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The export factor Yra1 modulates mRNA 3' end processing

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Abstract

The yeast mRNA export adaptor Yra1 binds the Pcf11 subunit of cleavage-polyadenylation factor CF1A linking export to 3'-end formation. We found a surprising consequence of this interaction is that Yra1 influences cleavage-polyadenylation. Yra1 competes with the CF1A subunit, Clp1, for binding to Pcf11, and excess Yra1 inhibits 3' processing *in vitro*. Release of Yra1 at the 3' ends of genes coincides with recruitment of Clp1, and depletion of Yra1 enhances Clp1 recruitment within some genes. These results suggest that CF1A is not necessarily recruited as a complete unit, but instead Clp1 can be incorporated co-transcriptionally in a process regulated by Yra1. Yra1 depletion causes widespread changes in poly(A) site choice particularly at sites where the efficiency element is divergently positioned. We propose that one way Yra1 modulates cleavage-polyadenylation is by influencing co-transcriptional assembly of the CF1A/B 3' processing factor.

Keywords

Yra1; cleavage-polyadenylation; mRNA export; Pcf11; Clp1; Sub2; alternative polyadenylation

INTRODUCTION

Messenger ribonucleoprotein particle (mRNP) biogenesis comprises transcription by RNA polymerase II (pol II), coupled to processing of the nascent pre-mRNA and loading of RNA binding proteins (reviewed in [1–4]). Coordination of mRNP export with 3' end processing by cleavage-polyadenylation is a link between two distinct steps in mRNP metabolism that is conserved between yeast and humans [5–9]. This link helps ensure that only properly matured mRNAs become export-competent. Although export marks the final step in the nuclear history of an mRNP, the acquisition of export competence begins much earlier [10] with the co-transcriptional recruitment of export factors such as Yra1 in budding yeast (*S. cerevisiae*) (Aly/REF in metazoans) and the DEAD box helicase Sub2 (UAP56/HEL) [11–13]. Yra1 binds over 1000 mRNAs [14] and its recruitment to transcribed genes requires the 3' end processing machinery [8,15]. Subsequently Yra1 is transferred to the nascent mRNA in a step facilitated

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AUTHOR CONTRIBUTIONS

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by the helicase Sub2 (ref. 16,17) and then it binds the export receptor Mex67/TAP that escorts the mRNP to the nuclear pore [1819]. Yra1 and Sub2 are also proposed to associate within the TREX complex that enhances transcription elongation (reviewed in [19]). It is not known how initial Yra1 recruitment and hand-off to the mRNA are integrated with 3' end processing. Previously Yra1 and Sub2 were localized on several genes [11,12,17,20] but there has not been a genome-wide analysis that compares them with 3' processing factors at high resolution. The yeast genome is widely transcribed into non-coding cryptic unstable transcripts (CUTs) and stable unannotated transcripts (SUTs) [21], but the role, if any, of mRNA export factors in their metabolism is not known.

mRNA cleavage-polyadenylation in yeast requires the RNA-binding protein Hrp1/Nab4 (CF1B) and two multi-subunit complexes: cleavage factor 1A (CF1A) and cleavage-polyadenylation factor (CPF) (reviewed in [22–24]). These proteins recognize several loosely conserved elements in the nascent mRNA: the efficiency element (EE), the A-rich positioning element (PE), the cleavage site, and two U-rich elements flanking the cleavage site [25,26]. CF1A comprises Rna14, Rna15, Pcf11 and Clp1 and binds the PE through Rna15 (refs. [22–24, 27]). Among the CF1A subunits, Clp1 is the least well studied. CF1B binds the EE and contacts Rna14 and Rna15 [28]. The importance of the spacing between EE and PE motifs for 3' end processing is unclear, however it is notable that CF1B can modulate poly(A) site selection [29,30]. The CF1A complex also plays a backup role in processing of snoRNAs, and possibly CUTs [31,32].

The cleavage-polyadenylation complex assembles by making contacts with the nascent pre-mRNA and the pol II C-terminal domain (CTD) comprising heptad repeats, YSPTSPS, that are dynamically phosphorylated during transcription (reviewed in [33]). The Pcf11 subunit of CF1A directly contacts Ser2 phosphorylated heptads [33]. Whether CF1A binds pol II as a complete pre-formed complex or assembles co-transcriptionally is unknown.

Previously we reported a direct interaction between the export factor Yra1 and the 3' end processing factor Pcf11, which is sufficient to recruit Yra1 to a transcribed gene [15]. How the Pcf11-Yra1 complex is disassembled following recruitment of Yra1 to the gene is unknown but one possible player is the DEAD box helicase Sub2, which interacts with Yra1 (ref. 34) and has been implicated in re-modeling of mRNP complexes at 3' ends [9,35]. Yra1 binds to a conserved segment of Pcf11 (ref. 15) that encompasses the Clp1 interacting domain [36], thus when Yra1 is bound to Pcf11, it may prevent Clp1 from assembling into the CF1A complex. These observations suggest the intriguing hypothesis that CF1A may not be recruited to genes as a complete, pre-formed complex, but instead requires some assembly on the TEC [37].

Alternative polyadenylation (APA) is emerging as a major modulator of gene expression in mammalian cells (reviewed in [23,38]). Large-scale shifts in poly(A) site usage coccur in cancer cells relative to normal cells, and in pluripotent stem cells relative to differentiated cells [39,40]. A recent RNA-seq study, showed that alternative polyadenylation (APA) is also widespread in yeast [41]. Little is known about whether the use of multiple poly(A) sites at yeast genes is stochastic or controlled by specific protein factors. One candidate modulator

of poly(A) site choice is Yra1, which associates with a CF1A subunit, but is not a canonical 3' end processing factor.

In this report we compared the genome-wide localization of mRNA export and 3' end processing factors in *S. cerevisiae* and investigated whether the Pcf11-Yra1 interaction influences CF1A assembly and 3' end formation. Our results show that Yra1 is a novel *in vivo* modulator of 3' end processing with widespread effects on poly(A) site choice.

RESULTS

Disruption of the Pcf11-Yra1 interaction by Clp1 and Sub2

Co-transcriptional remodeling of mRNP complexes is an important part of their maturation. We asked how the Pcf11-Yra1 complex might be disassembled after recruitment of Yra1 to the gene. Yra1 binds a conserved region of Pcf11 flanked by two zinc fingers (residues 420–608; termed ZCZ; Supplementary Fig. 1a) that also binds to Clp1 (ref. 36). Yra1 and Clp1 may compete with one another for binding to Pcf11, or alternatively, a stable ternary complex may form between Yra1, Clp1 and Pcf11. The same regions of Yra1 (residues 1–66 and 124–226) that interact with Pcf11 (ref. 15) also interact with the helicase Sub2 (ref. 12,34). *sub2* mutations can increase Yra1 occupancy on genes [15] suggesting that Sub2 could participate in disassembly of Pcf11-Yra1 complexes. To investigate these possibilities, we assembled the GST-Yra1:Pcf11(ZCZ-His_6) complex on beads and challenged it with recombinant Clp1, or RNA and Sub2 (Supplementary Fig. 1b). The experiment in Figure 1a shows that Clp1 effectively released Pcf11(ZCZ-His_6) from the immobilized complexes into the supernatant in a dose-dependent way (top panel, compare lanes 4–7), but buffer alone had no effect (lane 3). When challenged with total yeast RNA, the Yra1-Pcf11 complexes were also dismantled with release of Pcf11(ZCZ-His_6) into the supernatant and this effect was sensitive to RNAse (Fig. 1b, top panel, lanes 3–6). Excess Sub2 plus ATP failed to disassemble Yra1-Pcf11 complexes, however the same amount of Sub2 together with a low concentration of RNA did effectively release Pcf11(ZCZ-His_6) (Fig. 1b, lanes 7, 8). In summary, these results show that *in vitro* at least, Clp1, which binds Pcf11, and Sub2 plus RNA, which both bind Yra1, can dismantle Yra1-Pcf11 complexes. These experiments do not eliminate the possibility that intermediate complexes can form between Yra1 or Sub2 and CF1A, however Clp1 did not appreciably co-immunoprecipitate with Yra1, nor did Sub2 co-immunoprecipitate with Pcf11 (Supplementary Fig. 1c).

Yra1 inhibits cleavage and polyadenylation *in vitro*

If Yra1 competes with Clp1 for binding to Pcf11 as suggested by the experiment in Figure 1a, then formation of an active CF1A 3' end processing complex may be limited by Yra1 thereby impairing cleavage and or polyadenylation. To test this idea, we examined the effect of adding recombinant Yra1 to an *in vitro* cleavage-polyadenylation reaction. Full length GST-Yra1 inhibited coupled cleavage-polyadenylation of the *GAL7-1* substrate RNA (see Methods) by approximately 400% as compared to the Yra1-RRM control fragment (Fig. 1c lanes 1–3 and graph) that does not bind Pcf11 (Supplementary Figure 1a) and slightly activated processing (Fig. 1c) In the presence of cordycepin (3'dATP), that permits cleavage, but not polyadenylation, full-length Yra1 impaired processing by approximately

400%, whereas the RRM fragment had no inhibitory effect (Fig. 1d, lanes 1–3). The effect of Yra1 on polyadenylation uncoupled from cleavage was assayed using a pre-cleaved *GAL7* substrate. Full-length Yra1 inhibited the reaction approximately 300% in a dose-dependent way (Fig. 1d, lanes 5–7, Supplementary Fig. 1d). Notably the polyadenylation that did take place in the presence of excess full-length Yra1 produced longer poly(A) tails than in control reactions with the RRM domain alone (Fig. 1d, lanes 6, 7, Supplementary Fig. 1d). In summary, the inhibition of 3' processing by Yra1 *in vitro* is consistent with the model that binding of this export factor to Pcf11 impairs CF1A activity, however these experiments do not eliminate other potential inhibitory mechanisms.

Distinct Chip profiles for export and 3' processing factors at 3' ends

To investigate how Yra1 occupancy on genes is related to Clp1 occupancy, we compared the localization of these factors by genome-wide ChIP-Chip. We detected Yra1 ChIP signals greater than flanking intergenic regions on 2198 genes, including 399 previously identified targets of mRNA binding [14]. As expected, Yra1 was strongly enriched on the *YRA1* intron consistent with autoregulation of splicing [42] (Supplementary Fig. 2a). Analysis of 789 genes enriched for both Pcf11 and Yra1 (Supplementary Table 1) revealed that on average Yra1 and Sub2 peak near the 3' ends of ORFs (Fig. 2a, solid arrow) and then decline, whereas Clp1 and Pcf11 peak further downstream near poly(A) sites (Fig. 2a, dashed arrow) as previously reported [20]. This transition probably corresponds to the concerted transfer of Yra1 and Sub2 from the transcription elongation complex to the transcript [16,17] and is consistent with cooperative disassembly of Pcf11–Yra1 by Sub2 and RNA (Fig. 1b).

Notably, the average ChIP profiles of Clp1 and Pcf11 do not exactly parallel one another. Whereas Pcf11 increases fairly uniformly across most ORFs [43,44], Clp1 is often recruited specifically at 3' ends (Figs. 2a, b compare Pcf11 (black) vs Clp1 (green). The ChIP-Chip data therefore suggest that the Pcf11 and Clp1 subunits of CF1A are not always recruited together, and may be recruited sequentially.

mRNA export factors at non-coding RNA genes

Yra1 and Sub2 are not only recruited to protein coding genes, but also to non-coding pol II transcribed genes including CUTs, SUTs, and snoRNAs (Fig. 2c, Supplementary Fig. 2b). We also found that Yra1 and Sub2 are strongly enriched on tRNA genes, transcribed by pol III (Fig. 2c, Supplementary Fig. 2c). Yra1 at ncRNA and tRNAs genes could be associated with Pcf11 that is also enriched on these genes [43]. Clp1 is also enriched at snoRNAs and tRNAs and at lower levels on CUTs and SUTs, where its distribution differs from that of Yra1/Sub2 (Fig. 2c).

Depletion of Yra1 favors early recruitment of Clp1 relative to Pcf11

The interaction of Yra1 with Pcf11 could influence the recruitment of other CF1A components, notably Clp1, to transcribed genes. To explore this idea, we analyzed Pcf11 and Clp1 by ChIP-Chip in WT and isogenic Yra1-depleted cells using a GAL1-*YRA1* mutant (DBY1276-2) that was grown in glucose for 8 hours (Fig. 3a). Yra1 depletion caused a modest increase in Clp1 recruitment relative to Pcf11 that was most evident on long genes (>2000 bases Supplementary Table 1)(Figs. 3b, c). When Yra1 was depleted, Clp1 ChIP

signals relative to Pcf11 were most strongly increased near the middle of long ORFs (Figs. 3b, c). A similar increase in Clp1 relative to Pcf11 following Yra1 depletion was observed when the signals were normalized to pol II (data not shown). The average distributions of Pcf11, Clp1, Yra1 and Sub2 on this subset of long genes in WT cells closely resembled those in the larger cohort of genes shown in Fig. 2A (Supplementary Fig. 2d). These experiments therefore show that on some genes Yra1 depletion favors premature recruitment of Clp1 within the body of the gene before polymerase reaches the 3' end.

Depletion of Yra1 modulates poly(A) site choice

To investigate whether Yra1 modulates 3' end processing *in vivo*, we asked whether its depletion affected poly(A) site choice at *ACT1* that has five alternate processing sites (Fig. 4a, bottom). *ACT1* poly(A) sites were mapped by RNAse protection assay (RPA) of total RNA from WT or GAL-*YRA1* (yra1-dep) cells grown in glucose for 8 hours. Yra1 depletion caused a shift in poly(A) site choice at *ACT1* with reduced use of the distal site 5 relative to the proximal sites 1 and 2 (Figs. 4a, b). This result contrasts with the effects of mutants in CF1A subunits including Clp1 (Supplementary Fig. 3) that increase the use of distal *ACT1* poly(A) sites [45]. We conclude that the mRNA export factor Yra1 functions in normal *ACT1* poly(A) site choice.

Genome-wide effects of Yra1 on poly(A) site choice

To determine whether the effect of Yra1 on poly(A) site choice is general, we surveyed the 3' end transcriptome of normal and Yra1-depleted cells by RNA-sequencing (RNA-seq). Oligo dT primed cDNA libraries were made from isogenic WT and GAL-*YRA1* cells that were both shifted from galactose to glucose for 8 hours. The libraries were sequenced unidirectionally from the 3' end across the junction between the poly(A) tail and the 3' UTR. This approach provides an unambiguous readout for the direction of transcription and the precise cleavage site for each polyadenylated mRNA. Reads that mapped adjacent to runs of A's in the genome are a source of artifacts due to internal priming and were discarded. We obtained 2.70 million uniquely mapped reads for WT and 1.58 million for the Yra1-depleted sample. Poly(A) site mapping by this method in the WT strain was in excellent agreement with a similar data set obtained by direct RNA sequencing [41] (Supplementary Fig. 4a). Like Ozsolak et al. (2010), we observed pervasive use of multiple poly(A) sites. RNA-seq confirmed the ORF-proximal shift among poly(A) sites at *ACT1* when Yra1 was depleted (Fig. 4c). The agreement between RNA-seq and RPA analyses of *ACT1* poly(A) sites validates counting poly(A) sequence tags as a convenient measure of mRNA abundance. The RNA-seq data were analyzed (see Methods) to identify those 3' UTRs where the major cleavage site was shifted ORF-proximal or ORF-distal by Yra1 depletion. Among those genes which exceeded a threshold number of reads at the major poly(A) site (9 for WT, 7 for Yra1-dep, see Supplementary Methods) we identified 1430 which shifted their major poly(A) sites upon Yra1 depletion including 349 which shifted by ≥15 bases ORF proximally and 381 which shifted by ≥15 bases ORF distally. At 2782 other genes Yra1 depletion had no effect on\distal shifted genes are shown in Figure 5 and genes with "no change" are shown in Supplementary Fig. 4b. In some, cases like *SSC1* and to a small extent *PMA1*, earlier recruitment of Clp1 following Yra1 depletion is associated with

a 5’ shift in poly(A) site choice, but this is not always the case (compare Fig. 3c with Supplementary Fig. 5d).

In addition to the genes where poly(A) sites shifted within 3’ UTRs, we also observed that use of premature poly(A) sites within ORFs or introns was modulated by Yra1 depletion. We identified 823 genes (Supplementary Table 1) where relative use of ORF-internal cleavage sites decreased by a factor of at least 4 after normalizing for total read counts (see Methods) following Yra1 depletion, including the poly(A) site within *RNA14* (ref. 46) (Fig. 6a). In these cases Yra1 enhances processing at gene internal sites (Fig. 6a). We also identified 426 genes (Supplementary Table 1) where use of ORF-internal cleavage sites increased by a factor of at least 4 following Yra1 depletion, including *RVS167* and *ATG17* (Fig. 6b). In these cases Yra1 suppresses processing at gene internal sites. In many other cases utilization of a poly(A) site within a gene was unaffected by Yra1 depletion, including the *DBP2* intronic poly(A) site (Supplementary Fig. 4c). Yra1 depletion did not strongly decrease expression of any cleavage-polyadenylation factor mRNAs as measured by the abundance of 3’ RNA-seq tags after accounting for total read numbers (Supplementary Fig. 4d and data not shown), therefore it is unlikely that reduced expression of these factors indirectly affected poly(A) site choice.

We can not eliminate the possibility that some of the effects of Yra1 depletion on relative abundance of different polyadenylated mRNA species are due to changes in their relative stability or to other indirect effects including defective mRNA export. However we did not find evidence for any widespread uniform stabilization or destabilization as judged by the relative abundance of different mRNAs in the WT and Yra1-depleted samples, consistent with previous observations [14]. For example, the decay of *GAL* gene transcripts over the 8 hours following the shift from galactose- to glucose-containing media was not markedly affected by Yra1 depletion (Supplementary Fig. 5a)

In addition to protein coding genes, Yra1 is enriched on genes for ncRNAs (Fig. 2c and Supplementary Fig. 2b) and we investigated whether Yra1 influenced formation of polyadenylated 3’ ends of these transcripts. We identified 312 cases (Supplementary Table 1, column 7) where Yra1 depletion altered ncRNA poly(A) sites including SUTs and CUTs that overlap *HSP82*, *GAL10*, *EPL1*, *TFP3* and *FLO9* as well as snoRNAs, snR11 and snR33 (Fig. 6c, d, Supplementary Fig. 5b). Yra1 depletion also affected poly(A) site choice of some non-coding regulatory RNAs including those that overlap the 5' regions of *HRP1* and *NRD1*, but not the *SRG1* ncRNA that overlaps *SER3* (Fig. 6e, and 5c). In summary, Yra1 has widespread effects on poly(A) site choice, both enhancing and suppressing 3' end formation of different coding and non-coding transcripts.

Divergent positioning of EE motifs at Yra1-sensitive poly(A) sites

CF1A and CF1B cooperatively bind to adjacent PE and EE motifs in the RNA (consensus AAWAAA and UAYRUA respectively) upstream of the mRNA cleavage site[47,48] (Fig. 7a). We asked whether the positions of these motifs relative to the cleavage site differ between genes that change their major poly(A) site upon Yra1 depletion (“Yra1 sensitive”) and those that are unaffected (“Yra1 insensitive”). Initially we confirmed that EE and PE motifs are positioned approximately 40 and 20 bp upstream of the cleavage site at 2782 “Yra1

insensitive" genes (Fig. 7a). Next we examined 730 "Yra1 sensitive" genes where the predominant cleavage site shifted by more than 15 bases in either direction when Yra1 was depleted (see Supplementary Methods). The position of the EE, but not the PE motif, relative to the major cleavage site used in WT cells differs among "Yra1-sensitive" genes (Fig. 7b and data not shown). At genes where Yra1 depletion causes an ORF-proximal shift in poly(A) site (349 genes, Supplementary Table 1 column 2), the EE is positioned further upstream of the cleavage site than at genes where Yra1 depletion causes an ORF-distal shift (~381 genes Supplementary Table 1 column 3, compare red and green lines, Fig. 7b; univariate KS test pvalue < .0001, Wilcoxon Rank Sum test pvalue< 2.22 E–16). The distribution of EE elements relative to cleavage site is more diffuse for both proximal and distal shifted genes compared to non-shifters (compare Figs. 7a, b). When the positions of EE motifs in the same 730 "shifted" genes are plotted relative to the major cleavage sites used in Yra1-depleted cells (Fig. 7c), then the distance between the EE motif and the cleavage site approaches the consensus value of ~40 bases especially for proximally shifted poly(A) sites (Fig. 7c, red arrow). These results suggest that normally, Yra1 specifically promotes 3' processing at poly(A) sites with divergent or non-optimal positioning of EE elements, and it is these genes where the site of cleavage most frequently changes when Yra1 is depleted.

DISCUSSION

In this report, we asked whether the interaction between Yra1 and Pcf11 that links mRNA export with 3' end formation, could also modulate assembly of the CF1A 3' processing complex and cleavage-polyadenylation. Our results are consistent with a model where Yra1 and Clp1 compete for binding to a common partner, Pcf11 as predicted by Saguez and Jensen [37] (Fig.7d). Several lines of evidence support this model:1) Excess Clp1 can disrupt Yra1–Pcf11 complexes *in vitro* (Fig. 1); 2) Pcf11 and Clp1 occupancy increases in the 3' flanking region as Yra1 and Sub2 occupancy decreases (Fig. 2a, b) suggesting a concerted transfer of Yra1 and Sub2 from the polymerase complex onto the transcript [16,17] prior to poly(A) site cleavage; 3) Depletion of Yra1 *in vivo* can enhance Clp1 occupancy relative to Pcf11 within the body of long genes (Figs. 3b, c); 4) Full-length Yra1 inhibits cleavage and polyadenylation *in vitro* whereas a fragment that does not bind Pcf11 has no inhibitory effect (Fig. 1c, d). ChIP localization of Pcf11 and Clp1 suggests that these two factors can load onto the TEC separately. Whereas Pcf11 occupancy increases quite uniformly 5'–3' across many ORFs [43,44], Clp1 signals remain relatively low in the 5' region and then increase at the 3' end (Fig. 2a, b). We propose that Pcf11 and Yra1 may assemble into a "poised" complex on the TEC that is subsequently remodeled by Clp1 and Sub2 to achieve two important goals: 1) co-transcriptional assembly of CF1A through formation of the Clp1–Pcf11 contact and 2) transfer of Yra1 to the transcript (Fig. 7d).

Unexpectedly Yra1 and Sub2 are also enriched on genes for CUTs, SUTs, snoRNAs and tRNAs (Fig. 2c, Supplementary Fig. 2b, c) where Pcf11 is also present [43]. Yra1 and Sub2 may promote export of ncRNAs, as they do for mRNAs. In any event, our results suggest that the function of these two factors is not confined to mRNA metabolism.

A dynamic balance between Pcf11–Yra1 and Pcf11–Clp1 complexes could influence the decision between alternative poly(A) sites. This idea is supported by the fact that depletion of Yra1 alters poly(A) site selection at numerous coding and non-coding RNAs (Figs. 5, 6, Supplementary Fig. 5b, c). The heterogeneity of poly(A) site use at numerous yeast genes [41] is therefore not a purely stochastic phenomenon, but can be influenced by the export factor Yra1. Another yeast export factor, Npl3, has been shown to alter cleavage-polyadenylation by masking a cryptic processing site in the *GAL7* 3' UTR [49]. Similarly many metazoan RNA binding proteins affect 3' end processing by competing with core cleavage-polyadenylation factors[23,50,51]. Depletion of Yra1 does not simply activate cryptic sites, rather it can either enhance or suppress utilization of poly(A) sites in different contexts (Figs. 5–7, Supplementary Fig. 5b, c). We propose Yra1 influences poly(A) site choice by a regulated assembly mechanism in which interaction with Pcf11 regulates co-transcriptional formation of fully functional CF1A complexes (Fig. 7d). This model also suggests that, like Yra1, Sub2 could affect poly(A) site choice. Our results do not rule out the possibility that Yra1 might influence 3' end processing after it has been transferred to the nascent RNA and it remains possible that Yra1 modulates poly(A) site choice by multiple mechanisms.

We do not fully understand why Yra1 depletion affects poly(A) sites in different ways, but one distinction between Yra1-sensitive and -insensitive sites lies in the positioning of the EE motif that binds CF1B. Yra1-insensitive poly(A) sites have well-positioned EE motifs whereas at Yra1-sensitive sites, they are less precisely positioned relative to the cleavage site (Fig. 7b, c). We speculate that at such non-optimal poly(A) sites, interaction of CF1A and CF1B with one another and with the RNA [28] may be more sensitive to modulators such as Yra1. How does Yra1 affect the selection of poly(A) sites at these "sensitive" sites? It could delay formation of fully active CF1A complexes by competing with Clp1, thereby suppressing use of particular poly(A) sites. This mechanism is consistent with the effects of Yra1 depletion on genes like *ACT1* where poly(A) sites closer to the 5' end are favored (Figs. 4, 5a, 6b). It is also possible that Yra1 could enhance formation of properly folded CF1A complexes by acting as a chaperone. This possibility is suggested by the fact that Aly, the human homologue of Yra1, has chaperone activity that enhances the DNA binding of several transcription factors [52,53]. Such a scenario could explain why processing at some sites is suppressed by Yra1 depletion. At first glance a positive influence on co-transcriptional CF1A assembly is not consistent with inhibition of cleavage-polyadenylation by Yra1 *in vitro* (Fig. 1c, d), however this experiment does not assay transcription-coupled processing, which may be affected differently than uncoupled processing. If different poly(A) sites in a transcription unit compete for processing by CF1A, then an increase or a decrease in the rate of assembly of the complex when Yra1 is depleted could shift the balance such that some sites are used more frequently and competing sites are used less frequently.

Human Aly and Pcf11 interact with one another like their yeast homologues[15] suggesting that Aly could be a modulator of poly(A) site choice in metazoans. Human Pcf11 is not found in the CstF complex with the homologues of Rna14 and Rna15, but instead is a subunit of cleavage factor II_m ($CFII_m$); the other subunit is human Clp1 [54]. We speculate

that REF/Aly, like Yra1, may affect the huPcf11-huClp1 interaction, and in doing so perhaps alters the function of $CFII_m$.

Modulation of alternative polyadenylation in metazoans is an important means of regulating gene expression [23,38]. One way that such regulation can be achieved is through altered expression of core components of the 3' processing machinery. The archetypal example is control of immunoglobulin heavy chain poly(A) site choice by regulating expression of CstF64, the homologue of yeast Rna15 (ref. 55). Another recently discovered mechanism works through U1snRNP inhibition of cleavage at cryptic poly(A) sites, probably by interacting with core cleavage-polyadenylation factors [56]. Our results suggest a related mechanism for APA regulation; namely that an export adaptor, which interacts with the core 3' end processing machinery, but is not itself a cleavage-polyadenylation factor, can function as a general modulator of poly(A) site choice.

METHODS

GST-Yra1 (aa 1–226) [57], GST-Yra1 RRM (aa 77–167) and Pcf11(ZCZ-His_6) (aa 420–608) have been described [15]. Full length Sub2 (aa 1–446) was a gift from Dr. R. Zhao (U. Colorado), and GST-Clp1 (aa. 1–445) was expressed from pGEX6P1-Clp1 and cleaved with PreScission Protease (GE Healthcare) (Supplementary Figure 1).

Pcf11-Yra1 competition assays

GST-Yra1 full length and GST-Yra1 RRM on glutathione Sepharose were treated with RNAse and bound to yPcf11(ZCZ-His_6) [15], washed extensively and resuspended in buffer containing:1) (0.25, 1.0, 4.0 or 16.0 μg) of full length Clp1 (cleaved from the GST fusion) 2) 0.1, 1.0, or 10.0 μg of total yeast RNA (W3031B); 3) RNA pre-treated with RNAse (1 μg ml^{-1}); 4) 10 μg Sub2 + 1mM ATP; or 5) both 1μg RNA and 10μg Sub2 (+ 1mM ATP). After incubation for 30 minutes at room temperature supernatants were collected and immunoblotted for yPcf11 ZCZ-His_6 with anti-his antibody (Sigma).

Yeast strains and growth conditions

Yeast strains are described in Supplementary Table 2. Except where noted cells were grown in YPD to a density of OD_{600}= 0.8 for ChIP. For Yra1 depletion, W3031B DBY1276-2 (GAL-*YRA1*) or WT control cells were grown in SC medium containing 2% w/v galactose and 2% w/v raffinose to OD_{600}= 0.2, then grown for an additional 2–8 hours in SC+ 2% w/v glucose.

Antibodies

Rabbit polyclonal anti-panCTD, -yPcf11, -Yra1 and -Sub2 antibodies were described previously [15]. Rabbit anti-Clp1 was made against full length GST-Clp1. Monoclonal antibodies were anti-HA (12CA5), anti-Pgk1 (Molecular Probes), and anti-His (Sigma).

ChIP-Chip

ChIP samples were amplified by ligation mediated PCR (23 cycles) prior to hybridization to Nimblegen whole genome arrays (cat. no. C4214-00-01 with 378,684 50mers tiled every 32

bases) as described [43]. ChIP-ChIP data are available at GEO Series accession number GSE30706.

Plots for individual loci and averages for multiple loci were generated with ChIPViewer software as described [43]. For calculation of profiles for multiple genes, the data points within a gene were scaled and averaged over 10 equal intervals corresponding to 10 dots (e.g. Fig. 2a). One kb of 5' and 3' flanking sequence was included in each profile. ChIPViewer profiles will be made available on our searchable database at http://bit.ly/bentley-lab.

In vitro Cleavage-polyadenylation

Extract was prepared from W3031B cells grown in YEPD at 30°C to OD_{600} 2.0 and processing reactions were carried out as described [49] in 30μl (see Supplementary Methods). ^{32}P labeled GAL7 substrates (250,000 cpm per reaction) were made using linearized plasmids pJCGAL7-1 or pJCGAL7-9 [58]. Reactions were carried out in 5mM HEPES (pH 7.9), 0.05mM EDTA, 1mM MgOAc, 11mM KCl, 75mM KOAc, 0.1mM DTT, 20mM creatine phosphate, 2% w/v PEG8000, 0.4U RNA guard, and ATP (2mM) or cordycepin (2mM). GST-Yra1 full length or GST-Yra1 RRM (5μg) was pre-incubated with extract (5 min. at 25°C then 10 min. on ice). Products were separated on 6% denaturing gels and quantified by Phosphorimager (Molecular Dynamics).

RNA-seq of polyadenylated 3' ends

Oligo-dT primed libraries of 3' ends were made as described [59] with modifications outlined in Supplementary Methods and sequenced with the Illumina Genome Analyzer IIx. Single-end 25 base reads (after removing oligodT(12) and barcodes) were mapped to the UCSC yeast genome (sacCer2, SGD genome Jun 2008 assembly) with Bowtie version 0.12.5 as described in Supplementary Methods. Statistical methods for identification of poly(A) sites with altered usage in Yra1 depleted cells and motif analysis (Fig. 7a–c) are described in Supplementary Methods. RNA-seq data are available at GEO Series accession number GSE30706.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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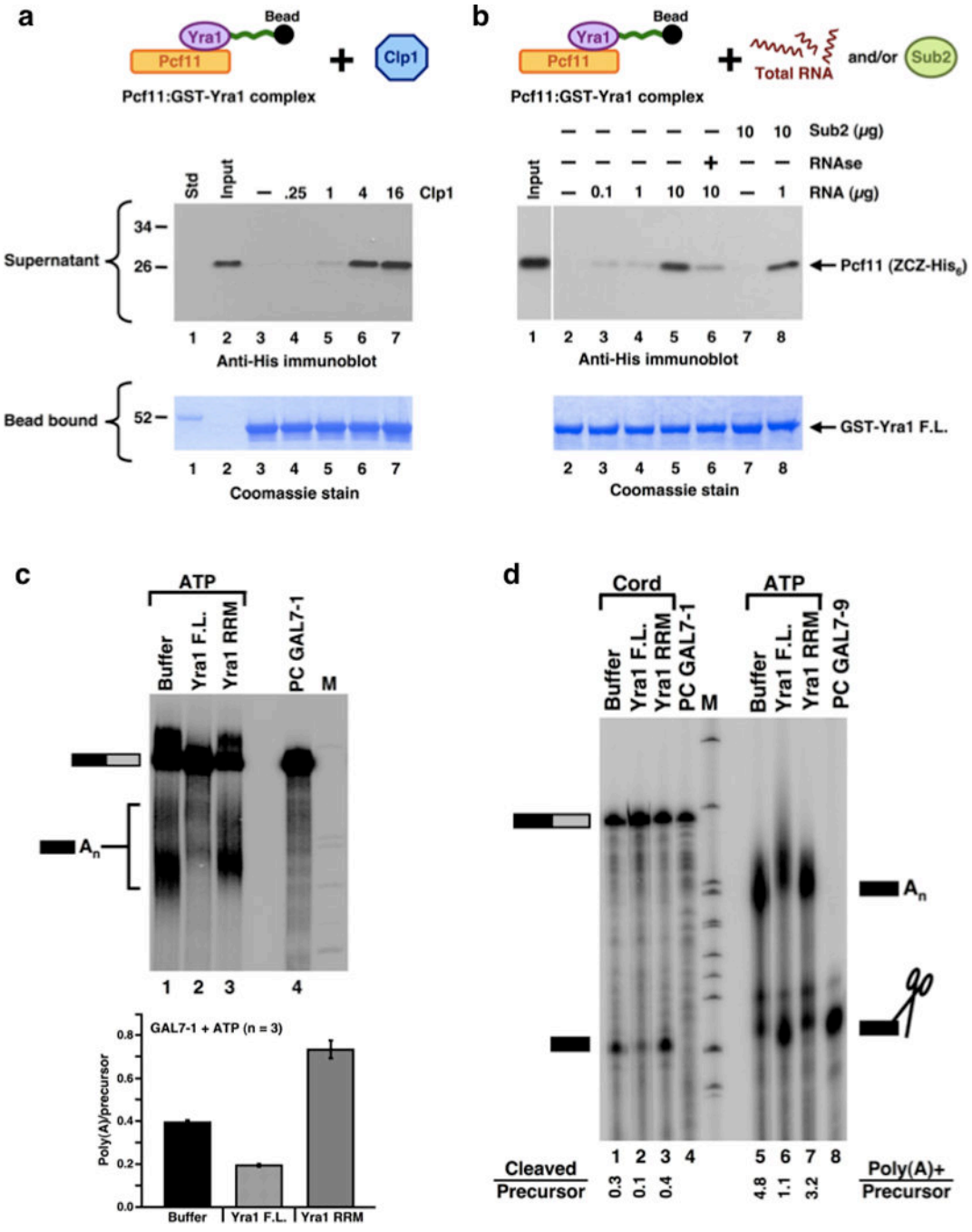

Figure 1.

Yra1 influences the cleavage-polyadenylation apparatus. **(a)** Clp1 disrupts the Pcf11-Yra1 interaction. GST-Yra1 beads (full length F.L.) bound to Pcf11(ZCZ-HisHis6) were incubated with RNAse-treated Clp1 (0.25–16μg) or buffer (lane 3), and Pcf11(ZCZ-His_6) released into the supernatant was immunoblotted (top panel). Coomassie stained GST-Yra1 is a loading control (bottom panel). **(b)** Sub2 and RNA disrupt the Pcf11-Yra1 interaction. Immunoblot of released Pcf11(ZCZ-His_6) as in panel **a**. **(c)** Yra1 inhibits coupled cleavage-polyadenylation of ^{32}P-labeled GAL7 RNA precursors (lane 4) (see Methods). GST-

Yra1F.L. or GST-Yra1RRM (5 μg) was added in lanes 2, 3. Products were quantified by Phosphorimager, and ratios of polyadenylated products:precursor calculated. Values are means, ± s.e.m. **(d)** Yra1 inhibits uncoupled cleavage and polyadenylation. Left: processing of GAL7 + cordycepin to inhibit polyadenylation. Right: polyadenylation of pre-cleaved GAL7 + ATP.

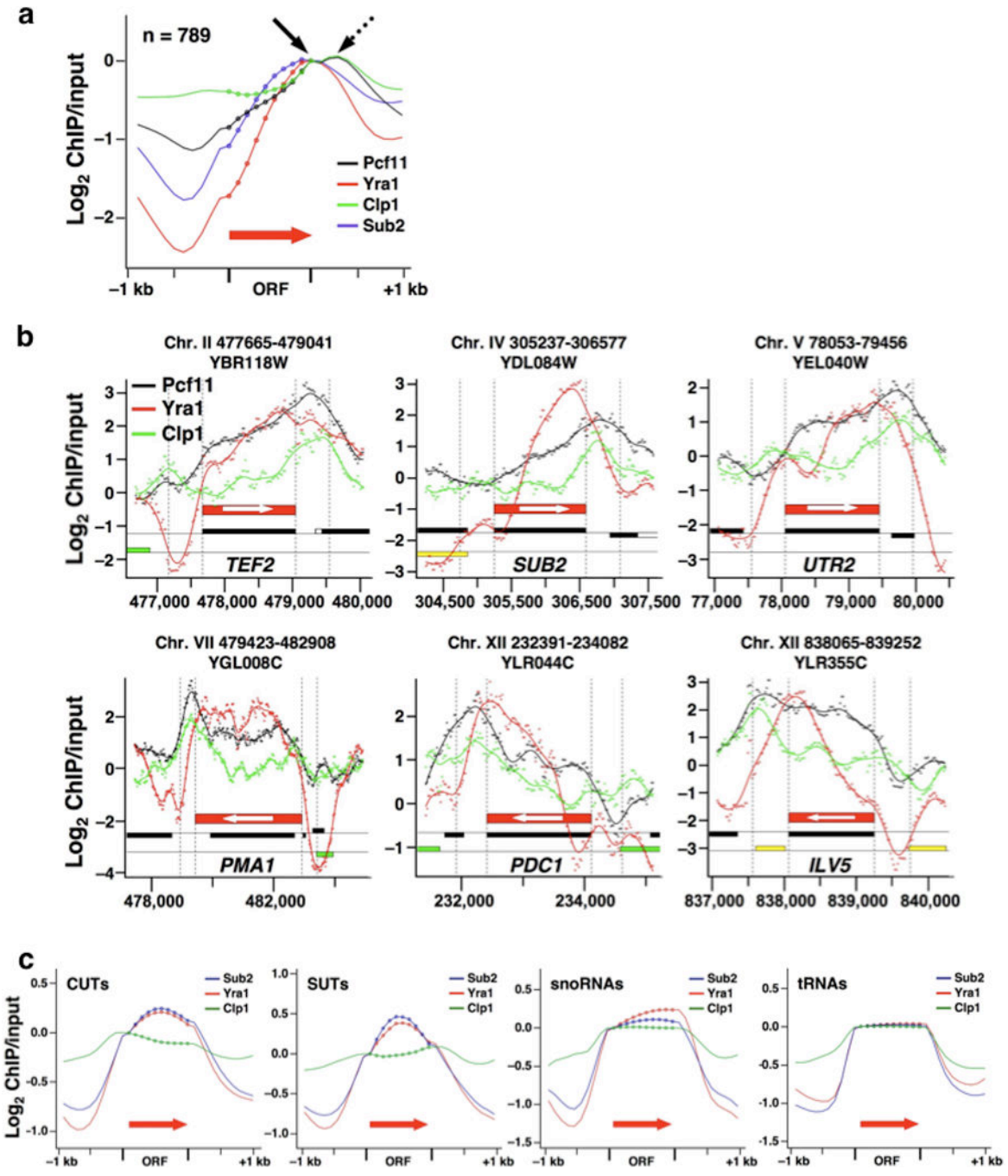

Figure 2.

Distinct Chip profiles for export and 3' processing factors. **(a)** Yra1 occupancy decreases as Clp1 occupancy increases at the 3' ends of genes. Average distributions of Pcf11, Clp1, Yra1 and Sub2 normalized to the values at the 3' end of the ORF on 789 protein-coding genes (Supplementary Table 1). Plot includes the ORF divided into 10 equal intervals and 1 kb of 5' and 3' flank. Variation between these proteins in relative signals upstream of the ORF after normalization results from differences in average signal:background ratios between the IPs. Note distinct transitions of export (solid arrow) and 3' processing factors

(dashed arrow) at 3' ends. Log2 ChIP signals relative to input DNA are shown for ORFs (dotted lines, red arrow) with 1 kb of 5' and 3' flanking sequence (smooth lines). **(b)** Representative ChIP profiles of Pcf11, Clp1, and Yra1 generated with ChIPViewer[43]. Each point is the average ChIP signal at 20-base intervals. Note distinct profiles of Pcf11 and Clp1 recruitment. **(c)** Average distributions as in (a) showing enrichment of Yra1, Sub2 and Clp1 on non-coding RNA genes as described [43].

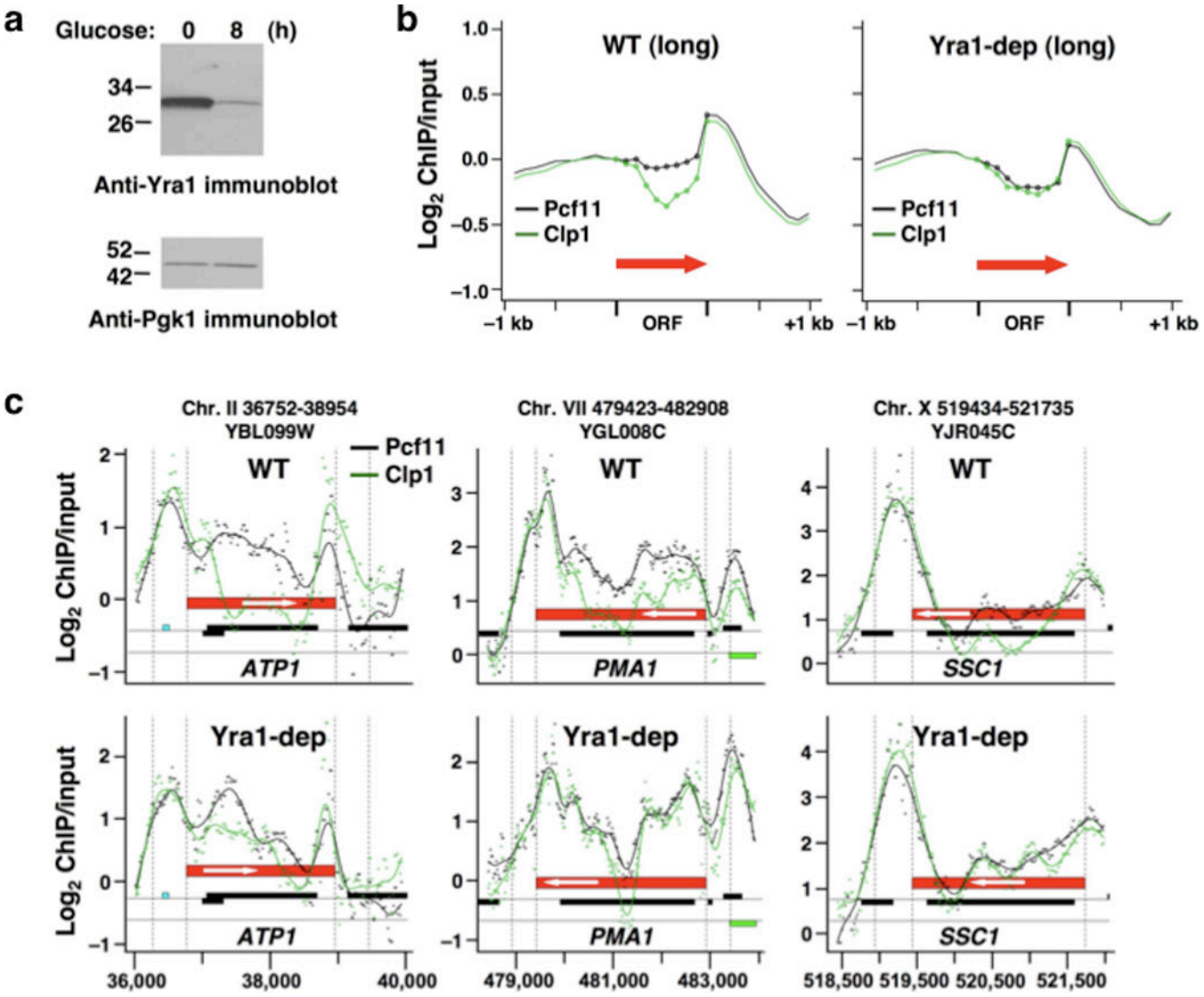

Figure 3.

Yra1 depletion enhances Clp1 recruitment on long genes. **(a)** Depletion of Yra1 in glucose grown GAL1-Yra1 cells (DBY1276-2). Immunoblots of Yra1 and Pgk1 loading control are shown. **(b)** Average ChIP-Chip profiles as in Fig. 2a of Pcf11 and Clp1 normalized to the values at the 5' end of the ORF on 128 long genes (>2000 bases, Supplementary Table 2) in isogenic WT and Yra1-depleted cells (8 hr in glucose). **(c)** Representative ChIP-Chip profiles of Pcf11 and Clp1 in WT (top row) and Yra1-depleted (bottom row) cells. Note enhanced Clp1 occupancy relative to Pcf11 within the ORFs in Yra1-depleted cells.

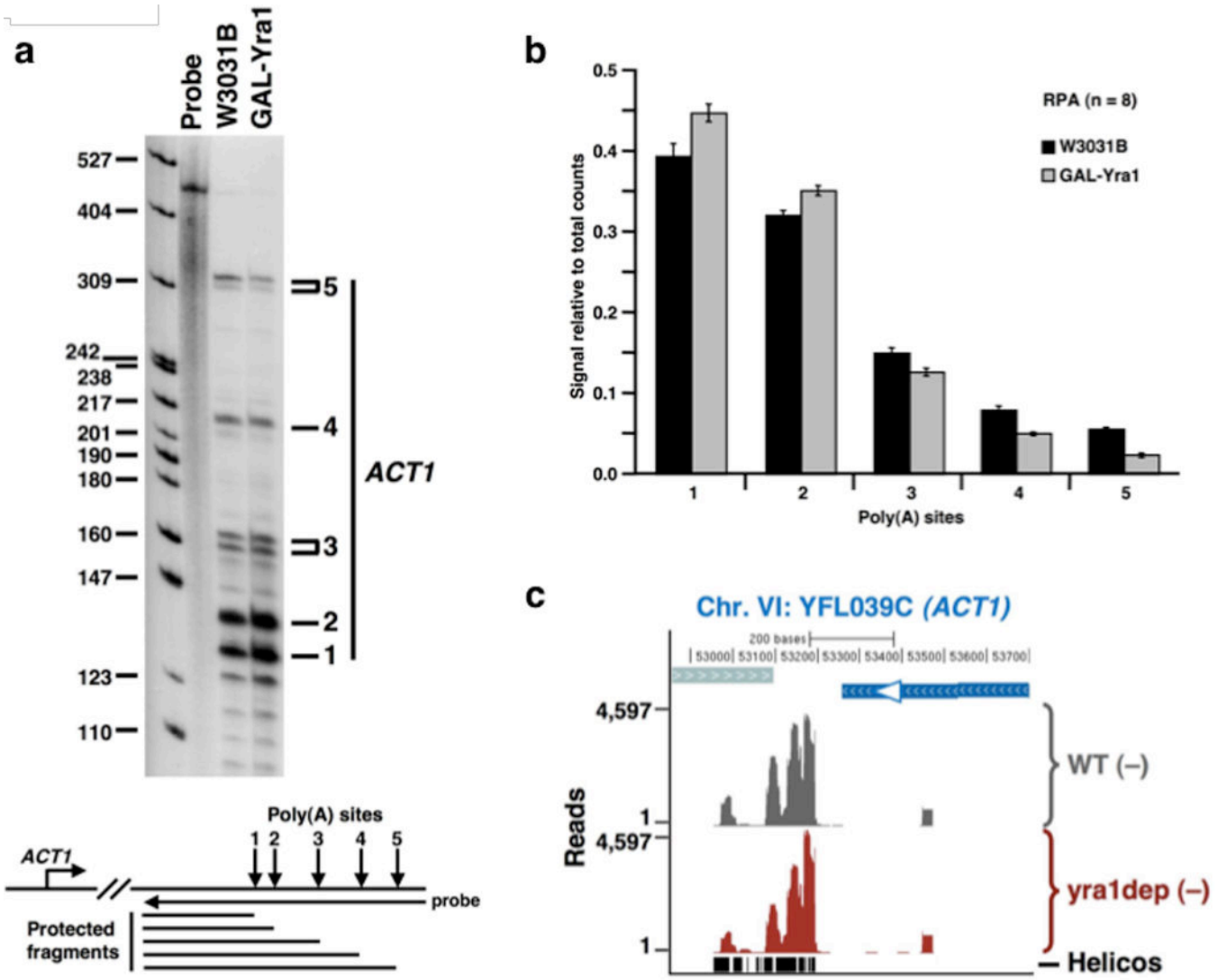

Figure 4.

Yra1 depletion affects alternative poly(A) site choice at *ACT1*. **(a)** RNAse protection assay of total RNA from WT and Yra1-depleted *GAL1-YRA1* cells (8 hr glucose as in Fig. 3a). A map of the major poly(A) sites is shown below. **(b)** Reduced ORF distal and enhanced proximal site usage in Yra1-depleted cells. Cleavage at each site is expressed as a fraction of total signal after normalization for the ^{32}P-U content of protection products 1–5. Values are means, ± s.e.m. **(c)** 3' RNA-seq (see Methods) confirms the shift in favor of ORF proximal *ACT1* poly(A) sites in isogenic WT and Yra1-depleted (Yra1dep) cells (8 hr in glucose, see Fig. 3a) and in the Helicos data set [41]. (−) refers to transcripts on the Crick strand. The Helicos track combines reads on both strands.

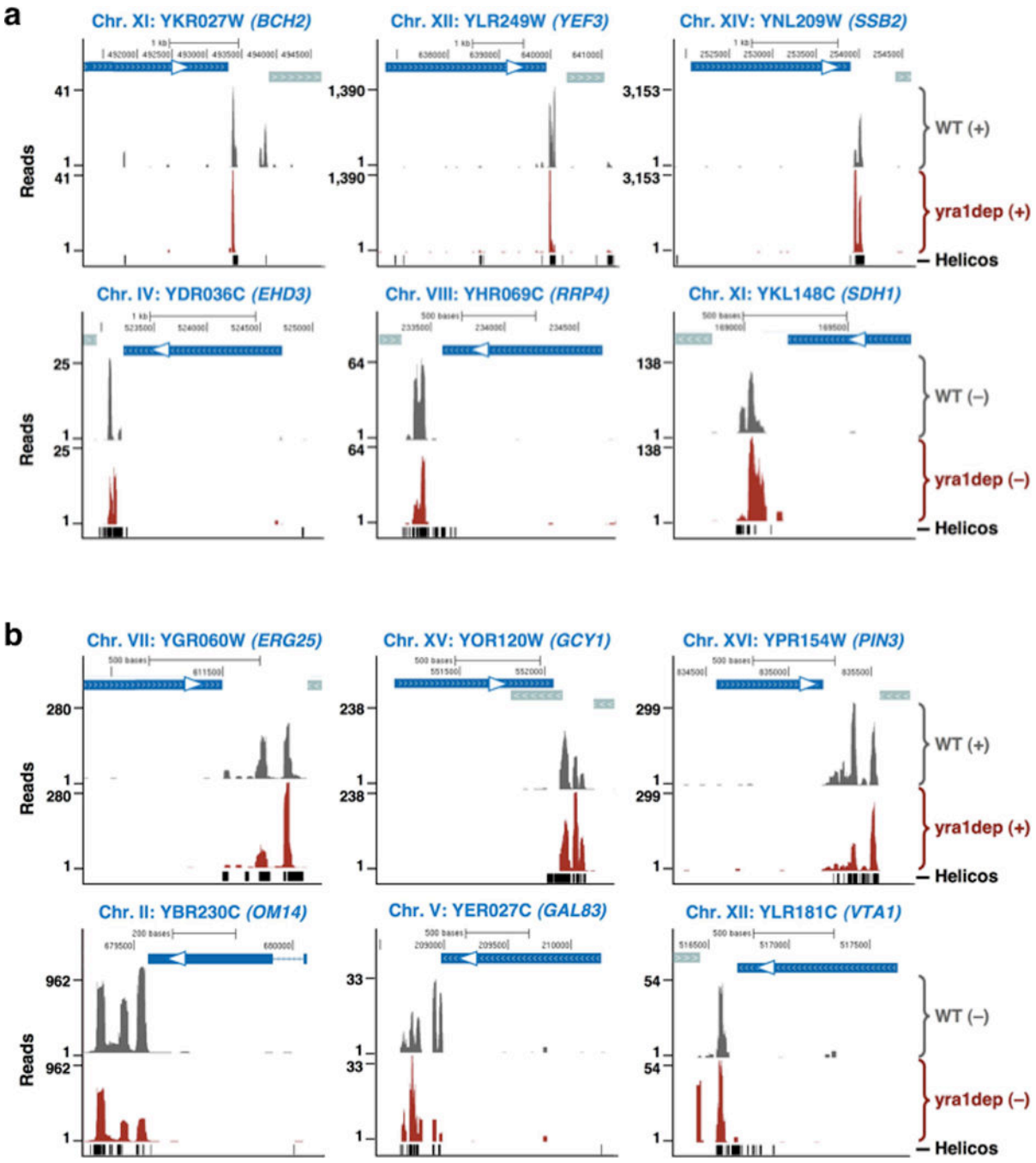

Figure 5.

Widespread effects of Yra1 depletion on alternative poly(A) site choice within 3' UTRs. **(a, b)** Genome browser views of RNA-seq reads across poly(A) sites in WT and Yra1-depleted *GAL1-YRA1* cells (8 hr in glucose) and in the Helicos data set [41]. (+) and (−) refer to transcripts of the Watson and Crick strands respectively. ORF proximal and ORF distal shifting of poly(A) site choice in Yra1-depleted conditions are shown in **(a)** and **(b)** respectively. Total read counts are 2.70 million for WT and 1.58 million for yra1-dep data sets.

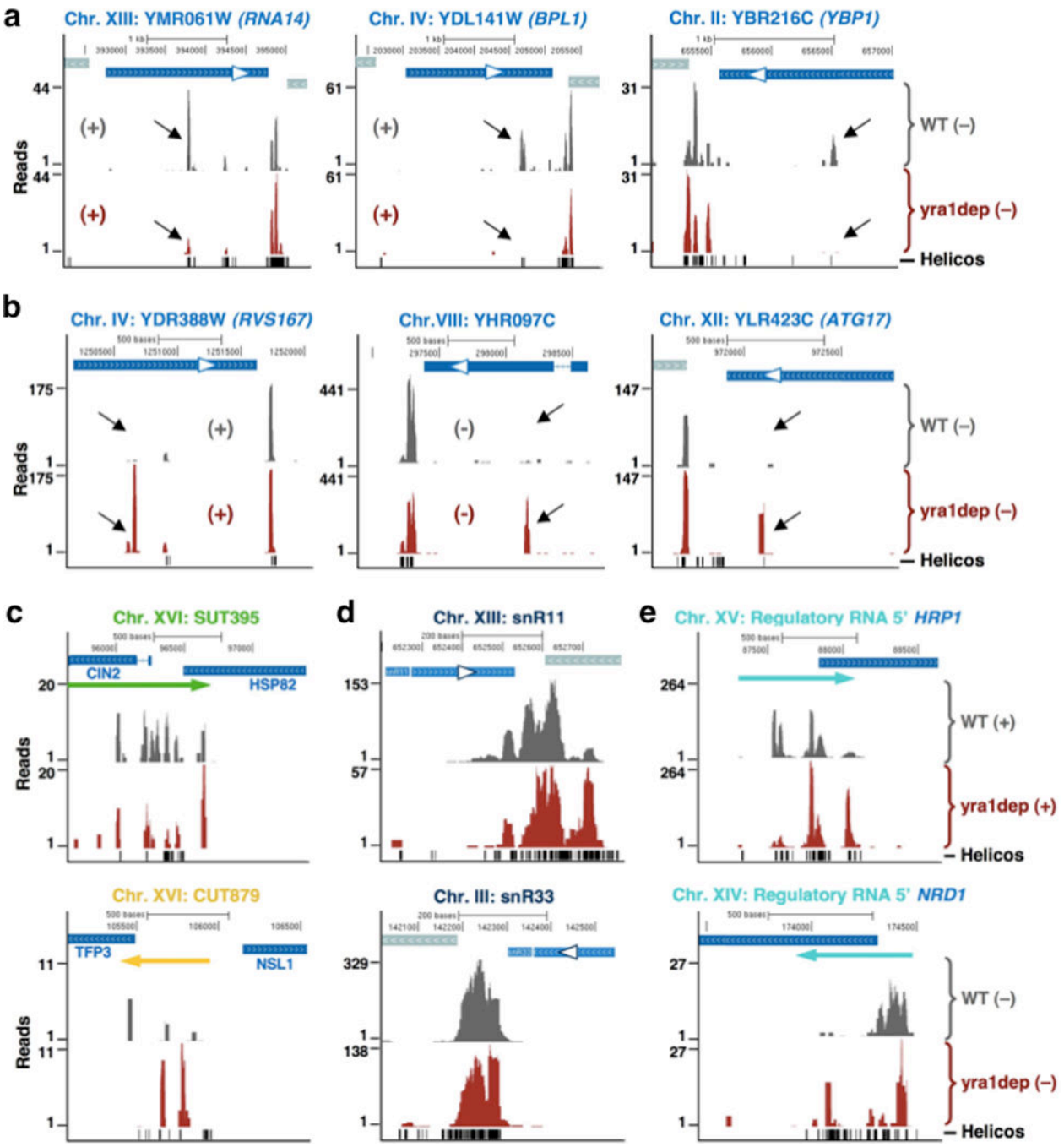

Figure 6.

Yra1 depletion alters use of poly(A) sites within genes and at ncRNAs. **(a–e)** Genome browser views of RNA-seq reads across poly(A) sites in WT and Yra1-depleted cells as in Fig. 5. **(a, b)** Premature poly(A) sites within ORFs that are reduced **(a)** or enhanced **(b)** by Yra1 depletion. **(c–e)** Altered poly(A) site use at ncRNAs following Yra1 depletion at SUTs/CUTs **(c)**, snoRNAs **(d)** and regulatory ncRNAs **(e)**.

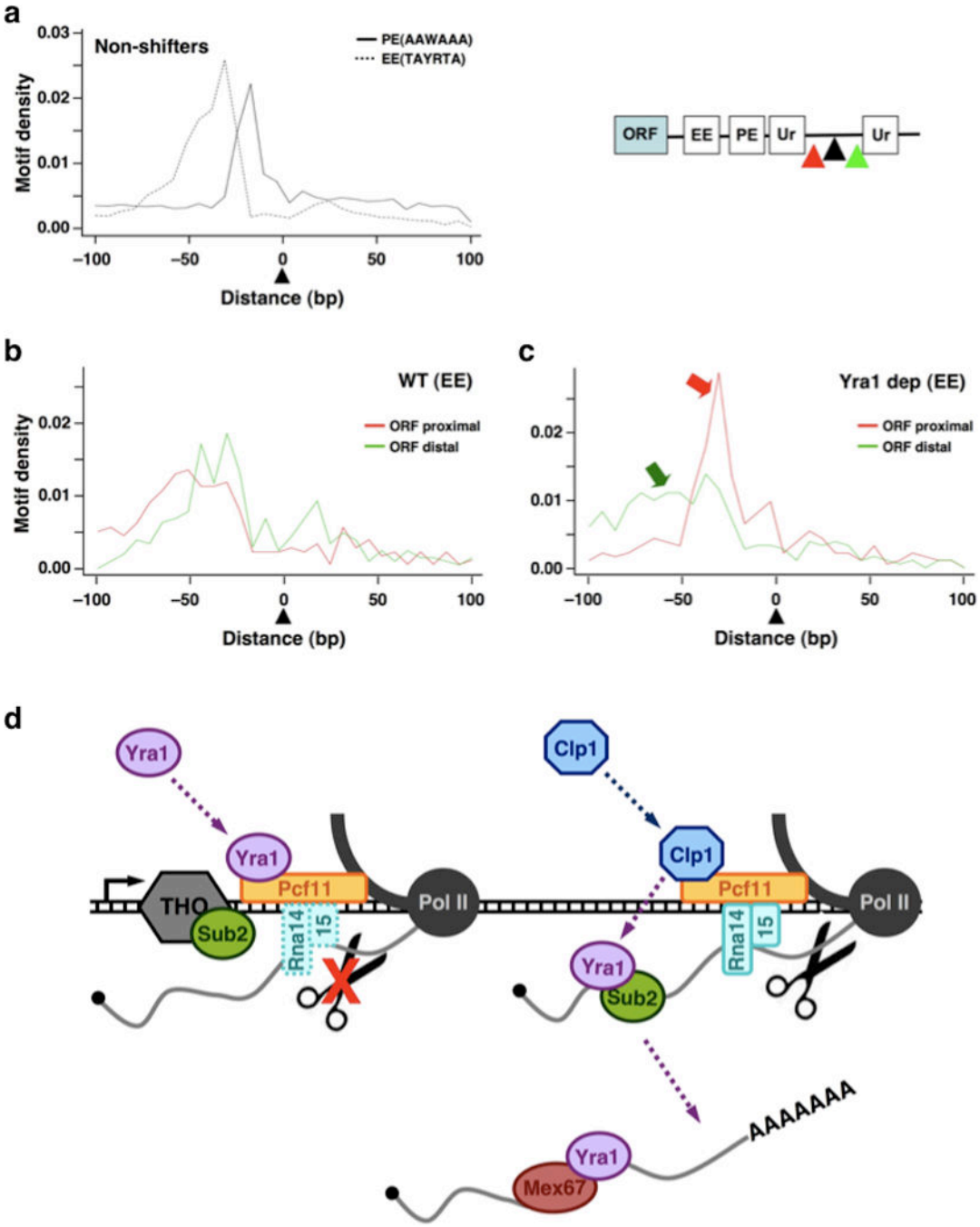

Figure 7.

Positioning of consensus efficiency element differs between “Yra1 sensitive” and “insensitive” poly(A) sites. **(a)** Distribution of positioning and efficiency element (PE and EE) consensus sites relative to cleavage sites (black arrowhead, position 0) used in WT cells on genes that are unaffected by Yra1 depletion (non-shifters, 2782 genes). When multiple elements were present, we chose the closest to the cleavage site. Map shows location of poly(A) site consensus elements including U-rich sequences (Ur). Red and green arrowheads mark poly(A) sites shifted upon Yra1-depletion. **(b)** Distributions of EE motifs

relative to cleavage sites used in WT cells at genes whose major poly(A) sites shift ≥15 bases following Yra1 depletion. Note the divergent positions of EE elements at genes that change their poly(A) site choice ORF proximally (349 genes, red line) or distally (381 genes, green line). **(c)** Distributions of EE motifs relative to cleavage sites used in Yra1-depleted cells at the same genes shown in (b). Note the change from WT cells (b) in the position of EE elements (arrows) relative to cleavage sites. **(d)** A regulated assembly model for how the export factor Yra1 could influence co-transcriptional 3' end processing. Left and right panels depict recruitment of Yra1 and CF1A subunits within genes and at 3' ends. Rna14 and Rna15 are dotted at left to indicate that the timing of their recruitment relative to Pcf11 is not known. 'Poly(A) site cleavage (scissors, right panel) becomes possible at 3' ends only after assembly of the complete CF1A complex.