

Defective histone supply causes changes in RNA polymerase II elongation rate and cotranscriptional pre-mRNA splicing

Silvia Jimeno-González^{a,1}, Laura Payán-Bravo^a, Ana M. Muñoz-Cabello^a, Macarena Guijo^a, Gabriel Gutierrez^b, Félix Prado^a, and José C. Reyes^{a,1}

^aCentro Andaluz de Biología Molecular y Medicina Regenerativa (CABIMER), Consejo Superior de Investigaciones Científicas, E-41092, Seville, Spain; and

^bDepartment of Genetics, University of Seville, 41080, Seville, Spain

Edited by Steven Henikoff, Fred Hutchinson Cancer Research Center, Seattle, WA, and approved October 26, 2015 (received for review April 8, 2015)

RNA polymerase II (RNAPII) transcription elongation is a highly regulated process that greatly influences mRNA levels as well as pre-mRNA splicing. Despite many studies in vitro, how chromatin modulates RNAPII elongation in vivo is still unclear. Here, we show that a decrease in the level of available canonical histones leads to more accessible chromatin with decreased levels of canonical histones and variants H2A.X and H2A.Z and increased levels of H3.3. With this altered chromatin structure, the RNAPII elongation rate increases, and the kinetics of pre-mRNA splicing is delayed with respect to RNAPII elongation. Consistent with the kinetic model of cotranscriptional splicing, the rapid RNAPII elongation induced by histone depletion promotes the skipping of variable exons in the *CD44* gene. Indeed, a slowly elongating mutant of RNAPII was able to rescue this defect, indicating that the defective splicing induced by histone depletion is a direct consequence of the increased elongation rate. In addition, genome-wide analysis evidenced that histone reduction promotes widespread alterations in pre-mRNA processing, including intron retention and changes in alternative splicing. Our data demonstrate that pre-mRNA splicing may be regulated by chromatin structure through the modulation of the RNAPII elongation rate.

chromatin | H3.3 | RNA polymerase II | transcription elongation | alternative splicing

The transcription process comprises several steps, including preinitiation complex formation, promoter escape, elongation, and termination (1). Recent reports indicate that elongation rates of RNA polymerase II (RNAPII) in mammals range from 0.5 to 4 kb/min, but which factors are responsible for these differences is still unclear (2–4). One obvious candidate for affecting transcription elongation is chromatin structure. The building block of chromatin is the nucleosome comprising 147 bp of DNA around a histone octamer formed by two H2A–H2B dimers and one H3–H4 tetramer. In vitro experiments have demonstrated that nucleosomes are a barrier for RNAPII transcription elongation (5, 6). We have reported that a nucleosome positioned in the body of a transcription unit impairs RNAPII progression in vivo (7). Furthermore, Weber et al. (8) have shown recently that RNAPII stalls in vivo at the entry site of essentially every transcribed nucleosome in *Drosophila*. Despite this evidence, it is still unclear whether changes in chromatin structure in different regions of a gene or between different genes can regulate the rate of transcription elongation.

Transcription and splicing are coupled processes (9, 10). Splicing occurs cotranscriptionally, and multiple lines of evidence indicate that transcription elongation and splicing influence each other. On one hand, it has been suggested that splicing factors are recruited to the template by the transcription machinery (11, 12). On the other hand, the rate of RNAPII elongation influences splicing. The kinetic model proposes that a slow elongation rate facilitates weak splice-site recognition, promoting the inclusion of alternative exons (13, 14). However, recent studies have extended

this view, showing that both slow and fast elongation affect splicing in complex ways not predicted by the model (15, 16). Interestingly, RNAPII accumulates at spliced exons (17, 18), and two independent studies recently have demonstrated that the elongation rate correlates negatively with exon density (2, 3). It has been proposed that this exon-dependent RNAPII accumulation might be influenced by chromatin, because increased nucleosome occupancy has been found at exons as compared with surrounding introns (19, 20). Recently, progesterone-dependent nucleosomal changes have been shown to correlate with changes in alternative splicing (21). In addition, some histone posttranslational modifications associated with elongation are enriched in exons. Based on these results the presence of nucleosomes per se or exon-specific histone posttranslational modifications have been proposed to affect splicing, either directly or indirectly by modulating RNAPII elongation (9, 22, 23).

To shed light on this subject, we decided to investigate how the RNAPII elongation rate is affected under conditions of histone depletion, when chromatin is not correctly structured, and whether this incorrect structure has an impact on splicing. Early studies of histone depletion in yeast by Grunstein and collaborators (24, 25) showed that nucleosome loss affects transcription initiation, although the elongation rate was not investigated. Histone depletion in higher eukaryotes has been hindered by the existence of large gene families encoding every histone subunit. Here, we decreased canonical histones expression in human cells by knocking down the expression of the stem-loop binding protein (*SLBP*) gene. SLBP controls stability, processing, nuclear export,

Significance

The study of the role of histones in transcription in mammals has been hindered by the existence of large gene families encoding every histone subunit. Here we reduce the level of canonical histones in a human cell line by silencing stem-loop-binding protein, a histone mRNA regulatory factor. We show that canonical histone depletion increases the RNA polymerase II (RNAPII) elongation rate and temporally separates transcription and splicing. Furthermore, histone depletion also causes several pre-mRNA splicing defects, including skipping of alternative exons and intron retention. Thus we demonstrate that the correct histone supply is required to control the RNAPII elongation rate and pre-mRNA splicing.

Author contributions: S.J.-G., F.P., and J.C.R. designed research; S.J.-G., L.P.-B., A.M.M.-C., and M.G. performed research; S.J.-G., L.P.-B., A.M.M.-C., M.G., G.G., F.P., and J.C.R. analyzed data; and S.J.-G. and J.C.R. wrote the paper.

The authors declare no conflict of interest.

This article is a PNAS Direct Submission.

Data deposition: The sequence reported in this paper has been deposited in the Gene Expression Omnibus (GEO) database, www.ncbi.nlm.nih.gov/geo (accession no GSE69307).

¹To whom correspondence may be addressed. Email: silvia.jimeno@cabimer.es or jose.reyes@cabimer.es.

This article contains supporting information online at www.pnas.org/lookup/suppl/doi:10.1073/pnas.1506760112/-DCSupplemental.

and translation of canonical histone mRNAs (26). Our data demonstrate that a reduction in the level of available canonical histones increases the RNAPII elongation rate in vivo and causes multiple splicing defects.

Results

SLBP Depletion Disrupts Chromatin Structure and Alters Histone Abundance.

To investigate how histone levels affect transcription elongation, we generated a stable cell line in HCT116 cells (named "HCT-shSLBP.1") that expresses a doxycycline (Dox)-inducible shRNA targeting the *SLBP* transcript. Dox treatment of HCT-shSLBP.1 cells for different time periods (3, 7, or 14 d) provoked a decrease in SLBP protein levels (Fig. S1A). As already reported, strong and prolonged SLBP depletion caused an accumulation of cells in S phase (Fig. S1B and C and ref. 27). However, we did not observe cell-cycle alterations 3 d after Dox treatment, and SLBP levels were moderately but significantly reduced. Therefore, to avoid cell-cycle effects, all experiments were performed 3 d after Dox treatment. Importantly, SLBP depletion caused a significant drop in soluble and chromatin-bound canonical H2B and H3 histone levels (Fig. 1A) with no significant changes in the level of DNA (Fig. 1A and by flow cytometry in Fig. S1B), suggesting that the histone:DNA ratio was reduced. Surprisingly, the levels of the chromatin-bound histone variants H2A.Z and H2A.X were reduced slightly, although their mRNAs either are not controlled (H2A.Z) or are only partially controlled (H2A.X) by SLBP (26). In contrast, levels of the chromatin-bound variant histone H3.3, which is also not controlled by SLBP, increased in Dox-treated cells (Fig. 1A), indicating that the H3.3 can partially replace canonical H3. SLBP depletion also increased chromatin accessibility to micrococcal nuclease I (MNase I), suggesting that the combined effect of the lower histone:DNA ratio and the modified histone composition promotes a more open chromatin configuration (Fig. 1B and C). However, nucleosome spacing was not altered by SLBP depletion.

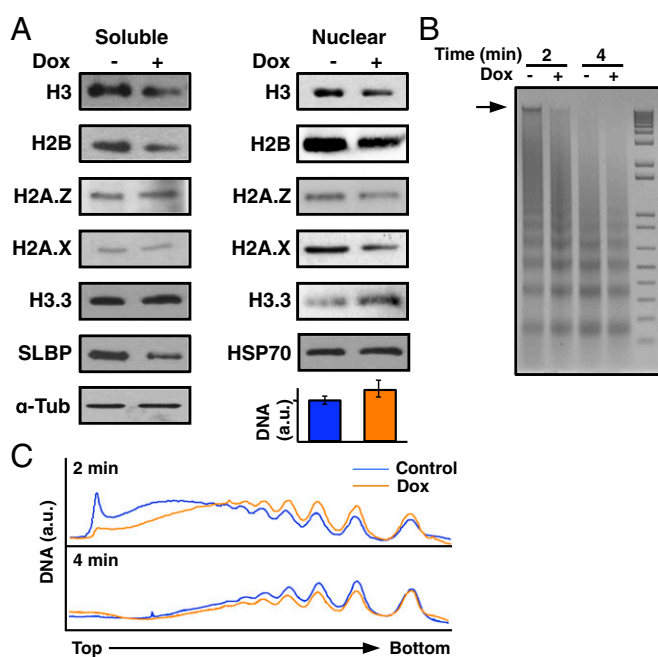


Fig. 1. Characterization of chromatin in SLBP-depleted cells. HCT-shSLBP.1 cells were cultured in the presence (+) or absence (–) of Dox for 72 h. (A) Levels of the indicated proteins in the soluble or chromatin-bound (nuclear) fractions were determined by Western blot. DNA content of the same nuclear preparation was measured by qPCR as control. (B) MNase I digestion of chromatin from isolated nuclei. Nuclei were digested for the indicated times. (C) Densitometric analysis of the lanes shown in B. a.u., arbitrary units.

Histone Depletion Accelerates RNAPII Elongation. We then studied the effect of histone depletion on the RNAPII elongation rate of two long human genes, utrophin (*UTRN*) and catenin beta-like 1 (*CTNNB1*), commonly used to analyze transcription elongation (28). First, we investigated by ChIP how SLBP depletion affects histone levels at different regions of these two genes. In agreement with the Western blotting data, SLBP silencing caused a decrease in the occupancy of total histone H3, H2A.Z, and H2A.X and an increase in the variant H3.3 at *UTRN* and *CTNNB1* genes (Fig. 2A). Histone changes were not identical in all analyzed regions, suggesting that some regions are more susceptible to histone depletion than others (compare, for example, the levels of H3 in exon 1 with those in exon 4, exon 5, and exon 6 of *CTNNB1*). To measure transcription elongation rate, initiating RNAPIIs were transiently inhibited with 5,6-dichlorobenzimidazole-1-β-D-ribofuranoside (DRB). Three h later DRB was washed off to resume transcription elongation (28). The velocity of the transcription wave was measured using quantitative RT-PCR (RT-qPCR) with primers spanning different exon–intron junctions of *UTRN* and *CTNNB1*. Exon 1 transcription in both genes was recovered 10 min after DRB removal from cells treated with Dox and from untreated cells. In contrast, expression of exon 3 of *UTRN* or exons 6 and 16 of *CTNNB1* was detected 10 min earlier in the presence of Dox than in the control cells (Fig. 2B and C), suggesting that the RNAPII elongation rate increases under conditions of canonical histone depletion.

Singh and Padgett (28) showed that pre-mRNA splicing of most introns occurs 5–10 min after transcription of the downstream exon. Because RNAPII elongates faster under histone depletion, we wondered whether splicing also was accelerated under these conditions. To measure the kinetics of pre-mRNA splicing, we determined the time between the new synthesis of an exon and the appearance of the splicing product of that exon and the immediately preceding exon by RT-qPCR (28). In agreement with previous results, introns 4 and 5 of the *CTNNB1* gene were spliced 5–10 min after synthesis of exons 5 and 6, respectively (Fig. 3). Interestingly, histone depletion delayed the splicing of these introns relative to the synthesis of the corresponding downstream exons (Fig. 3). In addition, *CTNNB1* intron 5 splicing was significantly impaired in the presence of Dox (Fig. 3B), suggesting that histone depletion promotes intron retention. Taken together, these results indicate that a correct chromatin organization is required for normal transcription elongation speed and for the temporary coupling between elongation and splicing.

Histone Depletion Promotes Exon Skipping at the *CD44* Gene. To characterize the consequences of histone depletion on splicing further, we examined the effect of histone depletion on the *CD44* gene as a well-known model of complex alternative splicing. *CD44* contains 10 constant and 9 clustered variable exons (Fig. 4A) and generates many splice variants (29). Histone depletion provoked a 20–40% decrease in total H3, H2A.Z, and H2A.X and a concomitant increase in H3.3 occupancy along the *CD44* gene (Fig. 4B and Fig. S2A). Once histone reduction was verified, the effect on *CD44* alternative splicing was analyzed. Fig. 4C shows that histone depletion decreased the levels of *CD44* mRNAs containing exons v4–v5 and v9–v10, indicating that a relaxed chromatin structure promotes skipping of the alternative exons. As a control, we verified that similar results were obtained in HCT-shRNA.2 cells, a stable cell line that expresses a different shRNA against *SLBP* (Fig. S2B and C).

Next, we investigated how histone depletion affects the distribution of RNAPII along the gene body of *CD44*. Under control conditions RNAPII occupancy increased at the variable region (Fig. 4D), in agreement with previous observations (30). However, RNAPII accumulation decreased drastically at this region under conditions of histone depletion, suggesting that chromatin has a role in modulating the progression and pausing of RNAPII at the variable exons. Importantly, histone depletion also decreased the levels of the splicing factor U2AF65 on the

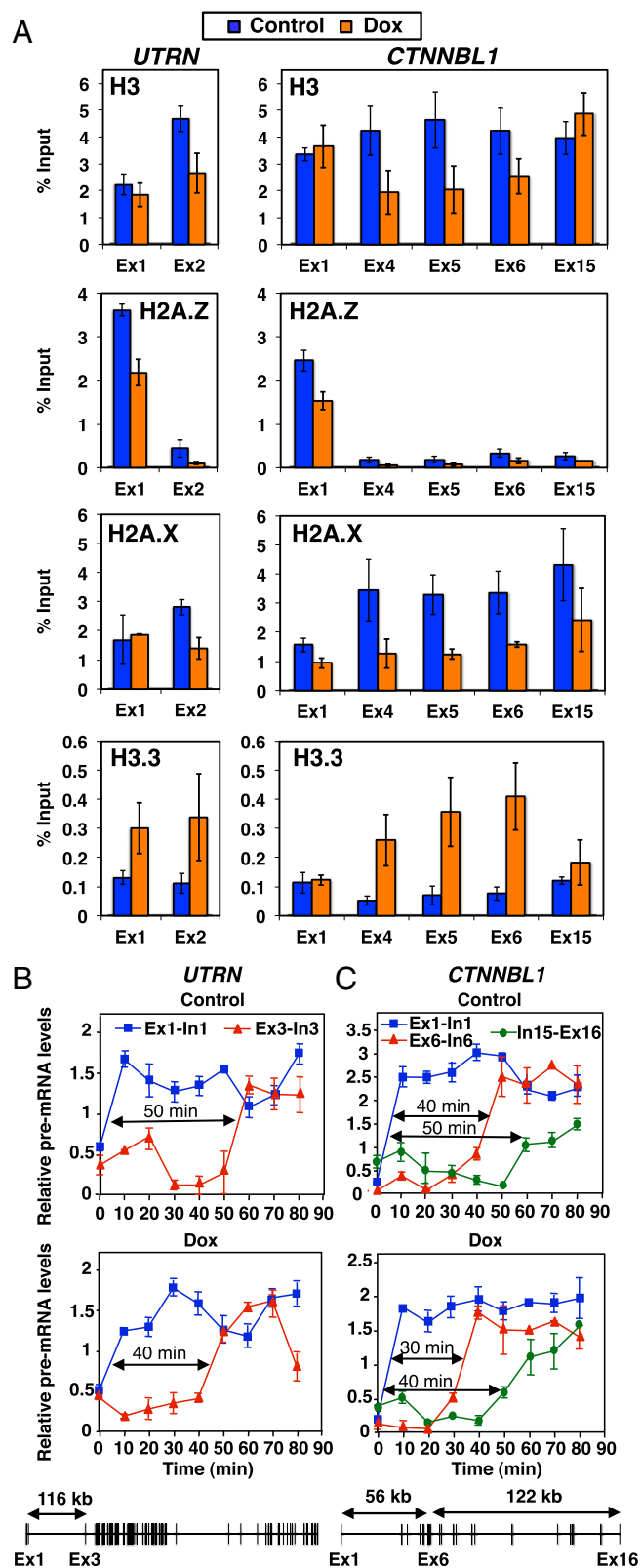


Fig. 2. Histone depletion increases the RNAPII elongation rate. (A) ChIP analysis of histone H3, H2A.Z, H2A.X, and H3.3 at the indicated regions of the *UTRN* and *CTNNB1* genes in Dox-treated and control HCT-shSLBP.1 cells. (B and C) Time course of transcription elongation for the *UTRN* (B) and *CTNNB1* (C) genes. Dox-treated or control HCT-shSLBP.1 cells were incubated with 100 μ M DRB for 3 h; then the DRB-containing medium was removed, and fresh medium was added. Levels of pre-mRNA at the indicated times were determined by RT-qPCR of the regions indicated below the

variable region of *CD44* (Fig. 4E), consistent with the skipping of variable exons observed under conditions of histone depletion.

Our data indicate that chromatin structure has an influence on alternative splicing. Two models (not mutually exclusive) have been proposed to explain how chromatin might affect alternative splicing (9, 22, 23): nucleosomes and histone posttranslational modifications may be involved in recruiting splicing factors, or, alternatively, nucleosomes might affect splicing by modulating the RNAPII elongation rate. We have shown that canonical histone depletion increases the RNAPII elongation rate, but we cannot exclude the possibility that chromatin has a role independent of elongation. To assess the relevance of transcription elongation rate on the skipping of *CD44* variable exons, we tested whether a “slow” mutant of RNAPII could rescue the splicing defects observed under conditions of histone depletion. We used the catalytic mutant of the large subunit of RNAPII, hC4, that causes a reduced transcription rate (14). hC4 and WT α -amanitin-resistant alleles were expressed in HCT-shSLBP.1 cells while endogenous RNAPII was inactivated by addition of α -amanitin (Fig. 4F). Fig. 4G shows that the slow hC4 RNAPII suppressed the skipping of variable exons promoted by histone depletion. Hence, we conclude that the effect of chromatin on *CD44* alternative splicing depends on the RNAPII elongation rate.

Histone Depletion Causes Widespread Splicing Defects. To determine the global effects of histone depletion in gene expression and splicing, we hybridized RNAs isolated from three biological replicates of HCT-shSLBP.1 cells, cultured either in the presence or absence of Dox, to splicing-sensitive Human Transcriptome Arrays (HTA) 2.0 from Affymetrix. This array contains probe sets covering 560,472 exons from protein-coding genes and 109,930 exons from non-protein-coding genes. Exons are covered by one to four probe sets. To perform standard microarray normalization procedures, we verified that SLBP depletion did not globally affect the total level of RNA (Fig. S3A). Analysis of the results at the gene level demonstrated that histone depletion caused only mild changes in transcripts levels (Fig. S3B). As expected, expression of 58 of the 74 canonical histone-encoding genes present in the array decreased in the presence of Dox compared with control conditions (Fig. S3B), confirming that SLBP silencing decreased histone mRNA levels. Histone depletion affected the expression of 290 genes ($P < 0.05$ and $|\text{linear fold change}| \geq 1.5$), of which 152 were down-regulated and 138 were up-regulated (Dataset S1). Only eight transcripts changed more than twofold; three were histone transcripts, and the other five were noncoding RNAs. Therefore, our data indicate that the canonical histone-depletion conditions used in our experiments caused only mild changes in gene expression.

Next we analyzed whether histone depletion promotes splicing defects. To do so, we calculated the splicing index of each probe set of the array. The splicing index represents the change in probe set inclusion (Dox versus control) normalized to the change of gene-level expression. A positive splicing index value indicates higher inclusion of this probe set with respect to the whole transcript; a negative splicing index indicates skipping of this probe set. Using a threshold of $|\text{splicing index}| \geq 1$ and $P < 0.05$, we identified significant splicing defects in 943 exon probe sets corresponding to 734 genes. One hundred eighty probe sets showed higher skipping in the presence of Dox than in control conditions; 763 probe sets showed higher inclusion (Dataset S1). Consistent with the results described above, several *CD44* variable exons presented negative splicing index values (Fig. S4). Positive splicing indexes were associated mainly with genes with a high level of expression ($P = 1.39 \times 10^{-22}$; hypergeometric test), whereas negative splicing indexes were present mostly in genes with medium or low levels of transcription ($P = 2.04 \times 10^{-24}$) (Fig. 5

panels. Pre-mRNA values are normalized to the values of the prior-DRB treatment sample, which was set to 1. Results are shown as means \pm SEM from three independent experiments.

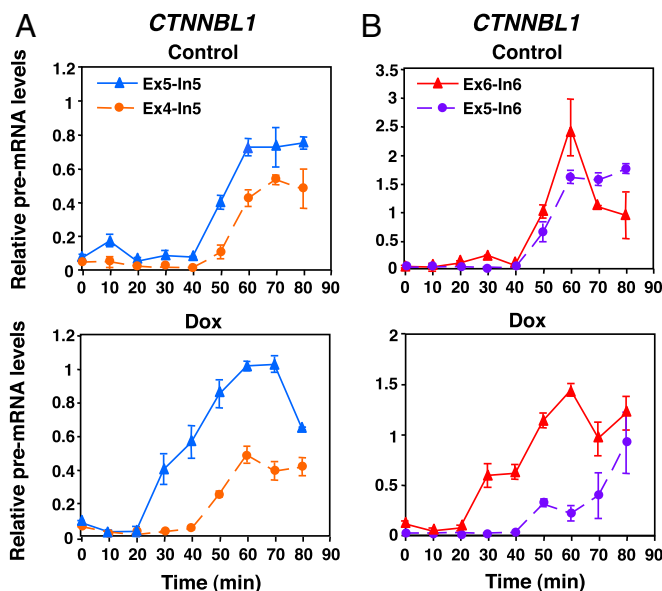


Fig. 3. Temporary coupling between elongation and splicing kinetics is impaired by histone depletion. Cells were processed as in Fig. 2B. The time course of transcription elongation in exon 5 (Ex5-In5) and the splicing of intron 4 (Ex4-In5) (A) and the transcription of exon 6 (Ex6-In6) and splicing of intron 5 (Ex5-In6) (B) of the gene *CTNNB1* in the absence (control, *Upper*) or presence of Dox (*Lower*). Results are shown as means \pm SEM from three independent experiments.

A and B), suggesting that the effect of canonical histone depletion on splicing depends on the transcription intensity.

As a second tool for studying alternative splicing, we used AltAnalyze software (31). In this case, using the default parameters of the MiDAS algorithm, 875 probe sets with splicing defects were identified in 702 genes (Dataset S1). Of the 875 misregulated splicing events, 644 showed a positive splicing index, and 231 had a negative splicing index. AltAnalyze incorporates a library of splicing annotations from University of California Santa Cruz (UCSC) KnownAlt database (Fig. S5A) (32). About 50% of the detected splicing defects were annotated in the KnownAlt database (Dataset S1). Alternative cassette exons were highly represented among the exons with a negative (57%) or positive (28%) splicing index. Importantly, ~40% of the exons with increased inclusions (splicing index >1) were annotated as intron retentions and bleeding exons (which also cause intron retention) (Fig. 5C and Fig. S5A). However, only 5% intron-retention and bleeding-exon annotations were found in skipped probe sets (splicing index <-1; Fig. 5C). Fig. S5B shows multiple examples of intron retentions and bleeding exons in *HNRNP*K, *UBAP2L*, and *FUS* genes. Manual examination of nonannotated exons showed that under conditions of histone depletion 42% of the up-regulated probe sets expanded regions cataloged as introns in the RefSeq database. In contrast, only 6% of the down-regulated probe sets expanded intronic regions, indicating a strong increase of intron-retention defects under histone depletion. Intron retentions and bleeding exons were found mostly in genes with high levels of expression (87%; $P = 1.3 \times 10^{-53}$; hypergeometric test) (Fig. S6). Increased levels of intron-containing pre-mRNAs in histone-depleted cells were confirmed by RT-qPCR at the *HNRNP*K, *UBAP2L*, and *FUS* genes (Fig. 5D). Therefore, our data suggest that depletion of canonical histones causes multiple types of splicing defects including increased or decreased inclusion of cassette exons and intron retention.

Discussion

How chromatin affects the RNAPII elongation rate in vivo is still not fully understood. Partial loss of core histones at gene bodies during intense transcription has been reported (33, 34), suggesting that a certain degree of histone removal is a prerequisite for or a

consequence of active transcription. However, recent genome-wide studies have shown either little (4) or no (3) negative correlation between nucleosome occupancy and elongation rates in mammals. To investigate the role of histones in transcription elongation in vivo, we reduced the levels of available canonical histones by SLBP silencing. SLBP-depleted cells showed a reduction in the total levels of H3, H2B, and H2A.X. Interestingly, expression of the variant histone H2A.Z, which is not controlled by SLBP, also was decreased, suggesting that reduction of H2B may cause loss of H2A.Z-H2B heterodimers from some nucleosomes. However, the levels of the histone variant H3.3 increased strongly in SLBP-silenced cells. H3.3 normally is enriched in active genes and in regulatory regions (35). In fact, H3.3-containing regions display a looser and more open chromatin structure (35, 36). Consistent with the partial reduction in the level of histones and the increased level of the

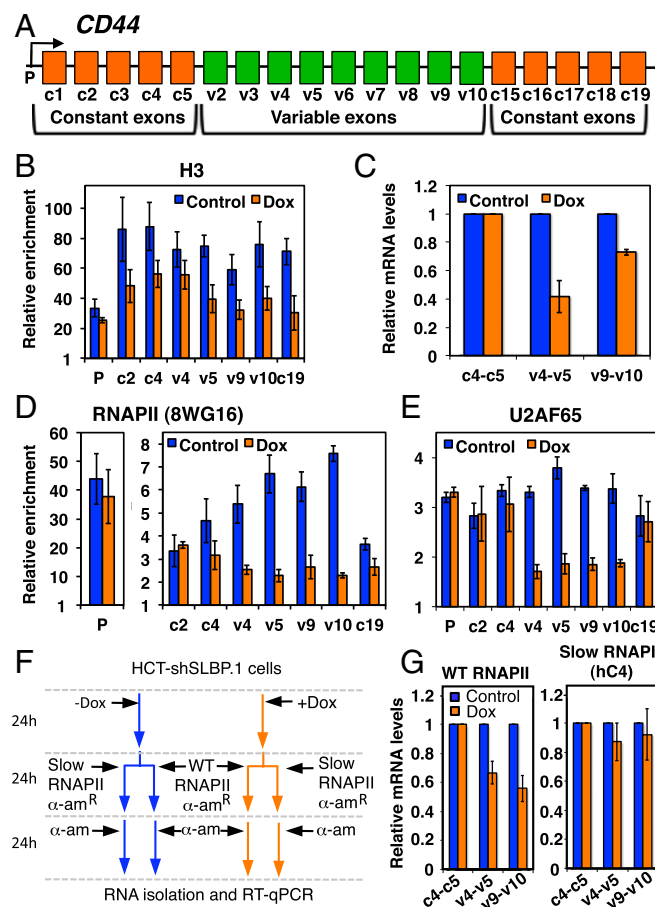


Fig. 4. Histone depletion promotes skipping of *CD44* variable exons. (A) Schematic representation of the exon-intron structure of the *CD44* gene. P, promoter. (B) ChIP analysis of histone H3 levels at the indicated regions of the *CD44* gene in Dox-treated and control HCT-shSLBP.1 cells. (C) RNA levels from the indicated exon couples (c4-c5, v4-v5, and v9-v10) were determined by RT-qPCR using RNA from control and Dox-treated cells. Exon inclusion is represented as the ratio between the indicated *CD44* exon couples and the constant exon couple c4-c5, with the signal from control samples being set to 1. (D and E) ChIP analysis of RNAPII (D) and U2AF65 factor (E) at the indicated regions of the *CD44* gene in Dox-treated and control HCT-shSLBP.1 cells. (F) Experimental setup of experiment shown in G. HCT-shSLBP.1 cells were treated with Dox for 24 h or were left untreated (control). Then plasmids expressing α -amanitin-resistant WT POLR2A (WT RNAPII α -am^R) or hC4 POLR2A mutant (slow RNAPII α -am^R) were transfected. Twenty-four hours later α -amanitin (α -am) was added, and cells were cultured for an additional 24 h to inhibit endogenous RNAPII. Then total RNA was isolated for RT-qPCR. (G) The levels of constant and variable exon couples were determined by RT-qPCR and are represented as indicated in C. Values in B-E and G are means \pm SEM of three independent experiments.

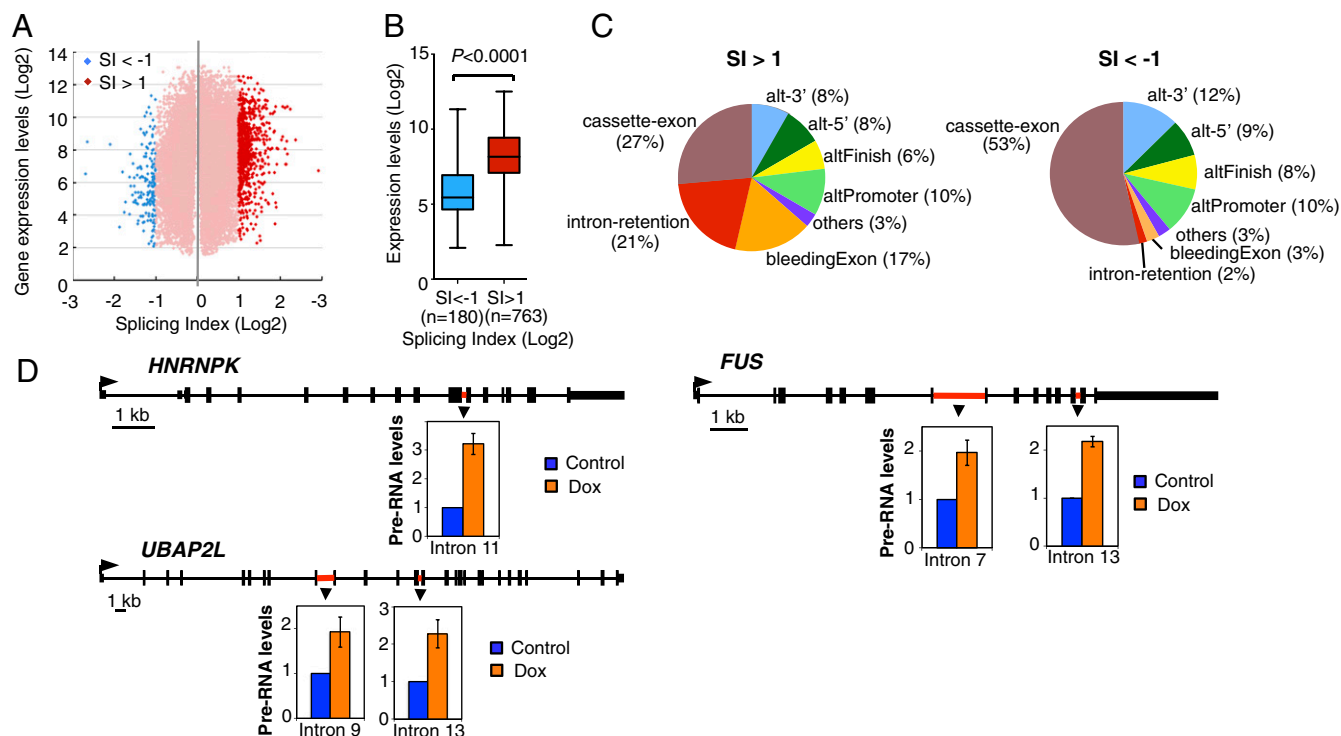


Fig. 5. Splicing changes upon histone depletion. (A) Relationship of the splicing index with the level of gene expression. Splicing indexes (abbreviated as “SI” in the figure) of exon probe sets were plotted against the level of expression of the corresponding gene under control conditions. (B) Box-and-whisker plot of gene expression of probe sets with splicing indexes < -1 or > 1. The P value (t-test) is provided. (C) Distribution of exon annotations of probe sets with splicing indexes > 1 (Left) and < -1 (Right) as provided by AltAnalyze. altFinish, alternate termination site; altPromoter, alternate promoter; alt-3', alternative 3' end of an intron; alt-5', alternative 5' end of an intron; bleedingExon, initial or terminal exons overlap an intron in another transcript; intron-retention, inclusion of intronic sequence. (D) Intron retention increases upon histone depletion. The level of pre-mRNA containing the indicated introns (highlighted in red) of *HNRNPK*, *UBAP2L*, and *FUS* genes was determined by RT-qPCR in Dox-treated and control HCT-shSLBP.1 cells. Data are normalized to the control. Values are means ± SEM of three independent experiments.

open-chromatin-specific H3.3, we observed that chromatin was more accessible to MNase I in SLBP-silenced cells (Fig. 1B). Importantly, these chromatin conditions, characterized by a general depletion of canonical histones together with an alteration in the level of non-canonical histones, caused a faster RNAPII elongation rate in two different genes, indicating that nucleosomes hinder RNAPII elongation in vivo as they do in vitro (5, 6). We also show that chromatin has an unanticipated role in the temporary coupling between transcription and splicing because histone depletion delayed pre-mRNA splicing from RNAPII elongation.

We have observed that canonical histone depletion produces skipping of the variable exons of *CD44* gene. RNAPII accumulates at the variable region of the *CD44* gene, a pause that facilitates variable exon inclusion (30, 37). We show here that variable exon inclusion and RNAPII accumulation decrease with histone depletion, suggesting that a correct chromatin structure is required for the inclusion of these exons. Why is this region so sensitive to histone depletion? It has been proposed that binding of the chromatin factors hBRM and HP1 γ to this region decreases the elongation rate of RNAPII (30, 37). Because hBRM and HP1 γ harbor histone-interaction domains able to interact with postranslationally modified histones (38, 39), it is possible that the absence of a correct nucleosomal structure under conditions of histone depletion impairs the recruitment of these factors to the chromatin. Our data also show that exon skipping in *CD44* is rescued by a slow mutant of RNAPII, indicating that the role of chromatin on *CD44* alternative splicing mostly depends on the RNAPII elongation rate and not on other putative roles of chromatin in recruiting splicing factors. This finding is consistent with the classical kinetic model that predicts that fast elongation rates can favor the skipping of alternative exons with weak splice sites (14). Certainly, we also detected a defect in the

recruitment of the splicing factor U2AF65 at the *CD44* variable region. However, because U2AF65 interacts with the C-terminal domain of RNAPII (11), this phenotype may result from the reduced occupancy of RNAPII observed at this region.

A severe histone reduction provokes strong replication defects, accumulation of cells in the S and G2 cell-cycle phases, and genetic instability (27, 40). However, the subtle histone depletion used in our work has allowed us to analyze the transcriptional phenotypes caused by histone depletion without affecting cell cycle. Under these conditions only mild changes in gene expression were observed (most of them less than twofold), splicing defects being the most prominent phenotypes observed. Genes encoding splicing factors were not significantly up- or down-regulated in the SLBP-silenced cells (Fig. S3C). However, a high proportion of splicing genes were slightly down-regulated in the presence of Dox (less than -1.4 lineal fold change). Although this effect can be attributed to the recently reported important role for RNAPII elongation rate in alternative splicing regulation of pre-mRNA splicing factor genes (13), it is too mild to be considered significant. Nevertheless, we cannot rule out the possibility that this subtle down-regulation acts as a positive feedback that increases splicing defects. Our genome-wide analysis showed that histone depletion caused different types of effects, including the firing of alternative promoters, changes in inclusion of cassette exons, alternative termination sites, and intron retention. Both increased and decreased cassette-exon inclusions were detected, in agreement with the proposal that the classical kinetic model can explain only part of the observed elongation-dependent splicing defects (15). Although we have shown that histone depletion promotes a faster transcription elongation rate, we cannot discard the possibility that some

splicing defects are caused directly by chromatin changes in splicing and not by the change in elongation rate.

The increase in intron retention with the depletion of canonical histones also is consistent with an extended view of the kinetic model in which elongation rate affects constitutive splicing (41). Thus, retention of intronic regions was observed mostly in highly expressed genes. It has been shown that highly transcribed genes have higher elongation rates (3, 4) and elevated histone replacement (42, 43). Therefore, the chromatin structure of highly expressed genes may be more severely affected by histone depletion, and, as a consequence, the RNAPII elongation rate might be further accelerated, affecting the recognition of constitutive splicing sites and hence promoting intron retention.

A reduction in canonical histones has been observed during replicative aging in yeast (44) and in senescent cells in yeast (45) and human fibroblasts (46). Furthermore, in senescent human fibroblasts depletion of canonical histones also is accompanied by an increase in histone H3.3 (47). It is currently unclear to what extent the transcriptional changes that occur during aging or/and senescence in mammals are a consequence of histone reduction. However, and interestingly, intron retentions are the most abundant age-related splicing changes found in the human brain (48). Therefore, our results prompt us to hypothesize that

the splicing alterations found during human aging might be caused by the associated histone depletion.

Materials and Methods

Detailed methods for plasmids, cell culture, generation of inducible cell lines, pre-mRNA analysis, MNase I treatment, and immunoblotting are provided in *SI Materials and Methods*. For exon array analysis, total RNA was isolated in triplicate from HCT-shSLBP.1 cells cultured in the presence or absence of Dox by using the RNeasy Mini Kit (Qiagen). Details about GeneChip HTA Array hybridization and data analysis are provided in *SI Materials and Methods*. Exon array data are available from the Gene Expression Omnibus database (accession number GSE69307). ChIP experiments were carried out as previously described (7). Five to ten micrograms of the indicated antibody (Table S1) were used per ChIP. Quantification of immunoprecipitated DNA was performed by qPCR, using three qPCR determinations per biological replica. Provided data are the average of three independent biological replicas \pm SEM. The primers used are described in Table S2.

ACKNOWLEDGMENTS. We thank A. Kornblihtt for providing reagents and E. Andújar and M. Pérez-Alegre from the Centro Andaluz de Biología Molecular y Medicina Regenerativa Genomic Unit for microarray expression hybridization and analysis. This work was funded by Spanish Ministry of Economy and Competitiveness (MINECO) Grants BFU-2011-23442 and BFU2014-53543-P (to J.C.R.) and Andalusian Government Grant P12CT52270 (to F.P.). S.J.-G. and A.M.M.-C. were supported by Juan de la Cierva Grants from MINECO. L.P.-B. was supported by the Asociación Española Contra el Cáncer.

- Kwak H, Lis JT (2013) Control of transcriptional elongation. *Annu Rev Genet* 47:483–508.
- Veloso A, et al. (2014) Rate of elongation by RNA polymerase II is associated with specific gene features and epigenetic modifications. *Genome Res* 24(6):896–905.
- Jonkers I, Kwak H, Lis JT (2014) Genome-wide dynamics of Pol II elongation and its interplay with promoter proximal pausing, chromatin, and exons. *eLife* 3:e02407.
- Danko CG, et al. (2013) Signaling pathways differentially affect RNA polymerase II initiation, pausing, and elongation rate in cells. *Mol Cell* 50(2):212–222.
- Hodges C, Bintu L, Lubkowska L, Kashlev M, Bustamante C (2009) Nucleosomal fluctuations govern the transcription dynamics of RNA polymerase II. *Science* 325(5940):626–628.
- Kireeva ML, et al. (2005) Nature of the nucleosomal barrier to RNA polymerase II. *Mol Cell* 18(1):97–108.
- Subtil-Rodriguez A, Reyes JC (2010) BRG1 helps RNA polymerase II to overcome a nucleosomal barrier during elongation, in vivo. *EMBO Rep* 11(10):751–757.
- Weber CM, Ramachandran S, Henikoff S (2014) Nucleosomes are context-specific, H2A.Z-modulated barriers to RNA polymerase. *Mol Cell* 53(5):819–830.
- Lucu RF, Allo M, Schor IE, Kornblihtt AR, Misteli T (2011) Epigenetics in alternative pre-mRNA splicing. *Cell* 144(1):16–26.
- Perales R, Bentley D (2009) “Cotranscriptionality”: The transcription elongation complex as a nexus for nuclear transactions. *Mol Cell* 36(2):178–191.
- David CJ, Boyne AR, Millhouse SR, Manley JL (2011) The RNA polymerase II C-terminal domain promotes splicing activation through recruitment of a U2AF65-Prp19 complex. *Genes Dev* 25(9):972–983.
- Muñoz MJ, de la Mata M, Kornblihtt AR (2010) The carboxy terminal domain of RNA polymerase II and alternative splicing. *Trends Biochem Sci* 35(9):497–504.
- Ip JY, et al. (2011) Global impact of RNA polymerase II elongation inhibition on alternative splicing regulation. *Genome Res* 21(3):390–401.
- de la Mata M, et al. (2003) A slow RNA polymerase II affects alternative splicing in vivo. *Mol Cell* 12(2):525–532.
- Fong N, et al. (2014) Pre-mRNA splicing is facilitated by an optimal RNA polymerase II elongation rate. *Genes Dev* 28(23):2663–2676.
- Dujardin G, et al. (2014) How slow RNA polymerase II elongation favors alternative exon skipping. *Mol Cell* 54(4):683–690.
- Kwak H, Fuda NJ, Core LJ, Lis JT (2013) Precise maps of RNA polymerase reveal how promoters direct initiation and pausing. *Science* 339(6122):950–953.
- Brodsky AS, et al. (2005) Genomic mapping of RNA polymerase II reveals sites of co-transcriptional regulation in human cells. *Genome Biol* 6(8):R64.
- Tilgner H, et al. (2009) Nucleosome positioning as a determinant of exon recognition. *Nat Struct Mol Biol* 16(9):996–1001.
- Schwartz S, Meshorer E, Ast G (2009) Chromatin organization marks exon-intron structure. *Nat Struct Mol Biol* 16(9):990–995.
- Iannone C, et al. (2015) Relationship between nucleosome positioning and progesterone-induced alternative splicing in breast cancer cells. *RNA* 21(3):360–374.
- Iannone C, Valcárcel J (2013) Chromatin's thread to alternative splicing regulation. *Chromosoma* 122(6):465–474.
- Gomez Acuna LI, Fiszbein A, Allo M, Schor IE, Kornblihtt AR (2013) Connections between chromatin signatures and splicing. *Wiley Interdisciplinary Reviews* 4(1):77–91.
- Kim UJ, Han M, Kayne P, Grunstein M (1988) Effects of histone H4 depletion on the cell cycle and transcription of *Saccharomyces cerevisiae*. *EMBO J* 7(7):2211–2219.
- Han M, Chang M, Kim UJ, Grunstein M (1987) Histone H2B repression causes cell-cycle-specific arrest in yeast: Effects on chromosomal segregation, replication, and transcription. *Cell* 48(4):589–597.
- Marzluff WF, Wagner EJ, Duronio RJ (2008) Metabolism and regulation of canonical histone mRNAs: Life without a poly(A) tail. *Nat Rev Genet* 9(11):843–854.
- Sullivan KD, Mullen TE, Marzluff WF, Wagner EJ (2009) Knockdown of SLBP results in nuclear retention of histone mRNA. *RNA* 15(3):459–472.
- Singh J, Padgett RA (2009) Rates of in situ transcription and splicing in large human genes. *Nat Struct Mol Biol* 16(11):1128–1133.
- Zöllner M (2011) CD44: Can a cancer-initiating cell profit from an abundantly expressed molecule? *Nat Rev Cancer* 11(4):254–267.
- Batsché E, Yaniv M, Muchardt C (2006) The human SWI/SNF subunit Brm is a regulator of alternative splicing. *Nat Struct Mol Biol* 13(1):22–29.
- Salomonis N, et al. (2009) Alternative splicing in the differentiation of human embryonic stem cells into cardiac precursors. *PLOS Comput Biol* 5(11):e1000553.
- Karolchik D, et al. (2008) The UCSC Genome Browser Database: 2008 update. *Nucleic Acids Res* 36(Database issue):D773–D779.
- Lee CK, Shibata Y, Rao B, Strahl BD, Lieb JD (2004) Evidence for nucleosome depletion at active regulatory regions genome-wide. *Nat Genet* 36(8):900–905.
- Schwabish MA, Struhl K (2004) Evidence for eviction and rapid deposition of histones upon transcriptional elongation by RNA polymerase II. *Mol Cell Biol* 24(23):10111–10117.
- Chen P, et al. (2013) H3.3 actively marks enhancers and primes gene transcription via opening higher-ordered chromatin. *Genes Dev* 27(19):2109–2124.
- Jin C, Felsenfeld G (2007) Nucleosome stability mediated by histone variants H3.3 and H2A.Z. *Genes Dev* 21(12):1519–1529.
- Saint-André V, Batsché E, Rachez C, Muchardt C (2011) Histone H3 lysine 9 trimethylation and HP1 γ favor inclusion of alternative exons. *Nat Struct Mol Biol* 18(3):337–344.
- Muchardt C, Yaniv M (1993) A human homologue of *Saccharomyces cerevisiae* SNF2/SWI2 and *Drosophila* brm genes potentiates transcriptional activation by the glucocorticoid receptor. *EMBO J* 12(11):4279–4290.
- Vakoc CR, Mandat SA, Olenchick BA, Blobel GA (2005) Histone H3 lysine 9 methylation and HP1 γ are associated with transcription elongation through mammalian chromatin. *Mol Cell* 19(3):381–391.
- Mejlvang J, et al. (2014) New histone supply regulates replication fork speed and PCNA unloading. *J Cell Biol* 204(1):29–43.
- Braberg H, et al. (2013) From structure to systems: High-resolution, quantitative genetic analysis of RNA polymerase II. *Cell* 154(4):775–788.
- Dion MF, et al. (2007) Dynamics of replication-independent histone turnover in budding yeast. *Science* 315(5817):1405–1408.
- Deal RB, Henikoff JG, Henikoff S (2010) Genome-wide kinetics of nucleosome turnover determined by metabolic labeling of histones. *Science* 328(5982):1161–1164.
- Feser J, et al. (2010) Elevated histone expression promotes life span extension. *Mol Cell* 39(5):724–735.
- Platt JM, et al. (2013) Rap1 relocalization contributes to the chromatin-mediated gene expression profile and pace of cell senescence. *Genes Dev* 27(12):1406–1420.
- O'Sullivan RJ, Kubicek S, Schreiber SL, Karlseder J (2010) Reduced histone biosynthesis and chromatin changes arising from a damage signal at telomeres. *Nat Struct Mol Biol* 17(10):1218–1225.
- Rai TS, et al. (2014) HIRA orchestrates a dynamic chromatin landscape in senescence and is required for suppression of neoplasia. *Genes Dev* 28(24):2712–2725.
- Mazin P, et al. (2013) Widespread splicing changes in human brain development and aging. *Mol Syst Biol* 9:633.
- Zhao X, McKillop-Smith S, Müller B (2004) The human histone gene expression regulator HBP/SLBP is required for histone and DNA synthesis, cell cycle progression and cell proliferation in mitotic cells. *J Cell Sci* 117(Pt 25):6043–6051.