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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 *MAT*a/α but not haploid *MAT*a or *MAT*α cells undergo gametogenesis, known as sporulation. We find that transcription of two long noncoding RNAs (lncRNAs) mediates mating-type control of sporulation. In *MAT*a or *MAT*α 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 *MAT*a/α diploids. Conversely, expression of the two lncRNAs abolishes sporulation in *MAT*a/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 *MAT***a**/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*, *i*nducer of *me*iosis *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 *MAT***a**/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 *MAT***a**/α diploid cells, *RME1* is not expressed. This is because the *MAT***a** locus encodes a1 and the *MAT*α locus α2, which together form the a1-α2 repressor complex that inhibits

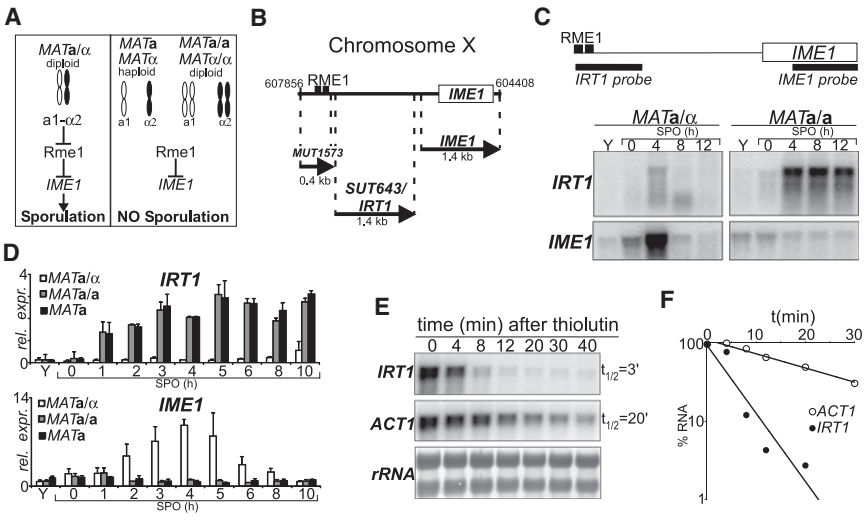

Figure 1. The Noncoding RNA *IRT1* Is Transcribed through the *IME1* Promoter

(A) Mating-type control of *IME1* expression. See text for details.

(B) Overview of the *IME1* locus. The locations of *IME1*, the noncoding RNA *IRT1* (formerly *SUT643*), and *MUT1573* are shown. The arrows show direction of transcription.

(C) *MAT***a**/α (A4962) and *MAT***a**/**a** (A28374) cells were grown to saturation in YPD (Y) for 24 hr followed by growth in BYTA medium overnight. Cells were then transferred into sporulation (SPO) medium to induce sporulation. Samples were taken at the indicated times to examine *IME1* and *IRT1* RNA levels. The cartoon above the blot indicates the locations of the probes used to detect *IME1* and *IRT1*.

(D) Haploid *MAT***a** (A4841), *MAT***a**/α (A4962), and *MAT***a**/**a** (A28374) diploid cells were induced to sporulate as described in (C), and *IRT1* and *IME1* RNA levels were analyzed at the indicated time points by RT-PCR. RNA levels were normalized to *ACT1* expression. The data are represented as mean ± SEM from multiple experiments. See also Figure S1.

(E and F) *MAT***a** (A4841) cells were induced to sporulate. After 6 hr, thiolutin (3 μg/ml) was added, and *IRT1* and *ACT1* RNA levels were determined at the indicated times.

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 *MAT***a** or *MAT*α 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 *MAT***a**/α diploid cells, the a1-α2 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 *MAT***a** or *MAT*α mating type but not in cells of the diploid *MAT***a**/α 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 *MAT***a** or *MAT*α mating type to sporulate as efficiently as *MAT***a**/α diploid cells. Conversely, expression of these two lncRNAs abolishes the ability of *MAT***a**/α 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 *MAT***a**/α mating type and only under sporulation-inducing conditions (Figures 1C and S1A). 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 *u*nannotated *t*ranscript 643 (*SUT643*) (Xu et al., 2009), is transcribed from the same strand as *IME1* (Figure 1B). This transcript is weakly expressed in *MAT***a**/α diploid cells upon induction of sporulation but highly expressed when *MAT*α/α diploid cells are incubated in SPO medium (Figure S1B). Northern blot and quantitative RT-PCR analyses confirmed this result (Figures 1C and 1D). In *MAT***a**/**a** diploid cells and *MAT***a** 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,

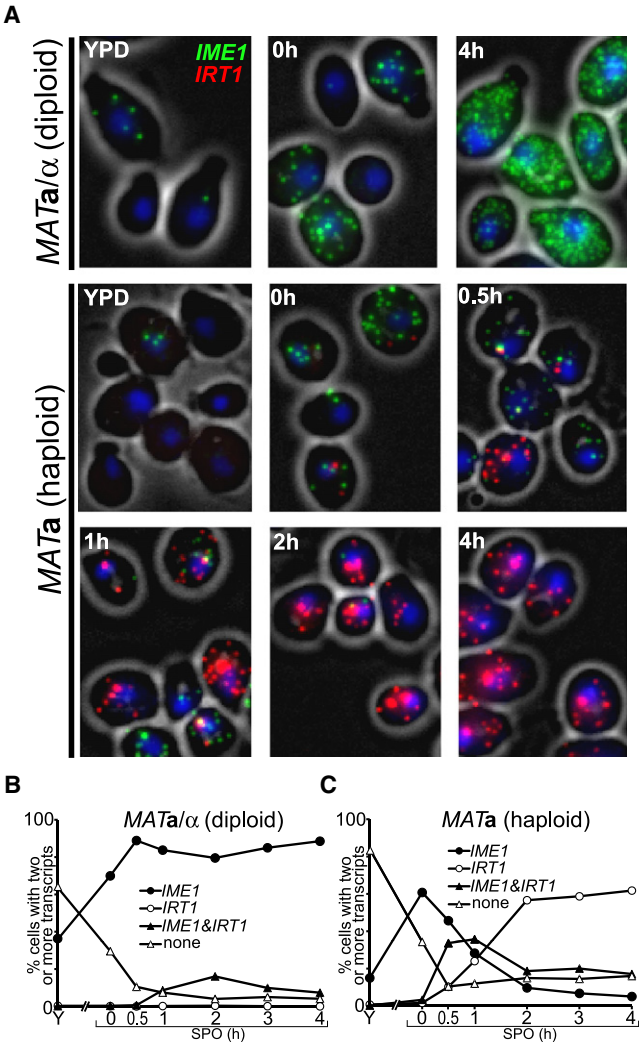

Figure 2. *IRT1* and *IME1* RNA Levels Are Mutually Exclusive

(A) *MAT***a**/α diploids (A24333) and *MAT***a** haploids (A10931) were induced to sporulate. Samples were taken at the indicated time points to examine *IME1* and *IRT1* RNA in single cells. Merged images of *IRT1* (red) and *IME1* (green) transcripts are shown. DNA is shown in blue.

(B and C) Quantification of the percentage of cells with no transcripts (open triangles) or with two or more transcript of *IRT1* (open circles), *IME1* (closed circles), or both (closed triangles) is shown. At least 450 cells were analyzed per time point (see Table S3).

See also Figures S2 and S3.

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 I*ME1 r*egulatory *t*ranscript *1*. We detected a second, shorter transcript upstream of *SUT643,* designated as *m*eiotic *u*nannotated *t*ranscript *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 *MAT***a**/α diploid and *MAT***a** 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 *MAT***a**/α cells harbor *IME1* transcripts. In contrast, *IRT1* RNA is barely detectable (Figures 2B and S3A).

In the *MAT***a** 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 *MAT***a** 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 *MAT***a** or *MAT*α 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, *MAT***a**/**a** diploid and *MAT***a** 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 *MAT***a** or *MAT*α mating type.

The transcription factor Rme1 is required for the repression of *IME1* in cells of the haploid *MAT***a** or *MAT*α 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).

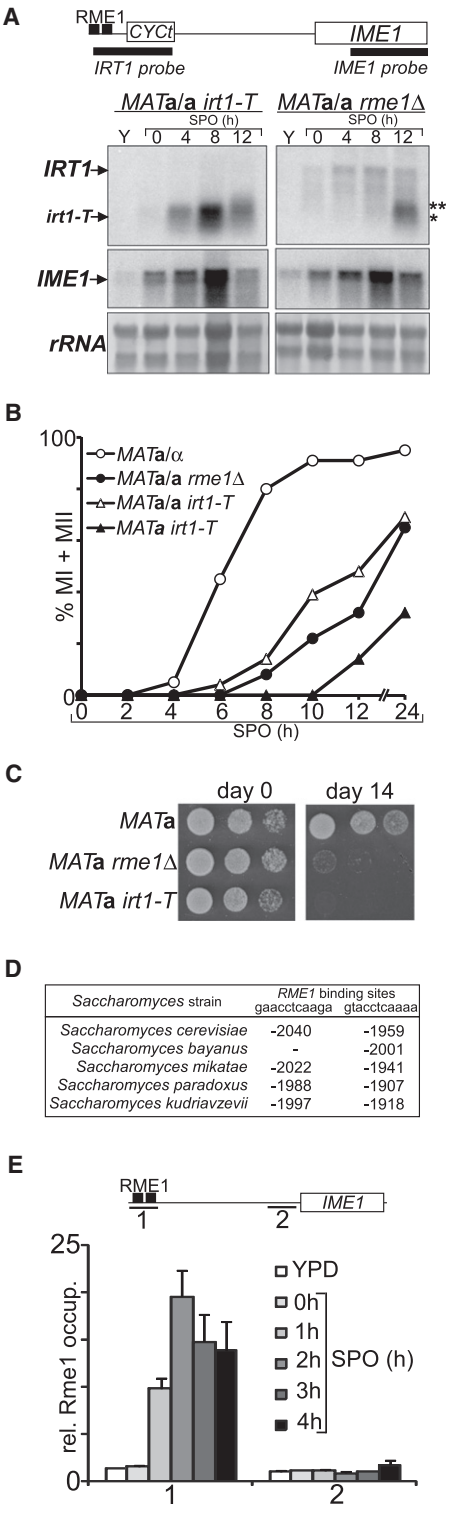

Figure 3. Rme1-Dependent *IRT1* Transcription Inhibits *IME1* Expression

(A) Analysis of *IRT1* and *IME1* expression in *MAT***a**/**a** *irt1-T* (A30070; truncated *IRT1*) and *MAT***a**/**a** *rme1*Δ (A30195) cells progressing through sporulation in a synchronous manner. RNA samples were taken from cells grown in YPD (Y) or SPO medium for 0, 4, 8, and 12 hr. *rRNA* is shown as a loading control. (*) marks the truncated version of the *IRT1* transcript. (**) marks the *MUT1573* transcript, which accumulates during late stages of sporulation. The *MAT***a**/*α* and *MAT***a**/**a** controls for this experiment are shown in Figure 1C as these experiments were performed at the same time.

(B) *MAT***a**/*α* (open circles; A4962), *MAT***a**/**a** *rme1*Δ (closed circles; A30195), *MAT***a**/**a** *irt1-T* (open triangles; A30070) diploid cells, and *MAT***a** *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) *MAT***a** (A4841), *MAT***a** *rme1*Δ (A30075), and *MAT***a** *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 *MAT***a** *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.

To test whether *RME1* is required for *IRT1* expression, we examined the consequences of deleting *RME1*. We found that *IRT1* expression was lost in *MAT***a** *rme1*Δ haploid and *MAT***a**/**a** *rme1*Δ diploid cells or *MAT***a** 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 *rme1*Δ strain were remarkably similar to that of *MAT***a**/**a** 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 *MAT***a** or *MAT*α mating type through activation of *IRT1* transcription. The observation that sporulation is not as efficient in *rme1*Δ or *irt1-T MAT***a**/**a** cells as it is in *MAT***a**/*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 *MAT***a** 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),

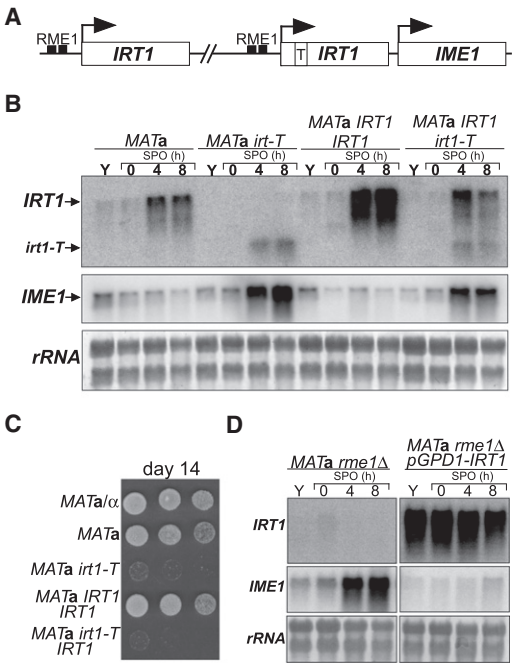

Figure 4. *IRT1* Represses *IME1* In *cis*

(A) Structure of the duplicated *IRT1* locus. The plasmid backbone harboring *URA3* and *lacZ* is located between the two *IRT1* genes.

(B) *MAT***a** *IRT1* (A4841), *MAT***a** *irt1-T* (A30067), *MAT***a** *IRT1 IRT1* (A30197), and *MAT***a** *IRT1 irt1-T* (A30199) cells were induced to sporulate. Samples were taken at the indicated times to determine the amount of *IME1* and *IRT1* RNA.

(C) Strains described in (B) were induced to sporulate. Cells were harvested either before transfer into SPO medium or after a 14 day incubation. 5-fold serial dilutions were spotted onto YPD plates.

(D) *MAT***a** *rme1*Δ (A30075) and *MAT***a** *rme1*Δ *pGPD1-IRT1* (A30134) cells were induced to sporulate. Samples were taken at the indicated times to determine the amount of *IRT1* and *IME1* RNA.

See also Figure S5.

IME1 was expressed in the *MAT***a** haploid strain, and cells underwent a lethal meiosis (Figures 4B and 4C).

The second way by which we tested the importance of *IRT1* location with respect to *IME1* regulation was by comparing the impact of constitutive expression of *IRT1* from its native locus versus an ectopic locus. Expression of *IRT1* from the constitutive *GPD1* promoter (*pGPD1-IRT1*) was sufficient to prevent *IME1* expression in *MAT***a** *rme1*Δ cells (Figure 4D). Furthermore, whereas *MAT***a**/**a** *rme1*Δ or *MAT***a**/α diploid cells readily sporulate, the same cells expressing *pGPD1-IRT1* at the *IRT1* locus showed poor sporulation (Figures S5D and S5E). Placing *pGPD1-IRT1* upstream of an ectopic locus, a *lacZ* reporter gene integrated at *URA3,* did not affect the kinetics of entry into sporulation of *MAT***a**/**a** *rme1*Δ or *MAT***a**/α diploid cells, but *lacZ* expression was affected (Figures S5D–S5F). Our results show that *IRT1* transcription represses *IME1* in *cis.*

IRT1 Prevents Transcriptional Activators from Binding to the *IME1* Promoter

How does *IRT1* transcription interfere with *IME1* expression? *IRT1* transcription could prevent the recruitment of *IME1* transcriptional activators from binding the *IME1* promoter. To test this possibility, we examined the effects of *IRT1* expression on the binding of known transcriptional activators to the *IME1* promoter. In a screen to be described in detail elsewhere, we identified *POG1* as being required for full *IME1* expression. Pog1 activates *CLN2* expression and binds to the promoters of genes encoding cell-cycle regulators (Horak et al., 2002; Leza and Elion, 1999). *POG1* is also needed for wild-type level expression of *IME1*. In a *pog1*Δ strain, *IME1* expression is reduced and entry into and progression through sporulation are delayed (Figures 5A and 5B). Furthermore, Pog1 associates with the *IME1* promoter in a region −750 and −1050 bp upstream of the translation start site. This binding is developmentally regulated, being low upon transfer into sporulation-inducing conditions but increasing as cells progress through early stages of sporulation (3 hr time point; Figures 5C–5E).

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 *MAT***a**/α diploid cells, Pog1 binding was induced under sporulation-inducing conditions (Figure 5E). In *MAT***a** 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 *MAT***a**/α 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 *irt1-T* 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 *MAT***a** haploid and *MAT***a**/α 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 *MAT***a** and *MAT***a**/α 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 *MAT***a** haploid and *MAT***a**/α diploid cells. Nucleosome occupancy was high in both *MAT***a** haploid and *MAT***a**/α 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 *MAT***a** haploid and *MAT***a**/α 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,

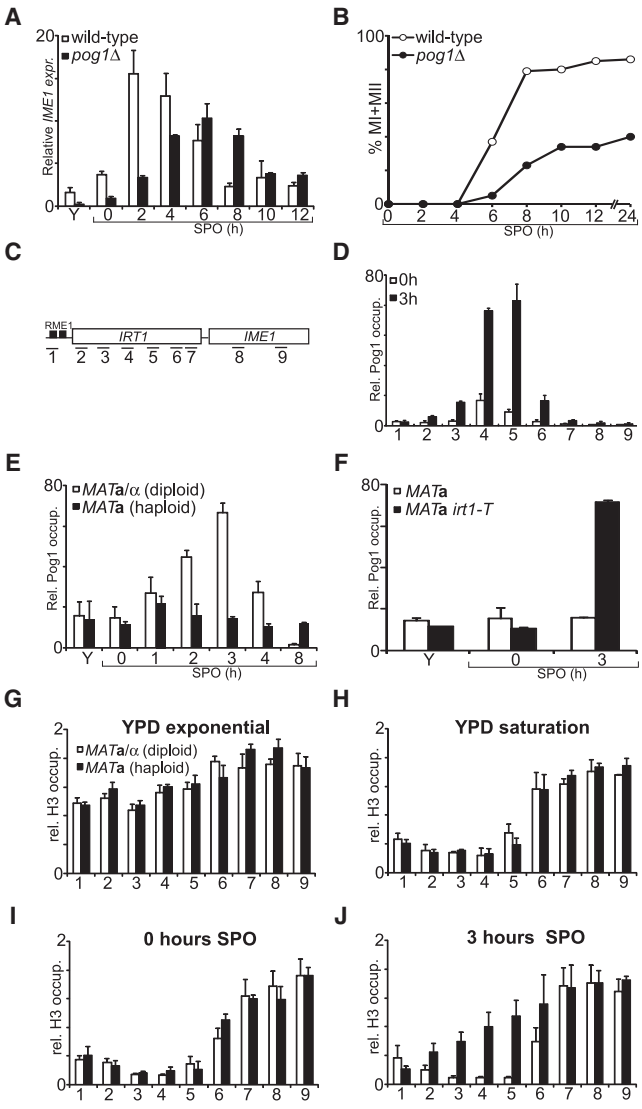

Figure 5. *IRT1* Transcription Inhibits Pog1 Recruitment and Increases Nucleosome Occupancy at the *IME1* Promoter

(A and B) Wild-type (A4962) and *pog1*Δ (A30194) *MAT***a**/*α* 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) *MAT***a**/*α* 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 *MAT***a**/*α* diploid and *MAT***a** haploid cells (A30235) at the indicated times. Primer pair 4 was used for this analysis.

(F) Wild-type (A30235) and *irt1-T MAT***a** 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 *MAT***a** haploid (A4841) and *MAT***a**/*α* diploid (A4962) cells. Cells were either grown in YPD (exponential phase or to saturation) or induced to sporulate for 0 or 3 hr.

high nucleosome occupancy was re-established in *MAT***a** haploid cells but not in *MAT***a**/*α* diploid cells (Figure 5J). These results show that nucleosome re-assembly at the *IME1* promoter occurs in *MAT***a** 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 *MAT***a** 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 *MAT***a** 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 S6D). *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*

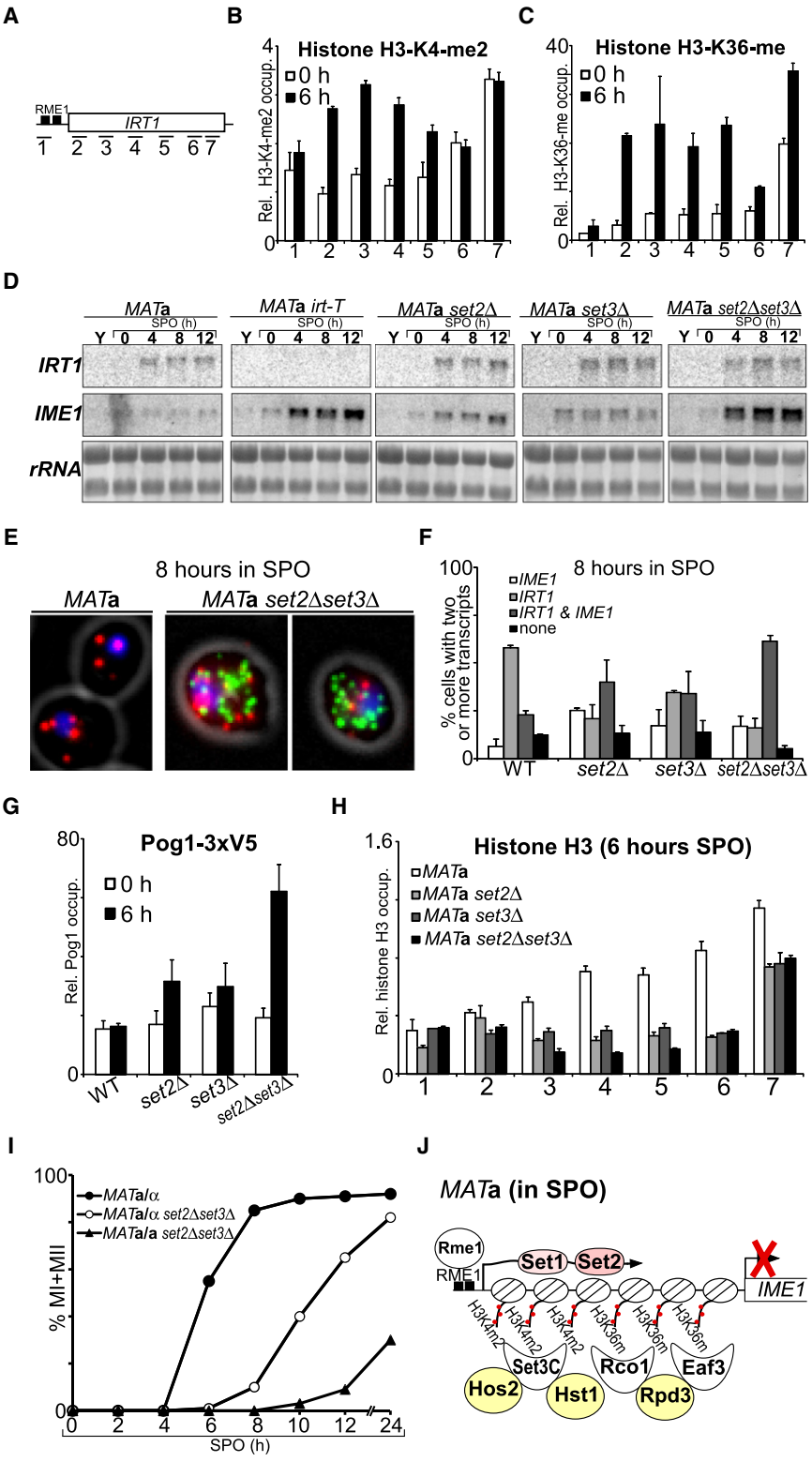

Figure 6. *SET2* and *SET3* Are Required for *IRT1* Transcription-Mediated Repression of the *IME1* Promoter

(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 *MAT***a** haploid (A4841) cells. The data are represented as mean ± SEM from multiple experiments.

(D) Wild-type (A4841), *irt1-T* (A30067), *set2*Δ (A31995), *set3*Δ (A31999), and *set2*Δ *set3*Δ (A32040) *MAT***a** 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), *set2*Δ (A31992), *set3*Δ (A31998), and *set2*Δ *set3*Δ (A32051) *MAT***a** haploid cells were induced to sporulate to examine *IME1* and *IRT1* RNAs in single cells. (E) shows *set2*Δ *set3*Δ 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), *set2*Δ (A32036), *set3*Δ (A32033), and *set2*Δ *set3*Δ (A32049) *MAT***a** 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) *MAT***a**/α (closed circles; A4962), *MAT***a**/α *set2*Δ *set3*Δ (open circles; A32041), and *MAT***a**/**a** *set2*Δ *set3*Δ (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.

single and double mutants. In contrast to wild-type *MAT***a** 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 6G). 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. *MAT***a**/α *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 *MAT***a**/**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

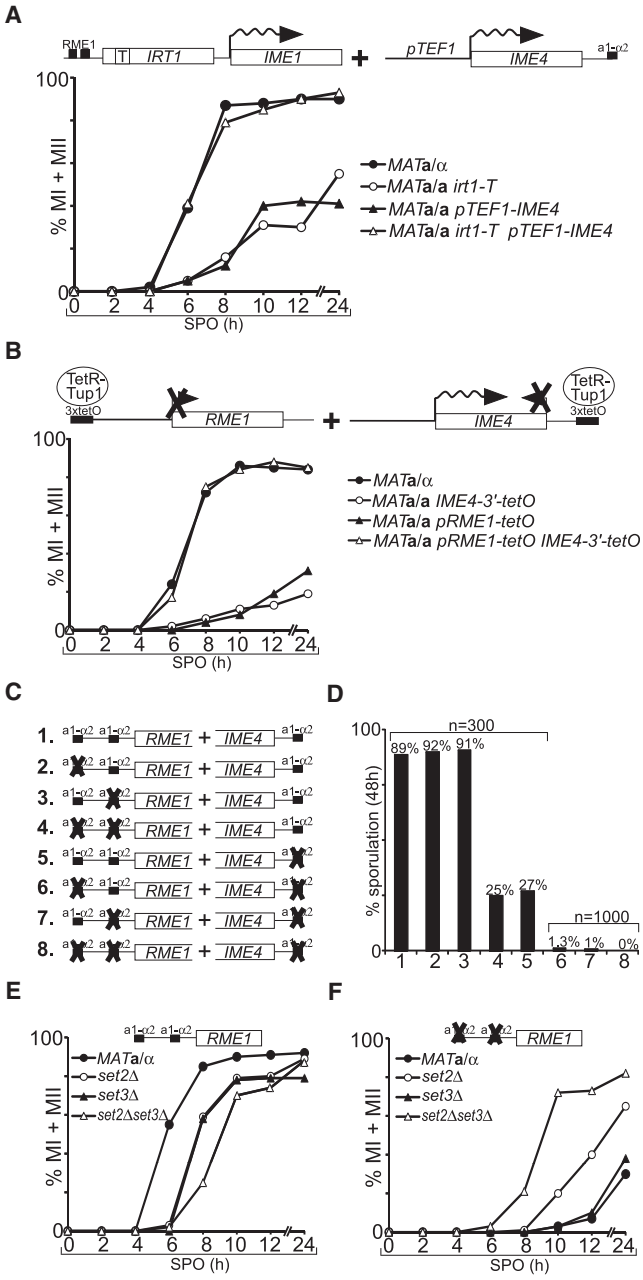

Figure 7. Transcription of Two lncRNAs Conveys Mating-Type Control of Sporulation

(A) *MAT***a**/α (A4962; closed circles), *MAT***a**/**a** *irt1-T* (A30070; open circles), *MAT***a**/**a** *pTEF-IME4* (A30133; closed triangles), and *MAT***a**/**a** *pTEF-IME4 irt1-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) *MAT***a**/α cells (closed circles), *MAT***a**/**a** cells in which the a1-α2-binding sites in the *IME4-AS* promoter were replaced by tetO sequences (*MAT***a**/**a** *pIME4-3′-tetO*; open circles; A30217), *MAT***a**/**a** cells in which the a1-α2-binding sites in the *RME1* promoter were replaced by tetO sequences (*MAT***a**/**a** *pRME1-tetO*; closed triangles; A30231), and *MAT***a**/**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).

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 6J). 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 *MAT***a** haploid and *MAT***a**/**a** diploid cells to induce *IME1* and to enter sporulation. However, these cells do not sporulate with the same kinetics and efficiency as *MAT***a**/α 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 *MAT***a**/α 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 *irt1-T* allele with an *IME4* allele driven from the constitutive *TEF1* promoter (*pTEF1-IME4*). Whereas each individual allele allowed 50% of *MAT***a**/**a** cells to sporulate with a delay, the combination of the two brought about sporulation efficiencies and kinetics seen in *MAT***a**/α diploid cells (Figure 7A).

We were also able to induce *MAT***a**/α levels of sporulation in *MAT***a**/**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; Bellí 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. *MAT***a**/**a** diploid cells that either harbor only

(C) *MAT***a**/α 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), *set2*Δ (A31996), *set3*Δ (A32001), and *set2*Δ *set3*Δ (A32041) *MAT***a**/α 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) *MAT***a**/α 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, *MAT***a**/**a** diploid cells formed spores with the same kinetics and efficiency as *MAT***a**/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 *MAT***a**/α levels of sporulation in *MAT***a**/**a** cells.

What are the effects of expressing *IRT1* and *IME4-AS* in *MAT***a**/α cells? In *MAT***a**/α cells, the a1-α2 repressor inhibits the transcription of the *IRT1* transcription factor *RME1* and *IME4-AS*. The *RME1* promoter harbors two a1-α2-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 *MAT***a**/α strains. Inactivating single a1-α2 sites in the *RME1* promoter had little effect on sporulation (Figure 7D). Inactivating both a1-α2-binding sites in the *RME1* promoter led to expression of *RME1* in *MAT***a**/α cells similar to what is seen in *MAT***a** cells, indicating that the *RME1* promoter is fully derepressed (Figure S7E, compare *MAT***a** 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-α2-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-α2-binding sites in the *RME1* promoter with a deletion of the a1-α2-binding site in the *IME4-AS* promoter. Deleting the *IME4-AS* a1-α2-binding site dramatically reduced sporulation in *MAT***a**/α cells (Figures 7C, 7D, and S7G) (Hongay et al., 2006), but inactivation of all three a1-α2-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 *MAT***a** or *MAT*α 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 lncRNA 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 lncRNA 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 lncRNAs has also been implicated in transcriptional

activation (Hirota et al., 2008; Houseley et al., 2008; Pinskaya et al., 2009; Uhler et al., 2007). It will be interesting to determine the relative importance of lncRNA-mediated transcriptional activation 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, Set2 and Set3, 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 transcription by Set2 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 *MAT***a** or *MAT*α 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 *MAT***a**/α diploid cells but also in cells expressing the *MAT***a** or *MAT*α 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 *MAT***a** haploids is 10-fold lower compared to *IME1* in *MAT***a**/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 prerequisite 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 *MAT***a** or *MAT*α 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 *MAT***a**/α diploid cells in both efficiency and kinetics. Conversely, deleting three a1-α2-binding sites, two at the *RME1* promoter and one in the *IME4-AS* promoter, abolished the ability for *MAT***a**/α diploid cells to sporulate. Thus, transcription of two lncRNAs is all that mediates mating-type control of sporulation. Why did budding yeast evolve the use of lncRNA transcription to govern this key cell-fate decision? Perhaps repression of complex promoters by lncRNA 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 lncRNA 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 lncRNA transcription is that repression is the default. Repression is alleviated only in *MAT***a**/α diploid cells, through the repression of *IRT1* and *IME4-AS*.

lncRNAs 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 lncRNAs 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 lncRNA transcription as a regulatory tool may impact biological processes beyond transcription. In fission yeast meiosis, the *sme2*$^+$ lncRNA 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. lncRNAs 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 lncRNAs in these systems too serve to inhibit transcription. The principles of cell-fate control by lncRNAs 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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