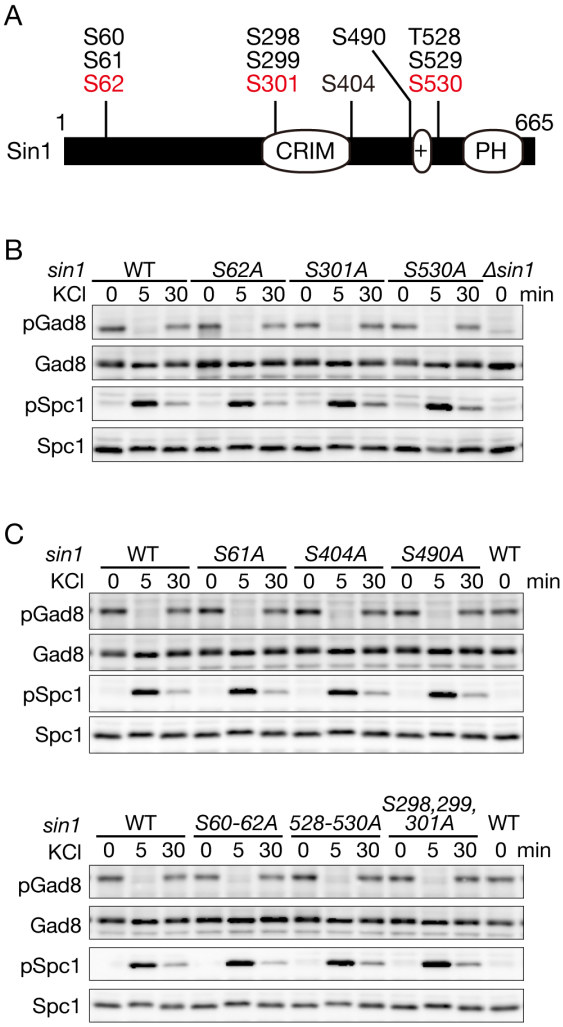

Figure S1. Mutations to the reported phosphorylation sites in Sin1 show no apparent effect on TORC2 activity.

(A) Sin1 phosphorylation sites identified by mass spectrometry (Hayashi *et al.*, 2007). Ser-62, Ser-301 and Ser-530 (in red) followed by proline are putative MAPK phosphorylation sites. "+" denotes the cluster of basic residues identified in this study (Fig. 1B).

(B) The putative MAPK phosphorylation sites shown in (A) were substituted by alanine, and TORC2-dependent phosphorylation of Gad8 (pGad8) and the activating phosphorylation of Spc1 MAPK (pSpc1) before and after high osmolarity stress of 0.6 M KCl were monitored by immunoblotting as in Fig. 4. S62A, *sin1-S62A* (CA10009); S301A, *sin1-S301A* (CA10017); S530A, *sin1-S530A* (CA10025); and *Δsin1* (CA9067).

(C) The other reported phosphorylation sites were analyzed by alanine substitutions. Upper panel: S61A, *sin1-S61A* (CA10622); S404A, *sin1-S404A* (CA11212); and S490A, *sin1-S490A* (CA10661). Lower panel: Multiple serine/threonine residues that are close to the putative MAPK phosphorylation sites were mutated. S60-62A, *sin1-S60,61,62A* (CA10630); 528-530A, *sin1-T528A,S529A,S530A* (CA11220); and S298,299,301A, *sin1-S298,299,301A* (CA10654).

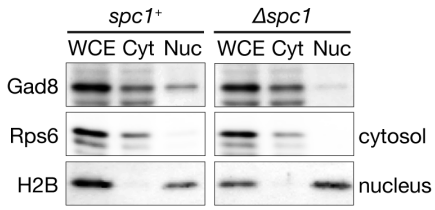

Figure S2. Nuclear-cytoplasmic distribution of the Gad8 protein.
The lysate of spheroplasts (whole cell extract, WCE) prepared from the wild-type and *Δspc1* strains was divided into the soluble cytosolic fraction (Cyt) and the nucleus-rich fraction (Nuc) as described by Cohen *et al.* (2016). Gad8 in each fraction was detected by immunoblotting using anti-Gad8 antibodies. The ribosomal subunit Rps6 and Histone H2B (H2B) were used as cytosolic and nuclear markers, respectively.

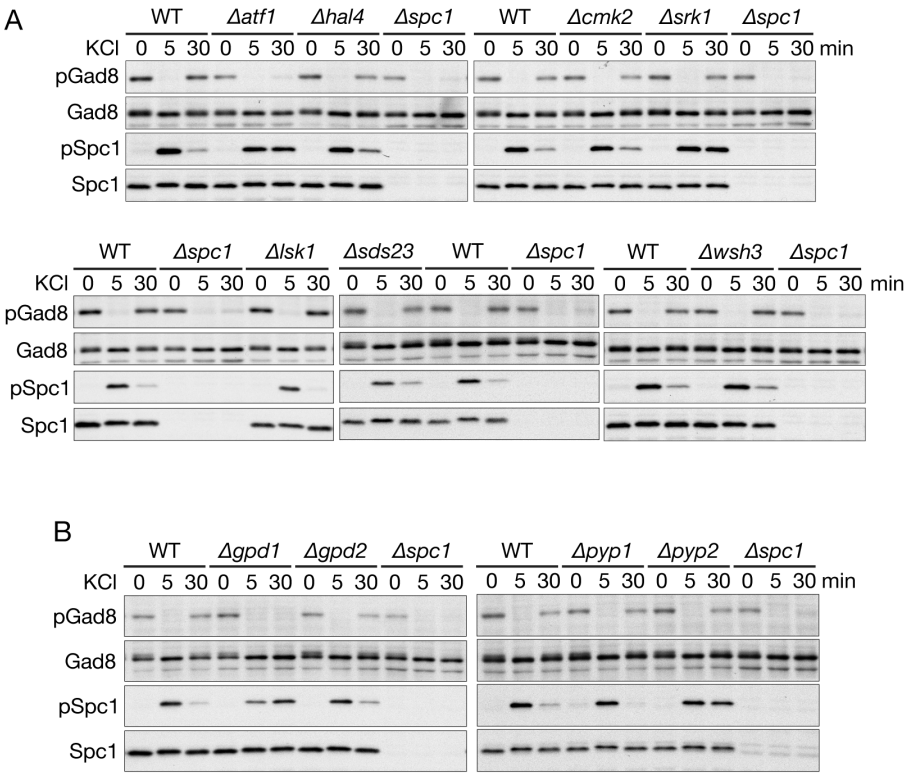

Figure S3. A search for genes required for reactivation of TORC2 after osmostress.
In the wild-type and indicated null mutant strains, TORC2-dependent phosphorylation of Gad8 (pGad8) and the activating phosphorylation of Spc1 MAPK (pSpc1) before and after high osmolarity stress of 0.6 M KCl were monitored by immunoblotting as in Fig. 4.

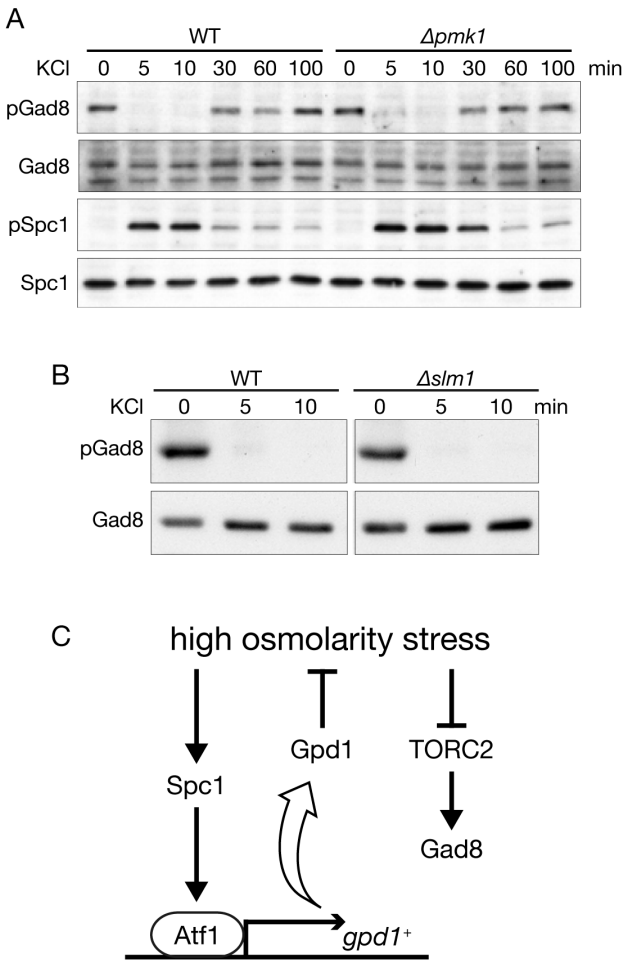

Figure S4. Osmo-response of TORC2 signaling in fission yeast does not involve Pmk1 MAPK nor Slm1.
(A) Pmk1 MAPK is not required for the osmo-inhibition of TORC2-Gad8 signaling. TORC2-dependent phosphorylation of Gad8 (pGad8) and the activating phosphorylation of Spc1 MAPK (pSpc1) in the wild-type and Δpmk1 mutant strains along the time course after high osmolarity stress of 0.6 M KCl were monitored by immunoblotting as in Fig. 4. (B) Fission yeast Slm1 is not required for TORC2 activity. TORC2-dependent phosphorylation of Gad8 (pGad8) was monitored by immunoblotting in the wild type (WT) and a strain lacking the only ortholog (slm1+; ORF, SPAC637.13c) of budding yeast Slm1/2 before and after high osmolarity stress of 0.6 M KCl. (C) Regulation of the Spc1-Atf1 and TORC2-Gad8 pathways in response to high osmolarity stress. Activation of the Atf1 transcription factor by Spc1 MAPK induces expression of the glycerol synthesis enzyme Gpd1 that promotes cellular adaptation to high osmolarity environment, mitigating the osmo-inhibition of TORC2-Gad8 signaling. Spc1 and Atf1 also positively regulate TORC2 in the absence of osmostress, but in a Gpd1-independent manner.

Table S1. *S. pombe* strains used in this study

Strain	Genotype	Source or reference
BG3847H	*sds23::kanR ura4-D18 leu1-32 ade6 h+*	Bioneer*
JP76	*srk1::ura4$^+$ ura4-D18*	Smith *et al*., 2002
KS1115	*pyp2::ura4$^+$ ura4-D18*	Shiozaki and Russell, 1995a
KS1616	*spc1::ura4$^+$ ura4-D18 h-*	Laboratory stock
KS1366	*spc1::ura4$^+$ ura4-D18*	Laboratory stock
KS1497	*atf1::ura4$^+$ ura4-D18*	Shiozaki and Russell, 1996
KS1533	*atf1::ura4$^+$ spc1::ura4$^+$ ura4-D18*	Shiozaki and Russell, 1996
KS1598		Laboratory stock
KS2060	*cmk2::ura4$^+$ ura4-D18*	Laboratory stock
KS2079	*wis1:myc(ura4$^+$) ura4-D18*	Shiozaki *et al*., 1998
KS2080	*wis1AA:myc(ura4$^+$) ura4-D18*	Shiozaki *et al*., 1998
KS2081	*wis1DD:myc(ura4$^+$) ura4-D18*	Shiozaki *et al*., 1998
PR37	*h- (972)*	Laboratory stock
PR253	*pyp1::ura4$^+$ ura4-D18*	Shiozaki and Russell, 1995a
TP319-31A	*pmk1::ura4$^+$ ura4-D18*	Toda *et al*., 1996
CA1788	*hal4::ura4$^+$ ura4-D18*	Wang *et al*., 2005
CA2527	*wsh3::ura4$^+$ ura4-D18*	Tatebe *et al*., 2005
CA4593	*tor1::ura4$^+$ ura4-D18*	Kawai *et al*., 2001
CA4776	*sin1:FLAG(kanR) spc1::ura4$^+$ ura4-D18*	This study
CA5123/CA9121	*sin1:FLAG(kanR)*	Tatebe *et al*., 2010
CA5126/NT475	*sin1::kanR*	Ikeda *et al*., 2008
CA5764	*slm1::kanR*	This study
CA5999	*NTAP:tor1 sin1:FLAG(kanR)*	Tatebe *et al*., 2010
CA6271	*ste20:FLAG(kanR)*	This study
CA6287	*NTAP:tor1 ste20:FLAG(kanR)*	This study
CA6407	*NTAP:tor1 wat1:FLAG(kanR)*	Tatebe *et al*., 2010
CA6437	*wat1:FLAG(kanR)*	Tatebe *et al*., 2010
CA6530	*(hph)FLAG:tor1*	Hayashi *et al*., 2007
CA6655	*ste20:3GFP(kanR)*	Tatebe *et al*., 2010
CA6743	*gad8:1GFP(kanR)*	This study
CA6764	*bit61:FLAG(kanR)*	Laboratory stock
CA7139	*ste20:FLAG(hph)*	This study
CA7209	*ste20:FLAG(hph) bit61:myc(kanR)*	This study
CA7813	*bit61:FLAG(kanR) spc1::ura4$^+$ ura4-D18*	This study
CA8227	*(hph)FLAG:tor1 spc1::ura4$^+$ ura4-D18*	This study
CA8576	*sin1-3KQ*	This study
CA9067	*sin1::ura4$^+$ ura4-D18*	This study
CA9141	*sin1-3KQ:FLAG(kanR)*	This study
CA9538	*sin1-3KQ:FLAG(kanR) spc1::ura4$^+$ ura4-D18*	This study
CA9552	*sin1:FLAG(kanR) spc1::ura4$^+$ ura4-D18*	This study
CA10009	*sin1-S62A*	This study
CA10017	*sin1-S301A*	This study
CA10025	*sin1-S530A*	This study
CA10622	*sin1-S61A*	This study
CA10630	*sin1-S60,61,62A*	This study
CA10654	*sin1-S298,299,301A*	This study
CA10661	*sin1-S490A*	This study
CA11212	*sin1-S404A*	This study
CA11220	*sin1-528-530A*	This study
CA13019	*gpd2::kanR*	Bioneer*
CA13029	*gpd1::ura4$^+$ ura4-D18*	This study
CA13232	*sin1ΔC:FLAG(ura4$^+$) ura4-D18*	This study
CA13421	*lsk1::kanR*	Bioneer*
CA13735	*wat1:FLAG(kanR) spc1::ura4$^+$ ura4-D18*	This study
CA13774	*ste20:FLAG(hph) spc1::ura4$^+$ ura4-D18*	This study
CA13783	*ste20:FLAG(kanR) spc1::ura4$^+$ ura4-D18*	This study
CA13881	*gad8:1GFP(kanR) spc1::ura4$^+$ ura4-D18*	This study
CA13883	*ste20:3GFP(kanR) spc1::ura4$^+$ ura4-D18*	This study
CA13885	*NTAP:tor1 sin1:FLAG(kanR) spc1::ura4$^+$ ura4-D18*	This study
CA13892	*NTAP:tor1 ste20:FLAG(kanR) spc1::ura4$^+$ ura4-D18*	This study
CA13893	*NTAP:tor1 wat1:FLAG(kanR) spc1::ura4$^+$ ura4-D18*	This study
CA13966	*gpd1::ura4$^+$ spc1::ura4$^+$ ura4-D18*	This study
CA13970	*ste20:FLAG(hph) bit61:myc(kanR) spc1::ura4+ ura4-D18*	This study

All strains are *h- leu1-32*, except for BG3847H, KS1616, PR37.

* *S. pombe* haploid deletion mutant library

Table S2. Primer DNAs used in this study

Product	Primer name	Sequence	PCR reaction
Spc1TA_1-313	NdeI-spc1_1-22	CTGACATATGGCAGAATTTATTCGTACAC	Amplification of *spc1TA* fragment from +1 to +939
	spc1_939pst1c	TACCTGCAGTTCATCAGCAACAGGCTCATCAG	
Spc1TA_2DN	spc1_908fwd	ATAATCCTACTAATGAGCCTGTTGCTGATG	Site-directed mutagenesis
	spc1_922rev	CATTAGTAGGATTATGGTATGGAGCAAGATA	
Spc1TA_DENQ	spc1_DEnqfwd	TAATCAAGTTTTTAACTGGTCATTCCAAGATA	Site-directed mutagenesis
	spc1_Denqrev	TTAAAAACTTGATTAGCAACAGGCTCATCAGT	
Spc1TAΔ299-313	spc1_delfwd	TAACTACGTATTTGACTGGTCATTCCAA	Site-directed mutagenesis
	spc1_delrev	TCAAATACGTAGTTATGAGCCAAAGCA	
Sin1_2-565	BamHI-sin1	CGCGGATCCGGAATTAACAAGAGAGAAAGTTCTTT	Amplification of *sin1* cDNA fragment from +4 to +1695
	Sin1-565Xh	CCGCTCGAGTTACCATACAAGAAATTCTTGATAGGTATTGC	
Sin1_2-523	BamHI-sin1	same as in "Sin1_2-565"	Amplification of *sin1* cDNA fragment from +4 to +1569
	sin1_1569apa1c	CTAGGGCCCGGTACTTCCTTTTTTATCGCGTACCTTC	
Sin1_2-400	BamHI-sin1	same as in "Sin1_2-565"	Amplification of *sin1* cDNA fragment from +4 to +1200
	SalI_sin1_1200-1178	GGGGTCGACTACTTCGATTTAAACGGGTAGGCAG	
Sin1_466-665	BmSin1-466	GCGGGATCCGGCTATGGTGTTGAACCAGGTG	Amplification of *sin1* cDNA fragment from +1396 to +1998
	ApaI-sin1	ATTGGGCCCTTAATTTATTTTTTTAACAGTATTCATCAGTG	
Sin1_540-665	sin1_1617bamh1	CACGGATCCTAAGAAAGATGCACAATCTTCAACATACAATGC	Amplification of *sin1* cDNA fragment from +1618 to +1998
	ApaI-sin1	same as in "Sin1_466-665"	
Sin1_3KQ	sin1_kqfwd	TCAACAGCAGGTTCGCGATAAAAAAGGAAGT	Site-directed mutagenesis
	sin1_kqrev	CGAACCTGCTGTTGAACAAGTTCTAGAGTTGG	
Sin1_RKHQ	sin1_rkhqfwd	TTCACGATCAACAAGGAAGTACCCAACAAT	Site-directed mutagenesis
	sin1_rkhqrev	CTTGTTGATCGTGAACCTTCTTTTTAACAAGT	
Sin1Δ511-523	sin1_1570xba1	CAGTCTAGAACAACAATTGCCAACCTCCTCACC	Amplification of *sin1* cDNA fragment from +1570 to +1998
	ApaI-sin1	same as in "Sin1_466-665"	
Sin1	sin1-497pst1nde1	AGTCTGCAGCATATGTCTAGCTTGGCGTTGTCGAGTG	Amplification of *sin1* + fragment from -497 to +2522
	sin1+2522sma1bamh1	TTCAGGATCCCGGGAAAGAGGAAAGCGAGTTTATGGACAGTG	
Sin1S62A	sin1s62a_fwd	TTTCTAGCGCTCCCCCGATTGTCGCTAATG	Site-directed mutagenesis
	sin1s62a_rev	GGGGAGCGCTAGAAAACGAAGTTTTAGA	
Sin1_S61A	sin161afwd	TTTCTGCTAGCCCCCCGATTGTCGCTAAT	Site-directed mutagenesis
	sin161arev	GGGGGCTAGCAGAAAACGAAGTTTTAGA	
Sin1_S60,61,62A	sin160-62afwd	GTTTGCGGCCGCTCCCCCGATTGTCGCTAA	Site-directed mutagenesis
	sin160-62arev	GGAGCGGCCGCAAACGAAGTTTTAGAATA	
Sin1_528,529,530A	sin1528-30afwd	GCCAGCGGCCGCACCACAAAATTCCGTTT	Site-directed mutagenesis
	sin1528-30arev	GGTGCGGCCGCTGGCAATTGTTGGGTACT	
Sin1_S301A	sin1s301a_fwd	GAGCGAGGCGCCTTCAAAGCCCTTATTTG	Site-directed mutagenesis
	sin1s301a_rev	GAAGGCGCCTCGCTCGAAGGAAAATAAATG	
Sin1_S530A	sin1s530a_fwd	AACCAGCGCTCCACAAAATTCCGTTTATG	Site-directed mutagenesis
	sin1s530a_rev	TGTGGAGCGCTGGTTGGCAATTGTTGGGT	
Sin1_S404A	sin1s404afwd	AACAGCTATTCCGGAAGCCAATAACAAAACGC	Site-directed mutagenesis
	sin1s404arev	TCCGGAATAGCTGTTGGATGCTTCGATTT	
Sin1_S490A	sin1490afwd	GTTGCCGGCGCTGATACTGTTTTACCAC	Site-directed mutagenesis
	sin1490arev	ATCAGCGCCGGCAACTCGCAGAGTATAC	

Table S3. Plasmids used in this study

For Y2H

Name	Expressed protein	
Bait plasmid		
pGBT8	GAL4 DNA-binding domain (BD)	Laboratory stock
pGBT8-spc1TA	BD-Spc1T171A(1-349, full length)	Laboratory stock
pGBT8-spc1TA_1-313	BD-Spc1T171A(1-313)	This study
pGBT8-spc1_1-109	BD-Spc1(1-109)	This study
pGBT8-spc1TAΔ299-313	BD-Spc1(1-298:314-349, ΔCD)	This study
pGBT8-spc1TA_2DN	BD-Spc1T171A,D304N,D307N(1-349)	This study
pGBT8-spc1TA_DENQ	BD-Spc1T171A,D312N,E313Q,D316N(1-349)	This study
Prey plasmid		
pGADGH	GAL4 activation domain (AD)	Laboratory stock
pGADGH-sin1	AD-Sin1(2-665, full length)	Laboratory stock
pGADGH-sin1_2-565	AD-Sin1(2-565)	This study
pGADGH-sin1_2-523	AD-Sin1(2-523)	This study
pGADGH-sin1_2-509	AD-Sin1(2-509)	This study
pGADGH-sin1_2-400	AD-Sin1(2-400)	This study
pGADGH-sin1_416-665	AD-Sin1(416-665)	Laboratory stock
pGADGH-sin1_466-665	AD-Sin1(466-665)	This study
pGADGH-sin1_509-665	AD-Sin1(509-665)	This study
pGADGH-sin1_540-665	AD-Sin1(540-665)	This study
pGADGH-sin1Δ511-523	AD-Sin1(2-510:524-665)	This study
pGADGH-sin1_3KQ	AD-Sin1K513Q,K514Q,K515Q(2-665)	This study
pGADGH-sin1_RKHQ	AD-Sin1R517H,K519Q,K520Q(2-665)	This study

For construction of strains with mutated *sin1*

Name	Mutation	
pBSIISK-sin1+	N/A	This study
pBSIISK-sin1S62A	S62A	This study
pBSIISK-sin1S61A	S61A	This study
pBSIISK-sin1S60,61,62A	S60A,S61A,A62A	This study
pBSIISK-sin1 528,529,530A	T528A,S529A,S530A	This study
pGADGH-sin1S301A	S301A	This study
pGADGH-sin1S530A	S530A	This study
pGADGH-sin1_S404A	S404A	This study
pGADGH-sin1_S490A	S490A	This study
pREP1-sin1 S298A S299A S301A:12myc	S298A,S299A,S301A	Laboratory stock