Modulation of TOR complex 2 signaling by the stress-activated MAPK pathway in fission yeast

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Abstract

Sin1 is a substrate-binding subunit of Target Of Rapamycin Complex 2 (TORC2), an evolutionarily conserved protein kinase complex. In fission yeast, Sin1 was also identified as a protein that interacts with Spc1/Sty1 stress-activated protein kinase (SAPK) and therefore, this study examined the relationship between TORC2 and Spc1 signaling. We found that the common docking (CD) domain of Spc1 interacts with a cluster of basic amino acid residues in Sin1. Although diminished TORC2 activity in the absence of the functional Spc1 cascade suggests positive regulation of TORC2 by Spc1, such regulation appears to be independent of the Sin1-Spc1 interaction. Hyperosmotic stress transiently inhibits TORC2, and its swift recovery is dependent on Spc1, the Atf1 transcription factor, and the Gpd1 glycelrol-3-phosphate dehydrogenase, whose expression is induced upon osmostress by the Spc1-Atf1 pathway. Thus, cellular adaptation to osmostress seems to be important for TORC2 reactivation, though Spc1 and Atf1 contribute to TORC2 activation also in the absence of osmostress. These results indicate coordinated actions of the SAPK and TORC2 pathways, both of which are essential for fission yeast cells to survive environmental stress.

Introduction

Stress-activated protein kinases (SAPKs) comprise a mitogen-activated protein kinase (MAPK) subfamily that is responsive to environmental stress conditions. Following the discovery of budding yeast Hog1 MAPK as a SAPK prototype (Brewster et al., 1993), identification of c-Jun N-terminal kinase (JNK) and p38 SAPKs demonstrated the evolutionary conservation of this stress-responsive subtype of MAPKs (Kyriakis and Avruch, 2001). Spc1 (also known as Sty1, Phh1) in the fission yeast *Schizosaccharomyces pombe* (Kato et al., 1996; Millar et al., 1995; Shiozaki and Russell, 1995a) is an ortholog of budding yeast Hog1 and mammalian p38, and plays a crucial role in cellular survival of diverse forms of stress, such as high osmolarity, oxidative stress, heat shock, and starvation (Nguyen and Shiozaki, 2002; Toone and Jones, 2004). In response to stress stimuli, Spc1 MAPK is phosphorylated by Wis1 MAPK kinase (MAPKK) (Shiozaki and Russell, 1995b; Warbrick and Fantes, 1991) and activated Spc1 then phosphorylates the Atf1 transcription factor (Gaits et al., 1998; Shiozaki and Russell, 1996; Wilkinson et al., 1996) to induce a set of stress resistance genes (Chen et al., 2003). Among the stress resistance genes regulated by the Spc1-Atf1 pathway is *gpd1*$^{+}$ that encodes glycerol-3-phosphate dehydrogenase, a key enzyme in glycerol synthesis (Aiba et al., 1995; Degols et al., 1996; Shiozaki and Russell, 1996; Wilkinson et al., 1996). Induced expression of *gpd1*$^{+}$ results in cellular accumulation of glycerol, which serves as a major cytoplasmic solute to counterbalance extracellular hyperosmotic stress (Ohmiya et al., 1995).

Sin1 (SAPK-interacting protein 1) was isolated by a yeast two-hybrid screen as a protein that interacts with Spc1 MAPK and proposed to regulate the Spc1-mediated expression of the stress resistance genes (Wilkinson et al., 1999). A later examination, however, found that the Spc1-dependent phosphorylation of Atf1 and its function in gene induction upon stress are not affected by the *sin1* null (Δ*sin1*) mutation, failing to confirm the functional link between Sin1 and the Spc1 MAPK cascade (Ikeda et al., 2008). A mammalian Sin1 ortholog, SIN1/MIP1, was also reported to interact with JNK SAPK as well as its upstream MAPKK kinase, MEKK2 (Cheng et al., 2005; Schroder et al., 2005). It was suggested that SIN1/MIP1 interacts with MEKK2 and prevents its dimerization and activation, leading to suppression of the SAPK cascade (Cheng et al., 2005).

On the other hand, Sin1 orthologs in budding yeast and higher eukaryotes have been identified as a component of TOR complex 2 (TORC2), a high-molecular weight protein kinase complex that contains the Target of Rapamycin (TOR) kinase as its catalytic subunit (Frias et al., 2006; Jacinto et al., 2006; Lee et al., 2005; Loewith et al., 2002; Wedaman et al., 2003; Yang et al., 2006). Mammalian TORC2 (mTORC2) functions as a key activator of a set of the AGC-family protein kinases, such as AKT, PKCα and SGK1, through phosphorylation of the "hydrophobic motif" conserved among these AGC kinases (García-Martínez and Alessi, 2008; Hresko and Mueckler, 2005; Sarbassov et al., 2004; Sarbassov et al., 2005). Also in fission yeast, mass spectrometry analysis of TORC2 identified Sin1 as a TORC2 component (Hayashi et al., 2007). Furthermore, the Δ*sin1* mutant shares phenotypes with strains lacking the other TORC2 subunits, such as Tor1 kinase and Ste20, a *S. pombe* ortholog of mammalian RICTOR; the Δ*sin1*, Δ*tor1* and Δ*ste20* mutants are sterile and exhibit hyper-sensitivity to environmental stresses, including high osmolarity (Ikeda et al., 2008; Kawai et al., 2001; Matsuo et al., 2003; Weisman and Choder, 2001; Wilkinson et al., 1999). In addition, these mutants are defective in phosphorylation and activation of the AGC-family Gad8 kinase, which is structurally related to mammalian AKT and SGK1 (Ikeda et al., 2008; Matsuo et al., 2003; Tatebe et al., 2010). These biochemical and genetic studies indicated that Sin1 is an essential subunit of TORC2 also in fission yeast and consistently, a more recent study has demonstrated that Sin1 serves as a substrate-binding subunit of TORC2 (Tatebe et al., 2017). Sin1 specifically binds Gad8 through a domain highly conserved among Sin1 orthologs, thus named the “CRIM (Conserved Region In the Middle) domain (Schroder et al., 2007). NMR analysis found that CRIM is a ubiquitin-fold domain of ~120 amino acid residues, and the CRIM domain of human SIN1 also binds specifically to the mTORC2 substrates, such as AKT, PKCα and SGK1 (Furuita et al., 2015; Kataoka et al., 2015; Tatebe et al., 2017). Mutations to CRIM impair TORC2 signaling both in fission yeast and human cells, demonstrating the critical role of Sin1 as a functional subunit of TORC2.

Contrary to the comprehensive characterization of Sin1 as a TORC2 subunit, the physiological significance of its interaction with the SAPK cascade remains unclear in both fission yeast and mammals. In this study, we further characterized the interaction between Sin1 and Spc1 MAPK as well as the role of Spc1 in the regulation of TORC2 signaling. Detailed analysis of the Sin1-Spc1 interaction suggested that the common docking (CD) domain of Spc1 MAPK interacts with a cluster of basic amino acid residues in Sin1. Interestingly, inactivation of Spc1 MAPK results in reduced TORC2 activity, however, independently of the Spc1-Sin1 interaction. TORC2 is transiently inhibited upon high osmolarity stress and the swift recovery of TORC2 activity after the stress is dependent on the Spc1-Atf1 pathway that induces the glycerol synthesis enzyme Gpd1 for cellular adaptation to osmostress. These results have uncovered coordinated actions of the SAPK and TORC2 pathways, both of which mediate cellular responses to changing environmental conditions.

Results

The CD domain of Spc1 MAPK and clustered basic residues in Sin1 mediate the Spc1-Sin1 interaction

Full-length Spc1 MAPK was used as a bait in the yeast two-hybrid screen that isolated Sin1 (Wilkinson et al., 1999). To further narrow down the Sin1-binding region within Spc1, truncated Spc1 fragments were tested for their interaction with Sin1 in yeast two-hybrid assays. Spc1 N-terminal fragments of 313 residues and 109 residues failed to interact with Sin1 (Fig. 1A), implying that the C-terminus of Spc1 is required. Interestingly, the region C-terminal to the kinase catalytic domain of Spc1 contains the common docking (CD) domain (residues 299-313), a sequence motif conserved among the MAPK family members (Tanoue et al., 2000). Because the CD domain is known to mediate interactions of MAPKs with their regulators and substrates, we examined whether mutations to the CD domain affects the interaction of Spc1 MAPK with Sin1. Deletion of the CD domain ("ΔCD" in Fig. 1A) as well as Asn substitutions of the conserved, critical Asp residues within the CD domain (Asp-304 and Asp-307); (Tanoue et al., 2000) abrogated the Spc1-Sin1 interaction ("2DN"), whereas mutations to the other acidic residues (Glu-308, Asp-312 and Glu-313) did not ("DENQ"). These results suggest that the CD domain of Spc1 MAPK is required for its interaction with Sin1.

Similar yeast two-hybrid assays using a series of Sin1 truncations (Fig. 1B) showed that the N-terminal 2-523 fragment as well as the C-terminal 509-665 fragment can interact with Spc1, indicating that residues 509-523 of Sin1 are required to bind Spc1. This region contains a cluster of positively charged amino acids, a known characteristics of the docking sites for the CD domains of MAPKs (Tanoue et al., 2000). Indeed, deletion of the basic stretch (Δ511-523 in Fig. 1B) prevented Sin1 from interacting with Spc1.

Moreover, the full-length Sin1 with mutations to the three consecutive Lys residues within this region (residues 513-515; asterisks in Fig. 1B) failed to interact with Spc1 (“3KQ” in Fig. 1B), though Arg-517, Lys-519, and Lys-520 appeared to be dispensable (“RKHQ”).

These results suggest that the interaction between Spc1 MAPK and Sin1 is mediated by the Spc1 CD domain with the acidic residues that may interact with a cluster of basic residues in Sin1, which resembles MAPK docking sites found in substrates and regulators of the MAPK family members (Tanoue et al., 2000).

Spc1 MAPK-dependent phosphorylation of Sin1

Sin1 has been reported as a phosphorylated protein, and its phosphorylation status is reflected by the electrophoretic mobility of the protein (Wilkinson et al., 1999). In SDS-PAGE, the Sin1 protein expressed from its chromosomal locus with the FLAG epitope tag ran as somewhat diffused bands (Fig. 2A, lane 1), which converged to a fast-migrating band by phosphatase treatment (lane 2). Disruption of the *spc1*$^{+}$ gene (Δ*spc1*) also resulted in appearance of a fast-migrating band, together with a slow-migrating band similar to that in wild-type cells; thus, some fraction of Sin1 appears to be hypo-phosphorylated in Δ*spc1* cells (lane 4). We observed no significant change to the electrophoretic mobility of the other TORC2 subunits, such as Tor1, Ste20, Wat1, and Bit61 (Fig. 2B). On the other hand, it was noticeable that the amounts of the Sin1, Wat1 and Bit61 proteins somewhat increased in Δ*spc1* cells when compared to those in wild-type cells.

Disassembly of mammalian TORC2 was reported as a regulatory mechanism for TORC2 signaling under starvation stress (Chen et al., 2013). In order to examine whether the TORC2 integrity is affected by the hypo-phosphorylation of Sin1 and/or the altered levels of the TORC2 subunits in the absence of the stress-responsive MAPK (Fig.

2B), the physical interactions among the TORC2 subunits were evaluated in the Δ*spc1* mutant. When the tandem affinity purification (TAP)-tagged Tor1 was collected onto IgG-beads from the wild-type and Δ*spc1* strains, no significant difference between the two strains was observed for the co-purification of Sin1 (Fig. 2C), Ste20 (Fig. 2D), Wat1 (Fig. 2E). Bit61 associates with the Ste20 subunit of TORC2 (Tatebe and Shiozaki, 2010), and their interaction was also not affected by the Δ*spc1* mutation (Fig. 2F). These observations suggest that Spc1 MAPK does not notably affect the TORC2 integrity.

Spc1 MAPK positively regulates TORC2 activity

Sin1 functions as a substrate-binding subunit of TORC2 by specifically recruiting Gad8, so that Tor1, the catalytic subunit of TORC2, phosphorylates the C-terminal hydrophobic motif of Gad8 (Tatebe et al., 2017). We found that the TORC2-dependent phosphorylation of Gad8 was significantly reduced in Δ*spc1* cells, suggesting that Spc1 MAPK positively regulates the TORC2 activity toward Gad8 (Fig. 3A).

In order to test whether the Spc1-Sin1 interaction is involved in the Spc1-dependent regulation of TORC2 activity, we constructed a fission yeast strain whose chromosomal *sin1* gene carries the "3KQ" mutation that disrupts the interaction with Spc1 in the yeast two-hybrid assay (Fig. 1B). No significant difference in the Gad8 phosphorylation was detected between the wild-type and *sin1-3KQ* mutant strains (Fig. 3B). In addition, the electrophoretic mobility of the Sin1-3KQ mutant protein is very similar to that of the wild-type protein both in *spc1*$^+$ and Δ*spc1* cells ("Sin1-FLAG" in Fig. 3B). Therefore, the Spc1-Sin1 interaction detectable in yeast two-hybrid assays does not appear to be essential for the Spc1-dependent TORC2 regulation nor for the Sin1 phosphorylation.

A previous mass spectrometry analysis of fission yeast TORC2 identified multiple phosphorylation sites in the Sin1 protein (Fig. S1A; Hayashi et al., 2007). Among those are Ser-62, Ser-301 and Ser-530 followed by proline, and they can be phosphorylated by MAPK. We mutated the chromosomal *sin1* gene to substitute these serine residues individually with alanine and the Gad8 phosphorylation in these strains was examined, but no significant difference was observed in comparison with that in wild-type strains (Fig. S1B). The other phosphorylation sites that do not match the MAPK phosphorylation site consensus were also mutated to alanine, with no apparent effect on the TORC2-dependent phosphorylation (Fig. S1C).

Cellular localization of TORC2 can be visualized by fusing three copies of GFP to Ste20, the fission yeast ortholog of the RICTOR subunit; Ste20-3GFP shows punctate signals throughout the cell surface as well as the cell division septum (Tatebe et al., 2010). Similar cortical localization of TORC2 was observed in Δ*spc1* cells (Fig. 3C), which are elongated due to a cell-cycle delay (Shiozaki and Russell, 1995a). Δ*spc1* cells also showed no significant change in the distribution of Gad8 tagged with a single copy of GFP at the C-terminus, and fluorescent signals were detectable throughout the cell except vacuoles, as in wild-type cells (Fig. 3C). Although it was reported that the majority of Gad8 was in the nuclear fraction of the cell lysate (Cohen et al., 2016), we did not observe such nuclear enrichment of untagged, endogenous Gad8 both in wild-type and Δ*spc1* cells (Fig. S2), consistent with our microscopy results (Fig. 3C).

Together, these results indicate that Spc1 MAPK positively regulates TORC2 activity, but the regulation is independent of the Spc1-Sin1 interaction and may be a rather indirect one.

The TORC2-Gad8 pathway responds to osmostress

Like the Spc1 MAPK cascade (Shiozaki and Russell, 1995a), the TORC2-Gad8 pathway is required for fission yeast cells to grow under high osmolarity stress (Ikeda et al., 2008; Tatebe et al., 2010). In addition, it was reported that the TORC2-dependent activation of Gad8 is inhibited in response to high osmolarity stress (Cohen et al., 2014). We therefore characterized the kinetics of the TORC2 osmo-response and found that the TORC2-dependent phosphorylation of Gad8 disappeared within 5 min after osmostress of 0.6 M KCl, followed by a gradual, somewhat oscillating recovery of the phosphorylation after 20 min ("pGad8" in Fig. 4A). The prompt inactivation of TORC2 upon osmostress seemed to be correlated to Spc1 activation, which was monitored through its activation loop phosphorylation ("pSpc1" in Fig. 4A). Therefore, we examined whether high osmolarity stress inhibits the TORC2-Gad8 pathway through activation of Spc1 MAPK. Like wild-type cells, Δ*spc1* mutant cells showed transient attenuation of the Gad8 phosphorylation upon osmostress, though the phosphorylation hardly recovered at later time points (Fig. 4B). A very similar osmo-response of the Gad8 phosphorylation was observed in the strain expressing Wis1AA, an inactive mutant Wis1 MAPKK that cannot phosphorylate Spc1 (Shiozaki et al., 1998) (Fig 4C). On the other hand, the strain expressing the constitutively active Wis1DD mutant MAPKK exhibited transient inactivation and recovery of TORC2 activity as in the wild-type strain (Fig. 4D). These results indicate that the Spc1 MAPK cascade is not required for the osmostress-induced inactivation of TORC2, but Spc1 activity promotes the re-activation of TORC2 after osmostress.

The Atf1 transcription factor and its target gene *gpd1*⁺ are important for reactivation of TORC2 after osmostress

Although active Spc1 MAPK plays a role in the reactivation of TORC2 after osmostress (Fig. 4), we found that the osmo-response kinetics of the Gad8 phosphorylation in the *sin1-3KQ* mutant is very similar to that in the wild type (Fig. 5A), negating the involvement of the Spc1-Sin1 interaction. As shown in Fig. 5B, this osmoregulation of TORC2 was not altered also in the *sin1ΔC* strain expressing Sin1 lacking the pleckstrin homology (PH) domain, which is implicated in the phosphoinositide-dependent regulation of mTORC2 activity (Liu et al., 2015).

In order to explore how Spc1 contributes to the recovery of TORC2 activity after osmostress, the Gad8 phosphorylation was monitored in the null mutants of the reported Spc1 MAPK targets, such as Atf1 (Shiozaki and Russell, 1996), Hal4 (Wang et al., 2005), Cmk2 (Sánchez-Piris et al., 2002), Srk1 (Smith et al., 2002), Lsk1 (Sukegawa et al., 2011), Sds23 (Jang et al., 2013; Yamada et al., 1997) and Wsh3/Tea4 (Tatebe et al., 2005). In wild-type cells, the TORC2-dependent phosphorylation of Gad8 starts recovering within 30 min after osmostress (Fig. 4A); however, re-phosphorylation of Gad8 was not observed even after 30 min in the Δ*atf1* strain among the null mutants tested (Figs. 5C, S3A).

Being phosphorylated and activated by Spc1 MAPK, the Atf1 transcription factor induces expression of a set of the genes important for cellular adaptation to stressful conditions (Chen et al., 2003; Shiozaki and Russell, 1996; Wilkinson et al., 1996). We tested some of the genes under the regulation of Spc1-Atf1 for their involvement in the recovery of TORC2 activity after osmostress. It was found that a strain lacking *gpd1*$^{+}$ failed to induce significant re-phosphorylation of Gad8 even after 100 min under osmostress (Figs. 5D and S3B). *gpd1*$^{+}$ encodes glycerol-3-phosphate dehydrogenase in biosynthesis of glycerol that is important for cellular adaptation to high osmolarity (Ohmiya et al., 1995). On the other hand, such a defect was not observed in a strain lacking *gpd2*$^{+}$, a *gpd1*$^{+}$ paralog with no apparent role in cellular osmo-resistance (Yamada et al., 1996) (Fig. S3B).

These results suggest that Spc1 MAPK promotes reactivation of TORC2 after osmostress through the Atf1 transcription factor, which induces expression of *gpd1*$^{+}$. Indeed, the Δ*spc1* Δ*atf1* and Δ*spc1* Δ*gpd1* double mutants showed defects similar to the respective single mutants, consistent with the idea that Spc1, Atf1 and Gpd1 function together during the recovery of TORC2 inactivated by osmostress (Fig. 6A, B). However, in normal osmolarity media, the Δ*gpd1* mutation did not affect the Gad8 phosphorylation (Fig. 6C), indicating that the positive regulation of TORC2 by Spc1 MAPK in the absence of osmostress (Fig. 3A) is not dependent on *gpd1*$^{+}$. On the other hand, as in Δ*spc1* cells, the Gad8 phosphorylation was reduced in Δ*atf1* cells (Fig. 6C). Unexpectedly, the Δ*spc1* and Δ*atf1* mutations appeared to be additive, and the Gad8 phosphorylation in the Δ*spc1* Δ*atf1* double mutant was significantly lower than those in the respective single mutants. It is likely that, under normal growth conditions without osmostress, Spc1 MAPK and the Atf1 transcription factor independently affect TORC2 activity.

Discussion

Genetic analysis in fission yeast demonstrated that the Spc1 MAPK cascade and the TORC2-Gad8 pathway are both required for cellular adaptation to high osmolarity stress, though the stress elicits opposite responses to these two signaling pathways; activation of the Spc1 cascade and inhibition of the TORC2 pathway (Cohen et al., 2014; Ikeda et al., 2008; Millar et al., 1995; Shiozaki and Russell, 1995b). Because Sin1 was identified as a SAPK-interacting protein (Wilkinson et al., 1999) and also as a TORC2 subunit (Hayashi et al., 2007; Matsuo et al., 2007), Sin1 seems to be a candidate molecule that links Spc1 MAPK to TORC2 in cellular stress response. Having found that TORC2 does not affect Spc1 signaling (Ikeda et al., 2008), we pursued in this study the question of whether Spc1 modulates TORC2 signaling.

We successfully reproduced the previously reported interaction between Spc1 and Sin1 in the yeast two-hybrid assay (Wilkinson et al., 1999), and further showed that the CD domain of Spc1 MAPK and a cluster of basic residues in Sin1 are involved in the interaction. The specificity of this Spc1-Sin1 interaction was further corroborated by a reciprocal yeast two-hybrid screen of a *S. pombe* cDNA library using a C-terminal Sin1 fragment of residues 401-665 as bait; a short, C-terminal Spc1 fragment that includes the CD domain (304-349) was identified in this screen (data not shown). Thus, the interaction of Spc1 MAPK with Sin1 may be similar to those of other MAPKs with their substrates and regulators (Tanoue et al., 2000). On the other hand, we failed in our attempt to detect the Spc1-Sin1 interaction by co-purification assays (data not shown), and the mass spectrometry analysis of fission yeast TORC2 detected Sin1, but not Spc1 (Hayashi et al., 2007). The interaction between Spc1 MAPK and Sin1 may not be stable enough for these biochemical approaches.

We found that mutational inactivation of Spc1 MAPK results in compromised TORC2-dependent phosphorylation of Gad8, indicating that Spc1 MAPK positively regulates the TORC2-Gad8 pathway. However, TORC2 activity is not altered by the *sin1-3KQ* mutation that disrupts the interaction of Sin1 with Spc1 and thus, the Sin1-Spc1 interaction is not required for the observed Spc1-dependent regulation of the TORC2 pathway. In addition, the loss of Spc1 has no apparent impact on the TORC2 integrity nor on the cellular localization of TORC2 and its substrate Gad8. These observations imply a rather circuitous regulatory mechanism by which Spc1 MAPK positively regulates TORC2-Gad8 signaling.

We found that TORC2 is inhibited upon high osmolarity stress in a manner independent of the stress-induced activation of Spc1 MAPK. Although Pmk1, another stress-responsive MAPK in fission yeast, is implicated in the negative regulation of the TORC2-Gad8 pathway (Cohen et al., 2014; Madrid et al., 2016), we found that Pmk1 is not required for the osmo-inhibition of TORC2 signaling (Fig. S4A). A recent study in budding yeast proposed that decreased plasma membrane tension under high osmolarity induces clustering of phosphatidylinositol-4,5-bisphosphate (PI(4,5)P_2), to which TORC2 is tethered as clumps segregated from its activators Slm1/2 (Riggi et al., 2018). Whereas high osmolarity stress induces prominent clustering of PI(4,5)P_2 also in fission yeast (Kabeche et al., 2015), the Slm1/2 ortholog in fission yeast has no apparent role in TORC2 activation both in the presence and absence of osmostress (Fig. S4B). In addition, Slm orthologs are not found in mammals, where inactivation of TORC2 signaling upon high osmolarity stress is also observable (Meier et al., 1998). Thus, the underlying mechanisms of the osmostress sensitivity of TORC2 may be different from species to species.

There may also be a difference between budding yeast and fission yeast in the process of TORC2 reactivation after osmostress. Whereas Hog1 SAPK is not important for the TORC2 reactivation in *S. cerevisiae* (Riggi et al., 2018), we found that Spc1 contributes to the recovery of TORC2 activity after osmostress through the Atf1 transcription factor and its target gene, *gpd1*$^+$. This observation is probably not surprising, considering the essential role of the Spc1-Atf1 pathway in cellular adaptation to hyperosmolarity through the induction of the glycerol synthesis enzyme Gpd1(Degols et al., 1996; Gaits et al., 1998; Ohmiya et al., 1999; Ohmiya et al., 1995; Shiozaki and Russell, 1996; Wilkinson et al., 1996). Unexpectedly, however, our study also uncovered a role of Atf1, but not Gpd1, in TORC2 activation under normal growth conditions (Fig. 6C). TORC2 activity is severely compromised in the Δ*spc1* Δ*atf1* double mutant, suggesting that the Atf1 transcription factor contributes to TORC2 activity independently of Spc1 MAPK, most likely through expression of unknown target genes.

In summary, the data presented in this paper shed light on the intertwining relationship between Spc1 MAPK and TORC2, both of which play critical roles in osmostress resistance of fission yeast cells. The Spc1-Atf1 pathway positively regulates TORC2 signaling both in the presence and absence of osmostress, independently of the Spc1-Sin1 interaction detectable in the yeast two-hybrid assay (Fig. S4C). Although both Hog1 MAPK and TORC2 in budding yeast are involved in the regulation of cellular glycerol accumulation during osmostress (Lee et al., 2012; Muir et al., 2015), crosstalk between the two signaling modules has not been reported. Further genetic studies in both yeast species may unravel a novel mode of interaction between the SAPK and TORC2 pathways that are highly conserved among diverse eukaryotes.

Materials and Methods

General *S. pombe* methods

S. pombe strains used in this study are listed in Table S1. Growth media and basic techniques for fission yeast were previously described (Alfa et al., 1993). Epitope-tagging of chromosomal genes was carried out by the PCR-based method (Bähler et al., 1998). Site-directed mutagenesis was performed using the PrimeSTAR Max DNA polymerase (Takara Bio Inc.) according to the supplier's manual. Oligo DNAs for PCR are listed in Table S2. Stress treatment of *S. pombe* cells was carried out as previously described (Shiozaki and Russell, 1997). For high osmolarity treatment, one-third volume of pre-warmed medium containing 2.4 M KCl was added to the culture. Protein concentrations were determined using Protein Assay Reagent (Bio-Rad Laboratories Inc.).

Protein-protein interaction

Yeast two-hybrid assay was performed as previously described (Tatebe et al., 2005). The ORF encoding the inactive form of Spc1 (Spc1-T171A) was subcloned in the bait plasmid, pGBT9 (Clontech Laboratories, Inc.) using *Nde*I and *Pst*I sites, as ectopic expression of active Spc1 causes a growth defect. The complementary DNA of Sin1 (nucleotides 3 to 1998) was subcloned in the prey plasmid, pGAD GH (Clontech Laboratories, Inc.) using *Bam*HI and *Apa*I sites. Plasmids used in the assay are listed in Table S3. HF7c budding yeast strain (Clontech Laboratories, Inc.) was used as host. Interaction was judged by histidine auxotrophy. Cells harboring either/both empty vector(s) were used as negative controls.

Co-purification of epitope-tagged proteins was performed (Morigasaki and Shiozaki, 2010) using buffers as described below. Lysis buffer containing 1xPBS, 10%(w/v) glycerol, 0.25%(w/v) Tween20, 10 mM NaF, 10 mM sodium pyrophosphate, 10 mM NaN_3, 10 mM beta-glycerophosphate 2Na, 10 mM *p*-nitrophenylphosphate 2Na, 1 mM PMSF, and 1/200-volume Protease inhibitor cocktail (P8849, Sigma-Ardrich Co.). The lysis buffer without protease inhibitors was used as washing buffer. Protein bound to beads was eluted with the Laemmli sample buffer without 2-mercaptoethanol for 15 min at room temperature. After removing the beads, the eluate was mixed with 1/19-volume of 2-mercaptoethanol and heated at 65°C for 15 min. IgG-Sepharose 6 Fast Flow (GE Healthcare) and EZview Anti-c-Myc Affinity Gel (Sigma-Aldrich, Co.) were used for precipitation of NTAP-Tor1 and Bit61-myc, respectively.

Preparation of TCA extract

Whole-cell protein extract was prepared by trichloroacetic acid (TCA) extraction. Yeast cells in early log phase (OD_{600}=0.4, 25 ml) were harvested on a 0.4 µm-porosity filter membrane and resuspended in 200 µL of 10%(w/v) TCA solution. Cells were disrupted by beating with glass beads (ø=0.5 mm) at 2500 rpm for 4.5 min (30 sec/ON and 30 sec/OFF, 9 cycles) using the Multibeads shocker (Yasui Kikai Co.). After removing glass beads, the cell homogenate was centrifuged for 10 min at 10,000 rpm at room temperature, and the precipitate was resuspended in 200 µL of the Laemmli sample buffer containing 0.5 M Tris-HCl, pH8.0. The sample was then heated at 65°C for 15 min and centrifuged for 10 min at 10,000 rpm at room temperature to remove cell debris. The supernatant was used as “TCA extract”. The protein concentration of the TCA extract was adjusted to 1 mg protein/mL with the standard Laemmli sample buffer.

Mobility shift assay

For the Sin1 mobility shift assay, the TCA extract of Sin1-FLAG-expressing cells was subjected to SDS-PAGE using 6.5%T/2.67%C polyacrylamide gel. Sin1 was detected by immunoblotting using anti-FLAG antibodies. Phosphatase-treatment was performed according to Tatebe and Shiozaki (2008) with some modification. Briefly, 10 µg protein of the TCA extract was diluted 180-times with the lambda-protein phosphatase (PPase) buffer. The dilution was dispensed into 3 tubes (A, B, and C). One-tenth volume of the buffer, 60 units of PPase (New England BioLabs Inc.) in the buffer, or 60 units of PPase + 10x phosphatase inhibitor mix in the buffer were added to dilution A, B or C, respectively. After mixing gently, the reaction mixtures were incubated at 30°C for 30 min. To stop the reaction, 1/7-volume of 100%(w/v) TCA was added. Protein was precipitated by centrifugation at 18700 x g for 10 min at 4°C, after standing on ice for 30 min. The precipitate was then resuspended in 20 µL of the Laemmli sample buffer containing 0.5 M Tris-HCl, pH 8.0 and heated at 65°C for 15 min. The 10x phosphatase inhibitor mix is composed of 20 mM Na_3VO_4, 100 mM NaF, 100 mM EDTA, 100 mM beta-glycerophosphate, 40 mM *p*-nitrophenylphosphate.

Antibodies/antisera for immunoblotting

The activating phosphorylation of Thr171 and Tyr173 in Spc1 (pSpc1), Spc1, phosphorylation of Ser546 in Gad8 (pGad8), and the Gad8 protein were detected by immunoblotting using rabbit polyclonal antisera (Tatebe et al., 2010; Tatebe and Shiozaki, 2003). Anti-histone H2B antiserum was a gift from Dr. M. Yanagida (Maruyama et al., 2006). Rps6 was detected with anti-RPS6 antibody (ab40820, Abcam plc.). For detection of FLAG-, HA-, and myc-tagged proteins, anti-FLAG (M2, Sigma-Aldrich, Co.), anti-HA (12CA5, Roche Diagnostics GmbH), and anti-c-myc (9E10, Covance Inc.) mouse monoclonal antibodies were used, respectively. NTAP-Tor1 was detected with anti-calmodulin binding protein epitope tag (Merch Millipore Ltd.). Anti-rabbit IgG (H+L) HRP-conjugate or anti-mouse IgG (H+L) HRP-conjugate (Promega Co.) were used as secondary antibodies.

Quantification of signal intensity of immunoblotting

In immunoblotting, Pierce^{TM} ECL Plus Western Blotting Substrate (Thermo Fisher Scientific) was used for detection. The image of chemiluminescence was obtained using the imaging analyzer LAS4000 (GE Healthcare) and the signal intensity was measured with the software Multi Gauge 3.0 (Fujifilm Co.). For quantification, the signal intensity of phospho-Gad8 (pGad8) was compensated by that of the Gad8 protein.

GFP-tagged protein localization

Cells were cultured in EMM medium until reaching early log phase in the dark and mounted on a thin layer of EMM+agar. Fluorescence images were taken with DeltaVision Elite Microscopy System (GE Healthcare) as described previously (Chia et al., 2017; Tatebe et al., 2010).

Preparation of nucleus-rich fractions

As reported by Cohen *et al*. (Cohen et al., 2016; Keogh et al., 2006), the nucleus-rich fraction was prepared from *S. pombe* cells: *972 h-* (PR37) and Δ*spc1* (KS1616). An aliquot of spheroplast was used as whole cell extract (WCE). After fractionation using 1.2 M sucrose cushion, the upper layer and pellet were collected as cytoplasmic (Cyt) and nucleus-rich (Nuc) fractions, respectively. Gad8 in each fraction was analyzed by immunoblotting using antiserum against Gad8. In addition, distribution of Rps6 and histone H2B (H2B) were analyzed as markers of cytosol and nucleus, respectively.

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Competing interests

The authors declare no competing or financial interests.

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References

Aiba, H., Yamada, H., Ohmiya, R. and Mizuno, T. (1995). The osmo-inducible *gpd1*$^{+}$ gene is a target of the signaling pathway involving wis1 MAP-kinase kinase in fission yeast. *FEBS Letters* **376**, 199-201.

Alfa, C., Fantes, P., Hyams, J. and Warbrick, E. (1993). Experiments with fission yeast. New York: Cold Spring Harbor Laboratory Press.

Bähler, J., Wu, J.-Q., Longtine, M. S., Shah, N. G., McKenzie, A., Steever, A. B., Wach, A., Philippsen, P. and Pringle, J. R. (1998). Heterologous modules for efficient and versatile PCR-based gene targeting in *Schizosaccharomyces pombe*. *Yeast* **14**, 943-951.

Brewster, J. L., Devaloir, T., Dwyer, N. D., Winter, E. and Gustin, M. C. (1993). An osmosensing signal transduction pathway in yeast. *Science* **259**, 1760-1763.

Chen, C.-H., Kiyan, V., Zhylkibayev, A. A., Kazyken, D., Bulgakova, O., Page, K. E., Bersimbaev, R. I., Spooner, E. and Sarbassov, D. D. (2013). Autoregulation of the Mechanistic Target of Rapamycin (mTOR) Complex 2 Integrity Is Controlled by an ATP-dependent Mechanism. *Journal of Biological Chemistry* **288**, 27019-27030.

Chen, D. R., Toone, W. M., Mata, J., Lyne, R., Burns, G., Kivinen, K., Brazma, A., Jones, N. and Bähler, J. (2003). Global transcriptional responses of fission yeast to environmental stress. *Molecular Biology of the Cell* **14**, 214-229.

Cheng, J., Zhang, D. Y., Kim, K., Zhao, Y. X., Zhao, Y. M. and Su, B. (2005). Mip1, an MEKK2-interacting protein, controls MEKK2 dimerization and activation. *Molecular and Cellular Biology* **25**, 5955-5964.

Chia, K. H., Fukuda, T., Sofyantoro, F., Matsuda, T., Amai, T. and Shiozaki, K. (2017). Ragulator and GATOR1 complexes promote fission yeast growth by attenuating TOR complex 1 through Rag GTPases. *eLife* **6**, e30880.

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Cohen, A., Kupiec, M. and Weisman, R. (2014). Glucose Activates TORC2-Gad8 Protein via Positive Regulation of the cAMP/cAMP-dependent Protein Kinase A (PKA) Pathway and Negative Regulation of the Pmk1 Protein-Mitogen-activated Protein Kinase Pathway. *Journal of Biological Chemistry* **289**, 21727-21737.

Cohen, A., Kupiec, M. and Weisman, R. (2016). Gad8 Protein Is Found in the Nucleus Where It Interacts with the MluI Cell Cycle Box-binding Factor (MBF) Transcriptional Complex to Regulate the Response to DNA Replication Stress. *Journal of Biological Chemistry* **291**, 9371-9381.

Degols, G., Shiozaki, K. and Russell, P. (1996). Activation and regulation of the Spc1 stress-activated protein kinase in *Schizosaccharomyces pombe*. *Molecular and Cellular Biology* **16**, 2870-2877.

Frias, M. A., Thoreen, C. C., Jaffe, J. D., Schroder, W., Sculley, T., Carr, S. A. and Sabatini, D. M. (2006). mSin1 is necessary for Akt/PKB phosphorylation, and its isoforms define three distinct mTORC2s. *Current Biology* **16**, 1865-1870.

Furuita, K., Kataoka, S., Sugiki, T., Hattori, Y., Kobayashi, N., Ikegami, T., Shiozaki, K., Fujiwara, T. and Kojima, C. (2015). Utilization of paramagnetic relaxation enhancements for high-resolution NMR structure determination of a soluble loop-rich protein with sparse NOE distance restraints. *Journal of Biomolecular NMR* **61**, 55-64.

Gaits, F., Degols, G., Shiozaki, K. and Russell, P. (1998). Phosphorylation and association with the transcription factor Atf1 regulate localization of Spc1/Sty1 stress-activated kinase in fission yeast. *Genes & Development* **12**, 1464-1473.

García-Martínez, J. M. and Alessi, D. R. (2008). mTOR complex 2 (mTORC2) controls hydrophobic motif phosphorylation and activation of serum- and glucocorticoid-induced protein kinase 1 (SGK1). *Biochemical Journal* **416**, 375-385.

Hayashi, T., Hatanaka, M., Nagao, K., Nakaseko, Y., Kanoh, J., Kokubu, A., Ebe, M.

and Yanagida, M. (2007). Rapamycin sensitivity of the *Schizosaccharomyces pombe tor2* mutant and organization of two highly phosphorylated TOR complexes by specific and common subunits. *Genes to Cells* **12**, 1357-1370.

Hresko, R. C. and Mueckler, M. (2005). mTOR center dot RICTOR is the Ser^{473} kinase for Akt/protein kinase B in 3T3-L1 adipocytes. *Journal of Biological Chemistry* **280**, 40406-40416.

Ikeda, K., Morigasaki, S., Tatebe, H., Tamanoi, F. and Shiozaki, K. (2008). Fission yeast TOR complex 2 activates the AGC-family Gad8 kinase essential for stress resistance and cell cycle control. *Cell Cycle* **7**, 358-364.

Jacinto, E., Facchinetti, V., Liu, D., Soto, N., Wei, S., Jung, S. Y., Huang, Q., Qin, J. and Su, B. (2006). SIN1/MIP1 maintains rictor-mTOR complex integrity and regulates Akt phosphorylation and substrate specificity. *Cell* **127**, 125-137.

Jang, Y.-J., Won, M. and Yoo, H.-S. (2013). Phosphorylations of Sds23/Psp1/Moc1 by stress-activated kinase and cAMP-dependent kinase are essential for regulating cell viability in prolonged stationary phase. *Yeast* **30**, 379-394.

Kabeche, R., Madrid, M., Cansado, J. and Moseley, J. B. (2015). Eisosomes Regulate Phosphatidylinositol 4,5-Bisphosphate (PI(4,5)P-2) Cortical Clusters and Mitogen-activated Protein (MAP) Kinase Signaling upon Osmotic Stress. *Journal of Biological Chemistry* **290**, 25960-25973.

Kataoka, S., Furuita, K., Hattori, Y., Kobayashi, N., Ikegami, T., Shiozaki, K., Fujiwara, T. and Kojima, C. (2015). H-1, N-15 and C-13 resonance assignments of the conserved region in the middle domain of *S. pombe* Sin1 protein. *Biomolecular NMR Assignments* **9**, 89-92.

Kato, T., Okazaki, K., Murakami, H., Stettler, S., Fantes, P. A. and Okayama, H. (1996). Stress signal, mediated by a HOG1-like MAP kinase, controls sexual development in fission yeast. *FEBS Letters* **378**, 207-212.

Kawai, M., Nakashima, A., Ueno, M., Ushimaru, T., Aiba, K., Doi, H. and Uritani, M. (2001). Fission yeast Tor1 functions in response to various stresses including nitrogen starvation, high osmolarity, and high temperature. *Current Genetics* **39**, 166-174.

Keogh, M. C., Mennella, T. A., Sawa, C., Berthelet, S., Krogan, N. J., Wolek, A., Podolny, V., Carpenter, L. R., Greenblatt, J. F., Baetz, K. et al. (2006). The *Saccharomyces cerevisiae* histone H2A variant Htz1 is acetylated by NuA4. *Genes & Development* **20**, 660-665.

Kyriakis, J. M. and Avruch, J. (2001). Mammalian mitogen-activated protein kinase signal transduction pathways activated by stress and inflammation. *Physiological Reviews* **81**, 807-869.

Lee, S., Comer, F. I., Sasaki, A., McLeod, I. X., Duong, Y., Okumura, K., Yates, J. R., Parent, C. A. and Firtel, R. A. (2005). TOR complex 2 integrates cell movement during chemotaxis and signal relay in *Dictyostelium*. *Molecular Biology of the Cell* **16**, 4572-4583.

Lee, Y. J., Jeschke, G. R., Roelants, F. M., Thorner, J. and Turk, B. E. (2012). Reciprocal phosphorylation of yeast glycerol-3-phosphate dehydrogenases in adaptation to distinct types of stress. *Molecular and Cellular Biology* **32**, 4705-4717.

Liu, P., Gan, W., Chin, Y. R., Ogura, K., Guo, J., Zhang, J., Wang, B., Blenis, J., Cantley, L. C., Toker, A. et al. (2015). PtdIns(3,4,5)P-3-dependent activation of the mTORC2 kinase complex. *Cancer Discovery* **5**, 1194-1209.

Loewith, R., Jacinto, E., Wullschleger, S., Lorberg, A., Crespo, J. L., Bonenfant, D., Oppliger, W., Jenoe, P. and Hall, M. N. (2002). Two TOR complexes, only one of which is rapamycin sensitive, have distinct roles in cell growth control. *Molecular Cell* **10**, 457-468.

Madrid, M., Vázquez-Marín, B., Franco, A., Soto, T., Vicente-Soler, J., Gacto, M. and

Cansado, J. (2016). Multiple crosstalk between TOR and the cell integrity MAPK signaling pathway in fission yeast. *Scientific Reports* **6**.

Maruyama, T., Nakamura, T., Hayashi, T. and Yanagida, M. (2006). Histone H2B mutations in inner region affect ubiquitination, centromere function, silencing and chromosome segregation. *EMBO Journal* **25**, 2420-2431.

Matsuo, T., Kubo, Y., Watanabe, Y. and Yamamoto, M. (2003). *Schizosaccharomyces pombe* AGC family kinase Gad8p forms a conserved signaling module with TOR and PDK1-like kinases. *EMBO Journal* **22**, 3073-3083.

Matsuo, T., Otsubo, Y., Urano, J., Tamanoi, F. and Yamamoto, M. (2007). Loss of the TOR kinase Tor2 mimics nitrogen starvation and activates the sexual development pathway in fission yeast. *Molecular and Cellular Biology* **27**, 3154-3164.

Meier, R., Thelen, M. and Hemmings, B. A. (1998). Inactivation and dephosphorylation of protein kinase Bα (PKBα) promoted by hyperosmotic stress. *EMBO Journal* **17**, 7294-7303.

Millar, J. B. A., Buck, V. and Wilkinson, M. G. (1995). Pyp1 and Pyp2 PTPases dephosphorylate an osmosensing MAP kinase controlling cell-size at division in fission yeast. *Genes & Development* **9**, 2117-2130.

Morigasaki, S. and Shiozaki, K. (2010). Two-component signaling to the stress MAP kinase cascade in fission yeast. In *Methods in Enzymology, Vol 471: Two-Component Signaling Systems, Part C*, (eds M. I. Simon B. R. Crane and A. Crane), pp. 279-289.

Muir, A., Roelants, F. M., Timmons, G., Leskoske, K. L. and Thorner, J. (2015). Down-regulation of TORC2-Ypk1 signaling promotes MAPK-independent survival under hyperosmotic stress. *eLife* **4**, e09336.

Nguyen, A. N. and Shiozaki, K. (2002). MAPping stress survival in yeasts: From the cell surface to the nucleus. In *Sensing, Signaling and Cell Adaptation*, (eds. Storey,

K. B. and Storey, J. M.), pp. 75-90. Amsterdam, The Netherlands: Elsevier Science.

Ohmiya, R., Kato, C., Yamada, H., Aiba, H. and Mizuno, T. (1999). Isolation of multicopy suppressors of the calcium sensitivity of a mutant lacking the bZIP transcription factor Atf1 in fission yeast. *Molecular and General Genetics* **261**, 297-306.

Ohmiya, R., Yamada, H., Nakashima, K., Aiba, H. and Mizuno, T. (1995). Osmoregulation of fission yeast: Cloning of two distinct genes encoding glycerol-3-phosphate dehydrogenase, one of which is responsible for osmotolerance for growth. *Molecular Microbiology* **18**, 963-973.

Riggi, M., Niewola-Staszkowska, K., Chiaruttini, N., Colom, A., Kusmider, B., Mercier, V., Soleimanpour, S., Stahl, M., Matile, S., Roux, A. et al. (2018). Decrease in plasma membrane tension triggers PtdIns(4,5)P-2 phase separation to inactivate TORC2. *Nature Cell Biology* **20**, 1043-1051.

Sánchez-Piris, M., Posas, F., Alemany, V., Winge, I., Hidalgo, E., Bachs, O. and Aligue, R. (2002). The serine/threonine kinase Cmk2 is required for oxidative stress response in fission yeast. *Journal of Biological Chemistry* **277**, 17722-17727.

Sarbassov, D. D., Ali, S. M., Kim, D. H., Guertin, D. A., Latek, R. R., Erdjument-Bromage, H., Tempst, P. and Sabatini, D. M. (2004). Rictor, a novel binding partner of mTOR, defines a rapamycin-insensitive and raptor-independent pathway that regulates the cytoskeleton. *Current Biology* **14**, 1296-1302.

Sarbassov, D. D., Guertin, D. A., Ali, S. M. and Sabatini, D. M. (2005). Phosphorylation and regulation of Akt/PKB by the rictor-mTOR complex. *Science* **307**, 1098-1101.

Schroder, W., Bushell, G. and Sculley, T. (2005). The human stress-activated protein kinase-interacting 1 gene encodes JNK-binding proteins. *Cellular Signalling* **17**, 761-767.

Schroder, W. A., Buck, M., Cloonan, N., Hancock, J. F., Suhrbier, A., Sculley, T. and

Bushell, G. (2007). Human Sin1 contains Ras-binding and pleckstrin homology domains and suppresses Ras signalling. *Cellular Signalling* **19**, 1279-1289.

Shiozaki, K. and Russell, P. (1995a). Cell-cycle control linked to extracellular environment by MAP kinase pathway in fission yeast. *Nature* **378**, 739-743.

Shiozaki, K. and Russell, P. (1995b). Counteractive roles of protein phosphatase 2C (PP2C) and a MAP kinase kinase homolog in the osmoregulation of fission yeast. *EMBO Journal* **14**, 492-502.

Shiozaki, K. and Russell, P. (1996). Conjugation, meiosis, and the osmotic stress response are regulated by Spc1 kinase through Atf1 transcription factor in fission yeast. *Genes & Development* **10**, 2276-2288.

Shiozaki, K. and Russell, P. (1997). Stress-activated protein kinase pathway in cell cycle control of fission yeast. In *Methods in Enzymology, Vol. 283: Cell Cycle Control*, (ed. W. G. Dunphy), pp. 506-520.

Shiozaki, K., Shiozaki, M. and Russell, P. (1998). Heat stress activates fission yeast Spc1/StyI MAPK by a MEKK-independent mechanism. *Molecular Biology of the Cell* **9**, 1339-1349.

Smith, D. A., Toone, W. M., Chen, D. R., Bähler, J., Jones, N., Morgan, B. A. and Quinn, J. (2002). The Srk1 protein kinase is a target for the Sty1 stress-activated MAPK in fission yeast. *Journal of Biological Chemistry* **277**, 33411-33421.

Sukegawa, Y., Yamashita, A. and Yamamoto, M. (2011). The fission yeast stress-responsive MAPK pathway promotes meiosis via the phosphorylation of pol II CTD in response to environmental and feedback cues. *PLoS Genetics* **7**, e1002387.

Tanoue, T., Adachi, M., Moriguchi, T. and Nishida, E. (2000). A conserved docking motif in MAP kinases common to substrates, activators and regulators. *Nature Cell Biology* **2**, 110-116.

Tatebe, H., Morigasaki, S., Murayama, S., Zeng, C. T. and Shiozaki, K. (2010). Rab-

Family GTPase Regulates TOR Complex 2 Signaling in Fission Yeast. *Current Biology* **20**, 1975-1982.

Tatebe, H., Murayama, S., Yonekura, T., Hatano, T., Richter, D., Furuya, T., Kataoka, S., Furuita, K., Kojima, C. and Shiozaki, K. (2017). Substrate specificity of TOR complex 2 is determined by a ubiquitin-fold domain of the Sin1 subunit. *eLife* **6**, e19594

Tatebe, H., Shimada, K., Uzawa, S., Morigasaki, S. and Shiozaki, K. (2005). Wsh3/Tea4 is a novel cell-end factor essential for bipolar distribution of Tea1 and protects cell polarity under environmental stress in *S. pombe*. *Current Biology* **15**, 1006-1015.

Tatebe, H. and Shiozaki, K. (2003). Identification of Cdc37 as a novel regulator of the stress-responsive mitogen-activated protein kinase. *Molecular and Cellular Biology* **23**, 5132-5142.

Tatebe, H. and Shiozaki, K. (2010). Rab small GTPase emerges as a regulator of TOR complex 2. *Small GTPases* **1**, 180-182.

Toone, W. M. and. Jones, N. (2004). Stress responses in *S. pombe*. In *The Molecular Biology of Schizosaccharomyces pombe*, (ed. R. Egel), pp. 57-72: Springer.

Wang, L. Y., Shimada, K., Morishita, M. and Shiozaki, K. (2005). Response of fission yeast to toxic cations involves cooperative action of the stress-activated protein kinase Spc1/Sty1 and the Hal4 protein kinase. *Molecular and Cellular Biology* **25**, 3945-3955.

Warbrick, E. and Fantes, P. A. (1991). The wis1 protein-kinase is a dosage-dependent regulator of mitosis in *Schizosaccharomyces pombe*. *EMBO Journal* **10**, 4291-4299.

Wedaman, K. P., Reinke, A., Anderson, S., Yates, J., McCaffery, J. M. and Powers, T. (2003). Tor kinases are in distinct membrane-associated protein complexes in *Saccharomyces cerevisiae*. *Molecular Biology of the Cell* **14**, 1204-1220.

Weisman, R. and Choder, M. (2001). The fission yeast TOR homolog, *tor1*$^{+}$, is required for the response to starvation and other stresses via a conserved serine. *Journal of Biological Chemistry* **276**, 7027-7032.

Wilkinson, M. G., Pino, T. S., Tournier, S., Buck, V., Martin, H., Christiansen, J., Wilkinson, D. G. and Millar, J. B. A. (1999). Sin1: an evolutionarily conserved component of the eukaryotic SAPK pathway. *EMBO Journal* **18**, 4210-4221.

Wilkinson, M. G., Samuels, M., Takeda, T., Toone, W. M., Shieh, J.-C., Toda, T., Millar, J. B. A. and Jones, N. (1996). The Atf1 transcription factor is a target for the Sty1 stress-activated MAP kinase pathway in fission yeast. *Genes & Development* **10**, 2289-2301.

Yamada, H., Ohmiya, R., Aiba, H. and Mizuno, T. (1996). Construction and characterization of a deletion mutant of *gpd2* that encodes an isozyme of NADH-dependent glycerol-3-phosphate dehydrogenase in fission yeast. *Bioscience Biotechnology and Biochemistry* **60**, 918-920.

Yamada, H., Ohmiya, R., Yamamoto, E., Aiba, H. and Mizuno, T. (1997). Characterization of multicopy suppressor genes that complement a defect in the Wis1-Sty1 MAP kinase cascade involved in stress responses in *Schizosaccharomyces pombe*. *Journal of General and Applied Microbiology* **43**, 209-215.

Yang, Q., Inoki, K., Ikenoue, T. and Guan, K.-L. (2006). Identification of Sin1 as an essential TORC2 component required for complex formation and kinase activity. *Genes & Development* **20**, 2820-2832.

Figures

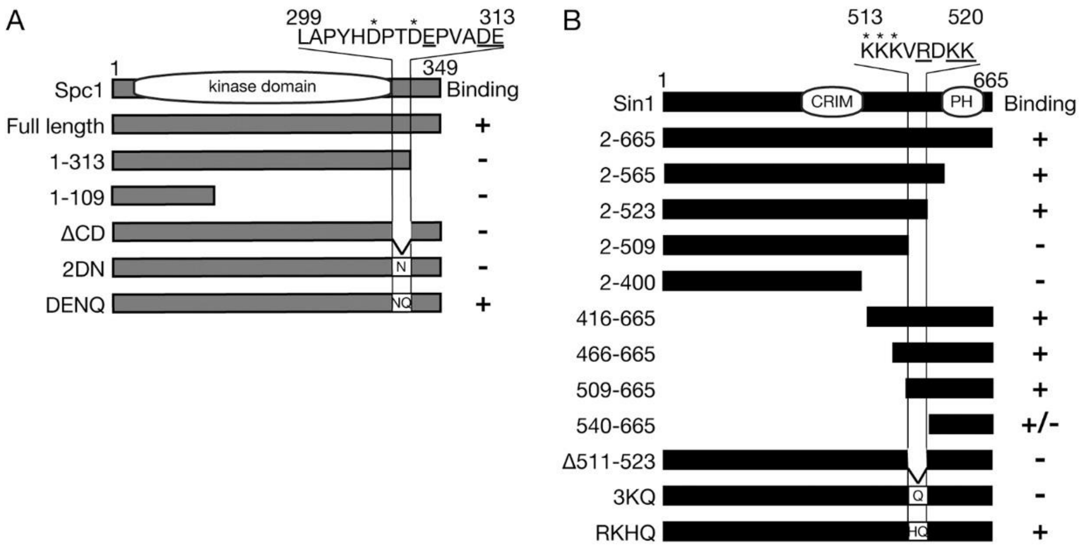

Figure 1. Yeast two-hybrid assays to characterize the interaction between Spc1 MAPK and Sin1.

(**A)** Various Spc1 fragments shown were expressed as bait together with Sin1(2-665) as prey in the budding yeast HF7c strain. The amino acid sequence of the putative CD domain (299-313) is shown at the top, where mutated residues in the 2DN and DENQ mutants are indicated by asterisks and underlines, respectively.

(B) Various Sin1 fragments shown were expressed as prey together with full-length Spc1 as bait as in (A). The amino acid sequence of a basic residue cluster (513-520) is shown at the top, where mutated residues in the 3KQ and RKHQ mutants are indicated by asterisks and underlines, respectively. +/-, 7 out of 12 clones examined were positive.

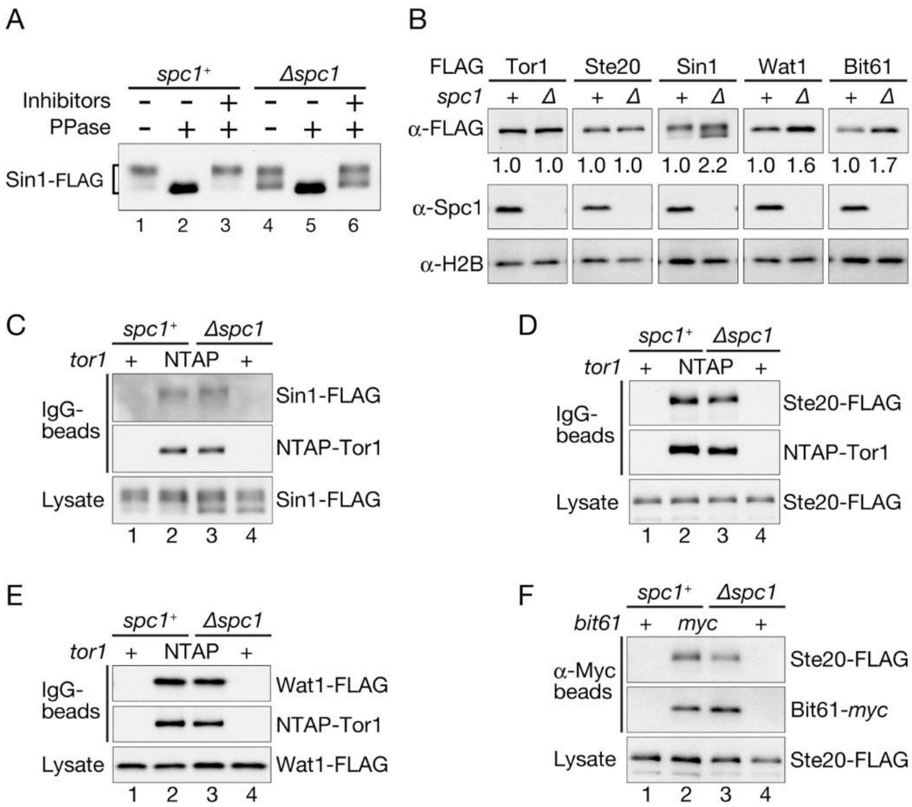

Figure 2. Spc1 MAPK-dependent phosphorylation of Sin1.

(A) Sin1 phosphorylation was examined by mobility-shift assays. The cell lysate of *spc1*$^+$ and Δ*spc1* strains carrying the *sin1:FLAG* allele was treated with lambda-protein phosphatase (PPase) in the presence and absence of phosphatase inhibitors, followed by SDS-PAGE and anti-FLAG immunoblotting.

(B) The lysate of *spc1*$^+$ and Δ*spc1* cells expressing FLAG-tagged Tor1, Ste20, Sin1, Wat1 and Bit61 from their respective chromosomal loci were analyzed by immunoblotting using anti-FLAG (α-FLAG), anti-Spc1 (α-Spc1), and anti-histone H2B (α-H2B) antibodies. Anti-FLAG signals normalized against anti-H2B signals are shown as values relative to the normalized values of the *spc1*$^+$ strains as 1.0.

(C-E) Physical interaction of NTAP-Tor1 with Sin1-FLAG (C), Ste20-FLAG (D), and Wat1-FLAG (E) was analyzed by co-affinity purification. NTAP-Tor1 was purified with

IgG-Sepharose beads from the cell lysate of *spc1*$^{+}$ *NTAP:tor1* and Δ*spc1 NTAP:tor1* strains expressing the FLAG-tagged regulatory subunits of TORC2 from their respective chromosomal loci (lanes 2 and 3). The *tor1*$^{+}$ strains expressing Tor1 without the NTAP tag were used as negative controls (lanes 1 and 4).

(F) Physical interaction between the Ste20 and Bit61 subunits was analyzed by co-immunoprecipitation. Bit61-*myc* was purified with Anti-c-Myc Affinity Gel from the cell lysate of *spc1*$^{+}$ *bit61:myc* and Δ*spc1 bit61:myc* strains expressing FLAG-tagged Ste20 from its chromosomal locus (lanes 2 and 3). The *bit61*$^{+}$ strains expressing untagged Bit61 were used as negative controls (lanes 1 and 4).

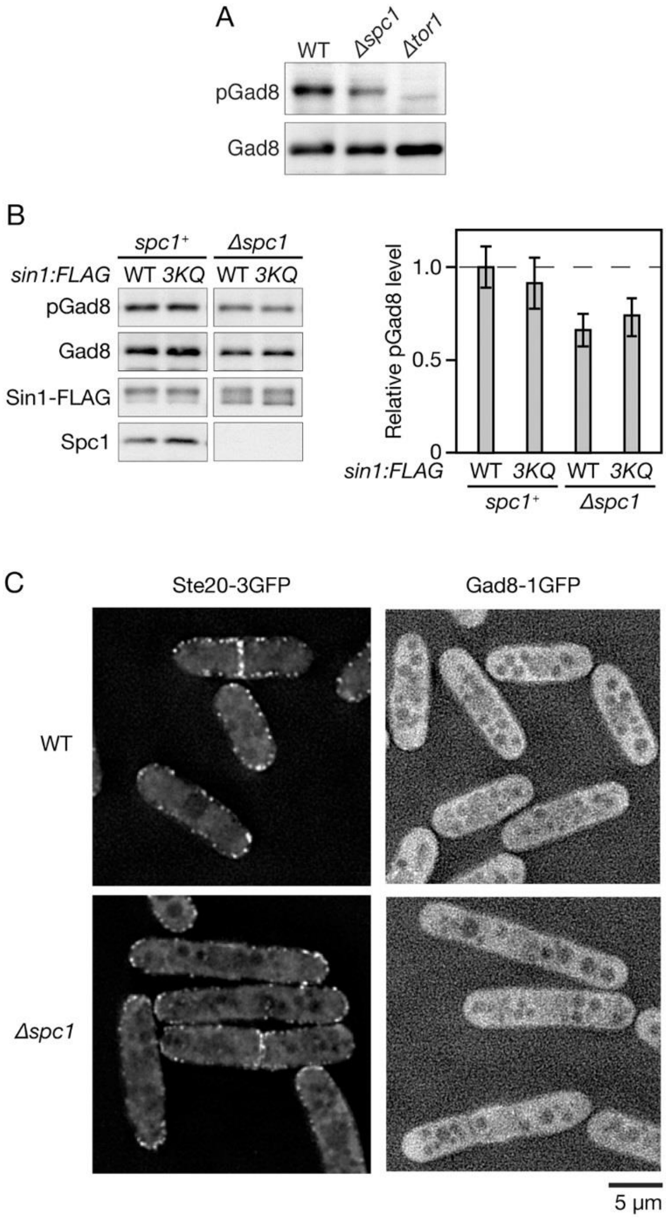

Figure 3. Spc1 MAPK positively regulates TORC2 activity.

(A) Gad8 phosphorylation levels in wild-type and Δ*spc1* cells were compared by immunoblotting using antibodies that specifically recognize phosphorylation of Ser-546 in the hydrophobic motif of Gad8 (pGad8) as well as those against the Gad8 C-terminus (Gad8). The Δ*tor1* strain, which lacks functional TORC2, was used as a negative control.

(B) TORC2 activity is not affected by the *sin1-3KQ* mutation that disrupts the Sin1-Spc1 interaction. The TORC2-dependent Gad8 phosphorylation in the *spc1*$^{+}$ and Δ*spc1* strains carrying the *sin1:FLAG* or *sin1-3KQ:FLAG* alleles were examined as in (A). The Sin1-FLAG and Spc1 proteins were detected by anti-FLAG and anti-Spc1 antibodies, respectively. Quantified pGad8 levels relative to that in the *spc1*$^{+}$ *sin1:FLAG* strain (mean±SD, n≥3) were shown as a bar graph on the right.

(C) The Δ*spc1* mutation does not significantly affect the cellular localization of TORC2 and Gad8. z-axial images of wild-type and Δ*spc1* strains expressing Ste20 or Gad8 from their chromosomal loci with the GFP tag were deconvolved and mid-section images are shown. Bar, 5 µm.

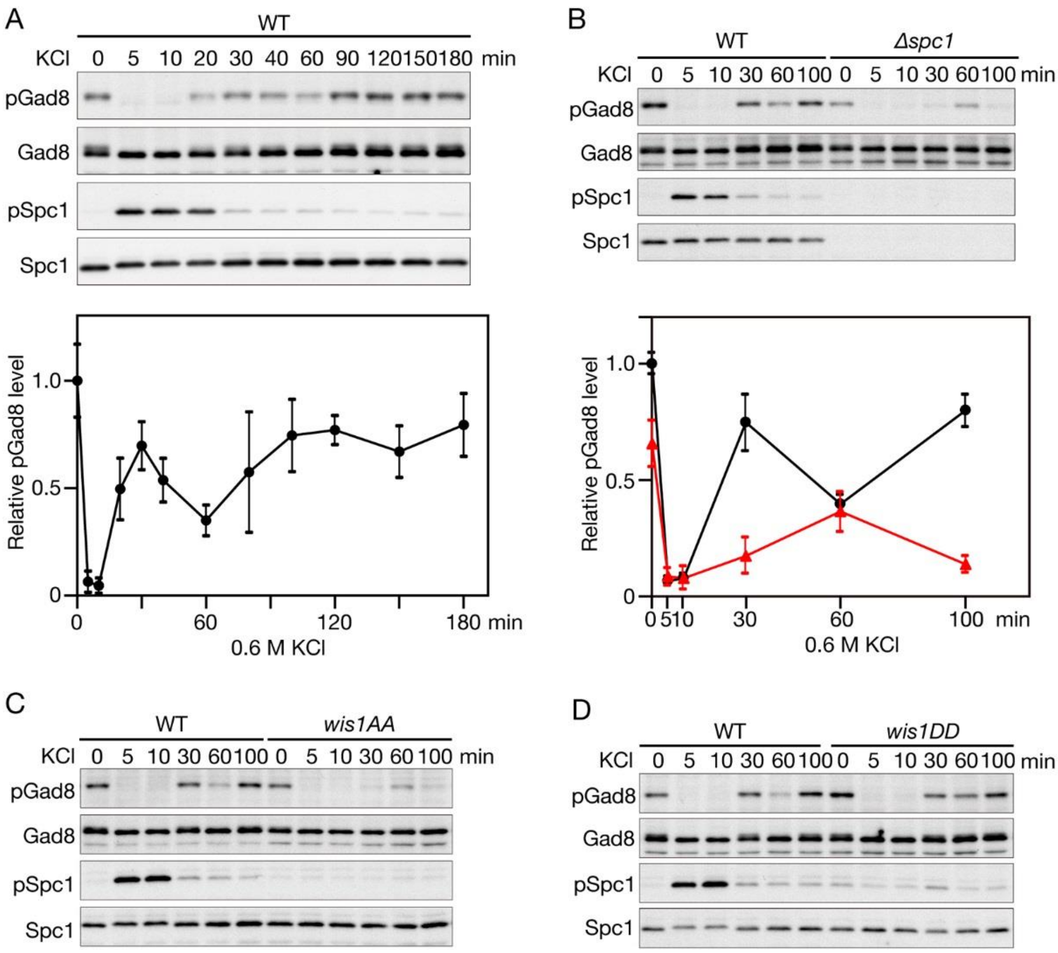

Figure 4. TORC2 activity responds to high osmolarity stress.

Cultures in early log-phase were treated with high osmolarity stress of 0.6 M KCl, and the TORC2-dependent phosphorylation of Gad8 Ser-546 (pGad8), the Gad8 protein level (Gad8), the activating phosphorylation of Spc1 Thr-171/Tyr-173 (pSpc1) and the Spc1 protein level (Spc1) were monitored along the time course in wild-type (A), Δ*spc1* (B), *wis1AA* (C) and *wis1DD* (D) strains. In (A) and (B), Gad8 phosphorylation levels after osmostress was quantified and plotted as values relative to that of non-stressed cells (mean±SD, n≥3). Black circle, wild-type; Red triangle, Δ*spc1*.

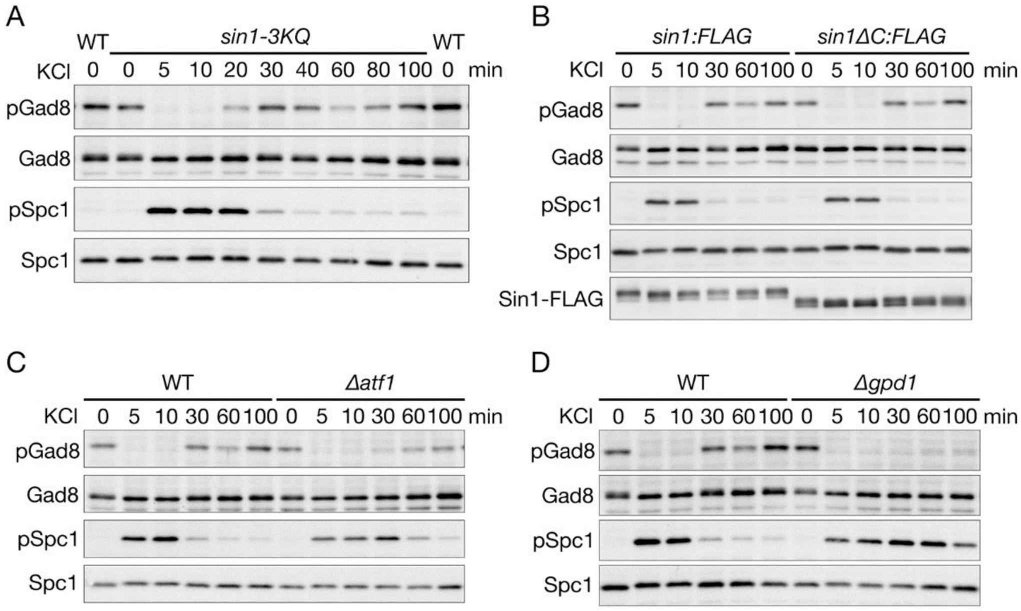

Figure 5. The Atf1 transcription factor and its target gene *gpd1*⁺ are important for reactivation of TORC2 after osmostress.

TORC2-dependent phosphorylation of Gad8 (pGad8) and the activating phosphorylation of Spc1 (pSpc1) in response to high osmolarity stress of 0.6 M KCl were monitored by immunoblotting as in Fig. 4 in the *sin1-3KQ* (A), *sin1∆C:FLAG* (B), ∆*atf1* (C) and ∆*gpd1* (D) strains. The *sin1∆C:FLAG* strain expresses the FLAG epitope-tagged Sin1 protein lacking the C-terminal 114 amino acid residues, which include the PH domain (see Fig. 1B for the domain structure of Sin1).

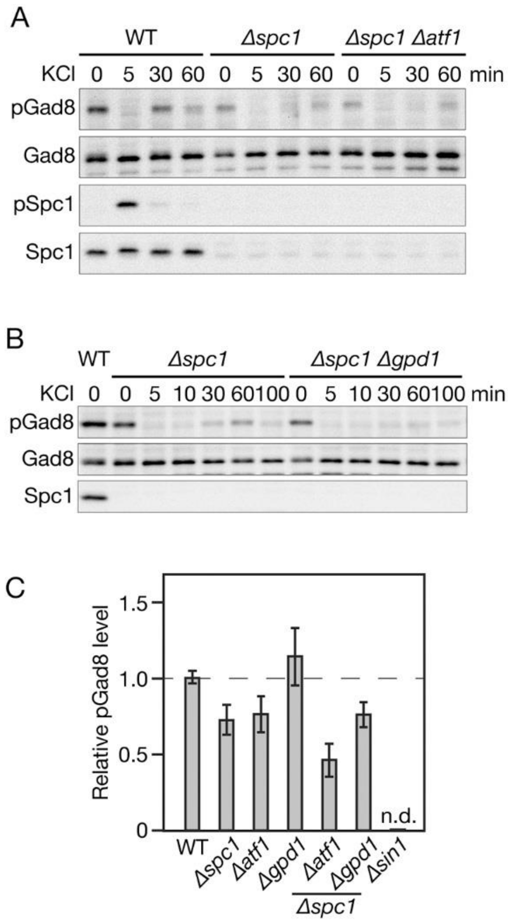

Figure 6. Regulation of TORC2 by the Spc1-Atf1-Gpd1 pathway in the presence and absence of osmostress.

(A, B) TORC2-dependent phosphorylation of Gad8 during the time course after high osmolarity stress of 0.6 M KCl was monitored by immunoblotting as in Fig. 4 in the Δ*spc1*, Δ*spc1* Δ*atf1* (A), and Δ*spc1* Δ*gpd1* (B) strains.

(C) Gad8 phosphorylation levels in the indicated strains under normal osmolarity were quantified and shown as values relative to that in the wild-type (WT) strain (mean±SD, n≥3). n.d., not detectable.

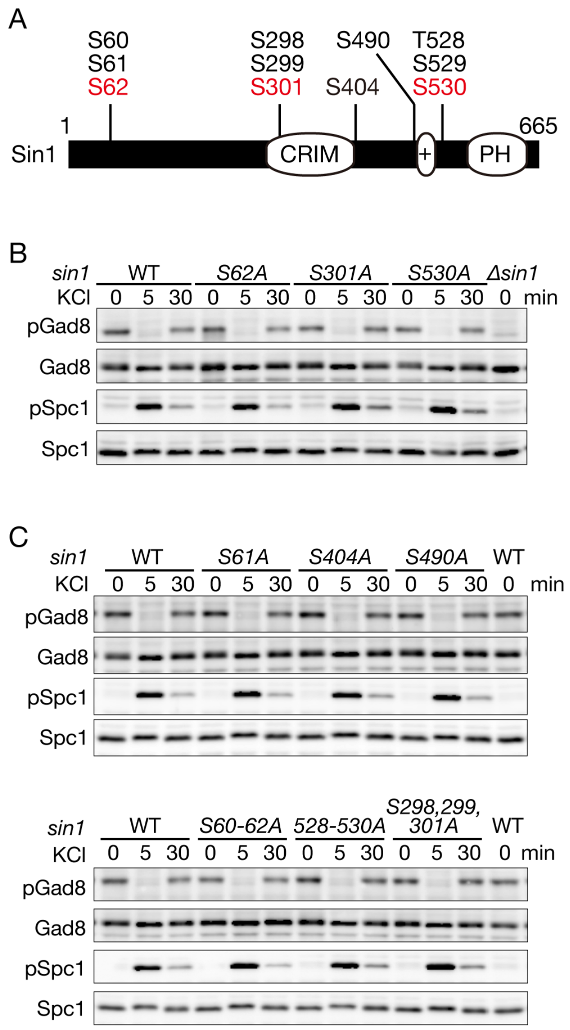

Figure S1. Mutations to the reported phosphorylation sites in Sin1 show no apparent effect on TORC2 activity.

(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).

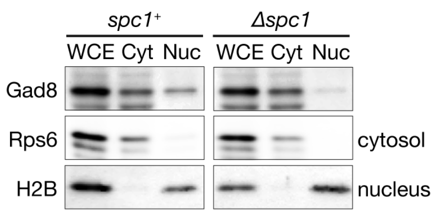

Figure S2. Nuclear-cytoplasmic distribution of the Gad8 protein.
The lysate of spheroplasts (whole cell extract, WCE) prepared from the wild-type and *Δspc1* strains was divided into the soluble cytosolic fraction (Cyt) and the nucleus-rich fraction (Nuc) as described by Cohen *et al.* (2016). Gad8 in 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.

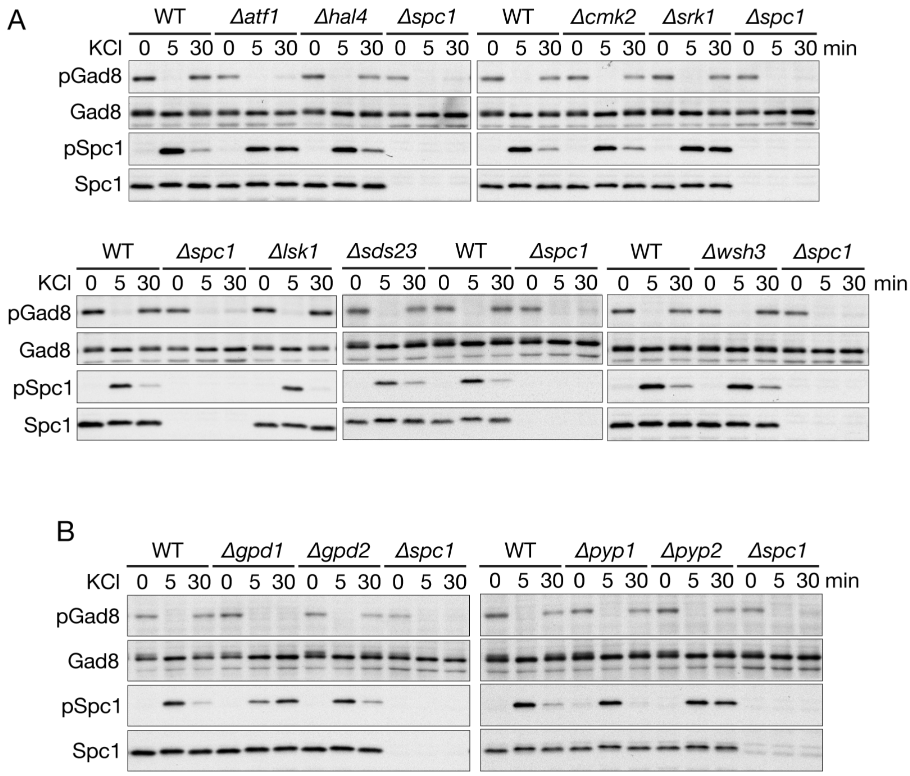

Figure S3. A search for genes required for reactivation of TORC2 after osmostress.
In the wild-type and indicated null mutant strains, TORC2-dependent phosphorylation of Gad8 (pGad8) and the activating phosphorylation of Spc1 MAPK (pSpc1) before and after high osmolarity stress of 0.6 M KCl were monitored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

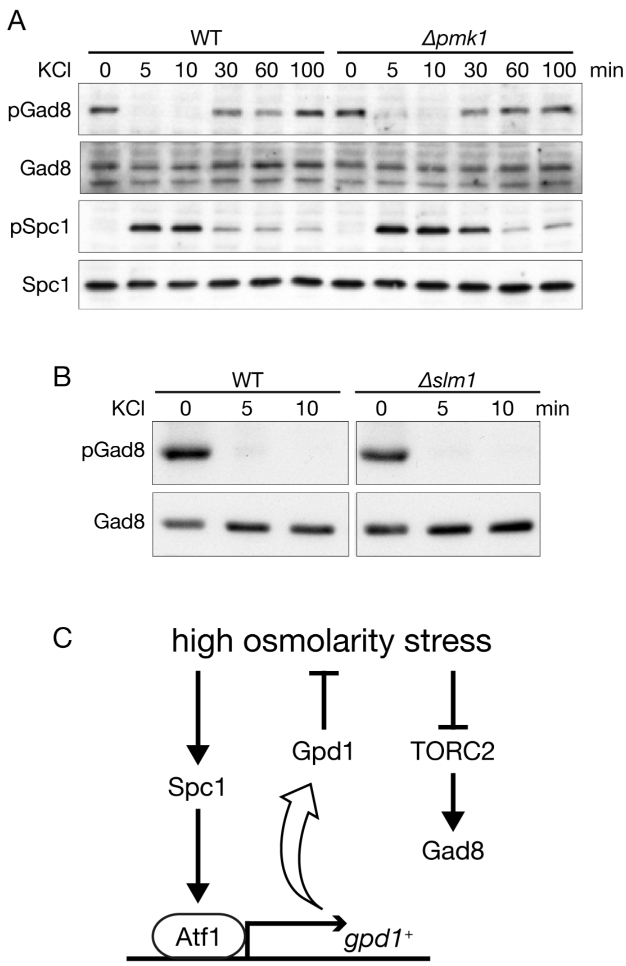

Figure S4. Osmo-response of TORC2 signaling in fission yeast does not involve Pmk1 MAPK nor Slm1.
(A) Pmk1 MAPK is not required for the osmo-inhibition of TORC2-Gad8 signaling. TORC2-dependent phosphorylation of Gad8 (pGad8) and the activating phosphorylation of Spc1 MAPK (pSpc1) in the wild-type and 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

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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