Unusual Processing Generates SPA LncRNAs that Sequester Multiple RNA Binding Proteins

Highlights
- Unusual processing generates 5' SnoRNA capped and 3' PolyAdenylated (SPA) IncRNAs
- SPA processing requires a fast Pol II and snoRNP protection from XRN2 trimming
- Two SPAs that sequester multiple RBPs in hESCs are absent in PWS patients
- Knockout SPAs in hESCs leads to altered patterns of RBPs binding and splicing

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In Brief
Wu et al. report SPA IncRNAs that are capped by snoRNAs and require snoRNP complexes to protect them from trimming by XRN2. Two SPAs associated with Prader-Willi syndrome can sequester multiple RBPs and regulate alternative splicing.

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Unusual Processing Generates SPA LncRNAs that Sequester Multiple RNA Binding Proteins

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SUMMARY

We identify a type of polycistronic transcript-derived long noncoding RNAs (lncRNAs) that are 5’ small nucleolar RNA (snRNA) capped and 3’ polyadenylated (SPAs). SPA processing is associated with nascent mRNA 3’ processing and kinetic competition between XRN2 trimming and Pol II elongation. Following cleavage/polyadenylation of its upstream gene, the downstream uncapped pre-SPA is trimmed by XRN2 until this exonuclease reaches the co-transcriptionally assembled snoRNP. This snoRNP complex prevents further degradation, generates a snoRNA 5’ end, and allows continuous Pol II elongation. The imprinted 15q11-q13 encodes two SPAs that are deleted in Prader-Willi syndrome (PWS) patients. These IncRNAs form a nuclear accumulation that is enriched in RNA binding proteins (RBPs) including TDP43, RBFOX2, and hnRNP M. Generation of a human PWS cellular model by depleting these lncRNAs results in altered patterns of RBPs binding and alternative splicing. Together, these results expand the diversity of IncRNAs and provide additional insights into PWS pathogenesis.

INTRODUCTION

In eukaryotic cells, nascent pre-mRNA processing, generally including capping, splicing, and cleavage/polyadenylation (C/P), is functionally associated with RNA polymerase II (Pol II) transcription. 7-methyl guanosine (m7G) capping at the 5’ end occurs during the initial phase of Pol II transcription. The cap structure increases mRNA stability, regulates pre-mRNA processing and nuclear export, and stimulates mRNA translation (Cowling, 2009).

The mechanism of 3’ end processing of nearly all Pol II transcribed RNAs in eukaryotic cells involves C/P of nascent transcripts. The C/P machinery first recognizes the AAUAAA hexanucleotide (or some variants), often together with a downstream G/U-rich sequence present in nascent transcripts, resulting in the endonucleolytic cleavage of the pre-mRNA by the cleavage and polyadenylation specificity factors (CPSFs) (Mandel et al., 2006). A poly(A) tail is subsequently added by poly(A) polymerase to the 3’ end of the transcript in a non-templated fashion.

The 3’ end maturation of a pre-mRNA is tightly connected with Pol II termination. Transcription of the poly(A) signal triggers the endonucleolytic cleavage of the nascent RNA, generating an upstream cleavage product that is immediately polyadenylated. The remaining downstream cleavage product, with an uncapped phosphate at its 5’ end, is highly unstable and is rapidly degraded (Shi et al., 2009). One model explains that the connection between Pol II termination and C/P is the torpedo model (West et al., 2004). The endonucleolytic cleavage at the poly(A) site creates an entry site for XRN2, the 5’ → 3’ exonuclease in humans, to degrade the RNA downstream of the cleavage site. Short nascent RNA induces Pol II elongation complex arrest and promotes termination (Rosonina et al., 2006).

The m7G cap and 3’ poly(A) are hallmark structures of eukaryotic mRNAs that usually contain multiple exons. These features are also true for most long noncoding RNAs (lncRNAs) (Cabili et al., 2011; Khalil et al., 2009; Ulitsky et al., 2011). However, recent studies have shown that some Pol II transcribed lncRNAs are processed in alternative ways. For example, the excised intron-derived sno-lncRNAs are ended by small nucleolar RNAs (snoRNAs) at both ends (Yin et al., 2012).

SnoRNAs are a family of conserved RNAs that are concentrated in Cajal bodies or nucleoli where they either function in the modification of small nuclear RNAs (snRNAs) or ribosomal RNAs (rRNAs), or participate in the processing of rRNAs during ribosomal subunit maturation (Boisvert et al., 2007; Kiss, 2001; Matera et al., 2007). The great majority of snoRNAs are encoded in the introns of protein-coding genes, and usually one intron contains one snoRNA gene (Filipowicz and Pogacic, 2002). SnoRNAs are processed from excised and debranched introns by exonucleolytic trimming and carry out their functions in complex with specific proteins by forming ribonucleoprotein complexes (snoRNPs) (Kiss, 2001). There are two main classes of...
Figure 1. Identification of SPA1s from the Imprinted Region of Chr15

(A) SPA1s from the imprinted region of chr15. Top: diagram of the region. Transcription of SNURF-SNRPN and downstream noncoding region (green bars) occurs only from the paternal chromosome. 70% of the PWS individuals contain the 15q11-q13 deletion up to 5–6 Mb (Cassidy et al., 2012), and the minimal chromosome deletions reported in four PWS individuals are indicated (gray lines, cases 1–4 (Bieth et al., 2015; de Smith et al., 2009; Duker et al., 2010; Sahoo et al., 2008)). SPA1, SPA2 (red or blue lines), and five sno-lncRNAs (black lines) (Yin et al., 2012) are shown underneath. Bottom: Fib RIP RNA-seq (red) and poly(A)+ RNA-seq revealed SPA1 in this region in PA1 cells. See also Figures S1 and S2 and Table S1.

(B) A schematic view of SPA1. SPA1 contains seven exons and is a lncRNA with the 5' snoRNA cap and a 3' poly(A) tail.

(C) Validation of SPA1 expression in hESCs H9. Total RNAs were isolated from H9 cells and resolved on an agarose gel. Probes for NB were shown in (A). Equivalent amounts of RNAs from H9 cells were loaded as indicated by 28S and 18S rRNAs. S, sense; AS, antisense.

(legend continued on next page)
snoRNAs: box C/D snoRNAs and box H/ACA snoRNAs. The box C/D snoRNP contains four key proteins: Fibrillarin (Fib), Nop56, Nop58, and 15.5k (Kiss, 2001). Sno-IncRNAs are formed when one intron contains two snoRNA genes. During splicing, the sequences between the snoRNAs are not degraded, leading to the accumulation of IncRNAs flanked by snoRNA sequences but lacking 5' caps and 3' poly(A) tails (Yin et al., 2012). The genomic region encoding the most abundant sno-IncRNAs is in human embryonic stem cells (hESCs) is specifically deleted in Prader-Willi syndrome (PWS) (Figure 1A) (Yin et al., 2012).

PWS is a neuro-developmental genetic disorder (Cassidy et al., 2012). The minimal deletion associated with PWS has been mapped to the 3' untranslated region (UTR) of SNURF-SNRPN at the imprinted region of 15q11-q13 (Figure 1A) (Bieth et al., 2015; de Smith et al., 2009; Duker et al., 2010; Sahoo et al., 2008). This critical deletion region that causes PWS is paternally transcribed and expresses a number of snoRNAs and IncRNAs (Figure 1A), but a detailed annotation of this important genomic region still has been lacking. Here we identified two SPAs, a type of previously unreported IncRNAs that are 5' capped by snoRNAs and 3' polyadenylated. These SPAs assemble into a striking nuclear accumulation and affect binding patterns of RNA binding proteins (RBPs) and alternative splicing in hESCs, suggesting a link between aberrant localizations of multiple RBPs and PWS pathogenesis.

RESULTS

Identification of SPAs

Since the minimal PWS deletion region contains many snoRNAs (Figure 1A), we reasoned that RNA-immunoprecipitation (RIP) of snoRNP-associated RNAs followed by RNA-sequencing (RNA-seq) would allow us to identify additional snoRNA-related IncRNAs, if any. Fibrillarin is one component of the box C/D snoRNP (Kiss, 2001). We carried out Fib-RIP followed by RNA-seq (Fib-RIP-seq) from the human ovarian carcinoma cell line PA1 cells using antibodies against Fib. The expressed box C/D snoRNAs (Table S1) and the known PWS region sno-IncRNAs (Figures S1A and S1B) could be readily precipitated by Fib-RIP-seq. Unexpectedly, we identified two SPAs (5' snoRNA capped and 3' polyadenylated IncRNAs) in this imprinted region (Figure 1A and Table S1).

SPAs are sequentially located downstream of the protein-coding bicistronic gene SNURF-SNRPN (Figure 1A). This region has been previously annotated as the 3' UTR of SNRPN. The 5' end of SPA1 correlates with the snoRNA SNORD107 (Figure 1A, bottom panel). SPA1 contains seven exons and is 34,000 nt in length (Figure 1B). We have confirmed the existence of SPA1 by northern blotting (NB) in H9 cells (Figure 1C) and in PA1 cells (Figure S1C). Furthermore, two phosphorothioate-modified antisense oligodeoxynucleotides (ASOs) that targeted different exons of SPA1 both reduced SPA1 to barely detectable levels (Figure 1D), revealing that SPA1 contains multiple exons. We further confirmed that SPA1 could only be precipitated with the anti-Fib antibody (Figure 1E), but not with an anti-m7G cap antibody (Figure 1F), and that SPA1 is only present in the polyadenylated fractionation in PA1 and H9 cells (Figure 1G and data not shown). Although capped by a snoRNP at the 5' end, we observed that the half-life of the polyadenylated SPA1 is similar to that of many m7G capped mRNAs (Figure S1D).

In both PA1 and H9 cells, SPA2 is also produced from the PWS deletion region (Figure 1A). The SPA2 gene is located 5.6 kb downstream of SPA1 and contains over 30 exons (Figure S2A). Its 5' end is capped by SNORD109A and is 16,000 nt in length (Figure S2A). The existence of SPA2 in H9 cells was confirmed by NB with probes that recognize its exons across splicing junctions or its last exon, the IPW region (Figure S2B), and by ribonuclease protection assay (RPA) (Figure S2C). It should be noted that the last exon of SPA2 was previously annotated as the IncRNA IPW (Wevrick and Francke, 1997). Together, our analyses show that the 3' UTR of SNURF-SNRPN (Kishore and Stamm, 2006; Runte et al., 2001) comprises two additional SPA IncRNAs (Figure S2A).

SPA Processing Requires an Intact SnoRNA and a Weak Upstream Poly(A) Signal

Nearly all Pol II transcribed exon-containing RNAs in mammals are capped by m7G at their 5' ends to protect RNAs from the 5' exonuclease degradation (Ross, 1995). However, the 5' end of SPA is not m7G capped (Figures 1A and 1F). We asked what mechanism is involved in the 5' end formation of SPA. Nascent RNA-seq in PA1 cells (Zhang et al., 2016) revealed that targeted different exons of SPA1 with its endogenous poly(A) signals, the intact 3.4 kb long internal sequence between SNURF-SNRPN and SPA1, and the first 800 nucleotides of SPA1 including its 5' end SNORD107, followed by a poly(A) signal embedded in the vector (Figure 2A, left panel). We expected that this vector would transcribe a 5,000 nt pre-RNA, which could be processed into a mRNA containing e9+e10 and SPA (Figure 2A, top right panel). Transfection of this plasmid into...
HeLa cells, which lack endogenous SNURF-SNRPN and SPA1, followed by NB revealed that both e9+e10 and SPA1 could be produced (Figure 2A, lane 1). Importantly, deletion of the intact SNORD107 or its box C, or box C'/D motifs that disrupt the snoRNP structure, could completely eliminate SPA formation, while the expression of e9+e10 remained unchanged (Figure 2A, lanes 2–5). Replacement of the endogenous SNORD107 with another box C/D or box H/ACA snoRNA still allowed SPA1 formation (Figure 2A, lanes 6 and 7). Moreover, RPAs also revealed that the 5' end of SPA1 is a snoRNA in H9 cells, as well as in HeLa cells that were transfected with the SPA1 vector (Figure S3A). These results together demonstrate that the essential motifs of a snoRNA at the 5' end are required for SPA processing.

Production of SPA requires continuous Pol II transcription of the downstream region including the snoRNA, after read through of the upstream poly(A) signal. We thus examined whether the strength of the upstream poly(A) signal would have an effect on SPA processing. Deletion of the upstream poly(A) signal, either the AAUAAA C/P recognition sequences (Figure 2B, lane 2), or the cleavage signal (Figure 2B, lane 3), or both the recognition and cleavage sequences (Figure 2B, lane 4), allowed the upstream RNA to be processed at different levels. A weaker poly(A) strength led to less processed e9+e10. Correspondingly, more pre-RNA accumulation could be observed and more SPA1 could be detected (Figure 2B, right panels). It is worth noting, however, that the complete deletion of the poly(A) signal of e9+e10, together with any possible cryptic poly(A) signals in the 3.4 kb internal sequence downstream, not only completely inhibited the maturation of the upstream e9+e10, but also significantly reduced SPA1 processing (Figure 2B, lane 5). These results suggest that the poly(A) signal of the upstream transcript is required for downstream SPA processing from a polycistronic transcript and that different strengths of the upstream poly(A) sequences can affect the downstream SPA processing. In fact, the replacement of endogenous poly(A) sequence with a strong poly(A) signal from the BGH gene led to the strong processing of the upstream e9+e10 but dramatically eliminated SPA1 formation (Figure 2B, lanes 6 and 6*). Altogether, these results reveal that

Figure 2. SPA Processing Requires cis-Elements

(A) SPA processing requires a snoRNA structure at its 5' end. Left: a schematic drawing of wild-type (WT) SPA1 and mutants in the expression vector; top right box shows the processing of SPA1 expression vector. Right: total RNA isolated from HeLa cells transfected with each indicated plasmid was resolved on agarose gels for NB with probe1 or probe2, and rRNAs were used as the loading control. See also Figure S3A. (B) SPA processing requires a weak upstream poly(A) signal. Left: a schematic drawing of a WT SPA1 and mutants containing upstream poly(A) signals in expression vectors. Right: total RNA isolated from HeLa cells transfected with each indicated plasmid was resolved on agarose gels for NB. Efficiency of plasmid transfection was indicated by the co-transfection with pEGFP-C1 vector and detected by NB with an egfp probe.
SPA formation requires an intact snoRNA, which is either a box C/D or a box H/ACA snoRNA and a weak poly(A) signal of its upstream gene in the polycistronic transcript.

**SPA Processing Requires Protein Factors Involved in the Cleavage/Polyadenylation of Pre-mRNA and the Torpedo Model of Pol II Termination**

Transcription of the poly(A) signal triggers endonucleolytic cleavage of the nascent RNA, generating an upstream cleavage product that is immediately polyadenylated (for example, the upstream e9+e10 mRNA shown in Figures 2A and 2B). The remaining downstream cleavage product, with an uncapped phosphate at its 5’ end, is unstable and rapidly degraded. Multiple factors, including CPSF73 and CPSF30, are responsible for the recognition of the poly(A) signal (Chan et al., 2014; Mandel et al., 2006).

To determine whether CPSFs play roles in SPA processing, we knocked down CPSF73 or CPSF30 in PA1 cells followed by the examination of the relative abundance of pre-SPA1 and SPA1. Depletion of CPSF73 led to the accumulation of pre-SPA1 after it was normalized to the upstream snurf-snrpn in the scramble and CPSF73 shRNA treated cells (Figures S3B and S3C). A similar result was obtained by RPA where the accumulation of pre-SPA1 was increased after CPSF73 knockdown (Figure 3A). In addition, depletion of CPSF30, another key factor involved in C/P, also led to the accumulation of pre-SPA1 (Figure S3D).

As the torpedo model explains the connection between Pol II termination and C/P (West et al., 2004), we next examined whether XRN2 is involved in SPA processing. Knockdown of XRN2 led to the accumulation of pre-SPA1 from the PWS deletion region, as revealed by qRT-PCR (Figure S3E) and RPA (Figure 3A) in PA1 cells, as well as by reporter assays in HeLa cells (Figure S3F). It should be noted that in all examined situations, knockdown of XRN2 did not significantly affect transcription or the processing of the upstream mRNA (Figures S3E and S3F), but only the ratio of the relative abundance of pre-SPA1 and SPA1 was altered (Figure S3F). Together, these results strongly suggest that SPA processing requires factors involved in C/P of the upstream mRNA and XRN2 involved in the torpedo model of Pol II termination.

**SPA Processing Is Associated with Fast Pol II Transcription Elongation**

Measuring the Pol II elongation speed by nascent RNA-seq (Zhang et al., 2016) revealed that the Pol II transcription elongation rate (TER) at this locus (~3.4 kb/min) was much higher than the average Pol II speed (~2.5 kb/min) in both H9 and PA1 cells (Figure 3B). This observation suggested that the fast Pol II elongation could allow polymerases to reach SNORD107 before XRN2, allowing the co-transcriptional assembly of a snoRNP to occur. This snoRNP would block the arriving XRN2 degradation from the 5’ end of nascent pre-SPA1 and allow the continuous transcription of the polycistronic transcript by fast Pol II. To test this hypothesis, we modulated Pol II TER by expressing the z-amanitin-resistance (Amr) wild-type, R749H (slow Pol II), or E1126G (fast Pol II) of the human Pol II large subunit implicated in elongation control (Fong et al., 2014; Zhang et al., 2016) in PA1 cells. After transfection with either wild-type or each Pol II mutant, z-amanitin was added to block endogenous Pol II elongation. We found that the R749H mutant reduced Pol II TER and the E1126G mutant promoted TER (Figure 3C) at the SNURF-SNRPN locus as expected (Figure 3C, left panel). Importantly, the relative abundance of the newly processed SPA1, when compared to its upstream snrn mRNA at the nascent level, was significantly decreased or increased in cells with slow or fast Pol II (Figure 3C, right panel). These results thus support our hypothesis that SPA processing requires a fast RNA Pol II TER to win the competition with XRN2.

Together, we propose a model for the PWS region SPA1 processing shown in Figure 3D. The paternal transcription at the 15q11-q13 region (Figure 1A) generates a 180 kb long snurf-snrpn polycistronic transcript. It can be processed into both mRNAs and different types of IncRNAs including SPAs and sno-IncRNAs from the previously thought 3’ UTR of snurf-snrpn. Fast Pol II transcription elongation coupled with a weak promoter-proximal poly(A) signal allows the continuous transcription of fast Pol II to the downstream region including a snoRNA gene located 3.4 kb away. Following C/P at the 3’ end of upstream snurf-snrpn, the nascent uncapped pre-RNA associates with XRN2, which chases the Pol II elongation complex in order to destabilize it. However, Pol II reaches SNORD107 first. XRN2 then encounters the co-transcriptionally assembled SNORD107 snoRNP that blocks further degradation of XRN2 and also allows continuous Pol II elongation. Such a mechanism allows the formation of the 5’ end snoRNP cap of SPA1. Meanwhile, Pol II continues its transcription until it encounters the next poly(A) signal to initiate another round of cleavage and polyadenylation that forms the 3’ end poly(A) tail of SPA1. In addition, SPA2, located 5.6 kb downstream of SPA1, is formed by the same mechanism as SPA1 (gray box, Figure 3D).

Since the SPA2 gene also comprises 29 copies of SNORD116s and 5 sno-IncRNAs (Yin et al., 2012) in its introns, we further examined whether SPAs are precursors of these sno-IncRNAs. Treated cells with ASOs that target snrpn mRNA, SPA1, or SPA2 did not significantly alter sno-IncRNA expression (Figure 3E), suggesting that these two types of IncRNAs do not have a precursor-product relationship. Indeed, snrn, SPAs, and sno-IncRNAs are all derived from the same polycistronic transcript (Figure 3D). SPA1 and SPA2 each contains multiple exons and is generated by a mechanism associated with polycistronic transcription, nascent RNA 3’ processing, and fast Pol II elongation rate, whereas sno-IncRNAs are derived from the excised introns of SPA2 (Figure 3D).

**The PWS Region LncRNAs Form a Striking Nuclear Accumulation**

We have measured the copy number of PWS region SPAs in hESCs. SPAs represent one of the most abundant IncRNAs in hESCs (Figure 3F). For instance, the abundance of SPAs is about one third of that of the IncRNA MALAT1 and is similar to the non-polyadenylated sno-IncRNAs in hESCs.

The nuclear/cytoplasmic RNA fractionation revealed that PWS region SPAs were nuclear retained (Figure S4A). By RNA fluorescence in situ hybridization (FISH) with tiling probes that are antisense to the full length of SPA1, SPA2, or sno-IncRNAs (Figure 4A), we found that these lncRNAs accumulated to a striking
nuclear accumulation in each nucleus of H9 and PA1 cells (Figures 4B and S4B). These accumulations were about 0.4–1.2 μm in diameter, and their volumes were 1–2 μm³ (Figure 4C). Their sizes were larger and greater in H9 cells than those in PA1 cells (Figure 4C). In each subnuclear accumulation, sno-lncRNAs appeared to be located in the middle, surrounded by SPA1 and SPA2 (Figures 4B and S4B).

Double DNA/RNA FISH showed that SPAs accumulated at or near their sites of processing on the paternal chromosome (Figure 4D). To exclude the possibility that the detected signals by RNA FISH were from the partially processed PWS region polycistronic transcript, we blocked Pol II transcription followed by examining the SPA accumulation. 3 hr in Act D or DRB treatment was sufficient to eliminate the polycistronic transcript, with only 5%–12% retaining a barely detectable signal revealed by an intron probe (Figure 4E). In contrast, the SPA1 RNA, although somewhat reduced, remained in 98%–100% of these same nuclei (Figure 4E). Thus, the persistence of PWS region IncRNAs in these transcriptionally inhibited interphase cells is not due to the unprocessed polycistronic transcript. Finally, we found that the striking subnuclear accumulation of SPAs and sno-lncRNAs also existed in induced pluripotent stem cells (iPSCs) derived from normal individuals but was absent in iPSCs derived from PWS patients (Yang et al., 2010) (Figure 4F).

Conservation of PWS Region SPAs
Comparison of human and mouse genomes revealed that the genome organization of SPA1 is largely conserved (Figure S5A). Similar to human SPA1, the 5’ end of mouse SPA1 (mSPA1) is Snord107. mSP contains 9 exons, and its predicted size is about 20,000 nt (Figure S5A). However, the homolog of 5’ end SNORD109A for the human SPA2 gene is not present in the mouse genome. Indeed, there is a gap missing in the mouse genome that corresponds to the SPA2 locus in human, suggesting that SPA2 IncRNA is not conserved (Figure S5A). Remarkably, although Snrpn is expressed in mouse embryonic stem cells (mESCs) and upon neuronal differentiation (Figure S5B), mSPA1 is largely absent in mESCs but highly expressed in neurons (Figure S5B). We further confirmed the processing of mSPA1 in the adult mouse hippocampus tissue, but not in mESCs by NB (Figure S5C). These analyses indicate a different processing of mSPA1 from human SPA1 and the non-conserved feature of the PWS region IncRNAs between mouse and human.

Generation of Allele-Specific Isogenic hESC Lines Lacking PWS Region LncRNAs
Because the PWS region SPAs are not conserved (Figure S5) and the current existing mouse models cannot fully recapitulate the clinical features of human PWS (Cassidy et al., 2012; Ding et al., 2008; Tsai et al., 1999), we decided to generate isogenic hESC lines lacking these IncRNAs by CRISPR/Cas9 to create an unbiased human PWS cellular model.

We designed sgRNAs to delete the entire 141 kb genomic region that comprises SPA1 and SPA2 in H9 cells (Figure 5A). As this locus is a genomic imprinting region only transcribed paternally, we selected H9 clones carrying the 141 kb paternal chromosome deletion for a minimum perturbation of the genome. Compared to wild-type (WT) cells, H9 clones having the paternal deletion (P-KO) did not express PWS region IncRNAs (Figures 5A and 5B). The lncRNA-enriched PWS accumulation also disappeared in P-KO cells (Figure 4C). It should be noted that this 141 kb genomic deletion inevitably removed the SPA2 intronic-located SNORD116 snoRNAs, which are orphans and may possibly lack functional potential (Bazely et al., 2008; Bratkovic and Rogelj, 2014).

H9 P-KO clones of the 141 kb paternal deletion appeared morphologically normal, expressed pluripotency markers, maintained normal karyotype, and could differentiate into all three germ layers (data not shown). RNA-seq and NB revealed little changes in snrpn expression in P-KO cells (Figures 5A and 5C), or few alterations in global gene expression (data not shown). These observations suggest that although the PWS region IncRNAs primarily accumulated to their sites of synthesis (Figure 4), they do not act in cis. Furthermore, knockout of SPAs had no significant effect on the CpG methylation status at the imprinting center located upstream of the SNURF-SNRPN locus (data not shown). Together, we conclude that these PWS region SPAs and sno-lncRNAs are not essential for hESCs, consistent with the fact that PWS patients lacking these lncRNAs are viable.

PWS Region LncRNAs Sequester RBPs with Distinct Binding Preferences
To further explore the role of SPA1, we set up RNA pull-down experiments to identify SPA1-interacting proteins. Since SPA1 is long (Figure 1B), we generated 1,000–2,000 nt sense and antisense biotin-labeled fragments that span almost the full SPA1. After incubation with nuclear extracts isolated from PA1 cells with the biotin-labeled control egfp, sense, or antisense SPA1 fragments, we identified three RBPs, TDP43, RBFOX2, and...
hnRNP M that interacted with the sense but not antisense SPA1 or egfp (Figures 5D and 5E). Additional co-localization of SPA1 and TDP43, RBFOX2, and hnRNP M (Figure 5F) also revealed that these proteins were strongly enriched in close proximity to SPA1. Interestingly, additional RNA pull-down assays revealed that SPA2 and sno-IncRNAs also interacted with these RBPs (Figure S6A).

We then performed super-resolution 3D structured illumination microscopy (SIM) to assess the relative localization of each RBP and PWS region IncRNAs. Using 3D SIM, we not only confirmed the co-localization of each RBP with these IncRNAs, but also found that greater than 1% of each RBP could be sequestered there (Figure 5G). Since the diameter of the PWS accumulation and the nucleus is about 0.4–1.2 μm and 10 μm,

Figure 4. Nuclear Localization of SPAs

(A) A schematic drawing of probes for RNA FISH and RNA/DNA FISH.
(B) PWS region SPA1, SPA2, and sno-IncRNAs form a nuclear accumulation. Triple-color RNA FISH of SPA1 (green), SPA2 (blue), and five sno-IncRNAs (red) by individual pooled tiling AS probes was performed in PA1 and H9 cells. Nuclei are shown by red dotted lines. See also Figures S4A and S4B.
(C) Statistics of PWS region IncRNA accumulation. The volume and the median distance from the geometric center of the accumulation to its surface’s pixels are shown by boxplots.
(D) SPA1 accumulates at a single chromosomal locus. RNA/DNA FISH of SPA1 (green, with the 1 kb long AS probe shown in A) and its DNA region (red) in PA1 cells.
(E) The persistence of PWS region IncRNA accumulation in interphase cells. Left: after treatment with two transcriptional inhibitors (ActD and DRB), the precursor RNA of SPA1 (pre-SPA1) disappeared, but the SPA1 IncRNA still stably accumulated in the nucleus. Right: statistics of RNA FISH signal of pre-SPA1 or SPA1. Error bars represent SD in triplicate experiments.
(F) Visualization of PWS region IncRNAs in iPSCs derived from a healthy adult and a PWS patient (Yang et al., 2010). Representative images are shown.
respectively, the PWS lncRNAs constitute about 0.02%~0.1% of the nuclear volume but can sequester greater than 1% of each RBP.

To further illustrate the binding capability of each RBP with these lncRNAs at the single-nucleotide resolution, we performed individual-nucleotide resolution UV cross-linking and
immunoprecipitation (iCLIP) with antibodies for TDP43, RBFOX2, and hnRNP M in WT and P-KO H9 cell lines (Figures S6B–S6D and S8). TDP43, RBFOX2, and hnRNP M in examined H9 cell lines all exhibited binding motifs similar to those previously reported (Huelga et al., 2012; Tollervey et al., 2011; Yeo et al., 2009) (Figure 6B). For example, TDP43 binds to clusters of UG-rich sequences (Tollervey et al., 2011), RBFOX2 binds to UGCAU and GCAUG sequences (Yeo et al., 2009), and hnRNP M binds to clusters of GU-rich sequences that often contain UU sequences within the motif (Huelga et al., 2012). In addition, comparison of the TDP43 iCLIP results in H9 cells between a previously published dataset (Tollervey et al., 2011), and ours revealed that over 70% of TDP43 targeted cDNAs were overlapped (Figure S6E). All of these analyses suggested successful iCLIP assays performed in our hands. For each antibody, we performed iCLIP in duplicate, and only changes detected in both iCLIPs were counted (Figure S6F).

iCLIP experiments confirmed that all three proteins directly and strongly interacted with SPA1, SPA2, and sno-IncRNAs in WT H9 cells, but no binding signals could be retrieved in P-KO H9 cells lacking these IncRNAs (Figures 6A and S6G). Calculation of putative binding motifs of these RBPs in these IncRNAs revealed remarkable enrichments of their respective motifs without preference (Figure 6C). Strikingly, a strong preference between IncRNA and RBP was revealed when the binding capability of each RBP on individual PWS region IncRNAs was counted: SPA1 preferred to interact with TDP43, sno-IncRNAs preferred to bind RBFOX2, while SPA2 did not have a preference to these examined RBPs (Figure 6D). SIM further confirmed the binding preference between TDP43 and SPA1 as well as the preference between RBFOX2 and sno-IncRNAs (Figure 6E). Remarkably, SPA1 bound to TDP43 with a strong bias toward its 5' end but interacted with RBFOX2 and hnRNP M more promiscuously (Figures 6F and 6G). Together, these results show that the PWS region IncRNAs form a striking nuclear accumulation that can sequester a significant proportion of RBPs with distinct preferences.

**hESCs Lacking PWS Region IncRNAs Have Altered Patterns of RBP Binding and Alternative Splicing**

The significant enrichment of these RBPs in the PWS region IncRNA-enriched nuclear accumulation in hESCs but not in PWS patients suggests that the lack of these IncRNAs might alter patterns of RBP binding in PWS patients. Comparing RNA targets of TDP43, RBFOX2, and hnRNP M in WT and P-KO H9 cells revealed changes in the proportion of their associated target RNAs (out of all cDNA counts mapping to the human genome in each iCLIP dataset after removal of PCR duplicates) (König et al., 2010) between WT and P-KO H9 cells (Figures S7A–S7C). We then selected transcripts that have altered RBP binding in P-KO H9 cells; meanwhile, we required that the expression of these RBPs associated transcripts remained unchanged in WT and P-KO H9 cell lines (Figures S7A–S7C and Tables S2, S3, and S4). Examples of such transcripts and the proportions of cDNAs that mapped to each transcript in iCLIPs are shown in Figures S7D–S7F.

We then analyzed whether the altered RBP binding would result in alternative splicing changes in P-KO hESCs lacking these IncRNAs. 348 altered splicing events repeatedly occurred from RNA-seq of three P-KO lines (Figure 7A and Table S5). 90 among 348 (26%) showed a corresponding change of RBPs binding to pre-mRNAs (Figures 7B–7E and Table S5). Some correlated only with either TDP43 or RBFOX2, and some with both (Figures 7B–7E and S7G and Table S5), but very few correlated with hnRNP M (Figure 7B). Furthermore, the binding of TDP43 and RBFOX2 to these altered pre-mRNAs could be validated in hESCs (Figure 7F). This observation indicates that SPA1 and sno-IncRNAs can regulate different populations of targets. Remarkably, some alternative splicing changes could be found in human iPSCs derived from a reported PWS patient (Yang et al., 2010) (Figure 7G). Intriguingly, a large number of altered binding sites of RBPs were located to genes that do not have cassette exons (Figure S7H), indicating that the PWS region SPAs and sno-IncRNAs can sequester RBPs away from their normal functional sites (Figure 7H).

**DISCUSSION**

Most well-characterized IncRNAs that contain multiple exons are capped by m7G. We report here previously unreported nuclear RNAs that are capped by a snoRNPs at their 5' ends. SPA processing in the chr15q11-q13 region is associated with fast Pol II transcription elongation and C/P of the upstream gene (Figures 6D and 7D, respectively). SPA1 or SPA2, and their interacting RBPs from triplicate experiments. See Figure 5G for details. (F) iCLIP binding density of TDP43, RBFOX2, and hnRNP M on SPA1. Note that there is a notable enrichment of TDP43 binding density at the 5' end of SPA1. The biotin-labeled pull-down probes for SPA1 used in (G) are shown in the bottom.

**Figure 6. PWS Region IncRNAs Exhibit Different Binding Preferences to RBPs**

(A) TDP43, RBFOX2, and hnRNP M specifically interact with PWS region IncRNAs. Left panels: iCLIP reads were enriched along the PWS deletion region, but not in the adjacent upstream or downstream regions in H9 cells. TDP43 (blue) mainly accumulated at SPA1, RBFOX2 (red) mainly accumulated at sno-IncRNAs, whereas hnRNP M (green) did not show an obvious preference. Right panels: in P-KO H9 cells, iCLIP reads of each RBP mapped to this region in WT H9 cells were not present in P-KO samples. Counts of iCLIP reads were calculated every 500 bp. See also Figures S6B–S6G.

(B) Z scores of pentamer occurrence within the 61 nucleotide (nt) sequence surrounding all cross-link sites (~30 to +30 nt) for iCLIP assays with individual TDP43 (left), RBFOX2 (middle), and hnRNP M (right) antibodies in WT and P-KO H9 cells. See also Tables S2, S3, and S4.

(C) Number of putative motifs of each RBP (TDP43, RBFOX2, and hnRNP M) in SPA1, SPA2, and sno-IncRNAs per kilobase.

(D) Binding capability of TDP43, RBFOX2, and hnRNP M to different PWS region IncRNAs. TDP43 prefers to bind to SPA1, RBFOX2 prefers to interact with sno-IncRNAs, while hnRNP M binds to all three IncRNAs without an apparent preference. Counts of iCLIP reads were calculated every 500 bp.

(E) PWS region IncRNAs interact with RBPs with preferences. SPA1 or sno-IncRNAs co-staining with TDP43 or RBFOX2 under SIM, respectively. Left panels: representative images of IncRNA and RBP co-staining under SIM. Right panels: statistics of SPA1 or sno-IncRNAs and their interacting RBPs from triplicate experiments. See Figure 5G for details.

(F) iCLIP binding density of TDP43, RBFOX2, and hnRNP M on SPA1. Note that there is a notable enrichment of TDP43 binding density at the 5' end of SPA1. The biotin-labeled pull-down probes for SPA1 used in (G) are shown in the bottom.

(G) TDP43 prefers to interact with the 5' end of SPA1. Proteins from PA1 cell extracts were pulled down with the biotin-labeled probes of different SPA1 fragments (F) and then subjected to immunoblotting with antibodies to TDP43, RBFOX2, and hnRNP M.
Figure 7. HESCs Lacking PWS Region LncRNAs Have Altered Patterns of Alternative Splicing and RBP Binding to Pre-mRNAs

(A) Altered splicing of cassette exons after depleting PWS region lncRNAs. Cassette exons with significant PSI changes (mean PSI in P-KO samples – PSI in WT sample) ≥ 0.2 were selected. Red points represent cassette exons with more inclusion in P-KO samples; blue points represent cassette exons with more exclusion in P-KO samples. Cassette exons shown in (C)–(G) are marked in black. See also Table S5.

(B) Analysis of changed alternative splicing events corresponding to altered RBP binding to pre-mRNAs in P-KO hESCs. See also Tables S2, S3, and S4 and Figures S7A–S7H.

(C–E) Examples of alternative splicing changes and their corresponding altered RBP binding in WT and P-KO hESCs. Left panels: wiggle tracks show the RNA-seq mapped reads to altered exons, inferred from the change of PSI on the right (red tracks, WT H9; orange tracks, P-KO H9 lines). Right panels: the altered binding of corresponding RBPs. Inclusion in RIMS2 corresponded with the altered binding of both TDP43 and RBFOX2 (C); the exclusion in DLG1 corresponded with the altered binding of RBFOX2 (D); whereas the inclusion in MAGI2 corresponded with the altered binding of TDP43 (E). See also Figure S7G.

(F) TDP43 or RBFOX2 interact with pre-mRNAs with altered patterns of alternative splicing shown in (C)–(E). RIP was performed in H9 cells using anti-TDP43 and anti-RBFOX2 antibodies followed by qRT-PCR. Bar plots represent fold enrichments of RNAs immunoprecipitated by anti-TDP43 and anti-RBFOX2 antibodies, and error bars represent SD in triplicate experiments.

(legend continued on next page)
At the SPA-produced SNURF-SNRPN locus, Pol II runs faster than its average speed in both PA1 and H9 cells (Figures 3B and 3C). Such a fast Pol II elongation at this locus would shorten the window of opportunity for the upstream 3′ end formation to occur and win the race with XRN2 degradation (Figure 3D). It has been very recently shown that fast transcription elongation could shift termination downstream (Fong et al., 2015). Remarkably, the assembly of snoRNPs downstream of cleavage sites impedes XRN2 degradation and delays Pol II termination (Figure 3E). These seemingly coupled processes together unexpectedly generate SPAs with 5′ snoRNP ends at the SNURF-SNRPN locus. As both box C/D and box H/ACA snoRNAs are capable of mediating SPA formation (Figure 2A), additional genome-wide analyses will be required to identify more SPAs and polycistronic transcripts of this type in different cells. For example, the analysis of publicly available ENCODE datasets revealed a few SPAs that were likely to be generated from the SNORD113 and SNORD115 regions (data not shown).

Bacterial and viral mRNAs are often polycistronic (Blumenthal, 1998). The use of alternative translation of bicistronic transcripts to generate protein diversity also exists in mammals (Brubaker et al., 2014), including the SNURF-SNRPN locus. Snurf-snrp is known as a bicistronic transcript encoding two proteins: Smn, a spliceosomal protein involved in mRNA splicing (Glenn et al., 1996), and a polypeptide SNURF with unknown function (Gray et al., 1999). However, snurf-snrp produces not only mRNAs and SNROD116 snoRNAs (Bazeley et al., 2008), but also different types of IncRNAs including SPAs and sno-IncRNAs (Figure 3D). While SNROD116s may lack functional significance (Bazeley et al., 2008; Bratkovic and Rogelj, 2014; Cassidy et al., 2014), the two SPAs and five sno-IncRNAs localize to a close proximity within the 1–2 μm² domain in the nucleus (Figures 4A and S4). This striking co-localization indicates that all of these IncRNAs originating from the snurf-snrp polycistronic transcript that are not expressed in almost all PWS patients (Figure 1A) might possess similar functions.

A least three important RBPs, TDP43, RBFOX2, and hnRNP M, are sequestered by PWS region IncRNAs (Figures 5 and 6). These RBPs are well known to be involved in multiple aspects of mRNA metabolism regulation (Cho et al., 2014; Lagier-Tourenne et al., 2012; Park et al., 2011; Shi et al., 2009; Tollerv et al., 2011; Wang et al., 2008; Yin et al., 2012). Their aberrant localization and expression have been reported to be associated with neurogenetic disorders, such as Amyotrophic Lateral Sclerosis (Lagier-Tourenne et al., 2012; Tollerv et al., 2011) and autism (Voineau et al., 2011; Weyn-Vanhentenryck et al., 2014). We have previously reported that PWS region sno-IncRNAs interact with RBFOX2 (Yin et al., 2012). Here we show that SPA1 exhibits a strong preference to TDP43 (Figure 6), suggesting a different role between sno-IncRNAs and SPA1.

Analysis of RNA-seq from H9 cells with or without these IncRNAs revealed altered splicing events corresponding to altered TDP43 and RBFOX2 cross-link clusters (Figure 7). Interestingly, gene ontology showed that some genes with altered cassette exons in P-KO hESCs lines were associated with synaptosome and neurotrophin signaling pathways (Figure S7A). For example, RIMS2 is a regulator of synaptic vesicles release, presynaptic plasticity, and insulin secretion (Kaeser et al., 2012; Kashi et al., 2001). DLG1 has a reported role in synaptogenesis (Bonnet et al., 2013; Parkinson et al., 2013). Importantly, altered cassette exons in RIMS2 and DLG1 mRNAs could be found in iPSCs derived from a human PWS patient (Figure 7G). These observations thus suggest a link between the mislocation of RBPs, alternative splicing, and PWS pathogenesis.

The observed outcomes in PWS region IncRNA-depleted hESCs are subtle. This is indeed consistent with the fact that PWS patients lacking these IncRNAs are viable. At the molecular level, such changes could be due to the requirement of the cooperativity and synergy between distinct RBPs that act in alternative splicing regulation. For example, we have observed that some alternative splicing events were co-regulated by both TDP43 and RBFOX2 (Figure 7 and Table S5). Alternatively, these RBPs might simply be poised in undifferentiated hESCs but could be more actively involved in a greater degree of splicing regulation upon differentiation. Future studies are warranted to investigate roles of these IncRNAs upon differentiation.

Finally, the PWS locus is very complex, with the expression of a number of different noncoding RNAs (SPAs, sno-IncRNAs, and snoRNAs) (Figures 1A and 3D). These RNAs are abundant and processed by unusual pathways. Understanding PWS pathology will thus require isolating the individual contributions of these transcripts, and the current work represents important progress in this direction. More work is needed to identify additional proteins, RNAs, and DNAs in this IncRNA-enriched nuclear accumulation.

**EXPERIMENTAL PROCEDURES**

**RIP**

Native RIP was carried out as described (Yeo et al., 2009; Yin et al., 2012). See the Supplemental Experimental Procedures for details.

**RNA FISH**

RNA FISH was carried out as described (Yin et al., 2012), and images were acquired on a DeltaVision Elite imaging system. See the Supplemental Experimental Procedures for details.

**3D SIM**

3D SIM on fixed samples was performed on a Deltavision OMX V4 system (GE Healthcare). SI image stacks were captured with a z-distance of 0.125 μm and with 5 phases, 3 angles, and 15 raw images per plane. The raw data were reconstructed with channel-specific OTFs, and a Wiener filter was set to 0.01 for DAPI channel and 0.002 for other channels using softWoRx 6.5. Images were registered with alignment parameters obtained from calibration measurements with 100 nm diameter TetraSpeck beads (Thermo Scientific). See the Supplemental Experimental Procedures for details.
Generating Allele-Specific SPA KO hESCs

Six sgRNAs (3 targeting upstream and 3 targeting downstream of the deletion) were designed (Table S6) and inserted into one CRISPR/Cas9-nuclease vector pX330A by Multiplex CRISPR/Cas9 Assembly System Kit (Addgene) (Sakuma et al., 2014). hESCs were transfected with pX330A-SPA using FuGENE HD Transfection Reagent (Roche), and clones were picked up in 2 weeks. Genomic DNA and total RNA were extracted to validate P-KO hESCs with primers listed in Table S6.

RNA Pull Down

Biotinylated RNA pull down was performed as described (Zhang et al., 2013). The co-immunoprecipitated proteins were analyzed by western blotting. See the Supplemental Experimental Procedures for details.

icLIP

icLIP assays were performed as described (König et al., 2010) with modifications. UV cross-linked immunoprecipitation was carried out with different primary antibodies. Linearized icLIP cDNA libraries were subjected to high-throughput deep sequencing. See the Supplemental Experimental Procedures for details.

High-Throughput Sequencing

Detailed information about RNA-seq, RIP-seq, and iCLIP-seq is summarized in Table S7.

ACCESSION NUMBERS

The accession numbers for the sequencing data reported in this study are NCBI Sequence Read Archive: GSE85851, GSE85852, and GSE85854.

SUPPLEMENTAL INFORMATION

Supplemental Information includes Supplemental Experimental Procedures, seven figures, and seven tables and can be found with this article online at http://dx.doi.org/10.1016/j.molcel.2016.10.007.

AUTHOR CONTRIBUTIONS


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