TOR complex 2 in fission yeast is required for chromatin-mediated gene silencing and assembly of heterochromatic domains at subtelomeres

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Adiel Cohen1, Aline Habib2, Dana Laor5, Sudhanshu Yadav3,1, Martin Kupiec5, and Ronit Weisman1,2

From the 1Department of Natural and Life Sciences, Open University of Israel, University Road 1, 4353701 Ranana, Israel and the 2Department of Molecular Microbiology and Biotechnology, Tel Aviv University, Ramat Aviv 6997801, Tel Aviv, Israel

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The conserved serine/threonine protein kinase target of rapamycin (TOR) is a major regulator of eukaryotic cellular and organismal growth and a valuable target for drug therapy. TOR forms the core of two evolutionarily conserved complexes, TOR complex 1 (TORC1) and TORC2. In the fission yeast Schizosaccharomyces pombe, TORC2 responds to glucose levels and, by activating the protein kinase Gad8 (an orthologue of human AKT), is required for well-regulated cell cycle progression, starvation responses, and cell survival. Here, we report that TORC2–Gad8 is also required for gene silencing and the formation of the heterochromatin at the S. pombe mating-type locus and at subtelomeric regions. Deletion of TORC2–Gad8 resulted in loss of the heterochromatin modification of histone 3 lysine 9 dimethylation (H3K9me2) and an increase in euchromatic modifications, including histone 3 lysine 4 trimethylation (H3K4me3) and histone 4 lysine 16 acetylation (H4K16Ac). Accumulation of RNA polymerase II (Pol II) at subtelomeric genes in TORC2–Gad8 mutant cells indicated a defect in silencing at the transcriptional level. Moreover, a concurrent decrease in histone 4 lysine 20 dimethylation (H4K20me2) suggested elevated histone turnover. Loss of gene silencing in cells lacking TORC2–Gad8 is partially suppressed by loss of the anti-silencer Epe1 and fully suppressed by loss of the Pol II–associated Paf1 complex, two chromatin regulators that have been implicated in heterochromatin stability and spreading. Taken together, our findings suggest that TORC2–Gad8 signaling contributes to epigenetic stability at subtelomeric regions and the mating-type locus in S. pombe.

The target of rapamycin (TOR)3 pathway is a central regulator of metabolic pathways, cell growth, and proliferation in response to nutritional and stress signals, and its deregulation is implicated in cancer, diabetes, neurodegenerative disease, and aging (1, 2). TOR is a serine/threonine protein kinase that is found in two structurally and functionally distinct complexes, TORC1 and TORC2. In mammalian cells, a single catalytic subunit, mTOR, is present in both TORC1 and TORC2 (mTORC1 and mTORC2). Many features of the two TOR complexes are conserved in evolution, and a substantial amount of research has suggested multiple conserved pathways by which TOR proteins control cellular growth, including regulation of transcription, translation, enzymatic metabolism, and autophagy (3, 4). TORC1 is inhibited by rapamycin and its derivatives and is well-known for its conserved role as a positive regulator of cellular growth and a negative regulator of starvation responses. TORC2 has been implicated in growth, metabolism, and survival; however, our understanding of TORC2 lags behind that of TORC1. This is partly due to the lack of TORC2-specific inhibitors, a disadvantage that is mitigated in genetically tractable organisms such as yeasts (5, 6).

Schizosaccharomyces pombe contains two TOR homologues, Tor1 and Tor2, which were numbered based on the order of their discovery (7). Tor2 interacts with the Raptor-like subunit, Mip1, to form TORC1, whereas Tor1 interacts with the Rictor-like subunit Ste20 and with Sin1 (mSin1 in humans) to form TORC2 (8, 9). S. pombe TORC1 (SpTORC1) promotes cellular growth in response to nitrogen and amino acid availability while inhibiting starvation responses (8, 10, 11). SpTORC2 is critical for several responses to starvation, including entrance into the sexual development pathway or stationary (quiescence) phase and for survival under osmotic or oxidative stress (7, 12), DNA-damaging, or DNA replication stress conditions (13, 14). More recently, it was demonstrated that SpTORC2 controls the sexual development pathway in coordination with SpTORC1 via a cross-talk mechanism that involves the PP2A-B55 phosphatase (15) and the SAGA (Spt–Ada–Gcn5 acetyltransferase) co-activation transcription complex (16). Under normal growth conditions, SpTORC2 plays a role in regulating entrance into mitosis (13, 17, 18), cytokinesis (19), and nutrient assimilation (11, 20). SpTORC2 mediates its known functions via phosphorylation and activation of the Gad8 kinase, the homologue of mammalian AKT or SGK1 kinases that lie down-

1 Present address: Dept. of Immunology and Oncology, National Biotechnology Centre, CSIC Darwin 3 Cantoblanco, Madrid 28049, Spain.
2 To whom correspondence should be addressed. Tel.: 972-9-7782188; Fax: 972-778278; E-mail: ronitwe@openu.ac.il.
3 The abbreviations used are: TOR, target of rapamycin; mTOR, mechanical target of rapamycin; TORC1 and -2, TOR complex 1 and 2, respectively; Paf1C, Paf1 complex; MBF, MluI cell cycle box–binding factor; H3 and H4, histone H3 and H4, respectively; K4, K9, K16, and K20, lysine 4, 9, 16, and 20, respectively; me, me2, and me3, methyl, dimethylation, and trimethylation, respectively; HP1, heterochromatin protein 1; kb, kilobase(s); Pol, polymerase; qRT-PCR, quantitative RT-PCR.
stream of mTORC2 (21, 22). Unlike SpTORC1, glucose, but not nitrogen, is required for activation of SpTORC2–Gad8 (23, 24).

Transcriptional profile analyses of cells lacking the catalytic subunit of SpTORC2 (Δtor1) revealed an extensive similarity with the transcriptomes of chromatin structure or function mutant cells, such as similarity with mutants of histone deacetylases (clr6-1 or Δclr3 clr6-1) or mutants of the SWI/SNF chromatin-remodeling complexes RSC (remodeling the structure of chromatin) (Δrsc58) (13). This finding led us to discover several defects in nuclear functions in TORC2–Gad8 mutant cells, including elongated telomeres, increased chromosomal loss, and elevated levels of Rad52 foci (13). Moreover, Tor1 and Gad8 are found in the nucleus in association with the chromatin fraction, physically interact with the Mlul cell cycle binding factor (MBF) transcription complex, and affect MBF-dependent gene transcription (25). These findings suggest the possibility that TORC2–Gad8 has direct effects on nuclear and chromatin functions.

Regulation of chromatin states is an efficient way to rapidly and reversibly control cellular growth in response to changing environmental conditions. Chromatin states primarily consist of silent and compact heterochromatic regions and less condensed, active euchromatic regions. Reversible transition between heterochromatic and euchromatic states is characteristic of facultative heterochromatin, which provides regulatory mechanisms for gene expression (26, 27). The dynamics between heterochromatic and euchromatic states plays an important role during early development and stem cell differentiation in higher eukaryotes (28), and its regulation is crucial for epigenetic stability, the process that ensures that the epigenetic state is faithfully transferred and maintained in daughter cells.

In S. pombe, constitutive heterochromatin is formed at highly repetitive sequences residing at the centromeres, telomeres, and the silent mating-type locus and is characterized by H3K9 methylation, hypo-acetylation, and low histone turnover (27). Facultative heterochromatin, enriched in stress-regulated and meiotic genes, is found at small blocks of heterochromatic islands dispersed along the chromosomes (29, 30). The establishment of heterochromatin at centromeres and repetitive elements is primarily regulated by the RNAi machinery that includes the argonaute, dicer, and RNA-dependent RNA polymerase (23, 24). The RNAi machinery recruits the Clr4–Rik1–Cul4 complex containing the histone methyl transferase Clr4 (Suvar9h in humans) (33). Clr4 is the sole H3K9 methylase responsible for di- and tri-H3K9 methylation (34, 35), and its activity provides a docking site for chromodomains proteins, including Swi6 (heterochromatin protein 1, HP1 in humans), Chp1, Chp2, and Clr4 itself (36). Transcription factors (37) and RNA processing and surveillance mechanisms (38, 39) also contribute to heterochromatin formation.

The spreading of the chromatin is restricted by several mechanisms. At the centromeres and mating-type locus, heterochromatin is confined by sequence-specific boundaries, such as the IRC and IR elements, respectively (26, 29). These boundaries are responsible for a sharp decrease of the H3K9me signature outside the heterochromatic region. At the telomeres, the borders between the heterochromatin and the euchromatin are not well-defined, and the H3K9me2 signature gradually declines as the heterochromatin spreads away from the telomeric repeats (29). The Jun monji C protein Epe1, which acts to antagonize histone modifications and functions.

### Results

**TORC2–Gad8 is required for silencing of a reporter gene embedded in the silent mating-type locus**

The mating-type region in S. pombe contains the mat1, mat2, and mat3 genes. The mat1+ gene is transcriptionally active and determines the mating type, whereas mat2+ and mat3+ lie within the constitutive heterochromatin, flanked by the two inverted IR boundary elements. The cenH element, which lies in between the two silent mating-type cassettes, acts as a heterochromatin nucleation center (see schematic presentation in Fig. 1A). We previously showed that loss of the catalytic subunit of TORC2 (Δtor1) results in de-silencing of the
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Figure 1. The TORC2–Gad8 pathway promotes silencing at the mating-type region. A, schematic representation of the mating-type locus in *S. pombe*. The mating-type region consists of three genes: *mat1*, *mat2*, and *mat3*. *mat1* is transcriptionally active and determines the mating type. *mat2-P* and *mat3-M* reside at the heterochromatic region shown within the gray box. This region is flanked by the two inverted repeats boundary elements IR-L and IR-R. The *ade6* reporter genes inserted at the SacI or BamHI sites are indicated. B, mutations in TORC2–Gad8 result in de-silencing at the mating-type locus. WT strain (*ade6*) and strains containing *ade6-M210* in addition to the indicated mutations, with or without the *mat2:BamHI::ade6* reporter gene, were spotted onto rich medium plates, supplemented with a standard amount of adenine or low adenine (*YE+ Ade*, *YE− Ade*) or minimal medium plates containing a standard amount of adenine or no adenine (*ADE+, ADE−*). White colonies growing on *ADE−* plates indicate de-silencing of the *ade6* gene. C, expression levels of the *ade6* gene inserted at the mating locus (*mat2:BamHI::ade6*); qRT-PCR analysis. The level of *act1* mRNA was used as a reference. Each value is the mean of at least three independent assays. D, stability of ectopic heterochromatin in mitotic cells. Five independent colonies of each strain were isolated on nondiverse medium (*YE+ Ade*) and replated on differential medium (*YE− Ade*), and their color was scored. N, number of scored colonies. E, hereditability of the repressed or active chromatin state in *Δtor1* cells. Five independent red or white *Δtor1* colonies were isolated on limiting adenine (*YE− Ade*) plates and replated on *YE-Ade* plates, and their color was scored. N, number of scored colonies.

*ade6* reporter gene inserted at the SacI site (13) that is located at the left IR boundary, IR-L (Fig. 1A). The *mat2::SacI::ade6* reporter gene is only weakly silenced in WT cells (40); thus, the de-silencing observed in *Δtor1* cells is not striking (13). Here, we employed the *mat2::BamHI::ade6* reporter gene that resides within the *mat2* gene (40). This reporter gene is completely silenced in WT cells, resulting in cells that cannot grow in medium lacking adenine and accumulate a red pigment under adenine-limited conditions (Fig. 1B). Remarkably, mutant cells carrying deletion mutations in TORC2–Gad8 (*Δtor1, Δsin1, Δste20, or Δgad8*) carrying *mat2::BamHI::ade6* grew on minimal medium lacking adenine, and they produced white colonies under limiting adenine conditions (Fig. 1B). Consistently, quantitative RT-PCR (qRT-PCR) analysis in *Δtor1* cells revealed a strong expression of the *ade6* transcript (~120 fold increase compared with WT), similar to the de-repression observed in *Δclr4* cells, lacking the sole H3K9 methyl transferase in *S. pombe* (34) (Fig. 1C). Similar de-repression of the *ade6* gene is observed in *Δgad8* mutant cells (Fig. S1A).

The repression of a euchromatic gene upon its insertion into a heterochromatic region is a universally conserved epigenetic phenomenon termed position effect variegation (PEV), which has been used to identify key regulators of heterochromatin-induced silencing (reviewed in Ref. 27). However, the sensitivity to silencing of the reporter gene is not identical to that of the endogenous mating-type cassettes. Thus, for example, disruption of *clr4* results in de-silencing of reporter genes at the mating-type locus but has little or no effect on the expression level of the silent *mat2* gene (52). Similarly, the *Δtor1* or *Δgad8* mutations had only a very limited effect on the expression of the endogenous *mat2* gene (Fig. S1B). Thus, TORC2–Gad8, similar to *Clr4*, strongly affects the spreading of heterochromatin across the euchromatic reporter gene but is incapable of overriding the heterochromatin-dependent silencing at the endogenous mating-type gene. This probably reflects the existence of redundant repressive mechanisms at the endogenous locus.

Instability of chromatin states in TORC2–Gad8 mutant cells

We noted that *Δtor1 mat2::BamHI::ade6* cells that grew under rich, nondefined media (*YE+ Ade* plates) produced a majority of white colonies but could also give rise to variegated/pink (35%) or red (10%) colonies under limiting adenine conditions (Fig. 1D). In comparison, *Δclr4* (lacking the H3K9 meth-
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TORC2–Gad8 is required for gene silencing at the subtelomeric region

We previously showed an extensive similarity with the transcriptional profiles of ΔtorI cells and the profiles of histone deacetylase mutant cells (Dclr3 or clr6-1) and nuclease-remodeling complex mutant cells (Dsrc8) but not RNAi mutant cells (such as Δdcr1 or Δago1) (13). Here, we have extended this analysis by performing RNA-Seq of ΔtorI and Δgad8 cells (Table S3). In agreement with the activation of Gad8 by Tor1, there is a significant overlap between the expression profiles of ΔtorI or Δgad8 mutant cells (Fig. 2A). The RNA-Seq analysis recapitulated several of our previous results for gene expression in ΔtorI cells (13). For example, the majority of the genes that are abnormally expressed are up-regulated (154 and 184 genes in ΔtorI and Δgad8, respectively), whereas a minority of the genes are down-regulated (53 and 24 genes in ΔtorI and Δgad8, respectively). As reported previously for ΔtorI cells (13), among the genes that are down-regulated, there is an enrichment for membrane transporters (Table S3). No enrichment for specific functional groups is detected within the abnormally up-regulated genes. However, when plotted along the chromosomes, we noted that genes located close to the telomeres of chromosomes I and II are up-regulated in ΔtorI or Δgad8 mutants (Fig. 2B). In contrast, the ΔtorI or Δgad8 mutations do not affect genes at the ends of chromosome III that contain ribosomal DNA repeats. The subtelomic region of chromosomes I and II consists of 20–40 kb of heterochromatin that is characterized by a gradual decline in H3K9me2 at the telomere-distal end (29). We also noted two clusters of up-regulated genes in ΔtorI or Δgad8 cells that reside in the middle of chromosomal arms I and II (see blue arrows in Fig. 2B). These clusters are enriched in noncoding RNA transcripts (Fig. S2), but no enrichment in H3K9me2 has been reported in these regions or was detected in our ChIP-Seq analysis (described below).

Using qRT-PCR with specific primers, we confirmed that the subtelomeric genes spac186.045+, spac186.055+, spac186.065+, spac750.015+ (chromosome I, right arm), spac977.025+ (chromosome I, left arm), spbc1348.035+ (chromosome II, left arm), and spbpb2b2.185+ (chromosome II, right arm) are up-regulated in cells carrying mutations in ΔtorI or Δgad8 or in Δryh1 cells, lacking the small Ryh1 GTPase that activates TORC2 (53) (Fig. 3, A and B). All of these subtelomeric genes are located at the regions characterized by intermediate levels of H3K9me2 (see schematic presentation in Fig. S3 and Refs. 29 and 54). Unlike Δclr4 cells, mutations in ΔtorI or Δgad8 did not result in de-silencing of tih1+/tih2+, the most telomere-proximal genes located at the high-level H3K9me2 subtelomeric region (Fig. 3C). The tih1+/tih2+ genes encode RecQ-like helicases and contain repeat elements in their coding regions, similar to the repeats at the centI element at the mating-type locus or the dg/dlh centromeric repeats (26). The de-silencing of subtelomeric genes but not tih1+/tih2+ is similar to the differential effect observed for cohesin mutant cells, the cohesin loader Mis4, or the cohesin core component Rad21 (54). Thus, similar to mutations in cohesin, TORC2 affects gene expression only at the subtelomeric region that is characterized by an intermediate level of H3K9me2.

Disruption of TORC2–Gad8 did not affect the level of expression of centromeric dg repeats (Fig. 3B) or the silencing of reporter genes inserted into the centromeric innermost repeats (imr1L) or outermost repeats (otr1L) (Fig. S4). Thus, our data indicate that TORC2–Gad8 is not required for silencing at the centromeric constitutive heterochromatin. We also found no effects of ΔtorI or Δgad8 cells on the expression levels of facultative heterochromatin islands encoding meiotic genes (our RNA-Seq analysis; see qRT-PCR for mei4+ in Fig. 3B), ruling out the involvement of complexes that are dedicated to meiotic mRNA decay, such as the Erh1-Mmi (EMC) and Mti1–Red1 core (MTREC) complexes (55).

Loss of TORC2–Gad8 is accompanied by loss of heterochromatic markers at subtelomeric regions

H3K9me2 is a key histone modification that characterizes heterochromatic regions. As our RNA-Seq experiments suggest that TORC2–Gad8 signaling has a global effect on gene expression that is associated with chromosomal location, we investigated the effects of ΔtorI or Δgad8 on genome-wide H3K9me2 levels by ChIP-Seq analysis. We found that the strongest effect of the ΔtorI or Δgad8 mutations was at the subtelomeric region of the right telomere of chromosome 1
where a 33-kb region that resides 30 kb away from the telomeric repeats became almost completely devoid of H3K9me2 in the mutant cells (Fig. 4). Using ChIP–qRT-PCR analyses, we confirmed that the level of H3K9me2 is reduced at the subtelomeric genes that reside within the 33-kb region of tel1R but is unchanged for the centromeric dg repeats (Fig. 4B). The subtelomeric genes that reside at the other chromosomal ends and exhibited elevated levels of transcription in /H9004 tor1 mutant cells (Fig. 3B) also showed a significant reduction in H3K9me2 levels (Fig. 4B). The exception was the most tel1R-proximal gene, spac750.01”, which retained Swi6 binding in Δtor1 cells.

The level of Swi6 binding at the dg repeats was not affected by Δtor1 (Fig. 4C), consistent with the lack of effect on H3K9me2 or transcription at the centromeric repeats.

Loss of TORC2–Gad8 elevates chromatin modifications associated with transcriptional active chromatin

We next examined changes in trimethylation of H3K4 (H3K4me3), a modification that is associated with transcriptionally active chromatin (56). We found an increase in H3K4me3 in all subtelomeric genes examined in Δtor1 mutant cells (Fig. 5A). The histone methyltransferase Set1, a component of the set1C–COMPSS complex, is the sole methyltransferase responsible for mono-, di-, or tri-methylation of H3K4 in S. pombe (57). Although Set1 is responsible for a histone modification that is associated with active euchromatin, Set1 acts as a transcriptional repressor at many chromosomal
loci, including the subtelomeric and mating-type regions (57). Indeed, we found that deletion of set1+/H11001 resulted in up-regulation of subtelomeric genes, very similar to that observed in /H9004tor1 cells (Fig. S5A). The double mutant /H9004tor1/set1+ cells showed no enhancement of the up-regulation of gene expression at the subtelomeric genes (Fig. S5A), suggesting that Tor1 and the repressive function of Set1 may lie on the same pathway. Because we observed an increase in the level of H3K4me3 in /H9004tor1 cells, it is unlikely that the elevation in H3K4me3 is responsible for the increase in gene transcription in /H9004tor1 cells. Rather, the increase in H3K4me3 is the result of elevated levels of transcription in this region.

The reduction in heterochromatic markers and the increase in euchromatic markers at the subtelomeric genes in /H9004tor1 mutant cells suggest a higher level of gene transcription. We therefore investigated the accessibility of RNA Pol II to the subtelomeric regions in WT compared with mutant cells. As RNA Pol II moves from the initiation site, the Rbp1 C-terminal domain becomes phosphorylated at Ser-2. We observed an accumulation of the elongating form of RNA Pol II at the subtelomeric genes, consistent with elevated transcription at this region (Figs. 3B and 5B). There is no change in RNA Pol II occupancy at the dg repeats, consistent with the lack of effect on transcription at this site (Figs. 3B and 5B). We also detected an increase in transcription-associated H4K16 acetylation (H4K16Ac) at the subtelomeric genes in /H9004tor1 cells (Fig. 5C). The H4K20me2 modification is carried out by Set9 and can be used as a marker for “old” histones, due to the progressive nature of Set9 and the absence of an H4K20me2 demethylase (58). We found a decrease of H4K20me2 at the subtelomeric genes, which suggests an increase in histone turnover (Fig. 5D).

Figure 3. TORC2–Gad8 is required for silencing at a specialized subtelomeric region but not for silencing of centromeric repeats or meiotic genes. A, schematic presentations of chromosomes I and II. The triangles on the chromosomal edges are magnifications of the subtelomeric regions. Dark and light gray boxes indicate high- or low-H3K9me2 regions, respectively. B, expression levels of subtelomeric genes on chromosome I (ChI) or chromosome II (ChII), /H11001, and the centromeric dg repeat were examined in WT or cells carrying mutations in /H9004tor1, /H9004gad8, /H9004ste20, or /H9004ryh1 cells. The numbers in parenthesis indicate the distance of each subtelomeric gene from the nearby telomeric repeats. Expression levels were determined by qRT-PCR. The level of act1+/H11001 mRNA was used as a reference. Each value is the mean of at least three independent assays, and the error bars indicate S.D. C, the levels of the tlh1+/tlh2+ genes that are located next to the telomeric repeats are unchanged or slightly reduced in TORC2–Gad8 mutant cells.
This increase may be the result of an elevated level of transcription at the subtelomeric region.

Finally, we considered the possibility that TORC2–Gad8 may affect gene silencing via degradation of the RNAs emerging from heterochromatic regions, a process that involves the exosome (38). Deletion of the core subunit of the exosome, rrp6Δ, resulted in de-silencing of the subtelomeric genes, but the effect was generally very different in its strength compared with that of the Δtor1 mutation (Fig. S5B). Moreover, combining the Δtor1 and Δrrp6 mutations resulted in an additive effect on gene de-silencing for three subtelomeric genes (spac186.04Δ, spac186.05Δ, and spac750.01Δ; Fig. S5B), suggesting that Rrp6 and Tor1 act in different pathways.

The Leo1–Paf1 subcomplex and the anti-silencer Epe1 antagonize the silencing activity of TORC2

The unstable chromatin states that we observed for TORC2–Gad8 mutant cells (Fig. 1D) are reminiscent of the phenotypes observed in Δepe1 (40) or mutations in the Leo1–Paf1 subcomplex of Paf1C (49). However, whereas TORC2–Gad8 functions to maintain the repressed state of the chromatin, Epe1 or the Paf1C complex functions to antagonize heterochromatin spreading. Accordingly, overexpression of Epe1 resulted in up-regulation of subtelomeric genes (42), whereas Δleo1 resulted in spreading of the heterochromatin at the subtelomeric regions (48). Therefore, we tested for genetic interactions between TORC2–Gad8 and Epe1 or the Leo1–Paf1 subcomplex. Deletion of epe1Δ partially suppressed the de-silencing defect of Δtor1 cells, as evidenced by the pink color of Δtor1Δepe1 double mutant cells carrying the mat2::BamHI::ade6+ construct (Fig. 6A). In contrast, Δleo1 completely suppressed the de-silencing defect of Δtor1 or Δgad8, resulting in fully red colonies (Fig. 6B). Similarly, qRT-PCR analyses show that the de-silencing defect in Δtor1 mutant cells at the subtelomeric region is partially suppressed by Δepe1 and fully suppressed by Δpaf1 (Fig. 6, C and D).

The partial suppression of the de-silencing defects in TORC2–Gad8 by Δepe1 could be explained if the global levels of Epe1 or the efficiency by which Epe1 is bound to the chromatin is altered in TORC2–Gad8 mutant cells. However, we did not detect changes in the protein level of Epe1-FLAG in

\[ \text{Figure 4. Loss of TORC2–Gad8 leads to a decrease in heterochromatic markers at the subtelomeric region. A, genome browser view showing ChIP-Seq analysis of H3K9me2 levels in WT (green), Δtor1 (red), and Δgad8 (orange) in log2 scale. B, levels of H3K9me2 by ChIP-quantitative PCR at subtelomeric genes. C, levels of Swi6 by ChIP–qRT-PCR at subtelomeric genes. Δclr4 cells, used as control for no Swi6 binding, showed no significant signal. Each value is the mean of at least three independent assays, and the error bars indicate S.D.} \]
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\[ \text{TORC2–Gad8} \]

\[ \Delta \text{tor} \text{1} \text{ or} \Delta \text{gad8} \text{ cells or abnormal accumulation of Epe1-FLAG in} \Delta \text{tor1} \text{ mutant cells at the mating-type locus (data not shown).} \]

\[ \text{TORC2–Gad8 may suppress Leo1–Paf1 activity or act via a distinct pathway that antagonizes Leo1–Paf1 activity. Distinguishing between these two possibilities requires further investigation.} \]

Discussion

The \textit{S. pombe} TORC2 complex is composed of well-conserved subunits: the catalytic subunit Tor1 and the auxiliary subunits Ste20 (Rictor in human) and Sin1 (mSin1 in humans). A highly conserved feature of TORC2 signaling is the phosphorylation and activation of members of the family of AGC kinases (protein A protein G protein C kinases), Gad8 in \textit{S. pombe} and AKT in human cells. Similar to mTORC2-AKT, TORC2–Gad8 is implicated in different aspects of growth and proliferation and plays critical roles in starvation responses, cell survival, and aging.

TOR signaling and the regulation of epigenetic information

TOR-dependent signaling often exerts its effects via transcriptional regulation. In particular, TORC1 was shown to regulate gene transcription via multiple mechanisms, including nuclear localization of transcription factors, the regulation of their activity, and regulation of the activity of RNA polymerases (59). TORC1 has also been implicated in chromatin-mediated transcriptional regulation. In \textit{S. cerevisiae}, TORC1 has been linked to histone H4 acetylation (60, 61) and affects the activity of the sirtuin deacetylases Hst3 and Hst4 (62). Functional links between mTORC1 and SIRT4 have also been reported (63). mTORC1 has also been shown to phosphorylate H2B, thereby mediating histone modifications required for early adipogenesis (64). As in many other aspects, our understanding of the roles of TORC2 in regulating gene expression lags behind that of TORC1. Our studies reveal for the first time that TORC2–Gad8 regulates chromatin-mediated gene expression, epigenetic states, and their stability.

We demonstrate that TORC2–Gad8 is required to silence a reporter gene embedded at the heterochromatic region of the mating-type locus \((\text{mat2::BamHI::ade6}^-\text{})\) and affects heterochromatic spreading and gene silencing at subtelomeres. The use of \(\text{mat2::BamHI::ade6}^-\) as a reporter for chromatin-dependent gene silencing revealed the unstable nature of the epigenetic states in TORC2–Gad8 mutant cells. Accordingly, the epigenetic state of \(\text{mat2::BamHI::ade6}^-\) in TORC2–Gad8 mutant cells can be found either in its expressed or repressed state, whereas WT cells or mutations in major histone modifiers, such as the Crl4 or Sir2, stably maintain the reporter gene in either its expressed or repressed state, respectively. Epigenetic instability is characteristic of mutations in \textit{epe1}, a heterochromatin boundary element or mutations in the RNA Pol II–associated complex, Paf1C (48, 49, 51). However, whereas loss of Epe1 or Paf1C results in an unstable extension of the heterochromatin, the deletion of TORC2–Gad8 results in contraction of the heterochromatin. Loss of function of Epe1 or Leo1–Paf1 suppresses the de-silencing defect in TORC2–Gad8 mutant cells, indicating that these chromatin factors act in an antagonistic manner to TORC2–Gad8.

Roles of TORC2–Gad8 in the formation of specific heterochromatic regions at subtelomeres

Our genome-wide transcription (RNA-Seq) and H3K9me2 (ChIP-Seq) analyses, followed by validations of specific subtelomeric genes, indicate that TORC2–Gad8 has a role in regulating heterochromatic spreading and gene silencing at subtelomeres of chromosomes I and II. \textit{S. pombe} contains three chromosomes, of which chromosomes I and II contain telomeric repeats followed by species-specific homologous DNA
sequences (SH sequences) and a variety of genes encoding for proteins. In contrast, arrays of ribosomal DNA repeats, which are typically transcribed by RNA polymerase I, lie next to the telomeres of chromosome III \((29, 65)\). The up-regulation of genes in \(\Delta tor1\) or \(\Delta gad8\) at the subtelomeric regions of chromosome I and II, but not chromosome III, is in agreement with previous reports that suggest that chromosomes I and II contain a distinct form of heterochromatin state at their edges \((29, 54, 65, 66)\).

The subtelomeric regions of chromosomes I and II can be divided into a telomere-proximal high-level H3K9me2 region and a telomere-distal low level H3K9me2 region \((29, 54)\). We detected elevated levels of gene expression only at the low-level H3K9me2 regions. The high- and low-H3K9me2 regions may serve different purposes. The high-level H3K9me2 regions may provide repressive structures, guarding the chromosomal ends from rearrangements that could result from illegitimate recombination between nearly identical sequences. The low-level H3K9me2 region may provide a way to regulate gene expression via chromatin-mediated mechanisms. Indeed, many genes that reside at the low H3K9me2 region become expressed in response to environmental changes, such as starvation and other stress conditions \((67)\). The seven subtelomeric genes that we monitored by qRT-PCR and ChIP–qRT-PCR in TORC2–Gad8 mutant cells encode for putative plasma membrane and transporter proteins \((\text{spac186.04}, \text{spac186.05}, \text{spbpb2b2.18}^+)\) or metabolic enzymes \((\text{spac186.06}^+, \text{spac750.01}^+)\). According to global gene expression databases, these genes are up-regulated...

**Figure 6.** The silencing defects in \(\Delta tor1\) or \(\Delta gad8\) mutant cells are partially suppressed by disruption of \(\text{epe1}^+\) and fully suppressed by disruption of the Paf1 complex. \(\text{A and B},\) suppression at the mat2 locus. Strains containing the indicated mutations were spotted onto low-adenine plates. WT cells that are prototrophic or auxotrophic to adenine were used as a control. \(\text{C and D},\) suppression at subtelomeres. Expression levels of the indicated genes were determined by qRT-PCR as described in the legend to Fig. 3.
in meiosis and/or in response to oxidative DNA damage or cadmium stress (see the Bähler Expression viewer) (67). The specific effect of TORC2–Gad8 at relatively low methylated H3K9me2 regions is remarkably similar to the effects of mutations in cohesin loader *mis4* or cohesin core component *rad21* mutations (54). Cohesin is recruited to silent chromatin via Swi6 (68, 69) but may also affect Swi6 binding, leading to a cooperative mechanism by which Swi6 levels are maintained at the low-H3K9me2 subtelomeric regions (54). Whether the effects of TORC2–Gad8 and cohesin at subtelomeric region are related remains to be investigated.

**TORC2–Gad8 affects gene silencing at the transcriptional level**

Loss of TORC2–Gad8 results in up-regulation of gene expression at the subtelomeric regions that is accompanied by an increase in histone modifications associated with elevated levels of transcription, including elevated levels of H3K4me3 and H4K16Ac and accumulation of the elongating form of RNA Pol II. Thus, our data suggest that Δtor1 mutant cells are defective in gene silencing at the transcriptional level. In contrast, mutations in the exosome produced an additive effect when combined with the Δtor1 mutation.

We observed an increase in H4K20me2 in subtelomeric genes in TORC2–Gad8 mutant cells, a modification change that indicates an increased level of histone turnover (58). Suppression of histone turnover is characteristic of heterochromatic regions and accompanies histone modifications that result in suppression of transcription (70). Both Epe1 and the Leo1–Paf1 subcomplex were suggested to mediate their effects on chromatin states through histone turnover (49). Because deletion of the Leo1–Paf1 subcomplex completely abolished gene de-silencing in TORC2–Gad8 mutant cells, it is possible that TORC2–Gad8 is also involved in the regulation of histone turnover. Alternatively, the elevated levels of transcription in TORC2–Gad8 mutant cells bring about an increase in histone turnover. These two possibilities are not mutually exclusive, as histone turnover and elevated levels of transcription are two closely related processes. Interestingly, phosphosite mutations of Ser-2 or Ser-7 at the C-terminal domain region of RNA Pol II resulted in up-regulation of *S. pombe* subtelomeric genes of chromosome I and II (66). Thus, the phosphorylation of these sites may provide a mechanism that links transcription and chromatin modifications. Moreover, phosphorylation of the CDT region of RNA Pol II plays a role in recruitment of the Set1–Compass complex and co-transcriptional methylation of H3K4 in *S. cerevisiae* or *S. pombe* (71, 72). The Paf1C complex has also been shown to play a role in recruitment of Set1–Compass in *S. cerevisiae* (73). Because we observed elevation of H3K4me3 in Δtor1 mutant cells, it is possible that the absence of TORC2 allows the access of RNA Pol II to the subtelomeric region, bringing with it accessory factors, such as the Paf1C complex and Set1–Compass. Alternatively, but not mutually exclusive, TORC2–Gad8 may affect directly RNA Pol II or its associated factors, leading to maintenance of the subtelomeric region in its heterochromatic form. The detailed molecular mechanism by which TORC2–Gad8 regulates gene silencing awaits the isolation of the relevant direct substrate(s).

**Experimental procedures**

**Strains and growth conditions**

*S. pombe* strains used in this study are listed in Table S1. Standard procedures were used for growth and genetic manipulation. Yeast cells were cultured in rich YE medium supplemented with adenine and uracil at 30 °C or in Edinburgh minimal medium (5 g/liter NH₄Cl) as described (7). Gene deletions and tagging were performed by standard PCR-based methods (74).

**ChIP**

50 ml of each strain was grown to *A*₆₀₀ ~ 1 in YE. 1.5 ml of formaldehyde (37% solution) was added for 15 min, and the formaldehyde was quenched with 2.5 ml of 2.5 M glycine for 5 min. Cells were harvested, washed once with 15 ml of cold PBS, and broken down for 10 min with glass beads in 600 µl of lysis buffer (50 mM HEPES-KOH, pH 7.5, 140 mM NaCl, 1 mM EDTA, 1% Triton X-100, 0.1% sodium deoxycholic acid). The supernatant was removed to a new tube (the lysate). The glass beads were washed with 500 µl of lysis buffer and centrifuged, and the supernatant was added to the lysate. The lysate was sonicated six times for 10 s at 80% amplitude with 1 min on ice between each time. The sonicated material was centrifuged for 30 min at 2500 rpm. The supernatant was used for immunoprecipitations. The sonicated proteins were preclared with 25 µl of protein A-Sepharose and protein G-Sepharose bead mixture (GE Healthcare), and the appropriate antibodies were added to the cleared extract and incubated overnight at 4 °C. Swi6-FLAG, H3K9me2, H3K4me3, Ser2P RPD1, H4K16Ac, H3, H4, and H4K20me2 were immunoprecipitated with 2–5 µg of anti-FLAG antibody (Sigma, F3165), anti-H3K9me2 (Abcam, ab1220), anti-H4K16Ac (Active Motif, 39167-AM), anti-H4 (Abcam, ab10158), anti-H4K20me2 (Abcam, ab9052), anti-H3 (Abcam, ab1791), anti-H3K4me3 (Abcam, ab8580), and anti-Ser2P RPD1 (Millipore, MABE953). A total of 10% of the extract was saved as input. The beads after the immunoprecipitations were washed once with lysis buffer, once with lysis buffer with 360 mM NaCl, once with washing buffer (10 mM Tris/HCl, pH 8, 0.25 mM LiCl, 0.5% Nonidet P-40, 0.5% sodium deoxycholic acid, 1 mM EDTA) and once with TE (10 mM Tris/HCl, pH 8, and 10 mM EDTA). The washed beads and the input were treated with elution buffer (50 mM Tris/HCl, pH 8, 10 mM EDTA, 1% SDS) overnight at 65 °C. The DNA was precipitated, resuspended in water, and used for PCR real-time analysis. All experiments are plotted as the average of at least three independent biological repeats, and each biological repeat is the average of three technical PCR repeats.

**qRT-PCR**

RNA extractions and qRT-PCR analysis were performed as described by Laor et al. (75), followed by treatment of DNase (RQ1, Promega). For RT-PCR, complementary DNA was synthesized using the ImPromII Reverse transcription system (Promega) with poly(dT) and analyzed by quantitative PCR using the StepOne real-time PCR system. Data were normalized against actin and investigated using the C(t) method.
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Sequences of the primers used for qRT-PCR analyses are listed in Table S2.

RNA-Seq

RNA was extracted from 50 ml of logarithmic cells using the RNeasy minikit (Qiagen, catalog no. 74104). 1000 ng of total RNA from nine samples was processed using the TruSeq RNA sample preparation kit version 2 (Illumina) (RS-122-2001). Libraries were evaluated by Qubit and TapeStation. Sequencing libraries were constructed with barcodes to allow multiplexing of nine samples on one lane. Single-end 60-bp reads were sequenced on an Illumina HiSeq 2500 V4 instrument. The sequence yield was between 26.6 and 30.5 million reads per sample.

ChIP-Seq

9–20 ng of ChIP DNA was processed as described previously (76). Libraries were evaluated by Qubit and TapeStation. Sequencing libraries were constructed with barcodes to allow multiplexing of 30 samples on one lane. Single-end 60-bp reads were sequenced to a median depth of ~9 million reads/sample on an Illumina HiSeq 2500 version 4 instrument. The reads were aligned uniquely to the S. pombe genome, version ASM294v2 (Ensembl), using Bowtie (version 1.0.0) (77). Differential binding was performed using cswa (78).

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