The IncRNA RZE1 Controls Cryptococcal Morphological Transition

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Abstract

In the fungal pathogen Cryptococcus neoformans, the switch from yeast to hypha is an important morphological process preceding the meiotic events during sexual development. Morphotype is also known to be associated with cryptococcal virulence potential. Previous studies identified the regulator Znf2 as a key decision maker for hypha formation and as an anti-virulence factor. By a forward genetic screen, we discovered that a long non-coding RNA (lncRNA) RZE1 functions upstream of ZNF2 in regulating yeast-to-hypha transition. We demonstrate that RZE1 functions primarily in cis and less effectively in trans. Interestingly, RZE1’s function is restricted to its native nucleus. Accordingly, RZE1 does not appear to directly affect Znf2 translation or the subcellular localization of Znf2 protein. Transcriptome analysis indicates that the loss of RZE1 reduces the transcript level of ZNF2 and Znf2’s prominent downstream targets. In addition, microscopic examination using single molecule fluorescent in situ hybridization (smFISH) indicates that the loss of RZE1 increases the ratio of ZNF2 transcripts in the nucleus versus those in the cytoplasm. Taken together, this lncRNA controls Cryptococcus yeast-to-hypha transition through regulating the key morphogenesis regulator Znf2. This is the first functional characterization of a lncRNA in a human fungal pathogen. Given the potential large number of lncRNAs in the genomes of Cryptococcus and other fungal pathogens, the findings implicate lncRNAs as an additional layer of genetic regulation during fungal development that may well contribute to the complexity in these “simple” eukaryotes.

Author Summary

The involvement of non-protein regulators in developmental processes in higher eukaryotes is an area that has come to light more recently. Earlier known as the dark matter of the genome, the non-protein coding genes are now recognized for their important regulatory roles in the life of eukaryotes. Using forward genetic screen, we identified RZE1 as a
IncRNA with key function in regulating morphogenesis in Cryptococcus. We further discovered that RZE1 regulates the transcription and transcript export of ZNF2, which encodes the key morphogenesis transcription factor. RZE1 is the first functionally characterized lncRNA in a human fungal pathogen. Given the potential large number of lncRNAs in Cryptococcus and other fungal pathogens, the RZE1-ZNF2 regulatory system could serve as a paradigm for the investigation of lncRNAs in development and virulence in eukaryotic pathogens.

Introduction

In many human fungal pathogens, the morphological transition from yeast to hypha plays a central role in pathogenesis [1, 2], as demonstrated in the ascomycetes Candida albicans, Penicillium marneffei, Histoplasma capsulatum, Coccidioides immitis, and Paracoccidioides brasiensis [3–6]. Different morphotypes also display different levels of pathogenicity in the basidiomycetous fungus Cryptococcus neoformans [1, 7], the causative agent of the deadly cryptococcal meningitis [8]. Although primarily considered as yeasts, Cryptococcus undergoes yeast-to-hypha transition during unisexual mating (self-fruited) or bisexual α-α mating [9–11].

The zinc finger transcription factor Znf2 ultimately controls this morphotype transition. During mating, Znf2 is activated by the pheromone MAPK pathway controlled by the HMG domain transcription factor Mat2 [12–15] (Fig 1A). Mat2 is essential for pheromone sensing and response, which leads to the cell fusion event. Hyphal growth commences after cell fusion and eventually gives rise to fruiting structures and meiotic spores [9, 16]. However, Mat2 does not control hyphal morphogenesis per se [12]. By contrast, Znf2 governs hypha generation and it is dispensable for the early mating events like cell fusion [12, 17] (Fig 1A). Under non-mating inducing conditions, Znf2 could be activated by the matri-cellular signal protein Cfl1 through a positive feedback regulation [18, 19]. It is unknown whether other host or environmental factors can also regulate Znf2 activity.

Znf2 is an anti-virulence factor in the mouse model of cryptococcosis [12, 20]. The deletion of the ZNF2 gene locks the fungal cells in the yeast form, making them more virulent [12]. Conversely, the activation of ZNF2 drives filamentation and attenuates Cryptococcus virulence [21, 22]. The ZNF2 overexpression cells, either in the live or heat-killed form, can protect the hosts from a subsequent challenge with otherwise lethal wild-type cells [22]. Thus manipulation of ZNF2 activity could be a potential means to alleviate cryptococcosis. Besides its anti-virulence effect during cryptococcal infection in a mammalian host, Znf2 also shapes cryptococcal interaction with other heterologous hosts, such as the soil amoeba Acanthamoeba castellani and the insect Galleria mellonella [23]. The essential role of Znf2 in Cryptococcus sexual cycle and its pivotal role in regulating cryptococcal interaction with various host species make this transcription factor a potential target for multi-layered regulation in response to various stimuli.

To identify the upstream regulators of ZNF2, we conducted a forward genetic screen to find mutations that cause similar phenotypes as those caused by the disruption of ZNF2. The screen led to the discovery of RZE1 that functions upstream of ZNF2. Further investigation revealed that RZE1 functions primarily as a cis-acting, and less efficiently a trans-acting, lncRNA. Furthermore, this lncRNA is functionally restricted to its native nuclei based on heterokaryon assay. We found that RZE1 exerts its impact on cryptococcal morphogenesis by regulating ZNF2 transcription and by influencing the nuclear versus cytoplasmic distribution of ZNF2 transcripts, which consequently affects ZNF2’s ability to get translated into protein.
Although genomic and transcriptomic data suggest the presence of lncRNAs showing infection- or tissue-specific expression in fungal species pathogenic to plants and animals [24], it is unclear if lncRNAs have any biological relevance in the life cycle or the development of human fungal pathogens.

**Results**

**Identification of the RZE1 gene for its importance in filamentation via forward genetic screen**

*Cryptococcus* undergoes filamentation in response to the mating signal and other environmental cues. Znf2 is the essential regulator of this morphological switch and it bridges morphogenesis and virulence in this fungal pathogen [12, 17–19]. To identify the regulatory network of the Znf2-controlled filamentation pathway, we conducted a random insertional mutagenesis screen for znf2Δ-like phenotype in the self-filamentous strain XL280 [25]. XL280 has been commonly used in morphogenesis studies [12, 20, 25]. It has a publically released genome sequence (~19 Mb, ~7000 genes) [26] and a well characterized congenic pair [21]. We generated 63,000 insertional mutants via *Agrobacterium*-mediated transformation in XL280 and screened these mutants on filamentation-inducing V8 medium for non-filamentous phenotypes. Among a set of selected non-filamentous mutants, 15 had their insertion site identified
by inverse PCR and sequencing (Table 1). Of the 15 insertion sites identified, one T-DNA insertion (Tn) in mutant X261 was found to be in the genetic locus that we named \textit{RZE1}. The \textit{RZE1} gene encodes a 1,268 nt long transcript in XL280 based on our primer walking and RACE PCR results (S1 Fig). Only one transcription start site and one transcription stop site were identified for \textit{RZE1} under the tested condition.

Disruption of \textit{RZE1} recapitulates the phenotypes caused by the deletion of \textit{ZNF2}

The \textit{rze1}\textsuperscript{Tn} mutant, like the \textit{znf2}\textsuperscript{Δ} mutant, is non-filamentous under mating-inducing conditions such as on V8 media (Fig 1B). To confirm the role of \textit{RZE1} in hyphal growth, we deleted the \textit{RZE1} gene in the XL280 background. The targeted deletion of \textit{RZE1} also abolished self-filamentation (Fig 1B). To ensure that the non-filamentous phenotype of the \textit{rze1}\textsuperscript{Tn} and the \textit{rze1}\textsuperscript{Δ} mutants was attributable to the disruption of \textit{RZE1} and not due to other cryptic mutations, a wild-type copy of the \textit{RZE1} gene was re-introduced ectopically into the \textit{rze1}\textsuperscript{Tn} and the \textit{rze1}\textsuperscript{Δ} mutants. The ectopically integrated \textit{RZE1} partially restored the filamentation defects in these \textit{rze1} mutants (S2 Fig). We also introduced the wild-type \textit{RZE1} back to its native locus in the \textit{rze1}\textsuperscript{Tn} and the \textit{rze1}\textsuperscript{Δ} mutants. The introduced \textit{RZE1} gene at its native locus effectively restored the ability of both mutants to filament (Fig 1B), indicating that \textit{RZE1} indeed is required for filamentation.

The pheromone sensing pathway is critical for filamentation under mating-inducing conditions. Disruption of the pheromone sensing pathway (e.g. the deletion of HMG domain transcription factor Mat2 or the MAPKK Ste7), even in one mating partner, will abolish filamentation produced by bisexual mating between \textit{a} and \textit{α} partners [12, 14]. The lack of the bisexual mating hyphae is due to lack of cell fusion. It is previously established that the disruption of \textit{ZNF2} does not impair pheromone pathway or abolish cell fusion [12]. Thus a unilateral cross involving the \textit{znf2}\textsuperscript{Δ} mutant and a wild-type partner still produces mating filaments, in
contrast to the unilateral cross involving one *mat2Δ* mating partner [12]. We then decided to assess if this also holds true for RZE1. To avoid the complication due to self-filamentation in the XL280 background, we chose to delete RZE1 in the non-self-filamentous strain H99. In the H99 background, hyphae can only result from bisexual mating following cell fusion between a and α cells activated by the pheromone pathway. We found that the unilateral crosses between the rze1Δ (or the rze1Tn) mutant with a wild-type mating partner produced mating filaments (Fig 1C), as in the znf2Δ mutant and unlike the mat2Δ mutant (Fig 1C). Consistently, the pheromone production in the rze1Δ mutant was at par with the wild type, indicating that RZE1, like ZNF2, is not required for pheromone sensing pathway.

Under non-mating inducing conditions like on the YPD medium, ZNF2 and thereby filamentation can be activated by the matricellular signaling protein Cfl1 through a positive feedback loop [18]. Znf2 is absolutely necessary for *Cryptococcus* to respond to exogenous Cfl1 signal to promote filamentation [20]. Similarly, we found that RZE1 is also required for the recipient strain to filament in response to the CFL1 protein released from nearby donor cells under mating-suppressing conditions (Fig 1D).

Morphological switch from the yeast state to the filamentous state is inversely correlated with virulence in *Cryptococcus*. Consequently, the loss of ZNF2 modestly increases fungal virulence [20]. Thus we hypothesize that the upstream regulator of ZNF2 might also influence cryptococcal virulence potential. Since mice are highly susceptible to XL280 (*C. neoformans* var. *neoformans*, serotype D) [21, 27] and H99 (*C. neoformans* var. *grubii*, serotype A) [28, 29], we decided to compare the virulence between the wild-type strain and the rze1Δ mutant made in both XL280 and H99 backgrounds using the fungal burden assays. We inoculated the mice with the rze1Δ mutants and their corresponding wild-type strains through inhalation and measured the fungal burdens in the lungs at day 10 post inoculation. The fungal burdens in the lungs infected by the rze1Δ mutants were close to 2 fold higher than those infected by the corresponding wild-type controls (Fig 1E), indicating enhanced virulence of the rze1Δ mutants. Given that an increase in virulence with gene disruption is rare, these results are consistent with the observed enhanced virulence of the znf2Δ mutant, which showed about 2.4 fold increase in lung fungal burden compared to the wild type at the same time point during infection [12, 20]. In line with our previous observations for the znf2Δ mutant [12, 20], the rze1Δ mutant did not show any apparent difference from the wild type in classic virulence traits such as melanization, capsule production, or thermo-tolerance (S3 Fig). We also did not observe any apparent alteration in the susceptibility of the rze1Δ mutant to stressors like SDS, caspofungin (inhibitor of β-1,3-glucan synthase), calcoflour white (inhibitor of chitin), iron chelator, UV radiation, or oxidative stress (H2O2) when compared to the wild-type control (S3 Fig). Taken together, these observations indicate that the disruption of RZE1 recapitulates *in vitro* and *in vivo* phenotypes caused by the deletion of ZNF2. Thus RZE1 might be a highly selective regulator of ZNF2 or a major target of ZNF2.

**RZE1 is physically and functionally upstream of ZNF2**

*RZE1* is located ~2.5 kb upstream of ZNF2. Although the average intergenic space is less than 800 bp in *Cryptococcus* [30], the physical location of RZE1 raises a concern that the RZE1 transcript could be part of the ZNF2 transcript and the disruption of RZE1 would cause the disruption of ZNF2 itself. To address this concern, we amplified and compared four regions (I—IV in Fig 2A) that cover RZE1, the beginning of the ZNF2 coding region, and the region between RZE1 and ZNF2, using templates of total cDNA or genomic DNA derived from the wild-type XL280 strain. Region II that lies between RZE1 and the 5’ region upstream of the ZNF2 ORF failed to yield any detectable amplicon when cDNA was used as the template (Fig 2A). This
suggests that \textit{RZE1} is likely produced as a separate transcript independent from \textit{ZNF2}. Furthermore, the ability of the ectopically introduced \textit{RZE1} to partially complement the \textit{rze1} \textit{Tn} and the \textit{rze1} \textit{Δ} mutants (S2A Fig) reinforces the idea that \textit{RZE1} is a functionally independent transcript from that of \textit{ZNF2}. In addition, since the phenotype of the \textit{znf2} \textit{Δ} mutant can be complemented effectively using an ectopic copy of \textit{ZNF2} with 1kb sequence upstream of the \textit{ZNF2} ORF that does not include \textit{RZE1} (S4 Fig), it is reasonable to conclude that \textit{RZE1} and \textit{ZNF2} encode separate transcripts that can function independently.

To investigate the relationship between \textit{RZE1} and \textit{ZNF2}, we examined the \textit{RZE1} transcript level in the wild type, the \textit{znf2} \textit{Δ} mutant, and the \textit{ZNF2} oe strain under three different growth conditions (YPD/rich medium, serum/host relevant, and V8/mating-inducing). The transcript level of \textit{RZE1} in the wild-type H99 strain was relatively stable, and there was no dramatic difference when cells were cultured in YPD medium, on V8 medium, or in serum (Fig 2C). Under the same culture conditions, the \textit{RZE1} transcript level remained constant among the different strains (black bars in Fig 2D–2F), even though the \textit{ZNF2} transcript level was drastically different among these strains (grey bars in Fig 2D–2F). This indicates that \textit{RZE1} is not responsive to changes in the \textit{ZNF2} transcript level. This result supports the earlier conclusion that \textit{RZE1} and \textit{ZNF2} encode separate transcripts that can function independently.
the partial restoration of filamentation to these rze1 mutants (Fig 2B), indicating that ZNF2 indeed functions downstream of RZE1. These observations are in accordance with the idea that RZE1 functions in the filamentation pathway upstream of ZNF2.

**RZE1 likely functions as a lncRNA**

Based on the *Cryptococcus* EST databases, the RZE1 transcript is present in all subspecies of the *Cryptococcus neoformans* species complex, namely *C. neoformans* var. *grubii* (serotype A), *C. neoformans* var. *neoformans* (serotype D), and the subspecies *C. gattii* (serotype B and C). An analysis of the RZE1 transcript sequence suggests that the RZE1 gene is not a typical protein-coding gene. Rather than encoding one open reading frame (ORF), as expected for most mRNAs in fungi, RZE1 contains 5 short potential ORFs (Fig 3A), with three potential translation start codons (ATG) in a poor translation context (S1 Fig). BLAST searches using these potential ORFs against fungal genome databases or GenBank did not yield any significant hits. Thus, these potential ORFs do not encode conserved protein products.

To test if products from any of the small ORFs confer RZE1 function in filamentation, we inserted the constitutive active GPD1 promoter [31] (Fig 3B) or the CTR4 inducible promoter [32] (S5 Fig) upstream of each of the five ORFs contained within the RZE1 transcript. We introduced these constructs into the rze1Δ mutant and then tested the transformants for their

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**Fig 3. RZE1 is a likely IncRNA.** (A) The diagram of the five putative small open reading frames in RZE1 is shown in the top row. (B) The constitutive (P<sub>GPD1</sub>) or overexpression (P<sub>CTR4-2</sub>, see S5 Fig) of each putative open reading frame of RZE1 does not restore filamentation in the rze1Δ mutant. Cells were cultured on V8 medium for 4 days. (C) Partial complementation of the rze1Δ mutant with RZE1 modified for each potential translation start codon. All the start codon mutated alleles generated by single nucleotide mutagenesis and the wild-type allele of RZE1 were integrated at its native genetic locus in the rze1Δ mutant background. The mutated alleles showed partial complementation. Better complementation was achieved by the wild-type allele of RZE1. Cells were cultured on V8 medium for 4 days.

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ability to filament. None of the P_GPD1-ORF transformants were able to produce filaments (Fig 3B). Similarly, none of the P_CTR4-2-ORF transformants were able to filament under inducing conditions (S5 Fig). The results suggest that the potential ORFs carried within the RZE1 transcript are unlikely to produce protein products that function in the filamentation pathway. We postulate that RZE1 may function as a lncRNA instead.

To further determine whether RZE1 functions as a protein or a transcript, we did site-directed single nucleotide mutagenesis to alter the translation start codon of each of the five potential ORFs in RZE1 (Fig 3C). We mutated ATG to ATA or AAG as such changes are known to almost abolish translation initiation in other fungi [33]. These codon changes are expected to prevent or considerably reduce the translation of these potential ORFs. We then introduced each of these mutated RZE1 alleles into the rze1Δ mutant and selected transformants with the mutated RZE1 allele integrated into the RZE1’s native locus. We then examined the ability of these transformants to produce hyphae. To our amazement, all the mutated RZE1 alleles were able to restore the filamentation defect of the rze1Δ mutant (Fig 3C), although to a lesser degree compared to the wild-type RZE1 allele integrated at the native locus (Fig 3C). This finding indicates that these RZE1 alleles are at least partly functional in promoting filamentation, even though the specific nucleotides that are potential start-codons are mutated. Collectively, our results strongly suggest that RZE1 is a long non-coding RNA regulating morphogenesis in Cryptococcus.

RZE1 is functionally restricted to the nucleus

As a lncRNA, it is possible to be functionally restricted to its target within its native nucleus, as XIST or MALAT-1 [34–37], or to be functional in the cytoplasm [38]. This is in contrast to protein-coding mRNAs, which need to be exported from the nucleus to the cytoplasm for translation to make the functional products. Proteins produced by mRNAs would be able to function with its targets produced by any nucleus that shares the same cytoplasm. To narrow down the potential mode of action of RZE1, we decided to first examine if RZE1 is functionally restricted to its native nucleus. For this purpose, we designed a heterokaryon assay where two cells conjugate and thus share the cytoplasm, but their nuclei do not fuse. Heterokaryon assay was previously used to show that the lncRNA MALAT1 is functionally restricted to the nucleus [39]. Forming heterokaryons is a natural process during the a-α bisexual mating in Cryptococcus [9, 40, 41] (Fig 4A). The formation of α-a dikaryon after mating conjugation brings the homeodomain proteins Sxi1α and Sxi2a together [42], which triggers the expression of ZNF2 and the production of dikaryotic mating hyphae [9, 12, 43]. It is important to note that RZE1 controls filamentation but not conjugation (cell fusion), as established previously for Znf2 (Fig 1C) [12, 17]. Furthermore, the dikaryon will be blocked from filamentation only when ZNF2 is disrupted in both mating partners [12, 43]. For this assay, we used cryptococcal strains in H99 background because H99 does not self-filament. As a result, filaments produced from the α-α bisexual mating are all derived from conjugated dikaryons. This allows us to examine if RZE1 produced by one nucleus could compensate the loss of RZE1 in the other nucleus that shares the same cytoplasm. The idea is that if the RZE1 from one nucleus was able to function in the cytoplasm or in another nucleus in the same conjugated cell, then it would have been able to regulate ZNF2 activity even if ZNF2 is produced by the other nucleus (Fig 4A).

As expected, the unilateral crosses rze1Δ α x a, znf2Δ α x a, and rze1Δznf2Δ α x a all filaments at a reduced level compared to the WT cross α x a (Fig 4A). However, the dikaryons generated from the cross rze1Δ α x znf2Δ a or the cross rze1Δ a x znf2Δ α failed to filament (Fig 4A). These dikaryons contain one nucleus with RZE1 but no ZNF2, and the other nucleus with ZNF2 but no RZE1. The inability of these dikaryons to filament indicates that RZE1 produced
by one nucleus failed to support the activity of \( \text{ZNF2} \) transcribed by the other nucleus even though the two nuclei shared the same cytoplasm. Because the diploid \( \alpha/\alpha \) wild-type cells in \( H99 \) background did not filament under this condition, it precludes us from further assessing whether the diploid cells derived from nuclear fusion of the \( \text{rze1} \Delta \alpha \times \text{znf2} \Delta \alpha \) heterokaryon could filament. The decreased ability of the heterozygous diploid cells \( \alpha/\alpha \) compared to the corresponding dikaryons \( \alpha-\alpha \) to continue through the mating program appears to be a common phenomenon in basidiomycetes. Nonetheless, these findings indicate that \( \text{RZE1} \) is functionally restricted to its native nucleus and as such it is highly unlikely for \( \text{RZE1} \) to directly affect the \( \text{ZNF2} \) translation or other post-translational processes that occur in the cytosol.

**Fig 4. **\( \text{RZE1} \) is functionally restricted to its native nucleus. (A) Heterokaryon assay showing the functional restriction of \( \text{RZE1} \) to the nucleus of its origin. The diagram above shows the rationale behind this heterokaryon assay. The strains used for this assay are all in the non-self-filamentous \( H99 \) background. The complete lack of filamentation in the \( \text{rze1} \Delta \alpha \times \text{znf2} \Delta \alpha \) cross compared to the reduced filamentation from the \( \text{rze1} \Delta \alpha \times \text{znf2} \Delta \alpha \) cross (gene dosage consideration) suggests that the \( \text{RZE1} \) from the second mating partner is unable to compensate the loss of \( \text{RZE1} \) from the first mating partner. Bisexual mating was assayed on \( V8 \) medium for about 3 weeks. The localization (B) and intensity (C) of the fluorescence signal from the \( P_{\text{CTR4-2}}-\text{Znf2-mCherry} \) strain cultured in \( \text{YPD+BCS} \) with or without \( \text{RZE1} \). \( p = 0.89 \) for 24h and 0.59 for 48h. (D) Working model for \( \text{RZE1} \)'s nuclear function in \( \text{Cryptococcus} \).
To further test the hypothesis that RZE1 does not play a direct role in translation or Znf2 protein localization to the nucleus, we used a system where the expression of an ectopically introduced ZNF2-mCherry is controlled by the CTR4 promoter. We compared the fluorescent intensity (indicative of protein level) and the subcellular localization of Znf2 in the presence or absence of RZE1. We found that the loss of RZE1 does not affect either the intensity or the localization of Znf2-mCherry (Fig 4B and 4C). This is consistent with the idea that RZE1 does not directly regulate ZNF2 at the level of translation or protein translocation. Taken together, these results strongly suggest that RZE1 is functionally restricted in its native nucleus, and is not directly involved in the Znf2 protein processing or translocation. In the nucleus, however, it is possible that RZE1 could directly or indirectly regulate ZNF2 at different levels (Fig 4D): (i) promote ZNF2 transcription or transcript stability, (ii) support the production of the functional ZNF2 transcript isoform through its influence on alternative start, alternative termination, or alternative splicing [28], or (iii) assist the export of the ZNF2 transcript from nucleus to cytosol. Regulatory activity of RZE1 in any of the above processes could result in an effect on the Znf2 protein level or activity in the wild type strain.

**RZE1 regulates the ZNF2 transcript level**

The evidence presented so far suggests that RZE1 functions in its native nucleus and it functions in a regulatory capacity upstream of ZNF2 (Fig 4D). We decided to examine the first hypothesis of transcriptional control. To obtain a holistic view of the effect of the RZE1 deletion on cryptococcal transcriptome, we first conducted a pilot comparative transcriptome analysis between the wild-type XL280 and the rze1Δ mutant undergoing self-filamentation by RNA sequencing (RNA-seq). A total of 265 genes with the 1.5 fold change as the cut-off threshold (P<0.05) were differentially expressed in the rze1Δ mutant relative to the wild type at 24h post inoculation on filamentation-inducing V8 media (S1 Table). These genes are classified into eight functional categories (Fig 5A). Among the enriched GO terms, genes involved in the septin complex, cell cycle, and DNA replication are known to affect morphogenesis [44-46], which is consistent with the regulatory function of RZE1 in morphogenesis. Based on the RNA-seq data, the transcript level of ZNF2 in the rze1Δ mutant was lower compared to the wild type at 24 hours post inoculation on filamentation-inducing V8 media (S1 Table). These genes are classified into eight functional categories (Fig 5A). Among the enriched GO terms, genes involved in the septin complex, cell cycle, and DNA replication are known to affect morphogenesis [44-46], which is consistent with the regulatory function of RZE1 in morphogenesis. Based on the RNA-seq data, the transcript level of ZNF2 in the rze1Δ mutant was lower compared to the wild type at 24 hours post inoculation (Fig 5B). The reduction of the transcript level of ZNF2 in the rze1Δ mutant was less obvious at 72 hours post inoculation compared to the 24h and 28h time points (S6B Fig). The lower ZNF2 transcript level in the rze1Δ mutant was further confirmed by quantitative real-time PCR (Fig 5C). These results suggest that RZE1, directly or indirectly, up-regulates ZNF2 at the transcriptional level.

Since filamentation is obvious at 24h in XL280 under inducing condition, we suspect that the ZNF2 gene expression might be induced earlier. We therefore expanded our examination of the ZNF2 transcript level in the rze1Δ mutant and the wild type to include more time points (12h, 18h, 24h, 48h, and 72h post inoculation on V8 medium) by qPCR. As expected [12], the ZNF2 transcript level in the wild-type strain of all the later time points examined was increased compared to the reference level at 12h (Fig 5C). To our surprise, the ZNF2 transcript level in the rze1Δ mutant is also increased, suggesting that the signal transduction to promote filamentation still occurs in the mutant. However, the level of ZNF2 transcripts in the rze1Δ mutant is lower than that in the wild type, with the biggest difference observed at 18h (~4 fold) and smaller differences at 48h and 72h (Fig 5C). This might be consistent with the observation that the number of differentially expressed genes in the rze1Δ mutant is much higher at the 24h time point than the later 48h and 72h time points based on RNA-seq (S1 Table). These observations suggest an intriguing possibility that RZE1 might play an important regulatory role in driving filamentation at the early stage of morphogenesis.
Given the modest effect on the ZNF2 transcript level by the disruption of RZE1, we were surprised by the blocked filamentation in the rze1Δ mutant. Other mutants with reduced levels of ZNF2 typically show reduced but not abolished filamentation. Thus it is enigmatic that the disruption of RZE1 could completely abolish self-filamentation. The paradox between the observed reduction in the transcript level of ZNF2 and the abolished filamentation in the rze1Δ mutant thus prompted us to examine the effect of RZE1 deletion on the previously characterized downstream targets of Znf2 that are known to be important for filamentation: CFL1, FAD1, and FAS1 [17, 18]. Both qPCR and RNA-seq results indicated that the transcript levels of these filamentation markers were considerably reduced in the rze1Δ mutant (Fig 5D and S7 Fig), often more comparable to those observed in the znf2Δ mutant (Fig 5D). The drastic reduction in the transcript level of CFL1, an auto-inducer working in positive feedback with ZNF2 [18], is also in accordance with the results of the confrontation assay (Fig 1D). Thus it appears that although the deletion of RZE1 only exerts a modest impact on the transcript level.
of ZNF2, it exerts a drastic impact on factors downstream of ZNF2 and may inactivate at least part of the ZNF2 regulon that controls filamentation. This raises the possibility that RZE1 may affect additional processes between the ZNF2 transcription and translation.

The deletion of RZE1 shows no obvious effect on ZNF2 transcript intron splicing

As transcript isoforms different from those in the wild type could be non-functional, we decided to examine the impact of RZE1’s disruption on ZNF2 isoforms (Fig 4D). In C. neoformans 99.5% of all expressed genes have introns and an average gene contains multiple introns [30]. Thus alternative splicing could be one major way of generating different transcript isoforms. Indeed, multiple introns were found in ZNF2 wild type allele in the serotype D strain XL280 (Fig 5B and S6A Fig) as well as in the serotype A strain H99 [28]. The RNA-seq data showed that introns of the ZNF2 transcripts were spliced in both the wild-type strain XL280 and in the rze1Δ mutant (Fig 5B). Consistently, amplicons of the transcript that covered the whole ZNF2 ORF region in the wild type and in the rze1Δ mutant by reverse transcription PCR showed no apparent difference in size, again supporting the idea that introns of the ZNF2 transcripts could be spliced in the absence of RZE1. To examine if there was any quantitative difference in splicing of the ZNF2 transcript at different regions, we analyzed the transcript level of five different regions of the ZNF2 transcripts in the wild type and in the rze1Δ mutant during self-filamentation. These five regions included two regions in the 5’UTR, the junction between exon 1 and 2, the junction between exon 3 and 4, and the exon 5 region (Fig 6A). The levels of all five tested regions of the ZNF2 transcripts were lower in the rze1Δ mutant relative to those in the wild type, again with the biggest difference observed at 18h and smaller differences observed at 48h and 72h (Fig 6A). The pattern was similar to the earlier observation based on the conserved exon 5 region using the P5 primer set (Fig 5C). Collectively, the results suggest that RZE1 does not affect intron splicing of the ZNF2 transcripts.

To test if RZE1 plays a role in other aspects of ZNF2 transcript processing, we performed northern blot and probed with the ZNF2 ORF. As ZNF2 basal expression is low and is induced during bisexual mating [12], we first examined purified poly(A)-RNAs extracted from wild type, the znf2Δ mutant, and the rze1Δ mutant at the 12h and 24h time points during bisexual mating. One band that was larger than 3,000 nt and close to 4,000 nt was detected in both wild type and the rze1Δ mutant, but not in the znf2Δ mutant (Fig 6B). The size is consistent with the predicted approximately 3,600 nt-long mature transcript for ZNF2. The reduction in ZNF2 transcript level in the rze1Δ mutant during bisexual mating was apparent at the 12h time point, but not at the 24h time point (Fig 5C and S6B Fig). This might be because the impact of RZE1 deletion on the ZNF2 transcript level becomes more modest at later time points, as we observed in the unisexual mating cells (S6B Fig). We also compared the ZNF2 transcript size by northern hybridization using purified poly(A) RNAs extracted from wild type, the znf2Δ mutant, and the rze1Δ mutant at 48h during self-filamentation. We found that the size of the ZNF2 transcript remained ~3.6kb in wild type and the rze1Δ mutant (Fig 6B).

Taken together, these results indicate that ZNF2 only has one transcript isoform under the conditions we tested. Furthermore, the loss of RZE1 does not appear to affect cryptococcal ability to process introns of the ZNF2 transcripts.

Disruption of RZE1 reduces the number of ZNF2 transcripts and altered their subcellular distribution

We decided to test the third hypothesis: the effect of RZE1 on the export of the ZNF2 transcripts from the nucleus to the cytosol (Fig 4D). For this purpose, we used the technique called
single molecule Fluorescent In Situ Hybridization (smFISH), which helps visualize and quantify transcripts in the cell [47]. Unlike qPCR or RNA-seq that measure the total transcript level from the whole population, smFISH can measure the level of a particular transcript in a single cell and can be used to examine the heterogeneity in gene expression in that population. This microscopy based technique can also reveal the subcellular localization of the examined transcript. Although this technique has been used in research in other organisms (e.g. mammalian cells and model yeasts) [47–49], it has not been applied to Cryptococcus because of the unique challenge of making spheroplast/protoplast in this organism due to the presence of cell wall and polysaccharide capsule. Thus, we did pilot studies to optimize the experimental conditions for Cryptococcus using the U2 spliceosomal RNA as the positive control. We chose the U2 snRNA as the control because of its abundance in intron-rich eukaryotic cells and its exclusive nuclear localization [50]. Consistently, we found abundant U2 snRNA in Cryptococcus nuclei (Fig 7A).

To further establish a control of messenger RNAs that will be localized to the nucleus (for transcription) and cytosol (for translation), we chose to examine the actin transcripts (ACT1)
in cells plated on mating media (V8 medium, 18h) (Fig 7B). We found that 62.7% of cells in wild type (n = 884) and 59.3% of cells in the rze1Δ mutant (n = 899) were positive for the actin probe, likely due to incomplete cell wall digestion and the consequent occlusion of the probe from the remaining cells (S2 Table). However, digestion for longer period led to visible damage in some cells and was thus undesirable for the purpose of assessment of the transcripts' subcellular localization. Thus we decided not to extend enzymatic digestion to prevent cellular damage. We therefore assumed ~50–60% efficiency in probe penetration of cells prepared under such conditions. Among the actin transcripts examined, 70–80% of them were found in the cytosol and 20–30% in the nucleus (S2 Table). Such predominant distribution in the cytosol is expected for messenger RNAs as they primarily serve as templates for translation. Interestingly but not surprisingly, the deletion of RZE1 did not affect the subcellular distribution of ACT1, with still 71% found in the cytosol in the rze1Δ mutant (Fig 7C and S2 Table).

We then proceeded to visualize the ZNF2 transcripts in cryptococcal cells in the absence or the presence of RZE1 under the filamentation-inducing condition that induces the expression of ZNF2 (Fig 7E and 7F). The znf2Δ mutant cells processed under the same conditions were used as the negative control. We found that 49.18% of cells in wild type (n = 612) and 43.9% of cells in the rze1Δ mutant (n = 664) expressed ZNF2 (S2 and S3 Tables.). We believe that the percentage of wild-type cells expressing the ZNF2 gene was an under-estimation. This is because some of the wild type cells expressing higher levels of ZNF2 were turning into hyphae under this condition. These hyphal cells were more likely to be damaged during cell collection from solid agar plates and during the fixation and digestion process. Thus, wild-type cells that likely expressed higher levels of ZNF2 were also more likely to be excluded from the analysis. Nonetheless, as expected for an mRNA, we observed ZNF2 transcripts in both the nucleus and the cytosol (Fig 7E and 7F). Interestingly, we observed a higher percentile of ZNF2 transcripts localized in the nuclei in the rze1Δ mutant (44.7% cytoplasmic: 55.2% nuclear distribution compared to that in the wild type (69.6% cytoplasmic: 30.4% nuclear distribution) (Fig 7G and S2 and S3 Tables). The increased ratio of nucleus versus cytosol distribution of ZNF2 transcripts in the rze1Δ mutant could be caused by a defect in exporting ZNF2 transcripts to the cytoplasm, or alternatively increased cytoplasmic degradation of ZNF2 transcripts. Improperly processed transcripts are known to be subject to degradation in the cytoplasm by RNA surveillance mechanisms [51]. However, we did not detect any apparent difference in intron splicing of the ZNF2 transcripts generated in the rze1Δ mutant (Fig 5B). If ZNF2 transcripts undergo increased degradation in the cytoplasm, the involvement of RZE1 in that process must be, if any, indirect. This is because RZE1 is functionally restricted to its native nucleus (Fig 4A). Thus, we favor the other possibility of increased nuclear retention of ZNF2 transcripts (or reduced ZNF2 transport) in the absence of RZE1. That said, although the direct effect of RZE1 may be in the nucleus, we cannot exclude any indirect effect happening in the cytoplasm. Future investigation is warranted to further distinguish these hypotheses.

It was previously shown that functionally nuclear restricted IncRNAs could be physically confined to nuclei (as exemplified by IncRNAs MALAT1 and XIST [36, 37]). IncRNAs with nuclear function could also be distributed both in the cytoplasm and nuclei, although the cytoplasmic transcripts are considered non-functional as deductible from their mode of action (e.g. FLO11 IncRNAs in S. cerevisiae [52]). We therefore decided to examine the subcellular localization of RZE1 transcripts in the wild-type cells under a mating-inducing condition. We found that RZE1 was predominantly localized in the nucleus (29.3% cytoplasmic: 70.6% nuclear distribution; n = 514) (Fig 7H and S2 Table). The predominant nuclear localization of RZE1 again is consistent with the evidence presented earlier supporting RZE1 acting as a transcript rather than as an mRNA.
Discussion

Non-coding RNAs constitute a large part of genomes in higher eukaryotes [53, 54]. Among ncRNAs, IncRNAs are typically regulatory ncRNAs arbitrarily defined as transcripts of over 200 nt with minimum protein-coding potential [55]. The first discovered IncRNA H19 [56] was classified as an IncRNA in 1990 after the analysis of cloned gene sequence revealed its poor coding potential and its lack of observed sedimentation with the ribosomes [57]. The famous Xist RNA was identified by virtue of its exclusive expression by the inactivated X chromosome and its association with the X-Inactivation Center in 1991 [58–61]. Xist was classified as an IncRNA a year later due to lack of conserved open reading frames in its sequence and its exclusive nuclear localization [62]. Among the functionally characterized IncRNAs in higher eukaryotes, many are involved in growth, development, and differentiation [63–68]. Consequently, mis-regulation of these IncRNAs could result in tumorigenesis [69, 70], cardiovascular disorders [71, 72], or neurological diseases like Alzheimer’s and Parkinson’s [73]. Some of these IncRNAs are being exploited for the detection and treatment of cancers and lung diseases [74–76]. The mode of action by Xist in silencing the whole duplicated chromosome is being explored to treat diseases caused by trisomies [77]. Thus the investigation into the function of IncRNAs contributes significantly to our understanding of eukaryotic biology and diseases.

In lower eukaryotes such as fungi, however, few IncRNAs involved in the development and stress response have been characterized, mostly in the model systems such as Saccharomyces cerevisiae and Schizosaccharomyces pombe [52, 78, 79]. In Neurospora crassa and Aspergillus...
flavus, natural antisense transcripts (NATs) and IncRNAs are found to be differentially expressed in response to specific stimuli that induce developmental changes [80, 81]. One N. crassa IncRNA named qrf is experimentally shown to be important in maintaining circadian clock rhythmicity and clock resetting [82]. Although IncRNAs have been identified in phytopathogenic oomycete Phytophthora, and NATs in Ustilago are implicated in its virulence [83], none have been functionally characterized [84, 85]. Unfortunately, the prevalence or the potential functions of IncRNAs is unknown for human fungal pathogens.

Here we discovered and functionally characterized the IncRNA RZE1, which controls cryptococcal morphogenesis through its regulation of ZNF2. An ortholog of RZE1 could not be identified outside of the Cryptococcus species complex by sequence similarity searches. Even within the Cryptococcus species complex, the level of sequence conservation of the RZE1 gene is lower than protein-coding genes such as ZNF2, GPD1, or ACT1. This is not unexpected as IncRNAs are known to evolve at a faster rate and are sometimes species-specific. This, however, does raise the possibilities that RZE1 either is confined to the Cryptococcus species, or it retains evolutionary conservation across different fungal species but does so without sequence conservation. In higher eukaryotes, instances of functional conservation of IncRNAs without sequence conservation have been observed [85–87]. Thus, a functional equivalent of RZE1 in related and distant relatives of Cryptococcus is a possibility worth exploring. Another intriguing possibility is that in Cryptococcus, RZE1 might have evolved to be a regulator dedicated to ZNF2 and/or some of Znf2’s downstream targets. Consistently, the loss of RZE1 does not affect the transcript level of genes CNAG_03365 and CNAG_03367 that are adjacent to ZNF2, reflective of RZE1’s selectivity towards ZNF2 and/or its regulon. This makes RZE1 different from some other IncRNAs that can act globally (e.g. Xist for the entire X chromosome [86–88]).

RZE1 appears to act on ZNF2 primarily through transcription regulation. There are several instances of IncRNAs in the model yeast Saccharomyces that regulate by turning on or off their target transcription directly or indirectly [48, 89]. In this case, the amplitude of ZNF2 transcription induction was reduced about 2–3 folds in general in the rze1Δ mutant. Thus the role of RZE1 in ZNF2 transcriptional regulation appears to be more of modulating role than that of a switch. Given that other mutants with reduced ZNF2 expression level only shows decreased but not blocked filamentation, it stands to reason that the regulatory function of RZE1 goes beyond its effect on ZNF2 transcription. Intriguingly, RZE1 directly or indirectly regulates the distribution of ZNF2 transcripts, although the exact mechanism of RZE1 in regulating Znf2 activity is yet to be established.

Another enigmatic aspect of RZE1 is its position effect. The genetic position of RZE1 seems to have more drastic effect on Znf2 activity than RZE1’s expression level per se. For instance, the introduction of RZE1 in the ‘cis’ position seems to restore filamentation to the rze1Δ mutant much more effectively than an ectopic copy of RZE1 or the multi-copy RZE1 in episomally maintained vector (Fig 1B and S2 and S5 Figs). When comparing the level of RZE1 transcripts in the ‘cis’ complemented strain to the strains complemented with the site-directed mutated RZE1 alleles in the cis position, there were wide variations in the transcript level (S8 Fig). Most of these strains expressed RZE1 at a level higher than that in the wild type, yet their transcript level does not correlate with their robustness in filamentation (Fig 3C and S8 Fig). Similarly, ectopically expressing RZE1 using the strong CTR4 or the GPD1 promoter in the rze1Δ mutant yielded poorer filamentation than the RZE1 integrated at its native locus (S5 Fig). Collectively, these observations indicate that the physical distance between RZE1 and ZNF2, and consequently their newly made transcripts, matters greatly for their functional efficiency.

While we speculate that RZE1 specifically regulates ZNF2, we did not find any specific repeats or regions in RZE1 that share sequence similarity with ZNF2. This suggests that their direct interaction, if it exists, is unlikely to be based on simple sequence pairing. The
identification of molecules directly interacting with this lncRNA might help solve that mystery. Nonetheless, RZE1 is the first of its kind identified in Cryptococcus. The RZE1-ZNF2 can serve as a paradigm and a stimulus for the investigation of other regulatory lncRNAs in fungal genomes. Our preliminary transcriptional analysis of XL280 and H99 strains suggests that there are more than one thousand conserved lncRNAs in serotype D and serotype A, an equivalent of 1 lncRNA gene for every 6 protein-coding genes in Cryptococcus. Thus, exploring the function of these lncRNAs may give novel insights into the regulatory networks that control the morphogenesis and virulence of this organism. Such an additional layer of genetic regulation might explain the complexity of life cycle and pathogenic strategies of these "simple" eukaryotes.

Materials and Methods

Ethics statement

All the animal experiments were performed according to the guidelines of NIH and Texas A&M University Institutional Animal Care and Use Committee (protocol numbers: 2011–22 and 2014–0049).

Strains and growth conditions

The strains and plasmids used in this study are listed in S4 Table. Yeast cells were grown routinely on YPD media unless otherwise specified. Mating assays were conducted on V8 agar medium in the dark at 22°C as previously described [12]. Cells collected for qPCR were grown on V8 media at 22°C in ambient air or in serum at 37°C in presence of 5% CO2. Transformed strains were obtained by electroporation [90] or biolistic methods [91] and they were selected on YPD with 100 μg/ml of nourseothricin (NAT) or neomycin (NEO). Strains carrying genes driven by the inducible promoter of the CTR4 gene [32] were grown in media supplemented with 25 μM of CuSO4 for suppression or 200 μM of bathocuproine disulphonate (BCS) for induction [32].

Insertional mutagenesis via agrobacterium-mediated transformation and mutant screen

Agrobacterium tumefaciencs strain EHA105 containing the Ti plasmid pPZP-NATcc was used for the insertional mutagenesis as described previously [12, 92]. Briefly, the bacterium was grown overnight at 22°C, washed twice with sterile water, and transferred to an induction medium containing 100μM acetosyringone and incubated for 6h. Overnight culture of Cryptococcus grown in liquid YPD was washed in induction medium and re-suspended to get 10^7 cells/ml. Fungal and bacterial aliquots (200μl each) were mixed and plated on induction medium and co-cultured for 3 days at 22°C. The co-cultured cells were scraped and plated onto selective medium of YPD containing the antibiotic cefotaxime to remove Agrobacterium and the selective drug NAT 100μg/ml for fungal transformants. A total of 63,000 transformants were generated and screened for filamentation defect on V8 juice agar media using an Olympus SZX16 stereoscope. The colonies showing filamentation defect were further tested for intact pheromone sensing pathway by using unilateral crosses with the mating type a reference strain JEC20. The insertion site in selected 15 transformants with znf2Δ phenotype was identified using inverse PCR and sequencing as described below.

Inverse PCR and sequencing

Genomic DNA from the selected insertion mutants having znf2Δ like phenotype was extracted, digested with restriction enzymes, and then self-ligated as described previously [12, 92].
Primers AI076 and AI077 were used for inverse PCR. The PCR amplicons were sequenced and the sequence was BLAST searched against the *C. neoformans* (JEC21) serotype D genome database at GenBank to identify the insertion site.

**In vitro phenotypic assays**

Phenotypic assays were performed as described previously [93]. The strains to be tested were grown overnight in YPD. The cells were washed, adjusted to the same cell density (OD$_{600} = 1.0$), and serially diluted. The dilutions were plated onto appropriate media for various tests. To analyze the capacity of the strain to melanize, the original culture and the dilutions were plated onto media containing L-dihydroxyphenylalanine (L-DOPA) and incubated at 37°C in the dark. To observe capsule production, cells were plated on Dulbecco’s Modified Eagle’s medium (DMEM) (Invitrogen) and grown at 37°C under 5% CO$_2$. Capsule was visualized by India ink exclusion and examined under a light microscope. For testing the ability of the fungus to grow at high temperatures and their sensitivity to UV radiation, equal number of cells were plated onto YNB agar media and grown at 37°C or exposed to 300 J/m$^2$ of UV for 1, 5, and 10s. Cells were then cultured at 22°C for additional 2 days. To test the tolerance of fungal cells to osmotic and other stressors, cells were grown at 22°C for 2 days on YNB medium that was supplemented with H$_2$O$_2$ (21mM), calcoflour white (200 μg/ml), iron chelator-bathophenanthroline disulfonic acid (300 μg/ml), SDS (0.1%, 0.01% and 0.001%/shown in SF3), or caspofungin (16 μg/ml).

**Confrontation assay**

Confrontation assays were performed as described earlier [18]. Donor cells (OD$_{600} = 3$) were dropped onto YPD plates 2.5 days before dropping the recipient. The recipient cells (3 μl) of OD$_{600}$ 0.8 were dropped in close proximity without touching. The colonies were observed 60h after dropping of the recipient and photographed with Olympus SZX16 stereoscope.

**Rapid Amplification of cDNA Ends (RACE) cloning**

Gene Racer kit (Invitrogen, CA, USA) was used to amplify 5’ and 3’ ends of *RZE1* and ZNF2 RNA. Full length transcript of *RZE1* was amplified using the primers indicated in S5 Table from RNA extracted from cells grown on V8 for 24h following the manufacturer’s instructions. All the 3 sets of 5’ and 3’ RACE primers yielded the same start and stop termini. The amplified bands were separated on 0.8% agarose gel and extracted using the gel purification kit (Invitrogen), cloned in TOPO 2.1 (Invitrogen) and sequenced.

**Gene manipulations**

The targeted deletion of *RZE1* (excluding the first 100 bp after the transcription start) was conducted by introducing the deletion construct with the 1 kb flanks of the gene and split part of the dominant marker NAT, by electroporation or biolistic transformation as described earlier. Mutants generated were confirmed by PCR and by the genetic linkage assay using meiotic progeny dissected from genetic crosses [94]. The deletion mutant in the *MATα* background was obtained by crossing the *MATαrze1Δ* strains with the corresponding congenic wild type *α* strains. For complementation, *RZE1* transcript with 1kb of promoter region was amplified by PCR, digested, and inserted into the plasmid pXL1 [20]. Overexpression construct was created by amplifying the entire ORF by PCR and similarly inserting into pXL1 behind GPD1 or CTR4 promoters as described before [31, 32]. The *RZE1* multi-copy expression strains were generated by cloning the *RZE1* with 1kb of its own promoter into the pPM8 vector [95]. The linearized
vector was introduced into the auxotrophic \textit{rze1}Δ\textit{ura5} strain by electroporation. The primers used for the generation of the mutants and for their analysis are listed in S5 Table.

**RNA purification, qPCR, and northern blot**

RNA extraction and real-time qPCR were carried out as we previously described \cite{20}. Briefly, total RNA was extracted using PureLink RNA mini kit (Life Technologies). DNase (Ambion) treated RNA samples were analyzed on a denaturing formaldehyde agarose gel for assessing quality and concentration. Superscript III cDNA synthesis kit (Life Technology) was used for the first strand cDNA synthesis following the manufacturer’s instructions. Constitutively expressing housekeeping gene \textit{TEF1} was used as endogenous control for the normalization of expression of the other genes studied.

For Northern blot, total RNA was extracted from cultures on V8 medium (pH = 7.0) from the indicated strains under the conditions as described in the texts. Poly(A) tailed RNAs were purified using the PolyATract mRNA Isolation System IV (Promega) according to the manufacturer’s instruction. PolyA tailed RNAs were then separated on denaturing formaldehyde agarose gels and then transferred to nylon membrane. The Random Primers DNA Labeling System (Life technologies) was used to generate probes. Primer sequences used to make gene-specific templates are listed in S5 Table.

**RNA-seq analysis**

Wild-type strain XL280 and the \textit{rze1}Δ mutant were cultured on YPD and V8 (pH = 7) media. Cells were collected at 24, 48 and 72h from mating media and used for isolation of total RNA. Strand-specific RNA-seq was performed at the TAMU genomic facility according to the standard protocol for Illumina Genome Analyzer IIx. Sequenced reads were aligned to the XL280 reference sequence [26] using Tophat [96]. Reads that aligned uniquely to the reference sequence were considered for gene expression quantification with Cufflinks [97]. Gene expression was normalized using the DESeq package [98] in R [99]. Differential expression analysis comparing mutant to wild type was performed with using a 5\% false discovery rate. RNA-Seq data is deposited at NCBI (BioProject ID PRGNA278291, accession 278291).

**Site-directed mutagenesis**

To introduce single nucleotide mutations in the potential translation start sites of \textit{RZE1}, Quick Change II XL site directed mutagenesis kit from (Agilent Technologies) was used following manufacturer’s instructions. Primers for introducing the mutations (S5 Table) were designed using the QuickChange primer design program from Agilent Technologies. \textit{RZE1} along with 1kb of its 5’ flank were amplified by PCR and cloned into pGEMT easy vector (Promega). The entire plasmid with insert was then amplified using specific primers to change each of the ATGs to AAGs or ATAs. The modified inserts were then amplified from the plasmid using PCR with high fidelity Phusion enzyme (New England Biolabs) and used as template for overlap PCR with part of NEO marker gene to obtain a merged construct of \textit{RZE1} with 5’ flank and part of the marker. Similar overlap PCR with 3’ flank of \textit{RZE1} and part of NEO was used to obtain the other half of insertion construct. The two parts of insertion construct were then mixed and introduced biolistically into \textit{Cryptococcus rze1}Δ mutant strain to replace the \textit{rze1}Δ deletion construct. The stable transformants that showed resistance to NEO (indicative of the integration of the mutant \textit{RZE1} allele) and sensitivity to NAT (indicative of the loss of the \textit{rze1}Δ construct) were selected and further analyzed by PCR to confirm the replacement.
Virulence assay using the inhalation infection model of murine cryptococcosis

Female A/J mice of 6–8 week were intranasally inoculated with 5x10^4 cells/mouse of serotype A strains or 5x10^5 cells/mouse of serotype D strains with 5 mice each group as we previously described [100–102]. The mice were sacrificed 10 days post inoculation. The organs from sacrificed animals were dissected and homogenized in phosphate buffered saline, serially diluted, and plated on YNB plates. After 2 days of incubation at 30°C, the CFUs were counted. One way ANOVA was used to analyze the fungal burden. All statistical analyses were performed using the Graphpad Prism 5 program and and P values lower than 0.05 was considered statistically significant.

Heterokaryon assay

All strains in H99 background were grown overnight in YPD liquid medium. Cells were washed and suspended in PBS with the same cell density (OD<sub>600</sub> = 1.0). Equal number of cells of each pair of strains as indicated in the figure and the text were mixed and spotted onto V8 agar pH 5.0 and incubated at 22°C in the dark for 2.5 weeks. The colony morphology was observed and photographed using an Olympus SZX16 stereo microscope.

Measurement of fluorescence intensity

The fluorescent intensity of Znf2 tagged with mCherry was measured using the Zen Lite software from Carl Zeiss. The microscopic images taken on a Zeiss Axioplan 2 microscope were analyzed using the interactive fluorescence measurement feature of Zen. Fluorescence intensity of Znf2-mCherry from forty cells in either the wild-type background or the rze1Δ mutant background was measured using the software and statistically analyzed using Prism V from Graphpad.

Single molecule FISH experiments

The smFISH experiments were performed as described before [103–105] with modifications for *Cryptococcus*. Strains either grown to exponential growth phase in YPD or the co-culture of the congenic mating pairs on V8 media were collected and fixed in 4% paraformaldehyde for 30 minutes at 22°C. The cells were digested in sphaeroplasting buffer (1M sorbitol, 10 mM EDTA, and 100 mM sodium citrate) using 100 mg/ml of lyzing enzyme (Sigma) for 45 min. Sphaeroplasted cells were permeabilized after being incubated in 70% ethanol at 4°C overnight. Cells were then hybridized with TAMRA labeled ZNF2 specific probes or ALEXA FLOUR labelled RZE1 probes (Stellaris, Biosearch Technologies) in buffer containing 10% formamide for 16h at 37°C. Counter staining to visualize nuclei was done using 10 mg/ml of DAPI. The cells were visualized on a Zeiss Imager M2 microscope and the z-stack images were then subjected to 3D deconvolution by using AutoQuant software (Media Cybernetics). After deconvolution, the images were imported in Zen Blue software and the channels were merged and analyzed.

Supporting Information

S1 Fig. Transcript of RZE1 identified in XL280. The potential translation start codons in the five potential open reading frames are marked. (TIF)
S2 Fig. (A) The defect in filamentation of the \textit{rze1Δ} mutant was partially restored by an ectopic copy of RZE1. Cells of the indicated strains were grown on V8 medium for 10 days. (B) RZE1 maintained episomally in the multi-copy vector pPM8 conferred the wrinkled colony phenotype (bottom image) but barely conferred any filamentation to the \textit{rze1Δ} mutant (upper image). Cells of indicated strains were grown on V8 medium for one week. (TIF)

S3 Fig. The \textit{rze1Δ} mutants were not different from the corresponding wild type strains in classic virulence traits like thermos-tolerance, melanization, and capsule production. There are no differences in response to cell wall stressors or oxidative agents or UV. (TIF)

S4 Fig. Ectopic complementation of the filamentation defect in the \textit{znf2Δ} mutant using ZNF2 with its 1kb sequences upstream of its ORF. The 1 kb sequences upstream of ZNF2’s ORF does not overlap with the RZE1 transcript. This ZNF2 construct successfully restored bisexual mating in H99 background (A and left panel in B) and self-filamentation in the \textit{znf2Δ} mutant in XL280 background (right panel in B). The bisexual mating for strains in H99 background were cultured on V8 medium for 5 days. Strains in XL280 background were cultured on V8 medium for 3 days. The ectopically integrated ZNF2 construct with 1 kb sequence upstream of its ORF restored the ability of the \textit{znf2Δ} mutant to form mating hyphae during bisexual mating in H99 background in either unilateral mating (mutant x wild type) or bilateral mating (mutant x mutant). (TIF)

S5 Fig. The overexpression (P\textit{CTR4-2}) of each putative open reading frame of RZE1 does not restore filamentation in the \textit{rze1Δ} mutant. Cells were cultured on V8 medium for 4 days. (TIF)

S6 Fig. (A) The localization and transcription orientation of ZNF2 and RZE1 in XL280 wild type strain based on manual annotation of the RNA seq data in this region. Chr: chromosome. (B) The RNA-seq reads of ZNF2 transcripts in wild-type XL280 and the \textit{rze1Δ} mutant cultured on V8 medium for 24 hours, 48 hours, and 72 hours. (TIF)

S7 Fig. The expression of the prominent Znf2 downstream targets CFL1, FAD1, and FAS1 in the wild type and the \textit{rze1Δ} mutant during self-filamentation (RNA-seq). (TIF)

S8 Fig. The RZE1 transcript level in the \textit{rze1Δ-RZE1} strain, the different single nucleotide mutated RZE1 alleles (ATG1-ATG5) integrated into its native locus in the \textit{rze1Δ} mutant strains, and the wild type on filamentation-inducing V8 medium for 18 hours. (TIF)

S1 Table. List of genes differentially expressed in the \textit{rze1Δ} mutant and the wild type. (XLSX)

S2 Table. Summary of expression and subcellular distribution of Actin, ZNF2, and RZE1 transcripts. (DOCX)

S3 Table. Subcellular distribution of ZNF2 transcripts in the wild type and the \textit{rze1Δ} mutant. (XLSX)
S4 Table. Strains used in the study.
(DOCX)

S5 Table. Primers used in the study.
(XLSX)

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Author Contributions
Conceived and designed the experiments: NC YZ LW JJC XL. Performed the experiments: NC YZ LW EY XL. Analyzed the data: NC YZ EY JJC XL. Contributed reagents/materials/analysis tools: JJC XL. Wrote the paper: NC YZ XL.

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