyryvRtSLDmlgsekss	259	NO
YPR196w	Unkown function	470	qpeivtdsRlSLDsflevikv	267	NO
YTA6	Assymetric protein	754	erktnyksRaSLDnfssssks	255	YES
			kidnslikRrSLDvvrtsger	278	YES
YUR1	Protein modification	428	yfpkvklsRnSYDdytlnytr	70	NO
ZFN1	Respiratory transition	465	qpefvtdpRlSLDsffemirv	267	NO

Supplemental table 2: **Table recapitulating all the peptides containing one or more RXSLD/E motif(s) in the *Saccharomyces Cerevisae* proteome.** The table provides (from the left to the right) the name of the protein, its function, its length in amino acids (aa), the sequence used for cloning i.e. the consensus motif surrounded by 5 aa on both sides, the position(s) in the sequence of the serine (S) present in the consensus motif, and whether this motif was phosphorylated *in vitro*.

REFERENCE

1. Hoepfner, D., Helliwell, S. B., Sadlish, H., Schuierer, S., Filipuzzi, I., Brachat, S., Bhullar, B., Plikat, U., Abraham, Y., Altorfer, M., Aust, T., Baeriswyl, L., Cerino, R., Chang, L., Estoppey, D., Eichenberger, J., Frederiksen, M., Hartmann, N., Hohendahl, A., Knapp, B., Krastel, P., Melin, N., Nigsch, F., Oakeley, E. J., Petitjean, V., Petersen, F., Riedl, R., Schmitt, E. K., Staedtler, F., Studer, C., Tallarico, J. A., Wetzel, S., Fishman, M. C., Porter, J. A., and Movva, N. R. (2014) High-resolution chemical dissection of a model eukaryote reveals targets, pathways and gene functions. Microbiol Res 169, 107-120