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Differentially-expressed, variant U1 snRNAs regulate gene expression in human cells.

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ABSTRACT

Human U1 small nuclear (sn)RNA, required for splicing of pre-mRNA, is encoded by genes on chromosome 1 (*1p36*). Imperfect copies of these U1 snRNA genes, also located on chromosome 1 (*1q12-21*), were thought to be pseudogenes. However, many of these 'variant' (v)U1 snRNA genes produce fully-processed transcripts. Using antisense oligonucleotides to block the activity of a specific vU1 snRNA in HeLa cells, we have identified global transcriptome changes following interrogation of the Affymetrix Human Exon ST 1.0 array. Our results indicate that this vU1 snRNA regulates expression of a subset of target genes at the level of pre-mRNA processing. This is the first indication that variant U1 snRNAs have a biological function *in vivo*. Furthermore, some vU1 snRNAs are packaged into unique ribonucleoproteins (RNPs) and many vU1 snRNA genes are differentially expressed in human Embryonic Stem Cells (hESCs) and HeLa cells, suggesting developmental control of RNA processing through expression of different sets of vU1 snRNPs.

The data discussed in this publication are accessible through GEO Series accession number GSE37623 (http://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?token=zjsvdamuuoaikru&acc=GSE37623)

Accession numbers assigned to the vU1 snRNA genes

vU1.2	JQ988795
vU1.2a	JQ988796
vU1.7	JQ988801
vU1.8	JQ988802
vU1.9	JQ988803
vU1.11	JQ988805
vU1.13	JQ988807
vU1.18	JQ988812

(Supplementary material is included with this article)

INTRODUCTION

U1 small nuclear (sn)RNA, as part of a ribonucleoprotein (RNP) complex, is an essential component of the spliceosome responsible for pre-mRNA splicing. The 5′ end of the U1 snRNA base pairs with the 5′ splice site (5′ss), located at the exon/intron junction at the 5′ end of introns, and defines where the first cleavage occurs. The removal of the intron and subsequent ligation of the flanking exons requires an additional further four snRNA-containing RNP complexes, U2, U4, U5 and U6, in addition to numerous other splicing factors (Wahl et al. 2009). However, unlike the other UsnRNAs, U1 snRNA has also been shown to associate with intron-less transcription units *in vivo*, indicating an additional biological role for this snRNA (Spiluttini et al. 2010). In agreement with this, U1 snRNA co-purifies with the cyclin H component of the TFIIH complex and promotes transcription initiation by enhancing the association of the general transcription factors to the promoters of protein-coding genes (Damgaard et al. 2008; Kwek et al. 2002; O'Gorman et al. 2005). In addition, U1 snRNP can inhibit polyadenylation when bound to cryptic 5′ss located downstream of terminal exons (Fortes et al. 2003; Gunderson et al. 1998). The U1.70K component of the U1 snRNP complex has been implicated in this process, which typically leads to degradation of the transcripts. More recently, it has been shown that the U1 snRNP also protects nascent transcripts from premature cleavage/polyadenylation events occurring at cryptic poly A sites throughout the body of pre-mRNA transcripts (Berg et al. 2012; Kaida et al. 2010; Vorlova et al. 2011). Thus, U1 snRNA can play regulatory roles in gene expression, which are independent of its constitutive role in splicing.

In humans, the U1 snRNA gene (*RNU1*) is encoded by a multigene family located on the short arm of chromosome 1, *1p36* (Naylor et al. 1984). In addition, numerous U1 snRNA pseudogenes are present throughout the genome. There are three distinct classes of the U1 snRNA pseudogenes, which have been generated by both DNA and RNA mediated mechanisms (Bernstein et al. 1985; Denison and Weiner 1982). The U1 snRNA class II and class III pseudogenes are scattered throughout the genome and have no homology in their flanking regions to each other or to the U1 snRNA gene. The U1 snRNA class I

pseudogenes, in contrast, have considerable 5′ and 3′ flanking sequence homology to the U1 snRNA genes and retain good homology to the two essential promoter elements, the distal sequence element (DSE) and proximal sequence element (PSE) (Denison and Weiner 1982). The pseudogenes map to a separate location on the long arm of chromosome 1 (*1q12-21*) (Lindgren et al. 1985). The term 'pseudogene' has been assigned to gene sequences that resemble real genes but contain base changes, deletions and/or insertions that disrupt its ability to encode a functional protein (Balakirev and Ayala 2003; Zheng et al. 2007). Many of the U1 pseudogenes have been classified as such since they also encode imperfect or incomplete copies of the U1 snRNA (Denison et al. 1981; Denison and Weiner 1982). Since the U1 snRNA is not translated into a protein, active pseudogenes could generate variant functional snRNAs. It has been known for some time that minor U1-like snRNAs, with variation to the human U1 snRNA, exist but to date, their function remains elusive (Kyriakopoulou et al. 2006; Lund 1988; Patton and Wieben 1987). We questioned whether variant U1 snRNAs, encoded by class I pseudogenes could play a role in regulating gene expression *in vivo*.

We annotated a total of 21 U1 snRNA class I pseudogenes within the *1q12-21* locus and demonstrate that many of the pseudogene promoters have active transcription marks in human cells. The majority of these pseudogenes express an snRNA *in vivo* and are differentially expressed in human Embryonic Stem Cells (hESCs) and HeLa tissue culture cells. Moreover, these variant U1 snRNAs undergo normal 3′end processing and are packaged into ribonucleoprotein (RNP) complexes. Using antisense oligonucleotides to target the most abundant variant U1 snRNA (vU1.8 snRNA) for degradation in HeLa cells, followed by high throughput analysis of total RNA hybridized to the Human Exon ST 1.0 array, we show that this variant U1 snRNA is required for correct expression of a subset of genes at the level of mRNA 3′end processing. These findings indicate that this new group of snRNAs, which are encoded by sequences previously considered to be pseudogenes, play important roles in cell function.

RESULTS

U1 snRNA class I pseudogenes are transcriptionally active, *in vivo*

We searched the human genome for sequences similar to the prototypical human HU1.1 snRNA gene, (Lund and Dahlberg 1984), using the UCSC genome browser BLAST-like alignment tool (blat)(http://genome.ucsc.edu/). 21 putative and 4 near perfect hits were identified within the U1 snRNA class I pseudogene locus (*1q12-21*) and the U1 snRNA gene locus (*1p36*), respectively (Figure 1A and Table S1). The U1 snRNA class I pseudogenes (hereafter termed variant (v)U1 snRNA genes) were further divided into 9 groups based on sequence conservation (Figure S1) and all were aligned to the U1 snRNA gene sequence (Figure 1B and Figure S2-3). The results of our analysis demonstrate that the human U1 snRNA class I pseudogene locus spans a genomic region of approximately 6.8 Mb. The 21 vU1 snRNA genes are not clustered in this region but instead are interspersed with protein-coding genes and numerous transfer (t)RNA genes. These retain large stretches of homology to the U1 snRNA genes within the promoter and 3′ flanking sequences (Denison and Weiner 1982). While some of the vU1 snRNA-encoding regions are identical to the U1 snRNA sequence, most deviate by base changes and small deletions (Figure 1B). Interestingly, all U1 and vU1 snRNA genes lack sequence identity immediately downstream of the snRNA gene-specific 3′ box RNA processing element while the majority regain sequence conservation after an additional 250 bp (Figure S3).

To investigate whether these vU1 snRNA genes are active *in vivo*, we performed chromatin immunoprecipitation (ChIP) experiments in HeLa cells with antibodies to factors involved in expression of the pol II-transcribed UsnRNA genes. These include the multi-subunit transcription factor PTF/PBP/SNAPc (PSE-binding transcription factor/PSE-binding protein/snRNA-activating protein complex), which recognises the PSE element located approximately 50 bp upstream from the transcription start site, and pol II phosphorylated on serine 7 (Ser7) of the carboxy terminal domain (CTD) heptapeptide (Egloff et al. 2008). In addition we used antibodies to pol II and acetylated H3 core histone proteins, which are primarily associated with promoter regions of actively transcribing genes (Shahbazian and Grunstein 2007). As shown in Figure 2A, almost half of the vU1 snRNA promoters contain acetylated H3 and the same vU1 snRNA genes (vU1.6, vU1.7+9, vU1.8, vU1.13-16, 19 and vU1.18) are occupied by pol II at levels similar to those observed at U1 snRNA gene promoters. The snRNA gene-specific transcription

factor PTF and phosphorylation of Ser7 of the CTD of the large subunit of pol II, which is found on active snRNA genes (Egloff et al. 2007), are also detected on the same vU1 snRNA genes (Figure 2A).

With the exception of the vU1.6, vU1.1+10 and vU1.8 snRNA genes, the levels of PTF-γ are similar for all vU1 snRNA genes tested. Recent data indicate that PTF-γ may be required to maintain a persistently open chromatin state at snRNA promoters to facilitate rapid transcription re-initiation, as failure to remove this factor results in chromosome fragility at metaphase (Pavelitz et al. 2008). The finding that PTF-γ is associated with vU1 snRNA gene promoters with low pol II occupancy (vU1.2a-2+11, vU1.3-5,12+20, and vU1.17 snRNA genes, respectively) suggests that some genes are regulated at a step downstream from PTF recruitment to the PSE.

vU1 snRNA genes that appear transcriptionally active in HeLa cells also generate transcripts that continue beyond the 3′ box, as detected by qRT-PCR analysis, with transcripts from vU1.7+9, vU1.13-16, 19 and vU1.18 snRNA genes detected at levels comparable to the level of nascent transcripts from U1 snRNA genes (Figure S4). Primer specificity, for ChIP and qRT-PCR analysis, was confirmed using cloned U1 or vU1 snRNA genes as templates (Figure S5). To determine the steady state levels of vU1 snRNAs in HeLa cells, we analysed the small RNA sequencing data from Fejes-Toth et al. 2009. Our analysis confirms expression of vU1 snRNAs in HeLa cells and estimates their level to be <0.2 % the level of U1 snRNA (Figure 2B). Interestingly, vU1.8 snRNA, which contains numerous base changes and a deletion of the U1.A binding site (Figure 1B) (Pomeranz Krummel et al. 2009), is the most abundant vU1 snRNA. Taken together, our results indicate that up to 50% of the previously considered U1 snRNA class I pseudogenes are transcriptionally active in HeLa cells and could therefore contribute to regulation of mRNA production.

Some vU1 snRNA genes are differentially expressed

Numerous reports have documented the expression of variant U1 snRNAs during development of mouse, sea urchin, frog and fly embryos (Cheng et al. 1997; Forbes et al. 1984; Lo and Mount 1990;

Santiago and Marzluff 1989), which differ from their corresponding U1 snRNAs by only a few bases. U1 snRNA is generally constitutively expressed at all stages of development, whereas the U1 snRNA variants appear to be restricted to early embryo development (Lund et al. 1985). To determine whether expression of any human vU1 snRNA gene is restricted to early developmental stages, pol II, PTF-γ and H3.Ac association and vU1 snRNA levels were compared in HeLa and human Embryonic Stem Cells (hESCs). Pol II association and the levels of nascent transcripts are comparable in both cell types for the vU1 snRNA genes active in HeLa cells (vU1.6, vU1.7+9, vU1.13-16+19 and vU1.18 snRNA genes) (Figure S6 and S7). Thus, several vU1 snRNAs may be constitutively expressed. Interestingly, some vU1 snRNA genes (vU1.2a,2,+11, vU.3-5,12+20, and vU1.8) have marks of active transcription and generate up to 30-fold more nascent transcript in hESCs than in HeLa with a corresponding increase in pol II occupancy at their promoters (Figure 3A and 3B). These may therefore represent a distinct class of vU1 snRNA genes that are differentially regulated. Furthermore, as noted above, the levels of PTF-γ do not correlate with the transcriptional status of vU1 snRNA genes in the different cell types (Figure 3A and Figure S6), re-enforcing the notion that control of expression is regulated at a step downstream from PTF-γ association.

More extensive analysis of the transcription units of vU1.2a snRNA, vU1.8 snRNA and vU1.18 snRNA genes confirms that the vU1.2a snRNA gene is inactive in HeLa cells but transcribed in hESCs (Figure 4). When hESCs are differentiated into Embryoid Bodies (EB) there is a drastic reduction in pol II occupancy within the snRNA encoding region of the vU1.2a snRNA gene relative to the other U1/vU1 snRNA genes analysed. In contrast to the pol II ChIP data however, qRT-PCR analysis of steady state levels of the vU1 snRNAs in the 4 day old hEBs indicate a marginal reduction in snRNA levels in these cells as compared to levels in hESCs (Figure S8). This is in agreement with previous studies in mice, which show that a reduction in the embryo-specific U1b snRNA level is only detectable in 11-19 day old embryos (Cheng et al. 1997). This might reflect that vU1 snRNAs are as stable as the U1 snRNA, which has an estimated half-life of 4-5 days (Sauterer et al. 1988). This data indicates that regulation of vU1 snRNA gene activity occurs early in the differentiation process, possibly allowing time for steady state levels of

vU1 snRNAs to reduce before the hEBs differentiate into different cell types. Importantly, down-regulation of the expression of some vU1 snRNA genes during differentiation suggests a stage-specific function of these vU1 snRNAs during development.

vU1 snRNAs are in ribonucleoprotein complexes.

U1 snRNA is synthesized as a precursor in the nucleus and exported to the cytoplasm for 3′ end processing and maturation (Will and Luhrmann 2001). Whilst in the cytoplasm, seven core proteins, known as the Smith (Sm) proteins assemble onto the 5′-AUUUGUG-3′ motif located towards the 3′ end of the U1 snRNA (see Figure 1B). The association of U1 snRNP-specific proteins (U1-70K, U1-A and U1-C) and nuclear import completes the biogenesis and enables the U1 snRNP to participate in pre-mRNA processing. To determine whether vU1 snRNAs are also associated with RNPs, RNA was isolated from RNP complexes purified from both HeLa and hESC whole cell extracts by immunoprecipitation with anti-Sm antibodies. Anti-Sm was the antibody of choice as this is the only conserved sequence motif across the representative groups of vU1 snRNA genes (Figure 1B). Immunoprecipitation with human anti-IgM was used as a negative control. The presence of vU1 snRNAs was determined by qPCR amplification using gene-specific primers that target the snRNA encoding region. vU1.8 and vU1.16 snRNAs, and vU1.3-5,12+20 snRNAs were chosen for further analysis as they are the only vU1 snRNAs expressed in HeLa and hESCs, respectively, that are sufficiently divergent from U1 snRNA to ensure specific amplification of the mature snRNA. The results of qPCR analysis indicate that these vU1 snRNA transcripts are present in the purified Sm-containing RNP complexes (Figure 5). Using a genomic DNA standard to normalize primer efficiencies between the different primer sets, the vU1 snRNAs were estimated to be present at 0.2% and 0.5% the level of U1 snRNA in Hela and hESCs, respectively. As expected, the 7SK snRNA is not specifically immunoprecipitated.

To determine whether the vU1 snRNAs are fully processed at the 3′end, a poly A tail was added to the Sm-purified snRNAs and individual vU1 snRNAs were amplified using gene-specific forward primers together with an oligo dT reverse primer. Sequencing of the cloned transcripts confirmed that at least

vU1.7+9 and vU1.8 snRNAs , and vU1.3 and vU1.20 snRNAs in HeLa and hESCs, respectively, are fully 3′ end processed (Figure S9). Notably, transcripts from vU1.7+9 snRNA genes, which differ from the U1 snRNA by one base at position 150, comprise 10-15% of the clones when primers specific to U1 snRNA are used for amplification. In addition, vU1.18 snRNA encodes an snRNA identical to U1 snRNA and could therefore contribute to the pool of U1 snRNA. A significant proportion of the total U1 snRNA in mammalian cells may therefore derive from vU1 snRNA genes.

To further characterize the vU1 snRNP complexes in HeLa cells, immunoprecipitation was performed with antibodies against the U1 snRNA-specific RNP proteins, U1.70K, U1-A, U1-C, and an antibody that recognises the UsnRNA-specific tri-methyl cap structure. U1 snRNA was specifically immunoprecipitated with all antisera tested (Figure S10). Importantly, the 7SK was not enriched in any of the immunoprecipitation reactions, as expected. qRT-PCR analysis of RNA purified from the immunoprecipitates, using primers specific for vU1.8 snRNA and vU1.3-5,12+20 snRNAs, indicates that both these snRNAs contain a tri-methyl cap structure and are associated with the U1-C protein (Figure S10). Anti-U1-70K and anti-U1-A precipitates very little vU1.8 snRNA (1.4% and 4.7%, respectively) and vU1.3-5,12+20 snRNA (5.7% and 6.3%, respectively), although this is significantly more than for the 7SK RNA negative control (0.43% and 0.37%, respectively). This may indicate that these proteins are not associated with these vU1 snRNAs or that the epitopes are not readily accessible. The absence of U1-70K and U1-A would suggest that vU1 snRNAs may exist in novel RNP complexes with unique roles *in vivo*. Nonetheless, the presence of the U1-C protein in all vU1 snRNAs tested indicates a potential role for vU1 snRNAs in pre-mRNA processing (Du and Rosbash 2002).

vU1 snRNAs regulate gene expression *in vivo*

In addition to vU1 snRNAs, variant U5 snRNAs have also previously been identified in fly and purified from spliceosomal complexes extracted from HeLa cells (Chen et al. 2005; Sontheimer and Steitz 1992). However, analysis of the potential function of vUsnRNAs has been hampered, in part, by the fact that they all deviate from the major species by only a few base changes, making specific knockdown and/or

target-gene identification difficult. In contrast, 52% of the vU1 snRNAs described here have base changes in the region complementary to the 5′ss and 54% of vU1 snRNAs diverge significantly from U1 snRNA. To explore the contribution that vU1 snRNAs make to the regulation of gene expression *in vivo,* we measured global transcriptome changes following knockdown of the vU1.8 snRNA. This vU1 snRNA was chosen as it is the most abundant vU1 snRNA expressed from a single copy gene in HeLa cells, is properly 3′end processed, packaged into an RNP complex and importantly for this analysis, the only vU1 snRNA expressed in HeLa cells that contains a number of consecutive base changes, which enables functional knockdown with specific antisense oligonucleotides (Figure 1B, 2B, 5, S9 and S10). As illustrated in Figure 6A, the 2′O Methyl (2′OMe) RNA/DNA antisense oligonucleotide targeting positions 11 to 33 of vU1.8 snRNA specifically reduces the level of this snRNA *in vivo* by 89%, following transfection into HeLa cells. Importantly, the control oligonucleotide has no effect on vU1.8 snRNA levels.

Having established the specificity of the chimeric RNA/DNA oligo, we were now in a position to address whether degradation of the vU1.8 snRNA *in vivo* would lead to global transcriptome changes. We analysed total RNA prepared from HeLa cells transfected with either control or vU1.8 snRNA antisense oligonucleotides, using the Affymetrix Human Exon ST 1.0 array. This array contains on average 40 probes per gene, targeting exonic, intronic and untranslated (UTR) regions of all well annotated protein coding genes. Variations in expression levels, alternative 3′ and 5′ UTR usage and changes in pre-mRNA splicing events can all be measured, allowing the full effect of vU1.8 snRNA knockdown on gene expression to be analysed. Four independent biological repeats were analysed. We initially chose to look for novel changes in alternative splicing events (ASE), using the Splicing Index (SI) approach (Yamamoto et al. 2009), since this is thought to be the primary function of U1 snRNA *in vivo*. 444 hits were identified, representing 214 genes that exceed the following thresholds: fold change ≥ 2, P-value < 0.01 (data not shown). However, the vast majority of these hits (>92%) indicated a change in probe intensity over intronic and/or inter-genic regions, with each gene represented by more than one hit. Following manual examination of all differentially-expressed probesets, using the UCSC genome browser, it became apparent

that additional probesets flanking these hits were also deregulated, but outside the strict selection criteria. Furthermore, there appeared to be a clear bias in signal intensities at the start of the transcript compared to the end. Consequently, we felt the results of this analysis do not support a major role for vU1.8 snRNA in pre-mRNA splicing but instead indicate a potential regulatory role in gene expression at the nascent transcript level. To address this bioinformatically, we profiled the average intronic probe signal relative to the distance from the transcription start site (TSS) genome-wide and revealed that 140 of the expressed genes (2%) (Table S2) exhibited more than a 2-fold drop in nascent signal after ~5kb from TSS in the vU1.8 snRNA knockdown cells. This result is exemplified in Figure 6B and Figure S11 shows the validation of some of the array predictions by reverse transcription followed by qPCR. These data suggest that the major role of vU1.8 snRNA *in vivo* is to regulate expression of a subset of target genes at the level of transcription elongation and not pre-mRNA splicing, as previously predicted. Interestingly, from the 8834 genes detected as expressed, 116 genes (~1.3%) showed significant (>2-fold, individual p-value <0.01) change in expression across the entire gene, as measured by signals from exonic probesets, following knockdown of the vU1.8 snRNA (Table S3). Although we chose to exclude these genes from further analysis, the GeneOntology terms enriched for this group of genes include female pregnancy, regulation of proliferation and/or response to hypoxia (Table S4), potentially consistent with a role for vU1 snRNA genes in cell differentiation during development.

A loss in nascent transcript towards the 3′end of the gene is consistent with the recent reports showing a similar loss of the 3′end of pre-mRNAs following functional knockdown of the U1 snRNA (Berg et al. 2012; Kaida et al. 2010). In the absence of U1 snRNA recruitment, the cleavage/polyadenylation machinery is able to cleave the pre-mRNA ~20 bp downstream of a cryptic polyadenylation site and subsequently stabilize the shortened RNA by polyadenylation. To assess whether similar events are occurring following degradation of vU1.8 snRNA, we first characterized cDNA fragments for a number of transcripts which show this pattern of expression to more precisely define the region within which RNA processing is affected. As illustrated in Figure 6C, using the VAPA ((vesicle-associated membrane protein)-associated protein A) and PRPF4B (pre-mRNA processing factor 4 homolog

B) transcripts as examples, the loss in nascent transcript typically occurs within the first intron, ~ 5-10 kb from the start of transcription. To determine whether these shortened transcripts are polyadenylated, we located the potential polyadenylation sites within the first intron of both of these genes using Polyadj (http://rulai.cshl.org/tools/polyadq/polyadq_form.html) (Figure 6C). 3′RACE products were generated from control and vU1.8 snRNA knockdown cells using gene-specific forward primers within the first intron, together with an oligo dT reverse primer. The endogenous NR3C1 (nuclear receptor subfamily 3, group C, member 1) pre-mRNA was used as a control since it has been shown to be a target of U1 snRNA ((Kaida et al. 2010). Only one predicted poly A site within the first intron of both the PRPF4B and the VAPA pre-mRNAs generated specific products, following cleavage of the vU1.8 snRNA (Figure 6D). Sequence analysis confirmed that these 3′ RACE products are polyadenylated (Figure 6D). Functional knockdown of U1 snRNA, using a specific 2′OMe RNA/DNA antisense oligonucleotide targeting bases 1-25, (Figure S12), results in a major premature cleavage and polyadenylation defect for the NR3C1 transcript only (Figures 6D). These results suggest that vU1.8 snRNA protects a subset of pre-RNAs from premature cleavage at cryptic poly A sites within introns, in a manner analogous to that reported for U1 snRNA.

Discussion

The human genome contains upwards of 20,000 pseudogenes (Harrow et al. 2012; Kalyana-Sundaram et al. 2012; Kapranov and St Laurent 2012; Torrents et al. 2003), which are generally classified as non-functional sequences within our genome. However, wherever studied, it seems that the inability to produce a protein does not necessarily imply non-functionality as recent studies have demonstrated that pseudogenes often exert their activity through RNA-based mechanisms, generating antisense transcripts which either interfere with translation of real genes or sense transcripts that act as miRNA decoys or compete for mRNA stabilizing factors (Chiefari et al. 2010; Korneev et al. 1999; Muro et al. 2011; Poliseno et al. 2010). For the past 30 years, the class I U1 snRNA pseudogenes were also considered to be non-functional since they would encode imperfect or incomplete copies of the U1 snRNA (Denison et al. 1981; Lindgren et al. 1985). However, the results presented here challenge this notion and suggest that

these 'imperfect' copies have a biological function. We have focussed our attention on the entire U1 snRNA class I pseudogene locus and shown that 50% of the vU1 snRNA genes annotated within this 6.8 Mb region express an snRNA in HeLa cells and that all 21 vU1 snRNA genes are active, at varying levels, in hESCs. In addition, three other vU1 snRNAs (U1A5, U1A6 and U1A7) were also previously isolated from HeLa cells (Kyriakopoulou et al. 2006). A 'blat' search of their sequences indicate that they map to different locations on the human genome. Interestingly, U1A5 snRNA and U1A7 snRNA have features characteristic of class II and class III, including a lack in homology to the U1 snRNA gene in the 3′ and 5′ flanking regions. This is a strong indication that many U1 snRNA pseudogenes may have been mis-classified and our results therefore highlight a novel aspect of snRNA gene expression, which has previously been overlooked.

The incorporation of fully processed vU1 snRNAs in snRNP complexes suggests that vU1 snRNAs are likely to participate in pre-mRNA processing events. In agreement with this, high-throughput analysis of total RNA extracted from control and vU1.8 snRNA knockdown cells revealed a primary role for vU1.8 snRNA in 3′ end processing of pre-mRNAs rather than pre-mRNA splicing, as initially predicted. Knocking down U1 snRNA, at varying levels, has a profound effect on both pre-mRNA splicing and mRNA length (Figure S12) (Berg et al. 2012; Kaida et al. 2010). In contrast, knocking down vU1.8 snRNA levels in HeLa cells affects only 3′end processing of a small subset of genes (Figure 6D), which is consistent with the fact that the level of vU1.8 snRNA in HeLa cells is 0.2% of the U1 snRNA level. Although much less abundant than the U1 snRNA, vU1.8 snRNA is present at of 2-500 copies per HeLa cell, which is comparable to many highly abundant mRNAs (Wickrema et al. 1991). Given that there are significant differences between vU1.8 snRNA and U1 snRNA at their 5′ends and likely also in their RNP composition (Figure S3 and S10), we propose that vU1.8 snRNA is selectively recruited to a subset of pre-mRNAs. Previous data have shown that inhibition of polyadenylation by U1 snRNP requires interaction of the U1-70K protein with the Poly A Polymerase (PAP) (Gunderson et al. 1998). Mutational and biochemical analysis indentified a number of sequence motifs within the SR region of the U1-70K that are necessary for this U1-70K-PAP interaction. However, such motifs are also present in a number of different

SR-containing and non-SR containing proteins. Since data presented in this study suggest that little U1-70K protein is associated with the vU1.8 snRNA, we suggest that a different SR-factor associates with this snRNA and inhibits the PAP activity in a manner analogous to the U1-70K. Moreover, since U1 snRNA can marginally affect the cleavage and polyadenylation of the same cryptic Poly A in the VAPA gene as the vU1.8 snRNA (Figure 6D), we propose that in some instances the vU1.8 snRNP can act cooperatively with the U1 snRNP.

The presence of the U1-C protein in the vU1.8 snRNP complex suggests that vU1.8 snRNA could also function in pre-mRNA splicing (Du and Rosbash 2002; Du et al. 2004; Rosel et al. 2011). Of the 444 ASE (214 genes) that we initially identified using the splicing index algorithm, only 8% (~17 genes) indicated a potential ASE. However, further analysis of some of these ASEs did not support a direct role for vU1.8 snRNA in splicing regulation (data not shown). Moreover, GO:TERM analysis of transcripts subject to premature 3′ end processing following knockdown of vU1.8 indicates an enrichment in factors involved in RNA binding and pre-mRNA processing. It is therefore plausible that the identified splicing defects are an indirect consequence of changes in expression of one or a combination of these factors. The absence of evidence supporting a role for vU1.8 snRNA in splicing regulation is possibly not surprising considering that this variant is the most divergent in sequence conservation from the U1 snRNA (see Figure 1B). In addition to numerous base changes, vU1.8 snRNA also contains a 24 bp deletion encompassing part of the U1-A binding site. Although U1 snRNP is active in splicing assays in the absence of U1-A (Heinrichs et al. 1990; Pomeranz Krummel et al. 2009), lack of this factor on the vU1.8 snRNA may favour an alternative function *in vivo*. Moreover, U1A7 snRNA was unable to rescue mutations at complementary non-canonical 5′ss *in vivo*, further supporting the notion that vU1 snRNAs is involved in processes other than splicing (Roca and Krainer 2009). However, we cannot rule out the possibility that the vU1.8 snRNA or other variants characterized in this and other studies do participate in pre-mRNA splicing events.

Profiling the pattern of expression of vU1 snRNA genes in different cell types indicates that the majority of these genes are active in hESCs and approximately 50% of them are specifically expressed in this cell type. Pervasive transcription is considered a common feature of ESCs and this could explain why

most of the vU1 snRNA genes are expressed in the HUES 2 cell line tested in this study. However, the finding that some vU1 snRNA genes are specifically down regulated upon differentiation to EBs suggests that the control of vU1 snRNA gene expression is a crucial step during the early stages of embryogenesis (Figure 4). Since pre-mRNA processing is known to play an important role during lineage differentiation of stem cells, promoting the production of different isoforms of mRNAs in different cell types, it is plausible that developmental control of vU1 snRNA expression is a critical mechanism controlling gene expression patterns throughout development (Wu et al. 2010). For example, vU1.2a expression is down-regulated as hESCs differentiate into hEBs. Further examination of late stage embryo's and/or embryo-derived differentiated cells, coupled with high throughput transcriptome analysis of control and vU1-specific knockdowns, should allow for a better understanding of the functional importance of this group of vU1 snRNAs in gene expression. Multiple vU1 snRNAs expressed at low levels in different cells at different stages in development could have a significant impact on proteome diversity. A 5 nt deletion in one of the multicopy mouse U2 snRNA genes cause ataxia and neurodegeneration, and 4 point mutations in the human $U4_{ATAC}$ is associated with brain and bone malformations and unexplained postnatal death (microcephalic osteodysplastic primordial dwarfism type 1) emphasizing the potential for variant snRNAs to exert tissue-specific effects. (Edery et al. 2011; He et al. 2011; Jia et al. 2012). Moreover, deletion of the chromosome region (*1q12-21*), which harbours the vU1 snRNA genes, causes severe neurological dysfunction (Mefford et al. 2008), reinforcing the notion that these snRNAs may be important for normal development.

METHODS

ChIP analysis

Chromatin immunoprecipitation (ChIP) analysis was carried out as previously described (O'Reilly et al. 2003). On average 0.5×10^6 HeLa and 1×10^5 human embryonic stem cells (hESCs) were used per immunoprecipitation. Anti-pol II (N-20), anti H3 (ab1791), anti-H3.Ac (06.598) and anti-PTFγ antibodies were obtained from Santa Cruz Biotechnology, Inc., Abcam, Upstate laboratories and Robert G Roeder and Jong-Bok Yoon (Yoon et al. 1995), respectively. Refer to Table S5 for primer sequences. Typical quantitative PCR reactions included a 95°C step for 15 min, 40 cycles at 95°C/15 s, 57°C/15 s and 72°C/25 s followed by a 10 min melt. % Input was calculated by (IP average – no antibody average) × 100/Input).

Plasmid construction

U1 snRNA and vU1 snRNA genomic constructs: genomic U1 and vU1 snRNA clones were generated to control for U1 snRNA and vU1 snRNA primer specificity in ChIP and qRT-PCR. Human whole blood genomic DNA (Promega) was amplified with snRNA gene specific primers (Table S6) to generate 1.3 kb fragments containing approximately 100 bp of the 5′ flanking sequence, the RNA-encoding region and approximately 1 kb of the 3′ flanking sequence. The fragments, which contain Eco RI and Xho I restriction sites at the 5′ and 3′ ends, respectively, were cloned into pcDNA3 (Invitrogen) and sequenced. Genomic constructs were generated for the U1 snRNA and vU1 snRNAs, including vU1.2a snRNA, vU1.7 snRNA, vU1.8 snRNA, vU1.13 snRNA and vU1.18 snRNA.

Cell culture and transfection

HeLa cells were grown in Dulbecco's Modified Eagle Media (Invitrogen) supplemented with 10% foetal calf serum, 50 μg of Penicillin per ml and 4 μM L-glutamine. Human ESC, HUES-2 (passages 16–38), was obtained from the HUES Facility, University of Harvard (Cowan et al. 2004). Work with this line was reviewed and approved by the UK Stem Cell Bank Steering Committee. hESCs were cultured in mTeSR1

medium (Stem Cell Technologies) on hESC-qualified Matrigel (Becton Dickinson). For differentiation to Embryoid Bodies (EBs), hESCs were dissociated with TrypLE Express (Invitrogen) and aggregated in mTeSR1 medium with 10μM ROCK inhibitor Y-27632 (Calbiochem) by spinning in an AggreWell (Stem Cell Technologies) according to the manufacturers' manual, to form EBs of 4000 cells/EB. The EBs were analysed at day 4 post-aggregation.

Transient transfections in HeLa cells were performed with lipofectAMINE 2000 reagent (Invitrogen) according to manufactures' instructions.

Knockdown experiments.

2′-O-Methyl (2′OMe) RNA/DNA oligonucleotides were purchased from Integrated DNA Technologies (IDT). 5 nucleotides at the 5′ and 3′ ends were substituted with 2′-O-Methyl ribonucleotides. All bases were phosphorothioate converted. The control oligonucleotide is sense to the U1 snRNA at position 11 to 32, the vU1.8 snRNA-specific and U1 snRNA-specific oligonucleotide are antisense to vU1.8 snRNA at position 11 to 33 and U1 snRNA at position 1 to 25, respectively, where 1 is the first base of the U1/vU1 snRNA sequence (Table S8). Knockdown experiments were performed in HeLa cells with lipofectAMINE 2000 reagent (Invitrogen) according to manufacturers' instructions. Typically, 1×10^7 cells were transfected with 600 pmoles of the 2′-OMe oligonucleotide and cells harvested 18 hrs later.

RT-PCR and amplification of cDNA

Total RNA was isolated from HeLa and hESCs using Trizol (Invitrogen), according to manufacturers' instructions. cDNA was generated at 50^{o}C from 0.5 μg of total RNA using Superscript III (Invitrogen), random hexamers (Roche) or oligo dT in a total volume of 20 μl. The reverse transcriptase enzyme was omitted in cDNA preparations to control for genomic DNA contamination. Real-time quantitative PCR was performed using a QuantiTect SYBER Green master mix (Qiagen) on the Corbett Rotor-gene 3000 system.

Typical reactions included 0.4 µl cDNA and 1 µM concentration for each primer (Table S7) in a total volume of 10 µl - amplified for 40 cycles at 15 s at 94°C, 15 s at 57°C and 45 s at 72 °C.

3′RACE and amplification of PCPA products

Total RNA was isolated from HeLa cells using Trizol (Invitrogen), according to manufacturers' instructions. cDNA was were generated at 50°C with 1.0 µg of total RNA, from control and knockdown cells, using Superscript III (Invitrogen) and an oligo dT_1 primer, in a total volume of 20 µl. Pre-mature cleavage and polyadenylation products (PCPA) products were amplified by nested PCR with gene-specific forward primers and the oligo dT_2 reverse primer for 25 cycles at 15 s at 94°C, 15 s at 57°C and 45 s at 72 °C. This reaction was diluted 10-fold and 1µl was re-amplified with another gene-specific forward primer and the oligo dT_2 reverse primer for a further 25 cycles at 15 s at 94°C, 15 s at 57°C and 45 s at 72 °C. See Table S7 for primer sequences.

Purification of Ribonucleoprotein (RNP) complexes and subsequent cloning of vU1 snRNA s

Anti-IgM (Sigma; A-4540) or anti-Sm (abcam; ab3138) antibodies were incubated overnight at 4°C with Protein G-agarose. Total cell extracts from 1×10^7 HeLa cells or 1×10^7 hESCs were prepared, as follows. Confluent monolayers of cells, in 9 cm dishes, were washed 2 times in ice-cold PBS and 2 times in ice-cold TBS (150 µM NaCl, 40 µM Tris-HCl - pH7.4). The cell pellet was resuspended in 1 ml ice-cold NET-2 buffer (150 µM NaCl, 0.05 % NP-40, 50 µM Tris-HCl - pH7.4), sonicated on ice - 3 times for 30 seconds with 1 min intervals, followed by centrifugation at 15,000 rpm at 4°C for 15 min. U1-RNPs were isolated from the cleared lysate by pre-incubation with the antibody/agarose suspension overnight at 4°C. Beads were washed, treated with DNase 1 and Proteinase K and extracted with phenol. RNA was recovered by precipitation.

To facilitate cloning, a poly-A tailing kit (Ambion) was used to add a poly A tail to the 3′ end of the purified RNA, according to manufacturers' instructions. cDNA was generated by reverse transcription

using Superscript III (Invitrogen) and oligo $dT_{(12-20)}$(Roche) primers, according to manufacturers' instructions. U1 snRNA sequences were PCR amplified using gene specific U1 snRNA or vU1 snRNA coding primers (forward) together with an oligo dT reverse primer (Table S6). The resulting amplicons were cloned into the pCR 2.1 TOPO cloning vector (Invitrogen) and sequenced.

U1-70K, U1-A and U1-C immunoprecipitation were carried out as follows. 300 µl of monoclonal hybridoma supernatant (kindly provided by Cindy Will and Reinhard Luhrmann), 1 µg of U1-A (abcam) or 1 µg U1-C (abcam) wee incubated with 10 µl of Protein G Dynabeads, in a total volume of 300 µl of ice-cold NET-2 buffer for 1 hr at 4^{o}C rotating. Following 2 washes in ice-cold NET-2 buffer, 25 µl of HeLa nuclear extract (CilBiotech) were added to the Dynabead-antibody complexes, in a total volume of 500 µl of ice-cold NET-2 buffer, and re-incubated for a further 3 hrs at 4^{o}C rotating. Beads were washed six times in ice-cold NET-2 buffer, RNA extracted with Trizol, treated with DNase 1 and re-extracted with phenol. RNA was recovered by precipitation. U1 and vU1 snRNA sequences were PCR amplified using gene-specific primers.

The immunoprecipitation of tri-methyl capped U1 and vU1 snRNAs were carried out as outlined above for the U1-70K immunoprecipitation with the exception that total RNA from HeLa extract (10 µg) was used. The anti-m3 antibody (R1131) was kindly received from Cindy Will and Reinhard Luhrmann.

Affymetrix Human Exon ST 1.0 Array analysis.

Array processing was carried out by Source Bioscience Plc. Data from 4 biological repeats (each for vU1.8 snRNA knock-down and control) were processed by Affymetrix Power tools (http://www.affymetrix.com) using DABG and RMA algorithms for background correction and quantile normalization. Signal values were expressed in log2-scale. Individual probe sets were called 'present' if detected above background (p-value < 0.01) in one group in three or more repeats and 'differential' when mean signal differed by more than 1 (~ 2-fold change in linear scale) with t-test p-value $p<0.01$ between the groups. The transcript was called 'expressed' if more than two thirds of probes overlapping its annotated exons were 'present' and

'differentially expressed' when their mean signal changed by more than 1 (~2-fold) with t-test p-value < 0.01 between the groups.

Splicing index analysis was performed as in Exon Array Analyser (Gellert et. al, 2007), with minor modifications. To generate the average profile of nascent transcription across genes (Figure 6B), signals from 'present' probes in intronic regions were averaged according to the distance from annotated transcription start site (TSS) and plotted in 1 kb bins. Only a subset of transcripts, which are expressed, non-overlapping and longer than 10 kb (3889 genes) was used for this analysis. See Table S9 for primer sequences used in validation experiments.

Gene annotations.

All analysis was performed using RefSeq annotation of NCBI36 (hg18) human genome assembly, downloaded from UCSC genome browser (http://genome.ucsc.edu/). For genes with multiple transcripts annotations only the first transcript is presented in analysis outputs. GeneOntology term enrichment analysis (Table S4) was performed using DAVID functional annotation tool (Huang et. al 2009).

vU1 snRNA quantification by RNA-seq

To find out whether stable vU1 snRNAs can be detected (Figure 2B) in RNA-seq experiments, we analysed HeLa cell small RNA sequencing data from Fejes-Toth et. al 2009 (2009), downloaded from GEO archive (accession number GSE14262). Reads from 6 sequencing experiments were mapped to the human genome using Bowtie aligner, allowing mapping to multiple locations, but no sequence mismatch. Reads matching with 0 or 1 (to count for the potential sequencing errors) mismatch to U1 snRNA genes were subsequently removed from vU1 snRNA counts, thus potentially underestimating vU1 snRNA abundance. Considering mapability as well as sequencing biases, the resulting values need to be understood as very rough estimates. New algorithms dealing with sequencing error-rates and mapability of individual variants are currently being developed, which will allow more precise vU1 snRNA profiling using RNA-seq in different cell-lines.

DATA ACCESS

The data discussed in this publication have been deposited in NCBI's Gene Expression Omnibus (O'Reilly *et al*. 2012) and are accessible through GEO Series accession number GSE37623 (http://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?token=zjsvdamuuoaikru&acc=GSE37623)

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FIGURE LEGENDS

Figure 1 **Annotation and sequence of the U1 snRNA class I pseudogenes**

A) Schematic of Chromosome 1 illustrating the location of U1 snRNA genes (*1p36.13*) and the U1 snRNA class I pseudogenes (*1q21.1*). The size of each locus is shown in kilobases (kb). Each gene is represented by a vertical line and a number. Light and dark boxes on Chromosome 1 refer to regions of high and low gene activity, respectively. **B)** Alignment of the snRNA sequence of the U1 snRNA genes (U1.1-4) with the 21 vU1 snRNA genes. Paired vU1 snRNAs have identical sequences. Non-conserved bases are denoted with the letter code representing one of the 4 nucleotide bases. – indicates the presence of a corresponding base, and a gap indicates the absence of a base at that position. The bases are numbered, beginning with 1 for the first base of the U1 snRNA sequence. Important features of the U1 snRNA are boxed and indicated underneath the alignment: 5′ splice site recognition motif (5′ss, blue box), U1-70K protein binding region (U1-70K, purple box); U1-A protein binding region (U1-A, yellow box); Sm binding site (Sm, green box).

Figure 2 **Several vU1 snRNA are transcribed in HeLa cells**

A) ChIP analysis of U1 snRNA and vU1 snRNA genes in HeLa cells with antibodies indicated at the right. Error bars in this and subsequent figures represent standard error of at least 3 repeats. Location of the primers used is indicated on the diagram. The Proximal Sequence Element (PSE), coding and 3′ box are specified by grey and black boxes, respectively, and start of transcription with an arrow. **B**) Quantification of vU1 snRNA steady state levels in HeLa cells, normalized to U1 snRNA. Only reads with 2 or more nucleotide differences from U1 snRNA were counted towards the vU1 snRNA levels. Experimental data from Fejes-Toth, K, *et al*., Nature, 2009 (2009).

Figure 3 **vU1 snRNA genes are up-regulated in hESCs**

A) ChIP analysis of the U1 snRNA and some vU1 snRNA genes in hESCs and HeLa cells with antibodies indicated at the right. **B)** qRT-PCR analysis of nascent transcripts from the corresponding U1 snRNA and vU1 snRNA genes in HeLa and hESCs. *Rel. levels*; the levels of vU1 snRNA nascent transcripts are expressed relative to U1 snRNA, normalized to 1.0 in each cell type.

Figure 4 **Some vU1 snRNA genes are differentially regulated.**

Pol II ChIP analysis of the U1, vU1.2a, vU1.8 and vU1.18 snRNA genes in HeLa and hESCs, before and after differentiation to Embryoid Bodies (EB). The position of the primers is indicated in the schematics.

Figure 5 **vU1 snRNAs are packaged into RNP complexes.**

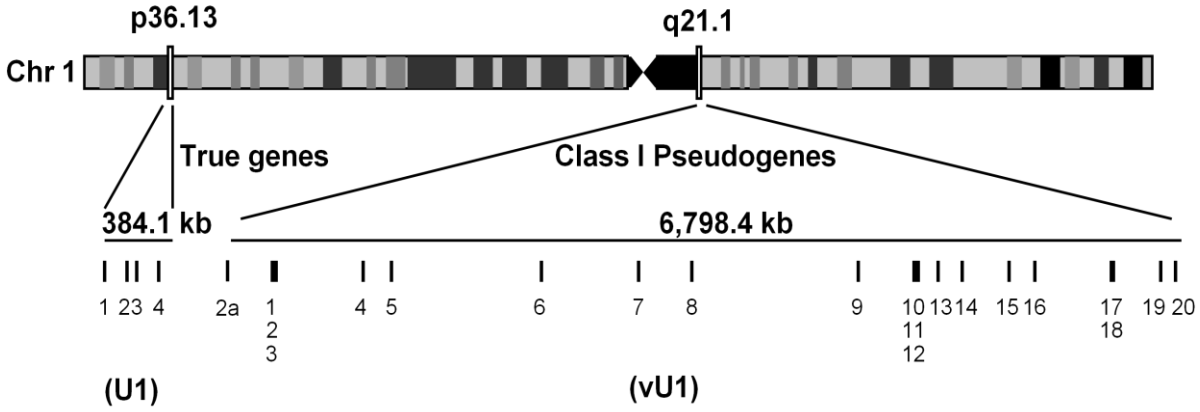
qRT-PCR analysis of U1 snRNA and vU1 snRNA levels in HeLa (A) and hESC (B) extracts, before and after immunoprecipitation with anti-Sm (Y-12) antibody. The non-Sm-containing 7SK RNA level was used as a negative control. Location of the primers is indicated in (A). The level of the U1 snRNA and vU1 snRNAs, enriched in the Sm-immunoprecipitate, is expressed relative to input at 100%. RT (reverse transcription) reactions without '–' or with '+' the addition of Superscript III. **Note**: although vU1.15/vU1.16 snRNA was enriched in RNP complexes molecular cloning, followed by sequencing, indicated that this variant was not properly processed at the 3′ end The levels of the vU1 snRNAs, enriched in the Sm immunoprecipitates, relative to the U1 snRNA were estimated using a genomic DNA standard to normalize primers efficiency.

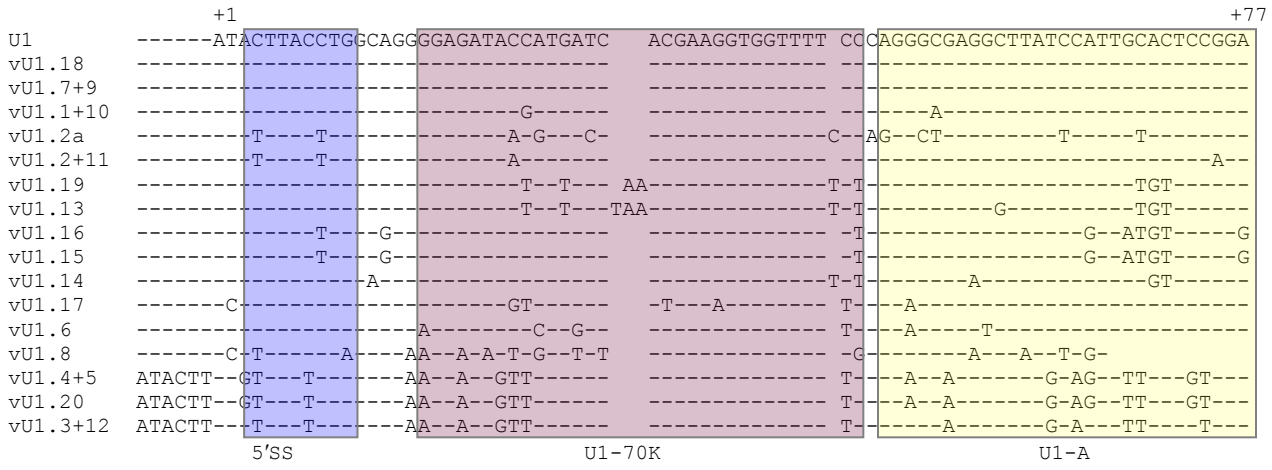
Figure 6 **Altering vU1.8 snRNA levels cause global transcriptome changes in HeLa cells**

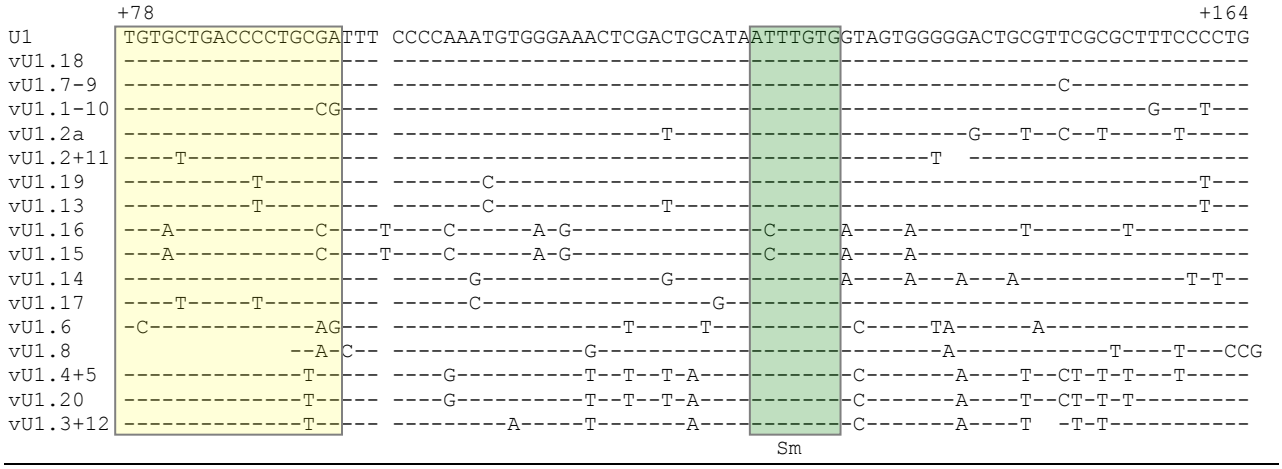
A) qRT-PCR analysis of vU1.8 and U1 snRNA levels in HeLa cell transfected with control or vU1.8 snRNA antisense oligonucleotides. **B)** Average profile of intronic probe signal difference between vU1.8 snRNA knockdown and Control cells across expressed non-overlapping genes, relative to the annotated transcription start sites. Each point represents average of more than 100 probes in the corresponding region. **C)** Validation of array results by qRT PCR, confirming the reduction in VAPA and PRPF4B pre-mRNA levels towards the 3′end. Gene structures are illustrated above the graphs, horizontal and vertical lines indicate introns and exons, respectively. Location of primers is denoted as horizontal lines below the schematics. Arrow indicates cryptic poly A sites within the first intron of VAPA and PRPF4B genes. Sizes of genes are indicated in kilobases. **D)** 3′RACE, using nested PCR, on total RNA from HeLa cells transfected with control, vU1.8 or U1 snRNA antisense oligonucleotides was performed on the endogenous PRPF4B, VAPA, and NR3C transcripts to detect polyadenylated mRNAs. PCPA denotes a pre-mature cleaved and polyadenylated mRNA product and * denotes mis-priming of the oligo dT primer at an A-rich region. Primers targeting the endogenous β-actin mRNA was used as a normalization control. Sequencing

results of the PCPA products for the PRPF4B and VAPA gene are shown on the right. The putative poly A sites are indicated with black bordered boxes.

A







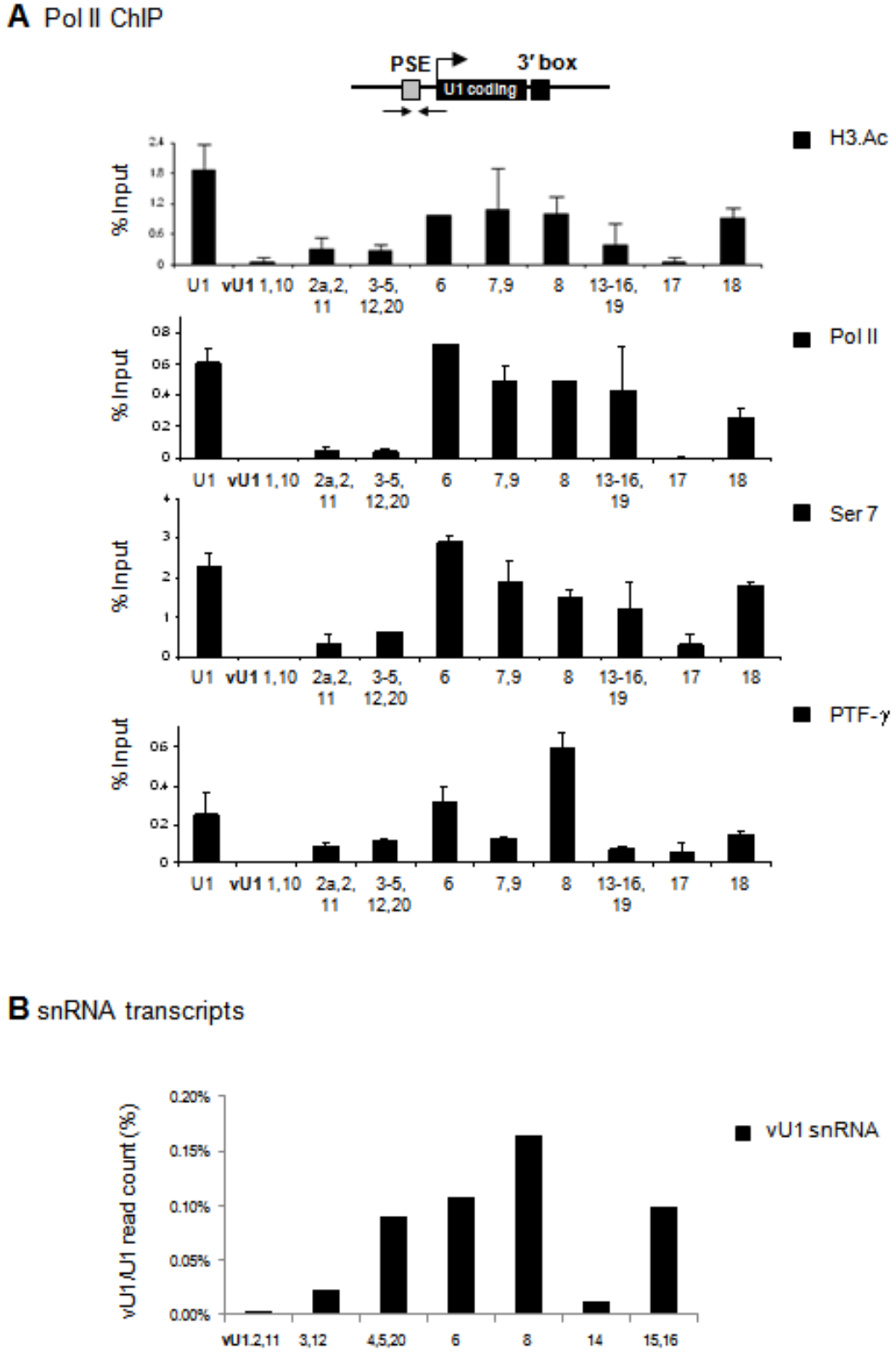


Figure 2

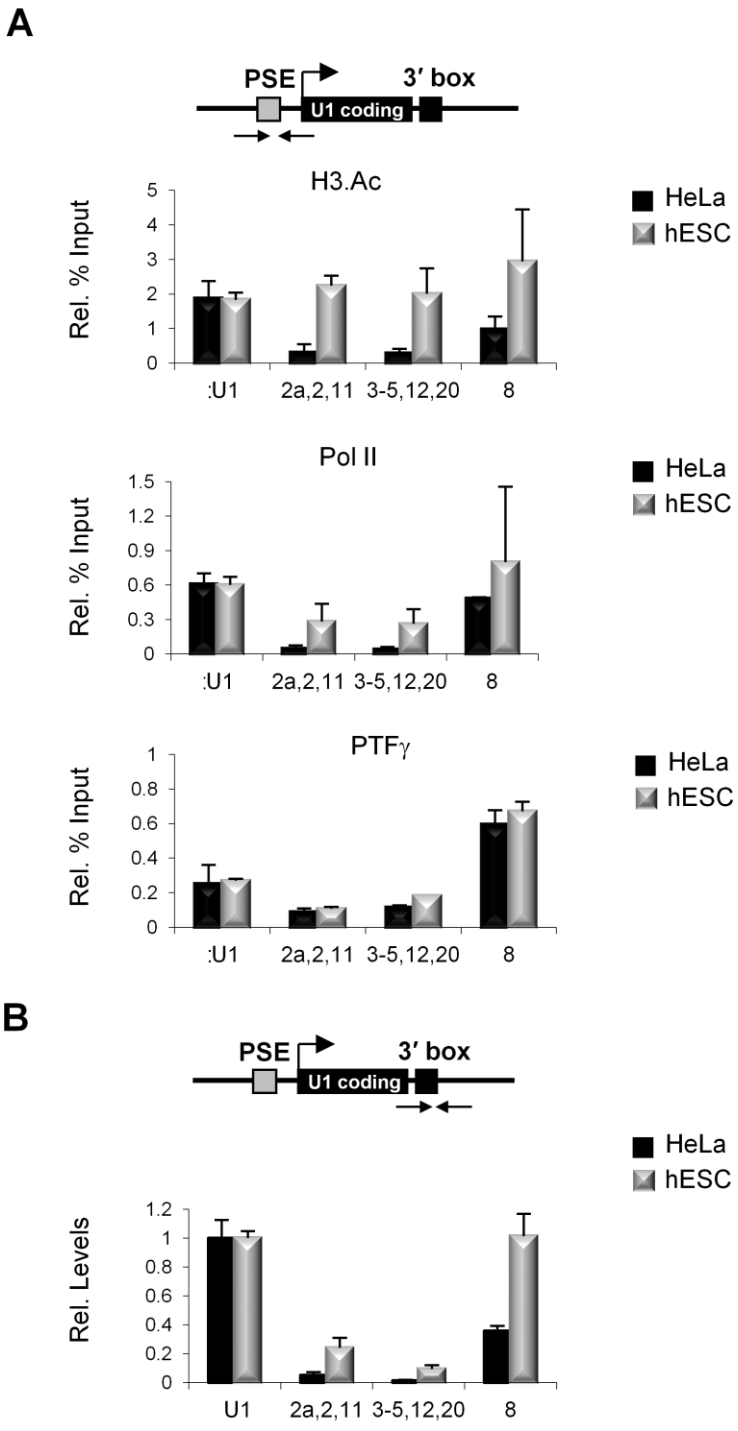
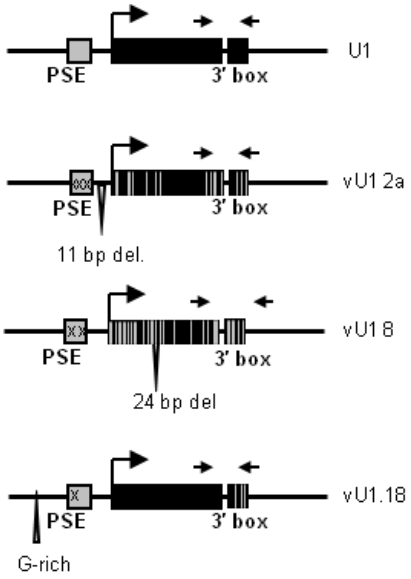


Figure 3



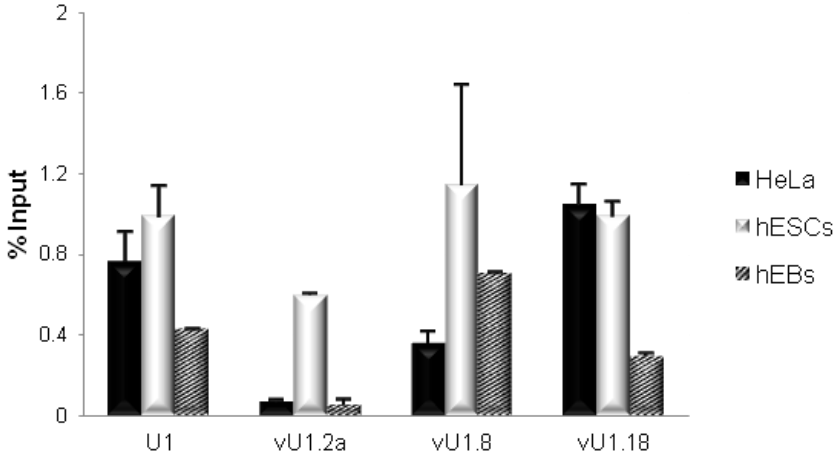


Figure 4

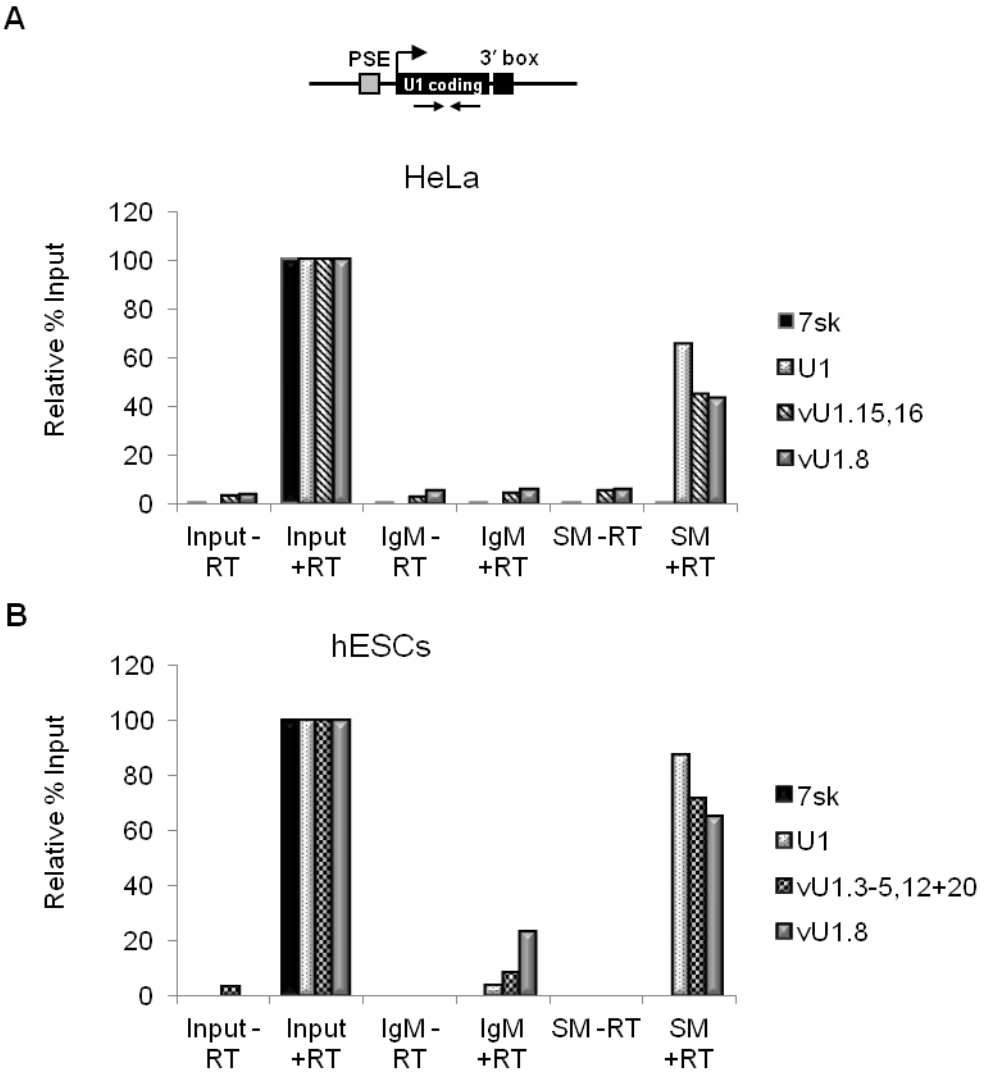


Figure 5

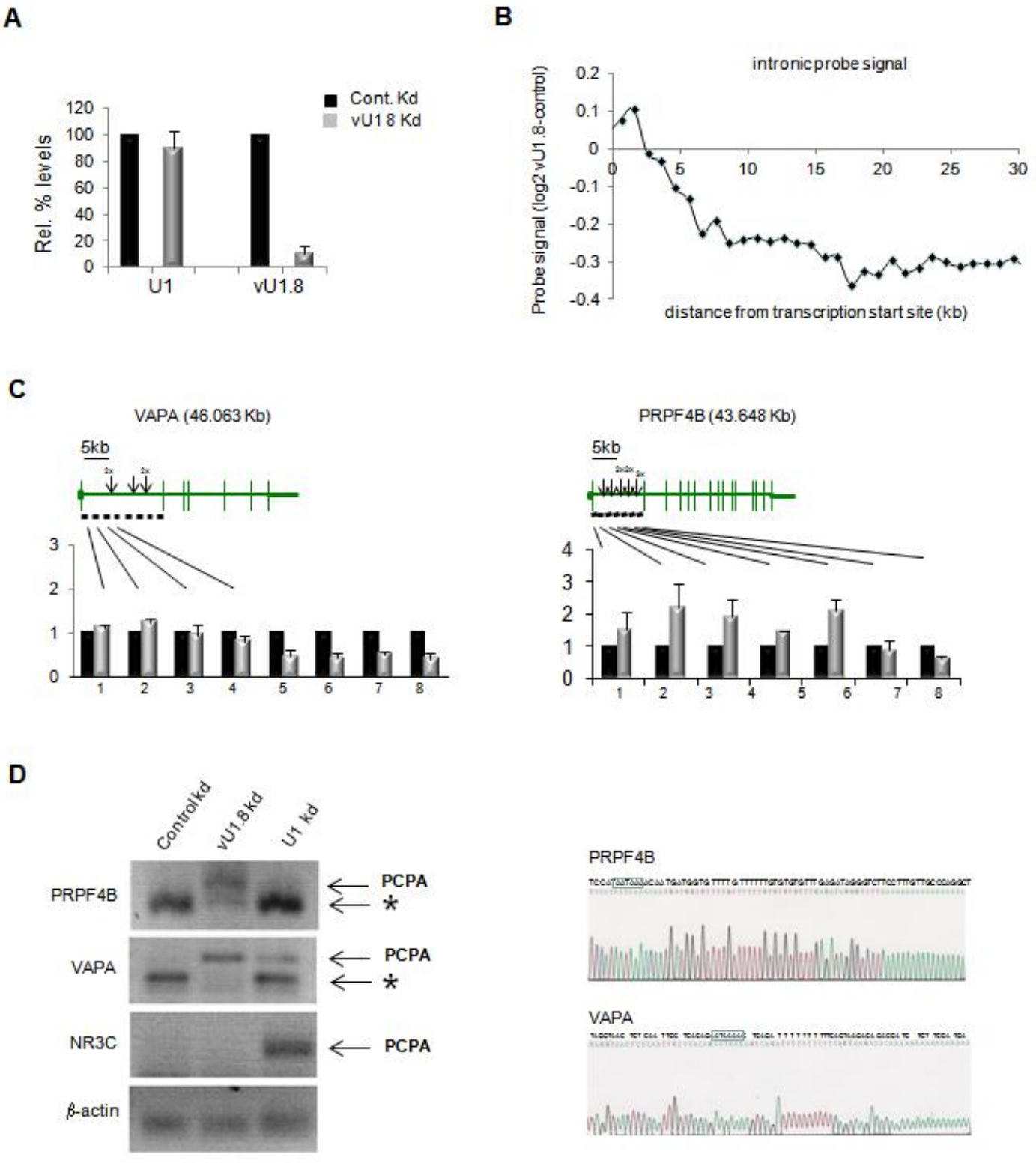


Figure 6

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