Transcription of Two Long Noncoding RNAs Mediates Mating-Type Control of Gametogenesis in Budding Yeast

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SUMMARY

The cell-fate decision leading to gametogenesis is essential for sexual reproduction. In S. cerevisiae, only diploid MATa/α but not haploid MATa or MATα cells undergo gametogenesis, known as sporulation. We find that transcription of two long noncoding RNAs (lncRNAs) mediates mating-type control of sporulation. In MATa or MATα haploids, expression of IME1, the central inducer of gametogenesis, is inhibited in cis by transcription of the lncRNA IRT1, located in the IME1 promoter. IRT1 transcription recruits the Set2 histone methyltransferase and the Set3 histone deacetylase complex to establish repressive chromatin at the IME1 promoter. Inhibiting expression of IRT1 and an antisense transcript that antagonizes the expression of the meiotic regulator IME4 allows cells expressing the haploid mating type to sporulate with kinetics that are indistinguishable from that of MATa/α diploids. Conversely, expression of the two lncRNAs abolishes sporulation in MATa/α diploids. Thus, transcription of two lncRNAs governs mating-type control of gametogenesis in yeast.

INTRODUCTION

Gametogenesis, the process of gamete formation, is central to sexual reproduction. In multicellular organisms, little is known about the molecular mechanisms whereby germ cells are induced to form gametes. Key determinants of this process have been identified in S. cerevisiae, making budding yeast an ideal model system to study entry into gametogenesis (reviewed in van Werven and Amon, 2011). In response to nutrient deprivation, diploid budding yeast cells undergo gametogenesis to form four stress-resistant haploid gametes, called spores. This process is known as sporulation and is comprised of a specialized cell division, meiosis, to produce haploid gametes from a diploid precursor and a developmental program that leads to the formation of spores.

Initiation of sporulation requires the convergence of multiple signals (reviewed in Honigberg and Purnapatre, 2003). First, sporulation only occurs in cells of the diploid MATa/α mating type. Second, sporulation is only initiated under starvation conditions. Fermentable sugars and nitrogen sources must be absent and a nonfermentable carbon source must be present for sporulation to be initiated. Finally, cells must be able to respire. All these signals converge on the promoter of IME1, the master regulator of gametogenesis. IME1, inducer of meiosis 1, encodes a transcription factor that sets the sporulation program in motion (Kassir et al., 1988). When IME1 is transcribed, cells enter gametogenesis (Deng and Saunders, 2001; Kassir et al., 1988; Mitchell and Bowdish, 1992). Thus, IME1 gene expression regulation lies at the heart of gametogenesis control in budding yeast.

The IME1 promoter is over 2 kb in length and is one of the most regulated promoters in S. cerevisiae (reviewed in Honigberg and Purnapatre, 2003; van Werven and Amon, 2011). Little is known about the transcription factors that bring about nutritional and respiratory control of IME1 expression, but the mechanism that restricts IME1 expression to MATa/α diploid cells has been partially elucidated (Figure 1A). The transcription factor Rme1 binds to two RME1-binding sites in the IME1 promoter (~2 kb upstream of the translation start site) and inhibits IME1 expression in haploid cells (Covitz and Mitchell, 1993; Shimizu et al., 1998). In MATa/α diploid cells, RME1 is not expressed. This is because the MATa locus encodes a1 and the MATα locus α2, which together form the a1–α2 repressor complex that inhibits...
RME1 expression (Figure 1A) (Covitz et al., 1991; Mitchell and Herskowitz, 1986). How Rme1 inhibits expression of IME1 in haploid cells is not understood.

IME1 is not the only inducer of sporulation whose expression is controlled by mating type. IME4 encodes an RNA methyltransferase that is essential for initiation of sporulation in some strain backgrounds and contributes to efficient entry in others (Clancy et al., 2002; Hongay et al., 2006; Shah and Clancy, 1992). In MATα or MATα diploid cells, IME4 is not expressed because an antisense transcript (IME4-AS, also known as RME2), initiated from the 3’ end of the IME4 locus, interferes with IME4 expression (Gelfand et al., 2011; Hongay et al., 2006). In MATα diploid cells, the a1–a2 complex inhibits the expression of the IME4 antisense RNA by directly binding to its promoter. Whether RME1 and IME4-AS are the sole mediators of mating-type control of sporulation is not known.

Here we describe the mechanism whereby the cell’s mating type regulates IME1 expression and hence gametogenesis. We find that Rme1 induces the expression of a long noncoding RNA (lncRNA) in cells expressing the haploid MATα or MATα mating type but not in cells of the diploid MATα/mating type. This lncRNA, termed IRT1, covers almost the entire IME1 promoter and functions in cis to prevent transcription factors from binding to the IME1 promoter. Interference with transcription factor binding is mediated by IRT1 transcription establishing a repressive chromatin state at the IME1 promoter. This requires the Set2 histone methyltransferase and the Set3 histone deacetylase complex (Set3C), indicating that cotranscriptional methylation of histones and recruitment of histone deacetylases are essential for IRT1-dependent silencing of the IME1 promoter. Furthermore, we define how the cell’s mating type regulates gametogenesis. Interfering with the expression of IRT1 and the antisense transcript at the IME4 locus is sufficient to allow cells expressing the haploid MATα or MATα mating type to sporulate as efficiently as MATα diploid cells. Conversely, expression of these two lncRNAs abolishes the ability of MATα diploid cells to sporulate. Our data demonstrate that transcription of two lncRNAs confers mating-type regulation of gametogenesis in budding yeast.

### RESULTS

#### Identification of Cell-Type-Specific Intergenic Transcripts in the IME1 Promoter

Recently, a detailed map of noncoding RNAs in sporulating cells revealed transcriptional activity in the IME1 promoter (Figure 1B and Figure S1 available online) (Lardenois et al., 2011). The IME1 gene itself is only expressed in cells of the MATα/mating type and only under sporulation-inducing conditions (Figures 1C and 1A). The gene is not expressed when nutrients are ample (Y). IME1 RNA begins to accumulate upon transfer of cells into sporulation-inducing medium (SPO medium; Figures 1C, 1D, and S1A), increases during early stages of sporulation, and declines thereafter.

Transcriptional activity was also detected in the IME1 promoter. A long promoter transcript, annotated as stable unannotated transcript 643 (SUT643) (Xu et al., 2009), is transcribed from the same strand as IME1 (Figure 1B). This transcript is weakly expressed in MATα diploid cells upon induction of sporulation but highly expressed when MATα diploid cells are incubated in SPO medium (Figure S1B). Northern blot and quantitative RT-PCR analyses confirmed this result (Figures 1C and 1D). In MATα diploid cells and MATα haploid cells, SUT643 transcription is strongly induced in SPO medium, and RNA levels remain high throughout the time course, despite the transcript being short-lived (Figures 1E and 1F). As expected,
IME1 is not expressed (Figures 1C and 1D). This result shows that SUT643 and IME1 exhibit cell-type-specific expression under sporulation-inducing conditions. In what follows, we show that SUT643 plays a key role in the control of IME1 expression. We therefore named the gene IRT1, for IME1 regulatory transcript 1. We detected a second, shorter transcript upstream of SUT643, designated as meiotic unannotated transcript 1573 (MUT1573), which was upregulated during later stages of sporulation (Figures 1B and S1C). The significance of this transcript in IME1 regulation is presently unclear.

**IRT1 and IME1 Expression Are Anticorrelated**

To define the relationship between IME1 and IRT1, we studied their expression in single cells using RNA fluorescence in situ hybridization (FISH) (Bumgarner et al., 2012; Raj et al., 2008). We measured IRT1 and IME1 RNAs in single MATa/α diploid and MATa haploid cells upon transfer into sporulation-inducing conditions (Figures 2A, S2, and S3). This analysis showed that 4 hr after transfer into SPO medium, IME1 is strongly expressed (average of ~44 transcripts per cell), and more than 90% of MATa/α cells harbor IME1 transcripts. In contrast, IRT1 RNA is barely detectable (Figures 2B and S3A).

In the MATa haploid strain, we observed that upon induction of sporulation, ~80% of cells transiently expressed low levels of IME1, as defined by the presence of at least two IME1 RNA molecules in cells (0–60 min time points; Figure 2C [combine IME1 and IRT1/IME1]; Figure S3B). The percentage of cells expressing IME1 decreased significantly at later time points. IRT1 expression was anticorrelated. The percentage of MATa cells expressing IRT1 was low upon transfer into SPO medium but increased to ~80% within 2 hr (Figure 2C). We further observed that at times when IME1 RNA levels declined and IRT1 levels rose (30 to 60 min after transfer into SPO medium), cells harbored both IME1 and IRT1 transcripts (Figures 2C and S3B). This observation together with the finding that in other stages of sporulation, IME1 and IRT1 RNAs are mutually exclusive (Figures 2A and 2C) indicates that IME1 is transiently induced upon starvation even in cells that express the haploid MATa or MATα mating type, but concomitantly with IRT1 induction, IME1 RNA levels decline in these cells.

**RME1-Dependent IRT1 Transcription Inhibits IME1 Expression**

The observation that IRT1 is expressed when IME1 is not raises the possibility that IRT1 transcription mediates the repression of IME1 transcription. To test this, we integrated the CYC1 transcriptional terminator 118 base pairs (bp) downstream of the transcription start site of IRT1 (henceforth irt1-T). This led to the loss of full-length IRT1. Instead, a shorter IRT1 transcript was detected (marked with *; Figure 3A). Importantly, MATa/α diploid and MATa haploid cells harboring the irt1-T allele expressed IME1 (Figure 3A). A fraction of cells also underwent meiosis, which is lethal in haploid cells (Figures 3B and 3C). Thus, full-length IRT1 transcription is required for the repression of IME1 in cells expressing the haploid MATa or MATα mating type.

The transcription factor Rme1 is required for the repression of IME1 in cells of the haploid MATa or MATα mating type (Covitz and Mitchell, 1993). However, Rme1 does not behave like a classic transcriptional repressor. Whereas Rme1 represses IME1 transcription, it functions as a transcriptional activator in the context of other promoters (Toone et al., 1995). The identification of IRT1 transcription as an inhibitor of IME1 expression raised the possibility that Rme1 activates IRT1 expression, thereby inhibiting IME1 expression. Consistent with this hypothesis is the observation that the two Rme1-binding sites are located immediately upstream of the IRT1 transcription start site, and that their position within the IME1 promoter is highly conserved across Saccharomyces species (Figure 3D).
To test whether RME1 is required for IRT1 expression, we examined the consequences of deleting RME1. We found that IRT1 expression was lost in MATa rme1Δ haploid and MATa/α rme1Δ diploid cells or MATa haploid cells lacking the RME1-binding sites (Figures 3A and S4). IME1 was induced in all these strains (Figures 3A and S4) (Covitz and Mitchell, 1993). The degree of IME1 expression and degree of sporulation observed in the rme1Δ strain were remarkably similar to that of MATa/α cells expressing the prematurely terminated irt1-T allele (Figures 3A and 3B). Chromatin immunoprecipitation (ChIP) analysis further showed that Rme1 binding to the IRT1 promoter only occurs under conditions supporting IRT1 expression (Figure 3E). During vegetative growth and upon transfer into SPO medium, Rme1 is not recruited to the IRT1 promoter and IRT1 is not expressed, but both events occur as cells enter the sporulation program. Our data show that Rme1 inhibits IME1 expression and hence sporulation in cells expressing the haploid MATa or MATα mating type through activation of IRT1 transcription. The observation that sporulation is not as efficient in rme1Δ or irt1-T MATa/α cells as it is in MATα/α cells further indicates that mating-type control of sporulation must be mediated by additional factors.

**IRT1 Represses IME1 Transcription In cis**

The IRT1 transcript harbors several putative short open reading frames with the longest encoding a protein of 74 amino acids. If an IRT1-encoded protein is responsible for IME1 repression, the location of the IRT1 gene within the yeast genome should not affect the ability of IRT1 to inhibit IME1 expression. We performed two experiments to test this possibility. First, we created a haploid MATa strain in which the IRT1 locus was duplicated (Figure 4A). In this strain IME1, expression was inhibited (Figures 4B and S5A–S5C). However, when the IRT1 locus immediately upstream of IME1 harbored the CYC1 terminator (irt1-T allele), control. (*) marks the truncated version of the IRT1 transcript. (**) marks the MHT1573 transcript, which accumulates during late stages of sporulation. The MATα/α and MATa/α controls for this experiment are shown in Figure 1C as these experiments were performed at the same time.

(B) MATa/α (open circles; A4962), MATa/α rme1Δ (closed circles; A30195), MATa/α irt1-T (open triangles; A30070) diploid cells, and MATα irt1-T (closed triangles; A30067) haploid cells were induced to sporulate. The number of cells that had undergone either one or both meiotic divisions was determined at the indicated times (n = 100).

(C) MATa (A4841), MATa rme1Δ (A30075), and MATα irt1-T (A30067) cells were induced to sporulate. Cells were harvested either before transfer into SPO medium or after a 14 day incubation in SPO medium. 5-fold serial dilutions were spotted onto YPD plates.

(D) Sequence conservation and position of the two RME1-binding sites with respect to the IME1 translation start site across different Saccharomyces species are shown.

(E) Analysis of Rme1 occupancy at the RME1-binding sites upstream of the IRT1 transcription start site (primer pair one), where Rme1 is known to bind, and at the transcription start site of IME1 (primer pair two), where Rme1 is not known to bind. Rme1 binding was determined at the indicated times in MATa RME1-3xV5 (A30108) cells grown in sporulation-inducing conditions. ChiP signals were normalized to the HMR locus, which does not bind Rme1.

The data are represented as mean ± SEM from multiple experiments. See also Figure S4.
serial dilutions were spotted onto YPD plates. either before transfer into SPO medium or after a 14 day incubation. 5-fold (C) Strains described in (B) were induced to sporulate. Cells were harvested taken at the indicated times to determine the amount of IME1 MAT was expressed in the IRT1 and (B) See also Figure S5.

The identification of a direct activator of IME1 expression allowed us to assess the effects of IRT1 transcription on transcription factor binding at the IME1 promoter. In MATα/a diploid cells, Pog1 binding was induced under sporulation-inducing conditions (Figure 5E). In MATα haploid cells, Pog1 binding was also slightly elevated as cells entered the sporulation program (1 hr after transfer into SPO medium) but never increased to levels seen in MATα/a diploid cells (Figure 5E). Importantly, Pog1 binding at the IME1 promoter was affected by IRT1. Pog1 was recruited to the IME1 promoter in haploid cells expressing the irr1-1 allele but not in cells expressing full-length IRT1 (Figure 5F). These results indicate that at least one transcriptional activator of IME1 is differentially recruited to the IME1 promoter in MATα haploid and MATα/a diploid cells. Furthermore, our data demonstrate that IRT1 transcription inhibits transcriptional activators from being recruited to the IME1 promoter.

**IRT1 Transcription Establishes a Silent Chromatin State**

Transcription of IRT1 could antagonize IME1 expression via two not mutually exclusive mechanisms. Movement of the transcription machinery through the IME1 promoter could interfere with transcription factor binding. It is also possible that transcription through the IME1 promoter establishes a repressive chromatin state.

To determine whether IRT1 transcription establishes a repressive chromatin state at the IME1 promoter, we examined nucleosome occupancy in MATα and MATα/a cells. Regions of low nucleosome occupancy, referred to as nucleosome-free regions (NFRs), are found in promoters of transcriptionally active genes and are thought to allow transcription factors to bind to promoters. High nucleosome occupancy at promoters is indicative of repressive chromatin (reviewed in Cairns, 2009). We observed that nucleosome occupancy, as measured by histone H3 occupancy (Figures 5G–5J), is differentially regulated between MATα haploid and MATα/a diploid cells. Nucleosome occupancy was high in both MATα haploid and MATα/a diploid cells during exponential growth when IME1 expression is low (Figure 5G). An NFR became apparent during starvation (saturated YPD and at the time of transfer into SPO medium) in both MATα haploid and MATα/a diploid cells, when IME1 is expressed at low levels in both cell types (compare Figures 5H and 5I with Figures 1 and 2). Shortly after transfer into SPO medium, IRT1 expression decreases (Figure 5I and Figure 5J).
high nucleosome occupancy was re-established in MATa haploid cells but not in MATa/Δ diploid cells (Figure 5J). These results show that nucleosome re-assembly at the IME1 promoter occurs in MATa cells at the time IRT1 is transcribed. Our data suggest that IRT1 transcription induces a repressive chromatin state, which prevents the recruitment of transcriptional activators to the IME1 promoter.

**IME1 Repression by IRT1 Transcription Requires SET2 and SET3**

How does IRT1 transcription establish a repressive chromatin state at the IME1 promoter? Two previous studies have implicated the histone methyltransferase Set2 and the Set3 histone deacetylase complex in IME1 regulation. Deletion of either gene increases sporulation efficiency (Deutschbauer et al., 2002). SET3 was also shown to dampen IME1 expression in certain strain backgrounds (Pijnappel et al., 2001). Set2 and Set3 are directly involved in establishing repressive chromatin structures within transcribed regions (Carrozza et al., 2005; Keogh et al., 2005; Kim and Buratowski, 2009) and could thus be critical for repression of IME1 by IRT1 transcription.

Set1 and Set2 travel with RNA polymerase to deposit the repressive lysine 4 dimethylation (H3-K4-me2) and lysine 36 methylation (H3-K36-me) marks, respectively, on histone H3 (Carrozza et al., 2005; Keogh et al., 2005; Kim and Buratowski, 2009; Xiao et al., 2003). After 6 hr in SPO, when IRT1 is expressed in MATa haploid cells, both marks were significantly enriched in the IME1 promoter (Figures 6A–6C) and, as expected, depended on SET1 and SET2 (Figures S6A–S6C). We conclude that histone modifications characteristic of repressive chromatin are present in the IME1 promoter in cells expressing a haploid mating type.

To determine whether SET2 and SET3 are required for IRT1-mediated repression of IME1, we measured the expression of IME1 and IRT1 levels in MATa haploid cells lacking either SET2 or SET3 or both genes (note, unlike in other strain backgrounds [Krogan et al., 2003], deleting SET2 and SET3 did not lead to significant growth defects in SK1 cells). IRT1 expression was not affected in all three mutants, but IME1 expression was (Figures 6D and 6E). IME1 levels were somewhat elevated in the set2 and set3 single mutants but reached levels similar to that of cells lacking IRT1 transcription (irt1-T cells) in the double mutant (Figures 6D and S6E). Analysis of IME1 and IRT1 RNAs in single cells further showed that the two RNAs are coexpressed in set2 set3 double mutants (Figures 6E and 6F). The fraction of cells only expressing IRT1 (two transcripts or more per cell) decreased in the set2 and set3 single mutants and was the lowest in the set2 set3 double mutant (Figure 6F). The fraction of cells only expressing IME1 increased somewhat in all mutants, suggesting that SET2 and SET3 may be necessary for full IRT1 expression. Deleting SET2 and SET3 had the largest effect on the category of cells that coexpress IRT1 and IME1. In the set2 set3 double mutant, almost 50% of cells harbor both IME1 and IRT1 transcripts. We conclude that repression of the IME1 promoter by IRT1 transcription is compromised in the set2 set3 double mutant.

To further study the role of Set2 and Set3 in IME1 expression, we analyzed the IME1 promoter architecture in set2 and set3 haploid cells. Although the Set2 and Set3 double mutant shows a smaller fraction of cells expressing both transcripts at 20 hr, a much smaller fraction of cells do not express both transcripts at 20 hr, even at 20 hr (Figure 5A). The data are represented as mean ± SEM from multiple experiments.

**Figure 5. IRT1 Transcription Inhibits Pog1 Recruitment and Increases Nucleosome Occupancy at the IME1 Promoter**

(A and B) Wild-type (A4962) and pog1Δ (A30194) MATaΔ diploid cells were induced to sporulate. IME1 RNA levels (A) and the percentage of cells that have undergone at least one meiotic division (B) were determined at the indicated times.

(C) Graphical overview of the IRT1/IME1 locus. The positions of the nine primer pairs used to determine Pog1 and histone H3 occupancy are shown.

(D) MATaδ diploid cells carrying a POG1-3xV5 fusion (A30236) were induced to sporulate. Pog1 binding throughout the IME1 promoter was determined after 0 or 3 hr in SPO medium. ChIP signals were normalized to the HMR locus, where Pog1 is not known to bind. The data are represented as mean ± SEM from multiple experiments.

(E) Pog1 binding to the IME1 promoter was determined in MATaδ diploid and MATa haploid cells (A30235) at the indicated times. Primer pair 4 was used for this analysis.

(F) Wild-type (A30235) and irt1-T MATa cells (A30246) were induced to sporulate, and Pog1 binding was determined at the indicated times.

(G–J) Relative histone H3 occupancy across the IRT1/IME1 locus in MATa haploid (A4841) and MATaδ diploid (A4962) cells. Cells were either grown in YPD (exponential phase or to saturation) or induced to sporulate for 0 or 3 hr.
single and double mutants. In contrast to wild-type MATa cells, Pog1 is recruited to the IME1 promoter in the set2 set3 double mutant cells and also to some extent in the single mutants (Figure 6AB C).

Figure 6. SET2 and SET3 Are Required for IRT1 Transcription-Mediated Repression of the IME1 Promoter
(A) The positions of the primer pairs used in ChIP experiments for (B), (C), and (H) are shown.
(B and C) Relative occupancy of histone H3 lysine 4 dimethylation (B) and lysine 36 methylation (using an antibody directed against histone H3 lysine 36 trimethylation) (C) across the IRT1/IME1 locus in MATa haploid (A4841) cells. The data are represented as mean ± SEM from multiple experiments.
(D) Wild-type (A4841), irt1-T (A30067), set2Δ (A31995), set3Δ (A31999), and set2Δ set3Δ (A32040) MATa cells were induced to sporulate. Samples were taken at the indicated times to determine the amount of IME1 and IRT1 RNA.
(E and F) Wild-type (A10931), set2Δ (A31992), set3Δ (A31998), and set2Δ set3Δ (A32051) MATa haploid cells were induced to sporulate to examine IME1 and IRT1 RNAs in single cells. (E) shows set2Δ set3Δ cells that harbor IRT1 (red) and IME1 (green) transcripts. DNA is shown in blue. (F) shows quantification of the percentage of single cells that harbor no transcripts or two or more transcripts of IRT1, IME1, or IRT1 and IME1 (n = 3; SEM). At least 450 cells were analyzed per strain.
(G) Wild-type (A30235), set2Δ (A32036), set3Δ (A32033), and set2Δ set3Δ (A32049) MATa haploid cells carrying a POG1-3xV5 fusion were induced to sporulate, and Pog1 occupancy in the IME1 promoter was determined.
(H) Relative histone H3 occupancy across the IRT1/IME1 locus after 6 hr in SPO medium.
(i) MATa/a (closed circles; A4962), MATa/a set2Δ set3Δ (open circles; A32041), and MATa/a set2Δ set3Δ (closed triangles; A32059) cells were induced to sporulate. Samples were taken at the indicated times to determine the number of cells that had undergone either one or both meiotic divisions.
(J) Model for IRT1-mediated repression of IME1 involving Set2 and Set3. See text for details. See also Figure S6.

Furthermore, an NFR became apparent in the single and double mutants (Figure 6H).

Deleting SET2 and SET3 even allowed some sporulation to occur in cells expressing a haploid mating type. MATa/a set2 set3 mutants undergo sporulation with delayed kinetics presumably because the two genes are needed for other aspects of the sporulation program (Figure 6I). Deleting SET2 and SET3, however, allowed a significant proportion of MATa/a cells to sporulate (Figure 6I), to produce viable spores (data not shown), and to induce a lethal meiosis in haploid cells (Figure S6F). These data demonstrate that IME1 repression by IRT1 transcription requires Set2 and Set3 to establish a repressive chromatin state in MATa haploid cells.
in the IME1 promoter to prevent transcription factor recruitment. We propose that transcription of IRT1 deposits histone methylation marks, which recruit histone deacetylase complexes to repress the IME1 promoter (Figure 5J). At the 5′ end of the IME1 promoter, the histone H3 lysine 4 dimethylation mark directly recruits Set3 together with Set3C containing the histone deacetylases Hos2 and Hst1 (Kim and Buratowski, 2009). Consistent with this model is the observation of Set3-dependent recruitment of Hos2 to the IME1 promoter (Figure S6G). IRT1 transcription is also required for cotranscriptional Set2-dependent methylation of histone H3 at lysine 36. This mark recruits the histone deacetylase complex Rpd3C(S) (Carrozza et al., 2005; Keogh et al., 2005). Thus, IRT1 transcription represses the IME1 promoter by recruiting histone deacetylases.

**Mating-Type Control of Sporulation Is Governed by Transcription of Two Noncoding RNAs**

Preventing IRT1 transcription allows MATa haploid and MATa/a diploid cells to induce IME1 and to enter sporulation. However, these cells do not sporulate with the same kinetics and efficiency as MATa/a diploids (Figure 3B). This observation indicates that other pathways exist that bring about mating-type control of sporulation. IME4 regulation could be such a parallel pathway. In cells harboring only one mating type, expression of an IME4 antisense (IME4-AS) RNA prevents the expression of IME4 (Hongay et al., 2006). In MATa/a diploid cells, IME4-AS is repressed by the a1→α2 repressor, and IME4 is expressed (Hongay et al., 2006).

To determine whether the IME4-AS and IRT1 transcripts collaborate to bring about mating-type control of sporulation, we combined the ir1-T allele with an IME4 allele driven from the constitutive TEF1 promoter (pTEF1-IME4). Whereas each individual allele allowed 50% of MATa/a cells to sporulate with a delay, the combination of the two brought about sporulation efficiencies and kinetics seen in MATa/a diploid cells (Figure 7A).

We were also able to induce MATa/a levels of sporulation in MATa/a diploid cells by simply repressing transcription of IRT1 and IME4-AS. We constructed a strain carrying a TetR repressor fused to the transcription repressor Tup1 (TetR-Tup1; Bellı´ et al., 1998). We then integrated tetO sites at the 5′ end of the RME1 promoter (386 bp upstream of the RME1 translation start site) and at the 3′ end of the IME4 gene (158 bp downstream from the IME4 stop codon) to replace the a1→α2-binding sites and hence a1→α2 regulation of RME1 and IME4-AS with that of the TetR-Tup1 fusion. MATa/a diploid cells that either harbor only

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**Figure 7. Transcription of Two IncRNAs Conveys Mating-Type Control of Sporulation**

(A) MATa/x (A4962; closed circles), MATa/a ir1-T (A30070; open circles), MATa/a pTEF1-IME4 (A30133; closed triangles), and MATa/a pTEF1-IME4 ir1-T (A30100; open triangles) cells were induced to sporulate. The percentage of cells that had completed at least one meiotic division was determined at the indicated times (n = 100).

(B) MATa/x cells (closed circles), MATa/a cells in which the a1→α2-binding sites in the IME4-AS promoter were replaced by tetO sequences (MATa/a pIME4-3′-tetO; open circles; A30217), MATa/a cells in which the a1→α2-binding sites in the RME1 promoter were replaced by tetO sequences (MATa/a pRME1-tetO; closed triangles; A30231), and MATa/a cells expressing both fusions (open triangles; A30219) all carrying a TetR-Tup1 fusion were induced to sporulate. The percentage of cells having completed at least one meiotic division was determined at the indicated times (n = 100).

(C) MATa/x strains carrying various combinations of a1→α2-binding site mutations are listed (#1 [A32019], #2 [A32020], #3 [A32021], #4 [A32022], #5 [A32023], #6 [A32024], #7 [A32025], and #8 [A32026]).

(D) The percentage of sporulated cells of strains in (C) was determined after 48 hr in SPO medium.

(E) Wild-type (A4962), set2Δ (A31996), set2Δ (A32001), and set2Δ set2Δ (A32041) MATa/a cells were induced to sporulate. The percentage of cells that had completed at least one meiotic division was determined at the indicated times (n = 100).

(F) MATa/a diploid cells carrying deletions in the two a1→α2-binding sites of the RME1 promoter (A32022, A32035, A32034, and A32057) were analyzed as in (E). See also Figure S7.
tetO sites or express the TetR-Tup1 fusion in the absence of tetO sites did not sporulate (Figures S7A and S7B). When TetR-Tup1 was tethered to either the RME1 promoter or the IME4 3′ end, a low percentage of cells sporulated (Figures S7A and S7B). However, when TetR-Tup1 was targeted to both sites simultaneously, MATα/α diploid cells formed spores with the same kinetics and efficiency as MATa/α diploids (Figure 7B). Similar results were obtained when the irt1-T allele was combined with the TetR-Tup1-repressible IME4-AS construct (Figure S7C). Our results show that inhibiting transcription of IRT1 and IME4-AS is sufficient to induce MATa/α levels of sporulation in MATa/α cells.

What are the effects of expressing IRT1 and IME4-AS in MATa/α cells? In MATa/α cells, the a1-a2 repressor inhibits the transcription of the IRT1 transcription factor RME1 and IME4-AS. The RME1 promoter harbors two a1-a2-binding sites; the IME4-AS promoter has one (Figures 7C and S7D). We examined the consequences of deleting individual and the combination of binding sites in MATa/α strains. Inactivating single a1-a2 sites in the RME1 promoter had little effect on sporulation (Figure 7D). Inactivating both a1-a2-binding sites in the RME1 promoter led to expression of RME1 in MATa/α cells similar to what is seen in MATa cells, indicating that the RME1 promoter is fully derepressed (Figure S7E, compare MATa with 4). Consistent with this effect on RME1 expression, progression through meiosis and sporulation efficiency was significantly reduced in this mutant (Figures 7C, 7D, and S7F). Deleting SET2 and SET3 suppressed the sporulation defect of cells with deletions of the a1-a2-binding sites in the RME1 promoter (Figures 7E and 7F), further confirming that SET2 and SET3 are required for IRT1-dependent repression of IME1.

Finally, we combined mutations in the a1-a2-binding sites in the RME1 promoter with a deletion of the a1-a2-binding site in the IME4-AS promoter. Deleting the IME4-AS a1-a2-binding site dramatically reduced sporulation in MATa/α cells (Figures 7C, 7D, and S7G) (Hongay et al., 2006), but inactivation of all three a1-a2-binding sites obliterated sporulation (Figures 7D and S7G; strain number 8). We conclude that transcription of two lncRNAs, IRT1 and IME4-AS, is the sole mediator of mating-type control of sporulation in budding yeast.

**DISCUSSION**

The decision of whether or not to enter the developmental program that leads to gamete formation is governed by multiple extracellular and intracellular signals. Here we describe how the cell’s mating type regulates gametogenesis. The control is remarkably simple: transcription of two noncoding RNAs prevents, via distinct mechanisms, the expression of two central regulators of the sporulation program in cells expressing the MATa or MATα haploid mating type.

**Mechanism of IME1 Repression by IRT1 Transcription**

Understanding how the expression of IME1 is controlled lies at the heart of gamete formation and serves as a model to understand signal integration at promoters. We have unraveled the mechanism whereby the cell’s mating type controls IME1 expression. Several lines of evidence indicate that IRT1 transcription interferes with IME1 expression by preventing transcription factors from binding the IME1 promoter. First, full-length transcription of IRT1 through the IME1 promoter is needed for IME1 repression. Second, IRT1 functions in cis to inhibit the expression of downstream genes. This repressive cis-acting function of IRT1 is observed at the native locus and at an ectopic site. Third, Rme1-dependent repression of IME1 requires two components of the RNA polymerase mediator complex, RGR1 and SIN4 (Covitz et al., 1994; Shimizu et al., 1997). Finally, we observe that an activator of IME1, Pog1, is displaced from its binding site when full-length but not a truncated version of IRT1 is expressed.

How does IRT1 inhibit IME1 expression? The IRT1 RNA itself is unlikely to contribute to the repression of IME1 expression. IRT1 RNA is highly unstable, and RNA FISH analysis showed that IRT1 transcripts do not localize to one region of the nucleus but are found throughout the cells. Furthermore, in the set2 set3 double mutant, IRT1 RNA is present in cells at levels seen in wild-type cells, yet IME1 is efficiently transcribed. Whether movement of the transcription apparatus through the IME1 promoter interferes with transcription factor binding is not yet known, but our data support a role for cotranscriptional chromatin modifications in establishing a repressive chromatin state at the IME1 promoter. IRT1 transcription is associated with an increase in nucleosome density and the repressive histone H3-K4-me2 and H3-K36-me marks at the IME1 promoter.

The inactive chromatin state at the IME1 promoter requires the Set2 histone methyltransferase and the Set3C. Previous studies showed that the Set2/Rpd3C(S) pathway is essential for repression of cryptic transcription within long genes (Carrozza et al., 2005; Keogh et al., 2005; Li et al., 2007). Set3C is required for the repression of histone acetylation at the 5′ ends of genes (Kim and Buratowski, 2009). We propose that in the context of the IME1 promoter, these functions are employed to regulate expression of a downstream gene via IncRNA transcription. In cells expressing a haploid mating type, IRT1 transcription recruits the Set1 and Set2 histone methyltransferases. At the 5′ end of the IME1 promoter, Set1-mediated histone H3 lysine 4 dimethylation recruits the Set3 complex containing the histone deacetylases Hos2 and Hst1 (Kim and Buratowski, 2009) (Figure 6J). IRT1 transcription also promotes cotranscriptional Set2-dependent methylation of histone H3 at lysine 36. This mark recruits the histone deacetylase complex Rpd3C(S) (Carrozza et al., 2005; Keogh et al., 2005), which, we propose, contributes to the repression of the IME1 promoter. This is, to our knowledge, the first example of Set2 and Set3C working together to silence a promoter through IncRNA transcription. This mechanism of gene regulation could be widespread. A recent genome-wide study suggests that the majority of Set3-regulated genes have overlapping ncRNA transcripts in yeast (Kim et al., 2012 [this issue of Cell]). It may also occur in other species. In fission yeast, transcription of long messenger RNA (mRNAs) has recently been shown to establish heterochromatin islands to silence meiotic genes during vegetative growth (Zofall et al., 2012). This raises the interesting possibility that transcription of all kinds of RNAs serves to establish a silent chromatin state to inhibit the expression of neighboring genes. Transcription of IncRNAs has also been implicated in transcriptional...
activation (Hirota et al., 2008; Houseley et al., 2008; Pinsky­
a et al., 2009; Uhler et al., 2007). It will be interesting to de­
termine the relative importance of IncRNA-mediated transcriptional ac­
vitation and repression in gene regulation and whether gene silencing mediated by long ncRNA transcription, as described here, also exists in higher eukaryotes.

The mechanism of IME1 repression by IRT1 has some parallels with what is observed at the SER3 locus. Like IRT1, SRG1, the noncoding RNA controlling SER3 expression, regulates its target in cis, increases nucleosome occupancy at the SER3 promoter, and prevents transcription factors from binding the SER3 promoter (Hainer et al., 2011; Martens et al., 2004). Nucleosome-remodeling proteins, such as Spt2, Spt6, and Spt16, are important for transcription-dependent repression of SER3 by SRG1 (Hainer et al., 2011; Thebault et al., 2011). Whether these remodeling factors are needed for IME1 repression is not yet known. However, Spt2 and Spt3, important for IME1 repression, do not play a role in SER3 repression (Hainer et al., 2011). This is perhaps not surprising, given that repression of intragenic tran­script by Spt2 predominantly occurs at longer genes (Li et al., 2007), and SRG1 is a relatively short ncRNA (~500 bp).

Rme1 Is a Transcriptional Activator

How Rme1 represses IME1 has been the subject of investigation for decades (Blumental-Perry et al., 2002; Covitz and Mitchell, 1993; Kassir et al., 1988; Mitchell and Herskowitz, 1986). Genetically, RME1 was shown to function as a repressor of IME1 expression but was found to activate transcription of CLN2 (Toone et al., 1995). Transcription reporter assays further showed that Rme1 functions as an activator or repressor depending on the position of the RME1-binding site within the promoter. A more distal binding site caused repression; location near the transcription start site brought about transcriptional activation (Covitz and Mitchell, 1993). Our findings provide a simple explanation for these results. Rme1 is an activator of transcription, which, when located at a distance from a transcriptional start site, can repress a target gene by inducing transcription through the promoter where it is located.

A Model for How IRT1 Regulates IME1 Expression

The single-cell analysis of IME1 and IRT1 transcripts sheds light onto how IRT1 transcription through the IME1 promoter represses IME1 transcription in cells expressing the MATa or MATα haploid mating type. Both IRT1 and IME1 expression is under nutritional control. Both transcripts are repressed during vegetative growth. IRT1 transcription continues to be repressed in presporulation medium and is activated only upon transfer into sporulation medium, which coincides with the recruitment of Rme1 to the IRT1 promoter. In contrast, IME1 transcription is already activated during growth in presporulation medium. Remarkably, this presporulation activation occurs not only in MATa/α diploid cells but also in cells expressing the MATa or MATα haploid mating type. Thus, IME1 is initially expressed in cells of all mating types in response to nutrient deprivation, but Rme1-mediated expression of IRT1 then downregulates IME1 expression in haploid cells. Interestingly, the maximal number of IRT1 molecules per cell in MATa haploids is 10-fold lower compared to IME1 in MATa/α diploid cells. This finding that a low level of IRT1 transcription is sufficient to repress IME1 expression is consistent with the idea that cotranscriptional silencing of the IME1 promoter by histone deacetylases is the major mechanism of IME1 repression. The observation that IRT1 is induced only after IME1 expression has been initiated, despite both promoters being under similar nutrient regulation, furthermore raises the interesting possibility that IME1 expres­sion may be a requisite for IRT1 expression. Further studies will be needed to determine whether IME1 is required for its own downregulation in cells expressing the haploid mating types.

Transcription of Two Noncoding RNAs Controls a Critical Cell-Fate Decision

Transcription of IRT1 and IME4-AS is essential to prevent MATA or MATα haploid cells from entering a lethal meiosis. Interfering with their expression is sufficient to induce mating-type-independent sporulation that is indistinguishable from that of MATA/α diploid cells in both efficiency and kinetics. Conversely, deleting three a1-a2-binding sites, two at the RME1 promoter and one in the IME4-AS promoter, abolished the ability for MATA/α diploid cells to sporulate. Thus, transcription of two IncRNAs is all that mediates mating-type control of sporulation. Why did budding yeast evolve the use of IncRNA transcription to govern this key cell-fate decision? Perhaps repression of complex promoters by IncRNA transcription is more effective than that by classic transcriptional repressors. The IME1 promoter is unusually long for an S. cerevisiae promoter (2.2 kb) and subject to complex regulation. Full repression of such a promoter would likely require the binding of repressors to multiple sites throughout the promoter. Repression by transcription of a IncRNA is simpler. It only requires two RME1-binding sites located upstream of the IME1 promoter. A similar rationale could apply to the use of antisense transcription to control the expression of genes with complex promoters. Antisense trans­cripts only require a single transcription initiation site at the 3′ end. Another advantage of gene repression by IncRNA transcription is that repression is the default. Repression is alleviated only in MATA/α diploid cells, through the repression of IRT1 and IME4-AS.

IncRNAs are widespread both in vegetatively growing and in sporulating budding yeast cells (Granovskaia et al., 2010; Larde­nois et al., 2011). Many genes important for progression through sporulation have been shown to harbor antisense transcripts that are expressed during vegetative growth (Zhang et al., 2011). Regulation of gene expression by IncRNAs also appears important for other developmental processes such as pseudo­hyphal growth or adaptation to changes in growth conditions (Bumgarner et al., 2009; van Dijk et al., 2011). The use of IncRNA transcription as a regulatory tool may impact biological processes beyond transcription. In fission yeast meiosis, the sme2+ IncRNA has recently been shown to be required for pairing at this locus (Ding et al., 2012). Perhaps sme2+ transcription establishes a heterochromatic state at this locus that facilitates pairing of homologous chromosomes. IncRNAs are also frequently found in mammalian promoters (Guttman et al., 2009). The regulation of mammalian promoters is often complex, and integration of multiple inputs is the norm rather than the
exception. Perhaps IncRNAs in these systems too serve to inhibit transcription. The principles of these cell-fate control by IncRNAs in budding yeast may thus also shed light onto complex developmental decisions in higher eukaryotes.

**EXPERIMENTAL PROCEDURES**

**Strains and Plasmids**

All strains used in this study are derivatives of SK1 and are listed in Table S1; plasmids are in Table S2. Gene or promoter deletions, tagging of genes, and plasmid constructions are described in the Extended Experimental Procedures.

**Growth Conditions**

Synchronous meioses were performed as described in Falk et al. (2010). To examine viability (Figures 3C, 4C, and S6G), cells were incubated for 14 days in sporulation medium at room temperature, before spotting 5-fold serial dilutions on YPD plates.

**Other Methods**

Northern blot analysis was performed as described (Hochwagen et al., 2005) with minor modifications (Extended Experimental Procedures). ChIP assays are as described in van Werven and Timmers (2006), and RNA FISH analyses were performed as described in Bumgarner et al. (2012) with minor modifications (Extended Experimental Procedures). β-galactosidase assays are described in Jambhekar and Amon (2008). Meiotic nuclear divisions were examined in cells fixed with 80% ethanol overnight and stained with DAPI. For each time point, 100 cells were counted. Meiosis I or meiosis II cells were defined as cells with two or four distinct DAPI masses, respectively.

**SUPPLEMENTAL INFORMATION**

Supplemental Information includes Extended Experimental Procedures, seven figures, and three tables and can be found with this article online at http://dx.doi.org/10.1016/j.cell.2012.06.049.

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