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The Spen Family Protein FPA Controls Alternative Cleavage and Polyadenylation of RNA

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SUMMARY

The spen family protein FPA is required for flowering time control and has been implicated in RNA silencing. The mechanism by which FPA carries out these functions is unknown. We report the identification of an activity for FPA in controlling mRNA 3′ end formation. We show that FPA functions redundantly with FCA, another RNA binding protein that controls flowering and RNA silencing, to control the expression of alternatively polyadenylated antisense RNAs at the locus encoding the floral repressor *FLC*. In addition, we show that defective 3′ end formation at an upstream RNA polymerase II-dependent gene explains the apparent derepression of the *AtSN1* retroelement in *fpa* mutants. Transcript readthrough accounts for the absence of changes in DNA methylation and siRNA abundance at *AtSN1* in *fpa* mutants, and this may explain other examples of epigenetic transitions not associated with chromatin modification.

INTRODUCTION

A key feature of flowering time control is the quantitative nature of the response, underpinned by precision in gene regulation, which enables plants to adapt to environmental change (Kobayashi and Weigel, 2007). Genetically separable pathways that promote, repress, or enable this developmental switch control flowering time. For example, flowering is promoted by the photoperiod pathway in response to day length (Kobayashi and Weigel, 2007), but this function is compromised by floral repressors such as the transcription factor FLC. The strength of this repressor is in turn controlled by vernalization and autonomous pathways, which effectively enable flowering by limiting *FLC* mRNA expression (Simpson, 2004).

FPA was first identified through the characterization of a late-flowering *Arabidopsis thaliana* mutant (Koornneef et al., 1991; Schomburg et al., 2001). As a component of the autonomous pathway, FPA enables flowering by preventing the accumulation of mRNA encoding FLC (Michaels and Amasino, 2001). FPA comprises three repeated RNA recognition motifs (RRM) located near the N terminus and a protein interaction SPOC (Spen paralog and ortholog C-terminal) domain at the C terminus. Together, this organization of protein domains defines the signature features of spen family proteins (Ariyoshi and Schwabe, 2003). The founding member of this family, the eponymous *split ends* (spen) gene, was identified as a lethal mutation perturbing neuronal development in *Drosophila* embryos (Kolodziej et al., 1995). A human spen protein, one twenty-two translocation (OTT) or RNA binding motif protein 15 (RBM15), is involved in the recurrent t(1;22)(p13;q13) chromosomal translocation that causes infant acute megakaryocytic leukemia (Ma et al., 2001). Spen proteins play a general role in cell fate specification during animal development (Kuroda et al., 2003; Raffel et al., 2009). The mechanisms by which spen family proteins mediate these effects are unclear, but a recurring theme has been regulation of transcription within notch signaling pathways (Jin et al., 2009; Oswald et al., 2002; Shi et al., 2001), while RBM15 can also function in RNA export (Zolotukhin et al., 2009).

In addition to FPA, the autonomous pathway comprises a combination of components associated with RNA binding/processing or chromatin modification (Simpson, 2004); for example, the plant-specific RNA binding protein FCA physically interacts with FY to control *FLC* expression (Simpson et al., 2003). FY is a conserved RNA 3′ end processing factor related to *Saccharomyces cerevisiae* Pfs2p (Ohnacker et al., 2000) and human cleavage and polyadenylation specificity factor (CPSF) WDR33 (Shi et al., 2009). In addition, FLD, which is related to human lysine-specific demethylase (He et al., 2003) and FVE, a homolog of yeast MSI (multicopy suppressor of IRA1) and mammalian retinoblastoma-associated proteins RbAp46/48 (Ausin et al., 2004), are members of the autonomous pathway that function in chromatin modification in other eukaryotes. Autonomous pathway components are therefore more widely conserved than FLC, the floral repressor they regulate in *A. thaliana,* raising the likelihood that these proteins function in processes other than flowering time control (Simpson, 2004). Consistent with this idea, FPA and other members of the autonomous pathway were recently found to be required for transgene-mediated RNA silencing and to control endogenous targets of RNA-mediated chromatin silencing effected by the RNA-directed DNA methylation (RdDM) pathway (Bäurle et al., 2007; Veley and Michaels, 2008). For example, the epigenetic silencing of the SINE (short interspersed element) retroelement *AtSN1* is apparently derepressed in *fpa* mutants (Bäurle et al., 2007; Veley and Michaels, 2008). The silencing of *AtSN1* mediated by the RdDM pathway depends on RNA Polymerase V (Pol V) transcription that guides locus-specific siRNAs complexed with ARGONAUTE 4 (AGO4) to the *AtSN1* locus. AGO4 subsequently recruits chromatin-modifying activities, including

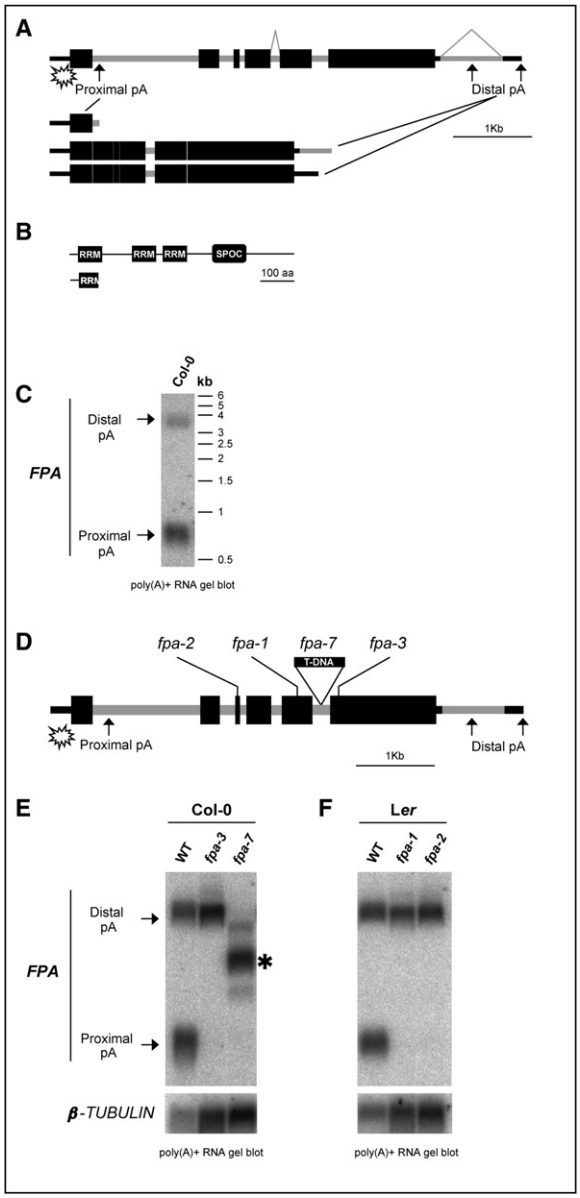

Figure 1. *FPA* Pre-mRNA Is Alternatively Polyadenylated in a Manner Dependent on Active FPA Protein

(A) Gene structure of *FPA*. Exons are denoted by black rectangles, UTRs by black lines, and introns by gray lines. Black arrows indicate cleavage and polyadenylation sites in pre-mRNA. Alternative splicing of intron 4 and 6 is indicated. The alternatively polyadenylated mRNAs of *FPA* are shown below.

(B) Domain organization of FPA protein encoded either by distally polyadenylated mRNA (above) or proximally polyadenylated RNA (below).

(C) RNA gel blot analysis of WT *A. thaliana* accession Columbia (Col-0) plants using poly(A)$^+$ purified mRNAs. Black arrows indicate the proximally and distally polyadenylated *FPA* mRNAs. A probe corresponding to the 5′UTR region of *FPA* mRNA (white star in [A]) was used to detect *FPA*-specific mRNAs. RNA size (kb) marker (Ambion).

(D) Schematic representation of the gene structure of *FPA* with point mutation or T-DNA insertion sites in mutant alleles indicated.

(E) RNA blot analysis of *FPA* mRNA in Col-0 WT and *fpa* alleles. Asterix indicates the major mRNA of *fpa-7* T-DNA insertion allele.

(F) RNA blot analysis of *A. thaliana* accession Landsberg *erecta* (*Ler*) WT and *fpa* mutant alleles.

the de novo DNA methyltransferase, DRM2, directing DNA methylation (Wierzbicki et al., 2008; Wierzbicki et al., 2009). In mutants defective in the RdDM pathway, *AtSN1*-specific siRNAs are not produced, DNA methylation is lost, and *AtSN1* RNA expression is upregulated (Wierzbicki et al., 2009). However, the upregulation of *AtSN1* in *fpa* mutants has features distinct from such mutants, as neither DNA methylation nor siRNA levels are affected (Bäurle et al., 2007). *fpa* mutants are not unique in this regard, as other silencing-defective *A. thaliana* mutants exist in which DNA methylation is unchanged (Nishimura and Paszkowski, 2007). The regulatory processes disrupted in these mutants are not yet understood (Nishimura and Paszkowski, 2007).

FPA is a nuclear protein, but the mechanism(s) by which it controls flowering and RNA silencing is unknown. We report here the identification of an activity for FPA in controlling alternative RNA cleavage and polyadenylation. This activity is similar to the function we previously identified for FCA (Quesada et al., 2003; Simpson et al., 2003). However, we have established that FCA and FPA promote poly(A) site selection in a genetically independent manner. By asking what implications such an activity might have for the function FPA performs in regulating flowering, we found FPA appears to function redundantly with FCA to control processing of antisense RNAs at the *FLC* locus. As FCA and FPA locate to *FLC* chromatin (Bäurle et al., 2007; Liu et al., 2007), our findings suggest they act directly to regulate expression of alternatively processed antisense RNA at this locus. When we investigated how an activity affecting RNA 3′ end formation could account for the function of FPA in RNA silencing, we discovered that defective RNA 3′ end formation at an RNA polymerase II (Pol II)-dependent gene upstream of *AtSN1* accounted for its apparent derepression in *fpa* mutants and could simultaneously explain the lack of change in siRNAs and DNA methylation at *AtSN1*.

RESULTS

FPA Controls Alternative Polyadenylation of *FPA* Pre-mRNA

FPA pre-mRNA is alternatively polyadenylated at promoter-distal sites within the 3′ end and at promoter-proximal sites in the first intron (Figure 1A). While distally polyadenylated RNAs would code for full-length FPA, the mRNA cleaved and polyadenylated within intron 1 codes only for the first RRM (Figure 1B). RNA gel blot analysis of wild-type *A. thaliana* poly(A)$^+$ RNA with a probe to the *FPA* 5′ leader revealed RNAs migrating at around 3.5 kb and 0.6 kb; these RNAs correspond to distally and proximally polyadenylated mRNAs, respectively, as judged by the size of sequenced full-length mRNAs and 3′ RACE (rapid amplification of cDNA ends) analysis (Figure 1C). The alternatively polyadenylated RNAs accumulate to similar levels, with quantification of RNAs polyadenylated at the proximal and distal sites revealing a ratio in the range of 1–3:1.

In contrast, the same analysis of an allelic series of *fpa* mutants in different *A. thaliana* accessions (Figure 1D) revealed a quite different pattern, as *FPA* RNA polyadenylated at the promoter-proximal site was almost undetectable (Figures 1E and 1F). Most of the alleles examined here have single base pair (bp) mutations that introduce a premature termination codon, while

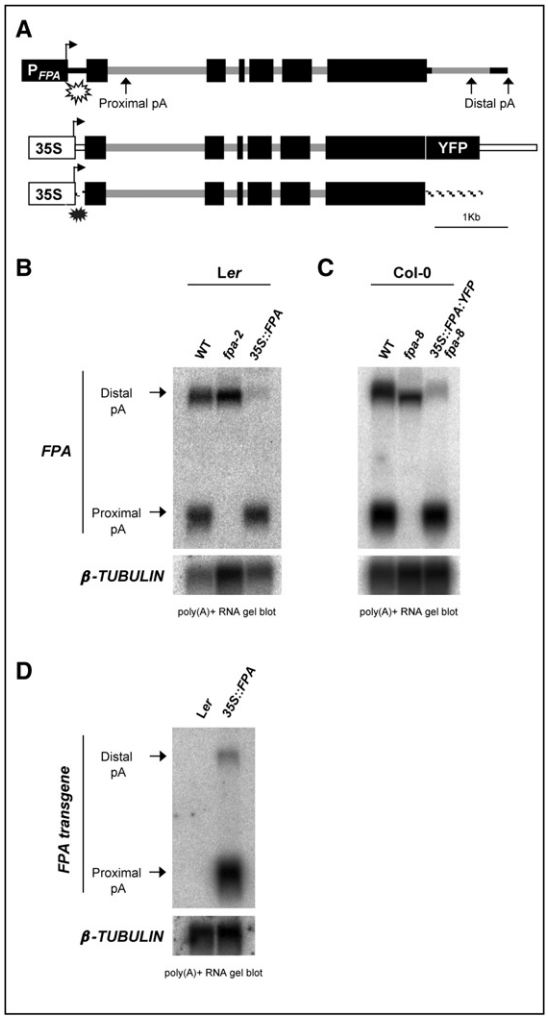

Figure 2. FPA Promotes Proximal Poly(A) Site Selection

(A) Schematic representation of endogenous *FPA* and *FPA* transgenes. White and dashed lines show the 5′ and 3′UTRs of transgenic *FPA* constructs. White star shows the region (5′UTR) used as a probe for endogenous *FPA*, black star shows region used as probe for detection of transgenic *FPA* RNA.

(B) RNA blot shows endogenous *FPA* specific mRNA accumulation in WT, *fpa* mutant, and transgenic plants overexpressing FPA protein in L*er* background.

(C) RNA blot shows endogenous *FPA* mRNA accumulation in WT, *fpa* mutant, and transgenic plants overexpressing FPA protein fused with YFP in Col-0 background.

(D) RNA blot analysis of plants overexpressing *FPA* from a transgene detected with a probe to the 5′ leader of the transgene (A).

fpa-7 contains a transfer (T)-DNA insertion within *FPA* intron 5 (Michaels and Amasino, 2001; Veley and Michaels, 2008). However, as the mutation that underpins each of these *fpa* alleles is found downstream of intron 1, the sequence of the truncated RNA produced from proximal polyadenylation within intron 1 would be indistinguishable from that found in wild-type (Figure 1D). Therefore, nonsense-mediated RNA decay cannot explain why the proximally polyadenylated RNAs are almost undetectable in *fpa* mutants. Instead, these findings lead us to suggest that proximal polyadenylation of *FPA* mRNA depends upon the expression of active FPA protein itself.

In order to test this idea, we overexpressed FPA from a transgene (Figure 2A) and asked how this would influence alternative polyadenylation of endogenous *FPA* pre-mRNA. *FPA* overexpression was driven by the cauliflower mosaic virus (CaMV) 35S promoter in stable transgenic lines. As the transgene had a different 5′ leader to that of endogenous *FPA*, a probe to this region allowed us to distinguish endogenous and transgene-derived RNAs (Figure 2A). The overexpression of FPA in these lines resulted in an almost complete switch to proximal poly(A) site selection within endogenous *FPA* pre-mRNA (Figure 2B). We repeated this experiment with a different line, overexpressing FPA from a different transgene in a different genetic background (Bäurle et al., 2007), and found the same switch in poly(A) site usage (Figure 2C). Together, these results indicate that FPA promotes proximal poly(A) site selection, rather than influencing RNA stability, because there is a reciprocal change in the detectable levels of alternatively polyadenylated RNAs in backgrounds either overexpressing or defective in FPA function.

Since RNAs cleaved and polyadenylated within intron 1 code for only the first RRM of FPA, this likely reflects a mechanism of negative autoregulation. To test this idea, a probe to the 5′ leader of overexpressed transgene-derived RNA (Figure 2A) was used to assess the relative use of proximal and distal poly(A) sites in RNA gel blot analysis (Figure 2D). An increase in the proportion of RNA polyadenylated at the proximal site, corresponding to a proximal:distal poly(A) site ratio of 30:1 for RNA expressed from the transgene compared to 1–3:1 for the endogenous gene, was detected (Figure 2D). Therefore, overexpression of full-length *FPA* is limited by cleavage and polyadenylation within intron 1 of *FPA* pre-mRNA, consistent with proximal poly(A) site selection mediating FPA autoregulation.

FPA and FCA Control Poly(A) Site Selection in a Genetically Independent Manner

Our findings with FPA are similar to our previous analysis of FCA: like FPA, FCA also autoregulates its expression by promoting proximal poly(A) site selection in its own pre-mRNA (Quesada et al., 2003). As both these proteins function in the same pathway of flowering time control (Simpson, 2004) and act to control the expression of targets of RNA-mediated chromatin silencing (Bäurle et al., 2007; Veley and Michaels, 2008), this raised the possibility that they worked together to control RNA 3′ end formation. To test this idea, we asked whether they were genetically required to control alternative polyadenylation of each other's pre-mRNAs.

We first studied *FPA* pre-mRNA poly(A) site selection in genetic backgrounds that lack *FCA.* However, in contrast to the clear requirement for active FPA, we found that *FCA* was not essential for proximal poly(A) site selection in *FPA* pre-mRNA (Figure 3A). FCA mediates poly(A) site selection by physically interacting with the cleavage and polyadenylation machinery via an interaction with FY (Quesada et al., 2003; Simpson et al., 2003), the homolog of *S. cerevisiae* Pfs2p and human CPSF WDR33 (Ohnacker et al., 2000; Shi et al., 2009). Although null alleles of *fy* are lethal, viable alleles that lack the C-terminal region required for its interaction with FCA exist that are late flowering (Henderson et al., 2005). We therefore asked whether FY was required for alternative polyadenylation of *FPA* pre-mRNA. However, RNA gel blot analysis of *fy-1* RNA revealed

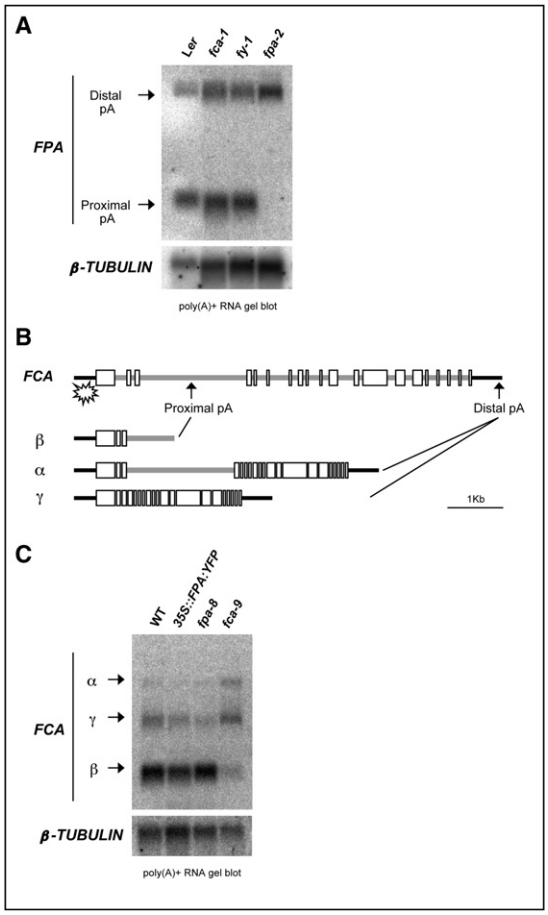

Figure 3. *FPA* and *FCA* Control Poly(A) Site Selection Genetically Independently

(A) RNA gel blot analysis of alternative polyadenylation of *FPA* mRNA accumulation in *fca* and *fy* mutants. Black arrows show alternatively polyadenylated *FPA* mRNAs.

(B) Schematic representation of *FCA* gene and alternatively polyadenylated *FCA* mRNAs.

(C) RNA gel blot analysis of alternative polyadenylation of *FCA* mRNA in *fpa* and *fca* mutants, and transgenic plants overexpressing FPA protein fused with YFP (in the *fpa-8* background). Black arrows show alternatively polyadenylated *FCA* mRNAs.

that FY was not essential for proximal poly(A) site choice in *FPA* pre-mRNA (Figure 3A).

We next asked the reciprocal question: could FPA influence alternative polyadenylation of *FCA* pre-mRNA? Using a probe to the *FCA* 5′UTR, it is possible to detect *FCA* RNAs cleaved and polyadenylated at a promoter-proximal poly(A) site within intron 3 (*FCA*β) and *FCA* RNAs cleaved and polyadenylated at a distal site at the 3′ end (*FCA*γ and α) (Quesada et al., 2003) (Figure 3B). Each of these RNAs were detected in wild-type plants (Figure 3C), and as we previously reported, there is a significant reduction in the proportion of RNA polyadenylated at the proximal poly(A) site in loss-of-function *fca* mutants (Quesada et al., 2003). However, no difference in alternative polyadenylation of *FCA* pre-mRNA was found in either loss-of-function *fpa* mutant backgrounds or lines overexpressing *FPA* from a transgene (Figure 3C). We therefore conclude that FCA and FPA control poly(A) site selection in a genetically independent manner on specific target mRNAs.

FPA Promotes Proximal Poly(A) Site Selection Directly

The genetic independence of FCA and FPA revealed that they must control poly(A) site choice through different mechanisms. Since FCA physically interacted with the core 3′ end cleavage and polyadenylation component FY (Pfs2p/WDR33), this raised the question of how directly FPA controlled alternative polyadenylation. Changing the expression of FPA affects flowering time through changes in the expression of mRNA encoding the floral repressor FLC (Michaels and Amasino, 2001). In order to determine whether the contrasting effects on poly(A) site selection within *FPA* pre-mRNA in genetic backgrounds differing in *FPA* activity might be mediated indirectly by changes in *FLC* expression, we used RNA gel blot analysis to investigate *FPA* poly(A) site selection in genetic backgrounds with either elevated levels of *FLC* (*fve-3, ld-1, fca-9, fpa-7*) or no functional FLC (*flc-3, flc-3 fpa-7*). No differences between *FPA* proximal or distal poly(A) site selection were detected in either type of background (see Figures S1A and S1B available online), revealing that changes in *FLC* expression that correlate with changes in FPA activity do not explain the distinct profiles of *FPA* alternative polyadenylation.

In order to determine how directly FPA was involved in poly(A) site selection, we next carried out chromatin immunoprecipitation (ChIP) experiments using antibodies that detect FPA specifically but which fail to cross-react with *fpa* mutant alleles (Figure S1C). Following FPA ChIP and using *fpa* mutants as negative controls, enrichment of *FPA* intron 1 sequence downstream of the proximal poly(A) site was detected (Figure 4), consistent with the idea that FPA controlled poly(A) site selection directly. The apparent absence of FPA association with the distal poly(A) sites in these ChIP experiments was also consistent with transgenic experiments that revealed the native 3′UTR was not required for FPA-mediated proximal poly(A) site selection (Figures 2A and 2D), suggesting the mechanism by which FPA controls poly(A) site choice does not involve inhibiting 3′ end formation at the distal sites in the conventional 3′UTR, with proximal poly(A) site selection then occurring by default.

We next asked whether FPA promoted proximal poly(A) site selection by promoting Pol II termination and thus preventing transcription of the distal poly(A) sites. However, ChIP analysis (Figure S1C) revealed no significant difference in Pol II association with the distal poly(A) sites between *fpa* mutant backgrounds that have predominantly distal *FPA* poly(A) site selection and FPA overexpression backgrounds that have little detectable distal poly(A) site usage. Overall, our findings suggest that FPA promotes proximal poly(A) site selection directly, independently from FCA, through an association with *FPA* chromatin downstream of this regulated site.

Alternative Polyadenylation of Naturally Occurring Antisense *FLC* RNAs Correlates with FPA Activity and *FLC* Expression

Having identified an activity for FPA in controlling RNA 3′ end formation, we were next interested in determining whether such a function had implications for understanding the mechanism by which FPA controls flowering time. It seems unlikely

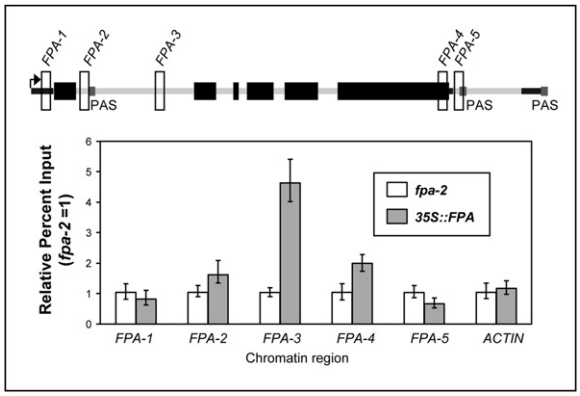

Figure 4. FPA Promotes Proximal Poly(A) Site Selection Directly
ChIP of FPA at the *FPA* locus. Schematic depiction of *FPA* locus with exons shown as black rectangles and introns as gray lines. Poly(A) sites (PAS) are indicated. The chromatin regions analyzed by qPCR are boxed. Plants expressing *35S::FPA* and *fpa-2* mutants were subjected to ChIP using anti-FPA antibodies followed by qPCR. Histograms show mean values ± SE obtained from four PCR amplifications. See also Figure S1.

that FPA plays a constitutive role in RNA 3′ end formation, as it did not affect *FCA* RNA 3′ end formation (Figure 3C). Consistent with this, we did not identify genome-wide changes in 3′ end formation in RNA from *fpa* mutants subjected to *A. thaliana* genome tiling array analysis (L.C.T. and G.G.S., unpublished data). As the function of FPA in flowering time control is explained by regulation of mRNA encoding the floral repressor FLC (Michaels and Amasino, 2001), we investigated whether FPA influenced 3′ end formation of *FLC* mRNA (Figure S2A). Although changes in the abundance of *FLC* mRNA were clearly detectable (Figure S2B), no evidence of alternative 3′ end formation of *FLC* mRNA was found. Another possibility was that improper RNA 3′ end formation or termination at loci adjacent to *FLC* in *fpa* mutants might result in increased *FLC* expression as a result of readthrough (Figure S2A). RT-qPCR was used to measure RNA expressed from the upstream gene At5g10150 and intergenic sequences between them. No significant difference in the expression of RNA from At5g10150 or of readthrough transcripts between these loci was detected in either *FPA* overexpression or *fpa* mutant backgrounds (Figure S2B).

We next investigated whether FPA affected the expression of naturally occurring *FLC* antisense RNAs: alternatively processed, capped, and polyadenylated RNAs are transcribed from a promoter downstream of the *FLC* cleavage and poly(A) site and from the opposite strand to *FLC* (Liu et al., 2007; Swiezewski et al., 2007). So-called class I transcripts are cleaved and polyadenylated within sequence antisense to *FLC* intron 6, while class II transcripts are cleaved and polyadenylated within a region antisense to the *FLC* promoter (Figure 5A). Our analysis identified further alternative processing events in these antisense RNAs (Figure 5A; see below). Using RT-qPCR, little difference in the expression of the class I RNA in *FPA* overexpression or mutant backgrounds was found (Figure 5B). However, reciprocal differences in the expression of the class II RNA were detected (Figure 5B): compared with wild type, there was an increase in the expression of the class II RNA in loss-of-function *fpa* mutant backgrounds, while there was a reduction in

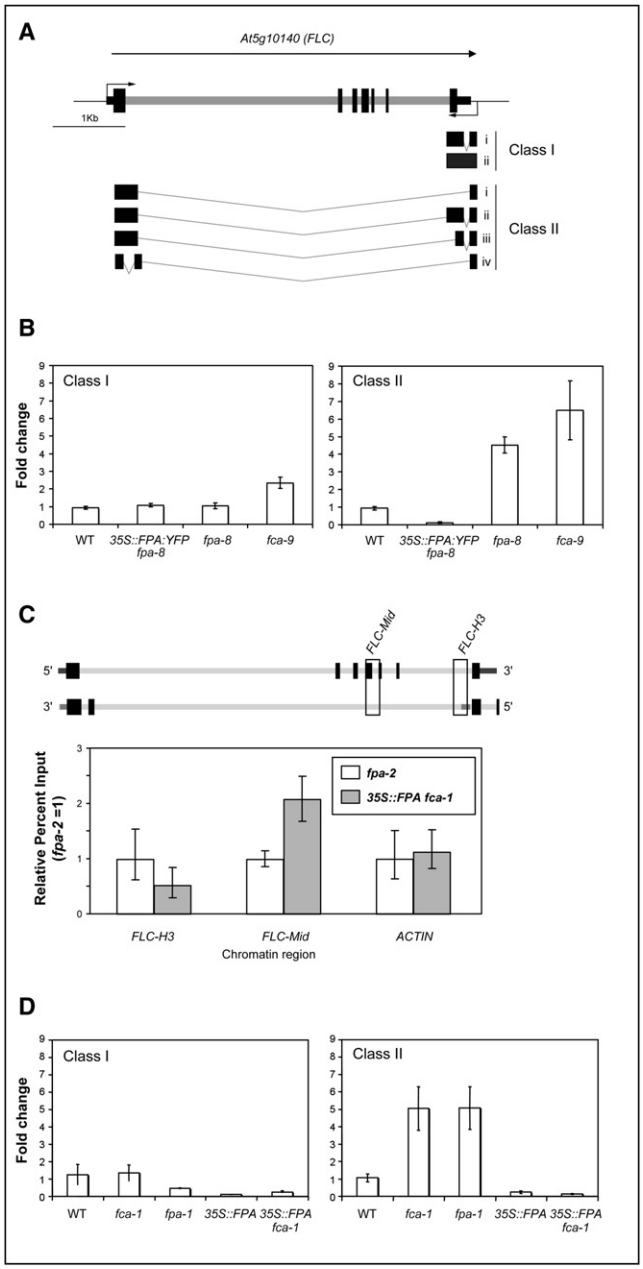

Figure 5. Alternative Polyadenylation of Naturally Occurring Antisense *FLC* RNAs Correlates with FPA Activity and *FLC* Expression
(A) Schematic representation of antisense RNAs at the *FLC* locus. Black boxes represent exons and lines represent splicing patterns.
(B) RT-qPCR analysis of class I and II antisense RNAs expressed at the *FLC* locus. Histograms show mean values ± SE for three independent PCR amplifications on three biological replicate samples.
(C) ChIP of FPA at the *FLC* locus. Schematic depiction of *FLC* locus with exons shown as black rectangles and introns as gray lines for sense and antisense strands. Poly(A) sites are indicated by smaller rectangles. The chromatin regions analyzed by qPCR are boxed. Lines expressing *35S::FPA* and *fpa-2* mutants were subjected to ChIP using anti-FPA antibodies followed by qPCR. Histograms show mean values ± SE obtained for four PCR amplifications.
(D) Class II antisense RNAs are redundantly controlled by FCA and FPA. Histograms show mean values ± SE for three independent PCR amplifications on three biological replicate samples. See also Figure S2 and Table S1.

expression of this RNA in lines overexpressing FPA from a transgene (Figure 5B). These data are consistent with FPA functioning to repress the formation of the class II RNA polyadenylated at the promoter-distal site. Therefore, FPA activity correlated with the expression of the distally polyadenylated *FLC* antisense RNAs and *FLC* expression level.

We coupled cloning and sequencing of antisense RNAs (Table S1) with fluorescently labeled primer RT-PCR and capillary electrophoresis to analyze these RNAs (Figure S2C). We identified polyadenylated class I RNAs in which intron 1 was either spliced or retained (class I i and ii, respectively) (Figure 5A and Figure S2C). In addition, we found that the most prominent class II antisense RNA (class II i) (Figure 5A and Figure S2C) resulted from splicing a 5′ splice site within intron 1 to a 3′ splice site in intron 2, thereby skipping exon 2, but we also found that the next most prominent processing event incorporated exon 2 (class II ii) (Figure 5A and Figure S2C). We therefore conclude that *FLC* antisense RNAs are alternatively spliced as well as alternatively polyadenylated.

A key next question was: how directly was FPA involved in the processing of *FLC* antisense RNA? Previous ChIP analyses using epitope-tagged FPA had indicated FPA associated directly with the *FLC* locus (Bäurle et al., 2007). In order to investigate this further, we used the antibodies we had raised against FPA itself in ChIP experiments to examine whether FPA associated specifically with the proximal poly(A) site on the antisense strand of the *FLC* locus. Our analysis revealed that FPA associated with *FLC* chromatin, but as with the *FPA* locus, we found FPA enriched downstream of the proximal poly(A) site it regulated (Figure 5C). We therefore conclude that FPA associates directly with the *FLC* locus to promote selection of a proximal poly(A) site within antisense RNA.

FPA Functions Redundantly with FCA to Control Expression of Class II Antisense *FLC* RNA

FCA and FPA control flowering in a somewhat redundant manner. *fca fpa* double mutants flower later than either single mutant alone (Koornneef et al., 1998), and the overexpression of FPA in an *fca* mutant background can suppress the late flowering phenotype of *fca* (Bäurle and Dean, 2008). As we have discovered that FCA and FPA control RNA 3′ end formation independently, we asked whether this could explain their redundancy in flowering time control. FPA was overexpressed from a transgene in *fca* mutant plants and the processing of *FLC* antisense RNAs analyzed by RT-qPCR. Like *fpa, fca* plants have elevated levels of class II antisense RNA. However, overexpression of FPA in *fca-1* mutants results in a repression of the class II isoform (Figure 5D). We therefore conclude that FCA and FPA act redundantly to prevent the expression of distally polyadenylated antisense RNAs at the *FLC* locus.

Transcript Readthrough Explains Increased RNA Expression at the *AtSN1* Locus in *fpa* Mutants

A function for FPA in controlling alternative polyadenylation seemed difficult to reconcile with its role in silencing the SINE retroelement *AtSN1*, as its expression likely depends on Pol III and it is silenced by the RdDM pathway (Wierzbicki et al., 2008). We therefore looked more closely at the proposed role of FPA in RNA silencing and asked whether FPA played a widespread role in silencing retroelements by determining how the expression of SINEs other than *AtSN1* was affected by FPA. We found that although the RdDM pathway regulated the SINE *SB2-17*, FPA did not (Figure S3A). We therefore conclude that FPA does not play a generic role in silencing SINEs, so there must be something specific about the *AtSN1* locus that rendered it susceptible to FPA-mediated control.

We measured RNA expression at annotated loci and intergenic regions adjacent to *AtSN1* in order to assess the specificity of FPA-mediated regulation of *AtSN1* in this region of chromosome III (Figure 6A). Consistent with previous reports (Bäurle et al., 2007; Veley and Michaels, 2008), increased levels of RNA expression at *AtSN1* in *fpa* and Pol V mutants (*drd3-7*) were detected (Figure 6B). However, increased RNA levels were detected in *fpa* mutants, but not Pol V mutants, at every other location in this region tested, with increased expression in *drd3-7* only being found at At3g44006 (Figure 6B). The boundary to this change in RNA expression was at At3g44010, the gene encoding ribosomal protein S29c; while no difference in RNA expression between wild-type and *fpa* mutants was detected upstream of, or at, At3g44010, an increase in detectable RNA expression was found at the intergenic region immediately downstream (Figure 6B). These data raised the possibility that defective RNA 3′ end formation at At3g44010 led to increased Pol II transcript readthrough into this region of chromosome III. Consistent with this idea, we detected increased levels of readthrough RNA at the 3′ end of At3g44010 in *fpa* mutants (Figures 6C and 6D). Furthermore, the upregulation of RNA signal detected at *AtSN1* was derived from the same strand as At3g44010 (Figure S3B), and contiguous RNAs that overlapped *AtSN1* from upstream and downstream were found in *fpa,* but not wild-type or Pol V mutants (Figures 6E and 6F). Readthrough in *fpa* mutants amounted to 3% of the total detectable At3g44010 RNA (Figures S3C and S3D). We therefore conclude that defective RNA 3′ end formation at the Pol II-dependent At3g44010 gene accounts for the upregulation of RNA signal at the *AtSN1* locus in *fpa* mutants. As a result, the expression of *AtSN1* itself likely remains silent, thereby explaining why there is no change in either DNA methylation or siRNA abundance at *AtSN1* in *fpa* mutants (Bäurle et al., 2007; Veley and Michaels, 2008).

DISCUSSION

Here we show that the spen family protein FPA controls alternative polyadenylation. The promotion by FPA of proximal poly(A) site selection in *FPA* pre-mRNA likely serves to negatively autoregulate expression, as the resulting RNAs lack an in-frame stop codon and encode only the first RRM of FPA. This discovery closely parallels our previous finding of alternative polyadenylation-mediated autoregulation by FCA (Quesada et al., 2003; Simpson et al., 2003) and suggests that FCA and FPA expression must normally be tightly controlled.

Having identified an activity for FPA in alternative polyadenylation, we next asked whether this had any functional implications for the role of FPA in flowering time control and RNA silencing. FPA and FCA control flowering by preventing the accumulation of mRNA encoding the MADS box transcription factor FLC (Michaels and Amasino, 2001), but the mechanism involved

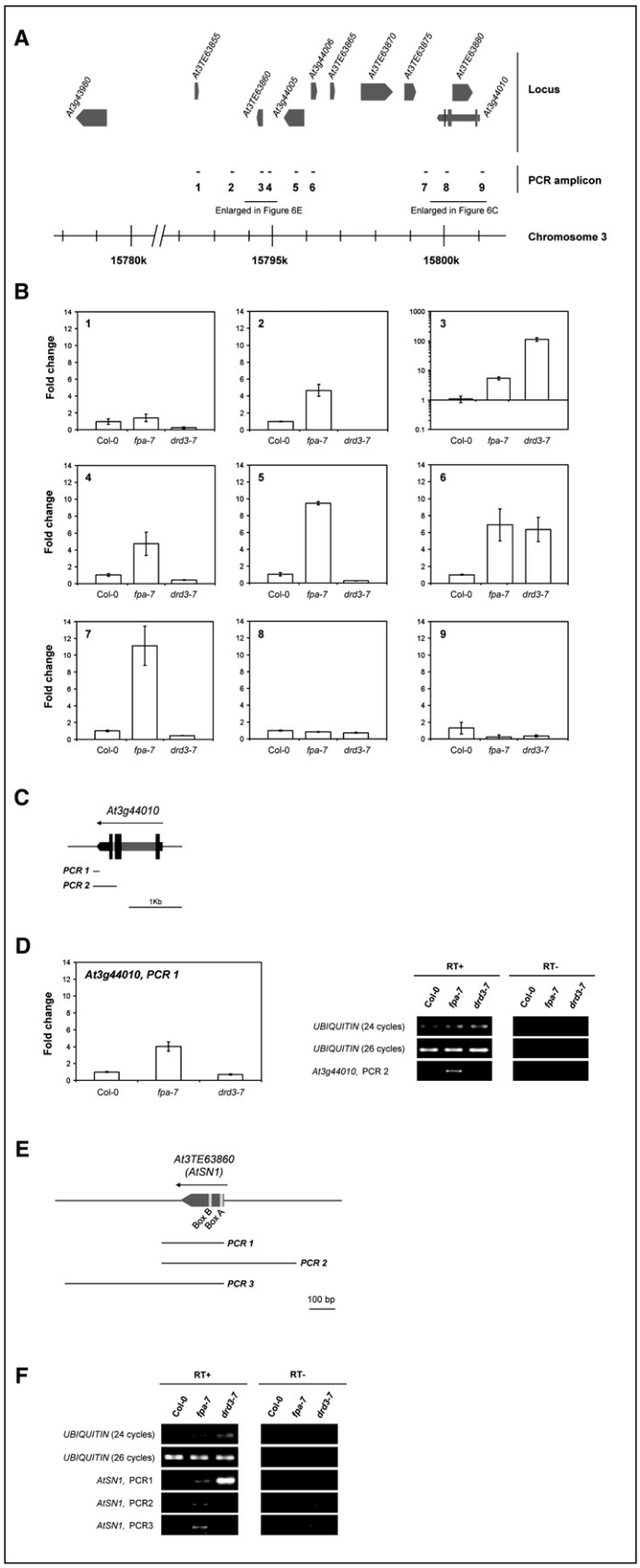

Figure 6. Transcript Readthrough Explains Increased RNA Expression at the *AtSN1* Locus in *fpa* Mutants

(A) Schematic representation of annotated loci adjacent to *AtSN1* (*At3TE63860*). Lines and numbers identify regions amplified in RT-qPCR.

(B) RT-qPCR analysis of RNA expressed from regions of chromosome III adjacent to *AtSN1*. Note $\log_{10}$ scale of panel 3 recording *AtSN1* expression. Histograms show mean values ± SE for three independent PCR amplifications on three biological replicate samples.

(C) Schematic of At3g44010 with regions amplified in RT-qPCR and RT-PCR analysis indicated.

(D) RT-qPCR quantification (left) and RT-PCR (right) analysis of read-through at the 3′ end of At3g44010. RT-PCR products were separated on agarose gels and stained with ethidium bromide. *UBIQUITIN* loading and amplification controls plus no RT controls are included.

(E) Schematic of *AtSN1* with regions amplified in RT-PCR analysis indicated.

(F) Identification of contiguous RNA upstream and downstream of *AtSN1*. RT-PCR products were separated on agarose gels and stained with ethidium bromide. *UBIQUITIN* loading and amplification controls plus no RT controls are included. See also Figure S3.

has remained elusive. FCA absolutely requires the RNA 3′ end processing factor FY (Pfs2p/WDR33) in order to repress *FLC* (Simpson et al., 2003). Our discovery, that FPA also mediates RNA 3′ end formation but genetically independently of FCA and FY, reinforces the importance of RNA 3′ end formation in this process. However, no changes in the 3′ end processing of *FLC* mRNA have been detected in backgrounds differing in FCA or FPA activity, preventing a straightforward, derived explanation for the mechanism by which they control *FLC* expression. Although such RNAs might be unstable, no clear changes in RNA expression were detected at *FLC* in *A. thaliana* exosome RNAi lines (Chekanova et al., 2007). Indeed, evidence suggests that FCA and FPA ultimately control *FLC* RNA expression at the transcriptional, not posttranscriptional, level (Bäurle et al., 2007). However, expression array analysis of *fpa* mutants did not identify evidence of FPA controlling expression of any factors known to regulate *FLC* (Veley and Michaels, 2008), and our own genome-wide tiling array analysis of *fpa* mutants was consistent with this (and furthermore, did not indicate a role for FPA in constitutive RNA 3′ end formation). We also asked whether the expression of genes adjacent to *FLC* were misregulated in *fpa* mutants in case the effects on *FLC* expression might be an indirect consequence of readthrough from improperly terminated RNAs due to lack of FPA activity. However, we found no evidence for this idea either. Instead, we discovered reciprocal changes in the processing of RNA expressed antisense to *FLC* that correlated with FPA activity and *FLC* expression. Since FCA and FPA associate with *FLC* chromatin (Bäurle et al., 2007; Liu et al., 2007), this finding is consistent with the idea that regulation by these proteins at the *FLC* locus is direct, but our findings suggest this involves processing of *FLC* antisense RNAs rather than *FLC* pre-mRNA.

How can the direct association of these regulators of RNA 3′ end formation result in *FLC* transcriptional control (Bäurle et al., 2007)? The processing of antisense RNA appears to be important, limiting readthrough to the distal poly(A) site antisense to the *FLC* promoter. This is because increased readthrough of class II antisense RNA correlates with high levels of *FLC* RNA expression. Therefore, although *cis*-acting antisense RNAs can inhibit sense-strand expression (Camblong et al., 2007; Hongay et al., 2006), the situation here is more reminiscent of the yeast *PHO5* gene (Uhler et al., 2007), where low-level antisense transcription through the promoter affects nucleosome exchange, facilitating chromatin remodeling and enhancing the rate of *PHO5* activation (Uhler et al., 2007). Consistent with this idea, T-DNA insertions in the 3′ end of *FLC* that may disrupt the antisense RNAs do not result in the same misregulation of *FLC* expression as autonomous pathway mutants (Swiezewski et al., 2007). However, the close correspondence of sites of transcription initiation and 3′ end formation between class II antisense RNA and *FLC* RNA (Liu et al., 2007; Swiezewski et al., 2007) mean such mutations may disrupt sense as well as antisense expression, making them difficult to interpret. This close correspondence of 5′ and 3′ ends might be relevant here. Paf1c (Pol II associated factor), which is essential for elevated *FLC* expression (He et al., 2004), also affects RNA 3′ end formation in *S. cerevisiae* (Penheiter et al., 2005) and humans (Rozenblatt-Rosen et al., 2009). Therefore, transcription and 3′ end formation of class II antisense RNA at the 5′ end of *FLC* may modify the nucleosomes and enhance recruitment of Paf1c to the *FLC* promoter and thus affect sense-strand transcription. Such interactions could be important in a gene loop conformation (Ansari and Hampsey, 2005; O'Sullivan et al., 2004), as this would juxtapose the 5′ and 3′ ends of class II antisense RNA and of *FLC*.

Regulated processing of antisense RNA by FPA and FCA in this way (and potentially other RNA processing factors of the autonomous pathway) may therefore provide poised and sensitive regulation of *FLC* transcription. This is important because *FLC* expression is limited, but not silenced, by the autonomous pathway, as *FLC* controls temperature-dependent germination (Chiang et al., 2009) and circadian clock function (Edwards et al., 2006) as well as flowering. Since chromatin modifications that slow the rate of Pol II elongation can affect alternative processing of pre-mRNA (Allo et al., 2009), it is conceivable that some of the chromatin modifying components of the autonomous pathway could also function to affect processing of *FLC* antisense RNA. In addition, it may be valuable to reexamine the misregulated *FLC* expression caused by *cis*-element deletions (He et al., 2003), since these simultaneously disrupt splice sites in antisense RNA and this, therefore, may be their primary effect. In order to test these possibilities, it will be necessary to experimentally separate the close correspondence of the 5′ and 3′ ends of *FLC* sense and antisense RNAs that effects codependency on expression.

At first glance, an activity for FPA in controlling alternative polyadenylation appeared difficult to reconcile with its proposed role in RNA silencing (Bäurle et al., 2007). However, our analysis clarifies previous findings. We have discovered increased levels of readthrough RNA at the 3′ end of the ribosomal protein gene At3g44010 in *fpa* mutants. Consequently, readthrough into the region downstream (where the retroelement, *AtSN1,* is located) results in detectable RT-qPCR signal corresponding to *AtSN1* sequences, but this does not necessarily reflect derepression of the epigenetic silencing of this retroelement per se. This explains why DNA methylation and siRNA abundance at *AtSN1* is unaltered in *fpa* mutants (Bäurle et al., 2007; Veley and Michaels, 2008) and reveals that even in the presence of Pol II transcript readthrough, the RdDM pathway, dependent on Pol V transcription, is still functional in this region of chromosome III (Wierzbicki et al., 2008). This raises the question as to whether transcriptional readthrough might also account for other epigenetic transitions in *A. thaliana* mutants not associated with changes in DNA methylation (Nishimura and Paszkowski, 2007).

Uncovering a role for FPA in RNA 3′ end formation has additional implications for understanding alternative polyadenylation and spen protein function. The importance of alternative polyadenylation in gene expression is increasingly well recognized (Danckwardt et al., 2008; Sandberg et al., 2008), most recently highlighted by its pervasive role in oncogene activation (Mayr and Bartel, 2009). However, we know surprisingly little of the mechanisms by which alternative polyadenylation is regulated. As neither FCA nor FPA are constitutive splicing or polyadenylation factors, they likely represent *trans*-acting regulators of RNA 3′ end formation distinct from the paradigm established for alternative polyadenylation of Immunoglobulin (M) in B cell development (Peterson, 2007). The existence of genetically independent *trans*-acting regulators of alternative polyadenylation

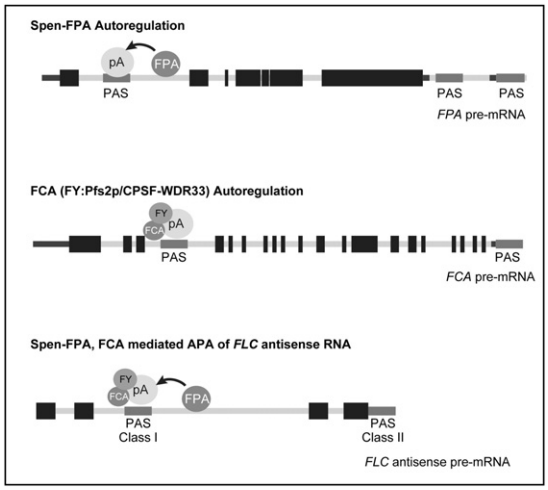

Figure 7. Alternative Cleavage and Polyadenylation Mediated by FCA and FPA

FPA and FCA function genetically independently to control poly(A) site selection in specific pre-mRNAs but function redundantly to affect alternative polyadenylation (APA) of antisense RNAs at *FLC*. FCA appears to associate with chromatin close to the site of the regulated poly(A) site and interacts with the 3′ end processing machinery (pA) via a physical interaction with FY. In contrast, FPA associates with chromatin downstream of the poly(A) sites it controls. Exons are depicted as black boxes, introns by gray lines. Poly(A) signals, PAS, are indicated.

in *A. thaliana* raises the likelihood that specific *trans*-acting factors also exist in human cells and contribute to the control of the widespread patterns of alternative polyadenylation.

We show here that FPA and FCA control alternative cleavage and polyadenylation independently on distinct pre-mRNAs and on antisense RNAs at the *FLC* locus (Figure 7). ChIP experiments reveal that FCA closely associates with the proximal poly(A) site of class I antisense RNAs at the *FLC* locus (Liu et al., 2007). This suggests that FCA is recruited to a binding site on the nascent transcript close to the weak poly(A) site and the subsequent physical association between FCA and the core cleavage and polyadenylation machinery, via FY (Pfs2p/CPSF WDR33), enhances the selection of this site (Simpson et al., 2003) (Figure 7). In contrast, ChIP analysis detected association of FPA with sequences downstream of the poly(A) sites it promoted (Figure 7). This resembles the ChIP pattern of core components of the human cleavage and polyadenylation machinery, which peak approximately 1 kb downstream of the poly(A) site, reflecting an association with paused Pol II (Glover-Cutter et al., 2008). In vivo cross-linking experiments with the brain-specific protein Nova reveal that it too binds RNA downstream of the poly(A) sites that it promotes (Licatalosi et al., 2008). Our analysis reveals that FPA functions genetically independently from FCA and FY (Pfs2p/CPSF WDR33) in controlling RNA 3′ end formation. Intriguingly, a double mutant between viable alleles of *fpa* and the RNA 3′ end processing factor *fy* is lethal (Koornneef et al., 1998). Fully understanding the different mechanisms by which FCA, FY, and FPA control poly(A) site selection should explain their redundant roles in *A. thaliana*, reveal different ways that alternative polyadenylation can be controlled, and clarify this example of sense/antisense gene regulation. Finally, our work suggests that understanding how spen family proteins control cell fate determination in animal development and how the fusion of the spen protein RBM15 to MKL1 causes infant acute megakaryoblastic leukemia (Ma et al., 2001) may benefit from the analysis of pre-mRNA processing.

EXPERIMENTAL PROCEDURES

RNA Gel Blot Analysis

RNA gel blot analysis of *FPA*- and *FCA*-specific mRNAs was carried out as described (Quesada et al., 2003), except that 1 mg total RNA was used as starting material for poly(A)$^+$ isolation. *FPA* mRNA was detected using a probe corresponding to the 5′UTR of *FPA* mRNA (using PCR product amplified with FPA probe For 5′-GTCTTCAAACTCAATCTAGGG-3′ and FPA probe Rev 5′-GGATTGTTTCAATTGACGATCC-3′). *FCA* was detected using a previously described probe (Quesada et al., 2003). To detect *FPA*-specific transgene mRNAs (*35S::FPA*), DNA oligonucleotide (5′-GGATCCTCTAGAGTCCCCCG TGT-3′) labeled with ATP [$\gamma-^{32}$P] by T4 polynucleotide kinase was used. RNA gel blots were visualized and quantified using a Fuji FLA7000 scanner.

RT-qPCR

RNA was isolated with TRIzol Reagent (Invitrogen). Q-PCR was carried out using SYBR Green I (QIAGEN), following reverse transcription (MMLV, Promega). The PCR program consisted of an initial activation step of 15 min at 95°C, followed by 45 cycles of 15 s at 95°C, 30 s at 54°C, and 30 s at 72°C. Fold change in expression was calculated relative to wild-type (WT) plants using *UBIQUITIN* (At5g25760) mRNA as a reference (Czechowski et al., 2005). Relative mRNA levels were calculated using the following equation: Relative mRNA level = $E(ct_{uc}-ct_{us})/E(ct_{rc}-ct_{rs})$, where E is the efficiency of the PCR (2 in our case), ct is the threshold cycle, u is the mRNA of interest, r is the reference gene (*UBIQUITIN*), s is the sample, and c is the WT control sample (Pfaffl, 2001).

RT-PCR

RNA was isolated with TRIzol Reagent (Invitrogen). PCR was carried out using FastStart Taq DNA Polymerase (Roche) following reverse transcription (MMLV, Promega). Strand-specific RT-PCR was performed as described (Wierzbicki et al., 2008). Quantification of absolute levels of readthrough RNA at At3g44010 was based on a standard curve derived from amplification of plasmid DNA containing cloned At3g44010 and downstream sequence.

Chromatin Immunoprecipitation

ChIP was performed as described (Wierzbicki et al., 2008). Anti-FPA antibodies were raised in rabbits against recombinant 6 x histidine-tagged fragment of FPA (residues 441-901) and affinity purified using the same protein. Pol II antibodies (8WG16) were from Cambridge Biosciences.

SUPPLEMENTAL INFORMATION

Supplemental Information includes three figures, one table, Supplemental Experimental Procedures, and Supplemental References and is available online with this article at doi:10.1016/j.devcel.2009.12.009.

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