Conserved protein Pir2^{ARS2} mediates gene repression through cryptic introns in IncRNAs

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Long non-coding RNAs (IncRNAs) are components of epigenetic control mechanisms that ensure appropriate and timely gene expression. The functions of IncRNAs are often mediated through associated gene regulatory activities, but how IncRNAs are distinguished from other RNAs and recruit effector complexes is unclear. Here, we utilize the fission yeast *Schizosaccharomyces pombe* to investigate how IncRNAs engage silencing activities to regulate gene expression in cis. We find that invasion of IncRNA transcription into the downstream gene body incorporates a cryptic intron required for repression of that gene. Our analyses show that IncRNAs containing cryptic introns are targeted by the conserved Pir2^{ARS2} protein in association with splicing factors, which recruit RNA processing and chromatin-modifying activities involved in gene silencing. Pir2 and splicing machinery are broadly required for gene repression. Our finding that human ARS2 also interacts with splicing factors suggests a conserved mechanism mediates gene repression through cryptic introns within IncRNAs.
ncRNAs dynamically regulate gene expression during development and in response to environmental conditions. Defects in gene regulation by IncRNAs are frequently linked to diseases including cancer. In many cases, IncRNAs govern gene expression by directing chromatin-modifying enzymes and other factors. This function of IncRNAs is mediated via their associated proteins, but the mechanisms by which the IncRNAs selectively engage gene regulatory activities have remained largely unknown.

*S. pombe* is a powerful genetic model system for studying IncRNAs and their roles in the regulation of gene expression. In addition to numerous annotated IncRNAs, several RNA processing factors that are missing in budding yeast are conserved from *S. pombe* to higher eukaryotes. Many IncRNAs control gene expression in response to environmental and developmental signals, including cis-acting IncRNAs that regulate the expression of nearby genes. Examples of regulatory IncRNAs include the *prt* IncRNA that represses the acid phosphatase *pho1* gene in the presence of phosphate, and the *nam1* IncRNA that silences the mitogen-activated protein kinase *byr2* gene essential for spore differentiation. Transcription termination and degradation of the IncRNAs prevents them from invading and repressing downstream genes. However, under specific growth conditions, readthrough transcription of IncRNAs leads to repression of downstream genes. Underscoring a direct role, cells defective in IncRNA production show de-repression of target genes. Although these and other IncRNAs play a critical role in mediating gene repression, the exact mechanism is not understood.

RNA processing factors that process diverse RNA species have been implicated in both posttranscriptional and transcriptional silencing. RNAi machinery processes transcripts into small RNAs (siRNAs), but is also critical for targeting chromatin-modifying activities, such as factors involved in heterochromatin assembly. The components of the RNAi pathway include the RNA-induced transcriptional silencing complex (RTS: Ago1, Chp1, and Tas3), the RNA-directed RNA polymerase complex (RDRC: Cid12, Hrr1 and Rdp1), and Dicer (Dcr1). In addition to playing a prominent role in processing centromeric repeat transcripts, RNAi targets various other loci, including retrotransposons, sexual differentiation genes, and genes encoding transmembrane proteins.

Additionally, *S. pombe* contains conserved machinery that promotes degradation of transcripts by the 3′ → 5′ exonuclease Rrp6. MTREC (Mtl1-Red1 core) is composed of the Mtr4-like RNA helicase Mtl1 and the zinc finger protein Red1 and serves as the molecular hub of an RNA processing network, related to NEXT and PAXT in mammals. MTREC and its associated factors preferentially target transcripts containing hexameric DSR (determinant of selective removal) elements, which are bound by a YTH family RNA-binding protein Mmii. Mmii physically interacts with the Erh1 protein to form a complex referred to as EMC (Erh1-Mmii Complex). EMC recruits MTREC to meiotic genes to prevent their untimely expression during vegetative growth, in addition to targeting cis-acting IncRNAs including *prt* and *nam1*. Mmii also mediates recruitment of the cleavage and polyadenylation factor (CPF) complex, which acts together with Rrp6 to trigger transcription termination and degradation of IncRNAs, thus preventing them from invading and repressing downstream genes. Despite these studies, a major unanswered question is how IncRNAs mediate gene repression.

In this study, we demonstrate that besides MTREC, cis-acting IncRNAs show enrichment of the highly conserved Pir2 protein in mammals. Remarkably, IncRNAs contain cryptic introns that provide a scaffold for splicing factors and Pir2, which are required for lncRNA-mediated gene repression. Our analyses show that the Pir2-splicing-machinery recruits silencing effector complexes to aid in the repression of target gene loci. We also find that ARS2 associates with splicing factors in human cells, suggesting that human ARS2 functions similarly to connect regulatory RNAs to gene silencing activities.

**Results**

**Pir2 is required for lncRNA-mediated repression.** We investigated if MTREC and its associated factors, including the Pir2ARS2 protein, are required for repression of *pho1* and *byr2* by IncRNA. Pir2ARS2 is an essential protein implicated in various aspects of RNA metabolism. Loss of the MTREC subunit Red1 resulted in the accumulation of longer readthrough transcripts (referred to as *prt-L* and *nam1-L*) (Fig. 1a), as was also observed in *mmi1Δ* and *rrp6Δ* cells (Fig. 1a and Supplementary Fig. 1a). By contrast, a mutation in *pir2* (*pir2-1*) did not affect the levels of *prt* and *nam1* IncRNAs (Fig. 1b). Surprisingly, *pir2-1* showed a drastic upregulation of *pho1* and *byr2* genes as compared to wild-type (WT). (Fig. 1b), similar to the effect observed upon deletion of the IncRNA (Supplementary Fig. 1b). Deletion of *prt* abolished Pir2 localization at the target locus (Fig. 1e).

Together, these results suggest that IncRNAs recruit Pir2 to repress their downstream genes. Supporting the function of Pir2 and IncRNA in the same pathway, we found no additive effect on *pho1* expression in the *pir2-1Δ* double mutant when compared to the effect in the single mutants (Fig. 1e).

The requirement for Pir2 in mediating the repressive effects of IncRNAs is a highly significant finding. We asked if Pir2 is also required for the repression of *byr2* that is observed upon the accumulation of *nam1* IncRNA in cells lacking Rrp6. Since *byr2* is required for meiotic induction, cells lacking Rrp6 are defective in sporulation (Fig. 1f). Remarkably, entry into meiosis and sporulation efficiency were restored in *pir2-1 Δ* cells (Fig. 1f). Similar results were obtained from a qualitative assay in which iodine vapor stains the starch-like compound produced by cells undergoing meiosis a dark brown color. Whereas *rrp6Δ* cells that are defective in meiotic induction due to *byr2* repression stained yellow, the *rrp6Δ pir2-1* double mutant colonies stained dark brown (Fig. 1f). These results support a role for Pir2 in mediating repression of *byr2* by *nam1* IncRNA.

**Pir2-CBC and splicing factors mediate gene repression.** We next tested whether Pir2-associated factors are also required for lncRNA-mediated gene repression. Consistent with co-purification of Pir2ARS2 with the cap-binding complex (CBC) in *S. pombe* and mammalian ([23,30–32]), Pir2 co-immunoprecipitated (co-IP) with CBC components (Supplementary Fig. 1e). Moreover, CBC co-fractionated with Pir2 in glycerol gradient analyses (Supplementary Fig. 2). To test whether CBC is required for Pir2-mediated gene repression we constructed a partial loss-of-function mutant allele of the *cbc1* gene (*cbc1-1*), which encodes an essential subunit of CBC. The IncRNA-mediated repression of *pho1* was impaired in *cbc1-1* cells (Supplementary Fig. 1e), suggesting that Pir2 likely acts together with CBC to promote gene repression.

In addition to CBC, Pir2-purified fractions also contain a subset of splicing factors including Cwf10 (EFTUD2 in human), which is a subunit of the U5 small nuclear ribonucleoparticle [33].
and Cwf21 (SRRM2 in humans)\textsuperscript{35}. Biochemical analyses showed that Pir2 indeed forms a complex with splicing factors. We confirmed their association by co-IP (Fig. 2a) and also found that Pir2 co-eluted with a subfraction of Cwf10 in a glycerol gradient (Fig. 2b). Cwf10 eluted in two major fractions, indicating the presence of a smaller complex containing Pir2 and a second larger complex corresponding to the active spliceosome and were not found in the smaller fraction with Pir2 (Supplementary Fig. 2).

Interestingly, our glycerol gradient analysis showed exclusive co-elution of Cwf21 and Pir2. Together, these results suggest that Pir2 forms a complex with splicing factors that are not part of the active spliceosome.

To determine whether Pir2 acts together with splicing factors to promote gene repression by lncRNAs, we performed northern blot analysis. A significant increase in the level of both \textit{pho1} and \textit{byr2} mRNAs in \textit{cwf10-1} as compared to WT confirmed that the splicing machinery indeed affects the expression of genes repressed by lncRNAs (Fig. 2c). We then performed epistasis analysis to test if Pir2 and the splicing machinery are components of the same silencing pathway. We found no cumulative increase in the expression of genes repressed by lncRNA in the \textit{pir2-1}
cwf10-1 double mutant as compared to the single mutants (Fig. 2d). Importantly, cwf10-1 rescued the sporulation defect observed in rrp6Δ caused by the silencing of the byr2 gene by nam1 lncRNA (Fig. 2e), similar to pir2-1 (Fig. 1f). These results confirm the biological significance of Pir2 association with splicing machinery and show that these factors collaborate to promote gene repression by lncRNAs.

LncRNA-mediated repression requires a cryptic intron. We considered that specific features of lncRNAs may be critical for gene repression by Pir2 and splicing machinery. Considering the involvement of splicing machinery, we searched for introns in the loci controlled by the lncRNAs. Despite the absence of annotated introns, examination of RNA-seq data from pir2-1 and pir2-1 rrp6Δ cells revealed “cryptic” introns, which contain consensus splice sites but are inefficiently spliced6, that map to the pho1 and byr2 loci (Fig. 3a). The detection of introns in these mutant cells likely reflects kinetic competition between splicing machinery and RNA processing factors. In cells lacking Pir2 and other factors such as Rrp6, defects in RNA degradation shift the balance in favor of splicing machinery, ultimately leading to splicing of cryptic introns. The region upstream of nam1 that showed Pir2 enrichment (Fig. 1c) also contained a cryptic intron (Fig. 3a). However, since splicing of the cryptic introns would disrupt the ORF, the possible biological significance of these introns was unclear. To address this, we generated two independent mutant strains containing deletions of the 5′ and 3′ splice sites of the cryptic intron within pho1 (Fig. 3b). Remarkably, strains carrying splice site mutations showed significant upregulation of the pho1 transcript (Fig. 3c), similar to the effect observed in pir2-1, cwf10-1 and prtΔ (Figs. 1b, 2c and Supplementary Fig. 1b). Importantly, splice site mutations affected target gene silencing but not the level of prt lncRNA (Fig. 3c), analogous to the results obtained with cwf10-1 or pir2-1 mutants (Fig. 1b). This effect is distinct from the changes observed upon deletion of other known Mmi1 binding sites that contain introns28.

To determine if Pir2 and the cryptic intron act together to maintain gene repression, we performed epistasis analysis. Combining pir2-1 with the mutant cryptic intron allele did not result in further accumulation of pho1 transcripts when compared to the single mutants, suggesting that Pir2 acts through the
cryptic intron to promote repression of *pho1* (Fig. 3d). Together with the requirement for lncRNA to invade into the gene body, these results implicate the inclusion of the cryptic intron in the regulatory RNAs as an essential element for repression via a mechanism involving Pir2 and splicing machinery.

**Pir2 and splicing machinery collaborate genome-wide.** Cooperation between Pir2 and Cwf10 might represent a common strategy employed at other loci. Comparison of the expression profiles of *pir2-1* and *cwf10-1* revealed 435 targets repressed by both Pir2 and Cwf10, accounting for more than 50% of Pir2 target transcripts (Supplementary Fig. 3a). The targets comprised 205 mRNAs and, interestingly, 230 ncRNAs (Supplementary Data 1). Amongst the loci upregulated in *pir2-1*, we detected cryptic introns in 204 transcripts (Supplementary Fig. 3b). This is likely an underestimation due to the difficulty of detecting inefficiently spliced cryptic introns. Cryptic introns were found in mRNAs and many ncRNAs that collectively show transcript upregulation as determined by comparing RNA-seq data from *pir2-1* and *cwf10-1* cells to WT (Supplementary Fig. 3c). Notably, we detected cryptic introns in transcripts arising from retrotransposon Tj2 elements in *pir2-1* cells (Supplementary Fig. 3d). Analysis of *pir2-1* and *cwf10-1* mutants revealed that expression of Tj2 elements increased in both mutant strains (Supplementary Fig. 3d, e), and RIP-seq analysis showed that Pir2 binds to Tj2 transcripts (Supplementary Fig. 3f). These results establish cryptic introns as a common feature among Pir2 targets and show that Pir2 and splicing machinery collaborate to repress genes and retrotransposons.

**Pir2-splicing machinery recruit RNAi proteins for repression.** How might Pir2 trigger repression by lncRNA containing cryptic introns? Although lncRNA production is important for loading silencing factors, the exact mechanism has remained unclear. Since Pir2 homologs are involved in RNAi and splicing machinery is implicated in siRNA production, we examined the association of Pir2 with RNAi machinery. Co-IP analysis showed Pir2 associates with the Hrr1 subunit of RDRC (Fig. 4a). Interestingly, this interaction was impaired in the *cwf10-1* mutant, indicating that splicing factors are required for association of Pir2 with Hrr1 (Fig. 4b). We then analyzed the role of Pir2 in siRNA production in cells lacking Rrp6, which show accumulation of lncRNAs and robust repression of their target loci. We found that siRNAs, which ranged in size from 20–24 nt and mapped to lncRNAs targeting *pho1* and *byr2*, were abolished in both *pir2-1* and *cwf10-1* mutant backgrounds (Fig. 4c and Supplementary Fig. 5). Based on these results, we conclude that Pir2 targets RNAi machinery to lncRNAs and other transcripts containing cryptic introns.

**Cryptic intron is required for siRNA production at lncRNA.** We next wondered whether cryptic introns are required for Pir2-dependent generation of siRNAs. Mutations of the *pho1* cryptic intron splice sites in *rrp6Δ* cells abolished the production of siRNAs mapping to the entire *pho1* lncRNA, including the region upstream of *pho1* (Fig. 4d). This result suggests that the cryptic intron acts as part of the *pho1* lncRNA to engage RNAi machinery. Importantly, siRNAs mapping to other loci were not affected (Fig. 4d and Supplementary Fig. 5), indicating that the observed
effect was specific to *prt-pho1*. This finding is consistent with our result showing that *pho1* is upregulated in cells carrying cryptic intron splice site mutations (Fig. 3c) and led us to examine the effects of RNAi factors on lncRNA-mediated gene repression. Cells lacking Ago1 showed a considerable increase in *pho1* transcript levels as determined by northern blot analysis (Fig. 4e), but the observed effect was weaker than in *pir2-1* or *cwf10-1*, suggesting that additional factors likely cooperate with Pir2-splicing machinery.

**Pir2 and splicing machinery target chromatin modifiers.** In addition to RNAi machinery, gene silencing by lncRNAs also requires Clr3 histone deacetylase (HDAC) and the FACT histone chaperone complex, which were detected in a Pir2-purified fraction. Therefore, we addressed whether Pir2 also recruits Clr3 and/or FACT. Clr3 and the Pob3 subunit of FACT associated with Pir2 in our biochemical analyses (Fig. 5a, b). The loss of Clr3 or Pob3 caused an increase in *pho1* transcript levels, consistent with their involvement in repression by lncRNA, but the extent of upregulation was less than in *pir2-1* (Fig. 5c). This could be due to the recruitment of multiple effectors by Pir2 to promote gene repression. To test this possibility, we compared *pho1* transcript levels in single and double mutants. Combining *pob3*Δ with other mutants resulted in severe growth defects, precluding their analysis. However, the *ago1Δ clr3Δ* double mutant showed cumulative de-repression of *pho1* (Fig. 5d). By contrast, the *pir2-1 clr3Δ* and *pir2-1 ago1Δ* double mutant strains showed no cumulative increase in transcripts as compared to the

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**Fig. 4** Pir2 interacts with RNAi machinery required for lncRNA-mediated gene repression. a, b Co-IP analysis of Hrr1-TAP and Pir2-GFP proteins in the indicated strains. Source data are provided as a Source data file. c The normalized number of siRNA reads are plotted in alignment with *pho1* and Tf2-3 loci. The signal above and below the line represent siRNAs that map to the top and bottom strands, respectively. TopHAT splice junctions of introns emerging in the strains are indicated by blue arcs. d Normalized number of siRNAs mapping to *pho1* and Tf2-3 loci in *rrp6Δ* (red) and *rrp6Δ* cells carrying the splice site mutation in the cryptic intron in *prt-pho1* (purple). The cryptic intron is marked by a blue box and stars indicate mutation sites. e Northern blot analysis of the *pho1* gene. Cells were grown in EMM medium with or without phosphate. The radioactive probe is described in Fig. 1a. Source data are provided as a Source data file.
Fig. 5 Pir2 recruits Clr3 HDAC and FACT to promote gene repression by lncRNA. a, b Co-IP analysis of Pir2-GFP with Pob3-MYC and Clr3-MYC. Source data are provided as a Source data file. c Northern blot analysis of transcripts produced from the pho1 locus. The radioactive probe is described in Fig. 1a. d RT-qPCR analysis of pho1 expression, normalized to leu1. The amplified region is indicated by the black line. Data are presented as mean values ± SD for n = 3 biologically independent samples. Student’s t-test (two-tailed) was used to calculate p-value. Between Pob3-MYC and Pob3-MYC pir2-1, p = 0.008851 (* p < 0.05). Between Clr3-MYC and Clr3-MYC pir2-1, p = 0.008851 (* p < 0.05). Mean data distribution is represented by white circles.

Pir2 and splicing machinery engage cryptic introns within lncRNA to promote repression. Model depicting the switch to co-transcriptional repression through the cryptic intron. For gene expression, lncRNA is terminated and degraded upstream of target loci by CPF, MTREC, and Rrp6, which are recruited by Mmi1 bound to DSR elements. Upon changes in growth conditions, the lncRNA extends into the neighboring region containing the cryptic intron, providing a scaffold for co-transcriptional recruitment of Pir2-splicing machinery, which in turn recruits multiple silencing effectors to repress gene expression (Fig. 6).

Fig. 6 Pir2 and splicing machinery engage cryptic introns within lncRNA to promote repression. Model depicting the switch to co-transcriptional repression through the cryptic intron. For gene expression, lncRNA is terminated and degraded upstream of target loci by CPF, MTREC, and Rrp6, which are recruited by Mmi1 bound to DSR elements. Upon changes in growth conditions, the lncRNA extends into the neighboring region containing the cryptic intron, providing a scaffold for co-transcriptional recruitment of Pir2-splicing machinery, which in turn recruits multiple silencing effectors including RNAi, HDAC, and FACT to promote gene repression. Mmi1 acts as part of the EMC to engage MTREC and other factors that together with CBC stabilize Pir2 association with lncRNAs and effector proteins.

single mutants, suggesting that Pir2 is epistatic to both RNAi and the Clr3 HDAC. Moreover, quantitative ChIP analyses showed enrichment of Clr3 and Pob3 at prr-pho1 in WT cells and a reduced localization in pir2-1 cells (Fig. 5e).

Given that lncRNAs mediate repression by chromatin modifiers and RNAi, we asked whether RNA was required to mediate interactions between Pir2 and its various interacting partners. Co-IP experiments performed in the presence of Benzonase, a DNA and RNA nuclease, revealed that except for Pob3, all other interactions with Pir2 were maintained (Supplementary Fig. 6). This suggests that Pir2 interacts with components of this pathway in an RNA-independent manner. However, RNA may have a role in promoting co-transcriptional association of Pob3 with Pir2. These results suggest that lncRNA with a cryptic intron provides a docking site for the Pir2-splicing complex, which in turn recruits multiple silencing effectors to repress gene expression (Fig. 6).

Pir2/ARS2-splicing factor connection is conserved in humans.

The conservation of Pir2/ARS2 suggested that its functional interactions and role in lncRNA-mediated repression might be relevant to mammalian systems. We asked if ARS2 formed similar interactions in human cells using RIME (rapid immunoprecipitation mass spectrometry of endogenous proteins), which can detect transient chromatin associated co-transcriptional interactions. In addition to the known ARS2 interaction partners CBC, NEXT, and PAXT31,32,40, RIME analysis identified additional proteins, including factors involved in nonsense-mediated decay (NMD), pre-mRNA 3’-end processing and chromatin modifiers such as FACT and HDACs (Supplementary Fig. 7a, b and Supplementary Data 3). Notably, the most abundant associating proteins, including factors involved in nonsense-mediated decay.

Effectors including RNAi, HDAC, and FACT to promote gene repression. Mmi1 acts as part of the EMC to engage MTREC and other factors that together with CBC stabilize Pir2 association with lncRNAs and effector proteins.
partners of ARS2 were splicing factors (Supplementary Fig. 7b, c). Indeed, splicing factors co-eluted with ARS2 in a glycerol gradient (Supplementary Fig. 7d), analogous to our observations in S. pombe (Fig. 2b). Moreover, we observed association of the human counterpart of Cwf10, EFTUD2, with ARS2, suggesting that the Pir2-splicing factor connection observed in fission yeast is conserved in humans (Supplementary Fig. 7a, e). Based on these findings, we envision that ARS2 bound to splicing machinery may function similarly to bridge regulatory RNAs to gene silencing activities.

Discussion

Our analyses reveal that a cryptic intron within the IncRNA is a crucial element for gene repression via a pathway involving Pir2ARS2 and splicing factors (Fig. 6). The readthrough transcription of intergenic IncRNA incorporates a cryptic intron and creates a scaffold for the co-transcriptional recruitment of the splicing machinery–Pir2 complex, which in turn engages silencing effectors. Our findings highlight a previously unrecognized mode of engagement of splicing factors to IncRNAs to dynamically control gene expression. To this end, we find that Pir2 associates with splicing factors such as Cwf21 and Cwf10 as part of a smaller complex and is not part of the larger spliceosome complex containing the NTC components. This finding suggests that the subset of splicing factors responsible for mediating gene repression is likely distinct from the active spliceosome.

Since cryptic intron-mediated silencing occurs specifically in the context of IncRNA but not the target gene transcript, additional factors bound to IncRNA are likely involved. Other factors that bind to IncRNA might help recruit and/or stabilize Pir2 with its associated silencing effectors. In this regard, we note that loss of ERH family protein, Erh1, that associates with IncRNAs and Mmi1 as part of EMC, severely affects repression of target gene loci29,45. Moreover, a mutation in Mmi1 that specifically disrupts EMC assembly without affecting its termination functions impairs IncRNA-mediated repression of neighboring genes.45 EMC bound to IncRNA may directly recruit Pir2 or may act in conjunction with other factors. Consistent with the latter possibility, EMC co-purifies with MTREC29, which forms a complex with Pir2,25,30. Therefore, MTREC, recruited by Mmi1/EMC bound to DSR elements, may act together with splicing factors engaged by the cryptic intron to promote Pir2 association with IncRNAs. In other words, the repressive effects of IncRNA require combinatorial and likely cooperative action of factors that bind to different elements embedded within regulatory RNAs (Fig. 6).

Once recruited to IncRNAs, Pir2 coordinates multiple effectors, including RNAi, to promote gene repression. In this regard, we note that Pir2 is the elusive factor that enables splicing machinery to selectively recruit RNAi to specific transcripts. RNAi processes transcripts and triggers assembly of repressive heterochromatin16–18,22. In addition, Pir2 promotes IncRNA-mediated recruitment of Clr3 HDAC and FACT, which increase nucleosome occupancy37,38,46 but may also engage additional factors to enforce gene repression. Pir2 and cryptic intron-based mechanisms repress targets throughout the genome and may be conserved in higher eukaryotes. Indeed, we note that ARS2 interacts with splicing factors in human cells, and IncRNAs such as XIST implicated in X-chromosome inactivation contain inefficiently spliced introns47,48. Considering that defects in IncRNA-mediated gene regulation contribute to human diseases including cancer and that ARS2 is commonly mutated in various types of cancer, our findings may shed light on pathways contributing to the misexpression of genes, ultimately leading to the development of specific therapeutic strategies.

Methods

Cell lines, strain construction, and growth conditions. The fission yeast strains used in this study are listed in Supplementary Table 1. Strains were generated through genetic crosses or were constructed using a PCR-based method. A DNA cassette containing a selection marker with or without epitope tag was amplified using long oligonucleotides with homology to target gene loci. The PCR product was transformed and transformants were grown on appropriate selection plates. Deletions tagged alleles were confirmed by PCR. Co-IP experiments were performed using standard protocols unless otherwise noted in the figures. Since pir2, cbc1, and cwf10 are essential genes, we used partial loss of function mutant alleles. pir2-1 and cbc1-1 were generated using an error prone PCR method6. The pir2-1 mutant allele carries two amino acid substitutions: F165L and S316P. The cbc1-1 mutant allele contains one amino acid substitution: L119P. cwf10-1 is a gift from R. Allshire. For the generation of cryptic intron mutants, a strain containing a urad4 selectable marker inserted at the pho1 locus was transformed with a DNA fragment containing the pho1 ORF with mutations in the splice sites. Splice site mutations were confirmed using Sanger sequencing and production of IncRNA was confirmed for RT-PCR. pir2-2 and cbc1-1 mutant strains were grown at 26 °C to an OD_600 0.5 prior to shifting to 33 °C for 5 h. Cells carrying the partial loss function cwf10-1 mutant were cultured under conditions that do not affect normal splicing.9 For experiments indicating phosphate or no phosphate growth conditions, Edinburgh minimal media (EMM) was prepared with or without 15.3 mM sodium phosphate and 20 mM potassium phosphate. HepG2 cells were purchased from ATCC (ATCC HB-8065). Monolayers of HepG2 cells were cultured in DMEM with 10% FBS at 37 °C and 5% CO2. According to ATCC, cells were authenticated and mycoplasma tests were done using Hoechst and direct culture method followed by microtechniques.

Sporulation assay. Cells were spotted onto EMM (Fig. 1f) or Pommbe minimal glutamate (PMG) medium (Fig. 2e) plates and grown at 30 °C for 3 days prior to exposure to iodine vapor. They were subsequently mounted on a 2% agarose pad for differential interference contrast (DIC) imaging using Hoffman modulation contrast software on a DeltaVision Elite fluorescence microscope (Applied Precision, GE Healthcare) with Olympus 100×/1.40 objective. Fiji V1.0 (Image), National Institutes of Health was used for processing the images and counting the sporulation frequencies. Sporulation efficiency was monitored in more than 1000 cells from three independent isolates for each strain.

Co-immunoprecipitations. For co-IP experiments in fission yeast, 1 L of S. pombe cells was grown overnight to OD_600 0.8 and cells were harvested by vacuum filtration. Cell pellets were flash frozen in liquid nitrogen. Cells were lysed using a CryoMill (Retsch) at a setting of 30 (frequency per second) for 1 min and repeated 3 times with 30 s intervals. Lysed cells were resuspended in lysis buffer (50 mM Tris–HCl pH 8.0, 1% NP40, 5 mM EDTA) containing Halt Protease Inhibitor Cocktail (Thermo Fisher Scientific). Lysates were incubated with anti-ARS2 antibody (Abcam, ab192999) for 2 h and washed 5 times with 1× sample buffer. Proteins bound to beads were washed and treated with 50 U/ml Benzonase for 30 min. Antibody–protein complexes were eluted off the beads using 1× sample buffer and heated to 95 °C for 5 min. For western blot analysis, samples were run on a 10% Tris-glycine gel, transferred to PVDF (Thermo Fisher Scientific) and probed using anti-FLATM (M2 Sigma, F1804) at dilution 1/1000, anti-GFP (Roche) at dilution 1/1000, or anti-c-MYC (Covance, 9E10) antibodies at dilution 1/250.

For co-IP experiments in mammalian cells, human HepG2 cells were grown to 70–90% confluency and the cell nuclei were isolated in hypotonic buffer (20 mM HEPES pH 7.4, 10 mM KCl, 2 mM MgCl2, 1 mM EDTA, protease inhibitors) and cell buffer (50 mM Tris pH 8, 0.3 M KCl, 0.5 mM EDTA/EGTA, 0.25% NP-40) by resuspending the nuclear pellet using a 20G needle. Lysates were incubated with anti-ARS2 antibody (Abcam, ab192999) for 2 h and washed 5 times with lysis buffer. Elution and western blotting were performed as described above. Anti-EFTUD2 (Abcam, ab72456) and anti-AR52 (Abcam, ab192999) antibodies were used to probe the western blots.

Glyceral gradient analysis. To prepare yeast cell lysates, cells were lysed using a CryoMill (Retsch) as described above and the cell powder was resuspended in 10% glycerol buffer (20 mM Tris pH 7.5, 137 mM NaCl, 10% Glycerol, 0.5% NP40, 2 mM EDTA). For human HepG2 cells, nuclear extract was prepared as described above and the nuclei were lysed in 10% glycerol buffer by passing through a 20G needle 10 times. The lysate was resolved by loading 150 μg onto a linear 20–50% glycerol gradient prepared in an ultracentrifuge tube (Beckman, 437357). The gradients were spun in an Optima TLX-ultracentrifuge (Beckman) at 81,400 × g for 19 h. Fractions were collected by pipetting and were resuspended on a 4–12% gradient gel (Invitrogen, NP0336BOX), followed by western blot analysis with ARS2 and EFTUD2 antibodies as described above. Western blotting to detect S. pombe proteins was performed with the following antibodies: GFP (Pir2-GFP detection; Roche, 11814460001) at dilution 1/1000, HA (Cdc5-HA and Spp42-HA detection; Roche, 11814460001) at dilution 1/1000, HA (Cdc5-HA and Spp42-HA detection; Roche, 11814460001) at dilution 1/1000, and CDK (Abcam, ab72456) at dilution 1/1000.
Northern blot analysis. For most experiments, cells were cultured in YEA medium that contains phosphate. In experiments comparing levels of poh1 expression in the presence or absence of phosphate, EMS was used with or without 15.5 mM sodium phosphate and 20 mM potassium phosphate. Total RNA was isolated by incubating cells in hot phenol heated to 65 °C for 10 min followed by 3 additional extractions using phenol-chloroform. RNA was precipitated using the sodium-acetate-ethanol method. Northern blots were performed according to the published protocol.18 10 µg of RNA was resolved on a 1% formaldehyde agarose denaturing gel and capillary transferred using NorthernMAX transfer buffer (Thermo Fisher Scientific) onto positively charged BrightStar-Plus nylon membrane (Ambion) and crosslinked using UV Stratalinker 2400 (Stratagene). The T7 in vitro transcription kit (Promega) was used to generate antisense T7 transcripts. The expression level of T7 transcript was analyzed using a Qubit fluorometer (Thermo Fisher Scientific). Libraries were directly generated from the IP sample. The total input sample was first subjected to ribosomal RNA removal before library preparation. The libraries for IP and input samples were prepared and analyzed as described above for RNA-seq. The libraries were sequenced using the NextSeq 500 platform (illumina).

RT-qPCR. Total RNA was extracted as described above and treated with RQ1 DNase (Promega) followed by phenol-chloroform extraction and ethanol precipitation. Strand specific reverse transcription was performed using Revertaid Reverse Transcriptase (Thermo Fisher Scientific) with gene specific reverse primers (Supplementary Table 2) and quantified by performing qPCR with iTaq Universal SYBR Green Supermix (Bio-Rad) on the QuanStudio 3 platform (Thermo Fisher Scientific).

ChiP and ChiP-sequencing. ChiP experiments were performed according to the published protocol.19 Cells were washed with OD560 buffer at OD560 < 0.1 and crosslinked using 1% formaldehyde at room temperature for 20 min. Cells were spun at 2000 x g for 10 min and pellets were lysed using glass beads and lysis buffer (50 mM HEPES/KOH, 140 mM NaCl, 1 mM EDTA, 1% Triton X100, 0.1% DOC plus protease inhibitors). Cell lysates were sheared using a Bioruptor-300 (Diagenode) to an approximate size of 500–1000 bp. The precipitation of protein of interest was performed using 50 µl of anti-c-MYC affinity gel (Sigma, A7470) or 5 µg of anti-GFP antibody (Abcam, ab299). Protein A magnetic beads (NEB) were used to capture the GFP-protein–antibody complexes. Beads were washed twice with lysis buffer, twice with lysis buffer containing 0.5 M NaCl and once in TE buffer pH 8. Chromatin–antibody complex was eluted using TE buffer (20 mM Tris–HCl pH 8, 10 mM EDTA, 1% SDS) and, along with whole-cell extract (WCE) input, were de-crosslinked by heating to 65 °C overnight and purified using PCR purification columns (Qiagen). Immunoprecipitated and input DNA were assessed using (i)aga-qPCR SYBR green supermix (Bio-Rad) according to the manufacturer’s recommendations. Sequencing libraries were prepared using the NEBNext Ultra II DNA Library Prep Kit for Illumina according to the manufacturer’s protocol and analyzed using an Agilent 4200 TapeStation system (Agilent). Sequencing was performed on the NextSeq 500 platform (illumina).

Small RNA-sequencing. Total RNA was isolated from a total of 4 OD600 units of log-phase cells using the Ribo-Zero Magnetic Kit for yeast (Illumina). Libraries were made using the NEBNext Ultra Directional RNA Library Prep kit for Illumina (NEB) according to the manufacturer’s instructions. Libraries were analyzed and sequenced on the MiSeq platform (illumina) as described above.

RNA immunoprecipitation. S. pombe cells expressing either GFP-tagged Pir2 (Pir2-GFP) or untagged Pir2 were grown in 30 ml of YE medium at 30 °C to an OD 600 of 0.5. Cells were then crosslinked by adding formaldehyde to a final concentration of 1% for 20 min with gentle shaking. After adding glycine to a final concentration of 0.2 M to stop crosslinking, cells were then resuspended in lysis buffer (50 mM HEPES pH 7.9, 140 mM NaCl, 1 mM EDTA, 10% glycerol, 0.5% NP-40, 0.25% Triton X-100) supplemented with complete EDTA-free protease inhibitors and RNase inhibitor. lysed by bead beating. The lysate was sheared by sonication using a Bioruptor-300 (Diagenode). 1.5% of the original lysate for RNA preparation was set aside as input. The rest of the lysate was pre-cleared using 0.9 µg of pre-washed protein G Dynabeads (Invitrogen, 10004D) at 4 °C for 1 h. Anti-GFP antibody (Abcam, ab299) was added to the lysate and incubated with gentle rotation at 4 °C overnight. Antibody–protein complexes were captured using 1.2 mg of protein G Dynabeads for 2 h at 4 °C. Beads were washed once in 900 µl of lysis buffer, once in 900 µl of lysis buffer with 300 mM NaCl, once in 900 µl of LiCl buffer (50 mM HEPES pH 7.5, 250 mM LiCl, 0.5% NP-40, 0.1% sodium deoxycholate), and once in 4× TE buffer pH 7. Beads were washed 7× in 25 µl of elution buffer (50 mM Tris pH 8, 10 mM EDTA, 300 mM NaCl, 1% SDS) at 37 °C for 10 min. To the 50 µl input samples, 100 µl of RIP elution buffer was added to a final volume of 150 µl. Then, 20 µg of proteinase K (Thermo Fisher Scientific, am2548) was added to both IP and input samples, and the mixtures were incubated at 37 °C for 1 h and then at 55 °C for 1 h to de-crosslink. The samples were then extracted once with phenol:chloroform and once with chloroform, precipitated with ethanol, and resuspended in 80 µl of DEPC treated water. The samples were further treated with 20 units of RNase-free DNase I (Thermo Fisher Scientific, am2222) for 1 h at 37 °C, extracted with phenol:chloroform:isoamyl alcohol (25:24:1), and the samples were resuspended in 30 µl and 100 µl water, respectively. RNA concentrations were determined using a Qubit fluorometer (Thermo Fisher Scientific). Libraries were directly generated from the IP sample. The total input sample was first subjected to ribosomal RNA removal before library preparation. The libraries for IP and input samples were prepared and analyzed as described above for RNA-seq. The libraries were sequenced using the NextSeq 500 platform (illumina).

RIME. Rapid immunoprecipitation mass spectrometry of endogenous proteins was performed according to the published procedure.20 Monolayers of IEB HepG2 cells were crosslinked with 0.8% formaldehyde for 8 min at room temperature. Crosslinking was quenched with 0.1 M (final) glycine and cell nuclei were isolated using buffer LBI (50 mM HEPES-KOH pH 7.5, 140 mM NaCl, 1 mM EDTA, 10% glycerol, 0.5% NP-40, 0.25% Triton X-100). Nuclear lysates were resuspended using buffer LB2 (10 mM Tris-HCL pH 8.0, 200 mM NaCl, 1 mM EDTA and 0.5 mM EGTA) and lysed with buffer L3 (10 mM Tris-HCL pH 8.0, 100 mM NaCl, 1 mM EDTA, 0.5 mM EGTA, 0.1% (wt/vol) sodium deoxycholate and 0.5% (vol/vol) N-lauroylsarcosine). A Bioruptor-300 (Diagenode) was used to sonicate the lysate to yield DNA fragments of size range 200–500 bp followed by full speed centrifugation at 4 °C for 10 min. The resulting supernatant was incubated with 10 µg of anti-ARS2 antibody (Abcam, ab192999) or 10 µg of rabbit IgG mock (Abcam, ab37415) bound to protein A magnetic beads (NEB) for 4 h at 4 °C with slow rotation. Beads were washed 10 times with RIP buffer (50 mM HEPES pH 7.5, 1 mM EDTA, 0.7% (wt/vol) sodium deoxycholate, 1% NP-40 and 0.5% LiCl) and twice with 100 mM ammonium hydrogen carbonate buffer. The protein–antibody complex was trypsinized overnight while on the beads and the peptides were extracted using Pierce C18 Spin Columns (Thermo Fisher Scientific). Extracted peptides were dried using a Vacufuge (Eppendorf). The resulting mass spectrometry data were acquired using an UltimaMate 3000 SRLCnano HPLC (Thermo Scientific). Peptides were separated at a flow rate of 300 nL/min followed by online analysis by tandem mass spectrometry using a Thermo Orbitrap Fusion mass spectrometer. Peptides were eluted into the mass spectrometer using a linear gradient from 96% mobile phase A (0.1% formic acid in water) to 55% mobile phase B (0.1% formic acid in acetonitrile) over 210 min. Parent full-scan mass spectra were collected in the Orbitrap mass analyzer set to acquire data at 120,000 FWHM resolution; ions were then isolated in the quadrupole mass filter, fragmented within the HCD cell, and the resulting normalized precursor ion mass was set as a variable modification. The precursor mass tolerance was 10 ppm, and the fragment mass tolerance was 0.6 Da. The Percolator node was used to score and rank peptide matches using a 1% false discovery rate. Protein false discovery rate was set at 1% and a minimum of 1 unique peptide required for protein identification. The mass spectrometry proteomics data are available in the ProteomeXchange Consortium via the PRIDE partner repository with the dataset identifier PXD018373 and 10.60199/PXD018373.

Bioinformatic analyses. ChiPseq sequences were aligned to the genome using Bowtie221. RIP and total RNA sequencing were aligned to the genome using TopHat2 and for RNA-seq, FPKM was calculated using cufflinks22. Normalized (RPKM) bedgraph files for aligned BAMs were generated using DeepTools23. Integrated Genomics assessments from sequencing reads in introns were derived by subtracting annotated and WT introns. Unlike annotated introns that are spliced in majority of reads, cryptic introns are inefficiently spliced in less than 5% of the total reads spanning the region containing the intron. Cryptic intron percentage was calculated by taking the number of reads that contained a split read, indicating a spliced event, and dividing that number by the total number of reads that overlap the intron junction. For RIP-seq, the signal obtained from the untagged strain was subtracted from Pir2-GFP signal. siRNA sequencing reads were aligned using Novoalign V2 (Novocraft) and aligned reads were processed to extract 21–24 nucleotide reads and generate SGR files using a python script. The resulting reads were normalized and a false discovery rate version using this study is AM2924v2. For RIME (Supplementary Fig. 7), p-values for peptide detection were estimated from the observed distribution of log2
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Author contributions
S.I.S.G. and G.T. designed the study. G.T., H.X., S.H., and J.D. performed experiments. H.X. performed RIP-seq and ultracentrifugation experiments. S.H. performed sporulation frequency experiment. J.D. generated the chc-1 mutant. D.W. performed statistical analysis of RIME and provided bioinformatics support. L.M.J. performed mass spectrometry analysis; G.T. performed all other experiments and bioinformatics analyses. S.I.S.G. and G.T. prepared data figures. S.I.S.G. and G.T. wrote the paper.

Competing interests
The authors declare no competing interests.

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