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Molecular mechanisms of substrate recognition and regulation of TOR complex 2

（TOR 複合体 2 の基質認識と活性制御の分子メカニズム）

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Functional characterization of the CRIM domain in Sin1, an evolutionally conserved subunit of TOR complex 2

The TOR Ser/Thr protein kinase is highly conserved from yeast to mammals. TOR forms two complexes, TOR complex 1 (TORC1) and 2 (TORC2). In fission yeast *Schizosaccharomyces pombe*, a genetically amenable eukaryotic model organism, TORC1 is composed of Tor2, Wat1, and Mip1, whereas TORC2 is composed of Tor1, Wat1, Bit61, Ste20, and Sin1. These complexes phosphorylate distinct substrates: TORC1 phosphorylates Psk1, a homologue of mammalian S6K that regulates protein synthesis, whereas TORC2 phosphorylates Gad8, a homologue of AKT that regulates numerous cellular events including glucose metabolism and cell growth.

In spite of extensive studies, it remained unclear how TORC2 specifically phosphorylates its own substrates. Since both TORC1 and TORC2 have the TOR kinase as the catalytic subunit, it is conceivable that TORC2 phosphorylates its substrates by physical interaction between its regulatory subunits and substrates. Recently, our laboratory found that the Sin1 subunit of TORC2 physically interacts with Gad8 via an amino acid region around CRIM (Conserved Region In the Middle), an evolutionarily conserved region in Sin1. Since interaction between Gad8 and CRIM is essential for TORC2 to phosphorylate Gad8, our laboratory concludes that CRIM recruits Gad8 to the Tor1 kinase in TORC2 to facilitate phosphorylation of Gad8. Considering that CRIM recruits Gad8 to TORC2, it is possible that CRIM enables TORC2 to selectively phosphorylate its own substrate but not those of TORC1 by interacting only with substrate of TORC2. Moreover, phosphorylation of Gad8 by TORC2 can be more efficient if CRIM binds to unphosphorylated Gad8 and release Gad8 following its phosphorylation by TORC2.

Our laboratory also found that the N-terminus of the CRIM domain is dispensable for Sin1 to interact with Gad8. Since amino acid residues in the N-terminus of the CRIM domain are highly conserved among Sin1 orthologues, it is expected that this region of Sin1 plays an essential role in TORC2 other than interacting with Gad8.

Here in CAPTER I, we characterized the molecular functions of Sin1 in fission yeast. We found that the CRIM domain of Sin1 did not bind to Psk1, a substrate of TORC1. We also found that the CRIM domain hardly binds to Gad8 that is phosphorylated by TORC2, comparing with

unphosphorylated Gad8. Moreover, Psk1 is phosphorylated by TORC2 when Psk1 was synthetically fused with Ste20, a subunit of TORC2, suggesting that the TOR kinase in TORC2 does not distinguish between Gad8 and Psk1 as its substrate. Therefore, we conclude that TORC2 specifically phosphorylates its own substrates by virtue of selective recruitment of Gad8 via Sin1 CRIM.

In the N-terminus of the CRIM domain, we identified a region that is significant for Sin1 to interact with Wat1. Interaction between Sin1 and Wat1 is required for Sin1 to be properly incorporated into TORC2 and for Gad8 to be phosphorylated by Tor1. In addition, in the N-terminus of the CRIM domain, we identified two evolutionarily conserved leucine residues that are required for Sin1 to bind to Wat1. Since interaction between Sin1 and Wat1 is required for Sin1 to interact with Tor1 and for TORC2 to phosphorylate Gad8, we conclude that Wat1 anchors Sin1 to the Tor1 kinase. Alanine substitution of these leucine residues attenuated interaction between Sin1 and Wat1. This was also the case in hSin1 and mLST8, the counterpart of Sin1 and Wat1 in mammals, suggesting that the molecular mechanism of interaction between Sin1 and Wat1 is conserved among eukaryotes. Combining all the results above, we concluded that Sin1 CRIM has two distinct functions: the one for recruiting Gad8 to TORC2 and the other for anchoring Sin1 in TORC2.

Rab GTPase responds to glucose in order to regulate TOR complex 2.

While Rheb and Rag small GTPases regulate TORC1, the Rab family small GTPase Ryh1 directly binds to and activates the TORC2-Gad8 pathway in fission yeast, presumably via the Bit61 subunit of TORC2. Extensive studies have demonstrated that Rheb and Rag are activated by various stimuli such as growth hormones and nutrients. On the other hand, what physiological stimuli activate fission yeast Ryh1 was unclear.

Here in CHAPTOR II, we demonstrated that the TORC2-Gad8 pathway was activated by glucose in fission yeast. In addition, to monitor *in vivo* activity of Ryh1, we developed the experimental system to detect active Ryh1. Using this technique, we successfully demonstrated that the amount of active Ryh1 decreased when cells were starved of glucose. In addition, cells expressing hyperactive Ryh1 showed sustained phosphorylation of Gad8 even after glucose starvation. Combining those results, we concluded that Ryh1 is activated by glucose to regulate the TORC2-Gad8 pathway. We also found that hyperactive Ryh1 failed to stimulate the TORC2-Gad8 pathway in the absence of Bit61, suggesting that Bit61 is required for Ryh1 to transmit glucose signals to TORC2. Since both Bit61 and Ryh1 orthologues are evolutionarily conserved among eukaryotes, it is possible that the TORC2 pathway in other species utilizes the same activation mechanism.

CHAPTER I

Functional characterization of the CRIM domain in Sin1, an evolutionally conserved subunit of TOR complex 2

INTRODUCTION I

TOR Ser/Thr specific protein kinase forms two distinct complexes, TORC1 and TORC2. TORC1 is composed of Raptor, mLST8, and mTOR, whereas TORC2 is composed of Rictor, hSin1, Protor, mLST8, and mTOR (Frias et al., 2006; Hara et al., 2002; Jacinto et al., 2006; Jacinto et al., 2004; Kim et al., 2002; Kim et al., 2003; Loewith et al., 2002; Sarbassov et al., 2004; Yang et al., 2006). These complexes phosphorylate distinct substrates. TORC1 phosphorylates numerous proteins including S6K and 4EBP to regulate protein synthesis (Figure 1. A) (Brown et al., 1995; Brunn et al., 1997). On the other hand, TORC2 phosphorylates AKT, SGK, and several isoforms of PKC to regulate various cellular events including glucose metabolism, cell growth, and survival (Figure 1. A) (Guertin et al., 2006; Sarbassov et al., 2005). All these substrates of TORC2 are AGC family protein kinase that composed of a kinase domain followed by a characteristic C-terminal extension. TORC2 phosphorylates the hydrophobic motif in C-terminal extensions in Akt, SGK, and several isoforms of PKC to stimulate catalytic activity of these protein kinases.

To characterize TOR complexes further, extensive studies have been conducted in a variety of model organisms. In fission yeast, a genetically amenable model organism, we as well as others identified the TORC1-Psk1 and TORC2-Gad8 pathways that are highly homologous to mammalian TORC1-S6K and TORC2-AKT or SGK pathways, respectively (Hayashi et al., 2007; Ikeda et al., 2008; Matsuo et al., 2003; Matsuo et al., 2007; Tatebe et al., 2010). Fission yeast TORC1 is composed of Mip1, Wat1, and Tor2, whereas TORC2 is composed of Ste20, Sin1, Bit61, Wat1, and Tor1 (Figure 1. B) (Matsuo et al., 2003; Hayashi et al., 2007; Matsuo et al., 2007; Ikeda et al., 2008; Tatebe et al., 2010.

Recently, our laboratory found that Sin1 physically interacts with Gad8 *in vitro*. Sin1 has the CRIM (Conserved Region In the Middle) domain, RBD (Ras Binding Domain), and PH domain that are highly conserved among eukaryotes (Cameron et al., 2011; Wang and Roberts, 2005). Our laboratory found that 281-400 amino acid residues in Sin1 surrounding the CRIM domain is the shortest fragment that interacts with Gad8. We also identified 352-361 amino acid residues in the Sin1 CRIM domain as an essential region for interaction between Sin1 and Gad8. These amino acid residues were highly conserved in hSin1 in human. Based on the structural analysis of Sin1 CRIM in fission yeast, this region was found in a surface area creating a negatively charged patch by accumulating aspartate and glutamate residues (Figure 2) (Kataoka et al., 2014). Thereby we named this region as “acidic region”. Interaction between Sin1 and Gad8 was crucial for phosphorylation of Gad8 and over-expression of the CRIM domain inhibits phosphorylation of Gad8 by TORC2,

presumably due to competition with endogenous TORC2 to bind to Gad8 (Murayama, unpublished data). From these observations, we propose a model that Sin1 binds to and recruits Gad8 toward Tor1 kinase to facilitate phosphorylation of Gad8.

Here we demonstrate that CRIM binds to unphosphorylated Gad8 but not to Gad8 that is phosphorylated by TORC2. From the observations, we concluded that the CRIM domain recruits unphosphorylated Gad8 to facilitate the phosphorylation by Tor1. In addition, we also found that N-terminus of the CRIM domain is required for Sin1 to be incorporated into TORC2 in a manner dependent on Wat1. This suggests that the CRIM domain is also indispensable for TORC2 assemble.

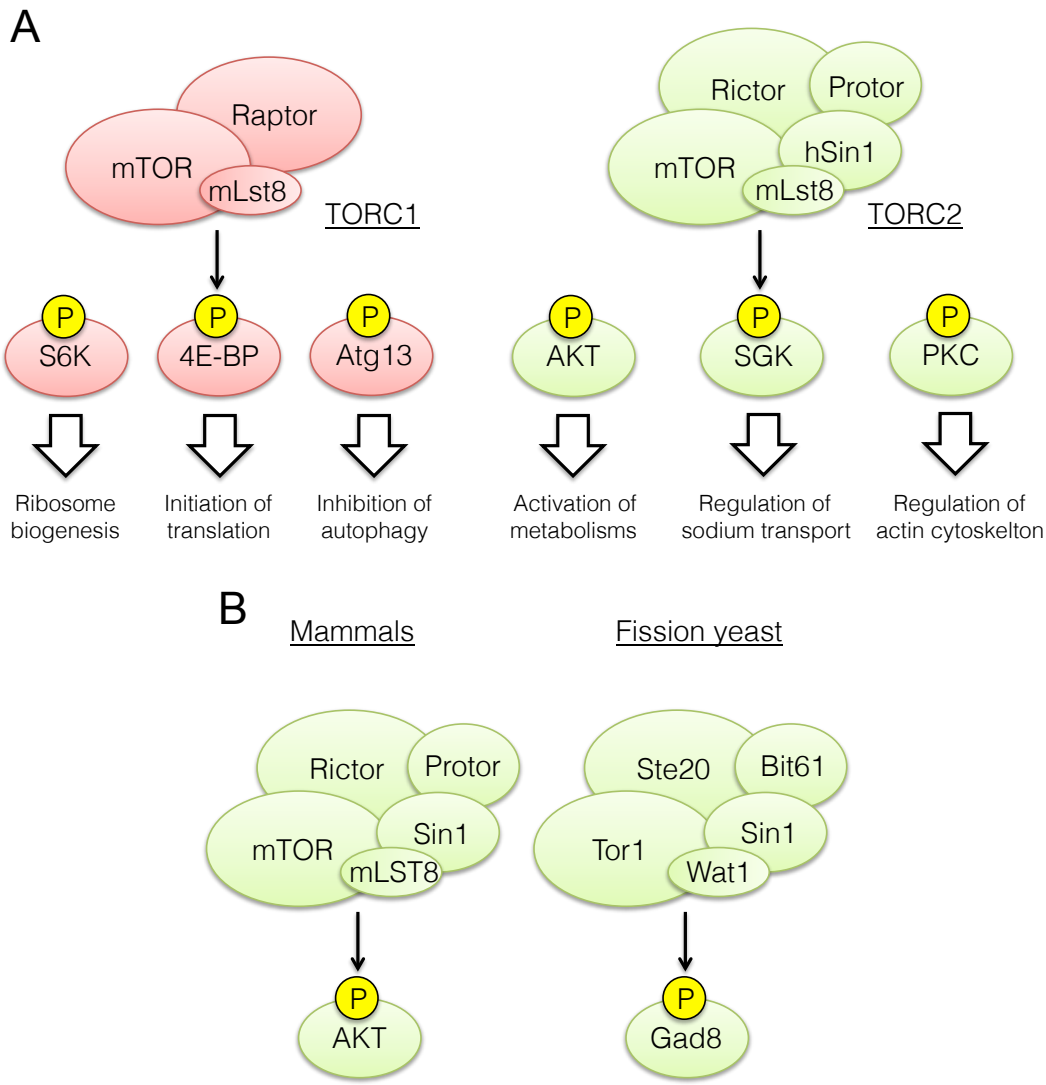

Figure 1. TOR complex 1 and 2

(A) Substrates of TOR complexes.

TORC1 phosphorylates S6K, 4E-BP, and Atg13, to regulate protein synthesis and degradation through regulation of translation and autophagy, respectively. TORC2 phosphorylates AKT, SGK, and several isoforms of PKC to regulate cellular metabolisms including glycolysis.

(B) TORC2 subunits are highly conserved from yeast to mammal.

Subunits identified in TORC2 in mammals are conserved in fission yeast. In mammals, TORC2 is composed of Rictor, hSin1, Protor, mLST8, and mTOR, and in fission yeast, these counterparts are identified as Ste20, Sin1, Bit61, Wat1, and Tor1, respectively. TORC2 in mammals and fission yeast phosphorylate Akt and Gad8, respectively.

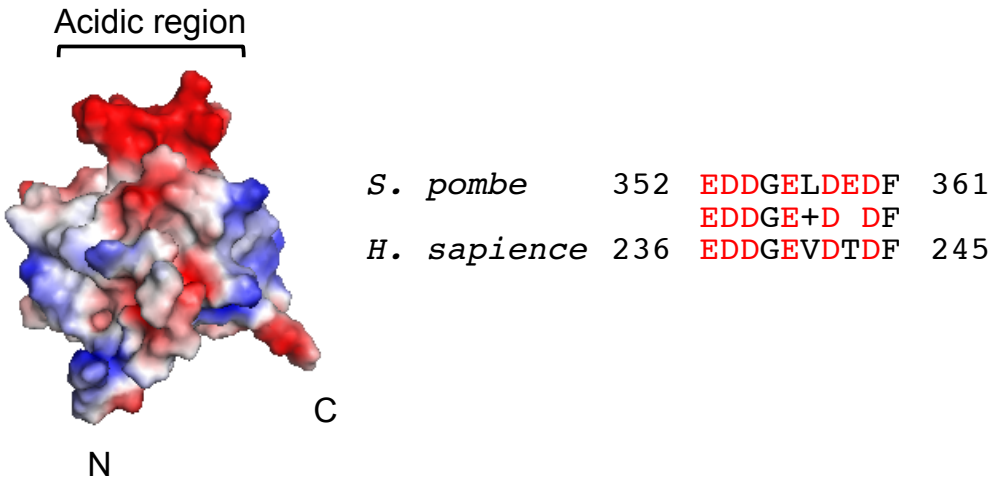

Figure 2. The structure of Gad8-interacting region in the CRIM domain

The NMR structure of 281-400 amino acid region of Sin1 that is responsible to bind to Gad8 was determined by Prof. Kojima's laboratory. This picture shows surface charge distribution of 281-400 amino acid region of Sin1. Charge marked with red or blue are corresponding to acidic or basic amino acids, respectively. The acidic residues are clustered in 352-361 amino acid-residues in Sin1 (referred as "acidic region") and this region is highly conserved among eukaryotes. Our previous study demonstrated that this region is indispensable for Sin1 to bind to Gad8.

MATERIALS AND METHODS I

Yeast strains and general techniques

Composition of media for yeast culture and genetic modification techniques of yeast cells were established previously (Bähler et al., 1998).

Antibodies

Antibodies used in this study are listed in the following table.

Antibody	Source (animals)	Supplier or Reference
Phosphorylated Gad8	Rabbit	(Tatebe et al., 2010)
Gad8	Rabbit	(Tatebe et al., 2010)
Phosphorylated p70 S6 Kinase	Mouse	#9205 (Cell Signaling Technology)
Psk1	Rabbit	Laboratory stock
FLAG epitope tag	Mouse	#F1804 (Sigma-Aldrich)
cMyc epitope tag	Mouse	#MMS-150P (COVANCE)
Rabbit IgG (HRP conjugated)	Goat	#W4011 (Promega)
Mouse IgG (HRP conjugated)	Goat	#W4021 (Promega)

Culturing yeast cells

Yeast growth was monitored as turbidity of culture with spectrophotometer (UVmini1240, SHIMAZU) at 600 nm wavelengths. All samples were prepared from the yeast cells in exponentially growing phase (OD600 value = ~0.4). Cells carrying plasmids were cultured in synthetic media {Edinburgh minimum media (EMM)} to maintain plasmids because yeast expression vectors used in this study were selected with complementation of leucine auxotrophy.

For nitrogen starvation, cells were cultured in EMM liquid media until exponentially growing phase and harvested on a membrane filter (#A045A0.25A*, ADVANTEC). Cells on a filter were re-suspended into fresh EMM media lacking (NH_4Cl).

Preparation of crude cell extract from yeast

To analyze phosphorylation status of Gad8 or Psk1, cells were harvested on a nitrocellulose filter membrane with 0.45 μm pore with 10% trichloroacetic acid (TCA) solution at room

temperature. Cells were disrupted by beads-beating with 0.5 mm glass beads (AZ ONE) and whole cell lysate were extracted with sodium dodecyl sulfate (SDS) sample buffer (550 mM Tris-HCl [pH 8.0], 2% SDS, 4.5% glycerol, 0.01% bromophenol blue, 0.7 M 2-mercaptoethanol).

Growth assays with 1 M KCl or 0.1 M $CaCl_2$ solid media

For assessing stress sensitivity of yeast cells, YES solid media containing 1 M KCl or 0.1 M $CaCl_2$ were prepared. Cells were inoculated into standard YES or EMM solid media and cultured at 30˚C for 24 hours. Cells on media were streaked onto fresh YES media (as control) or YES media containing 1 M KCl or 0.1 M $CaCl_2$. Cells on these solid media were incubated at 30˚C for few days and photographed with LAS4000 image analyzer (GE Healthcare Life Sciences).

The yeast two-hybrid assay

HF7c strain (CLONETEC) transformed with pGBT8 and pGADGH vectors were selected with culture media without supplementation of leucine and tryptophane. Transformants were cultured on media without leucine, tryptophane, and histidine with or without supplementation of 3-aminotriazole (3-AT), an inhibitor of histidine biosynthesis gene product. If objective pairs of gene products physically interacted, *HIS3* gene was expressed and cells were able to grow regardless of histidine supply into culture media.

Co-immunoprecipitation with FLAG or *myc* epitope tag

Following experiments was performed on ice. For co-precipitation of FLAG-Tor1 with Sin1-*myc* proteins, whole cell extracts were prepared in lysis buffer composed of 20 mM HEPES-KOH (pH 7.5), 150 mM sodium glutamate, 10% glycerol, 0.25% Tween 20, 10 mM sodium fluoride, 0.1 mM sodium orthovanadate, 10 mM *beta*-glycerophosphate, 10 mM sodium pyrophosphate, 10 mM *p*-nitrophenyl phosphate, di-sodium salt, 1 mM phenylmethylsulfonyl fluoride (PMSF), and the protease inhibitor cocktail for use in purification of Histidine-tagged proteins (#8830 SIGMA- ALDLICH). Cell extract containing FLAG-Tor1 and Sin1-*myc* were incubated with EZview Red Anti-c-Myc Affinity Gel (#E6654, SIGMA-ALDLICH) for 1 hour and washed extensively with lysis buffer without PMSF and the protease inhibitor cocktail before the elution process. Precipitates were eluted with the sample buffer without 2-mercaptoethanol and then the supernatants were mixed with 0.7 M 2-mercaptoethanol.

GST/MBP pull-down assays

GST or MBP fused proteins were expressed in bacteria as follows. *E. coli* transformants carrying plasmids encoding target proteins were selected with 50 mg/l of ampicillin and cultured in lysogeny broth (LB) media at 37˚C. For inducing proteins of our interests, cells cultured in LB containing 0.1 mM isopropyl β D-1-thiogalactopyranoside (IPTG) and 50 mg/l of ampicillin at 16˚C for 16 hours. Resulting cells were harvested and stored at -80˚C.

Proteins were affinity-purified as follows. 30 OD cells were suspended into ice-cold 900 μl of TBS with 1 mM PMSF and disrupted with sonication. 1% Triton X-100 was added and lysate was centrifuged at 15,000 rpm for 15 minutes. The supernatants were mixed with 20 μl of affinity beads {glutathione-sepharose (#17-0756-01) or amylose resin (#E8021S) supplied from Amersham or New England BioLabs, respectively} and incubated for 1 hour at a cold room. Beads were extensively washed with tris-buffered saline (TBS) with 1% Triton X-100 and stored 4˚C for couples of hours before use for pull-down assays with lysate prepared from yeast or HEK293T human cultured cells.

For analyzing physical interaction of yeast proteins, yeast cell lysates were prepared as follows; 20 OD cells were suspended into lysis buffer composed of washing buffer with 1 mM PMSF and 1/200 volume of the protease inhibitor cocktail for use in purification of histidine-tagged proteins (#8830, SIGMA-ALDLICH). Cell lysate was incubated with GSH-sepharose or amylose resin pre-loaded with bacterially expressed recombinant proteins and incubated for 1 hour at a cold room. Beads were washed extensively with lysis buffer without PMSF and the protease inhibitor cocktail before elution processes. Precipitates were eluted with sample buffer.

To assess interaction to Gad8-FLAG purified from yeast, proteins were prepared as follows. Yeast cells expressing Gad8-FLAG were harvested and immediately froze at -80˚C. Before use, cells were washed once with washing buffer composed of 20 mM HEPES-KOH (pH 7.5), 150 mM sodium glutamate, 10% glycerol, 10 mM sodium fluoride, 0.1 mM sodium orthovanadate, 10 mM *beta*-glycerophosphate, 10 mM sodium pyrophosphate, 10 mM *p*-nitrophenyl phosphate, di-sodium salt. Then cells were suspended into lysis buffer composed of washing buffer with 0.25% tween20, 1 mM PMSF, and the protease inhibitor cocktail for use in purification of histidine-tagged proteins (#8830, SIGMA-ALDLICH). Preparation schemes for yeast cell lysates were described above and resulting lysate was subjected to immunoprecipitation with anti-FLAG M2 magnetic beads (#M8823, SIGMA-ALDLICH). The Gad8-FLAG proteins on the beads were eluted with

3xFLAG peptides (#F4799, SIGMA-ALDLICH) in lysis buffer. Eluates were mixed with the GSH beads pre-loaded with GST-Sin1 CRIM and pull-down assays were performed as mentioned above.

For pull-down assays with FLAG-mLST8, lysate from HEK293T were prepared as follows. Confluent HEK293T cells cultured in D-MEM (High Glucose) media with L-Glutamine and Phenol Red (#044-29765, Wako) supplemented with antibiotics and 10% fatal bovine serum (FBS) on 10 cm dish were washed once with 5 ml of phosphate-buffered saline (PBS) (-) and seeded into 10 ml culture at 20×10^4 cells /ml in 10 cm dish. Cells were cultured for 24 hours post-seeding and mixed with 12 μg of the plasmid caring FLAG-mLST8 gene with 180 μl of polyethylenimine and 500 μl of PBS (-). Cells were incubated at 37˚C for 6 hours and then media were replaced with fresh D-MEM (High Glucose) media with L-Glutamine and Phenol Red (#044-29765, Wako) supplemented with antibiotics and 10% fatal bovine serum (FBS). Cells were further cultured for 24 hours for expression of the FLAG-mLST8 proteins. Cells were washed once with 5 ml of ice-cold PBS (-) prior to lyse in the 800 μl of lysis buffer. Pull-down assays were performed as described before.

Table-1. Fission yeast strains used in this study

Strain ID	Genotype	Source or Reference
CA101	*h- leu-1-32*	Laboratory stock
CA5126	*h- leu-1-32 sin1::kanMX6*	(Ikeda et al., 2008)
CA5687	*h- leu-1-32 wat1::kanMX6*	Laboratory stock
CA5765	*h- leu-1-32 ura4-D18 tor2-13:ura4*	(Uritani et al., 2006)
CA6271	*h- leu-1-32 ste20:5FLAG(kanMX6)*	Laboratory stock
CA6281	*h- leu-1-32 gad8:5FLAG(kanMX6)*	Laboratory stock
CA6323	*h- leu-1-32 tor1D2137A*	Laboratory stock
CA6437	*h- leu-1-32 wat1:5FLAG(kanMX6)*	Laboratory stock
CA6530	*h- leu-1-32 (hph)3FLAG:tor1*	(Hayashi et al., 2007)
CA6755	*h- leu-1-32 (hph)3FLAG:tor1 ste20:5FLAG(kanMX6) sin1:5FLAG(kanMX6)*	Laboratory stock
CA6767	*h- leu-1-32 (hph)3FLAG:tor1 bit61:5FLAG(kanMX6) wat1:5FLAG(kanMX6)*	Laboratory stock
CA6870	*h- leu-1-32 (hph)3FLAG:tor1 sin1::kanMX6*	Laboratory stock

CA6904	*h- leu-1-32 (kanMX6)3FLAG:tor2*	Laboratory stock
CA6912	*h- leu-1-32 sin1:13myc(kanMX6)*	Laboratory stock
CA7092	*h- leu-1-32 (hph)3FLAG:tor1 sin1:13myc(kanMX6)*	Laboratory stock
CA7109	*h- leu-1-32 sin1:13myc(kanMX6) tor1D2137A*	Laboratory stock
CA7124	*h- leu-1-32 (hph)3FLAG:tor1 wat1::kanMX6*	Laboratory stock
CA7200	*h- leu-1-32 (hph)3FLAG:tor1 sin1:13myc(kanMX6) wat1::kanMX6*	Laboratory stock
CA7517	*h- leu-1-32 sin1L348S:13myc(kanMX6)*	Laboratory stock
CA7519	*h- leu-1-32 sin1L364S:13myc(kanMX6)*	Laboratory stock
CA7548	*h- leu-1-32 (hph)3FLAG:tor1 ste20::kanMX6 wat1::kanMX6*	Laboratory stock
CA7641	*h- leu-1-32 ura4-D18 wat1:5FLAG(kanMX6) tor1::ura4 ste20::kanMX6*	Laboratory stock
CA7654	*h- leu-1-32 ura4-D18 ste20:5FLAG(kanMX6) wat1::kanMX6 tor1::ura4*	Laboratory stock
CA7664	*h- leu-1-32 wat1::kanMX6 sin1::kanMX6*	Laboratory stock
CA8070	*h- leu-1-32 psk1:5FLAG(kanMX6)*	Laboratory stock
CA8437	*h- leu-1-32 ura4-D18 tor1::ura4 tor2-13:kanMX6*	Laboratory stock
	h- leu-1-32 sin1L258A:13myc(kanMX6)	Laboratory stock
	h- leu-1-32 sin1L258S:13myc(kanMX6)	Laboratory stock
	h- leu-1-32 sin1L258P:13myc(kanMX6)	Laboratory stock

RESULTS I

While TORC1 phosphorylates a TORC2 substrate Gad8 when it is artificially recruited to TORC1, TORC2 phosphorylates a TORC1 substrate Psk1 when recruited to TORC2

TORC2 phosphorylates Gad8 but not Psk1, a substrate of TORC1. Therefore, there is possibility that the CRIM domain, which is required for recruiting Gad8 to TORC2, does not bind to Psk1. 281-400 amino acid residues in Sin1 was fused to GST (GST-CRIM) and expressed bacterially (Figure 3. A). GST-CRIM carrying amino acid substitution of all glutamate and aspartate in 352-361 amino acid residues ("acidic region") was also prepared as a negative control (GST-CRIM polyNQ) (Figure 3. A). GST pull-down assays demonstrated that GST-CRIM did not bind to Psk1-FLAG, suggesting that substrates of TORC1 do not bind to the CRIM (Figure 3. B).

Since CRIM binds to Gad8 but not to Psk1, we speculated that TORC2 relies on interaction between CRIM and Gad8 for phosphorylating Gad8 selectively and the catalytic subunit TOR kinases in TORC1 and TORC2 do not distinguish their substrates. To test this possibility, we investigated whether TORC2 phosphorylates Psk1 or not when Psk1 was targeted to TORC2 by fusion to Ste20 (Ste20-Psk1). We found that the Ste20-Psk1 protein was phosphorylated (Figure 3. C). To test whether the phosphorylation of Ste20-Psk1 was dependent on TORC2 but not TORC1, we expressed Ste20-Psk1 in a temperature sensitive mutant of *tor2* ("*tor2-ts*") that lose catalytic activity at the restrictive temperature. At the restrictive temperature (35 ˚C), Ste20-Psk1 but not endogenous Psk1 was phosphorylated (Figure 3. C). The phosphorylation of Ste20-Psk1 was diminished in *Δtor1 tor2-ts* cells at the restrictive temperature, suggesting that Ste20-Psk1 was phosphorylated in a manner dependent on TORC2.

Then we tested whether TORC1 phosphorylates Gad8 or not when Gad8 was targeted to TORC1 by fusion to Mip1, an essential subunit of TORC1 (Mip1-Gad8). We found that the Mip1-Gad8 was phosphorylated in *Δsin1* cells that lack TORC2 activity, indicating that Mip1-Gad8 is phosphorylated in a TORC2 independent manner (Figure 3. D). The phosphorylation of Mip1-Gad8 is reduced in *tor2-ts* cells at the restrictive temperature, indicating that Tor2 phosphorylates Mip1-Gad8 (data not shown). To test whether the phosphorylation of Mip1-Gad8 was dependent on physical interaction between Mip1-Gad8 and Tor2, we constructed Mip1-Gad8 that does not bind to Tor2. Previously, Kim *et al.* identified several amino acid regions in Raptor that are required for interaction between Raptor and mTOR (Kim et al., 2003). Based on this information, 316-318 or 366-369 amino acid resides of Mip1 were substituted to alanine to create Mip1-Gad8 mutants (Mip1

DLF_AAA-Gad8 or Mip1 NWIF_AAAA-Gad8, respectively). Co-immunoprecipitation demonstrated that physical interaction between Mip1-Gad8 and Tor2 were attenuated in Mip1-Gad8 carrying these mutations (Figure 3. E). Importantly, the phosphorylation of Mip1-Gad8 was diminished in these mutated Mip1-Gad8, suggesting that Mip1-Gad8 is phosphorylated in a manner dependent on interaction with Tor2 (Figure 3. D). From these results, we concluded that catalytic subunit TOR kinases in TORC1 and TORC2 do not distinguish their substrate.

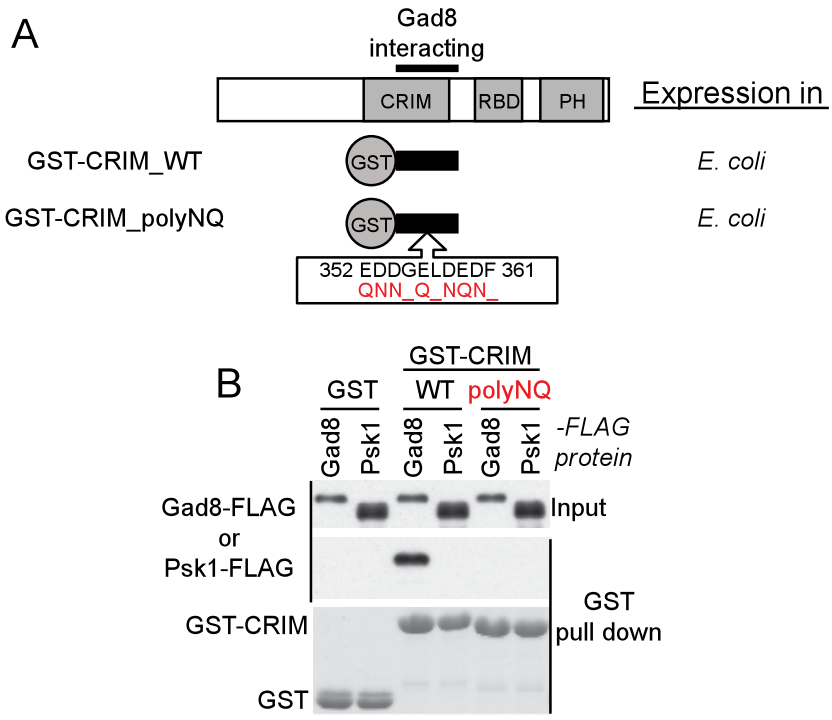

Figure 3. While TORC1 phosphorylates a TORC2 substrate Gad8 when it is artificially recruited to TORC1, TORC2 phosphorylates a TORC1 substrate Psk1 when recruited to TORC2

(A) Sin1 truncations and mutants used in this study

GST-CRIM contains 281-400 amino acid residues of Sin1 that interact with Gad8. GST-CRIM polyNQ has amino acid substitution of all glutamate and aspartate residues in the "acidic region" in Sin1. These proteins were used in Figure 3. B, 4. A, and 4.C.

(B) The Sin1 CRIM domain did not interact with Psk1.

Bacterially expressed GST-CRIM or GST-CRIIM poly NQ were immobilized on GSH beads and GST pull-down assays were performed with lysate prepared from yeast cells expressing FLAG tagged Gad8 or Psk1. GST protein was detected in coomassie brilliant blue staining. Gad8-FLAG or Psk1-FLAG was detected with anti-FLAG antibodies.

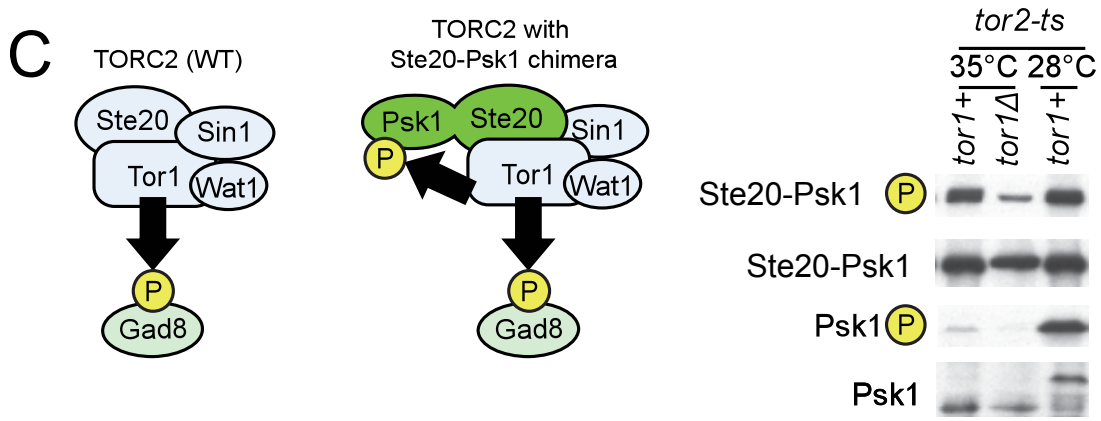

Figure 3. While TORC1 phosphorylates a TORC2 substrate Gad8 when it is artificially recruited to TORC1, TORC2 phosphorylates a TORC1 substrate Psk1 when recruited to TORC2

(C) TORC2 phosphorylated Psk1 when it was fused to Ste20.

Psk1 was fused to Ste20, an essential subunit of TORC2, and the fusion protein (Ste20-Psk1) was ectopically expressed in a temperature sensitive mutant of *tor2* (*tor2-13*, referred as "*tor2-ts*") or *tor2-ts Δtor1* double mutant. The phosphorylation of endogenous Psk1 or Ste20-Psk1 was detected with the antibodies against phosphorylated human S6 kinase (phosphorylated p70 S6K). Total Psk1 or Ste20-Psk1 was detected with anti-Psk1 antibodies.

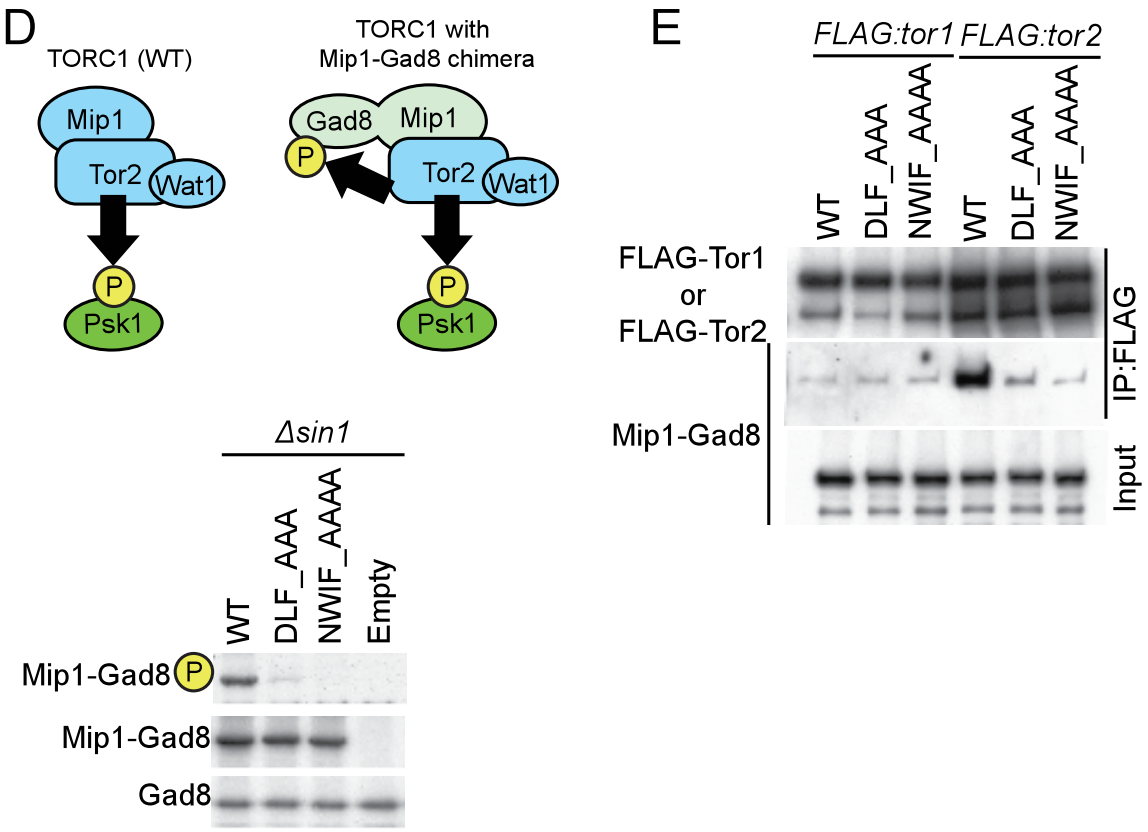

Figure 3. While TORC1 phosphorylates a TORC2 substrate Gad8 when it is artificially recruited to TORC1, TORC2 phosphorylates a TORC1 substrate Psk1 when recruited to TORC2

(D), (E) Gad8 fusion to Mip1 bypassed the requirement of Sin1.

(D) Gad8 was fused to Mip1, an essential subunit of TORC1, and the fusion protein (Mip1-Gad8) was ectopically expressed in *Δsin1* cells. 316-318 or 366-369 amino acid residues of Mip1 were substituted to alanine to create Mip1-Gad8 mutant (Mip1 DLF_AAA-Gad8 or Mip1 NWIF_AAAA-Gad8, respectively). Phosphorylation of Gad8 of the fusion proteins was monitored with immunoblotting with antibodies against phosphorylated Gad8 or Gad8.

(E) Wild-type or Mip1-Gad8 mutants were expressed in *FLAG:tor1* or *FLAG:tor2* cells. Anti-FLAG immunoprecipitation was performed and co-purification of wild-type or mutated Mip1-Gad8 were detected with anti-Gad8 antibodies.

The CRIM domain binds to unphosphorylated Gad8 but less to phosphorylated Gad8

As previously described, Sin1 binds to and recruits Gad8 to TORC2 to facilitate phosphorylation of Gad8. It seems efficient for TORC2 to phosphorylate Gad8, if CRIM binds to unphosphorylated Gad8 rather than phosphorylated Gad8. Therefore we tested whether phosphorylated Gad8 interacted with the CRIM domain or not. Gad8-FLAG expressed in yeast was precipitated with GST-CRIM or anti-FLAG antibodies. If the CRIM domain does not bind to phosphorylated Gad8, we cannot detect phosphorylated Gad8 in the precipitate derived from GST pull-down assays with GST-CRIM. On the other hand, since Gad8-FLAG can be immunopurified with anti-FLAG antibodies regardless of their phosphorylation, we may detect phosphorylated Gad8 in the precipitate derived from anti-FLAG immunoprecipitation. As expected, phosphorylation of Gad8 was detected less in Gad8-FLAG precipitated with GST-CRIM comparing to the immunopurified Gad8-FLAG (Figure 4. A). By normalization with total Gad8-FLAG, in Gad8-FLAG precipitated with anti-FLAG antibody, phosphorylation level of Gad8 was about twenty-times higher than that precipitated with GST-CRIM (Figure 4. A). There are many phosphatase in yeast and it is possible that some of them bind to GST-CRIM unexpectedly to dephosphorylate Gad8 during pull-down assays. To exclude this possibility, Gad8-FLAG was immunopurified, followed by pull-down assays with GST-CRIM (Figure 4. B). By normalization with the total Gad8-FLAG, phosphorylated Gad8 was hardly detected in the precipitate with GST-CRIM, although we successfully detected the phosphorylated Gad8-FLAG in input (Figure 4. B). These results indicate that CRIM does not bind to Gad8 that is phosphorylated by TORC2.

Since Gad8 is phosphorylated at the C-terminal hydrophobic motif by TORC2, and phosphorylated Gad8 barely interacts with the CRIM domain, we suspected that CRIM binds to unphosphorylated C-terminal region of Gad8. To test this possibility, we performed pull-down assays using GST-CRIM and Gad8 lacking the C-terminus including the hydrophobic motif that is phosphorylated by TORC2 (Gad8ΔC). Unexpectedly, we found that CRIM bound to Gad8 regardless of the C-terminal region of Gad8, indicating that the C-terminus of Gad8 is not required for the CRIM domain to bind to Gad8 (Figure 4. C).

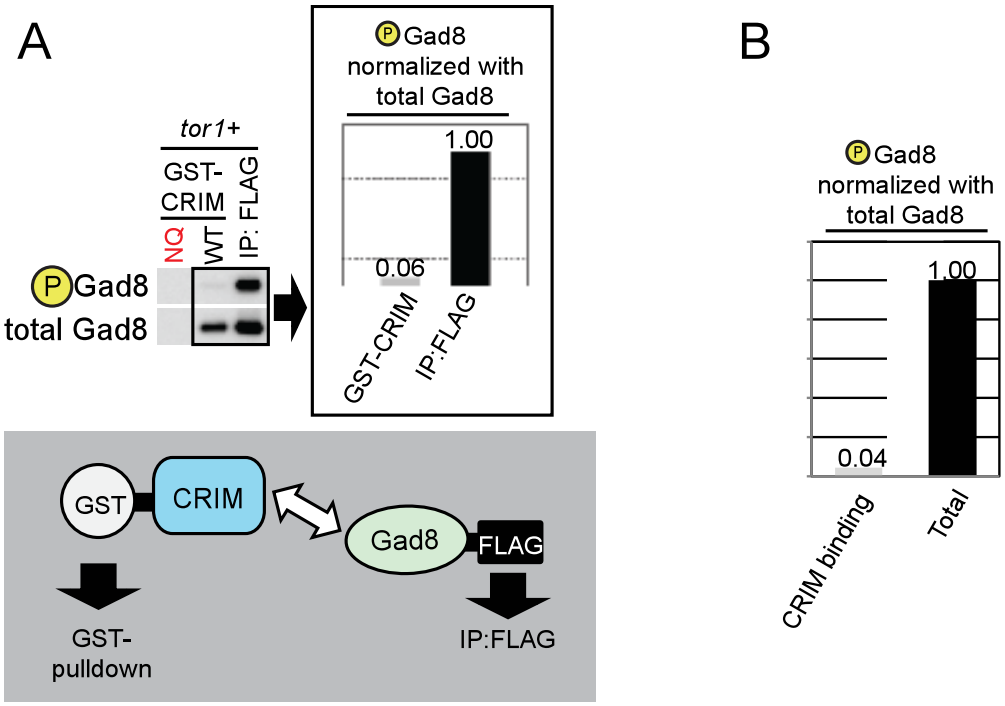

Figure 4. The CRIM domain binds to unphosphorylated Gad8 but less to phosphorylated Gad8.

(A, B) Gad8-FLAG interacting with the CRIM domain was barely phosphorylated.

(A) Gad8-FLAG in yeast lysate was precipitated with GST-CRIM or anti-FLAG antibodies. Phosphorylation levels of Gad8 were analyzed with immunoblotting and then quantified and normalized with levels of total Gad8.

(B) Gad8-FLAG was immunopurified with anti-FLAG antibodies and used for pull-down assays with GST-CRIM. Phosphorylation levels of Gad8-FLAG in input ("Total") and precipitate ("CRIM binding") were quantified and normalized with levels of total Gad8.

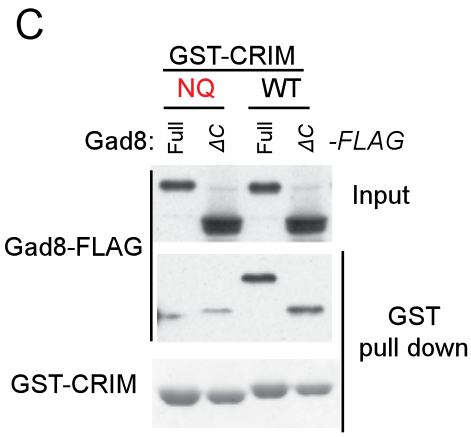

Figure 4. The CRIM domain binds to unphosphorylated Gad8 but less to phosphorylated Gad8.

(C) Gad8 lacking the C-terminal phosphorylation site bound to the CRIM domain.

Wild-type Gad8 and Gad8 lacking the C-terminal extension containing the hydrophobic motif (1-498 amino acid residues in Gad8, referred as "*ΔC*") were fused with 3 copies of FLAG epitope tag at their C-termini and expressed from multi-copy plasmids under the control of the *gad8*$^{+}$ native promoter. GST-CRIM pull-down assays were performed as described in Figure 3. B.

The N-terminal region of the CRIM domain is required for Sin1 to interact with other subunits of TORC2.

To further assess molecular functions of Sin1 in TORC2, we analyzed significance of domains in Sin1 by complementation of *Δsin1* cells with plasmids encoding Sin1 truncations (Figure 5. A). In fission yeast, loss of function of the TORC2-Gad8 pathway severely impairs cell growth under the stressful conditions including high osmorality and oxidative stress. Indeed, *Δsin1* cells carrying an empty vector showed severe growth defect in media containing 1 M KCl or 0.1 M $CaCl_2$ (Figure 5. B). *Δsin1* mutants transformed with plasmids expressing full-length Sin1 protein ("*sin1 full*"), 1-530 amino acid residues of Sin1 ("*sin1 1-530*"), and 1-390 amino acid residues of Sin1 (*"sin1 1-390")* grew well under these stress conditions (Figure 5. A and B). On the other hand, plasmids expressing 1-290 amino acids of Sin1 ("*sin1 1-290*") or Sin1 lacking 291-390 amino acid residues *("sin1 Δ291-390")* did not complement *Δsin1* phenotypes (Figure 5. A and B). Consistent with the stress sensitivity of the transformants, *sin1 1-290* and *sin1 Δ291-390* transformants that express Sin1 lacking the CRIM domain diminished phosphorylation of Gad8, while expression of Sin1 lacking the PH domain ("*sin1 1-530"*) or the PH domain and RBD ("*sin1 1-390"*) restored phosphorylation of Gad8 (Figure 5. C).

In fission yeast, the CRIM domain is located in 247-390 amino acid residues in Sin1 and 247-280 amino acid residues are dispensable for Sin1 to interact with Gad8, although this region is highly conserved among Sin1 orthologues (Figure 7. A; Murayama, unpublished data). Therefore, there is a possibility that the N-terminus of CRIM (247-280 amino acid residue of Sin1), described hereafter as "N-CRIM", has unknown functions in TORC2. To test significance of N-CRIM, N-terminal truncation series of Sin1 were expressed in *Δsin1* cells. The plasmid expressing Sin1 lacking 1-250 amino acid residue ("*sin1 251-665*") complemented stress sensitivity of *Δsin1* cells (Figure 5. D). In addition, phosphorylation of Gad8 was recovered partially in these cells. On the other hand, *Δsin1* cells expressing 261-665 amino acid residues of Sin1 ("*sin1 261-665*"), 271-665 amino acid residues of Sin1 ("*sin1 271-665*"), 281-665 amino acid residues of Sin1 ("*sin1 281-665*") did not grow in media containing 1 M KCl or 0.1 M $CaCl_2$ (Figure 5. D), and did not restore phosphorylation of Gad8 (Figure 5. E). From these results, 251-260 amino acid resides in Sin1 are functionally critical for TORC2. Then Sin1 lacking N-CRIM (Sin1 ΔN-CRIM, referred as "*sin1 Δ251-280*") was expressed in *Δsin1* cells and growth of the *Δsin1* mutant cells in media containing 1 M KCl or 0.1 M $CaCl_2$ and phosphorylation of Gad8 were tested (Figure 5. F and G). We found that growth under stress conditions and TORC2 dependent phosphorylation of Gad8 were not rescued when N-CRIM was excluded from Sin1 (Figure 5.

F and G). This indicates that the N-CRIM region, which is dispensable for Sin1 to bind to Gad8, has irreplaceable functions for TORC2 to phosphorylate Gad8. In addition, co-immunoprecipitation assays revealed that interaction between Sin1-*myc* and FLAG-Tor1 was impaired in Sin1 ΔN-CRIM (Figure 5. H). *In vitro* GST pull-down assays also demonstrated that Sin1 ΔN-CRIM interacts with FLAG-Tor1, Ste20-FLAG, Wat1-FLAG, or Bit61-FLAG less than wild-type Sin1 (Figure 5. I). Therefore, N-CRIM is important for Sin1 to interact with other subunits of TORC2.

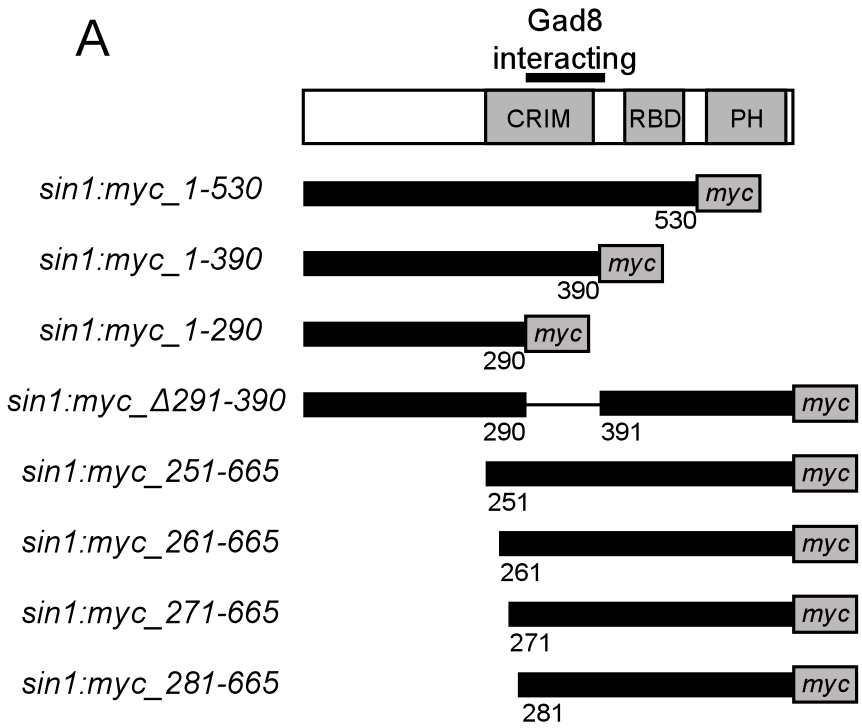

Figure 5. The N-terminal region of the CRIM domain is required for Sin1 to interact with other subunits of TORC2.

(A) *sin1* truncations used in this study

Sin1 truncations lacking the PH domain ("*1-530*"), the RBD and PH domains ("*1-390*"), the Gad8 interacting region the RBD and PH domain ("*1-290*"), or the Gad8 interacting region only ("*Δ291-390*") were used in Figure 5. B and C.

251-665 amino acid residue of Sin1 ("*251-665*"), 261-665 amino acid residue of Sin1 ("*261-665*"), 271-665 amino acid residue of Sin1 ("*271-665*"), 281-665 amino acid residue of Sin1 ("*281-665*") were used in Figure 5. D and E.

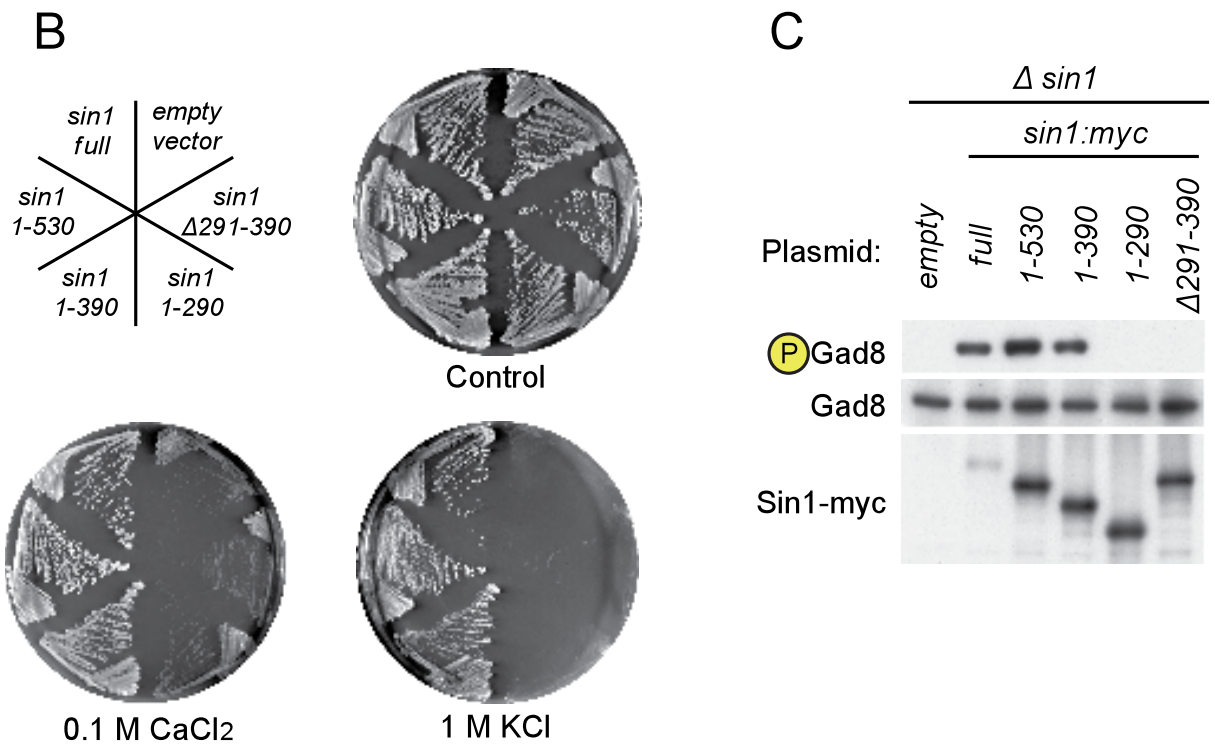

Figure 5. The N-terminal region of the CRIM domain is required for Sin1 to interact with other subunits of TORC2.

(B and C) The CRIM domain rather than the PH domain and RBD was indispensable for Sin1 function in TORC2.

Sin1 C-terminal truncations fused with 12 copies of the *myc* epitope tag (listed in A) were ectopically expressed under the control of the *sin1* native promoter in *Δsin1* mutant cells.

(B) Transformants were grown on YES solid media (as control) or YES containing 1 M KCl or 0.1 M $CaCl_2$.

(C) Cells were cultured and harvested with TCA followed by proteins extraction. Immunoblotting was performed with anti-phosphorylated Gad8, Gad8, or *myc* antibodies.

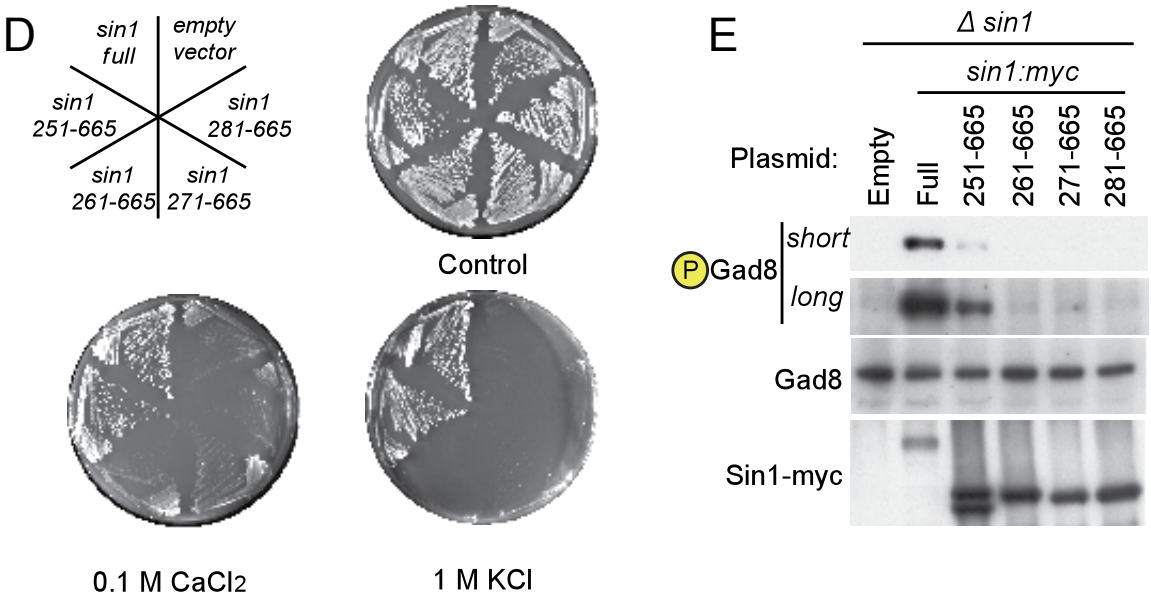

Figure 5. The N-terminal region of the CRIM domain is required for Sin1 to interact with other subunits of TORC2.

(D and E) Expression of 251-665 but not 261-665 amino acid residues of Sin1 rescues phenotypes of *Δsin1*.

Sin1 N-terminal truncations fused with 12 copies of the *myc* epitope tag (listed in A) were ectopically expressed in *Δsin1* cells.

(D) Growth phenotypes were tested in control or stress media as shown in Figure 5. B.

(E) Immunoblotting was performed as shown in Figure 5. C.

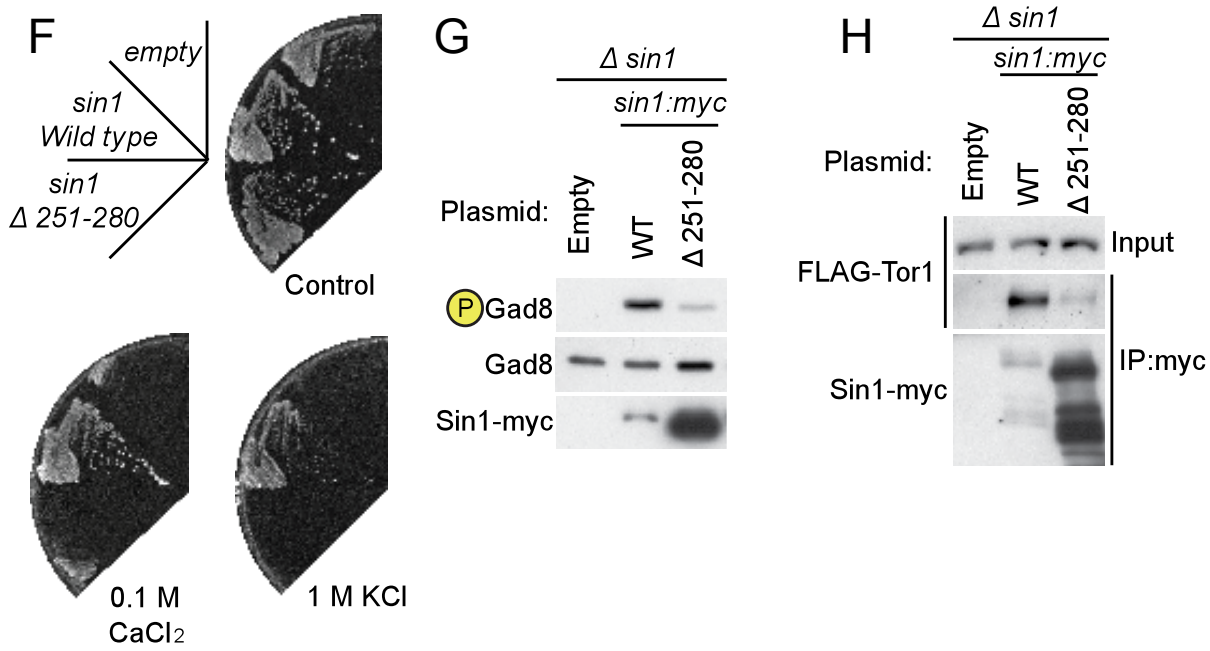

Figure 5. The N-terminal region of the CRIM domain is required for Sin1 to interact with other subunits of TORC2.

(F and G) Deletion of N-CRIM in Sin1 impaired TORC2 function.

Sin1 lacking 251-280 amino acid residues fused with 12 copies of the *myc* epitope tag (listed in A) was ectopically expressed in *Δsin1* cells.

(F) Growth phenotypes were tested in control or stress media as shown in Figure 5. B.

(G) Immunoblotting was performed as shown in Figure 5. C. Expression of Sin1 lacking N-CRIM (Sin1 ΔN-CRIM) was quite higher than that of wild-type by unknown reasons.

(H) Loss of ΔN-CRIM reduced interaction between Sin1 and Tor1.

Co-immunoprecipitation with anti-*myc* antibodies was performed using lysate prepared from yeast cells expressing FLAG-Tor1 and C-terminally *myc*-tagged wild-type Sin1 ("WT") or Sin1 ΔN-CRIM ("Δ251-280").

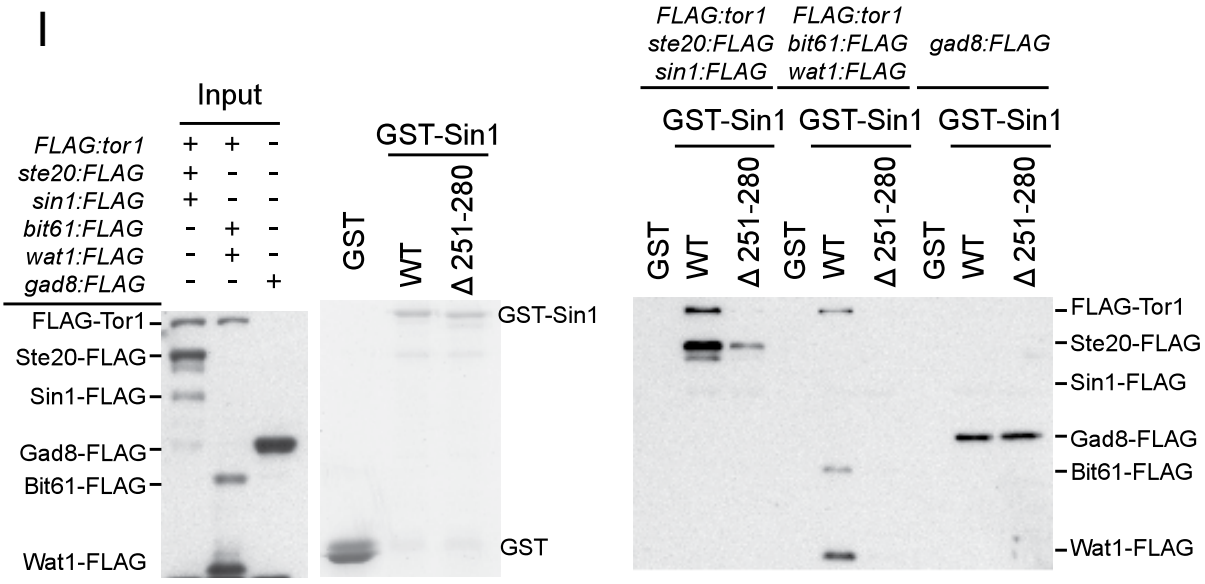

Figure 5. The N-terminal region of the CRIM domain is required for Sin1 to interact with other subunits of TORC2.

(I) Loss of N-CRIM inhibited Sin1 from binding to other subunits of TORC2.

GST pull-down assays were performed with bacterially expressed GST-Sin1 (WT) or Sin1ΔN-CRIM ("Δ251-280") and yeast lysate prepared from cells expressing FLAG-tagged Gad8 or TORC2 subunits Tor1, Ste20, Sin1, and Bit61.

Sin1 binds to Wat1 in a manner dependent on N-CRIM.

Since mLST8, the mammalian orthologue of Wat1, is required for Sin1 to interact with mTOR in human cultured cells (Guertin et al., 2006; Wang et al., 2012), we tested if Wat1 is also required in fission yeast. Since Sin1 is highly degraded in *Δwat1* cells, Sin1-*myc* was over expressed under the control of the thiamine-repressive *nmt1* promoter. When the amount of intact Sin1-*myc* protein in *Δwat1* was comparable to that in wild-type (Figure 6. A), FLAG-Tor1 was co-precipitated with Sin1-*myc* less than in wild-type. This result suggests that interaction between Sin1 and Tor1 is reduced in the absence of Wat1 (Figure 6. A). In GST pull-down assays, interaction between Sin1 and Tor1 is attenuated in Sin1 ΔN-CRIM (Figure 6. B). In *Δwat1*, the amount of Tor1 is partially decreased as shown in "Input" (Figure 6. B). In this mutant, interaction between Sin1 and Tor1 was also detected but this interaction was completely lost in *Δwat1 Δste20*, indicating that both Wat1 and Ste20 contribute to Sin1 to bind to Tor1 (Figure 6. B). Importantly, in *Δwat1*, interaction between Sin1 and Tor1 was not attenuated in Sin1 ΔN-CRIM (Figure 6. B). On the contrary, in *Δste20*, interaction between Sin1 and Tor1 was reduced and this interaction was additionally decreased in Sin1 ΔN-CRIM (data not shown). Therefore, Wat1 rather than Ste20 is essential for N-CRIM dependent interaction between Sin1 and Tor1 (Figure 6. B).

Then we expected that Sin1 binds to Wat1 in a manner dependent on N-CRIM. The yeast two-hybrid assay successfully detected interaction between Wat1 and wild-type Sin1 in the presence ("High" selective condition) or absence ("Low" selective condition) of 3-aminotriazole (3-AT), whereas Wat1 showed interaction to ΔN-CRIM only in the absence of 3-AT (Figure 6. C), suggesting that interaction between Wat1 and Sin1 in the yeast two-hybrid assay reduced by loss of N-CRIM. In GST pull-down assays, we detected physical interaction between Wat1 and Sin1 in wild-type and *Δtor1Δste20* cell lysate, although interaction between Wat1 and Sin1 was decreased in *Δtor1Δste20* than wild-type. By loss of N-CRIM, interaction between Sin1 and Wat1 was reduced in wild-type and abolished in *Δtor1Δste20,* indicating that N-CRIM is required for Sin1 to interact with Wat1 (Figure 6. D). On the other hand, wild-type and ΔN-CRIM GST-Sin1 comparably bound to Ste20-FLAG, indicating that N-CRIM is dispensable for interaction between Sin1 and Ste20. (Figure 6. E). Combining all the results above, N-CRIM is required for Sin1 to bind to Wat1.

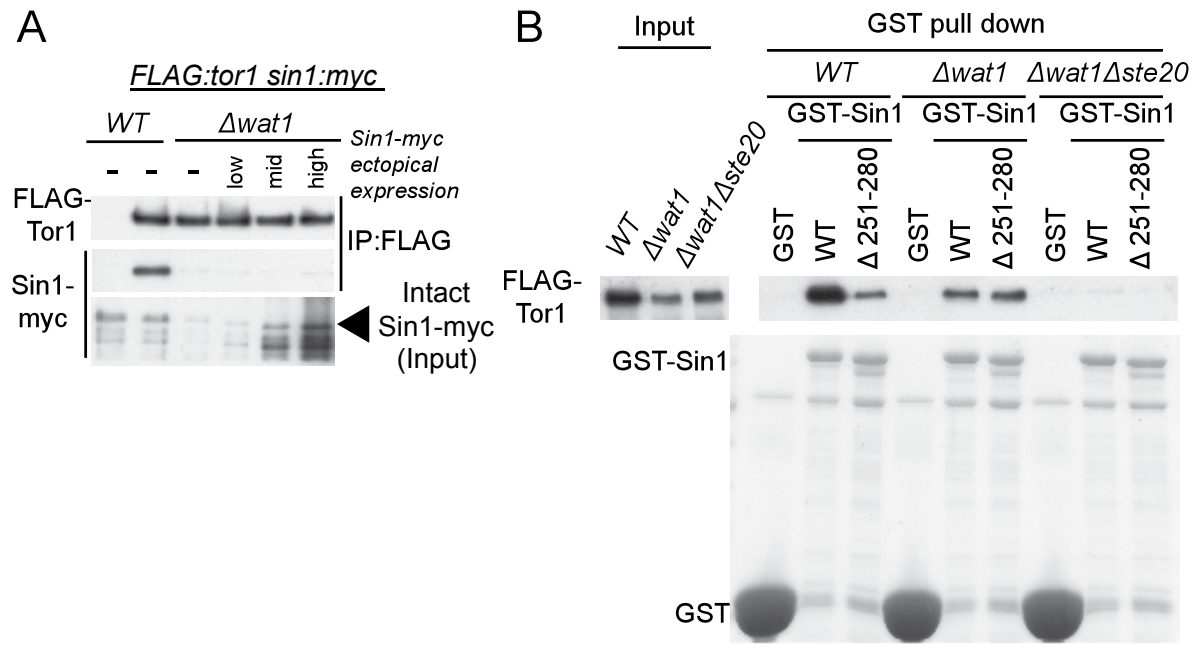

Figure 6. Sin1 binds to Wat1 in a manner dependent on N-CRIM.

(A) Interaction between Sin1 and Tor1 was reduced in the absence of Wat1.

Co-immunoprecipitation was performed using lysate prepared from yeast cells expressing FLAG-Tor1 and Sin1-*myc*. In *Δwat1*, Sin1-*myc* was ectopically expressed under the control of the thiamine-repressive *nmt1* promoter. Over-expression of Sin1-*myc* was induced for 0 hour (low), 12 hours (mid), or 14 hours (high) after thiamine removal in growth media. "-" indicates the negative control with the empty vector. Arrowhead indicates intact Sin1-*myc* in input.

(B) Wat1 is required for N-CRIM dependent interaction between Sin1 and Tor1.

GST pull-down assays were performed as described in Figure 5. I with lysate prepared from wild-type (*WT*), *wat1Δ*, or *wat1Δste20Δ*.

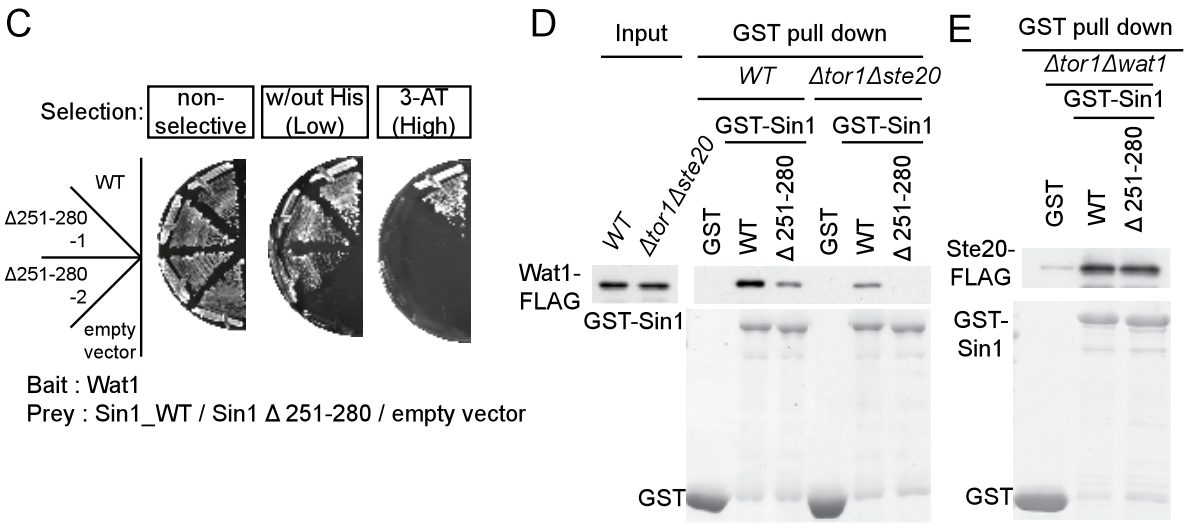

Figure 6. Sin1 binds to Wat1 in a manner dependent on N-CRIM.

(C) Wat1 interacted with Sin1 in the yeast two-hybrid assay.

The budding yeast HF7c strain carrying pGBT8-*wat1* as the bait plasmid was transformed with pGADGH-*sin1* (WT), or ΔN-CRIM (Δ251-280). Expression of the reporter gene was assessed by growth in the absence of histidine (Low) or in the presence of 4 mM 3-AT (High), an inhibitor of the histidine reporter gene product.

(D) Sin1 physically interacted with Wat1 in *Δtor1Δste20* in a manner dependent to N-CRIM.

GST pull-down assays were performed as described in Figure 5. I with cell lysate prepared from wild-type (*WT*) and *Δtor1Δste20* expressing Wat1-FLAG.

(E) N-CRIM is dispensable on interaction between Sin1 and Ste20.

GST pull-down assays were performed as described in Figure 5. I with *Δtor1Δwat1* expressing Ste20-FLAG.

Two conserved leucine residues in N-CRIM play a key role in interaction between Wat1 and Sin1 in fission yeast and between mLST8 and hSin1 in human.

To identify amino acid residues that are required for Sin1 to bind to Wat1, alanine scanning was performed throughout N-CRIM. 251-255, 256-260, 261-265, 266-270, 271-275, or 276-280 amino acid residues in Sin1 were replaced with 5-consecutive alanine to create Sin1 mutants 251-255A, 256-260A, 261-265A, 266-270A, 271-275A, or 276-280A, respectively. These mutants were fused with MBP and bacterially expressed to examine interaction between Sin1 and Wat1. MBP pull-down assays demonstrated that 251-255A, 256-260A, or 261-265A mutant of Sin1 showed decreased interaction to Wat1. Especially the 256-260A or 261-265A mutants showed severe defect in this interaction. On the other hand, 266-270A, 271-275A, or 276-280A mutations in Sin1 did not reduce interaction between Sin1 and Wat1. These results indicate that the region composed of the 251-265 amino acid residues are required for Sin1 to bind to Wat1 (Figure 7. B). This region includes two leucine residues at 258th and 262nd (L258 and L262, respectively) that are highly conserved among eukaryotes (Figure 7. A and B). When either of these two leucine residues in N-CRIM was substituted to alanine (L258A and L262A), the mutant Sin1 proteins bound to Wat1 less efficiently than wild-type Sin1. In addition alanine substitution of L261 residue that is weakly conserved among eukaryotes showed partial reduction of interaction between Sin1 and Wat1 (Figure 7. C). When all the leucine residues were simultaneously substituted to alanine (AAA), the Sin1 AAA mutant bound to Wat1 no less than L258A or L262A single mutants. These results suggest that L258 and L262 in N-CRIM are important for interaction between Wat1 and Sin1. Then, we expressed Sin1 carrying these mutations in *Δsin1* cells to assess significance of these residues *in vivo*. Expression of Sin1-*myc* possessing AAA or L258A mutations severely impaired cell growth in stressful conditions (1 M KCl or 0.1 M $CaCl_2$) as well as Gad8 phosphorylation (Figure 7. D and E). When we introduced alanine, proline, or serine substitution in the 258th leucine residue of Sin1 at the chromosomal *sin1* locus, Sin1 was expressed comparably in wild-type and all *sin1* mutants but Gad8 phosphorylation was significantly reduced in any *sin1* mutants (Figure 7. F). Furthermore, all the *sin1* mutants showed sensitivity to 1 M KCl, which was rescued by expressing Gad8 K128E (Figure 7. G), a hyperactive mutant previously obtained in our laboratory (Murayama, unpublished results). These results indicate that L258 and L262 residues of Sin1 are required for TORC2 to phosphorylate Gad8 in fission yeast.

Since N-CRIM is highly conserved between fission yeast and human, next we examined if N-CRIM is involved in interaction between human mLST8 and hSin1. In MBP pull-down assays with bacterially produced hSin1 fused with MBP (MBP-hSin1) and

FLAG-tagged mLST8 expressed in HEK293T human culture cells, the hSin1 fragment composed of the residues 1-160 (MBP-hSin1 1-160) interacted with mLST8 as well as full length MBP-hSin1. This indicates that the N-terminal 160 residues of hSin1 containing N-CRIM are sufficient to interact with mLST8 (Figure 7. H). Importantly, serine or proline substitution at L137 and L141 residues in hSin1 (L137S L141S or L137P L141P), corresponding to L258 and L262 of fission yeast Sin1 (Figure 7. A), reduced interaction between MBP-hSin1 and FLAG-mLST8. This result indicates that the conserved leucine residues in N-CRIM are important for hSin1 to bind to mLST8 (Figure 7. I).

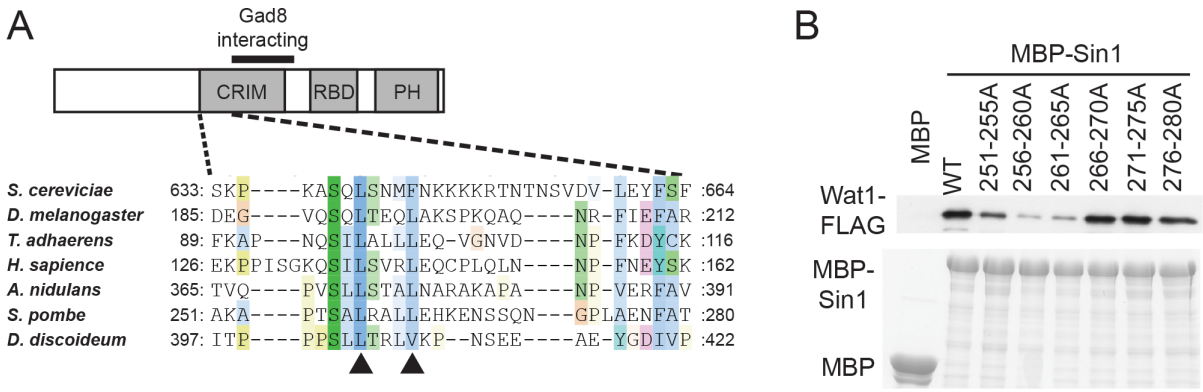

Figure 7. Two conserved leucine residues in N-CRIM play a key role in interaction between Wat1 and Sin1 in fission yeast and between mLST8 and hSin1 in human.

(A) Two leucine residues in N-CRIM are conserved among eukaryotes.

Multiple alignment of N-CRIM was performed with the clustalW2 program. Arrowheads indicate two conserved leucine residues. For the analysis, following organisms were chosen: *Saccharomyces cereviciae* (budding yeast), *Drosophila melanogaster* (fruit fly), *Tricohplax adhaerens* (placozoa), *Homo sapience* (human), *Aspergillus nidulans* (filamentous fungi), *Schizosaccharomyces pombe* (fission yeast), and *Dictyosterium discoideum* (amoeba). Dr. Tatebe in Shiozaki laboratory performed this informatics analysis.

(B) Alanine substitution in the residues 251-265 of Sin1 compromised interaction between Sin1 and Wat1.

251-255, 256-260, 261-265, 266-270, 271-275, or 276-280 amino acid residues in Sin1 were replaced with 5-consecutive alanine residues to create Sin1 mutants (251-255A, 256-260A, 261-265A, 266-270A, 271-275A, or 276-280A, respectively).

Bacterially produced, MBP-fused Sin1 (WT) or alanine substitution mutants were immobilized on the amylose resin and MBP pull-down assays were performed with Wat1-FLAG expressed in *Δtor1Δste20*.

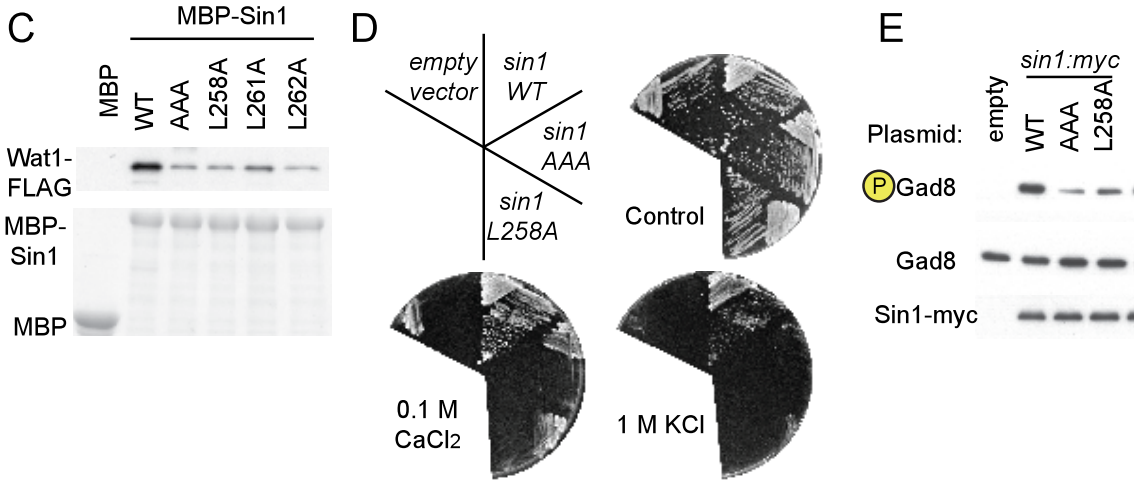

Figure 7. Two conserved leucine residues in N-CRIM play a key role in interaction between Wat1 and Sin1 in fission yeast and between mLST8 and hSin1 in human.

(C) Alanine substitution at L258 or L262 severely attenuated interaction between Sin1 and Wat1.

L258, L261, or L262 amino acid residue in N-CRIIM was substituted with alanine to construct L258A, L261A, or L262A mutant of Sin1. Sin1 mutant possessing L258A, L261A, and L262A was also constructed (referred as "AAA").

MBP pull-down assays were performed as described in (B) with MBP-Sin1 wild-type (WT), L258A, L261A, L262A, or AAA and Wat1-FLAG expressed in *Δtor1Δste20*.

(D and E) Expression of the *sin1 L258A* mutant did not rescue Δ*sin1*.

(D) Sensitivity to 0.1 M $CaCl_2$ or 1 M KCl was tested as described in Figure 5. B.

(E) Gad8 phosphorylation was tested as described in Figure 5. C.

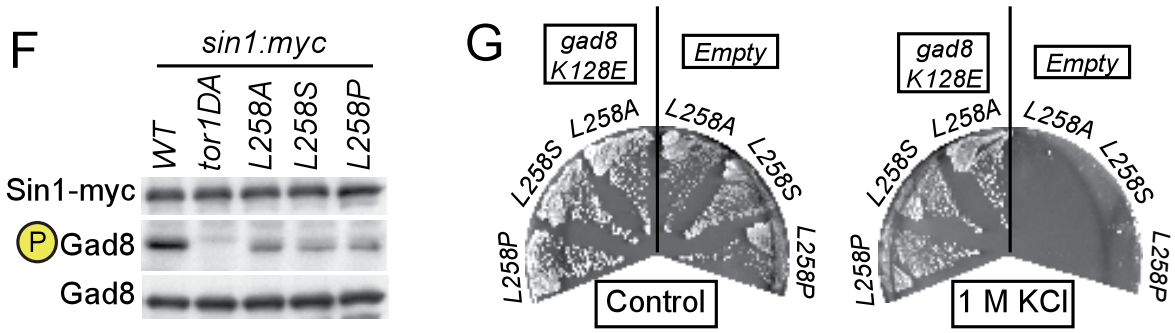

Figure 7. Two conserved leucine residues in N-CRIM play a key role in interaction between Wat1 and Sin1 in fission yeast and between mLST8 and hSin1 in human.

(F) Chromosomal integration of the *sin1 L258A*, *S*, or *P* mutations inhibited phosphorylation of Gad8 by TORC2.

Chromosomal *sin1*$^{+}$ gene was tagged with a PCR fragment encoding Sin1 with 13 copies of the *myc* epitope (*sin1:myc* WT). L258 residue was substituted with alanine, serine, or proline to create *sin1* L258A, L258S, or L258P, respectively.

Phosphorylation of Gad8 was analyzed in cells expressing *sin1*$^{+}$ (*WT*), *sin1L258A*, *L258S, L258P* as described in Figure 5. B. A *tor1* kinase-dead mutant *tor1D2137A* (*tor1DA*) was included as a negative control.

(G) Stress sensitive phenotypes in the *sin1 L258A*, *S*, or *P* mutants were suppressed by expression of *gad8 K128E* , a hyperactive mutant of *gad8*.

The *sin1* mutants were transformed with the empty vector or the pGNP-*gad8 K128E* plasmid that expresses a hyperactive Gad8 mutant under the control of the *gad8* native promoter. Cell growth was tested as described in Figure 5. C.

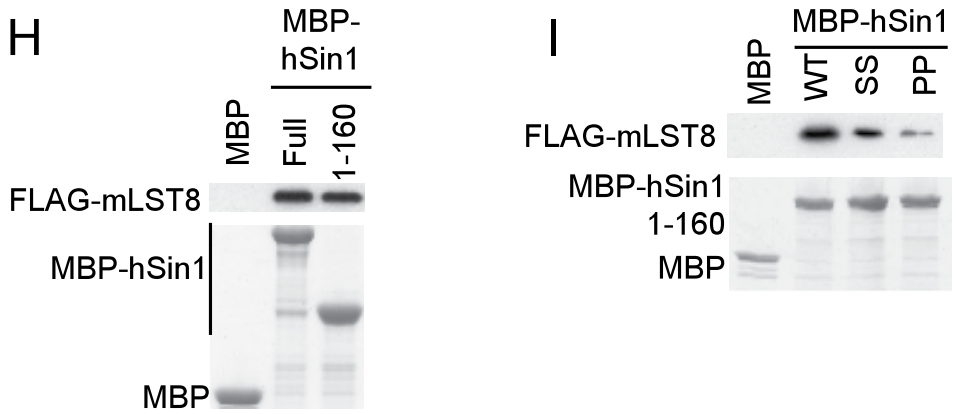

Figure 7. Two conserved leucine residues in N-CRIM play a key role in interaction between Wat1 and Sin1 in fission yeast and between mLST8 and hSin1 in human.

(H) 1-160 amino acid residues in hSin1 bound to mLST8.

hSin1 or its fragment composed of the 1-160 residues was fused to MBP (MBP-hSin1 or MBP-hSin1 1-160, respectively). MBP-hSin1 (referred as "Full") or MBP-hSin1 1-160 ("1-160") was immobilized on the amylose resin and MBP pull-down assays were performed as described in Figure 7. B. with FLAG-mLST8 protein. FLAG-mLST8 was expressed in HEK293T human cultured cells by transient transfection.

(I) Interaction between hSin1 and mLST8 was impaired by amino acid substitution of the conserved leucine residues in N-CRIM.

L137 and L141 residues in MBP-hSin1 1-160 (referred as "WT") were substituted to serines or prolines to create MBP-hSin1 1-160 L137S L141S or L137P L141P (referred as "SS" or "PP" respectively). MBP pull-down assays were performed as described in Figure 7. H.

Wat1 is indispensable for TORC2 to phosphorylate Gad8.

Although both TORC1 and TORC2 contain Wat1 orthologues in eukaryotes (Figure 8. A) (Ikeda et al., 2008; Matsuo et al., 2007), Wat1 orthologues in mice or flies (mLST8 or Lst8, respectively) were reported to be dispensable for phosphorylation of substrates of TORC1 but indispensable for substrates of TORC2 (Guertin et al., 2006; Wang et al., 2012). To evaluate significance of Wat1 in TORC1 and TORC2 in fission yeast, we analyzed phosphorylation of Gad8 and Psk1 in wild-type and *Δwat1* cells (Figure 8. B). In *Δwat1* cells, phosphorylation of Gad8 was severely reduced, suggesting that Wat1 is required for TORC2 to phosphorylate Gad8. Phosphorylation of Psk1 was slightly decreased, suggesting that Wat1 partially contributes to TORC1 activity (see "Discussion I"). Since fission yeast TORC1 is activated by nitrogen sources (Nakashima et al., 2012), we tested whether or not Wat1 is required for TORC1 to respond to nitrogen. Although phosphorylation of Psk1 was attenuated partially in the presence of nitrogen in *Δwat1*, phosphorylation of Psk1 was further reduced upon nitrogen withdrawal, suggesting that TORC1 is still responsive to nitrogen in the absence of Wat1.

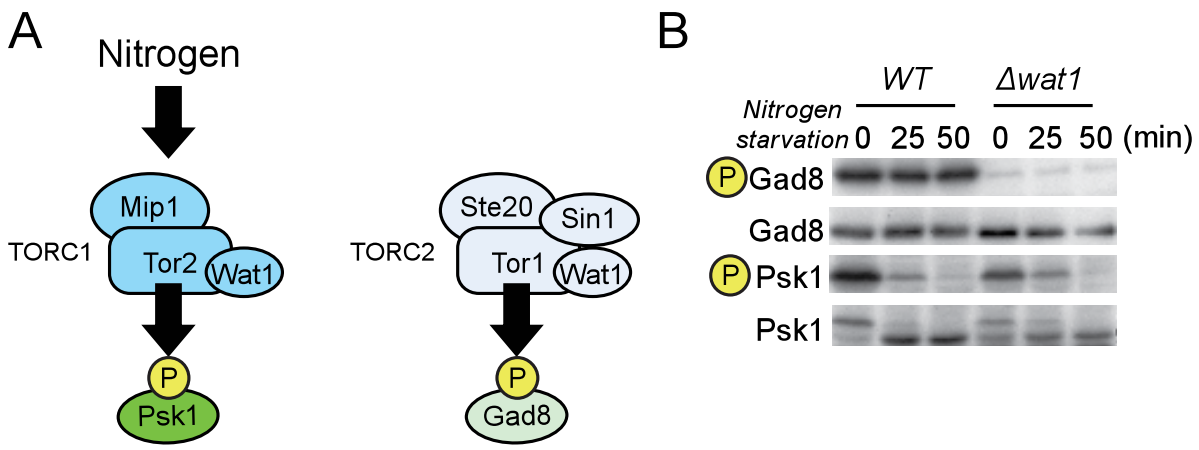

Figure 8. Wat1 is indispensable for TORC2 to phosphorylate Gad8

(A) Both TORC1 and TORC2 contain Wat1.

Wat1 is in both TORC1 and TORC2 (Matsuo et al., 2007; Nakashima et al., 2012). In fission yeast, only TORC1 responds to nitrogen sources.

(B) Disruption of *wat1* severely reduced phosphorylation of Gad8.

Wild-type and *Δwat1* cells were cultured in EMM and starved of the nitrogen source (NH_4Cl). Phosphorylated Gad8, phosphorylated Psk1, Gad8, and Psk1 were assessed as described before.

TORC2 is enzymatically active in the absence of Sin1 and Wat1

As mentioned earlier, our laboratory previously found that the CRIM domain in Sin1 is essential for Gad8 to be recruited to TORC2. In addition, loss of phosphorylation of Gad8 in *Δsin1* cells was rescued when Gad8 was fused to Ste20 (Ste20-Gad8) (see "*Δsin1* + Ste20-Gad8" in Figure 9. A). Since Wat1 is required for Sin1 to be incorporated into TORC2, an important function of Wat1 is to recruit Gad8 to TORC2 via Sin1. However, we could not exclude the possibility that Wat1 also has additional functions in TORC2 other than recruitment of Gad8 to TORC2 via Sin1. Therefore we expressed Ste20-Gad8 in *Δwat1* or *Δsin1 Δwat1* cells to bypass the requirement of Wat1 and Sin1 for recruiting Gad8 to Tor1 (Figure 9. B). It is important to note here that in fission yeast, Ste20 binds to Tor1 both in the presence or in the absence of Wat1 or Sin1 (data not shown). Since Ste20-Gad8 was hardly phosphorylated in cells expressing a Tor1 kinase-dead mutant (*tor1D2137A,* referred as "*tor1DA*"), the phosphorylation of Ste20-Gad8 was completely dependent on catalytic activity of Tor1 (Figure 9. B). Importantly, phosphorylation of Ste20-Gad8 was detected in *Δwat1* and *Δsin1 Δwat1* cells, although these cells lost phosphorylation of endogenous Gad8 (Figure 9. B). In addition, in *Δsin1 Δwat1* cells, phosphorylation of Ste20-Gad8 was comparable to those in each single mutant, *Δwat1* or *Δsin1*. These results indicate that Tor1 is still catalytically active in the absence of Wat1 and Sin1 and a fundamental role of Wat1 is to anchor Sin1 to Tor1 kinase to recruit Gad8 to TORC2.

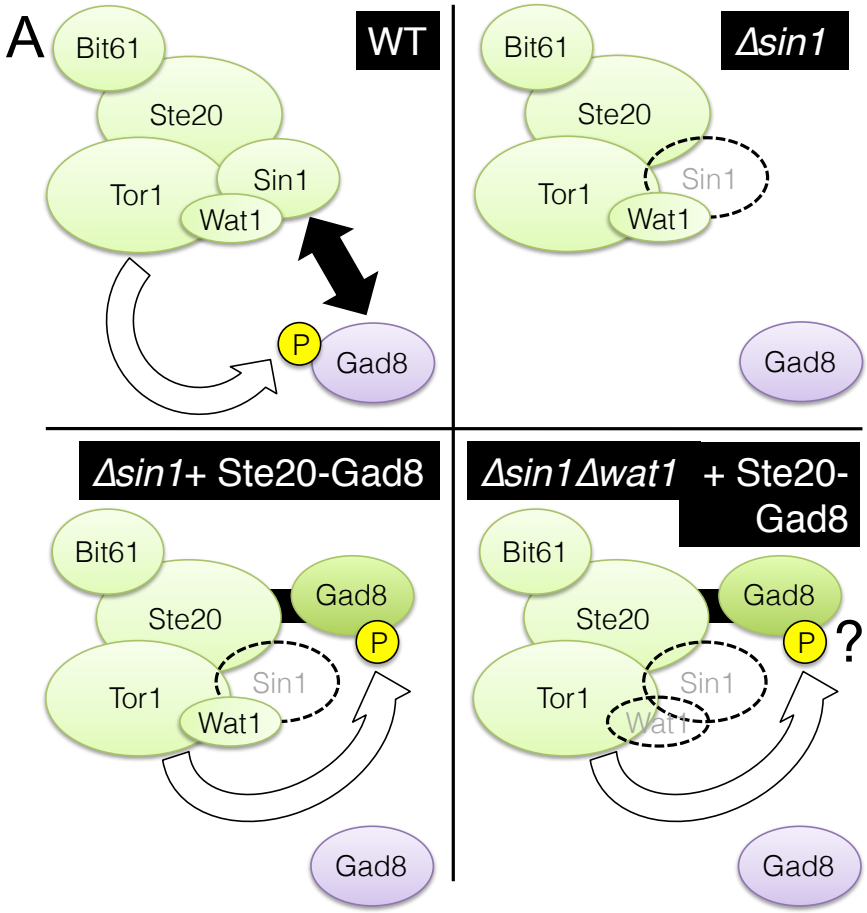

Figure 9. Wat1 anchors Sin1 to the Tor1 kinase.

(A) When Gad8 is fused to Ste20, Gad8 is phosphorylated even in the absence of Sin1.

Sin1 binds to Gad8 to recruit Gad8 to Tor1. Interaction between Sin1 and Tor1 requires Wat1 (WT). In *Δsin1* cells, TORC2 does not phosphorylate Gad8 (*Δsin1*) but the loss of phosphorylation of Gad8 in *Δsin1* cells is rescued when Gad8 is fused to Ste20 (Ste20-Gad8) (*Δsin1* + Ste20-Gad8). If the molecular function of Wat1 is to recruit Gad8 to TORC2 by anchoring Sin1 to Tor1, Wat1 and Sin1 are not required for phosphorylation when Gad8 is fused to Ste20 (*Δsin1 Δwat1* + Ste20-Gad8).

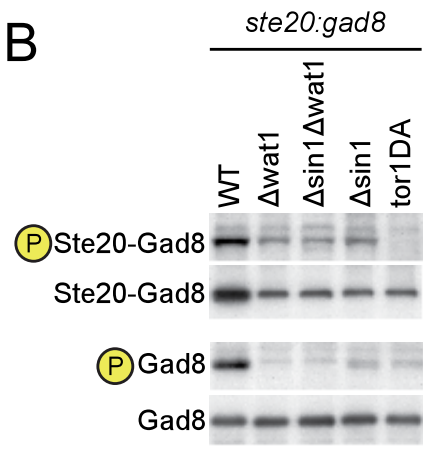

Figure 9. Wat1 anchors Sin1 to the Tor1 kinase.

(B) Gad8 was phosphorylated in *Δsin1* and *Δwat1* when Gad8 was fused to Ste20.

Wild-type ("*WT*"), *Δwat1*, *Δsin1Δwat1*, *Δsin1*, and *tor1D2137A* ("*tor1DA*") were transformed with a plasmid expressing Ste20-Gad8 under the control of the *sin1*$^{+}$ original promoter. Phosphorylation of Ste20-Gad8 was analyzed using antibodies against phosphorylated Gad8 or Gad8.

DISCUSSION I

In CHAPTER I, we demonstrated that the CRIM domain in Sin1 binds to unphosphorylated Gad8 rather than phosphorylated Gad8 or Psk1, a substrate of TORC1. This biochemical feature of CRIM seemed to contribute to selective recruitment of TORC2 substrate to Tor1 kinase to facilitate phosphorylation of TORC2 substrates. In addition, we also found that CRIM also have an additional function that is required for Sin1 to be incorporated into TORC2.

TORC2 phosphorylates its substrates including Gad8 (fission yeast) and Akt (mammals) rather than substrates of TORC1, although both TORC1 and TORC2 utilize TOR kinase as the catalytic subunit. Our laboratory previously found that Sin1 physically interacts with Gad8 via the CRIM domain and interaction between CRIM and Gad8 is essential for TORC2 to phosphorylate Gad8 *in vivo*, suggesting that Sin1 recruit Gad8 to Tor1 to facilitate phosphorylation of Gad8 by Tor1. In this study, we found that the CRIM domain does not bind to Psk1, suggesting that Sin1 recognizes substrates of TORC2. This CRIM function seems conserved in mammals because hSin1 CRIM also binds to TORC2 substrates rather than S6K, a substrate of TORC1 (Yonekura, unpublished data). Since artificial targeting of Psk1 to TORC2 induces the phosphorylation of Psk1 by TORC2, we concluded that TOR kinases do not distinguish the substrates of TORC1 and TORC2.

In mammalian TORC1, Raptor subunit that acts as substrate recruitment subunit in TORC1 preferentially binds to unphosphorylated substrates of TORC1 (Hara et al., 2002; Kim et al., 2003; Eguchi et al., 2006). We found that Sin1 CRIM binds to unphosphorylated Gad8 rather than phosphorylated Gad8, suggesting that Sin1 recognizes phosphorylation status of substrate and selectively recruits unphosphorylated substrates to TORC2. What are molecular mechanisms for Sin1 to preferentially interact with unphosphorylated Gad8? One possible explanation of this is due to conformational change of Gad8 induced by phosphorylation of the hydrophobic motif. Akt, a mammalian homolog of Gad8, is activated by phosphorylation of the hydrophobic motif in the C-terminus. The phosphorylated C-terminal region associates with the kinase domain to induce dramatic conformational change in the kinase domain, resulting in catalytically activation of Akt (Frödin et al., 2002; Yang et al., 2002). Since many AGC family kinases including Gad8 are activated by phosphorylation of the hydrophobic motif, Gad8 may also change its conformation by the phosphorylation (Frödin et al., 2002). We speculate that this dramatic conformational change of Gad8 induced by the phosphorylation reduces interaction between Sin1 and Gad8. Further studies are needed to test this possibility (Figure 10).

In the CRIM domain, 247-280 amino acid regions (N-CRIM) that has highly conserved amino acid residues is dispensable for Sin1 to interact with Gad8 (Murayama, unpublished data). We found that this region is required for Sin1 to interact with Wat1. In *Δwat1* cells, interaction between Sin1 and Tor1 is diminished, indicating that Wat1 anchors Sin1 on the Tor1 kinase (Figure 11). Consistently, in *Δwat1* cells, phosphorylation of Gad8 was severely attenuated and the reduction of phosphorylation of Gad8 in *Δwat1* cells was rescued by targeting Gad8 to Ste20. These results indicate that TORC2 is enzymatically active in the absence of Wat1.

We identify the amino acid residues in Sin1 N-CRIM that is required for Sin1 to interact with Wat1. 258th and 262nd leucine residues in N-CRIM that is highly conserved among Sin1 orthologues are required for Sin1 to interact with Wat1. Corresponding residues in hSin1 are also significant for interaction between hSin1 and mLST8, indicating that biochemical feature of interaction between Sin1 and Wat1 in fission yeast are evolutionally conserved. An alpha-helix secondary structure is predicted to be formed between L258 and L262 residues (Kojima, unpublished data). There is possible this predicted hydrophobic alpha-helix is significant for interaction between Sin1 and Wat1.

Although both TORC1 and TORC2 contain Wat1, *Δwat1* cells show dramatic reduction of phosphorylation of Gad8 by TORC2 with marginal reduction of phosphorylation of Psk1 by TORC1. Recently, our laboratory found that loss of the TORC2-Gad8 pathway attenuates phosphorylation of Psk1 by TORC1 (Hatano, Maenishi, and Matsuda, unpublished data). Therefore, the slight reduction of phosphorylation of Psk1 is due to lack of activity in the TORC2-Gad8 pathway.

In summary, our study has revealed the molecular mechanisms of Sin1 and Wat1, which are required for phosphorylation of Gad8 by TORC2. TORC2 distinguish its substrate by virtue of selective recruitment of TORC2 substrates by Sin1 CRIM. Since Wat1 is required for Sin1 to be incorporated into TORC2, we conclude that Wat1 acts as an anchor for Sin1 to interact with Tor1. TORC2 signaling was implicated in cellular response to hormones and tumorigenesis (Sarbassov et al., 2005). Indeed, prostate specific loss of functional TORC2 suppresses development of cancer in mice lacking PTEN, a tumor suppressor (Guertin et al., 2009), and in human, somatic mutations of the TORC2 components that sustained the kinase activity of the complex at higher level were identified in cancer patients (Liu et al., 2013). From these observations TORC2 signaling, whose specific inhibition system has not been developed pharmacologically, is attractive for developing the novel cures for cancer. We expect that interaction between hSin1 and mLST8 is potentially a novel target for cures for cancer. Further investigation of the molecular function of subunits in TORC2 will also

provide us novel ways for therapeutic development.

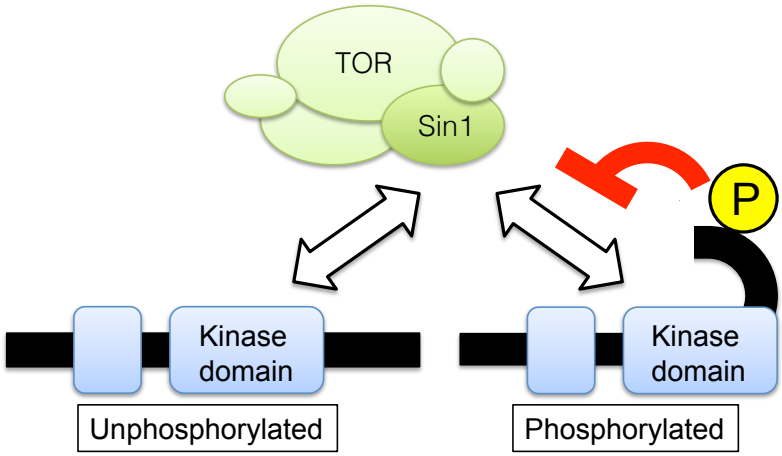

Figure 10. A model for preferential interaction of Sin1 to unphosphorylated Gad8

Here we depict a schematic model for the reduction of interaction between Sin1 and Gad8 by phosphorylation of the hydrophobic motif in Gad8.
When the C-terminus of Gad8 is phosphorylated, Gad8 turns to be in catalytically activated conformation and this change in conformation attenuates interaction between Sin1 and Gad8.

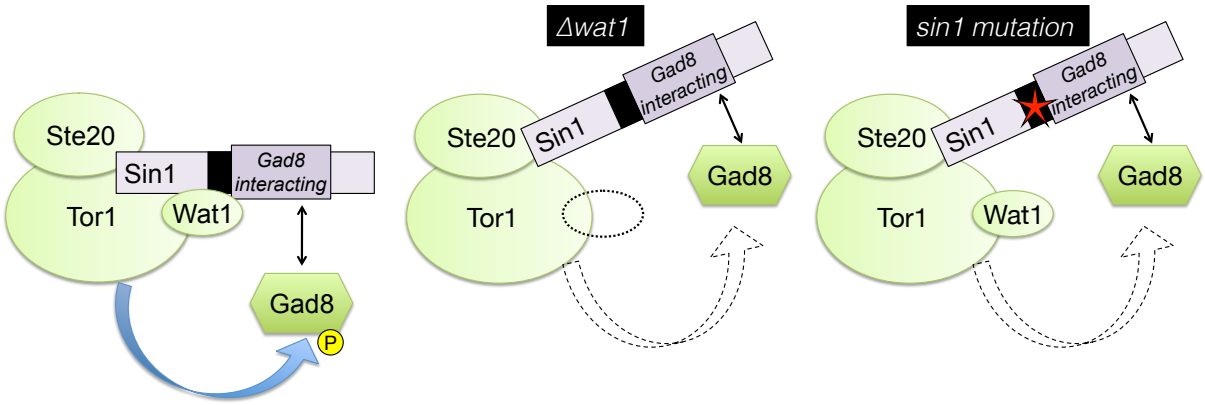

Figure 11. A model for incorporation of Sin1 into TORC2 and recruitment of Gad8 toward Tor1

N-CRIM in Sin1 is a region that is required for interaction between Sin1 and Wat1. When Wat1 is absent or Sin1 has mutations that diminish interaction to Wat1, Sin1 is no longer incorporated precisely into TORC2 and Sin1 may not recruit Gad8 to Tor1 properly. Therefore, we propose that Wat1 and Sin1 act in a coordinated manner to recruit Gad8 to Tor1.

CHAPTER II

Rab GTPase responds to glucose in order to regulate TOR complex 2.

INTRODUCTION II

TOR Ser/Thr specific protein kinase forms two different complexes, TORC1 and TORC2, and these are highly conserved among eukaryotes. By extensive studies, now it is accepted that TORC1 responds to amino acids and hormones such as insulin and growth factors to regulate protein synthesis and degradation (Avruch et al., 2009; Dunlop and Tee, 2009). The activation of TORC1 is mediated through two distinct small GTPases, Rheb and Rag. Rheb binds to TOR kinase and activates the catalytic activity, whereas Rag recruits the complex on the lysosomal membrane where Rheb exists by binding to the TORC1 (Bar-Peled et al., 2013; Bar-Peled et al., 2012; Sancak et al., 2008; Zoncu et al., 2011). On the contrary to the knowledge for the TORC1 regulation, we have only limited information about activation mechanisms of TORC2.

Fission yeast *Schizosaccharomyces pombe* is a genetically amenable model system with non-essential TORC2. This organism has the TORC2-Gad8 pathway that is highly homologous to the mammalian TORC2-AKT or SGK pathways (Figure 1. A in CHAPTER I) (Hayashi et al., 2007; Ikeda et al., 2008; Matsuo et al., 2003; Matsuo et al., 2007; Tatebe et al., 2010). In fission yeast, TORC2 is composed of Ste20, Sin1, Wat1, and Tor1 that are homologous to mammalian Rictor, hSin1, mLST8, and mTOR, respectively (Figure 1. A in CHAPTER I). To identify proteins that activate the TORC2-Gad8 pathway, our laboratory previously performed a genetic screening with fission yeast, and found that the Rab family small GTPase Ryh1 activates the TORC2-Gad8 pathway in fission yeast (Tatebe et al., 2010). Small GTPases function as molecular switches and cycle between an active, GTP-bound state and an inactive, GDP-bound state. Expression of the GTP-bound Ryh1Q70L mutant increases phosphorylation of Gad8 in a manner dependent on TORC2, whereas the GDP-bound Ryh1T25N mutant reduces phosphorylation of Gad8 (Tatebe et al., 2010). However physiological stimuli that regulate Ryh1 and TORC2 have not been understood.

Here, in the Chapter II, we demonstrate that TORC2 is inactivated upon glucose starvation and this response is mediated through Ryh1. In addition, we also found that Bit61 is required for Ryh1 to activate TORC2.

MATERIALS AND METHODS II

Yeast strains, media, and general techniques

Media composition for the yeast culture and genetic modification techniques of the yeast cells were established previously and described in CHAPTER I.

Antibodies

Antibodies used in this study are listed in following table.

Antigen	Source (animals)	Supplier or Reference
Phosphorylated Gad8	Rabbit	(Tatebe et al., 2010)
Gad8	Rabbit	(Tatebe et al., 2010)
Phosphorylated p70 S6 Kinase	Mouse	#9205 (Cell Signaling Technology)
FLAG epitope tag	Mouse	#F1804 (SIGMA-ALDRICH)
cMyc epitope tag	Mouse	#MMS-150P (COVANCE)
Rabbit IgG (HRP conjugated)	Goat	#W4011 (Promega)
Mouse IgG (HRP conjugated)	Goat	#W4021 (Promega)

Nutrient starvation

Cells were cultured as described in CHAPTER I.

For the glucose depletion, cells were cultured in EMM or YES media and transferred to fresh media without supplemented glucose following to collecting cells on filter membranes (#A045A0.25A*, ADVANTEC). Because removal of glucose from culture media reduces its osmorality, glucose was replaced with sorbitol, a non-fermentable sugar for fission yeast. To release from starvation of glucose, culture was split in to two at 20 minute and cells in an aliquot of culture were re-incubated in glucose media for 10 minutes. Cells were harvested after ten minutes incubation in media containing glucose.

Analysis of phosphorylation of Tor1

For analysis of the phosphorylated Tor1, phosphorylated affinity gel was used for SDS-PAGE prior to immunoblotting with anti-FLAG antibody. SDS-PAGE was performed with gels following content; 2.9% acrylamide, 0.1% *bis*-acrylamide, 0.5% agarose, 20 μM phos-tag (AAL-107, NARD institute), 40 μM $MnCl_2$. After electrophoresis, gel was washed with buffer containing 10 mM EDTA prior to the transferring procedure.

Phosphatase treatment of the FLAG-Tor1

Cells expressing FLAG-Tor1 were harvested on a nitrocellulose filter membrane with 0.45 μm pore (#A045A0.25A*, ADVANTEC) with 10% trichloroacetic acid (TCA) solution. Cells were disrupted by beads-beating and whole cell lysate were extracted with SDS sample buffer (550 mM Tris-HCl [pH 8.0], 2% SDS, 4.5% glycerol, 0.01% BPB, 0.14 M 2-mercaptoethanol).

50 μl of lysates were diluted into 950 μl of buffer composed of 25 mM Tris, 150 mM NaCl, and 1% NP-40 at pH 7.6 and the final concentration of SDS in buffer was 0.1%. This Buffer was subjected to immunoprecipitation with anti-FLAG M2 magnetic beads (#M8823, SIGMA-ALDLICH) at room temperature. Resulting FLAG-Tor1 on beads were subjected with lambda-phosphatase treatment with following buffer condition; 50 mM HEPES, 10 mM NaCl, 2 mM DTT, 0.01% Brij 35 with lambda phosphatase. For inhibiting phosphatase activity, its inhibitor cocktail was added as described previously (Tatebe et al., 2008).

Reaction mixtures were incubated at 30˚C for 30 minutes and FLAG-Tor1 were eluted from magnetic beads following to washing with buffer composed of 25 mM Tris, 150 mM NaCl, 0.1% SDS, and 1% NP-40 at pH 7.6.

GST pull-down assays with mammalian Rab6 effecter

Bicaudal D2 (BICD2) is a well-known effecter protein of Rab6 in a human counterpart of fission yeast Ryh1 and whose binding region to Ryh1 is identified (Bergbrede et al., 2009). Based on this information, 706-814 amino acid regions in BICD2, the effecter domain for Rab6, was fused with GST and expressed in bacteria at 16 ˚C. Resulting recombinant protein (GST-BICD2) was purified with glutathione-sepharose beads (#17-0756-01, Amersham) in lysis buffer (25 mM Tris-HCl [pH 7.5], 137mM NaCl, 2.7 mM KCl, 1% triton X-100, and 1 mM PMSF).

Yeast cells expressing FLAG-Ryh1 proteins were cultured in YES liquid culture media and harvested before or after starvation of glucose. The cells were washed once with ice-cold water and stored at -80 ˚C before using pull-down assays.

Following procedures were performed on ice. To analyze physical interaction between FLAG-Ryh1 and GST-BICD2, harvested yeast cells were suspended into ice cold phosphate buffered saline (PBS) containing 10 mM $MgCl_2$, 0.5% Tween 20, 1 mM PMSF, and the protease inhibitor cocktail for use in purification of histidine-tagged proteins (#8830, SIGMA-ALDRICH). Cells were disrupted by beads-beating with 0.5 mm glass beads (AZ ONE) and lysate were cleared by centrifugation at 20,400 x g for 15 minutes to remove cell

debris and insoluble fractions. Resulting supernatants were loaded on to GST-BICD2 proteins immobilized on glutathione beads and incubated for 45 minutes prior to wash 3 times with PBS containing 10 mM $MgCl_2$ and 0.5% Tween 20 and elution of the GST-BICD2 and FLAG-Ryh1 on beads.

Co-immunoprecipitation with FLAG or *myc* epitope tag

Following experiments in this paragraph was performed on ice. For purification of Ste20-*myc*, Sin1-*myc*, or Wat1-*myc*, whole cell extracts were prepared in lysis buffer composed of 20 mM HEPES-KOH (pH 7.5), 150 mM sodium glutamate, 10% glycerol, 0.25% Tween 20, 10 mM sodium fluoride, 0.1 mM sodium orthovanadate, 10 mM *beta*-glycerophosphate, 10 mM sodium pyrophosphate, 10 mM *p*-nitrophenyl phosphate, di-sodium salt, 1 mM PMSF, and the protease inhibitor cocktail for use in purification of histidine-tagged proteins (#8830, SIGMA-ALDRICH). Cell extract containing FLAG-Tor1 with Ste20-*myc*, Sin1-*myc*, or Wat1-*myc* were incubated with EZview Red Anti-c-Myc Affinity Gel (#E6654, SIGMA-ALDRICH) for 1 hour and washed extensively with lysis buffer without PMSF and the protease inhibitor cocktail. Precipitated proteins were eluted with sample buffer.

Table-2. Fission yeast strains used in this study

Strain ID	Genotype	Source or Reference
CA1	*h-*	Laboratory stock
CA101	*h- leu-1-32*	Laboratory stock
CA14	*h- leu1-32 ura4-D18 spc1::ura4*	Laboratory stock
CA238	*h- leu1-32 ura4-D18 mcs4::ura4*	Laboratory stock
CA3	*h- leu1-32 ura4-D18*	Laboratory stock
CA35	*h- leu1-32 ura4-D18 wis1::ura4*	Laboratory stock
CA4593	*h- leu1-32 ura4-D18 tor1::ura4*	(Matsuo et al., 2003)
CA473	*h- leu1-32 ura4-D18his7-366 wis4::his7 win1::ura4*	Laboratory stock
CA5021	*h- leu1-32 ste20::kanMX6*	(Ikeda et al., 2008)
CA5126	*h- leu1-32 sin1::kanMX6*	(Ikeda et al., 2008)
CA5142	*h- leu1-32 ura4-D18 gad8::ura4*	Laboratory stock
CA5603	*h- leu1-32 ura4-D18 pka1::ura4*	Laboratory stock

CA5605	*h- leu1-32 ura4-D18 cgs2::ura4*	Laboratory stock
CA5647	*h- leu1-32 ura4-D18 cyr1::ura4*	Laboratory stock
CA5657	*h- leu1-32 ura4-D18 git3::ura4*	Laboratory stock
CA5669	*h- leu1-32 ura4-D18 gpa2::ura4*	Laboratory stock
CA5764	*h- leu1-32 slm1::kanMX6*	Laboratory stock
CA6530	*h- leu1-32 (hph)FLAG:tor1*	(Hayashi et al., 2007)
CA6568	*h- leu1-32 (hph)FLAG:tor1 ryh1::kanMX6*	Laboratory stock
CA6652	*h- leu1-32 (hph)FLAG:tor1D2137A(kanMX6)*	Laboratory stock
CA6795	*h- leu1-32 ura4-D18 bit61::ura4*	(Ikeda et al., 2008)
CA6809	*h- leu1-32 3FLAG:ryh1*	Laboratory stock
CA6815	*h- leu1-32 ura4-D18 3FLAG:ryh1 bit61::ura4*	Laboratory stock
CA6817	*h- leu1-32 3FLAG:ryh1Q70L*	Laboratory stock
CA6822	*h- leu1-32 ura4-D18 3FLAG:ryh1Q70L bit61::ura4*	Laboratory stock
CA6826	*h- leu1-32 ura4-D18 3FLAG:ryh1T25N bit61::ura4*	Laboratory stock
CA6828	*h- leu1-32 3FLAG:ryh1T25N*	Laboratory stock
CA6855	*h- leu1-32 (hph)FLAG:tor1 bit61:13myc(kanMX6)*	Laboratory stock
CA6859	*h- leu1-32 bit61:13myc(kanMX6)*	Laboratory stock
CA6870	*h- leu1-32 (hph)FLAG:tor1 sin1::kanMX6*	Laboratory stock
CA6876	*h- leu1-32 (hph)FLAG:tor1 ste20::kanMX6*	Laboratory stock
CA7087	*h- leu1-32 (hph)FLAG:tor1 ste20:13myc(kanMX6)*	Laboratory stock
CA7092	*h- leu1-32 (hph)FLAG:tor1 sin1:13myc(kanMX6)*	Laboratory stock
CA7107	*h- leu1-32 ura4-D18 (hph)FLAG:tor1 bit61::ura4*	Laboratory stock
CA7130	*h- leu1-32 (hph)FLAG:tor1 gad8::ura4*	Laboratory stock
CA7185	*h- leu1-32 ura4-D18 (hph)FLAG:tor1 ste20:13myc(kanMX6) bit61::ura4*	Laboratory stock
CA7213	*h- leu1-32 (hph)FLAG:tor1 wat1:13myc(kanMX6)*	Laboratory stock
CA7313	*h- leu1-32 ura4-D18 (hph)FLAG:tor1*	Laboratory stock

	sin1:13myc(kanMX6) bit61::ura4	
CA7317	*h- leu1-32 ura4-D18 (hph)FLAG:tor1 wat1:13myc(kanMX6) bit61::ura4*	Laboratory stock
CA7801	*h- leu1-32 (hph)FLAG:tor1 3FLAG:ryh1*	Laboratory stock
CA7803	*h- leu1-32 (hph)FLAG:tor1 3FLAG:ryh1Q70L*	Laboratory stock
CA7807	*h- leu1-32 (hph)FLAG:tor1 3FLAG:ryh1T25N*	Laboratory stock
CA8350	*h- leu1-32 ura4-D18 its3-1*	(Zhang et al., 2000)

RESULTS II

The TORC2-Gad8 pathway responds to glucose.

The stimuli that regulate the TORC2-Gad8 pathway in fission yeast were not identified. Recently our laboratory found that *Δgad8* cells showed severe growth defects under lower glucose condition (Figure 12. A), indicating that the TORC2-Gad8 pathway is essential for cells to grow in limited glucose condition. Therefore, we examined whether the TORC2-Gad8 pathway responds to glucose or not. We found that phosphorylation of Gad8 by TORC2 was reduced when exponentially growing cells were shifted from growth media containing 3 percent glucose to the same media lacking glucose (Figure 12. B), indicating that activity of the TORC2-Gad8 pathway is under the control of glucose.

Synthetic media for fission yeast (EMM minimal media) contain 2 percent glucose as the sole carbon source. Fission yeast grows efficiently under this condition (~3 hour for a cell division), but growth of fission yeast is not significantly different in EMM media containing 0.2 percent glucose. However, when the glucose concentration is reduced to 0.02 percent, fission yeast cells stop their growth with their intracellular ATP level maintained over 1 hour (Pluskal et al., 2011). Correlating with the growth under media containing 0.2 or 0.02 percent glucose, when cells growing in 2 percent glucose were transferred to media containing 0.2 percent glucose, phosphorylation of Gad8 was not altered (Figure 12. C). On the other hand, phosphorylation of Gad8 was reduced when cells were transferred to media containing 0.02 percent glucose, suggesting that the TORC2-Gad8 pathway responds to glucose concentration that affects cell growth (Figure 12. C).

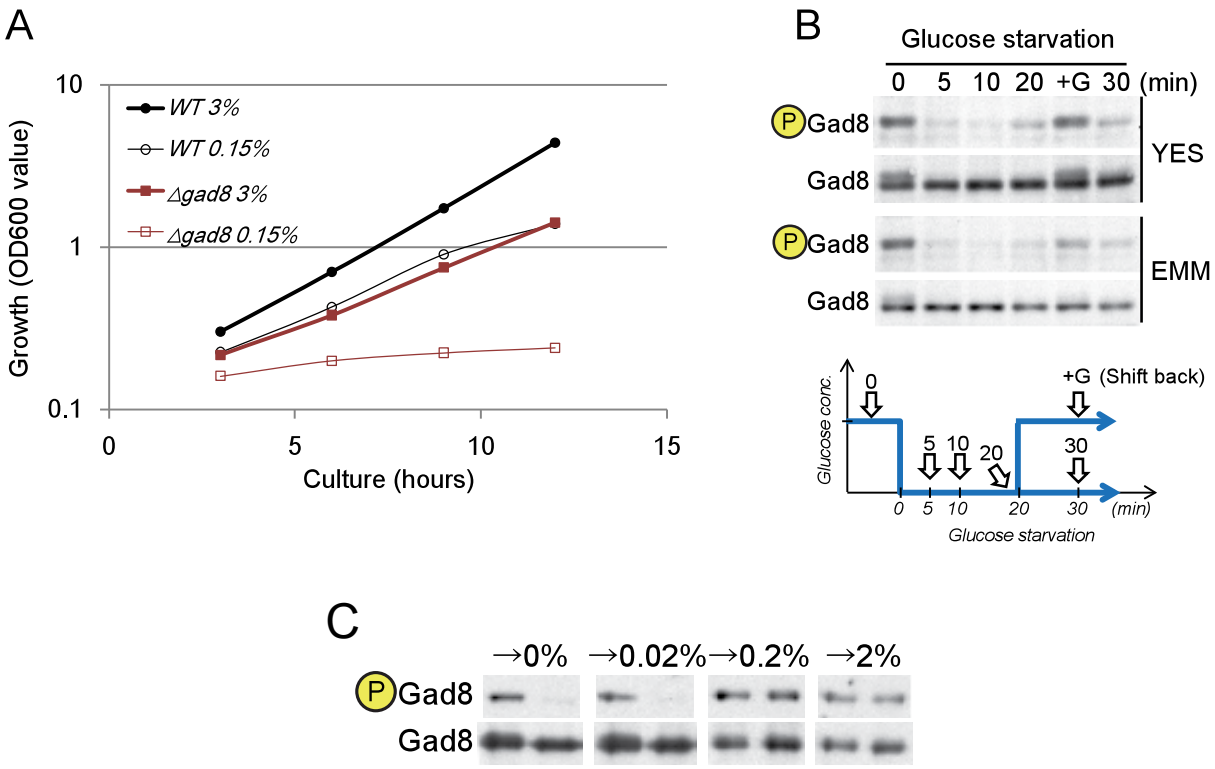

Figure 12. The TORC2-Gad8 pathway responds to glucose.

(A) Δ*gad8* cells showed poor growth in media containing low glucose.

Wild-type (black) and *Δgad8* (red) cells were cultured in YES media containing 3 or 0.15 percent glucose and their growth were monitored.

Wild type: circles (● and ○ represent 3% and 0.15%, respectively)

Δgad8: squares (■ and □ represent 3% and 0.15%, respectively)

(B), (C) Phosphorylation of Gad8 was reduced in response to glucose starvation.

(B) Cells cultured in YES or EMM liquid media were harvested and starved of glucose. The samples at 0 minute were taken just prior to glucose starvation. The culture was split into two at 20 minutes and cells in an aliquot of the culture were re-incubated in glucose media for 10 minutes (referred as "+G"). Cells were harvested with TCA, followed by proteins extraction. Immunoblotting was performed with anti-phosphorylated Gad8 or Gad8 antibodies.

(C) Cells cultured in standard EMM media (2% glucose) were transferred to media containing 0, 0.02, 0.2, or 2% glucose. Phosphorylated Gad8 or Gad8 were analyzed as described in (B).

Ryh1 responds to glucose to regulate the TORC2-Gad8 pathway.

Since our laboratory previously found that a small GTPase Ryh1 activates TORC2 in fission yeast, we tested the possibility that Ryh1 mediates glucose signals to the TORC2-Gad8 pathway. At first, we developed an experimental system to detect the GTP-bound state of Ryh1 to monitor *in vivo* activity of Ryh1. Ryh1 is a homologue of human Rab6, whose interaction with bicaudalD2 (BICD2), an effecter protein of Rab6, was well-characterized biochemically (Bergbrede et al., 2009; Matanis et al., 2002). Based on this information, the effecter domain of BICD2 was fused with GST (GST-BICD2) and expressed in bacteria. GST pull-down assays revealed that GST-BICD2 could precipitate GTP-bound, active FLAG-Ryh1 Q70L but not GDP-bound, inactive FLAG-Ryh1 T25N (Figure 13. A). FLAG-Ryh1 ("*WT*") in lysate prepared from cells grown in media containing glucose bound to GST-BICD2, whereas interaction between GST-BICD2 and FLAG-Ryh1 was attenuated when the cells were starved of glucose. These results indicate that GTP-bound Ryh1 decreased upon glucose starvation. To test significance of activation of Ryh1 by glucose for regulating the TORC2-Gad8 pathway, we examined phosphorylation of Gad8 in cells expressing constitutively activated Ryh1 ("*GTP-bound*") in the presence or absence of glucose. Although glucose deprivation impaired phosphorylation of Gad8 in wild-type cells ("*WT*"), phosphorylation of Gad8 remained high even after glucose deprivation in Ryh1Q70L cells (Figure 13. B). From these observations, we conclude that Ryh1 is a major regulator of the TORC2-Gad8 pathway in response to glucose.

Other than the TORC2-Gad8 pathway, there are several glucose responsive pathways in fission yeast. It has been well known that the PKA pathway is activated by extracellular glucose (Figure 13. C) (Hoffman, 2005). The PKA pathway senses glucose via a G-protein coupled receptor Git3, hetero-trimetric G-protein alpha subunit Gpa2, adenylate cyclase Cyr1, and cyclic AMP activated protein kinase Pka1 (Figure 13. C). In addition, the stress activated mitogen-activated protein kinase (MAP kinase) Spc1 (SAPK) is activated upon glucose starvation (Madrid et al., 2004; Shiozaki and Russell, 1995), and Spc1 is reported to interact with Sin1, an essential subunit of TORC2 (Wilkinson et al., 1999). Therefore, we speculated that the PKA or SAPK pathways were related to regulation of TORC2 in response to glucose. However, phosphorylation of Gad8 in cells lacking components of the PKA pathway was comparable to that of wild-type cells in the presence of glucose (Figure 13. C). In addition, we also tested phosphorylation of Gad8 in null mutants of components of the SAPK pathway (*Δmcs4*, *Δwis4Δwin1*, *Δwis1*, and *Δspc1*) in the presence or absence of glucose. Like wild-type cells, mutants of the SAPK pathway reduced phosphorylation of Gad8 after glucose starvation (Figure 13. D). From these results, the

Ryh1-TORC2-Gad8 pathway responds to glucose independently of the PKA and SAPK pathways.

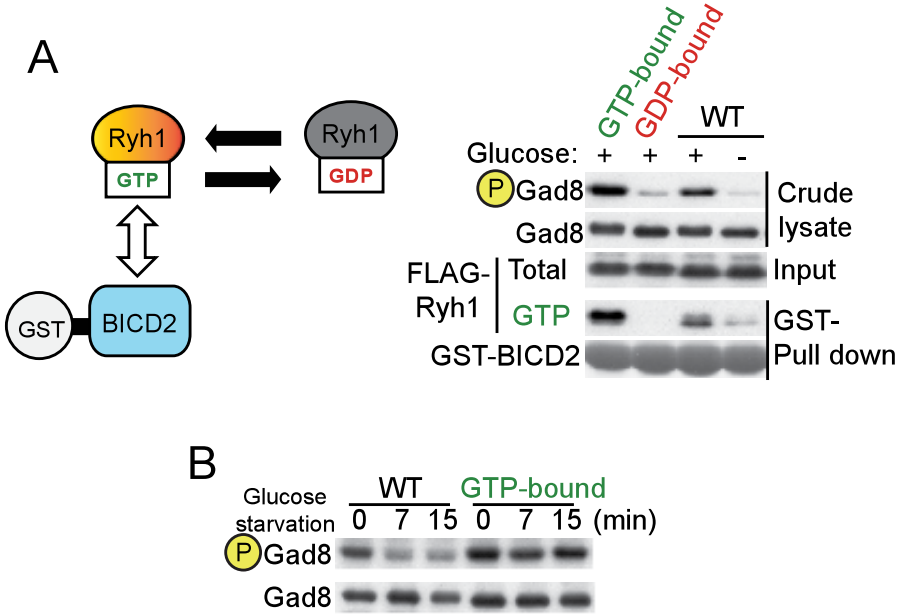

Figure 13. Ryh1 responds to glucose to regulate the TORC2-Gad8 pathway.

(A) GTP-bound Ryh1 was reduced upon glucose depletion.

Bacterially expressed BICD2 fragment fused with GST (GST-BICD2) was immobilized on glutathione beads and GST pull-down assays were performed with lysate prepared from yeast cells expressing FLAG-Ryh1 ("WT"), FLAG-Ryh1Q70L ("GTP-bound"), or FLAG-Ryh1T25N ("GDP-bound") in media containing glucose. WT lysate was also prepared after starvation of glucose for 5 minutes (shown as "-"). GST-BICD2 in precipitates was detected in coomassie brilliant blue staining. FLAG-Ryh1 in input or precipitate (referred as "Total" or "GTP", respectively) was detected with anti-FLAG antibodies. Phosphorylation of Gad8 was analyzed as described in Figure 12. B.

(B) Gad8 remained phosphorylated in the absence of glucose in the cells expressing a constitutively activated allele of *ryh1*.

FLAG-ryh1 ("WT") and *FLAG-ryh1Q70L* ("GTP-bound") cells cultured in YES media were harvested at indicated time points after being transferred to YES without glucose. Phosphorylation of Gad8 was analyzed as described in Figure 12. B.

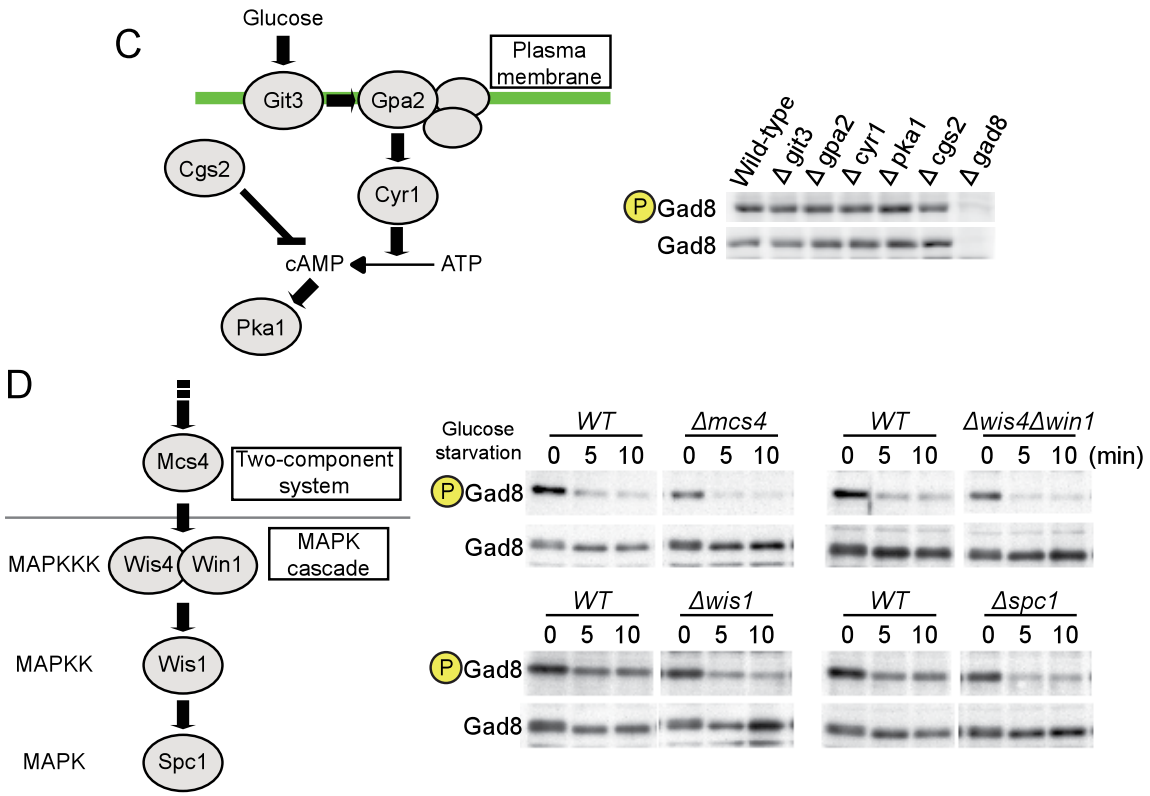

Figure 13. Ryh1 responds to glucose to regulate the TORC2-Gad8 pathway.

(C) The PKA pathway was dispensable for the phosphorylation of Gad8 in the presence of glucose.

(Left) The PKA pathway in fission yeast is depicted. The PKA pathway senses glucose via a G-protein coupled receptor Git3, hetero-trimetric G-protein alpha subunit Gpa2, adenylate cyclase Cyr1, and cyclic AMP activated protein kinase Pka1 (Hoffman, 2005).

(Right) Phosphorylation of Gad8 was monitored in wild-type ("*WT*"), *Δgit3*, *Δgpa2*, *Δcyr1*, *Δpka1*, *Δcgs2*, and *Δgad8* cells as shown in Figure 12. B.

(D) The SAPK signaling was dispensable for glucose dependent phosphorylation of Gad8.

(Left) The SAPK pathway in fission yeast is depicted. The SAPK pathway is composed of Spc1 (MAPK), Wis1 (MAPKK), and heterodimer of Wis4 and Win1 (MAPKKK) (Morigasaki et al., 2008; Nguyen et al., 2000; Shiozaki and Russell, 1995, 1996; Shiozaki et al., 1997). Mcs4, a response regulator of the two-component system, stably interacts with heterodimer of Wis4 and Win1 to stabilize the integrity of the heterodimer (Morigasaki et al., 2013; Morigasaki and Shiozaki, 2013).

(Right) Wild-type ("*WT*"), *Δmcs4*, *Δwis4Δwin1*, *Δwis1*, and *Δspc1* cells were cultured in EMM liquid media and starved of glucose for 5 or 10 minutes. Phosphorylation of Gad8 was analyzed as described in Figure 12. B.

Ryh1 regulates the TORC2-Gad8 signaling in a manner dependent on Bit61.

Bit61 is a subunit of TORC2 that binds to Tor1 in a manner dependent on Ste20. We found that, like Ryh1, Bit61 is dispensable for interaction of Tor1 with Ste20, Sin1, or Wat1 (Figure 14. A). In addition, similar to *Δryh1* cells, *Δbit61* cells reduced but not abolished phosphorylation of Gad8, indicating that Bit61 and Ryh1 are required for full activation of TORC2 (Figure 14. B) (Tatebe et al., 2010; Tatebe and Shiozaki, 2010). Importantly, in *Δryh1 Δbit61* double mutant, the phosphorylation level of Gad8 is equivalent to that in *Δryh1* or *Δbit61* mutants (Tatebe et al., 2010; Tatebe and Shiozaki, 2010), suggesting that these genes are epistatic for activating TORC2. Therefore, we speculated that Bit61 is required for Ryh1 to activate TORC2. Phosphorylation of Gad8 in *Δbit61* cells was reduced even in the presence of glucose (Figure 14. B). In cells expressing active Ryh1Q70L (*"GTP-bound"*), Gad8 is highly phosphorylated but this activation was cancelled in the absence of Bit61 (Figure 14. C). On the other hand, GTP-bound Ryh1 similarly decreased upon glucose starvation in wild-type and *Δbit61* cells (Figure 14. D). From these observations, we conclude that Bit61 is required for Ryh1 to activate TORC2 in response to glucose.

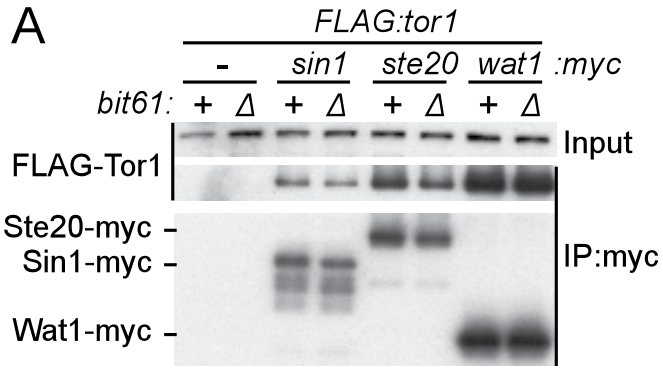

Figure 14. Ryh1 regulates the TORC2-Gad8 signaling in a manner dependent on Bit61.

(A) Bit61 was dispensable for interaction between Tor1 and Sin1, Ste20, or Wat1.

Cells expressing FLAG-Tor1 with Sin1-*myc*, Ste20-*myc*, or Wat1-*myc* were cultured in YES media and co-immunoprecipitation assays were performed with anti-*myc* antibodies. Precipitates and inputs were detected with antibodies against FLAG or *myc*.

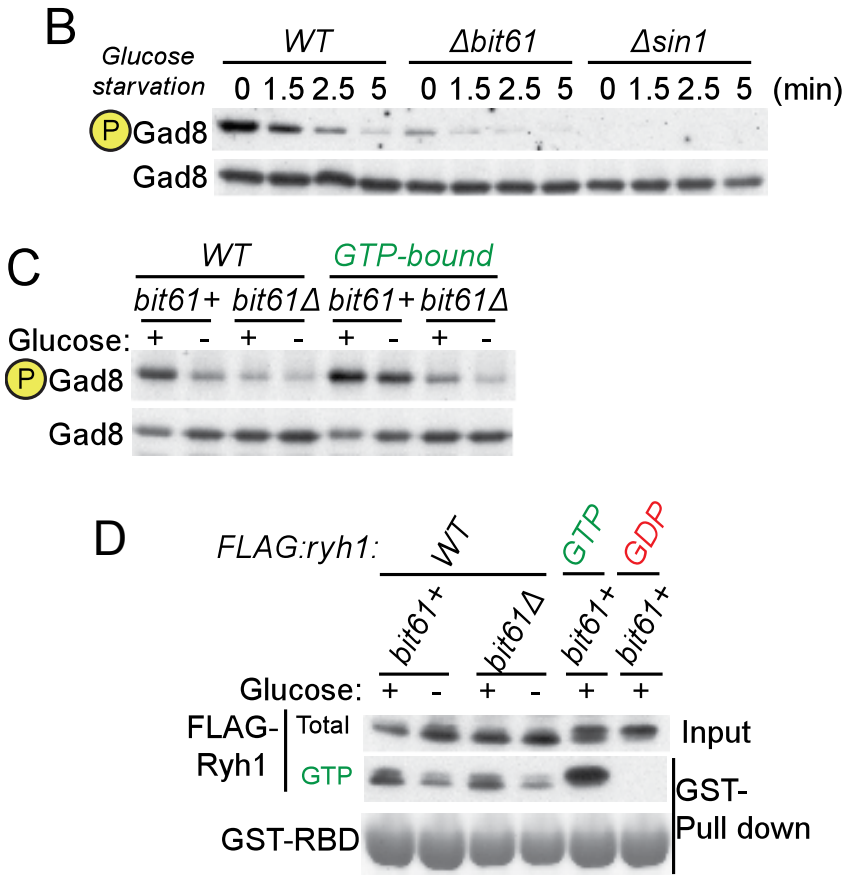

Figure 14. Ryh1 regulates the TORC2-Gad8 signaling in a manner dependent on Bit61.

(B) The TORC2-Gad8 pathway was hardly activated by glucose in *Δbit61* cells.

Wild-type, *Δbit61*, and *Δsin1* cells were cultured in YES media and starved of glucose. The cells were harvested at indicated time points after the starvation and immunoblotting was performed with antibodies against phosphorylated Gad8 and Gad8 as described in Figure 12. B. The sample at 0 minute was harvested just prior to glucose starvation.

(C) Loss of Bit61 cancelled activation of TORC2 by Ryh1.

Cells expressing FLAG-Ryh1 or FLAG-Ryh1Q70L ("*WT*" and "*GTP-bound*", respectively) were cultured in YES media and harvested for 5 minutes after glucose starvation (indicated by "-"). The samples indicated by "+" were harvested just prior to glucose starvation. Phosphorylation of Gad8 was monitored as described in Figure 12. B.

(D) Ryh1 responds to glucose similarly in wild-type and *Δbit61* cells.

Pull-down assays were performed with GST-BICD2 to monitor activity of FLAG-Ryh1 as described in Figure 13. A.

Ryh1 regulates phosphorylation of Tor1.

Then we questioned how Ryh1 and Bit61 promote phosphorylation of Gad8 by TORC2. Previously we reported that *in vitro* kinase activity of Tor1 prepared from *Δryh1* cells was comparable to that of Tor1 from wild-type (Tatebe et al., 2010). However, in that experimental system, we used recombinant PHAS-I as substrate, although PHAS-I is not a physiological substrate of TORC2 in fission yeast. It is possible that this experimental design is inappropriate for monitoring catalytic activity of TORC2 *in vitro*. Indeed, Tor1 phosphorylated PHAS-I similarly when Tor1 is immunopurified from wild-type or *Δste20* cells, although phosphorylation of Gad8 was abolished in *Δste20* cells (Morigasaki and Hatano, unpublished data). Therefore we monitored activity of TORC2 by another approach. It is already reported that in human, mTOR auto-phosphorylates the serine residue at the 2481st amino acid position (Ser2481) and phosphorylation of Ser2481 correlates with catalytic activity of TOR complexes. Thus, phosphorylation of Ser2481 is a good marker for monitoring activity of TOR complexes in cells (Soliman et al., 2010). Therefore, we established a method to monitor phosphorylation of Tor1 in fission yeast using phosphate-affinity gel electrophoresis (Kinoshita et al., 2006). In this strategy, the FLAG-Tor1 protein was detected as two individual bands (Figure 15. A). To test whether the upper band corresponds to phosphorylated Tor1 or not, FLAG-Tor1 was immunopurified and treated with lambda phosphatase (Figure 15. A). We found that the upper band was abolished by treating with lambda phosphatase, indicating that the upper band corresponds to phosphorylated FLAG-Tor1. Interestingly, in *FLAG:tor1 D2137A* mutant cells ("*DA*") expressing a kinase dead mutant of Tor1 (FLAG-Tor1 D2137A), phosphorylation of FLAG-Tor1 D2137A was abolished (Figure 15. A). Ectopic expression of Tor1 in *FLAG:tor1 D2137A* cells rescued phosphorylation of Gad8 but did not rescue phosphorylation of FLAG-Tor1D2137A, indicating that Tor1 is phosphorylated independently of activity of Gad8 (Figure 15. B). These results suggest that Tor1 phosphorylates itself. Correlating with phosphorylation of Gad8, phosphorylation of Tor1 is reduced upon glucose starvation (Figure 15. A and B). In addition, expression of Ryh1Q70L increased phosphorylation of Tor1 and this phosphorylation remained in the absence of glucose (Figure 15. D and E). On the contrary, *Δryh1* cells or cells expressing Ryh1T25N reduced phosphorylation of Tor1 regardless of glucose (Figure 15. D and F). From these results, we proposed that phosphorylation of Tor1 reflects catalytic activity of Tor1 *in vivo*.

To test whether subunits of TORC2 are required for phosphorylation of Tor1 or not, phosphorylation of Tor1 was tested in *Δste20*, *Δsin1*, *Δbit61*, and *Δgad8* cells. Like *Δryh1* or *ryh1T25N*, phosphorylation of Tor1 was reduced in *Δbit61* cells (Figure 15. C). This is

consistent with previous result that *ryh1* and *bit61* are epistatic (Tatebe and Shiozaki, 2010). In addition, like *Δbit61* cells, phosphorylation of Tor1 is diminished in *Δste20* cells. This is consistent with previous reports that Ste20 is required for Bit61 to be incorporated into TORC2 (Tatebe and Shiozaki, 2010). It is also important to note here that Tor1 was phosphorylated in *Δsin1* cells (Figure 15. C). It is possible that Sin1 contributes less to the catalytic activity of Tor1, although *Δsin1* cells completely diminish phosphorylation of Gad8 by TORC2. This is consistent with the previous findings that a function of Sin1 is to recruit Gad8 toward TOR kinase and Tor1 still has catalytic activity in *Δsin1* cells (see chapter I). In *Δgad8* cells, Tor1 was phosphorylated (Figure 15. C), supporting our notion that Tor1 auto-phosphorylates itself (Figure 15. B).

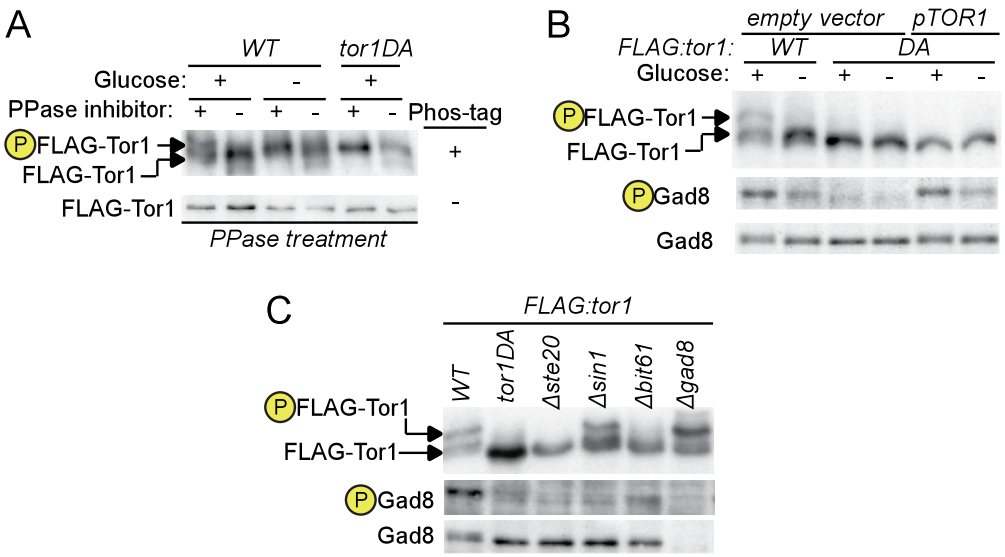

Figure 15. Ryh1 regulates phosphorylation of Tor1.

(A) FLAG-Tor1 is phosphorylated and this phosphorylation was dependent on kinase activity of Tor1.

Cells expressing FLAG-Tor1 ("*WT*") and FLAG-Tor1D2137A ("*tor1DA*") were cultured in EMM media and starved of glucose for 10 minutes (indicated by "-"). The sample indicated by "+" was harvested just prior to glucose starvation. Phosphatase treatments were performed in the presence or absence of phosphatase inhibitors. The mobility shift of FLAG-Tor1 was analyzed by immunoblotting following to SDS-PAGE with phosphate-affinity gel.

(B) Phosphorylation of FLAG-Tor1D2137A was not recovered by simultaneous expression of functional Tor1.

Cells expressing FLAG-Tor1 ("*WT*") and FLAG-Tor1D2137A ("*DA*") with empty or pSP-*tor1* ("*pTOR1*") plasmids were cultured in EMM media and starved of glucose for 10 minutes (indicated by "-"). The sample indicated by "+" was harvested just prior to glucose starvation. Phosphorylated Tor1 was analyzed as described in (A). Phosphorylation of Gad8 and Gad8 were analyzed as described in Figure 12. B.

(C) Phosphorylation of Tor1 is dependent on Ste20 and Bit61 but not on Sin1 or Gad8.

Cells expressing FLAG-Tor1 in the background of wild-type ("*WT*"), *tor1D2137A* ("*tor1DA*"), *Δste20*, *Δsin1*, *Δbit61*, and *Δgad8* were cultured and phosphorylation of FLAG-Tor1 was assessed as shown in (A). Phosphorylation of Gad8 was analyzed as described in Figure 12. B.

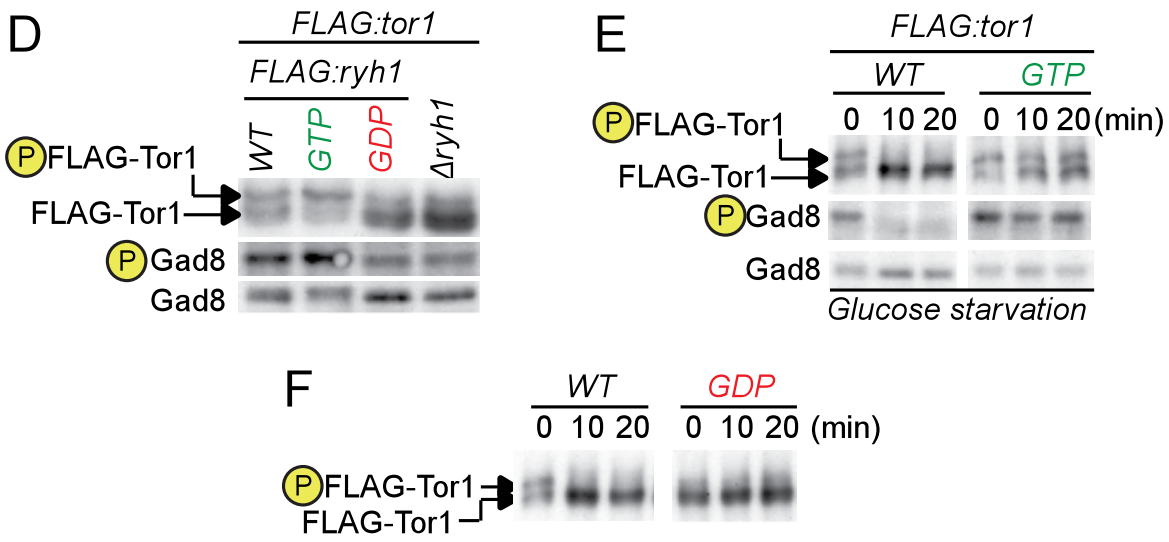

Figure 15. Ryh1 regulates phosphorylation of Tor1.

(D) Ryh1 regulated auto-phosphorylation of Tor1.

Cells expressing FLAG-Tor1 in the background of FLAG-Ryh1 (*WT*), FLAG-Ryh1Q70L ("*GTP*"), FLAG-Ryh1T25N ("*GDP*"), and *Δryh1* were cultured and FLAG-Tor1, phosphorylated Gad8, and Gad8 were detected by immunoblotting.

(E and F) Tor1 auto-phosphorylation was correlated with TORC2 dependent phosphorylation of Gad8.

Cells expressing FLAG-Tor1 in the background of FLAG-Ryh1 ("*WT*"), FLAG-Ryh1Q70L ("*GTP*"), or FLAG-Ryh1T25N ("*GDP*") were cultured and starved on glucose for indicated terms. FLAG-Tor1, phosphorylated Gad8, and Gad8 were detected by immunoblotting.

DISCUSSION II

Before this study, we have limited knowledge about what stimuli regulate the TORC2-Gad8 pathway in fission yeast. Our laboratory found that loss of function of the TORC2 signaling impairs cell growth in culture media containing low concentration of glucose (Figure 11. A), and phosphorylation of Gad8 by TORC2 responds to acute glucose deprivation (Figure 11. B). In fission yeast, it is already reported that, when glucose is completely eliminated from culture media (0 percent glucose), intracellular ATP concentration is immediately reduced below 50 percent in few minutes and exhausted within 2 hours (Pluskal et al., 2011). In contrast, when cells were transferred into media containing 0.02 percent of glucose, intracellular ATP concentration is remained over 1 hour (Pluskal et al., 2011). Phosphorylation of Gad8 is reduced in media containing both 0.02 and 0 percent glucose, indicating that activity of the TORC2-Gad8 pathway and intracellular ATP level are not correlated. From these results, we conclude that the TORC2-Gad8 pathway responds to glucose (or its derivatives) rather than intracellular ATP level.

We found that the PKA pathway that senses extracellular glucose is dispensable for phosphorylation of Gad8. In addition, we also found that the SAPK pathway, which is activated upon glucose starvation, is dispensable for response of the TORC2-Gad8 pathway to glucose. Instead, we demonstrated that Ryh1 is responsible for activating TORC2 upon glucose. However, inconsistent with our findings, Cohen et al. reported that the PKA pathway acts as an activator of the TORC2-Gad8 pathway (Cohen et al., 2014). We think that they obtained such inconsistent results due to their inappropriate experimental condition. They monitored phosphorylation of Gad8 following immunoprecipitation without any phosphatase inhibitors, although the intracellular phosphatase activity may be significantly different among the mutant they tested. We suspect that phosphorylation of Gad8 was altered during immunoprecipitation in their experiments.

Since Ryh1 is a major activator of TORC2, we are now interested in regulatory mechanisms of Ryh1 in response to glucose. Small GTPases are activated or inactivated by GEF (Guanine nucleotide Exchange Factor) or GAP (GTPase Activating Protein). Although a heterodimer composed of Ric1 and Rgp1 proteins was identified as a GEF for Ryh1, its GAP has not been indentified(Ma et al., 2010; Tatebe et al., 2010). Since in budding yeast, the Gyp6 protein was identified as a GAP for Ypt6, a homologue Ryh1, it is possible that fission yeast homologue of Gyp6 acts as a GAP for Ryh1 (Strom et al., 1993). Since Ryh1 is activated in the presence of glucose and immediately inactivated upon starvation of glucose, it

is possible that the GEF or GAP regulates Ryh1 in response to glucose. Further studies are needed to understand how glucose activates Ryh1.

How does Ryh1 regulate phosphorylation of Gad8 by TORC2? Previously our laboratory found that over expression of Ryh1Q70L increases interaction between TORC2 subunit Sin1 and Gad8 (Tatebe et al., 2010). In addition, our laboratory also found that Ryh1Q70L binds to TORC2 more stably than Ryh1T25N or WT (Tatebe et al., 2010). The increased affinity of Ryh1Q70L toward TORC2 is cancelled by the mutation in the effecter domain of Ryh1, suggesting that TORC2 is an effecter of Ryh1 (Tatebe et al., 2010). As mentioned in CHAPTER I, Sin1 acts as a substrate recruitment subunit in TORC2. Therefore, it is possible that Ryh1 promotes interaction between Sin1 and Gad8 (Tatebe et al., 2010). In addition, we found that Ryh1 regulates phosphorylation of Tor1 in response to glucose. Since phosphorylation of Tor1 depends on its kinase activity, we propose that Tor1 auto-phosphorylates itself and it is possible that phosphorylation of Tor1 reflects *in vivo* activity of TORC2. As phosphorylation of Tor1 is increased in cells expressing GTP-bound Ryh1, it is possible that Ryh1 promotes catalytic activity of TORC2. Now we hypothesize that in the presence of glucose, Ryh1 activates the TORC2-Gad8 pathway both by promoting recruitment of Gad8 toward TORC2 via Sin1 and by stimulating catalytic activity of Tor1. Since Bit61 is required for Ryh1 to activate TORC2, it is possible that Bit61 activate TORC2 by following two independent mechanisms. (1) Bit61 increases interaction between Sin1 and Gad8 to promote recruitment of Gad8 to TORC2. (2) Bit61 promotes catalytic activity of Tor1. As Ryh1 and Bit61 are evolutionally conserved among eukaryotes, we expect that further studies of Bit61 enable us to understand universal mechanisms of regulation of TORC2 in eukaryotes.

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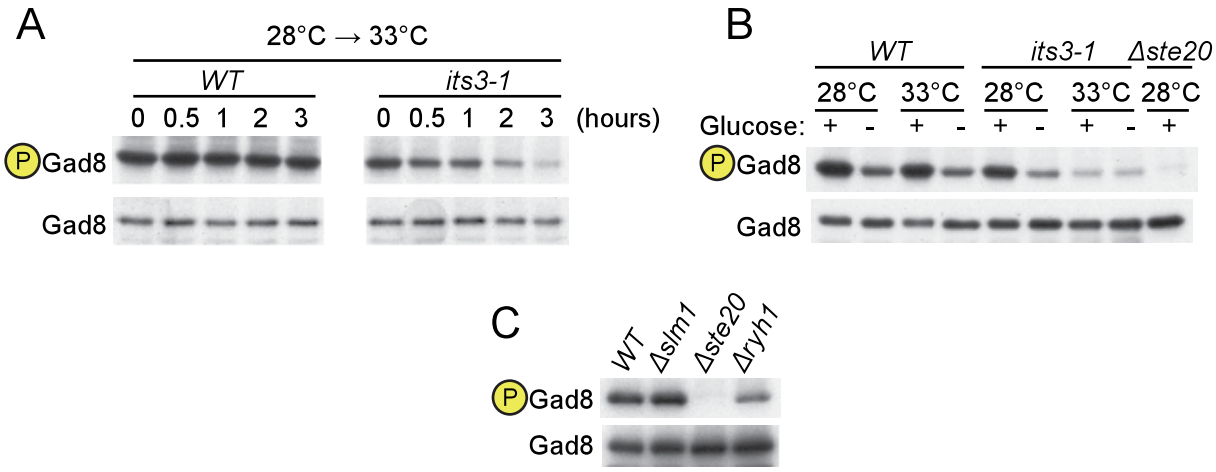
APPENDIX

PtdIns(4,5)P is required for glucose response of TORC2-Gad8 pathway.

TORC2 is thought to localize on the plasma membrane and membrane lipids are implicated into the regulation of TORC2 signaling pathway by following observations. (1) In mammals, AKT, a substrate of TORC2, binds to phosphatidylinositol 3, 4, 5-trisphosphate [PtdIns(3,4,5)P] via its N-terminal PH domain (Najafov et al., 2012) and this interaction promotes TORC2 dependent phosphorylation of AKT. (2) In budding yeast, Ypk1 and Ypk2, counter parts of Gad8, bind to Slm1 or Slm2 proteins that contain the PH domain. The PH domains in Slm1/2 are known to bind to phosphatidylinositol 4, 5-bisphosphate [PtdIns(l4,5)P], which mainly distributes on the plasma membrane and Ypk1/2 are enriched on the membrane, where TORC2 is located (Berchtold et al., 2012; Berchtold and Walther, 2009; Fadri et al., 2005; Niles et al., 2012; Niles and Powers, 2012). This Slm dependent recruitment of Ypk1/2 toward TORC2 is essential for the TORC2-Ypk signaling, therefore, functional connection between TORC2 signaling and PtdIns(4,5)P are predicted in this organism (Niles et al., 2012; Niles and Powers, 2012). To understand the upstream signaling of TORC2 in fission yeast, I focused on *its3* gene, which encodes PtdIns(4,5)P generator. Its3 is lipid kinase, which phosphorylate phosphatidylinositol 4-phosphate [PtdIns(4)P] to produce PtdIns(4,5)P (Zhang et al., 2000). Because *its3* is essential for the viability of fission yeast cells, temperature sensitive mutant of this gene (*its3-1*) was used in this study. Interestingly, at the restricted temperature, Gad8 is less phosphorylated in *its3-1* mutant cells and the phosphorylation was insensitive to glucose (Figure 15. A and B). This results indicate that PtdIns(4,5)P is required for the glucose dependent activation of TORC2. Importantly although *slm1 or slm2*-like gene was also found in fission yeast genome, disruption of the gene did not affect TORC2 dependent phosphorylation on Gad8, indicating that unknown mechanism which links PtdIns(4,5)P and TORC2-Gad8 pathway are exist in this organism (Figure 15. C).



PtdIns(4,5)P is required for glucose response of TORC2-Gad8 pathway.

(A) *its3-1*, a temperature sensitive mutant, showed reduced phosphorylation of Gad8 at restricted temperature.

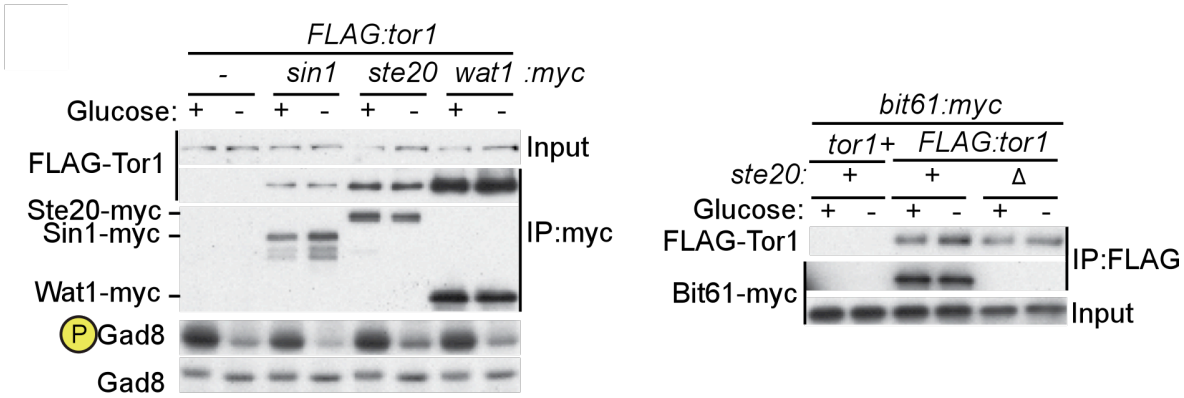
Wild-type ("*WT*") and *its3-1* mutant were cultured at 28 ˚C and then they are incubated at 33 ˚C for indicated times.

(B) Glucose response of TORC2-Gad8 pathway was diminished by inhibition of *its3* gene.

Strains in (A) were cultured at 28˚C and then shifted the temperature to 33 ˚C for 4 hours. Glucose starvation was performed before or after the temperature shift and phosphorylated Gad8 level were monitored.

(C) *slm1* was dispensable for the TORC2 dependent phosphorylation of Gad8.

Wild-type, *Δslm1*, *Δste20*, and *Δryh1* cells were cultured and phosphorylated Gad8 were monitored.



TORC2 components were stably associated regardless of glucose.

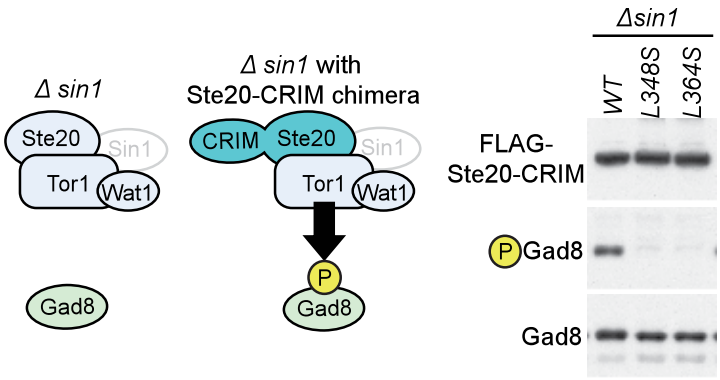
(Right panel)

Cells expressing FLAG-Tor1 with C-terminal myc tagged TORC2 components Sin1, Ste20, and Wat1 were cultured in YES medium and harvested pre- or post-starvation (5 minutes) on glucose. Co-immunoprecipitation was performed with anti-myc antibody and precipitants and inputs were detected with antibody against FLAG. *FLAG:tor1* cell was used as a negative control of the anti-myc immunoprecipitation.

For the phospho-Gad8 and Gad8 blots, cells were quenched before preparing crude cell lysate (see MATERIALS AND METHODS II).

(Left panel)

bit61:myc with *tor1+* , *FLAG:tor1* , and *FLAG:tor1 Δste20* cells were cultured in YES medium and starved on glucose for 5 minutes. Co-immunoprecipitation was performed with anti-FLAG antibody and precipitants were detected with antibody against *myc* tag.



The requirement of Sin1 was bypassed by covalently anchoring of the CRIM domain onto TORC2.

The Ste20-CRIM chimera protein was expressed in the background of *Δsin1*. As negative control, the fusion protein possessing *L348S* or *L364S* mutations were used. Immunoblotting using anti-phospho-S546 Gad8, Gad8, or FLAG anti bodies were performed.