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The chromatin remodeling and mRNA splicing functions of the Brahma (SWI/SNF) complex are mediated by the SNR1/SNF5 regulatory subunit

Claudia B. Zraly[1] and Andrew K. Dingwall[1,2,*]

[1]Cardinal Bernardin Cancer Center, Oncology Institute and [2]Departments of Pathology and Microbiology and Immunology, Stritch School of Medicine, Loyola University of Chicago, 2160 S. First Avenue, Maywood, IL 60153, USA

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ABSTRACT

Nucleosome remodeling catalyzed by the ATP-dependent SWI/SNF complex is essential for regulated gene expression. Transcriptome profiling studies in flies and mammals identified cell cycle and hormone responsive genes as important targets of remodeling complex activities. Loss of chromatin remodeling function has been linked to developmental abnormalities and aggressive cancers. The Drosophila Brahma (Brm) SWI/SNF complex assists in reprogramming and coordinating gene expression in response to ecdysone hormone signaling at critical points during development. We used RNAi knockdown in cultured cells and transgenic flies, and conditional mutant alleles to identify unique and important functions of two conserved Brm complex core subunits, SNR1/SNF5 and BRM/SNF2-SWI2, on target gene regulation. Unexpectedly, we found that incorporation of a loss of function SNR1 subunit led to alterations in RNA polymerase elongation, pre-mRNA splicing regulation and chromatin accessibility of ecdysone hormone regulated genes, revealing that SNR1 functions to restrict BRM-dependent nucleosome remodeling activities downstream of the promoter region. Our results reveal critically important roles of the SNR1/SNF5 subunit and the Brm chromatin remodeling complex in transcription regulation during elongation by RNA Polymerase II and completion of pre-mRNA transcripts that are dependent on hormone signaling in late development.

INTRODUCTION

The SWI/SNF ATP-dependent chromatin remodeling complex facilitates early events in transcription through interactions with DNA binding transcription factors and recruitment of RNA polymerase II (PolII) (1). The complex assists transcription events by interacting with nucleosomal substrates to alter DNA:histone contacts (2) and facilitates DNA translocation within the nucleosome (3). The SWI/SNF complex associates with sites of PolII accumulation (4,5) and it may form transient associations with PolII (6). Remodeling activities allow for transcription at promoters to initiate, while early elongation events are thought to be mediated by alternate chromatin remodeling activities (7). However, recent studies of transcription elongation and splicing regulation suggest there is even greater diversity in the modes of SWI/SNF regulation (8,9). For instance, possible links between mammalian SWI/SNF complex remodeling activities, the rate of RNA PolII movement, and alternative pre-mRNA splicing regulation have been reported. However, it has not yet been possible to obtain a clear understanding from these studies what regulatory mechanisms are in place to help coordinate either the timing and/or limit the extent of SWI/SNF complex remodeling activities in the developing organism.

The SWI/SNF complex is composed of a conserved set of core subunits, including SNF2/SWI2, SWI3 and SNF5 which are required for *in vitro* remodeling (10), and a set of accessory subunits (8–11 total). SWI/SNF counterparts have been identified in Drosophila (Brm) and vertebrates (BRG1/HBRM) (11) and have critically important functions in development (12), reprogramming somatic cells during differentiation (13), as well as establishment and maintenance of pluripotent and multipotent identities of murine embryonic stem cells (14–17). Mouse knockouts of

*To whom correspondence should be addressed. Tel: +1 708 327 3141; Fax: +1 708 327 3342; Email: adingwall@lumc.edu

genes encoding the core SWI/SNF subunits (SNF5/SMARCB1 and SWI2/BRG1) are associated with peri-implantation lethality (17) and genetic studies in flies revealed that the complex is required throughout development (11,18). Loss of SWI/SNF complex components has also been directly linked to a number of aggressive cancers (19), including malignant rhabdoid tumors associated with SNF5/SMARCB1 loss (20,21), and melanomas, prostate, lung and breast cancers linked to BRG1/HBRM loss (19,22).

Genetic and transcriptome profiling studies of the Drosophila Brahma (Brm) complex using dominant-negative mutant alleles of genes encoding the conserved components, SNR1(SNF5) and BRM(SNF2/SWI2), allowed for analyses of loss of subunit or complex function under conditions where the mutant subunit was properly assembled (18,23). During Drosophila wing vein and intervein cell development, *snr1* and *brm* elicit opposite mutant phenotypes revealing that SNR1 serves to block BRM ATPase functions in intervein cells in cooperation with histone modifying proteins and gene-specific repressors (24,25). We previously identified the hormone regulated *Eig* (ecdysone inducible) genes as direct targets of Brm complex regulation (26). The *Eig* genes comprise a cluster of five divergently transcribed pairs with short intergenic regions (~260 bp). Peak expression occurs during early pupariation correlating with elevated hormone levels (27). Many of the *Eig* genes encode for proteins related to mammalian defensins that may function as secreted antimicrobial polypeptides involved in host defense during metamorphosis. This type of sensor-mediated innate immune response system is strongly conserved between flies and mammals (28). The *Eig* genes were generally upregulated in response to reduced *snr1* function, but downregulated if *brm* function was reduced suggesting that remodeling activity was necessary for activation, while SNR1 was important for negatively regulating that activity (26). Brm complex regulation of the *Eig* genes mimics the interactions observed in the Drosophila wing suggesting a common mode of control. Thus, SNR1 acts as a regulatory subunit to restrict Brm complex-dependent chromatin remodeling activities during development on select target genes that control growth, differentiation and patterning, including genes that respond to hormone signaling (5,25,26).

To better understand how Brm complex remodeling activities are regulated within the context of a developing organism, we undertook a genetic approach to uncover the functional role of SNR1 and the Brm complex using the dominant negative $snr1^{E1}$ and brm^{K804R} mutants. We found that repression of ecdysone inducible gene expression by the Brm complex is due to restraint of complex chromatin remodeling activity leading to the stalling of PolII elongation. Further, we make the novel finding that the restraint is mediated through the functions of SNR1, while BRM ATPase activity is required for subsequent chromatin remodeling following release of the stalled polymerase. We found that promoter distal nucleosome structures can impede or stall PolII on certain developmentally regulated genes. In contrast to prevailing views, a consequence of Brm complex-dependent polymerase stalling is reduced splicing and the retention of introns that may function to control gene expression at a post-transcriptional level. This aspect of Brm (SWI/SNF) complex function has never been described and thus our findings represent an important advance in our understanding of *in vivo* developmental functions of chromatin remodeling.

MATERIALS AND METHODS

Genetic manipulations and strains

Fly strains and manipulations for $snr1^{E1}$, $UASBrm^{K804R}$ and *UAS-shNELF* have been described (23,26,29). $RpII215^{C4}$ and *UAS-shDSIF* stocks were from the Bloomington Drosophila Stock Center. Expression of UAS_{GAL4} transgenes was controlled using *P(GawB)69B*-GAL4 driver with crosses performed at 29°C. Animals were staged according to standard guidelines using morphological landmarks (30). Staging was independently verified by monitoring expression of the ecdysone inducible gene, *Eip93F* (31) (Supplementary Figure S1). Blue gut and clear gut larvae were differentiated by growth on food supplemented with 0.05% bromophenol blue (32).

Drosophila cell culture and RNAi

S2 cells were cultured on standard medium, with addition of 20-hydroxyecdysone (Sigma) to a final concentration of 1 μM for 24 or 48 h. RNAi mediated knockdowns of *snr1* and *brm* in cultured cells were performed with double-stranded RNA as described (26).

RNA analysis

Total RNA was prepared and semi-quantitative RT-PCR was performed as described (26). SYBR Green quantitative real time PCR on reverse transcriptase reactions (qRT-PCR) was performed in triplicate using GoTaq qPCR Master mix (Promega). The qPCR reactions were carried out in triplicate. Levels of mRNA were analyzed by comparative *Ct* method. The Drosophila *rp49* ribosomal gene was chosen as the endogenous reference. Primers used for splice form analysis were designed similar to the strategy described by Hargreaves *et al.* (56). Primers within a single 3′ exon were used to determine total expression (precursor). Exonic primers that crossed an intron were used for detection of spliced transcripts. Primer sequences are listed in Supplementary Table S1.

CHART-PCR Assay

Nuclease accessibility assays were performed essentially as described (33). Treated and untreated DNAs were used to perform SYBR Green real time PCR in triplicate. A standard curve was generated with serial dilutions of genomic DNA to correlate *Ct* values obtained from real time PCR to percent accessibility. MNase accessibility was calculated as a percentage of the accessibility observed in the untreated DNA (34).

Chromatin immunoprecipitation

Chromatin immunoprecipitation (ChIP) was performed as described in both S2 cells (26) and Drosophila-staged animals (35). For qPCR analysis, fold enrichment was measured against IgG negative control and values were normalized to ChIP input. Antibodies included αSNR1 and αBRM (5); αH3-trimethyl-K4 (07-473), αH3–trimethyl K27 (07-449), αH3-acetyl K9,14 (06-599), αH3-dimethyl K36 (07-274) from Upstate (Lake Placid, NY, USA); αH3 (ab1791), αIgG (ab27478), αH3-trimethyl K9 (ab1186) from Abcam (Cambridge, MA, USA); αOSA from DSHB (University of Iowa, Department of Biology, Iowa City, IA, USA). RNA PolII antibodies (8WG16, H5 and H14) were from Covance (Berkeley, CA, USA). Antibodies to Cyclin T (D. Price), NELF-E (D. Gilmour) and POLYBROMO (P. Verrijzer) were gifts.

RESULTS

Eig genes are poised for activation

Our previous gene expression profiling study using conditional dominant-negative mutant alleles of *snr1* and *brm* identified the hormone regulated *Eig* genes as significant *in vivo* targets of Brm complex regulation, and cultured cell studies confirmed that the regulation was direct (26). *Eig71Ef–i* gene transcripts are normally undetectable in cultured Drosophila Schneider (S2) cells in the absence of ecdysone hormone and transcripts first appear ~48 h after hormone addition (26); however, RNAi knockdown of *snr1* prior to adding hormone resulted in the accelerated appearance of the completed *Eig71Ef* transcript within 24 h (Figure 1A, left panel). Other *Eig* genes showed an identical response (data not shown), consistent with a function for SNR1 in repressing *Eig* expression.

Close relationships between chromatin remodeling and epigenetic histone modifications involved in gene regulation have been described (36). Since the upregulation of the *Eig* genes following RNAi depletion of *snr1* in S2 cells was similar to the *in vivo* mutant effects of $snr1^{E1}$ (26), we sought to determine if these effects were the result of changes in histone modifications. ChIP analyses revealed (26) no obvious differences in the profile or relative levels of histone modification marks tested on *Eig71Eh* chromatin (and *Eig71Ei, Ef* and *Eg*) upon *snr1* knockdown compared to control cells (data not shown). However, in both cases, addition of ecdysone led to the appearance of epigenetic marks associated with active transcription (H3-acetyl K9, K14 and H3-K4me3) coexisting with repressive histone marks (H3-K9me3 and H3-K27me3) suggesting that the *Eig* genes were poised for activation. Surprisingly, dimethylated histone H3-K36, a covalent modification frequently associated with transcription elongation (37), was detected within the *Eig* gene coding exons 24 h after hormone addition in normal S2 cells, suggesting that transcripts had been initiated but not fully elongated (Figure 1A, right panel). Therefore, hormone triggers the appearance of epigenetic marks associated with active transcription, while full-length transcripts are delayed. Depletion of the Brm complex using RNAi did not cause dramatic changes in these marks, but accelerated the appearance of completed transcripts.

The Brm complex regulates *Eig* transcript elongation

The accelerated appearance of completed *Eig* transcripts following *snr1* knockdown could result from elevated transcription initiation and/or elongation by RNA PolII. We focused our analyses of Brm complex functions on *Eig71Eh* as *Eig71Eh–Ei* represent the most robustly regulated divergent gene pair within the *Eig* gene cluster (26) and they share common promoter regulatory elements (Figure 1B). ChIP experiments using PolII antibodies that recognize the phosphorylation status of the carboxyl-terminal domain (CTD) revealed that a paused PolII (Ser5P-CTD) is situated at the 5′ *Eig71Eh–i* promoter proximal region 24 h after ecdysone treatment (Figure 1C). Depletion of *snr1* by RNAi in the presence of hormone resulted in reduction of the Ser5P-CTD form within the promoter and subsequent transcript elongation, consistent with negative regulation by the Brm complex (26). These results suggest that the accelerated accumulation of transcripts resulted from RNA polymerase elongation, and that the Brm complex contributes to stabilizing the initiating form of PolII (Ser5P-CTD) on chromatin.

To approximate the location of the intrinsic block to elongation in *Eig* chromatin, we examined transcripts using RT-PCR primers specific for *Eig* exonic regions (Figure 1B). Following ecdysone addition to S2 cells, a 5′ exon specific *Eig71Eh* transcript was detected within 24 h (Figure 1D, control), while the 3′ exon transcript was not detected until 48 h (Figure 1E, right, see gel analysis and 24 versus 48 h 5′ and 3′ exon specific qPCR measurements). In contrast, both 5′ and 3′ exon transcripts were detected within 24 h in S2 cells (+ hormone) depleted of Brm complex components (Figure 1D). Quantitative PCR measurements of 5′ and 3′ exonic regions revealed 3- to 4-fold enrichment in the ratio of 3′- versus 5′-ends in *snr1* and *brm* RNAi depleted cells after 24 h compared to control (Figure 1D, right). These results suggest that ecdysone stimulated *Eig* transcripts initiate but then PolII normally stalls within the first exon, consistent with the accelerated accumulation of full length transcripts we observed by RNAi depletion of *snr1* and *brm*.

We hypothesized that if *Eig* transcription is regulated during elongation, then pausing factors should be recruited to the *Eig71Eh* genomic region in a hormone-dependent manner. To address this question, we performed ChIP analyses using antibodies against Drosophila NELF, CyclinT (CycT) and PolII phosphorylated forms. CycT associates with the Cdk9 kinase to form P-TEFb that is required for the phosphorylation of Ser2-CTD, NELF and DSIF and the release of paused PolII. Bulk chromatin was isolated from untreated S2 cells and cells exposed to prolonged incubation with ecdysone. There was no significant accumulation of PolII or elongation factors in untreated cells (Figure 1E, left). The *Eig71Eh* 5′ exon transcript was detected after 24 h of treatment coincident with the appearance of the Ser5P-CTD, CycT (P-TEFb) and pausing factor, NELF

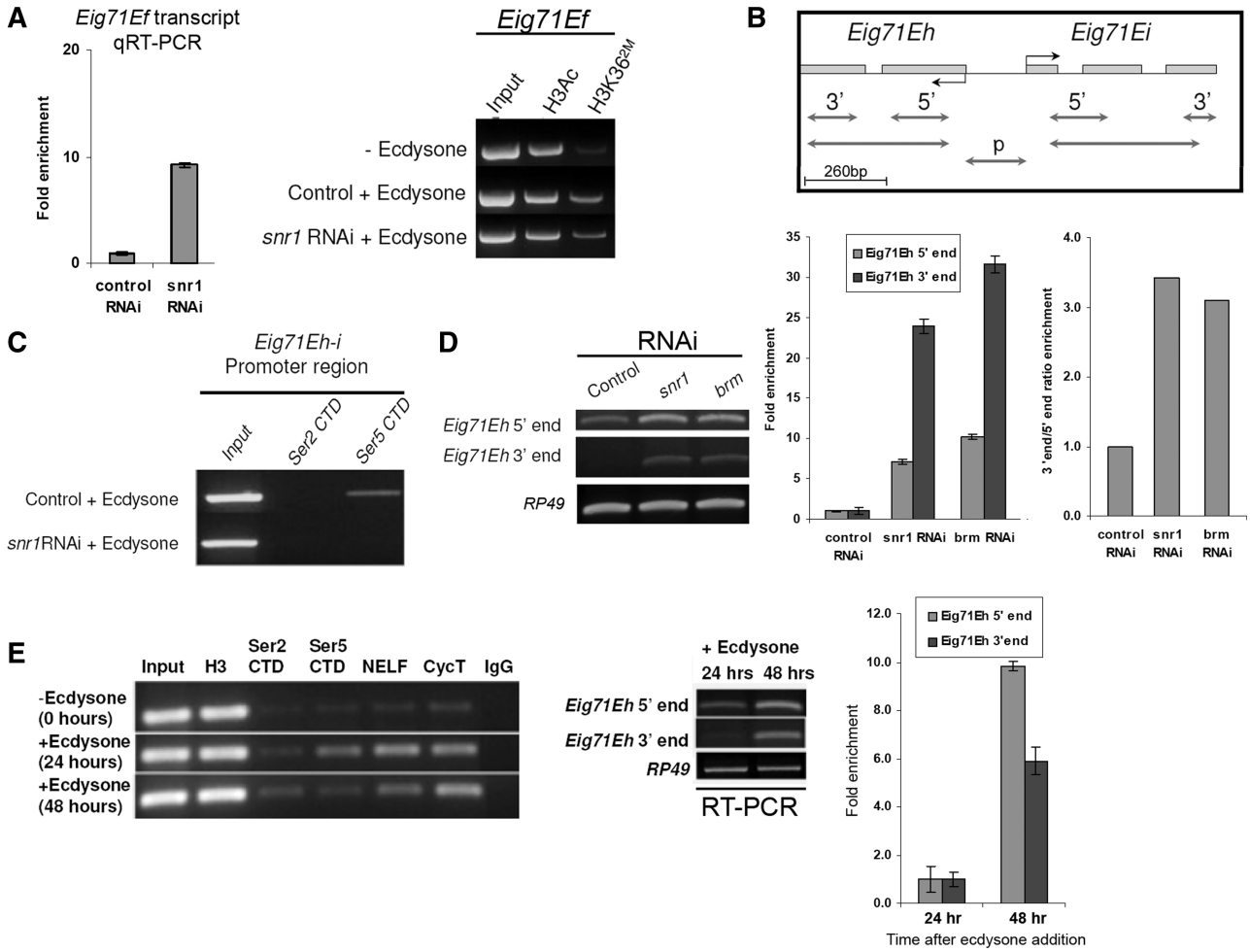

Figure 1. *Eig* genes are poised for activation. (**A**) Quantitative RT-PCR was used to detect *Eig71Ef* expression in S2 cells treated with control (CG10465), and *snr1* RNAi (left) after ecdysone treatment for 24 h. ChIP analysis of *Eig71Ef* coding region (+/− ecdysone, right). (**B**) *Eig71Eh–Eig71Ei* transcribed regions and primers used for RTPCR analysis. Primers spanning the promoter regions (p) and 5′ and 3′ exonic regions are depicted. (**C**) ChIP analysis of PolII phosphorylated forms on *Eig71Eh–i* promoter. S2 cells treated with control or *snr1* RNAi plus ecdysone followed by ChIP using antibodies specific to Ser2P-CTD (elongating) or Ser5P-CTD (initiating) PolII and PCR using *Eig71Eh–i* promoter primer pairs (p). (**D**) RNA analysis of S2 cells treated with ecdysone and RNAi for *snr1*, *brm* and control. Agarose gel analysis of RT-PCR products using primers specific to 5′ and 3′ coding exons as depicted in (A) and RP49 control. Quantitative RT-PCR of 5′ and 3′ exons performed (left); ratios 3′-end/5′-ends plotted (right). (**E**) Hormone-dependent recruitment of elongation factors to *Eig71Eh* genomic region. ChIP analysis of cultured S2 cells (−/+) ecdysone. Agarose gel analysis of RT-PCR products (center panel) and qRT-PCR (right) of *Eig71Eh* 5′- and 3′-ends from S2 cells 24 and 48 h after ecdysone treatment.

(Figure 1E, right). After 48 h of ecdysone treatment, the 3′ exon appeared (Figure 1E, right) indicating a full-length transcript. Importantly, histone H3 was present throughout prolonged ecdysone treatment suggesting no significant changes in nucleosome density occurred within the *Eig* chromatin.

We next sought to determine whether the *Eig* genes were regulated through control of transcription elongation during normal development. The *Eig71Eh–i* transcripts accumulate at the white prepupal (WPP) and early prepupal (EP) stages in response to rising hormone levels (27) (Figure 2A; see also http://flybase.org). During this time period, both NELF and CycT localize to *Eig71Eh* chromatin similar to the pattern in S2 cells following prolonged ecdysone treatment (Figure 2A, lower panel). As hormone levels drop in late prepupae (LP), the *Eig* gene transcripts are undetectable (see EP versus LP control lanes in Figure 3B).

We and others have shown that RNAi depletion of individual core subunits of the Brm complex in S2 cells resulted in complex instability and possible degradation of subunits (24,26,38). In this report, we find that loss of the Brm complex by RNAi depletion of either *snr1* or *brm* in S2 cells mimics effects on *Eig* regulation observed with our conditional $snr1^{E1}$ mutant *in vivo* (26), in which transcription was upregulated in response to reduced SNR1 function. In contrast, we had previously found that the BRM subunit exerts a positive role in *Eig* gene regulation *in vivo* using a dominant-negative *brm* mutant allele (brm^{K804R}) that is impaired in ATPase function but still assembled into Brm complexes (23). To more precisely uncover the role of the Brm complex, in particular the

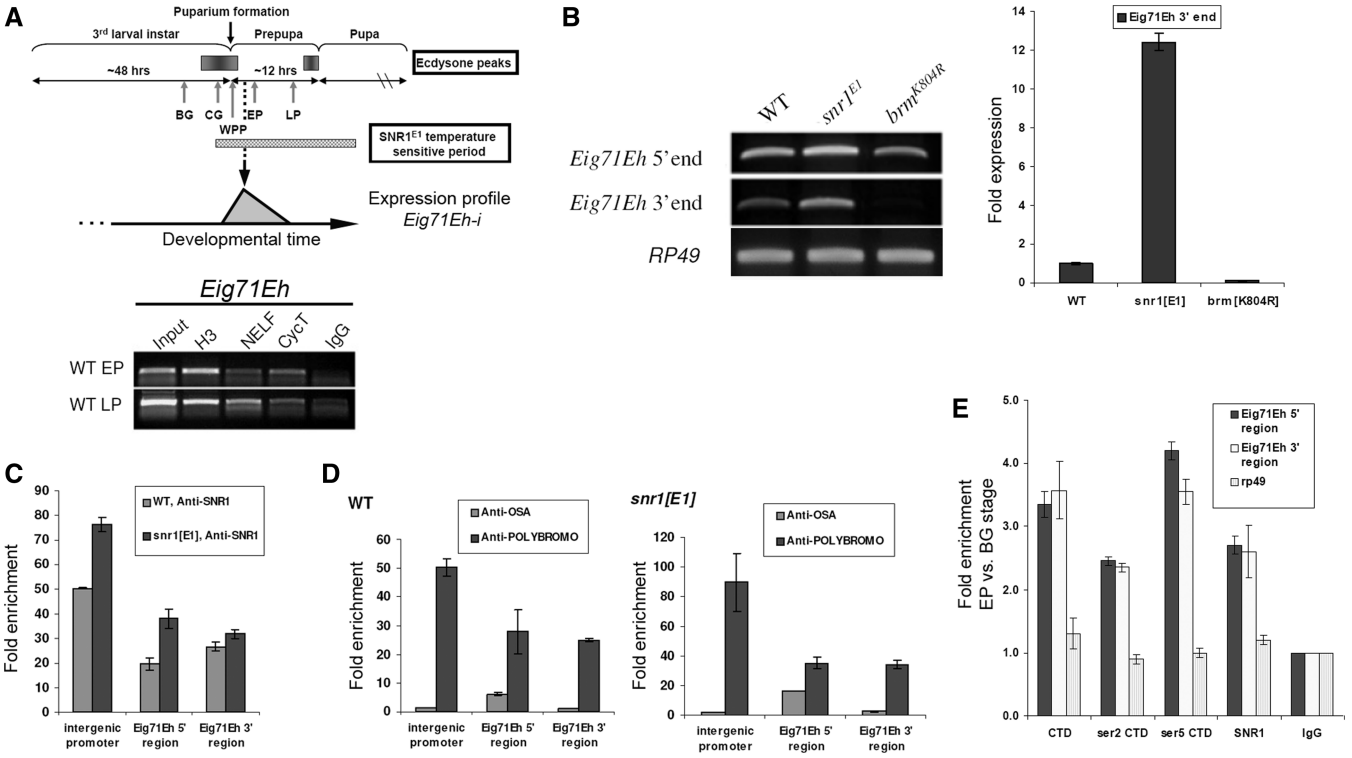

Figure 2. *Eig* genes are developmentally regulated by Brm complex during transcription elongation. (**A**) Depicted in the diagram are the major ecdysone pulses during metamorphosis, time points used (BG, blue gut; CG, clear gut; WPP, white prepupal; EP, early prepupal; LP, late prepupal), the $snr1^{E1}$ temperature sensitive period and the pattern of *Eig71Eh–i* transcript accumulation (27). (Lower panel) Recruitment of elongation factors to *Eig71Eh* genomic region during pupal transition. ChIP analysis of EP and LP animals was performed as in (Figure 1E) with antibodies to elongation factors. (**B**) RNA analysis of wild-type and mutant ($snr1^{E1}$ and brm^{K804R}) animals during the EP stage (left). RTPCR performed as in (Figure 1D); qRT-PCR (right). (**C**) Distribution of SNR1 on *Eig71Eh* chromatin during EP stage by ChIP and qPCR. (**D**) PBAP complexes regulate *Eig* transcription elongation. WT (left panel) and $snr1^{E1}$ mutant (right panel) chromatin analyzed by ChIP/qPCR during EP stage using αOSA and αPB antibodies. Note different scales were used to depict fold changes in the WT and mutant graphs. (**E**) Developmental enrichment of SNR1 to *Eig71Eh* chromatin. ChIP/qPCR analysis of *Eig71Eh* exonic regions and *RP49* from BG-and EP-staged animals. Antibodies included total RNA PolII, Ser2P-CTD and Ser5P-CTD, SNR1 and IgG. Shown is fold enrichment of qPCR values for EP compared to BG-staged animals.

SNR1 subunit, in regulating transcription elongation during development we used $snr1^{E1}$ and brm^{K804R} dominant negative mutants and carried out RT-PCR analyses of *Eig71Eh* during the EP stage that corresponds to the $snr1^{E1}$ temperature-sensitive period (Figure 2A and B). Thus, unlike the RNAi depletion studies in tissue culture, the conditional *snr1* and *brm* dominant negative mutant alleles afforded us the opportunity to assess the function of the individual subunits within the assembled complex (18,23). Comparing to wild-type (WT), we found that the *Eig71Eh* transcript was elevated at both 5′- and 3′-ends in the $snr1^{E1}$ mutant; however, the 5′-end transcript was much reduced and transcription of the 3′-end was undetectable in a brm^{K804R} mutant. Quantitative RT-PCR using 3′ exon-specific primers verified that transcription was upregulated 13-fold in the *snr1* mutant and reduced 8-fold in the *brm* mutant compared to WT. It is important to note, ChIP analyses of histone marks in S2 cells and in early pupae (Figures 1E and 2A) indicated that histones were present throughout *Eig* transcribed regions including those that correlated with polymerase stalling.

The requirement for BRM ATPase activity in transcription elongation suggests that nucleosomes need to be remodeled for release of the stalled polymerase and that the Brm complex would need to be present at nucleosome positions embedded within distal exons. Therefore, we next sought to examine the distribution of the Brm complex on the *Eig71Eh* genomic region. ChIP analyses using antibodies to SNR1 revealed the protein was present throughout the transcribed region, consistent with a possible role in regulating transcript elongation (Figure 2C). Since transcription of the *Eig* genes appears to be negatively regulated by SNR1, we addressed whether the upregulation we observed in a $snr1^{E1}$ mutant might be due to altered chromatin binding of the Brm complex. The $SNR1^{E1}$ protein is stably incorporated into Brm complexes where it functions as a dominant negative subunit due to loss of SNR1 function (5). ChIP analyses revealed that $SNR1^{E1}$ was associated with *Eig71Eh* in a pattern identical to WT SNR1 (Figure 2C); although, the mutant protein accumulated at higher levels within promoter and 5′ exon regions, possibly reflecting the elevated transcription observed in the $snr1^{E1}$ mutant.

We next addressed whether misregulation of the *Eig* genes in a $snr1^{E1}$ mutant could possibly be the result of alterations in Brm complex accessory subunit composition. Two subclasses of the SWI/SNF complex exist in a number

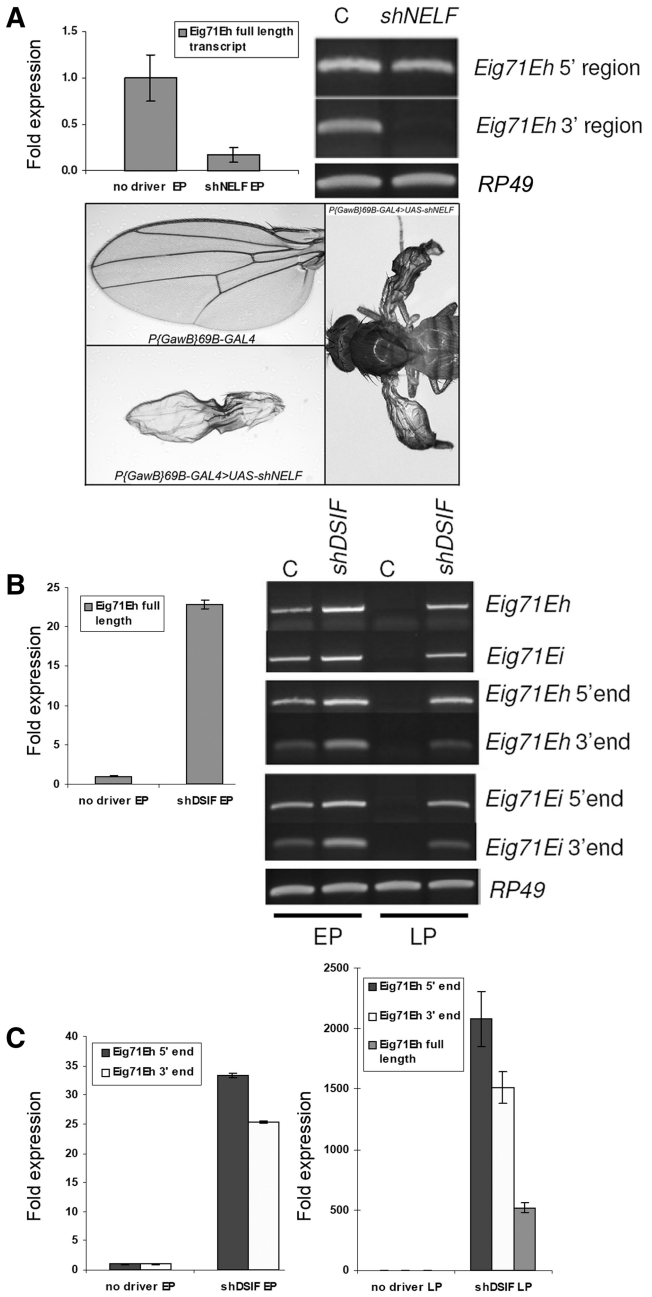

Figure 3. *Eig's* are regulated by PolII pausing factors. (**A**) *NELF* positively regulates *Eig's*. (Top left) qRT-PCR analysis of *Eig71Eh* full length transcripts in EP expressing shRNA to *NELF* or control with no driver. Depletion of *NELF* in early pupae causes an unanticipated reduction of *Eig71Eh* 3′ transcript. (Top right) Agarose gel analysis of RT-PCR products. (Bottom) shRNAi depletion of *NELF* causes developmental wing defects. Shown are representative wings from *P(GawB)69B-GAL4* driver alone and *P(GawB)69B-GAL4 > UAS-shNELF*. A dorsal view of an *shNELF* adult is shown on the right. (**B**) *DSIF* negatively regulates *Eig's*. (Left) RNA analyzed from *shDSIF* and control (no driver alone) EP animals by qRT-PCR. (Right) Agarose gel analysis of RT-PCR products from EP and LP animals with specific primers. (**C**) qRT-PCR analysis from EP (left) and LP (right) animals described in panel (B).

of organisms, including Drosophila, that share seven common core subunits while alternate subunits contribute to functional specificity. OSA/BAF250 subunit is found within mammalian BAF or Drosophila BAP complexes, while POLYBROMO (PB)/BAF180 and BAP170/BAF200 are subunits of mammalian PBAF or Drosophila PBAP complexes (38,39). It is not known which subcomplex participates in *Eig71Eh–Ei* gene regulation. To address these questions, quantitative ChIP of *Eig71Eh* chromatin was performed using PB and OSA antibodies in both WT and mutant animals. These experiments revealed that PB protein, in contrast to OSA, is enriched at the promoter and throughout the transcribed regions in a pattern identical to SNR1 in both WT and $snr1^{E1}$ mutant (Figure 2D). PB enrichment was observed during the EP stage when *Eig71Eh–Ei* transcripts were accumulating, suggesting that PBAP complexes most likely regulate *Eig71Eh–Ei* transcription elongation. Thus, misregulation of *Eig71Eh* transcription in the $snr1^{E1}$ mutant is not due to either altered accessory subunit composition or impaired Brm complex binding to chromatin.

Our data suggests that, while the BRM subunit ATPase activity is required for chromatin remodeling following release of the paused polymerase, the Brm complex-dependent restraint on transcription elongation is mediated through the functions of SNR1. In this context, SNR1 could act either as a physical block to PolII movement or to restrain remodeling activities of the Brm complex. If SNR1 acts as a barrier to PolII movement, we would predict an inverse correlation between binding of the Brm complex to chromatin and active elongation. As an example, NELF dissociates from the *Hsp70* gene upon activation by heat shock and a stalled PolII is released (40). Alternatively, if SNR1 functions to restrain remodeling during elongation, we would expect enhanced recruitment of the complex coincident with hormone-dependent activation. We examined the distribution of SNR1 and PolII CTD-phosphorylated forms on chromatin at the larval BG stage before transcription of *Eig71Eh* was initiated and compared that with the EP stage when transcripts were accumulating. SNR1 and PolII (Ser2P-CTD and Ser5P-CTD) were further enriched throughout transcribed regions in the EP stage compared with the earlier BG stage (Figure 2E) consistent with recruitment of the Brm complex to assist in elongation events when transcription was maximal. Interestingly, Ser5P-CTD showed the greatest change in recruitment during transcription of the *Eig* genes.

Hormone-dependent recruitment of elongation factors

We sought to more directly examine the individual roles of elongation factors on *Eig* regulation and, in particular, determine whether loss of NELF and DSIF had similar effects on *Eig* transcription as Brm complex mutants. We performed *in vivo* RNA knockdown of *NELF* and *DSIF* using short hairpin containing transgenes (shRNAi). NELF has been shown to have both positive and negative roles in regulating polymerase elongation (40). Expression of a *shNELF* driven by the GAL4 expressing *P(GawB)69B* driver produced crumpled wing phenotypes indicating important functions in tissue development (Figure 3A). Depletion of *NELF* in the EP stage caused an unanticipated reduction of full length transcripts by 6-fold (Figure 3A), with the 3′ mRNA almost

undetectable, suggesting that NELF has a positive function in regulating *Eig71Eh* transcription. Other Brm complex targets were upregulated by knockdown of *NELF* (data not shown) (40). Thus, the Brm complex and NELF are both required for production of *Eig71Eh* 3′ transcripts. In contrast, expression of *shDSIF* resulted in a 23-fold upregulation of *Eig71Eh* expression in EP-staged animals compared to controls (Figure 3B) suggesting a negative regulatory role. Similar upregulation was observed for other *Eig* genes (Figure 3B; data not shown). RT-PCR using exon-specific primers for *Eig71Eh* and *Eig71Ei* verified the transcripts were elevated at both 5′- and 3′-ends compared to control (Figure 3B gel analysis and Figure 3C qPCR measurements). Surprisingly, at the LP stage when transcripts are normally absent in WT, strong expression was observed upon *shDSIF* knockdown. Inappropriate transcription was also observed in the LP stage $snr1^{E1}$ mutant (data not shown). Thus, loss of the negative pausing factor DSIF shows a similar enhancement of *Eig* transcription as $snr1^{E1}$ mutant flies. The promiscuous transcription of *Eig* genes in both $snr1^{E1}$ and in the *shDSIF* knockdown flies in late development suggests that stalling of PolII at distal exons in response to nucleosome impediments may be also required for the return to a transcriptionally inactive state (41).

SNR1 negatively regulates splicing

In human cell lines, the SWI/SNF complex regulates RNA splicing by decreasing the PolII transcription elongation rate resulting in increased accumulation of PolII on variant exons and by interacting directly with splicing factors (8,9). Since a decreased elongation rate represents a form of RNA polymerase pausing, we looked in $snr1^{E1}$ mutants for altered splicing regulation. Our expectation was that if PolII was not appropriately paused in the $snr1^{E1}$ mutant, an increased transcription elongation rate might result in a pool of RNA species with retained introns. Examination of *Eig* RNA accumulation at developmental stages subject to ecdysone regulation revealed that predominantly non-spliced RNA first accumulates at the WPP stage in WT animals (Figure 4A). Conversion to a smaller spliced form is most evident during the later EP stage even though a non-spliced RNA subpopulation persists. No RNA is detected later in pupariation (see LP, Figure 3C). Thus, the ratio of spliced to non-spliced *Eig* RNA steadily increases over developmental time, indicating that *Eig* splicing is developmentally regulated. Unexpectedly and in contrast to WT, analysis of RNA accumulation in the $snr1^{E1}$ mutant revealed that the spliced form predominated at all developmental stages where the transcript was detected (Figure 4A and B). The largest differential between $snr1^{E1}$ and WT was measured at the WPP stage, in which we observed 79-fold increased spliced product and 23-fold greater total expression. By early pupal development, the difference in spliced product in $snr1^{E1}$ is 11-fold over WT with 28-fold greater total expression. Thus, the enhanced intron removal of *Eig71Eh* and *Eig71Ei* RNAs in the $snr1^{E1}$ mutant reveals an unanticipated role for SNR1 in regulating splicing/RNA maturation.

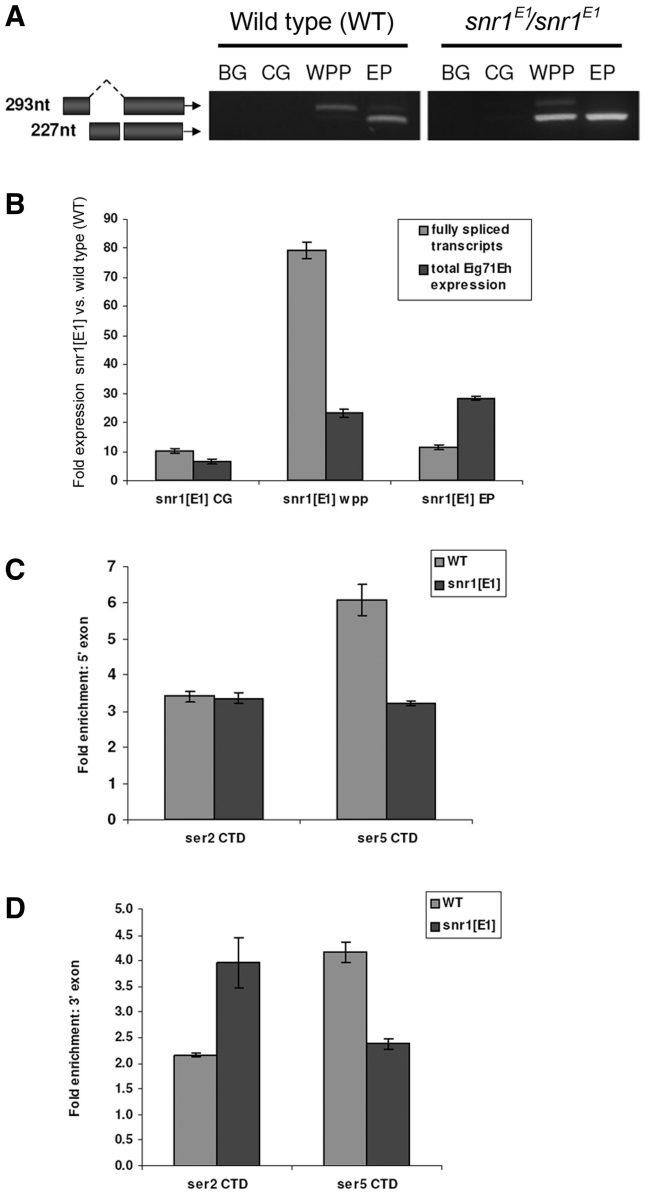

Figure 4. SNR1 negatively regulates expression and splicing of *Eig71Eh* transcripts. (**A**) Developmental splicing regulation of *Eig71Eh* transcripts. Agarose gel analysis of RT-PCR products from WT and $snr1^{E1}$ using *Eig71Eh* primers designed to detect spliced and unspliced *Eig71Eh* transcripts. Samples were normalized to a control transcript (ribosomal protein gene *rp49*; data not shown). Note *Eig71Eh* transcript is not expressed during the BG stage. (**B**) Comparison of *Eig71Eh* RNA splice forms. qRT-PCR analysis of $snr1^{E1}$ and WT described in (A). Graph indicates fold abundance of spliced form in $snr1^{E1}$ mutant compared to WT (+/+). (**C** and **D**) PolII Ser2P-CTD predominates on *Eig71Eh* 3′ exon in $snr1^{E1}$ mutants. ChIP/qPCR of 5′ (C) and 3′ regions (D) using antibodies to Ser2P-CTD and Ser5P-CTD PolII on EP stage chromatin from $snr1^{E1}$ and WT.

Splicing of the *Eig* genes does not depend on the PolII elongation rate

It has been suggested that the mammalian hBRM (SWI/SNF) complexes might regulate splicing through 'slowing down' RNA Polymerase on gene templates (8).

RNA polymerases paused near gene promoters frequently exhibit Ser5P-CTD, with increased Ser2P-CTD observed upon release of the pause (42). The Ser2P-CTD form is processive and can associate with splicing factors. PolII associated with hBRM changes from a predominantly Ser2P-CTD status on *CD44* gene constant exons to the Ser5P-CTD form on variant exons during alternative splicing events in HeLa cells, suggesting that mammalian SWI/SNF complexes participate in affecting CTD phosphorylation patterns on variant exons and contribute to the recruitment of elongation/pausing factors (8).

To account for the enhanced *Eig* splicing efficiency we observed, we first determined whether the phosphorylation pattern of the CTD was altered in the $snr1^{E1}$ mutant EP animals. In WT animals, Ser5P-CTD was detectable in both 5′ (Figure 4C) and 3′ (Figure 4D) exonic regions of *Eig71Eh*. In striking contrast, a reciprocal profile is exhibited in the 3′ exonic region in $snr1^{E1}$ compared to WT, as the PolII found there is predominantly enriched for Ser2P-CTD phosphorylation.

In previous studies, co-purification of RNA PolII with the mammalian SWI/SNF complex has been reported (43), though not in stoichiometric quantities. In addition, it has been reported that the association of PolII with the Drosophila salivary gland chromosomes is drastically impaired in a *brm* mutant, suggesting that Brm complex remodeling is important for stabilizing PolII on chromatin (4). The influence of the Brm complex on PolII could be direct through assisting PolII binding and processivity, or indirect through remodeling of nucleosomes ahead of the elongating polymerase, thus increasing chromatin accessibility. Based on the differences in PolII CTD phosphorylation status we observed on the 3′ exon of *Eig71Eh* we questioned whether the enhanced *Eig* splicing in the $snr1^{E1}$ mutant could be due to direct effects of the mutant Brm complex on PolII processivity. To address this question, we examined whether the elongation rate of PolII was important for *Eig71Eh* splicing regulation using a 'slow' RNA polymerase mutant ($RpII215^{C4}$) in Drosophila that exhibits a lower elongation rate, the human equivalent of which has been shown to affect alternative splicing (44,45). The single point mutation in the largest PolII subunit ($RpII215^{C4}$) impairs its ability to read through intrinsic elongation blocks, resulting in defective movement of the polymerase (46). We found that the lower elongation rate in the 'slow' RNA PolII mutant resulted in reduced *Eig71Eh* expression compared to WT by the EP stage in development, the time point in which the spliced form of the *Eig71Eh* transcript typically predominates. As development proceeded from the WPP to EP stage in WT, total *Eig* expression increased 4-fold with a 47-fold increase in the spliced product. During this same developmental transition period in the $RpII215^{C4}$ homozygous mutant, total *Eig71Eh* expression decreased by half with only a 7-fold enrichment in spliced transcript (Figure 5A). However, a fold comparison of spliced versus total *Eig71Eh* expression between the two stages in WT and $RpII215^{C4}$ mutant revealed nearly identical ratios (Figure 5B). Importantly, since the *Eig71Eh* splicing pattern in the $RpII215^{C4}$ slow RNA polymerase mutant animals was identical to WT, these results are most consistent with the view that PolII elongation rate or processivity during transcription is not the crucial determinant for splicing outcome.

Chromatin in the $snr1^{E1}$ mutant exhibits altered nuclease accessibility

Our results comparing the WT and slow mutant polymerases suggest that the presence of a nucleosomal barrier, rather than the rate of transcription elongation, contributes more directly to *Eig* splicing and removal of the barrier is dependent on Brm complex remodeling activity. If the changes in splicing that result from loss of SNR1 function are not due to alterations in PolII processivity or rate, then the *Eig71Eh* splicing enhancement we observed in the $snr1^{E1}$ mutant animals could be attributed to effects on nucleosome remodeling of the underlying chromatin, thereby altering interactions of PolII with the chromatin landscape. To test this hypothesis, we examined and compared the chromatin structure within the *Eig71Eh* genomic region in both WT and the $snr1^{E1}$ mutant. Three nucleosomes have been mapped to the *Eig71Eh* genomic region (47). The first is centered at +122 within the first exon, the second is centered at +347 spanning the intron and proximal portion of the second exon, and the third is centered at +529 including the distal portion of second exon and transcription termination site (Figure 6A). Development associated changes in chromatin accessibility were examined by micrococcal nuclease digestion and quantitative CHART assay. The intergenic promoter and 5′ primer 1 region that includes 59 nt upstream of the start of transcription were found to be highly accessible to nuclease digestion in both WT and $snr1^{E1}$ mutant backgrounds (Figure 6B and C). The WT chromatin was largely inaccessible within the first exon (5′ primer 2 and 3 regions) around the mapped nucleosome site. There was partial accessibility within the 3′ exon and intron in WT CG and WPP stages, which then diminished by the EP stage. In contrast, *Eig71Eh* chromatin was substantially more accessible in $snr1^{E1}$ compared to WT at all developmental stages. Thus, the increased nucleosome accessibility in the $snr1^{E1}$ mutant would likely minimize barriers to transcription, allowing elongation by PolII to proceed with little or no stalling. These results further strengthen the hypothesis that SNR1, acting within the context of the Brm complex, restrains PolII elongation through effects on chromatin. Thus, the transcription and splicing enhancement we observed in the $snr1^{E1}$ mutant are most consistent with the misregulation of Brm complex chromatin remodeling events.

DISCUSSION

Approximately 20–30% of genes may be enriched for PolII near their 5′-ends and release of the paused polymerase is a rate-limiting step for transcript completion (48,49). Paused polymerases allow for a rapid transcriptional response to stimuli, hormones and developmental cues

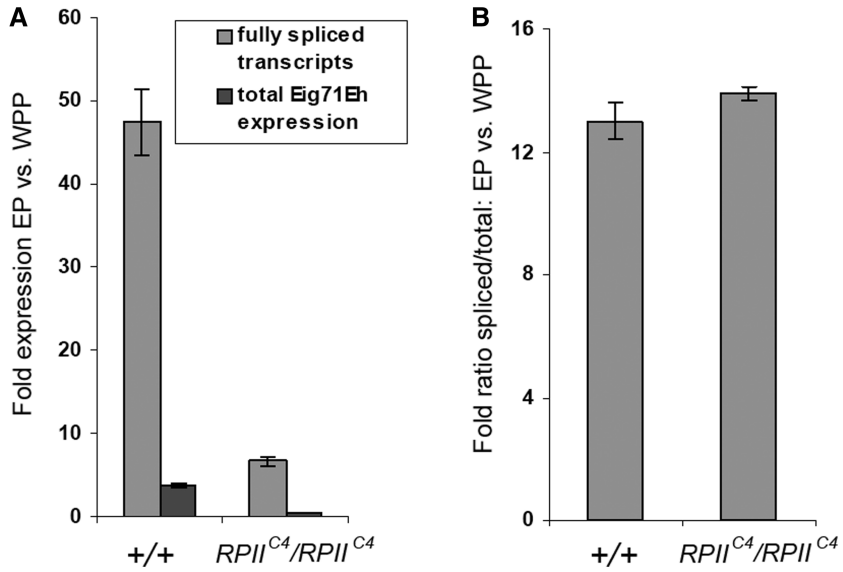

Figure 5. Splicing regulation of *Eig71Eh* in a defective PolII mutant. (**A**) Fold expression changes in EP versus WPP stages from $RpII215^{C4}$ homozygotes and WT (+/+) tested by qRT-PCR. Fully spliced RNAs were compared relative to total *Eig71Eh* RNA expression for each stage. (**B**) Fold comparisons of qRT-PCR values of spliced/total expression for EP versus WPP stages.

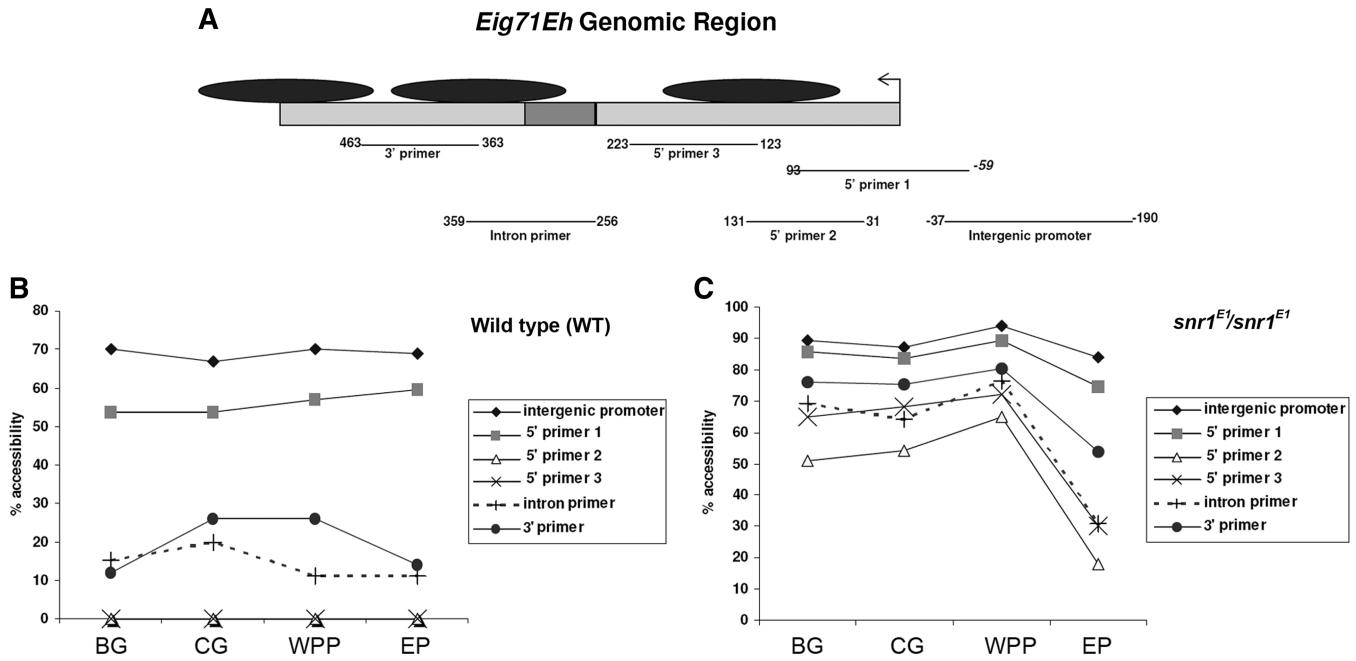

Figure 6. Altered chromatin accessibility of *Eig71Eh* in $snr1^{E1}$ mutant. (**A**) Schematic of qPCR primer sets spanning *Eig71Eh* genomic region. Arrow indicates start of transcription and single intron is shown. Black ovals represent deduced nucleosome positions (47). (**B** and **C**) CHART assay of *Eig71Eh* genomic region from various developmental stages prepared from WT (B) and $snr1^{E1}$ (C) using primers depicted in (A). Note values for 5′ primer 2 and 3 pairs overlay at 0% accessibility in WT at the limiting micrococcal nuclease concentration used in this analysis.

and are typically detected within 30–50 nt from the transcription start site (50–52).

We have identified a class of genes that appears to undergo two pausing/stalling events. The first pause is NELF and DSIF dependent and further stabilized by the Brm complex as evidenced by NELF and DSIF recruitment in both tissue culture and *in vivo*, NELF and DSIF RNAi knockdown and *Eig* transcription regulation *in vivo*, as well as evidence which suggests that the Brm complex stabilizes the Ser5-CTD form of PolII in the vicinity of the transcription initiation region. We have also identified a second PolII elongation block within the transcribed region regulated by Brm complex remodeling functions that is mechanistically distinct from a typical

promoter proximal pause. Using a *brm* dominant negative conditional mutant, we found that chromatin remodeling is necessary for release of the second elongation block and subsequent completion of the 3′-end of the transcript, suggesting that promoter distal nucleosome structure acts as a barrier to elongation. A crucial finding from our work is that Brm complex remodeling function at this distal elongation barrier is regulated by the SNR1 subunit. In the present study, CHART analysis revealed that chromatin in the $snr1^{E1}$ mutant was highly accessible even at developmental stages preceding hormone-dependent induction of transcription, leading us to speculate that a premature nucleosome remodeling event had occurred during early development as a consequence of the $snr1^{E1}$ mutant's inability to restrain Brm complex activities. As a result, nucleosomes in the *Eig71Eh* region would no longer be a barrier to future transcription in the $snr1^{E1}$ mutant and elongation would proceed unimpeded once the NELF/DSIF-dependent promoter proximal pause is alleviated during early metamorphosis. These data are consistent with our previous findings that *snr1* had an important role in transcription repression through effects on chromatin (5).

An unanticipated finding is that the Brm complex negatively regulates pre-mRNA splicing. Recent studies have highlighted the importance of positioned nucleosomes in exon definition and pre-mRNA splicing (53,54). Moreover, SWI/SNF remodeling complexes have been associated with splicing factors (8,9) and it has been suggested that hBrm promotes inclusion of alternative exons by decelerating PolII (8). If elongation rate is important for splice site recognition, then unimpeded transcription should produce a pool of unspliced message. Unexpectedly, we found that the $snr1^{E1}$ mutant exhibits enhanced splicing of the *Eig71Eh* transcript at all developmental time points when transcripts are detected. In contrast to existing models (55), our data suggests that SNR1-dependent pausing of PolII enhances retention of the intron, producing unspliced transcripts in WT animals.

A reduced rate of transcription elongation by PolII associated with hBrm in alternative exons is accompanied by an alteration in the phosphorylation status of PolII from predominately Ser2P-CTD to Ser5P-CTD, thus resembling the status of paused polymerases. Our results with Brm complex knockdown indicated that the complex likely serves to stabilize the initiating form of PolII within the 5′ transcribed region. The Ser5P-CTD predominates in all regions of *Eig71Eh* in WT animals even though we detect Ser2P-CTD in both the 5′- and 3′-ends. We cannot differentiate whether transcription of the *Eig* genes occurs primarily with Ser5P-CTD or whether there is an eventual conversion to Ser2P-CTD as the chromatin becomes remodeled. In contrast, the $snr1^{E1}$ mutant showed enrichment for Ser2P-CTD within the 3′ transcribed region, suggesting that SNR1 may directly affect associations of the Brm complex with PolII. However, studies employing a PolII 'slow' mutant in combination with the CHART analysis of the *Eig* region are consistent with the view that the effects on transcription elongation and splicing we observe in the $snr1^{E1}$ mutant are most likely the result of alterations in the chromatin landscape. Full-length transcription with Ser5P-CTD is not unprecedented. Primary response genes use Ser5P-CTD PolII to produce unspliced full length transcripts in the absence of stimulation and thus lack cotranscriptional processing (56). The generation of processed transcripts in this case, however, required the signal-dependent recruitment of P-TEFb and Ser2P-CTD of PolII. In contrast, for the *Eig* genes we found that an ecdysone pulse is required even before unspliced transcripts can be detected. Thus, restraining PolII elongation through pausing by SNR1 at nucleosomal barriers to transcription and subsequent remodeling by the Brm complex ultimately determines the quality of the transcript produced. Our results strongly suggest that nucleosome remodeling regulation and chromatin architecture can play important roles in determining whether initial messages produced from a transcribed region will be co-transcriptionally processed.

Based on our findings we propose the following model (Figure 7). In WT blue gut larvae prior to the ecdysone pulse that induces metamorphosis, the Brm complex and PolII are localized within promoter regions, with the PolII CTD phosphorylated on Ser5 and paused near the promoter. This pause is likely NELF and DSIF dependent and Ser5P-CTD PolII is stabilized by the Brm complex. During the clear gut stage P-TEFb and additional Brm complex are recruited, DSIF and NELF become phosphorylated and the promoter proximal pause is released. The CTD converts to Ser2P and transcription proceeds along the template until PolII approaches and encounters a nucleosome within exon one when it decelerates and stalls, as the nucleosome acts as an intrinsic barrier to transcription. As a consequence of stalling, Ser5P-CTD predominates and splicing factors likely disengage from the polymerase. Brm complex remodeling of the nucleosome is necessary for subsequent PolII elongation and the SNR1 subunit functions to prevent inappropriate remodeling, thereby stabilizing the nucleosome barrier. As the ecdysone titer rises by the WPP stage, SNR1 inhibitory function on remodeling is alleviated, the stall is released and elongation proceeds with Ser5P-CTD PolII. Elongation by Ser5P-CTD is not processive and co-transcriptional splicing may not occur so the majority of the transcripts have retained introns. Several different mechanisms, not addressed in this work, may account for the transition from mainly unspliced to spliced transcripts as development proceeds. In one scenario, as the chromatin becomes remodeled, stalling is alleviated and during subsequent transcription a population of PolII is eventually phosphorylated on serine 2, and splicing factors associate. A pool of co-transcriptional spliced products is generated *de novo,* and continues to accumulate up until the EP stage. In the second scenario, introns are removed from pre-existing messages post-transcriptionally and spliced transcripts slowly accumulate through the EP stage.

In $snr1^{E1}$ larval BG animals, we envision the first NELF- and DSIF-dependent pause occurs normally with a Ser5P-CTD paused at its promoter proximal position. After the ecdysone pulse at larval CG stage,

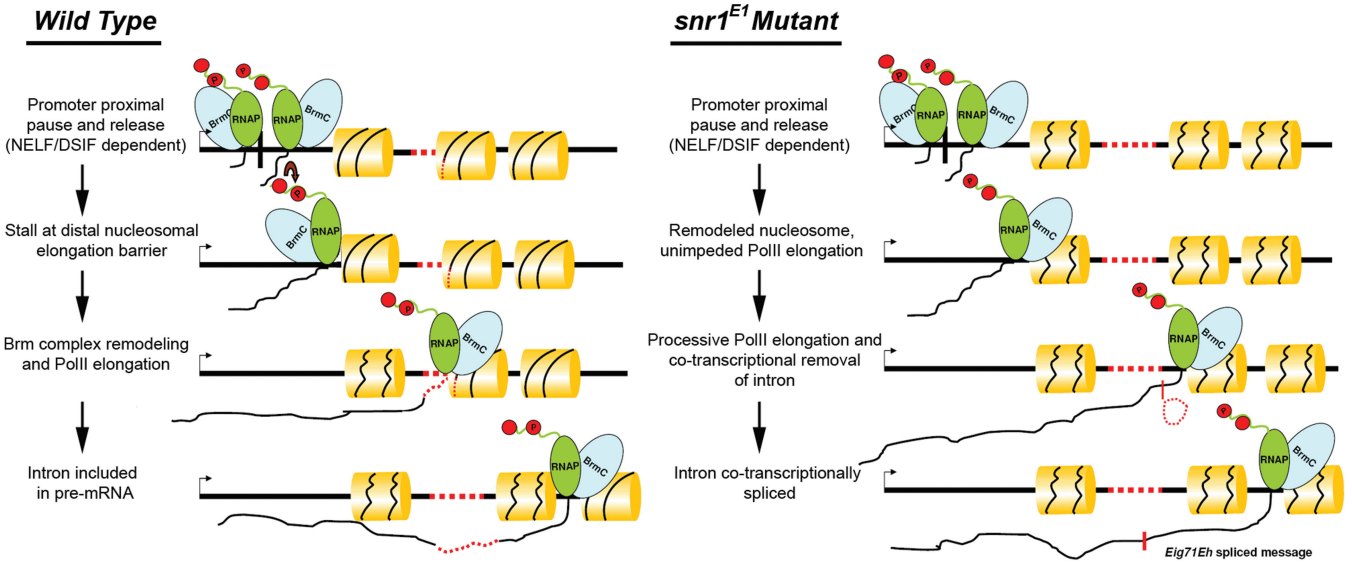

Figure 7. Model for SNR1 and Brm complex functions on transcription elongation and pre-mRNA splicing of *Eig71Eh* in wild-type and $snr1^{E1}$ backgrounds. See text for explanation.

PolII becomes phosphorylated on serine 2 and transcription proceeds to the first nucleosome within exon one. In contrast to WT, as a consequence of increased nucleosome accessibility in $snr1^{E1}$ animals (possibly due to a prior remodeling event earlier in development), Ser2P-CTD PolII does not stall at the first nucleosome. Furthermore, since there is no stall, PolII does not convert back to the Ser5P-CTD form. Elongation continues to be processive with Ser2P-CTD and proceeds with little or no impediments. RNA processing factors are engaged with Ser2P-CTD PolII so splicing occurs co-transcriptionally. Transcription continues unimpeded and the yield of fully spliced transcripts continues to increase until the EP stage.

What are the possible biological implications of SNR1's role in the regulation of paused target genes? The Brm complex appears to promote a developmental delay in splicing of the *Eig* genes resulting in the retention of introns during early transcription. Intron retention in pre-mRNAs is a post-transcriptional form of gene regulation that enhances gene expression (57) and promotes mRNA stability (58). Unspliced transcripts are enriched in quiescent hematopoietic stem cells (HSC) (59) and serve as a means to control HSC activation post-transcriptionally. Additionally, intron retention is a form of alternative splicing (60). However, translation of *Eig* intron containing transcripts would generate non-functional proteins. Since *Eig* genes encode components of the humoral immune response the regulation of paused transcripts by the Brm complex could result in a ready reserve of *Eig* pre-mRNA that could be spliced, allowing for a rapid response to infection during the vulnerable metamorphic period when massive tissue histolysis occurs. Interestingly, recent transcriptome analyses in Drosophila indicate that ~6000 transcripts have retained introns, suggesting that intron retention may represent an important developmental mechanism of post-transcriptional gene regulation (61,62).

What are the implications of our findings for human disease? Highly aggressive malignant rhabdoid tumors (MRT) result from inactivation of *hSNF5/Ini1/SMARCB1*, the human counterpart of Drosophila *snr1* (20). Almost all cases of MRT are associated with bi-allelic loss of *SMARCB1*, and inactivation of murine *mSNF5* resulted in rapid tumor formation with 100% penetrance that was accelerated by loss of p53 (63), classifying the gene as a potent tumor suppressor. We previously found that the patterning defects resulting from reduced SNR1 function could be compensated by reduced BRM activity (18,26,64), suggesting that rhabdoid tumors may result from oncogenic activation of hSWI/SNF targets. Our current data support this view and reveals that SNR1 has vital functions in regulating SWI/SNF complex targets through restraint of chromatin remodeling activities. These findings highlight the unique and essential role that the SNR1 and hSNF5/INI1 subunit plays in regulating target genes in normal development and cancer.

SUPPLEMENTARY DATA

Supplementary Data are available at NAR Online: Supplementary Table 1 and Supplementary Figure 1.

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