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A link between nuclear RNA surveillance, the human exosome and RNA polymerase II transcriptional termination

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ABSTRACT

In eukaryotes, the production of mature messenger RNA that exits the nucleus to be translated into protein in the cytoplasm requires precise and extensive modification of the nascent transcript. Any failure that compromises the integrity of an mRNA may cause its retention in the nucleus and trigger its degradation. Multiple studies indicate that mRNAs with processing defects accumulate in nuclear foci or 'dots' located near the site of transcription, but how exactly are defective RNAs recognized and tethered is still unknown. Here, we present evidence suggesting that unprocessed β-globin transcripts render RNA polymerase II (Pol II) incompetent for termination and that this quality control process requires the integrity of the nuclear exosome. Our results show that unprocessed pre-mRNAs remain tethered to the DNA template in association with Pol II, in an Rrp6-dependent manner. This reveals an unprecedented link between nuclear RNA surveillance, the exosome and Pol II transcriptional termination.

INTRODUCTION

In eukaryotes, the production of mature messenger RNA that exits the nucleus to be translated into protein in the cytoplasm requires precise and extensive modification of the nascent transcript (1). The initial primary transcripts synthesized by RNA polymerase II (pre-mRNAs) undergo several processing steps that include addition of a 7-methylguanosine cap at the 5′-end, splicing of introns, and 3′-end formation by cleavage and polyadenylation. During processing, pre-mRNA assembles together with RNA binding proteins into ribonucleoprotein particles [mRNPs; (2,3)]. Mature particles are exported to the cytoplasm and several lines of evidence indicate that mRNPs move from the sites of transcription to the nuclear pores by random Brownian motion (4–10). As diffusion cannot be regulated, traffic control of newly synthesized mRNA molecules is thought to rely on retention at dedicated sites within the nucleus (11). A failure that compromises the integrity of an mRNA may cause its retention in the nucleus and trigger its degradation. Such a surveillance mechanism operates in close proximity to the gene template and, at least in yeast, at the nuclear pore (12).

Multiple studies indicate that mRNAs with processing defects accumulate in nuclear foci or 'dots' located near the site of transcription, in mammalian cells (13) as well as yeast (14–17). In *Saccharomyces cerevisiae*, the nuclear exosome contributes to post-transcriptional tethering of faulty mRNAs within dots that are adjacent to the transcription site (15,18–20), but how exactly are defective RNAs recognized and tethered is still unknown.

The exosome is a multiprotein complex with ribonucleolytic activity that has a large number of substrates and participates in a wide range of cellular functions. In the cytoplasm, the exosome catalyzes mRNA turnover, nonsense-mediated mRNA decay and nonstop decay, whereas in the nucleus, the exosome is involved in the maturation of rRNA, snoRNA and snRNA, and in the degradation of incorrectly processed RNA precursors (21,22). More recent studies reveal additional roles of the exosome in gene silencing and in regulating the expression of non-coding RNAs (23). The human exosome consists of a nine-subunit core devoid of ribonuclease catalytic activity, and two associated hydrolytic RNases, Rrp44 (Dis3) and Rrp6 (PM/Scl-100). Although the structure and function of the exosome is widely conserved, Rrp44 copurifies with the rest of the core in *S. cerevisiae* and in *Drosophila melanogaster*, but not in *Homo sapiens* or *Trypanosoma brucei* (24). Furthermore, Rrp6 associates

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only with the nuclear exosome in yeast (25), whereas in human cells it is present both in the nucleus and in the cytoplasm (26). It remains unclear whether these differences reflect distinct functional properties of the exosome across species.

Here, we investigated in more detail and at higher resolution the relation between the exosome and unprocessed β-globin transcripts retained near the site of transcription in human cells. Our results show that deficiently processed pre-mRNAs remain tethered to the DNA template in association with RNA polymerase II (Pol II), in an Rrp6-dependent manner.

MATERIALS AND METHODS

Cell culture and transfections

The Flp-In™ T-REx™-293 cell line was purchased from Invitrogen Life Technologies, and grown as monolayer in Dulbecco's modified Eagle medium (DMEM) supplemented with 10% fetal bovine serum and 2 mM L-glutamin (all cell culture reagents were from Gibco). Isogenic, inducible stable cell lines were generated through Flp recombinase-mediated integration by cotransfecting the Flp-In™ T-REx™-293 host cell line harboring a single Flp recombination target site with a plasmid expressing the Flp recombinase (pOG44, Invitrogen) and pcDNA5/FRT/TO-βWT or pcDNA5/FRT/TO-βIVS1 constructs at a 9:1 ratio using FuGENE® 6 Transfection Reagent (Roche). Expression of βWT or βIVS1 was induced with 1 μg/ml tetracycline. The murine erythroleukemia (MEL) cell lines were previously described (13).

Gene constructs

pcDNA5/FRT/TO-βWT and pcDNA5/FRT/TO-βIVS1 were constructed by inserting a NcoI–Acc*65*I (blunted) fragment containing the human β-globin (*HBB*) gene (from ATG to ~1800 bp past the poly(A) site) into the KpnI (blunted) site of pcDNA5/FRT/TO (Invitrogen). The wild-type (βWT) and mutant (βIVS1) *HBB* genes were described previously (27,28).

RNA interference

To reduce the levels of exosome proteins by RNA interference (RNAi), we used siRNA duplexes and shRNA-expressing lentiviruses. For experiments using siRNA oligomers, both single and double transfections were performed. As unspecific siRNA control a sequence targeting the firefly luciferase gene (GL2) was used (29). Cells were plated 1 day before transfection such that they were 30–50% confluent at the time of transfection. The siRNA duplexes were transfected using Lipofectamine 2000 transfection reagent (Invitrogen) according to the manufacturer's protocol. Cells were either harvested 1 day after transfection, or re-transfected with the same siRNA duplex and harvested 2 days later. RNAi experiments using shRNA-expressing lentiviruses were performed as described (30) with the following modifications: cells were seeded at a density of 1.8×10^5 cells per well in a 24-well plate, infected with 20 μl of unconcentrated shRNA lentivirus supernatant from the 96-well plate viral production, incubated for 48 h before addition of 5 μg/ml puromycin for selection and harvested after 3–4 days of selection. The siRNA and shRNA sequences are listed in Supplementary Table S1.

RNA isolation and fractionation

Total cellular RNA was extracted using TRIzol® (Invitrogen). Nuclear and cytoplasmic RNA fractions were isolated as described (31). Nuclear RNA was further fractionated into chromatin-associated and nucleoplasmic fractions as described (32–35).

RT–PCR

cDNA was made using Superscript II Reverse Transcriptase (Invitrogen) according to the manufacturer's instructions. PCR products were separated by gel electrophoresis, detected by ethidium bromide, scanned with Typhoon (GE Healthcare) and analyzed with the ImageQuant software (Molecular Dynamics, Sunnyvale, CA, USA). Real-time quantitative PCR analysis was performed in the 7500 Real-Time PCR System (Applied Biosystems, Foster City, CA, USA), using SYBR Green PCR master mix (Applied Biosystems), as previously described (36). Gene-specific primer pairs are presented in Supplementary Table S2.

In situ hybridization and immunofluorescence analysis

Cells were fixed in 3.7% paraformaldehyde in PBS for 10 min and permeabilized with 0.5% Triton X-100/2 mM VRC in PBS. Fluorescence *in situ* hybridization (FISH) was carried out as described (13). The probe used for FISH was complementary to the 5′-region of the *HBB* gene up to the BamHI site in exon II. The probe was labelled either by nick-translation with digoxigenin-11-dUTP (Roche) or by *in vitro* transcription with digoxigenin-11-UTP (Roche). Digoxigenin was detected using a Cy3-conjugated mouse anti-digoxin antibody (1:200, Jackson ImmunoResearch Labs, Inc.). Simultaneous detection of RNA and protein was as previously described (37) with the exception that immunofluorescence preceded FISH. Images were acquired on Zeiss LSM 510 META confocal microscope using the PlanApochromat 63×/1.4 objective. AlexaFluor488 fluorescence was detected using the 488 nm line of the argon ion laser. Cy3 was excited with the DPSS 561-10 laser. Line profiles were obtained from unprocessed images using the LSM 510 software.

Western blotting

Whole-cell lysates were prepared, resolved and transferred as described (38). Incubations with primary antibodies were followed by incubations with the appropriate secondary antibodies (BioRad) and by detection using enhanced luminescence (Amersham).

Chromatin immunoprecipitation

ChIP was performed according to Listerman *et al.* (39) with some modifications. Cell extracts were sonicated with a Sanyo Soniprep 150 at an amplitude of 10 microns with six 20 s bursts, resulting in 200–400 bp chromatin fragments. The DNA fragments crosslinked to proteins were enriched by immunoprecipitation with rabbit polyclonal antibodies (anti-RNA Pol II, N20; anti-CBP80; anti-EXOSC10) and protein A sepharose beads (Sigma). Control (mock) immunoprecipitations were performed with anti-HA antibody. DNA from immunoprecipitated and input samples was extracted with UltraPure Phenol:Chloroform:Isoamyl Alcohol 25:24:1 (Invitrogen) and analyzed by quantitative real-time PCR, with the input consisting of a chromatin amount equivalent to that used for immunoprecipitation. The total number of cells was kept constant between experimental batches in order to yield similar amount of input chromatin and therefore avoid variability due to changes in precipitation efficiency (40). Primers used for PCR are listed in Supplementary Table S2. The relative occupancy of the immunoprecipitated protein at each DNA site was estimated as follows: $2^{\wedge}(C_{t\text{mock}} - C_{t\text{specific}})$, where $C_{t\text{mock}}$ and $C_{t\text{specific}}$ are mean threshold cycles of PCR done in triplicate on DNA samples from mock and specific immunoprecipitations (41).

Antibodies

The following primary antibodies were used: rabbit polyclonal antibodies anti-RNA Pol II, N20 (Santa Cruz Biotechnology); anti-CBP80 (kindly provided by Dr Elisa Izaurralde, Max Planck Institute for Developmental Biology, Tübingen, Germany); anti-PM/Scl-100, anti-hRrp40, anti-hRrp41, anti-hRrp46 (kindly provided by Dr Ger Pruijn, University of Nijmegen, The Netherlands); anti-EXOSC10 (ab50558; Abcam); anti-HA (Y-11; from Santa Cruz Biotechnology); and mouse monoclonal anti β-actin (Sigma).

RESULTS

Human exosome subunit Rrp6 is required for retention of unprocessed β-globin RNA at the transcription site

To investigate the molecular mechanisms involved in quality control of deficiently processed pre-mRNA, we used the well characterized human β-globin (*HBB*) gene as a model system. We engineered 293 human embryonic kidney cell lines to stably express a single tetracycline-inducible β-globin transgene integrated in the genome through site-specific DNA recombination. The β-globin transgene was either wild-type (βWT) or a mutant variant (βIVS1) that lacks completely the second intron (Figure 1A). We have previously shown that the βIVS1 mutation abolishes splicing of the first intron, reduces efficiency of 3′-end cleavage and causes retention of the RNA at the transcription site (13,37). Here, we were interested in determining whether the exosome subunit Rrp6 is involved in preventing release of these aberrantly processed transcripts from the site of transcription. We first examined the distribution of βWT and βIVS1 RNA by FISH. Tetracycline induces expression of the transgene and nascent transcripts are detected as a focus in the nucleus (Figure 1B), as previously described in stably transfected MEL cells (13,37). The wild-type mRNA is exported from the nucleus and progressively accumulates in the cytoplasm (Figure 1B). In contrast, the mutant RNA is not detectable by FISH in the cytoplasm (Figure 1B). Within 2 h after removal of tetracycline, nuclear foci of wild-type β-globin RNA are no longer detected in most cells, consistent with transcriptional shutdown of the transgene. We have previously shown that treating MEL cells with the transcription inhibitor actinomycin D for a short period of time (5–15 min) causes a dramatic, rapid reduction in the relative number of cells that contain a detectable focus of βWT transcripts within the nucleus, whereas the percentage of cells harboring βIVS1 RNA foci remained largely unaltered, suggesting that these mutant RNAs were not being released from the site of transcription (13). Here, we observe a similar result by specifically inhibiting transcription of the transgene by removal of tetracycline (Figure 1C).

To study the role of Rrp6, we reduced the levels of the protein by RNA interference (RNAi). To find the most effective conditions, a number of small interfering RNA (siRNA) duplexes were synthesized, and both single and double transfections were performed. Two different synthetic siRNAs resulted in similar phenotypes to those described below. As unspecific siRNA control a sequence targeting the firefly luciferase gene (GL2) was used (29). Western blot analysis shows that after 3 days of siRNA treatment, Rrp6 levels are reduced to 20% (Figure 1D). The relative number of cells that contain a detectable nuclear focus of βWT transcripts is similar in control and Rrp6 knockdown cells, following both tetracycline induction and tetracycline removal. In marked contrast, the percentage of cells with βIVS1 RNA foci is drastically reduced in the absence of Rrp6 (Figure 1E).

Next, we used a biochemical approach to obtain quantitative information on nascent β-globin transcripts that are associated with chromatin and transcripts that have been released into the nucleoplasm. Briefly, cells are lysed and nuclei separated from the cytoplasmic fraction. After treatment of nuclei with urea and a mild detergent, centrifugation pellets RNAs associated with Pol II and chromatin, whereas RNAs found in the supernatant are considered to have been released from the DNA template into the nucleoplasmic fraction (32–35). In agreement with previous studies (35), immunoblot analysis revealed that tubulin was exclusively detected in the cytoplasmic fraction, histone H3 was only detected in the chromatin fraction, RNA Pol II was predominantly detected in the chromatin fraction and the spliceosomal protein U2AF65 was detected in both nucleoplasmic and chromatin fractions (Supplementary Figure S1). Equal amounts of RNA were taken from cytoplasmic, chromatin-associated and nucleoplasmic fractions, then reverse transcribed with random primers and PCR amplified using primer pairs that detect total, spliced and unspliced β-globin RNA, as indicated in Figure 2. The abundance of

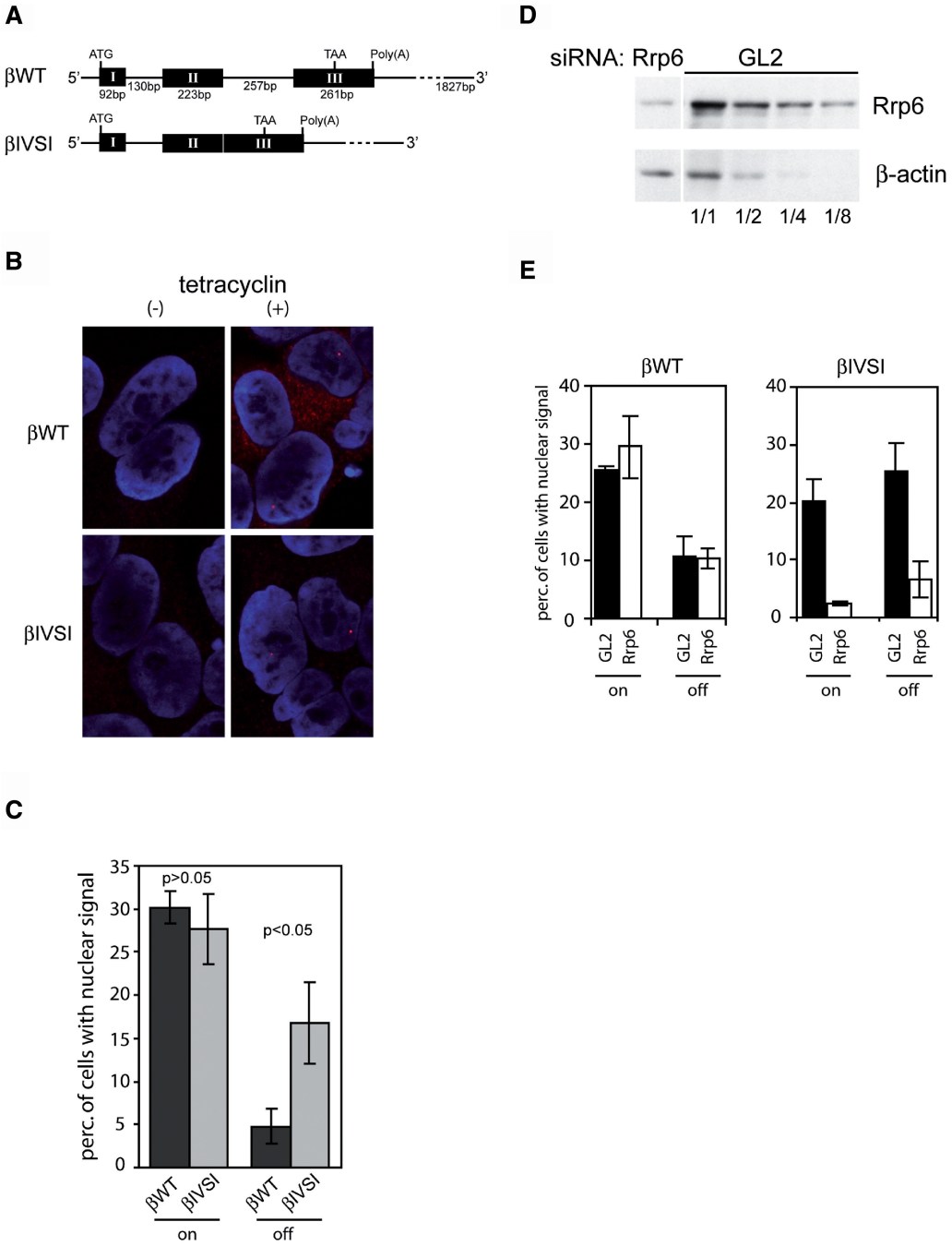

Figure 1. Human exosome subunit Rrp6 is required for retention of mutant β-globin RNA at the transcription site. (**A**) Schematic representation of wild-type (βWT) and mutant (βIVS1) human β-globin constructs. Exons are numerated (I, II and III). (**B**) RNA transcribed from βWT and βIVS1 transgenes was visualized by FISH, before (–) and after (+) tetracycline induction. Cells were double-labeled for β-globin RNA (red staining) and total DNA (blue staining). After tetracycline induction, nascent transcripts are detected as a focus in the nucleus. The wild-type mRNA is exported from the nucleus and accumulates in the cytoplasm, whereas the mutant RNA is not detectable in the cytoplasm. (**C**) The proportion of cells with a nuclear RNA focus was estimated in three independent experiments. Cells were treated with tetracycline for 8 h (transcription on). Tetracycline was then removed and cells analyzed 2 h later (transcription off). The mean proportion of cells with a nuclear RNA focus is plotted (error bars represent standard deviation). For each experimental condition, 10 microscopic fields were counted, with a total of 500–700 cells. After transcriptional shutoff, very few cells contain a focus of wild-type transcripts; in contrast, foci of mutant transcripts are still visible in a large proportion of cells. (**D**) Western blot analysis of Rrp6 and β-actin proteins in whole lysates from cells treated with siRNA against Rrp6 or negative control (GL2). The first two lanes contain equivalent amount of total cell protein, as shown by similar levels of β-actin. The subsequent lanes contain progressive 2-fold dilutions of protein from control cells to allow estimation of degree of knockdown. (**E**) Proportion of cells with a nuclear RNA focus in control and Rrp6-depleted cells. Cells were treated with siRNAs against Rrp6 and control siRNA (GL2). After tetracycline induction for 8 h (transcription on), tetracycline was removed and cells analyzed 2 h later (transcription off). The histogram depicts mean and standard deviation for three independent experiments. Total of 500–700 cells counted for each experimental condition. In the absence of Rrp6, there is a drastic reduction in the relative number of cells with a focus of mutant transcripts.

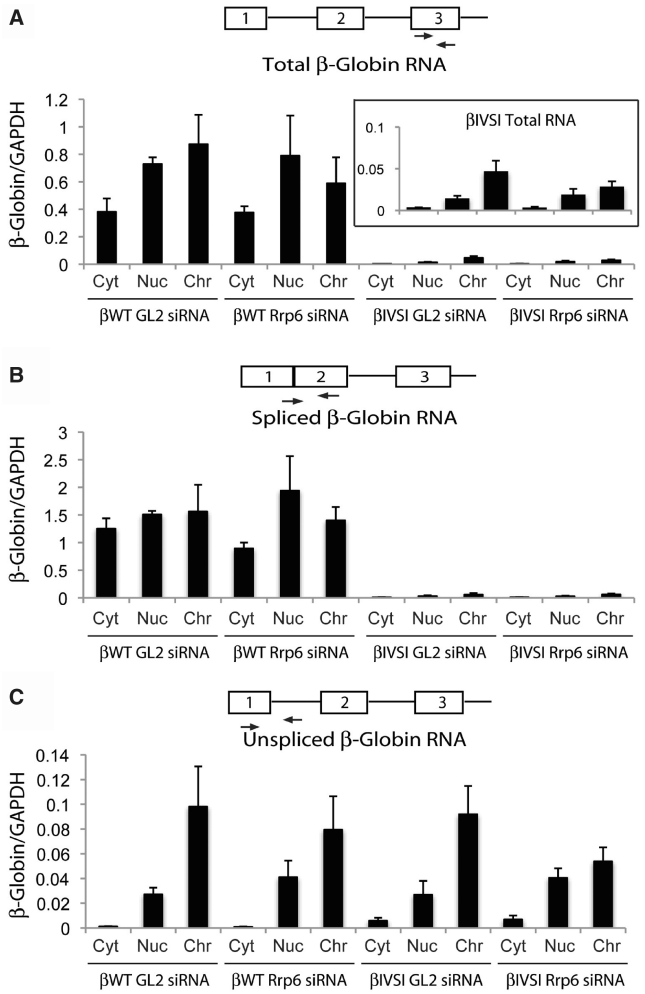

Figure 2. Rrp6 is required for retention of unspliced RNA in association with chromatin. RNA transcribed from βWT and βIVS1 transgenes was isolated from cytoplasmic (Cyt), nucleoplasmic (Nuc) and chromatin-associated (Chr) fractions of control (GL2 siRNA) and Rrp6 depleted cells (Rrp6 siRNA). The RNA was reverse transcribed with random primers and PCR amplified using primer pairs that detect total (**A**), spliced (**B**) and unspliced (**C**) β-globin RNA as illustrated in the diagrams. The amount of PCR product in each fraction was estimated by quantitative real-time PCR and normalized to the level of GAPDH RNA detected in the same fraction. The histograms depict mean and standard deviation for three independent experiments.

PCR product in each fraction is normalized to the level of GAPDH RNA detected in the same fraction. Total and spliced βWT RNA is detected in cytoplasmic, nucleoplasmic and chromatin-associated fractions (Figure 2A and B), consistent with our *in situ* results indicating that these transcripts are efficiently exported to the cytoplasm (Figure 1B). In contrast, the βIVS1 construct failed to express significant amounts of RNA, as previously reported (27). Moreover, we do not detect spliced βIVS1 RNA in any of the fractions (Figure 2B), consistent with our previous observation that these transcripts fail to be spliced (37). As expected, no significant amount of unspliced RNA is present in the cytoplasm (Figure 2C). In the nucleus, unspliced RNA appears enriched in the chromatin-associated fraction, consistent with the view that most wild-type β-globin introns are removed co-transcriptionally (13). Similar results are obtained for βWT transcripts in GL2 and Rrp6 knockdown cells, indicating that Rrp6 is not required for splicing and export of these RNAs. In control cells, unspliced mutant RNA is also predominantly found in the chromatin fraction (Figure 2C). However, in Rrp6 knockdown cells βIVS1 transcripts become equally abundant in chromatin-associated and nucleoplasmic fractions (Figure 2A and C). These results strongly suggest that Rrp6 is required for retention of unspliced mutant RNA in association with chromatin, but loss of Rrp6 is not sufficient to trigger export of unspliced RNA to the cytoplasm.

Deficiently processed β-globin RNA is stalled in association with Pol II on the gene template

Studies in *S. cerevisiae* suggest that errors in the mRNP biogenesis pathway lead to post-transcriptional retention of RNAs within 'dots' that are adjacent to but distinct from their transcription site (18,19). However, in mouse cells we detect defectively processed β-globin RNAs accumulated in discrete foci that are indistinguishable from the transcription sites (13). To investigate whether retention of unprocessed β-globin RNA occurs co-transcriptionally, we carried out chromatin immunoprecipitation (ChIP) with antibodies specific for the CBP80 subunit of the cap-binding complex (CBC). Because CBC binds the 5′-end of Pol II transcripts and newly synthesized RNAs lie adjacent to the gene template, formaldehyde-induced cross-links are able to capture nascent RNA–protein complexes (39). Cellular DNA was sonicated to 200- to 400-bp fragments, and immunoprecipitated fragments were analyzed by quantitative real-time PCR using primer pairs across the promoter (1), the open reading frame (2,3) and within the 3′-flanking region (4,5) of the β-globin gene (Figure 3A). In order to measure the relative occupancy of a protein of interest at distinct target genomic sequences, quantitative PCR data can be expressed as percent precipitated DNA relative to input. However, using this method alone a differential enrichment of core histones at specific genomic sites was observed in control experiments lacking antibody or using antibodies directed against non-histone proteins (42). This unexpected effect may be caused by the fact that different relative levels of DNA from distinct genomic loci are recovered during the extraction procedure (43). Thus, it is important to normalize ChIP data relative to control immunoprecipitations (41,42). Distributions of ChIP signals expressed as percent precipitated DNA relative to input with anti-CBP80 and control antibodies are depicted in Figure 3B. These results show a significantly higher ChIP signal for CBP80 compared to control antibody. Furthermore, there is no differential enrichment of ChIP signals along the β-globin gene in control immunoprecipitations. Distributions of CBP80 relative occupancy following normalization to control immunoprecipitations are depicted in Figure 3C. When compared to uninduced cells, a 2-fold increase in CBP80 occupancy is detected across

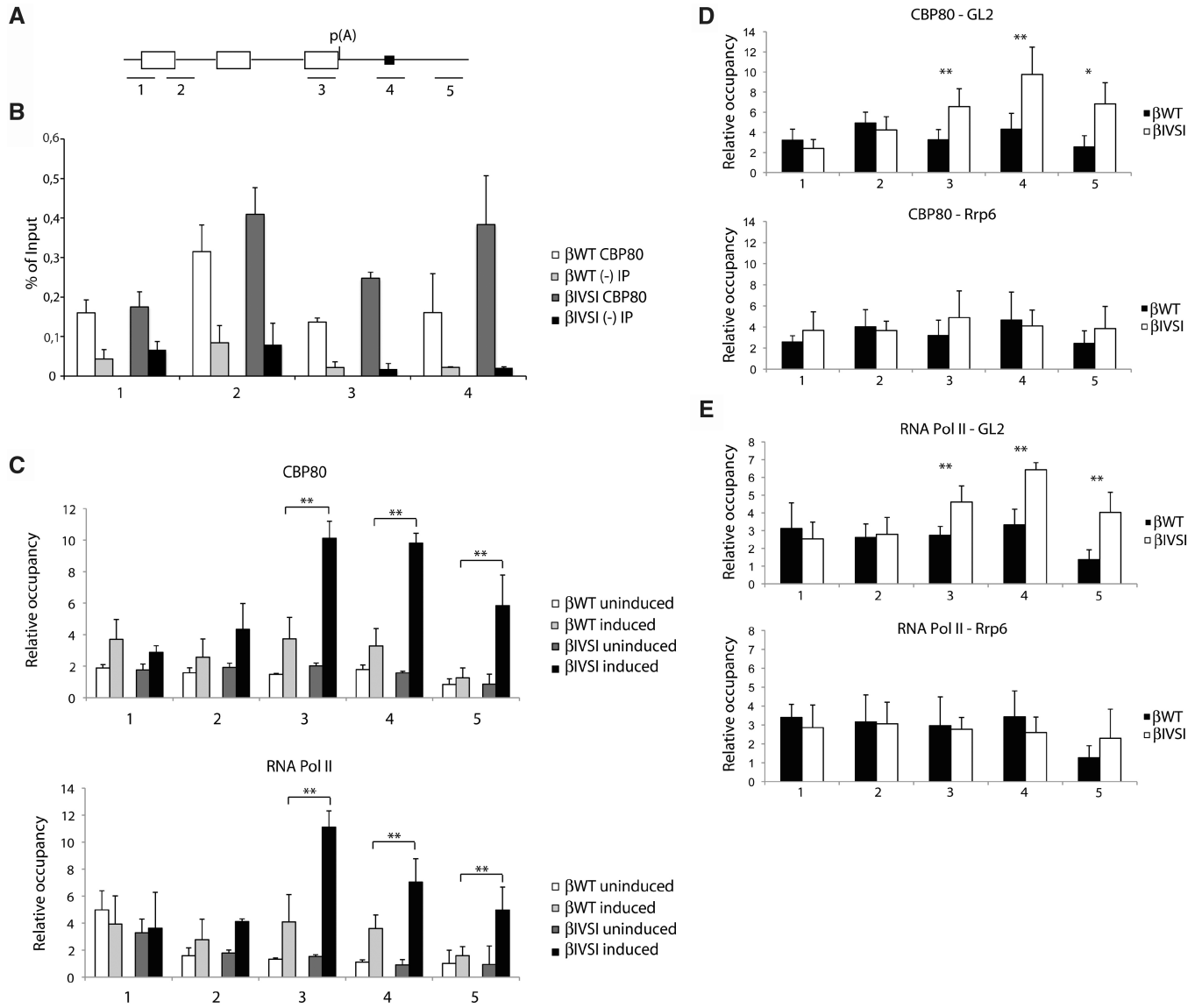

Figure 3. Rrp6-dependent stalling of mutant β-globin RNA on the gene template in association with Pol II. (**A**) Schematic diagram indicating the gene regions amplified by primer sets. The black box represents the CoTC sequence**.** (**B**) Aliquots of sheared chromatin from the same number of cells were used for ChIP with anti-CBP80 and control (anti-HA) antibodies (-IP). Data are expressed as percent precipitation relative to input chromatin [calculated as (amount of ChIP DNA)/ (amount of input DNA) ×100]. (**C**) ChIP was performed in cells expressing βWT and βIVS1 transgenes, using anti-CBP80 and anti-Pol II antibodies. Transcription was induced with tetracycline. Results are expressed as 'relative occupancy', calculated as enrichment from specific immunoprecipitations normalized to enrichment from control immunoprecipitations. (**D** and **E**) Relative occupancy of CBP80 and Pol II in control and Rrp6-depleted cells after induction with tetracycline. All histograms depict mean and standard deviation for at least three independent experiments. The asterisks denote statistically significant differences: $*P < 0.05$; $**P < 0.01$.

the β-globin gene after induction with tetracycline (Figure 3C), consistent with previously reported data for other mammalian genes (39). However, we note that while in the wild-type gene (βWT) all primers show signals of similar intensity, in the mutant gene (βIVS1) the primers positioned towards the 3′-end give much higher signal (Figure 3C). We also performed ChIP with polyclonal antibodies that recognize the N-terminal region of the large subunit of Pol II (N20). These antibodies bind Pol II in a phosphorylation-independent manner, i.e. they recognize Pol II with either the hyperphosphorylated or the hypophosphorylated CTD. Before transcriptional activation, we detect a significant amount of Pol II loaded in the promoter-proximal region (Figure 3C), as previously reported for other mammalian genes (39,44). Upon transcriptional induction with tetracycline, a significantly increased signal of Pol II is detected in downstream regions of both βWT and βIVS1 genes (Figure 3C). Along the βWT gene, Pol II signal remained high past the poly(A) site, but then decreased after the CoTC sequence consistent with the view that this element is essential for transcriptional termination of the human β-globin gene (45,46). A significantly higher Pol II occupancy towards the 3′-end is observed in βIVS1 compared

to βWT, including the 3′-flanking region past the CoTC sequence (Figure 3C). Taking into consideration that the ChIP analysis provides information about CBP80 and Pol II density across the two genes, and assuming that the association of capped nascent RNA with chromatin is mediated by the association of transcribing Pol II with DNA, these data suggest that polymerase release downstream of the CoTC is inefficient in the βIVS1 mutant gene.

Rrp6 regulates termination of unprocessed RNA

Because studies in *S. cerevisiae* indicate that Rrp6 regulates post-transcriptional RNA tethering (20), we asked whether Rrp6 plays any role in co-transcriptional RNA retention. In the wild-type gene, knockdown of Rrp6 has no apparent effects on the distribution of CBP80 and Pol II (Figure 3D and E). However, we observe significantly reduced occupancy with antibodies against CBP80 (Figure 3D) and Pol II (Figure 3E) at the 3′-end of the βIVS1 gene in Rrp6-depleted cells compared to the negative control knockdown. A similar result is observed when we examine the distribution of CBP80 and Pol II expressed as percent precipitated DNA relative to input (Supplementary Figure S2). Taken together with the results depicted in Figure 2C, these data indicate that in the absence of Rrp6 both Pol II and unspliced βIVS1 transcripts are released from the DNA template. As βIVS1 transcripts were previously found to be deficiently cleaved at the 3′-end (37), we asked whether down-regulation of Rrp6 causes release of uncleaved RNA. We used a primer downstream from the cleavage site to examine the amount of β-globin RNA that is uncleaved at the poly(A) site in the cytoplasmic, nucleoplasmic and chromatin fractions (Figure 4).

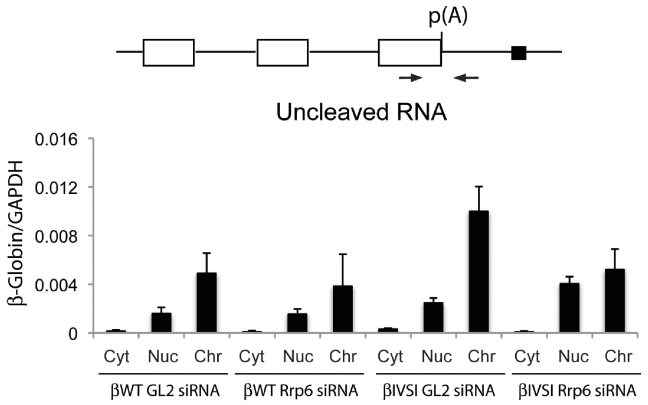

Figure 4. Rrp6 is required for retention of uncleaved RNA in association with chromatin. RNA transcribed from βWT and βIVS1 transgenes was isolated from cytoplasmic (Cyt), nucleoplasmic (Nuc) and chromatin-associated (Chr) fractions of control (GL2 siRNA) and Rrp6 depleted cells (Rrp6 siRNA). The RNA was reverse transcribed with random primers and PCR amplified using a pair of primers that detects uncleaved β-globin RNA, as illustrated in the diagram (the black box represents the CoTC sequence). The amount of PCR product in each fraction was estimated by quantitative real-time PCR and normalized to the level of GAPDH RNA detected in the same fraction. The histogram depicts mean and standard deviation for three independent experiments.

As expected, both βWT and βIVS1 RNAs that are uncleaved at the poly(A) site are enriched in the chromatin fraction. However, following Rrp6 knockdown the levels of uncleaved βIVS1 RNAs become essentially the same in chromatin and nucleoplasmic fractions (Figure 4). Therefore, we conclude that Rrp6 knockdown promotes release of uncleaved β-globin transcripts from the chromatin template.

Unprocessed transcripts are slowly degraded in the nucleus

Because the exosome participates in selective elimination of RNA molecules that are not properly processed (47), we asked whether the turnover of unprocessed mutant RNA is faster compared to wild-type RNA. Tetracycline was added to the culture medium to induce transcription of the transgenes, and removed after 3.5 h. The levels of unspliced and spliced RNA were measured at 1, 2 and 4 h after tetracycline removal. As shown in Figure 5A, tetracycline induction results in accumulation of similar levels of unspliced RNA from wild-type and mutant β-globin transgenes. The levels of unspliced RNAs persist for ~1 h after transcriptional shutoff, and decrease progressively thereafter. At 4 h after transcriptional shutoff, the levels of unspliced transcripts is similar to that detected in uninduced cells. In parallel, tetracycline induces a significant increase of spliced βWT mRNA, while the levels of βIVS1 mRNA do not change relative to uninduced cells (Figure 5B). These results show that unspliced βIVS1 RNA takes longer than βWT RNA to return to uninduced levels, suggesting that the kinetics of degradation of unprocessed pre-mRNA is slower than the normal processing of pre-mRNA into mRNA. Degradation of the unprocessed RNA is slower, perhaps because it requires dissociation of the stalled transcript from Pol II in order to provide entry sites for exonucleases. Indeed, while unspliced and uncleaved pre-mRNAs are stalled in association with Pol II, they provide no entry sites for exonucleases. The mutant transcripts that are not cleaved at the poly(A) site do not have a poly(A) tail covered by poly(A)-binding proteins. Therefore, once they dissociate from Pol II these transcripts will expose unprotected 3′-ends and will be most probably rapidly degraded. Thus, the data suggest that defects in pre-mRNA processing delay but do not irreversibly block Pol II transcription termination. In conclusion, the results show that unprocessed β-globin RNA is recognized by the exosome and retained at the transcription site. However, the subsequent degradation of unprocessed transcripts is a relatively slow process that takes longer than the normal turnover of wild-type RNA.

Rrp6 and core exosome subunits are recruited to β-globin transcription sites

A direct role of the exosome in co-transcriptional RNA surveillance is supported by studies in *Drosophila* showing that exosome subunits are recruited to transcriptionally active genes (48). To examine recruitment of the exosome to β-globin transcription sites, we combined FISH with immunofluorescence using antibodies against Rrp6 and the core subunits (Figure 6A and B). We detect

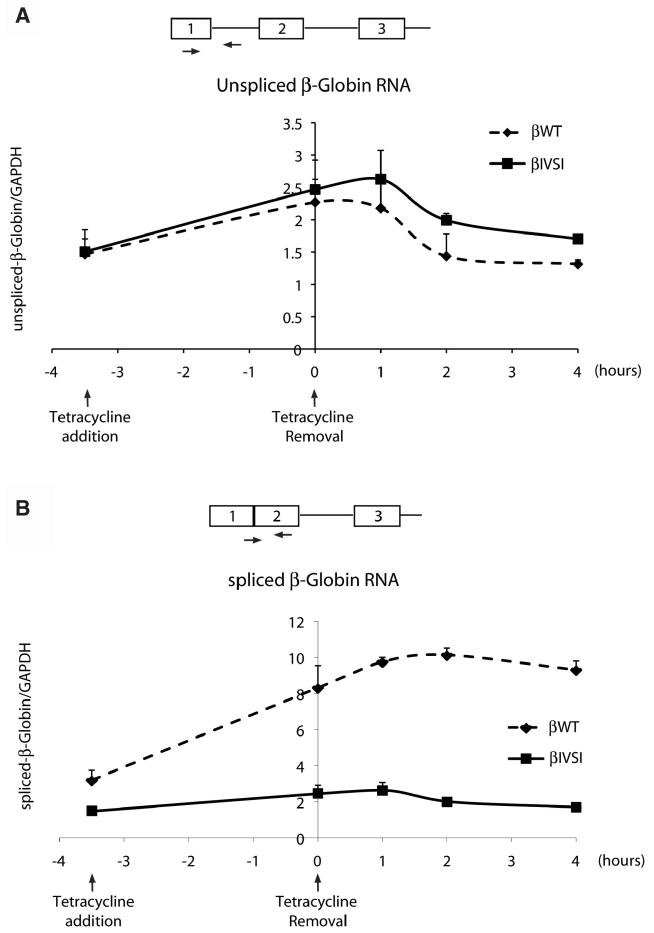

Figure 5. Turnover rates of wild-type and mutant transcripts. Time-course analysis of βWT and βIVS1 RNA by qRT–PCR. The upper diagrams show the primers used to distinguish unspliced (**A**) and spliced (**B**) transcripts. Tetracycline was added to the culture medium to induce transcription for 3.5 h and then removed, as indicated. Error bars show standard deviation from three independent experiments.

all proteins distributed throughout the nucleoplasm, with high concentration at the site of β-globin transgene transcription. Similar results are observed in cells that express the wild-type or mutant transgene. Because recent reports suggest that Rrp6 can act independently of the exosome core (49,50), we performed RNAi of Rrp6 and the core component Rrp46. As shown by western blot analysis, knockdown of Rrp6 results in depletion of this exosome subunit only, with no effect on the amount of Rrp46 in the cell (Figure 6C). In contrast, knockdown of Rrp46 also reduces the levels of Rrp6 (Figure 6C). A reduction of Rrp6 upon knockdown of the core components Rrp40 and Rrp41 was also recently reported (51). The stability of a component of multiprotein complexes often depends on the presence of the other components, so that when one protein is depleted, the others become unstable and show reduced cellular levels. We therefore infer that the majority of Rrp6 in the cell is in a complex with the core exosome.

We next examined the association of Rrp6 and β-globin transgenes at higher resolution using ChIP. As observed previously in *Drosophila* heat-shock genes (48), we detect Rrp6 distributed along the wild-type and mutant β-globin transgenes (Figure 6D). However, quantitative analysis shows a higher recruitment of Rrp6 to the promoter compared to the body of βWT and βIVS1 genes. Most important, similar levels of Rrp6 are recruited to the 3′-end of both βWT and βIVS1 genes, in marked contrast to the increased density of Pol II and CBP80 observed on the mutant gene. Thus, although Rrp6 most likely associates with the Pol II elongating complex, the protein does not seem to remain stably bound to stalled Pol II molecules on the mutant gene. Yet, in the absence of Rrp6 both Pol II and unprocessed transcripts are released from the DNA template (Figure 3C and D). Possibly, Rrp6 participates in the exchange of factors that associate with the elongation complex as transcription proceeds through the poly(A) site and that contribute to render Pol II termination competent.

DISCUSSION

Since our original observation that incorrectly processed β-globin mRNA precursors persist accumulated in close proximity to the gene template after inhibition of transcriptional activity (13), it has been unclear how are these RNAs tethered into discrete foci or dots within the nucleus. We now present evidence suggesting that unprocessed β-globin transcripts render Pol II incompetent for termination and that this quality control process requires the integrity of the nuclear exosome. Using ChIP experiments to measure the density of polymerases and nascent transcripts throughout the β-globin gene, we detect termination defects in a variant form of the gene harboring a deletion mutation that reduces the efficiency of pre-mRNA splicing and 3′-end cleavage. We also show that unprocessed pre-mRNAs remain tethered to the gene in association with Pol II by a mechanism that involves Rrp6.

Transcriptional termination of Pol II is the ultimate step in the transcription cycle, during which the polymerase dissociates from the DNA template and releases the RNA transcript. In mammals, termination by Pol II can occur from a few base pairs to several kilobases downstream from the cleavage and poly(A) site. Two alternative models have been proposed >20 years ago to explain this process, but the current view is that most likely both models contribute to the termination mechanism (52). According to the allosteric or anti-terminator model, transcription through the poly(A) site leads to conformational changes of the elongation complex whereas the torpedo model postulates that cleavage creates an entry site for an exonuclease at the uncapped 5′-monophosphate that will degrade the RNA and somehow induce dissociation of Pol II. Termination is intimately coupled to formation of the 3′-end of a mature mRNA, which occurs in a two-step reaction involving endonucleolytic cleavage at the poly(A) site and subsequent addition of adenine residues to form a poly(A) tail. The 3′-end processing machinery results from assembly of two multimeric complexes, the cleavage and polyadenylation specificity factor (CPSF)

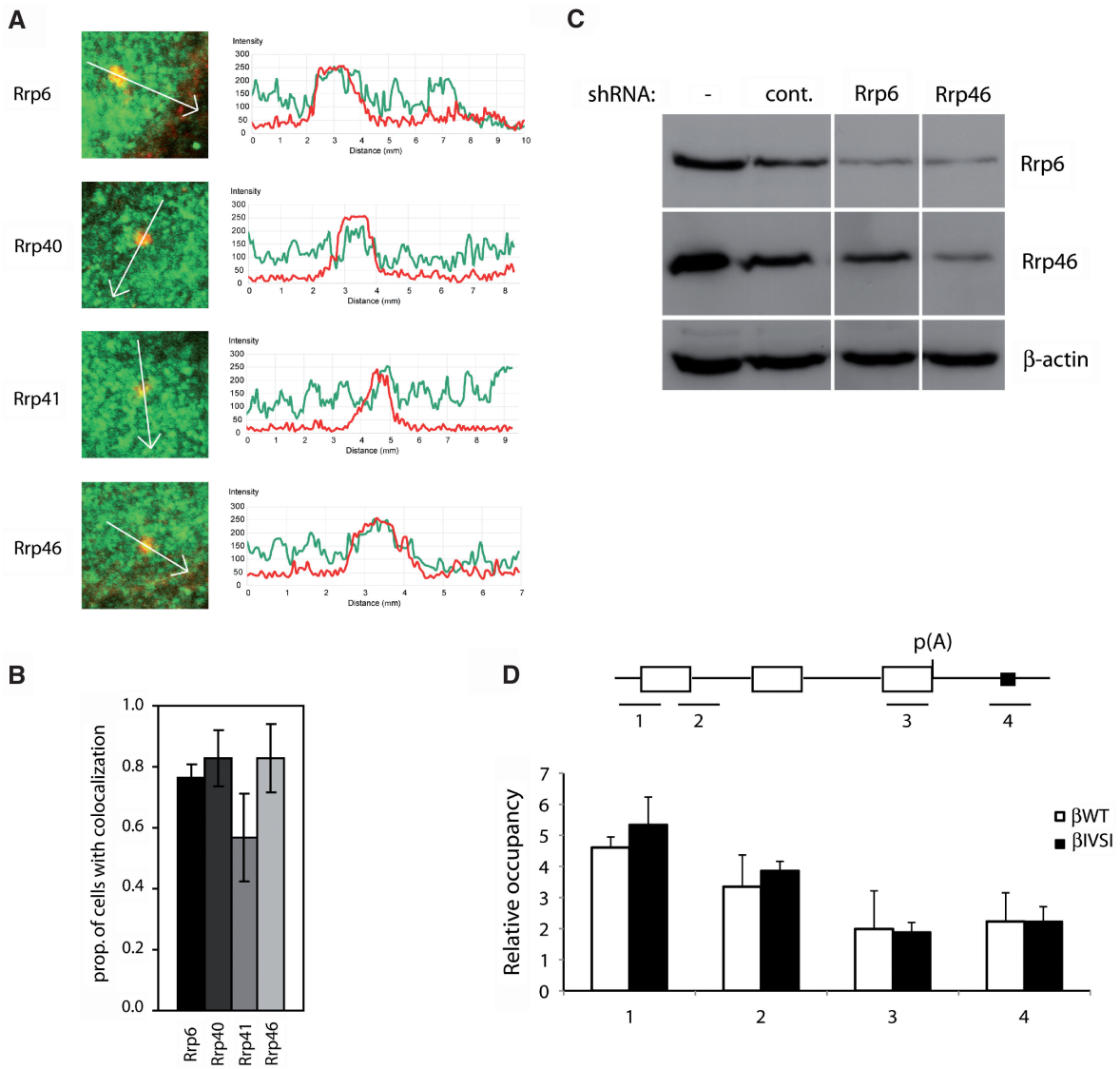

Figure 6. Rrp6 and core exosome subunits are recruited to β-globin transcription sites. (**A**) Cells expressing the wild-type transgene were double-labeled for β-globin RNA by FISH (red staining) and for protein by indirect immunofluorescence (green staining) using antibodies specific for exosome subunits, as indicated. The recruitment of proteins to transcription sites was analyzed drawing a test line across the βWT transcription site and measuring the relative fluorescence intensity along the line (in arbitrary units) for both the RNA signal and the protein signal. We considered a positive recruitment when the intensity of the protein signal at the transcription site was >2-fold higher than the global nucleoplasmic signal. The color of the lines in the graphics corresponds with the color of the detection of RNA and protein in the cell. (**B**) The proportion of cells exhibiting recruitment of exosome proteins to βWT transcription sites was quantified. Values represent average from at least 30 transcription sites analyzed in three independent experiments. Error bars denote standard deviation. (**C**) Western blot analysis of whole lysates from cells infected with individual short hairpin RNAs targeting the indicated exosome components. The blot was probed with antibodies against the indicated exosome subunits and β-actin. (**D**) Rrp6 relative occupancy in cells expressing βWT and βIVS1 transgenes after tetracycline induction. The histogram depicts mean and standard deviation for three independent ChIP experiments.

and the cleavage-stimulating factor (CstF). CPSF recognizes a conserved hexanucleotide termed the polyadenylation signal located 10–30 nt upstream of the poly(A) site, and CstF binds a less conserved U- or GU-rich region located up 30 nt downstream of the poly(A) site (52,53).

Mechanistically, transcriptional termination of the human β-globin gene has been extensively characterized and can be summarized in the following sequence of events (54). Pol II transcribes the β-globin gene past the poly(A) site until it synthesizes the terminator sequence, which is cleaved by an activity termed co-transcriptional cleavage or CoTC (45,46,55). This results in exposure of an unprotected RNA 5′-end, the degradation of which by the Xrn2 exonuclease leads to dissociation of the polymerase from the template as predicted in the torpedo model (46). Depletion of Xrn2 exonuclease results in a decrease in termination efficiency of both β-globin and β-actin genes, as shown by an increase in transcripts and Pol II density beyond the CoTC sequence (46,56). Moreover, it has been proposed that Pol II termination of the β-globin gene can occur prior to cleavage at the poly(A) site (54).

Here, we validate this model by showing that in Rrp6-depleted cells uncleaved transcripts are released from the DNA.

It is important to note that the presence of a CoTC element has only been identified in human globin and mouse serum albumin genes (52,57). For most genes, Pol II termination is thought to be more tightly coupled with poly(A) site cleavage. Yet, recent ChIP studies on multiple human genes reveal extensive co-localization of 3′-end processing factors with Pol II in regions more than a kilobase downstream of poly(A) sites (44). Most probably, there are differences in the mechanism of 3′-end processing and Pol II termination between genes and across species (58,59). Therefore, it is likely that cells have also different mechanisms to control faulty nascent transcripts. It remains to be established whether in other mammalian genes the surveillance of deficiently processed RNAs involves termination as described here for the β-globin gene, or occurs post-transcriptionally as observed in *S. cerevisiae* (18–20).

In yeast, defects in mRNA 3′-end formation cause accumulation of aberrant transcripts in foci near the sites of transcription, but in the absence of Rrp6 aberrant pre-mRNAs are no longer retained (14,15,60). This led to the proposal that release of newly synthesized mRNA from the transcription site may act as an exosome-dependent checkpoint for mRNA integrity (61). Here, we show that the involvement of Rrp6 in retention of aberrant pre-mRNAs near the sites of transcription is conserved from yeast to mammals. Most important, we find that Rrp6 is required to prevent transcription termination of an RNA processing mutant gene, which implies a novel cellular role for this protein in addition to its well-characterized exoribonuclease activity. Despite evidence that Rrp6 can act independently of the exosome core (49,50), we observe co-localization of Rrp6 and core subunits at β-globin transcription sites. This observation is consistent with a previous study in *Drosophila* showing that the whole exosome complex associates with elongating Pol II (48). Moreover, we observe that knocking down a core subunit leads to reduction of Rrp6, suggesting that the majority of Rrp6 in the cell is in a complex with the core exosome.

How can the exosome interfere with termination? One possibility is that the exosome associates with the elongating polymerase (48) and contributes to trigger termination upon recruitment of the cleavage and polyadenylation machinery to the poly(A) signal. It is generally assumed that the emergence of the poly(A) signal on the nascent transcript changes the properties of the elongating complex (probably by dissociation and/or recruitment of specific factors), rendering the polymerase competent for termination. In the case of the RNA processing defective mutant, failure to assemble a functional cleavage and polyadenylation complex is somehow sensed by the exosome and termination becomes less efficient.

A major breakthrough in our understanding of gene expression was the recent realization that individual steps from transcription to mRNA translation and decay are extensively interwoven and integrated (1). Many aspects of transcription and pre-mRNA processing are known to be coupled, and in particular, West and Proudfoot (54) found that termination enhances pre-mRNA splicing. The data presented in this study show that defects in splicing and 3′-end cleavage reduce termination efficiency, arguing for a bi-directional communication between pre-mRNA processing and transcriptional termination. Although further studies are needed to establish the molecular links involved in this cooperation, a recent study in *Caenorhabditis elegans* identified an SR splicing protein, SRp20, as implicated in termination (62).

SUPPLEMENTARY DATA

Supplementary Data are available at NAR Online.

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