8592–8609 *Nucleic Acids Research, 2021, Vol. 49, No. 15* *Published online 31 July 2021*
https://doi.org/10.1093/nar/gkab649

The splicing regulator SLU7 is required to preserve DNMT1 protein stability and DNA methylation

Miriam Recalde[1], María Gárate-Rascón[1], María Elizalde[1], María Azkona[1], M. Ujue Latasa[1,2], Marina Bárcena-Varela[1], Bruno Sangro[2,3,4], Maite G. Fernández-Barrena[1,2,3], Matías A. Ávila[1,2,3], María Arechederra[1,2,*] and Carmen Berasain[1,2,3,*]

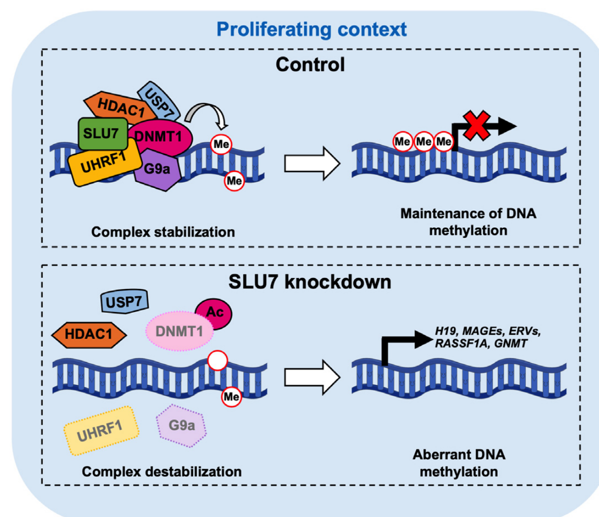
[1]Program of Hepatology, Center for Applied Medical Research (CIMA), University of Navarra, Pamplona 31008, Spain, [2]IdiSNA, Navarra Institute for Health Research, Pamplona 31008, Spain, [3]National Institute for the Study of Liver and Gastrointestinal Diseases (CIBERehd, Carlos III Health Institute), Madrid 28029, Spain and [4]Hepatology Unit, Navarra University Clinic, Pamplona 31008, Spain

Received March 22, 2021; Revised July 01, 2021; Editorial Decision July 19, 2021; Accepted July 21, 2021

ABSTRACT

Gene expression is finely and dynamically controlled through the tightly coordinated and interconnected activity of epigenetic modulators, transcription and splicing factors and post-translational modifiers. We have recently identified the splicing factor SLU7 as essential for maintaining liver cell identity and genome integrity and for securing cell division both trough transcriptional and splicing mechanisms. Now we uncover a new function of SLU7 controlling gene expression at the epigenetic level. We show that SLU7 is required to secure DNMT1 protein stability and a correct DNA methylation. We demonstrate that SLU7 is part in the chromatome of the protein complex implicated in DNA methylation maintenance interacting with and controlling the integrity of DNMT1, its adaptor protein UHRF1 and the histone methyl-transferase G9a at the chromatin level. Mechanistically, we found that SLU7 assures DNMT1 stability preventing its acetylation and degradation by facilitating its interaction with HDAC1 and the desubiquitinase USP7. Importantly, we demonstrate that this DNMT1 dependency on SLU7 occurs in a large panel of proliferating cell lines of different origins and in *in vivo* models of liver proliferation. Overall, our results uncover a novel and non-redundant role of SLU7 in DNA methylation and present SLU7 as a holistic regulator of gene expression.

GRAPHICAL ABSTRACT



INTRODUCTION

Multiple mechanisms are involved in the different -but interconnected- regulatory layers of gene expression, switching on and off transcription and widening the diversity of gene transcripts in a tissue and temporal manner. Among them, epigenetic marks are reversible modifications that without altering gene sequence determine chromatin structure and accessibility, resulting in transcription activation or repression (1,2). DNA methyltransferases (DNMTs) are the enzymes that add methyl groups to the 5′ position of cytosine residues of CpG dinucleotides (3). CpG islands (CGIs) are generally located at promoter regions and when methylated interfere with transcription factor binding and recruit repressor complexes (1). DNA methylation patterns are faithfully inherited from the mother DNA molecule to

*To whom correspondence should be addressed. Tel: +34 948194700; Fax: +34 948194717; Email: cberasain@unav.es
Correspondence may also be addressed to María Arechederra. Tel: +34 948194700; Fax: +34 948194717; Email: macalderon@unav.es

© The Author(s) 2021. Published by Oxford University Press on behalf of Nucleic Acids Research.
This is an Open Access article distributed under the terms of the Creative Commons Attribution-NonCommercial License (http://creativecommons.org/licenses/by-nc/4.0/), which permits non-commercial re-use, distribution, and reproduction in any medium, provided the original work is properly cited. For commercial re-use, please contact journals.permissions@oup.com

the daughters after each cell division (3,4). In particular, the DNA methyltransferase 1 (DNMT1) is the enzyme responsible for maintaining DNA methylation patterns during the S-phase of the cell cycle (5,6). Moreover, it is well established that DNA methylation plays a relevant role in maintaining genome stability (7–9). Indeed, deregulated DNA methylation is one of the early events in cell transformation (10,11), being DNMT1 essential for cancer cell proliferation and survival (12). In this context, DNMT1 levels are dynamically controlled by multiple mechanisms which mainly include allosteric interactions and post-translational modifications (13,14). DNMT1 phosphorylation, methylation and/or acetylation, among others, affect protein interactions, location and stability (13–15) in a dynamic and finely tuned fashion.

We have identified the splicing factor SLU7 as essential for maintaining the gene expression patterns required for securing differentiation, quiescence and metabolic function of the liver (16). In this context, SLU7 is controlling not only alternative splicing mechanisms but also the activity of transcriptional complexes, interacting with the RNA-polymerase and cAMP responsive proteins such as CREB and CBP (15). Moreover, we have demonstrated that SLU7 is required to preserve genome integrity and to secure correct cell division preventing the accumulation of R-loops, DNA damage and errors in chromosome segregation (17). Accordingly, the inhibition of SLU7 expression in pathological situations, such as liver cirrhosis or hepatocellular carcinoma (HCC) (18), would contribute to the process of hepatocarcinogenesis. On the other side, we have demonstrated that SLU7 knockdown in transformed cells represents a cancer cell vulnerability. Our results show that the viability of tumor cells of multiple origins is compromised by the induction of mitotic stress upon SLU7 silencing (19).

Altogether our results and other published data demonstrate that SLU7 and DNMT1 control gene expression at different levels, but both play essential roles maintaining genome integrity, cell proliferation and cell survival. Recent results have demonstrated a narrow connection and interplay between the different mechanisms implicated in the regulation of gene expression including DNA methylation and alternative splicing (20,21). Having demonstrated that SLU7 regulates both splicing and transcription, we decided to investigate whether SLU7 also regulates DNA methylation, representing a new hub integrating different levels of gene expression regulation.

In the present work, we demonstrate for the first time that SLU7 is required for DNA methylation maintenance. We found that SLU7 is part in the chromatome of the protein complex implicated in maintaining DNA methylation as it physically interacts at the chromatin level with DNMT1, its adaptor protein ubiquitin-like with PHD and RING finger domains-1 (UHRF1) and the histone methyl-transferase G9a. Moreover, we demonstrate that SLU7 is needed to maintain the association of the complex with chromatin. Interestingly, our results demonstrate that SLU7 is required to maintain DNMT1 protein levels in proliferating cells, both *in vitro* and *in vivo*. In fact, SLU7 knockdown induced DNMT1 degradation in multiple cell lines of different origin and *in vivo* in models of liver regeneration after partial hepatectomy or liver damage. Mechanistically we found that SLU7 is needed for the binding of histone deacetylase 1 (HDAC1) to DNMT1, preventing DNMT1 acetylation, and securing DNMT1 interaction with the deubiquitinating enzyme Ubiquitin-Specific Protease 7 (USP7) to stabilize DNMT1 protein. In summary, our present work demonstrates for the first time a role of SLU7 in DNA methylation, being essential to maintain the stability of DNMT1 protein regulating its post-translation modifications. These results uncover an unexpected function of SLU7 and demonstrate that SLU7 represents a regulator of gene expression with pleiotropic functions.

MATERIALS AND METHODS

Cell culture and treatments

All cell lines were obtained from the ATCC. The HCT116 *DNMT1*$^{-/-}$ cell line was kindly provided by Dr Rob M. Ewing (Southampton, UK) and described in (22). Human HCC cell lines PLC/PRF/5 and HepG2, human cervical carcinoma cell line HeLa, human melanoma cell line A375 and human embryonic kidney immortalized cell line HEK293T were grown in DMEM (Gibco-Life Technology, Madrid, Spain) supplemented with 10% FBS, glutamine and antibiotics. Human non-small cell lung cancer cell line H358 and human adenocarcinoma cell line A549 were grown in RPMI (Gibco-Life Technology) supplemented with 10% FBS, glutamine and antibiotics. Human colon cancer cell line HCT116 and HCT116 *DNMT1*$^{-/-}$ were grown in McCoy's 5A (Gibco-Life Technology) supplemented with 10% FBS, glutamine and antibiotics. Human breast cancer cell line MCF7 was grown in MEM (Gibco-Life Technology) supplemented with 10% FBS, insulin and antibiotics. Human glioblastoma cell line U87 was grown in DMEM/F12 (Gibco-Life Technology) supplemented with 10% FBS and antibiotics. All cells were grown in a humidified atmosphere at 37°C and 5% CO_2.

Where indicated, cells were treated before harvesting with the proteasome inhibitor 10 μM MG-132 (Calbiochem, CA, USA, 474790) for the last 20 h or with 20 μM MG-132 for the last 12 h; with the protein synthesis inhibitor 10 μ/ml cycloheximide (Sigma-Aldrich, MO, USA, C-7698) for 8 h and 12 h; with cell cycle drugs 20 μM lovastatin (Sigma-Aldrich PHR1285) or 2 μg/ml aphidicolin (Sigma-Aldrich A078) for 60 h or with 330 nM nocodazole (Sigma-Aldrich M1404) for 12 h or 60 h; with the demethylating agent 10 μM 5-aza-2′-deoxycytidine (Sigma, St. Louis, MO, USA) during 7 days.

siRNAs and plasmids transfections

Transfections with siRNAs were performed with Lipofectamine RNAiMAX reagent (Invitrogen, Carlsbad, CA, USA) following the manufacturer's instructions. Individual siRNAs were used at 75 nM. In those cases where combined siRNAs transfections were performed, each specific siRNA was used at 56.25 nM. Sequence of siRNAs will be provided upon request. Human siSLU7-2 and siSLU7-3 are two independent siRNAs directed to 2 different regions of SLU7. For overexpression experiments, plasmids were transfected with Lipofectamine 2000 (Invitrogen) following

the manufacturer's instructions. pcDNA6.1A-SLU7 plasmid was from GenScript BioTech (Piscataway, NJ, USA) and pcDNA3.1-HDAC1-Flag was kindly provided by Dr Donald E. Ayer (University of Utah, USA).

Total DNA isolation

Total DNA from cell lines was extracted using the automated Maxwell RSC Instrument with cultured cells DNA kit (Promega, Madison, WI, USA). DNA purity and concentration were measured using a NanoDrop spectrophotometer (Thermo Fisher Scientific, Waltham, MA, USA).

Reduced representation bisulfite sequencing (RRBS)

Genome-wide DNA methylation analysis was carried out using the reduced representation bisulfite sequencing method (23) by Active Motif (Carlsbad, CA, USA). One μg of genomic DNA from 72 h PLC/PRF/5 siGL, siSLU7 and siDNMT1 transfected cells was sent for RRBS analysis. Briefly, DNA was digested with the methylation insensitive restriction enzyme MspI, which cleaves DNA at CCGG sites creating fragments with high CpG content. Samples were then end-repaired and A-tails were added according to manufacturer's conditions. Once the adapters were ligated, DNA was loaded on an agarose gel, and 40–220 bp length fragments were isolated for bisulfite conversion. The bisulfite converted DNA was amplified by PCR (library preparation) and sequenced on the Illumina HiSeq™ instrument. Upon bioinformatic analysis, reads were mapped to the human reference genome hg38 and the differential methylation analysis was generated using DMAP (24). The resulting regions were then annotated using the HOMER software package (25). The subsequent analyses only include CpGs covered by 3 or more unique reads. Chi-squared test was then applied. CpG sites were considered differentially methylated when the methylation difference between siSLU7 or siDNMT1 compared to siGL sample was higher than 0.6. CpGs were localized within or outside a CGI using the CpG island bedfile identification (UCSC website; https://genome.ucsc.edu/).

Methylation-specific PCR

One μg of genomic DNA was modified upon bisulfite treatment using the EZ DNA Methylation Kit (Zymo Research, Irvine, CA, USA) following manufacturer instructions. Methylation-specific PCR (MSP) was performed with 1/10 bisulfite-converted DNA, the Phusion U Hot Start DNA Polymerase kit (F-555S, Thermo Fisher Scientific, Waltham, MA, USA), and specific methylated (M) and unmethylated (U) primers for the targeted regions of *TRIM47*, *TFF3*, *DLG3*, *RASSF1A* and *GNMT*. PCR products were electrophoresed and visualized in GelRed Nucleic Acid (Biotium, Fremont, CA, USA) stained gels (2% agarose) under UV light. The sequence of primers used in the study will be provided upon request.

Methylation quantification ELISA kit

Quantification of global levels of 5-methylcytosine (5-mC) was performed using the MethylFlash Global DNA Methylation (5-mC) ELISA Easy Kit (Colorimetric) (Epigentek, Farmingdale, NY, USA) following manufacturer's instructions, including a previous DNA denaturation step at 98°C for 5 min.

Total RNA isolation and PCRs

Total RNA from cell lines and tissues was extracted using the Maxwell RSC Instrument with simplyRNA tissue kit (Promega). RNA samples were treated with DNAse to degrade all possible traces of contaminating genomic DNA (gDNA). Reverse transcription was performed as previously described (26). Real-time PCRs were performed using an iCycler (Bio-Rad, Hercules, CA, USA) and the iQ SYBR Green Supermix (Bio-Rad) as previously described (27). To monitor the specificity final PCR products were analyzed by melting curves and the amount of each transcript was expressed relative to the housekeeping gene *RPLP0* as $2^{\Delta Ct}$, where ΔCt represents the difference in threshold cycle between the control and target genes, as described (28). To analyzed HCT116 *DNMT1*$^{-/-}$ cells, PCR using primers located on different exons of *DNMT1* and gel electrophoresis was performed. The sequence of primers used in the study will be provided upon request.

Immunofluorescence

For immunofluorescence, cells were cultured on coverslips and 72 h after transfection with siGL and siSLU7 were fixed with ice-cold methanol for 15 min at room temperature and washed twice with PBS. Quenching was performed with 50 mM NH_4Cl in PBS for 10 min. After washing three times with PBS, cells were permeabilized with 0,2% Triton X-100 for 5 min at 4°C and DNA was denatured with 4 M HCl for 15 min followed by 100 mM Tris–HCl pH 8,5 for 10 min. After washing, coverslips were blocked with Superblocking buffer (Thermo Fisher Scientific) for 1 h at room temperature and incubated overnight at 4°C with anti-5-methylCytosine (Eurogentec, Seraing, Belgium BI-MECY 0100) diluted in 1% BSA in PBS. After washing, cells were incubated with fluorophore-conjugated secondary antibodies in 1% BSA in PBS for 1 h at room temperature, washed and stained with vectashield (Vector laboratories, Burlingame, CA, USA) containing DAPI. Images were obtained using the Zeiss Axio Imager.M1 microscope (Zeiss, Oberkochen, Germany).

Protein extraction and Western blot analysis

For protein extraction, all cell lines were lysed in RIPA buffer: 150 mM NaCl, 50 mM Tris pH 7,5, 0,1% SDS, 1% Triton X-100, 0,5% deoxycholate, protease inhibitor cocktail Complete (Roche, Basel, Switzerland), 1 mM orthovanadate, 10 mM NaF, 100 mM β-glycerophosphate and sonicated. Lysates were cleared by centrifugation at 12 000 rpm for 20 min at 4°C. Protein concentration was measured using the BCA assay (Pierce Technologies, Rockford, IL, USA) according to manufacturer's specifications. Protein extracts were subjected to Western blot analyses as reported (26). Antibodies used were SLU7 (Novus, Biologicals, Centennial, CO, USA NBP2-20403; 1:1000), DNMT1 (Cell Signaling, Danvers, MA, USA 50325; 1:1000), DNMT1

(Abcam, Cambridge, UK ab13537; 1:1000), DNMT3B (Cell Signaling, 3598; 1:1000), DNMT3A (Cell Signaling, 672595: 1:1000), PCNA (Santa Cruz Biotechnology, Santa Cruz, CA, USA SC56; 1:1000), H3S10P (Cell Signaling, 97015; 1:1000), HDAC1 (Santa Cruz, 7872; 1:1000), G9a (Cell Signaling, 33065; 1:1000), UHRF1 (Cell Signaling, 123875; 1:1000), Acetylated-lysine (Cell Signaling, 9441S; 1:1000), HAUSP (USP7) (Cell Signaling, 4833S;1:1000), Histone 3 (Millipore, Burlington, MA, USA, 05–928; 1:3000), Cyclin A (Santa Cruz, sc-751; 1:1000), H3K9ac (Millipore, 06-911; 1:2000). Blots were probed with anti-ACTIN (Sigma-Aldrich, A2066; 1:6000) or anti-GAPDH (Cell Signaling, 21185: 1:6000), or stained with Ponceau S solution (Sigma-Aldrich) to demonstrate equal protein loading. Representative images are shown throughout the study.

Immunoprecipitation

For immunoprecipitation (IP) assays, cells were lysed in IP buffer with protease-phosphatase inhibitors (20 mM Tris–HCl pH 8, 137 mM NaCl, 1% NP-40 and 2 mM EDTA) at 4°C for 30 min under constant rotation. Lysates were cleared by centrifugation at 12 000 rpm for 20 min at 4°C. Protein concentration was determined and 800–1000 μg of proteins were pre-cleared with 25 μl of Dynabeads G (Invitrogen) for 2 h under constant rotation at 4°C. In parallel, 5 μg of primary antibody and the corresponding control IgG were incubated with 20 μl of Dynabeads G for 2 h under constant rotation at room temperature. Finally, the Dynabeads with the antibodies were washed three times with citrate phosphate pH5 buffer with 0,01% Tween-20 and incubated with the pre-cleared samples overnight at 4°C under constant rotation. The day after, samples were washed three times with PBS (Gibco-Life Technology, Waltham, MA, USA) with proteases and phosphatases inhibitors and boiled in Laemmli buffer at 95°C for 5 min. Pelleted beads were separated using a magnetic separation rack and the supernatants were used for subsequent Western blot analysis. Antibodies used were: SLU7 (BD Bioscience, Franklin Lakes, NJ, USA 612604; 5 μg), DNMT1 (Abcam, ab13537; 5 μg), IgG mouse (Santa Cruz, SC2025; 5 μg).

Subcellular fractionation

Subcellular fractionation was performed to separate cytoplasm, nucleoplasm and chromatin-bound fraction for subsequent immunoprecipitation analysis. It was carried out using the NE-PER Nuclear and Cytoplasmic Extraction Reagents Kit (Thermo Fisher Scientific, #78835) following manufacturer's instructions. Nuclear extracts were incubated in NP40 buffer (15 mM Tris–HCl pH 7,4, 1 mM EDTA, 250 mM NaCl, 1 mM $MgCl_2$, 10% Glycerol, 0,1% NP40 and protease-phosphatase inhibitors) at 4°C for 30 min under constant rotation. The nucleoplasm and the chromatin bound fraction were separated by centrifugation at 2500 rpm for 10 min at 4°C. Chromatin bound fraction was then treated with 250 U/ul benzonase (Sigma-Aldrich) in a buffer containing 20 mM Tris–HCl pH 7,4, 60 mM NaCl, 1,5 mM $MgCl_2$, 0,1% NP40 and protease-phosphatase inhibitors, at 4°C for 30 min and then centrifuged at 13 000 rpm for 10 min at 4°C to get the supernatant.

Histone extraction

Histones were isolated as previously described (29). Briefly, cells were lysed in a buffer containing 10 mM Tris–HCl pH 7.4, 10 mM NaCl and 3 mM $MgCl_2$. After centrifugation at 2500 rpm for 10 min at 4°C supernatants were removed and pellets were lysed in the previous buffer but containing 0.5% NP40 on ice for 10 min with gentle stirring. Nuclei were pelleted by centrifugation at 2500 rpm for 10 min at 4°C and resuspended in 5 mM $MgCl_2$ and 0.8 M HCl. Nuclei were incubated in this buffer during 30 min at 4°C to extract the histones. Samples were then centrifuged at 14 000 rpm for 10 min at 4°C to pellet debris and supernatants were transferred to a clean tube where trichloroacetic acid (TCA) 50% was added to precipitate the histones. After washing the pellets with acetone, they were air-dried and resuspended in 100 mM Tris–HCl pH 7.5, 1 mM EDTA and 1% SDS. The histone concentration in the extract was measured using the BCA assay (Pierce Technologies, Rockford, IL, USA) according to manufacturer's specifications.

Animal experiments

All animal used received humane care according to the criteria outlined in the 'Guide for the care and Use of Laboratory Animals' prepared by the National Academy of Sicences and published by the National Institutes of Health (NIH publication 86-23 revised 1985). Protocols were also approved and performed according to the guidelines of the Ethics Committee for Animal Testing of the University of Navarra (CEEA-062/16) and Navarra Government (GN 2016/325511).

C57BL/6J 8-week-old male mice (Harlan, Indianapolis, IN, USA) were injected i.v. with control (AAV-Ren) and liver expressing shSLU7 (AAV-shSLU7) adenoassociated virus (1×10^{11} pfu) constructed, produced and purified as described (16). Experiments were performed 15 days after AAV injection with at least 5 animals per group and were repeated at least twice. Mice were maintained under fed conditions with regular chow. Two-thirds partial hepatectomy (PH) and sham operations were performed as described previously (28). Animals were killed 24, 48 or 72 h after surgery. For induction of acute and chronic liver damage, mice were i.p. injected with a 1:1 mixture of CCl_4 in corn oil (0,6 μL CCl_4/g body weight, i.p.) twice a week during 1 week or during 5 weeks, respectively as described (30,31). Twenty-four or forty-eight hours after the last injection mice were humanely sacrificed and livers were collected for RNA and protein extraction.

Statistical analysis

Statistical analysis was performed using the GraphPad Prism software (version 8). Data were represented as mean ± standard error of the mean (SEM). Statistically significant differences were estimated by applying two-sided unpaired Student's *t*-test or Mann–Whitney *U*-test according to sample distribution. All experiments were performed

at least three times in duplicate. A *P*-value <0.05 was considered significant.

RESULTS

SLU7 knockdown leads to aberrant DNA methylation

In view of the fundamental roles played by SLU7 in the preservation of genome integrity and the correct cell cycle division, we decided to explore whether this factor could also impact on DNA methylation inheritance. We first measured the levels of 5-methylcytosine (5-mC) in control and SLU7 depleted cancer cell lines using a global DNA methylation assay. Remarkably, SLU7 knockdown significantly decreased global 5-mC content in the hepatoma cell lines PLC/PRF/5 and HepG2, the colorectal carcinoma cell line HCT116 and the lung cancer cell line H358 (Figure 1A). The loss of 5-mC upon SLU7 knockdown was confirmed by immunofluorescence, using a labeled anti-5-mC antibody, in PLC/PRF/5 and HepG2 SLU7 cells (Figure 1B). To better characterize the effect of SLU7 knockdown on DNA methylation we performed reduced representation bisulfite sequencing (RRBS) to determine genome-wide CpG sites methylation changes. DNMT1 knockdown was performed in parallel as control and for comparison (Supplementary Figure S1A). Considering a methylation difference higher than 0.6 we identified a total of 13.128 and 9.143 differentially methylated CpGs in siDNMT1 and siSLU7 transfected PLC/PRF/5 cells respectively (Figure 1C and D). In both cases, these CpGs were homogeneously distributed amongst all the autosomal and sex chromosome pairs (Supplementary Figure S1B). According with published data on DNMT1 activity inhibition (32) or even *Dnmt1* knockout (33), among the differentially methylated CpGs, 84% were hypomethylated and 16% hypermethylated in the absence of DNMT1 (Figure 1C). Those percentages were 62% and 38% respectively in the absence of SLU7 (Figure 1D). Interestingly, the knockdown of both genes induced the hypomethylation of scattered CpGs distributed along the genome, with a ratio of ∼1.2 CpGs per gene (Figure 1E). Moreover, although only a small proportion of CpGs were located within CpG islands (CGIs; Figure 1E), 63% in siSLU7 and 46% in siDNMT1 were at promoter regions. When we compared the list of genes hypomethylated after SLU7 and DNMT1 knockdown we found 2.104 common genes (Figure 1F, top). However, the overlap of single hypomethylated CpGs only included 993 sites (Figure 1F, bottom). Altogether, these findings suggest that SLU7 is important for DNA methylation process. More specifically, the transient depletion of SLU7 results in a bulk hypomethylation of scattered single CpGs along the genome, apparently without impacting on specific gene regions, comparable to what is found in DNMT1 depleted cells.

SLU7 knockdown activates gene expression silenced by DNA methylation

As mentioned above, the majority of the differentially methylated CpGs were scattered along the genome, however, bioinformatic analyses identified a set of genes for which more than 6–9 CpGs within the same genomic region were hypomethylated in both siSLU7 and siDNMT1 PLC/PRF/5 cells. This was the case of the E3 ubiquitin-protein ligase *TRIM47* a member of the tripartite motif-containing (TRIM) proteins superfamily, the trefoil factor *TFF3*, and the membrane-associated guanylate kinase protein disc large homolog *DLG3* (Figure 2A). To validate these RRBS results, we carried out methylation-specific PCR (MSP) analyses at these identified regions. As shown in Figure 2B, we confirmed the loss of DNA methylation in *TRIM47* and *DLG3* and the increase of DNA demethylation in *TFF3* upon both SLU7 and DNMT1 knockdown in PLC/PRF/5 cells. Among the three regions analyzed, those from *TRIM47* and *DLG3* were located within CGIs in the promoter and exon 1, whereas that of *TFF3* was at the gene body and not within a CGI (Supplementary Figure S2A). Promoter hypermethylation is associated with gene silencing whereas promoter demethylation is linked to gene activation (34). To further validate the extent of SLU7 impact on DNA methylation, we tested whether SLU7 knockdown may also activate the expression of methylated genes. We thus tested the expression of *TRIM47* and *DLG3* and found that SLU7 silencing resulted in the upregulation of both mRNAs (Figure 2C).

We next studied the impact of SLU7 knockdown in the regulation of genes characteristically hypermethylated and silenced in cancer such as *RASSF1A* (35) and *GNMT* (36,37). SLU7 silencing in PLC/PRF/5 cells induced DNA demethylation and gene upregulation of both genes (Figure 2D and E). *GNMT* promoter demethylation and gene reactivation after SLU7 knockdown were also confirmed in HepG2 and HCT116 cells (Figure 2F and G). Furthermore, it has been described that global DNA hypomethylation induces innate and acquired immunity (38) through different mechanisms, including the induction of expression of endogenous retroviruses (ERVs) (39) and cancer-testis antigens (CTAs) (40). Interestingly, we observed the induction of *MAGE-A1*, *MAGE-A7* and *envFc2* upon SLU7 silencing in the four human cancer cell lines analyzed (Figure 2H). Similarly, up-regulation of the *H19* imprinted gene was also observed upon SLU7 knockdown (Figure 2H). Comparable data were observed in PLC/PRF/5 cells treated with the demethylating agent 5-Aza-2′-deoxycytidine (Supplementary Figure S2B).

Collectively, these findings demonstrate that SLU7 knockdown is associated with the up-regulation of genes transcriptionally silenced by DNA methylation, and therefore highlight the contribution of SLU7 to epigenetic regulation of gene expression.

SLU7 knockdown reduces DNMT1 protein levels in proliferating cells

Next, we set out to characterize the mechanisms involved in the regulation of DNA methylation by SLU7. As SLU7 silencing mimics DNMT1 knockdown, we decided to evaluate the level of expression of DNMT1 upon SLU7 knockdown. Interestingly, depletion of SLU7 in PLC/PRF/5 cells resulted in a dramatic and consistent decrease of DNMT1 protein levels, reaching a 60% reduction as soon as 24h after siSLU7 transfection, an effect that was maintained

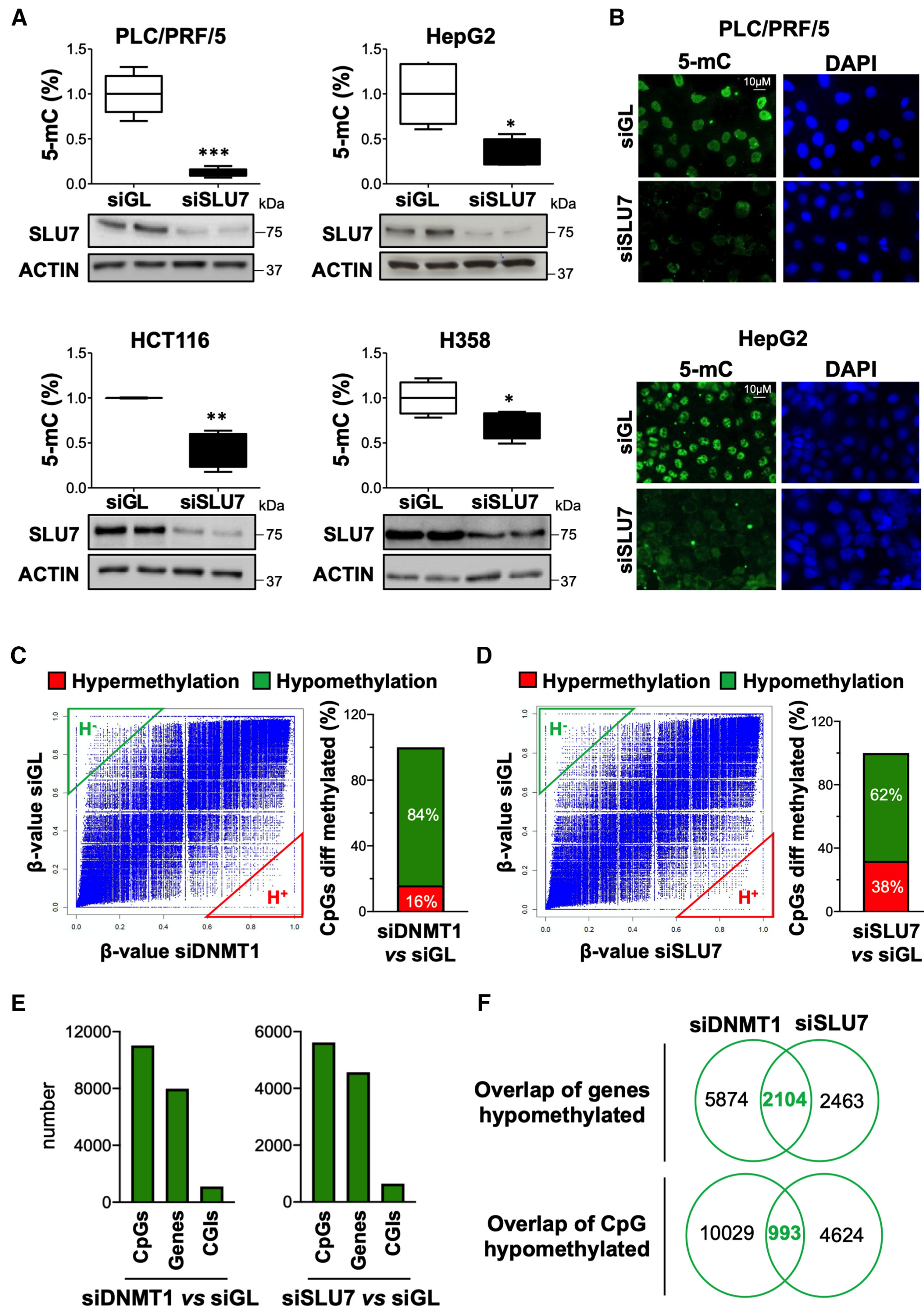


Figure 1. SLU7 knockdown compromises DNA methylation. (**A**) SLU7 knockdown induces a decrease in 5-methyl-cytosine (5-mC) content. DNA methylation levels were analyzed by ELISA 72 h after transfection with siSLU7 or control siGL in the human hepatoma cell lines PLC/PRF/5 and HepG2, the colorectal carcinoma cell line HCT116 and the lung cancer cell line H358. The percentage of 5-mC content is indicated. $*P < 0.05$, $**P < 0.01$, $***P < 0.001$. Western blot analysis confirms SLU7 knockdown. ACTIN was used as loading control. At least three independent experiments in duplicate were performed for each cell line. (**B**) Representative images of 5-methyl cytosine (5-mC) immunodetection (in green) in PLC/PRF/5 (top) and HepG2 (bottom) cells 72 h after transfection with siSLU7 or control siGL. Nuclei were stained with DAPI (in blue). Scale bar: 10 μm. (**C**, **D**) Scatter plots of reduced representation bisulfite sequencing (RRBS) DNA methylation β-values of siGL *versus* siDNMT1 (C) or siGL *versus* siSLU7 (D) PLC/PRF/5 cells 72 h after transfection. Fold change methylation differences higher than 0.6 were highlighted within a green triangle (hypomethylated) or a red triangle (hypermethylated). Graphs represent the percentage of hypomethylated (FC < 0,6) and hypermethylated (FC > 0,6) CpGs. (**E**) Number of single CpGs, genes with CpGs and CpGs within CpG islands (CGIs) found hypomethylated (FC < 0.6) in PLC/PRF/5 cells 72 h after transfection with siDNMT1 (left) or siSLU7 (right) compared to control siGL cells. (**F**) Venn diagram of the overlap between genes (top) or CpGs (bottom) found hypomethylated (FC < 0,6) in siSLU7 and siDNMT1 PLC/PRF/5 transfected cells when compared with control siGL cells.

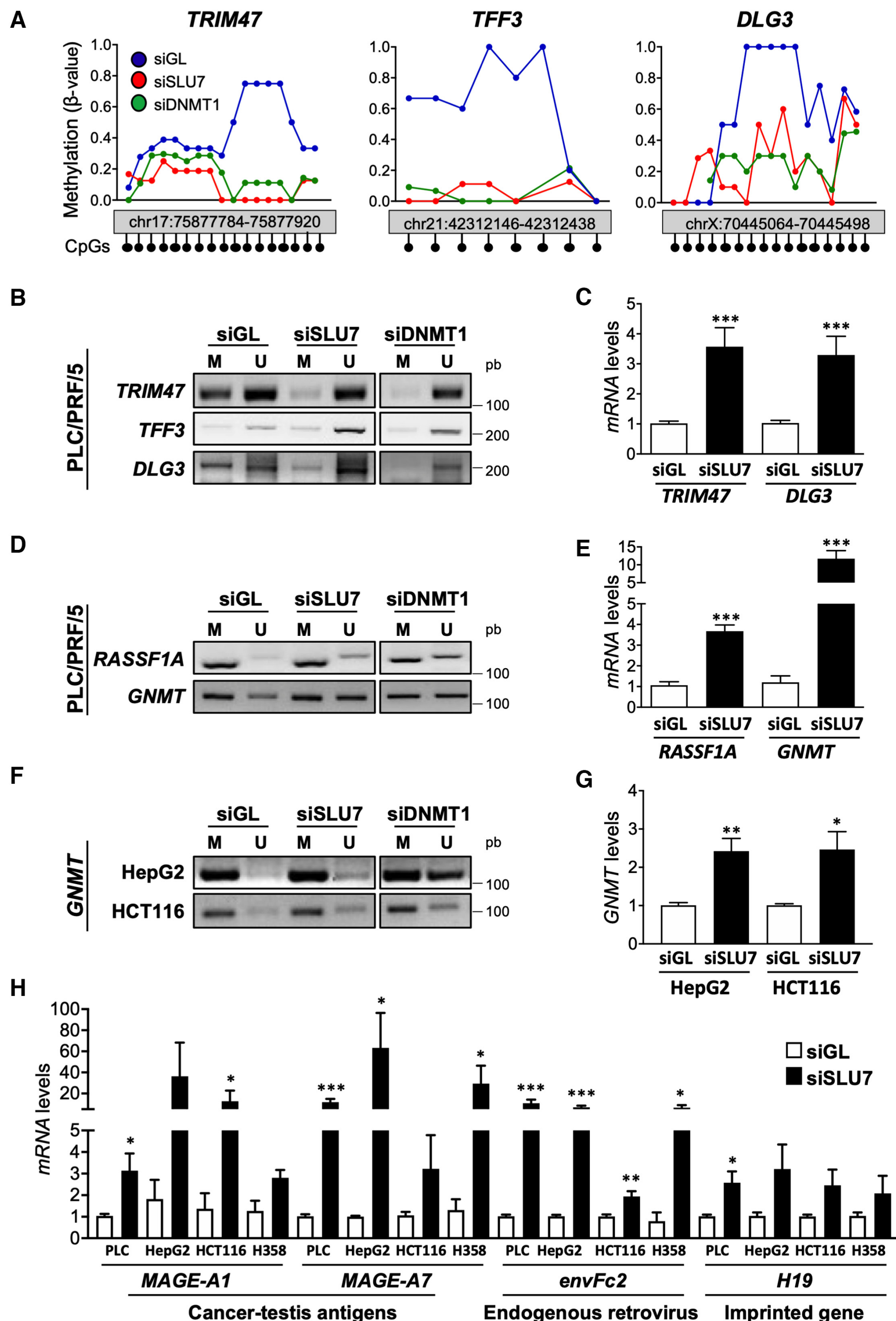


Figure 2. SLU7 knockdown activates transcription of genes silenced by DNA methylation. **(A)** Graphs reporting DNA methylation levels (β-values obtained by Reduced Representation Bisulfite Sequencing (RRBS) analysis) of *TRIM47*, *TFF3* and *DLG3* gene regions found hypomethylated in both SLU7 (siSLU7, in red) and DNMT1 (siDNMT1, in green) knockdown PLC/PRF/5 cells compared to control siGL (in blue) cells 72 h after transfection. **(B)** Representative methylation-specific PCR (MSP) results for DNA methylation analysis of *TRIM47*, *TFF3* and *DLG3* regions shown in (A). U: unmethylated and M: methylated. **(C)** Expression by RT-qPCR of *TRIM47* and *DLG3* in PLC/PRF/5 cells 72 h after transfection with siSLU7 or control siGL. (**D**, **E**) DNA methylation analysis by MSP of *RASSF1A* and *GNMT* promoter regions (D) and *RASSF1A* and *GNMT* expression by RT-qPCR (E) in PLC/PRF/5 cells 72 h after transfection with siSLU7, siDNMT1 or control siGL. U: unmethylated and M: methylated. (**F**, **G**) DNA methylation analysis by MSP of *GNMT* promoter region (F) and *GNMT* expression by RT-qPCR (G) in HepG2 and HCT-116 cells 72 h after transfection with siSLU7, siDNMT1 or control siGL. U: unmethylated and M: methylated. (**H**) Expression by RT-qPCR of cancer testis antigens (*MAGE-A1 and MAGE-A7*), endogenous retroviruses (*envFc2)* and imprinted genes (*H19)* in PLC/PRF/5, HepG2, HCT116 and H358 cells 72 h after transfection with siSLU7 or control siGL. *RPLP0* expression was used as housekeeping gene in (C, E, G, H). *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$. All experiments were performed at least three times with biological duplicates per condition.

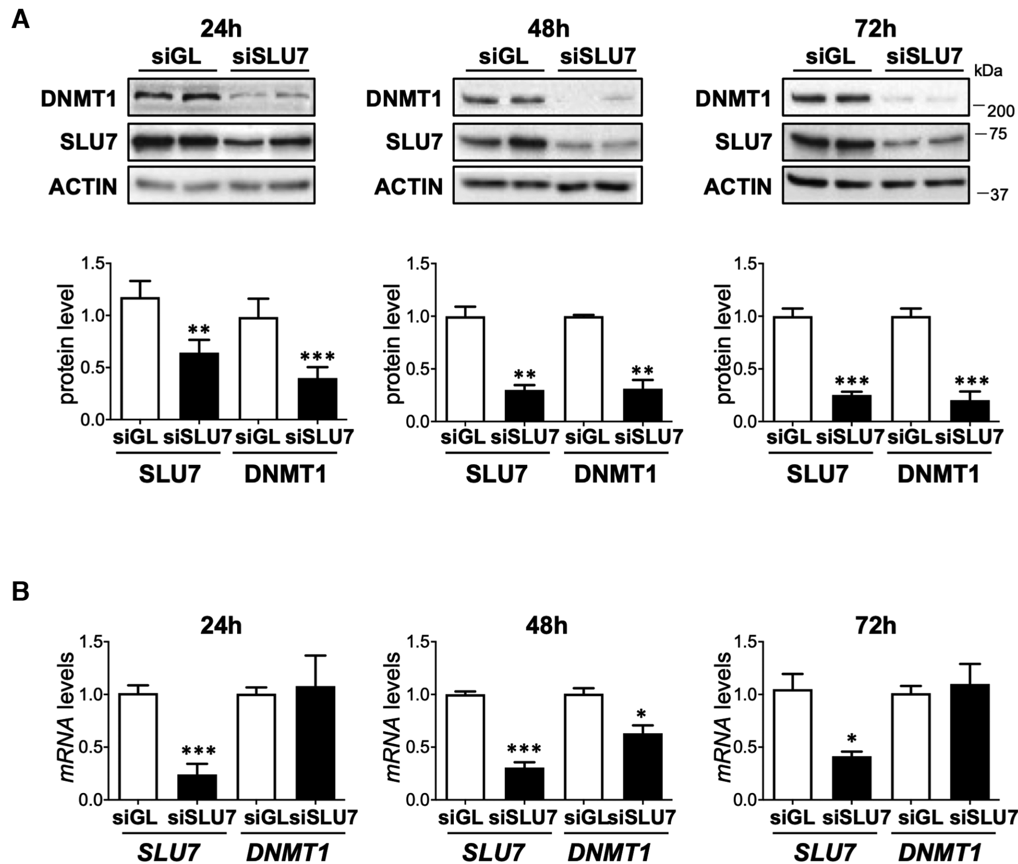


Figure 3. SLU7 knockdown decreases DNMT1 protein levels in proliferating cells. (**A**) Representative Western blots (top) of DNMT1 and SLU7 in PLC/PRF/5 cells 24, 48 and 72 h after transfection with siSLU7 or control siGL. ACTIN expression was shown as loading control. SLU7 and DNMT1 protein levels were quantified using Image J software from at least three independent experiments with biological duplicates per condition (bottom). (**B**) Histograms reporting *SLU7* and *DNMT1* mRNA levels by real time PCR in the same experiments analyzed in panel A. *RPLP0* expression was used as housekeeping gene in (B). $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$.

or even increased after 48h and 72h (Figure 3A). Quantitative RT-PCR analysis showed that the quick reduction of DNMT1 protein was not associated with a decrease in *DNMT1* mRNA levels (Figure 3B), suggesting that SLU7 directly impacts on DNMT1 protein stability. We also evaluated the levels of the other known DNA methyltransferases, DNMT3A and DNMT3B, and found that changes in DNMT3A and DNMT3B mRNA and/or protein levels upon SLU7 depletion were less consistent and occurred at later time points (Supplementary Figure S3A and B). Based on these results, we decided to focus our study on the regulation of DNMT1 by SLU7.

As mentioned before, SLU7 is a splicing regulator essential in the second catalytic step during the pre-mRNA splicing process (41). Furthermore, we have recently shown that reduced SLU7 expression may lead to the emergence of truncated protein forms due to splicing alterations (17). Therefore, it was important to rule out the possibility that the reduced levels of DNMT1 protein observed after SLU7 knockdown were associated with the generation of smaller DNMT1 protein forms by alternative splicing. Both, the PCR analysis performed with primers located on exon 1 and exon 17, and the Western blot analyses performed with antibodies directed against two different epitopes of DNMT1 protein (Supplementary Figure S3C and D), revealed the absence of truncated DNMT1 isoforms after SLU7 silencing.

These observations suggested the involvement of other mechanisms apparently independent of the activity of SLU7 as a splicing regulator. It has been described a cell-cycle phase-dependent regulation of DNMT1 levels (42,43) and we have previously shown that SLU7 knockdown induces a significant cell cycle arrest at G2/M phase in cancer cell lines of different origins (17). We therefore wondered whether the observed reduction in DNMT1 upon SLU7 knockdown might be attributable to the arrest of the cells at G2/M phase. However, we did not observe changes in DNMT1 protein in PLC/PRF/5 cells arrested at G2/M after overnight nocodazole treatment (Supplementary Figure S4A). This allowed us to conclude that the reduction in DNMT1 protein levels observed upon SLU7 knockdown is independent of SLU7-induced G2/M arrest. We then evaluated whether DNMT1 reliance on SLU7 expression was maintained along the different phases of the cell cycle. To do so, we arrested PLC/PRF/5 cells at G1, S and G2/M phases by lovastatin, aphidicolin and nocodazole treatment, respectively (Supplementary Figure S4B). Similar to non-synchronized cells, decreased level of DNMT1

protein was found at G1, S and G2/M arrested cells upon SLU7 depletion (Supplementary Figure S4C). We also arrested cells at G0 resting phase through cell contact inhibition by confluence together with FBS starvation before siGL and siSLU7 transfection. As already described (42), it is important to note that DNMT1 levels were significantly reduced when cells were arrested at G0 (Supplementary Figure S4D). However, in this condition, SLU7 knockdown did not significantly affect DNMT1 levels (Supplementary Figure S4D). Finally, to discriminate whether DNMT1 loss triggered by SLU7 depletion was linked to the transformed status of PLC/PRF/5 cells, or whether it was associated with the proliferating state of the cells, we tested DNMT1 levels after SLU7 knockdown in the widely used non-transformed HEK293T cell line. Similar to cycling PLC/PRF/5 cells, we observed a significant reduction in DNMT1 protein contents in HEK293T cells after SLU7 knockdown (Supplementary Figure S4E). Moreover, when HEK293T cells were arrested at G0, SLU7 depletion did not affect DNMT1 levels (Supplementary Figure S4E). All these data demonstrate that DNMT1 protein dependency on SLU7 levels is a general event in proliferating cells.

SLU7 is required to maintain DNMT1 protein stability in proliferating cells of very different origins

We next studied whether DNMT1 dependency on SLU7 levels might occur in different cell types. To this end, we analyzed DNMT1 protein levels after SLU7 knockdown in a wide panel of human cancer cell lines from different origins, including the hepatoma cell line HepG2, the colorectal carcinoma cell line HCT116, the lung cancer cell lines H358 and A549, the cervical carcinoma cell line HeLa, the breast carcinoma cell line MCF7, the glioblastoma cell line U87 and the melanoma cell line A375. In all the cell lines examined, independently of their origin, SLU7 knockdown drastically reduced DNMT1 protein levels (Figure 4A). To confirm specificity, these data were confirmed using two independent SLU7 siRNAs in a set of cell lines (Supplementary Figure S5A). Again, and in agreement with Figure 3B, *DNMT1* mRNA levels were not affected by SLU7 knockdown (Supplementary Figure S5B). On the other hand, ectopic overexpression of SLU7 resulted in the elevation of DNMT1 protein contents in PLC/PRF/5, HepG2 and HCT116 cells (Figure 4B and Supplementary Figure S5C), and had no effect on DNMT1 mRNA levels (Figure 4C).

Next, we conducted a series of experiments to elucidate the fundamental processes through which SLU7 could control DNMT1 protein steady state levels. First, we tested if reduced SLU7 expression could lead to DNMT1 protein degradation. To this end, we treated different cell lines with the proteasome inhibitor MG-132 in the face of SLU7 knockdown. We observed that the decrease in DNMT1 protein observed after SLU7 silencing was blunted by the addition of MG-132 in all the cell lines analyzed (PLC/PRF/5, HCT116 and HEK293T; Figure 4D). On the other hand, in order to exclude that SLU7 was required for DNMT1 protein synthesis, we treated siGL and siSLU7 cells with the protein synthesis inhibitor cycloheximide. We found that SLU7 knockdown resulted in DNMT1 downregulation even when protein synthesis was inhibited (Supplementary Figure S5D). Taken together, these data indicate that SLU7 enhances DNMT1 protein stability, preventing its degradation, through mechanisms that are conserved among cell lines of different origins, including non-transformed cells.

SLU7 is a new partner essential for the stability of the DNMT1/UHRF1/G9a complex

Our results so far demonstrate that SLU7 is required to secure DNMT1 protein stability and function. As a first step towards understanding the mechanism by which SLU7 stabilizes DNMT1, we sought whether SLU7 and DNMT1 could physically interact. As illustrated in Figure 5A, co-immunoprecipitation assays performed in different cell lines, both with anti-SLU7 and anti-DNMT1 antibodies, revealed the interaction between endogenous SLU7 and DNMT1 proteins (Figure 5A). Moreover, and considering that upon SLU7 silencing the 5-mC levels drastically fell down (Figure 1), we analyzed SLU7/DNMT1 interaction at the chromatin level, as well as the interaction of SLU7 with other members of the chromatin protein complex responsible of DNA methylation maintenance which include the adaptor protein UHRF1 and the histone methyl-transferase G9a (44–46). Firstly, we prepared different cellular fractions, including cytoplasm, nucleoplasm and chromatin-bound fractions. As expected, we found that all the proteins analyzed were predominantly localized at the chromatin-bound fraction (Figure 5B input). Co-immunoprecipitation assay on chromatin fraction confirmed the interaction between SLU7 and DNMT1, and strikingly revealed that, as observed for DNMT1, SLU7 also interacted with G9a and UHRF1 (Figure 5B). Since SLU7 knockdown reduced DNMT1 protein levels, we wondered whether the level of the other members of the complex would also depend on SLU7. We therefore analyzed the levels of DNMT1, G9a, UHRF1, together with PCNA and histone H3 as controls in the chromatin-bound fraction, and found that knockdown of SLU7 markedly reduced the association of DNMT1, G9a and UHRF1 with the chromatin (Figure 5C). These results show that SLU7 not only interacts with DNMT1, G9a and UHRF1 in the chromatome but also that SLU7 is required to maintain their association with chromatin.

To further explore the relevance of SLU7 in maintaining DNMT1 levels, we next examined whether DNMT1 stability was also governed by other members of the complex. Consistent with previous observations (44,45,47), we found that knockdown of G9a and UHRF1 did not affect DNMT1 abundance both in PLC/PRF/5 and HCT116 cells (Figure 5D), highlighting the relevant and non-redundant role of SLU7. Moreover, the effect of SLU7 depletion on DNMT1 stability cannot be attributed to its release from chromatin, as it has been described that UHRF1 silencing reduces the association of DNMT1 with chromatin without affecting its levels (45). Interestingly, our results evidenced that SLU7 is required to maintain G9a and UHRF1 global levels (Figure 5D), suggesting that SLU7 knockdown results in a so drastic disruption of the protein

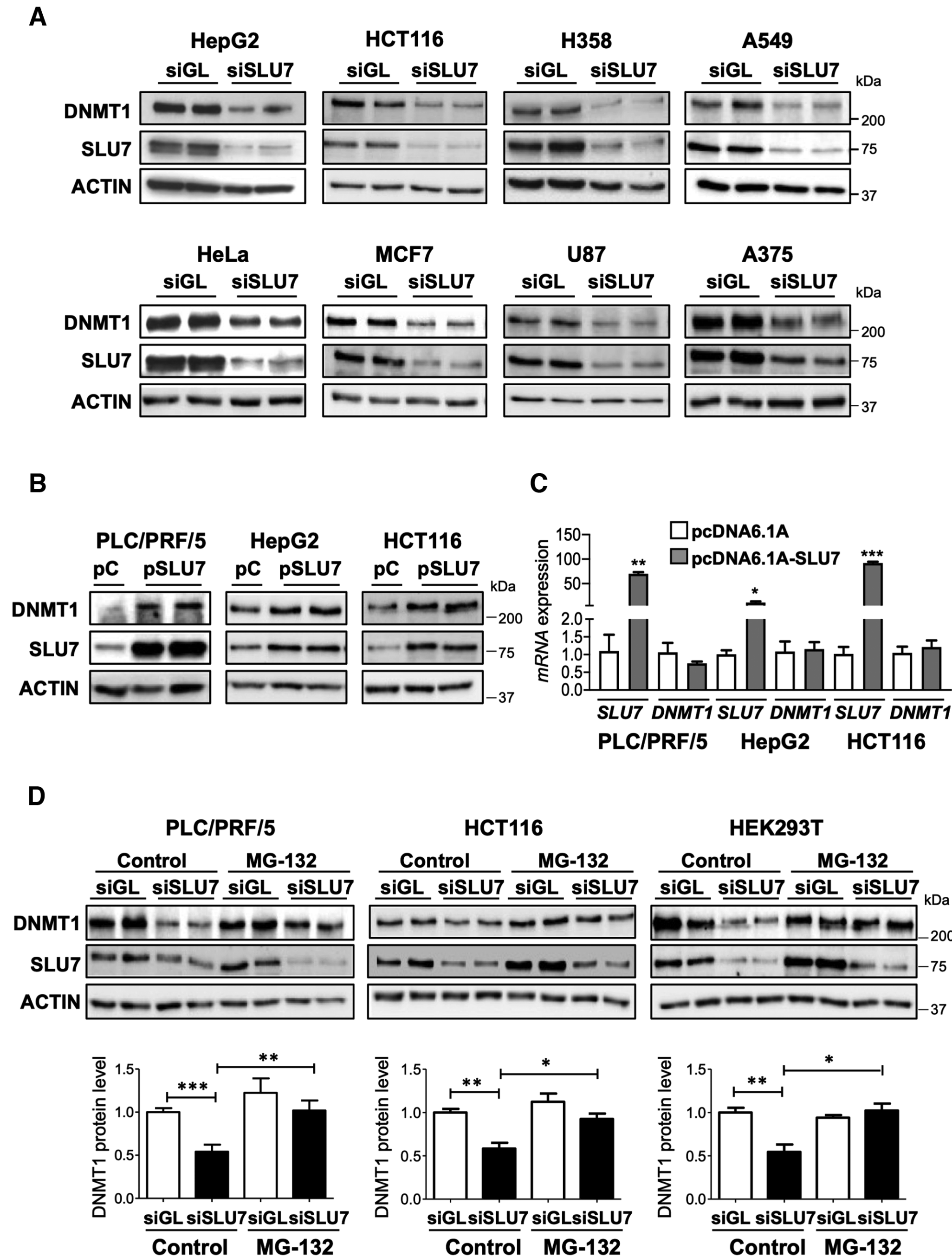


Figure 4. SLU7 is required for DNMT1 protein stability in a wide panel of cell lines from different origins. (**A**) Western blots of DNMT1 and SLU7 in HepG2, H358, A549, HCT116, HeLa, MCF7, U87 and A375 cancer cell lines 48h after transfection with siSLU7 or control siGL. ACTIN expression was shown as loading control. (**B**, **C**) pcDNA6.1A empty plasmid (pC) or the corresponding plasmid to express SLU7 (pSLU7) were transiently transfected for 48 h in PLC/PRF/5, HepG2 and HCT116 cells followed by Western blot (B) and real time PCR (C) analyses of DNMT1 and SLU7. ACTIN expression was shown as loading control in (B). *RPLP0* expression was used as housekeeping gene in (C). (**D**) PLC/PRF/5 and HEK293T cells transfected with siGL or siSLU7 for 48 h and treated with 10 μM MG-132 for the last 20 h were harvested to analyze DNMT1 and SLU7 expression by Western blot. HCT116 cells were transfected with siGL or siSLU7 for 36 h and treated with 20 μM MG-132 for the last 12 h before harvesting. The expression of ACTIN was analyzed as loading control. DNMT1 protein levels were quantified using ImageJ software from at least three independent experiments with biological duplicates per condition (bottom). $*P < 0.05$, $**P < 0.01$, $***P < 0.001$.

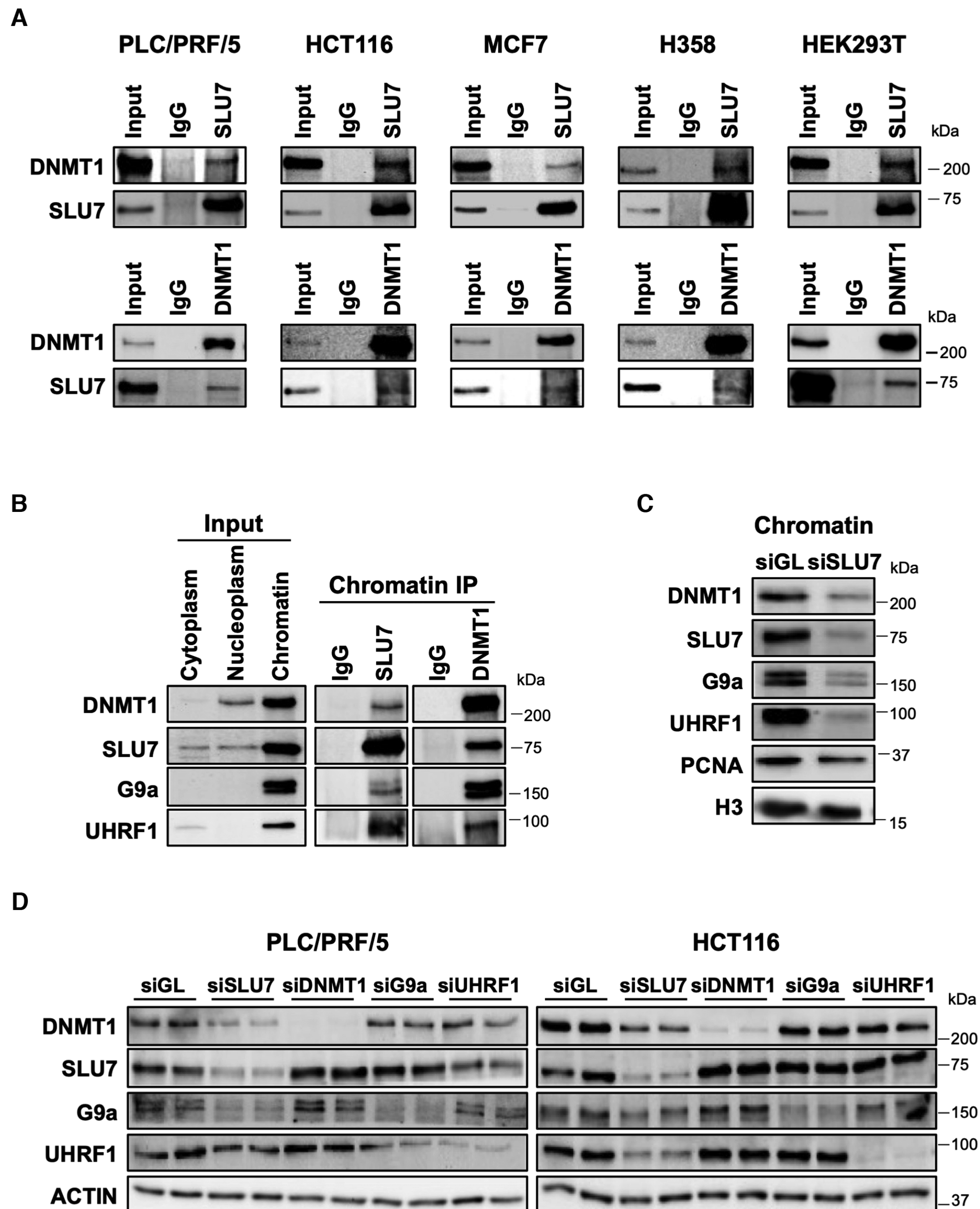


Figure 5. SLU7 is part of the methylation complex DNMT1/UHRF1/G9a and it is required for its stability on the chromatin. (**A**) SLU7 and DNMT1 were coimmunoprecipitated with each other in PLC/PRF/5, HCT116, MCF7, H358 and HEK293T cells. Pre-immune IgG immunoprecipitation and Inputs were shown as control. (**B**) Chromatin-bound cell extracts from PLC/PRF/5 cells were immunoprecipitated with anti-SLU7 and anti-DNMT1 antibodies or pre-immune IgG and blotted with DNMT1, SLU7, G9a and UHRF1 antibodies. Western blot on inputs from the different subcellular fractions were shown as control. (**C**) Chromatin extract for PLC/PRF/5 cells 48 h after transfection with siSLU7 were analyzed by western blot with antibodies against DNMT1, SLU7, G9a and UHRF1. HISTONE 3 and PCNA were used as loading control. (**D**) DNMT1, SLU7, G9a and UHRF1 protein levels were analyzed by western blot in PLC/PRF/5 and HCT116 cells 48 h after transfection with control siGL, siSLU7 siDNMT1, siG9a and siUHRF1. ACTIN was used as loading control.

complex that compromises the stability of all the components.

SLU7 knockdown induces DNMT1 acetylation

Many of the proteins interacting with DNMT1 are implicated in adding or removing post-translational modifications affecting its stability, therefore we decided to explore whether SLU7 could modulate DNMT1 covalent modifications. The post-translational modifications that regulate DNMT1 protein stability include phosphorylations, methylations and acetylations (48–52). For instance, S143 phosphorylation (50) stabilizes DNMT1, while K142 monomethylation (53) and several acetylations (52,54,55) destabilize this protein. The mentioned phosphorylation and methylation reactions occur at the DNMT1 region lost in the widely used HCT116 *DNMT1*$^{-/-}$ cell line which expresses a truncated DNMT1 generated by homozygous deletion of Ex3-Ex5 (56) (Supplementary Figure S6A and B). As shown in Figure 6A, and similar to the wild type cell line (Figure 4A), SLU7 knockdown in HCT116 *DNMT1*$^{-/-}$ cells reduced DNMT1 protein levels as fast as 24 h after siSLU7 transfection (Figure 6A) without affecting its mRNA (Figure 6B). These results suggest that SLU7 regulation of DNMT1 stability is independent of the post-translational modifications occurring in the amino-terminal region, as it is absent in this truncated DNMT1 protein.

Ruling out the implication of the described phosphorylation and methylation modifications, we next evaluated DNMT1 acetylation upon SLU7 knockdown. As shown in Figure 6C, immunoprecipitation of endogenous DNMT1 in control and SLU7 knockdown PLC/PRF/5 and HEK293T cells demonstrated an increase in DNMT1 acetylation after SLU7 depletion (Supplementary Figure S6C). It has been described that DNMT1 acetylation impairs its interaction with USP7 (54). Accordingly, we observed a decrease in USP7-DNMT1 interaction in SLU7 silenced cells (Figure 6C). These results suggest that SLU7 prevents DNMT1 acetylation. As it has been shown that the histone deacetylase 1 (HDAC1) deacetylates and protects DNMT1 from proteasomal degradation (51,57), we decided to examine HDAC1 levels in SLU7 knockdown cells, observing that HDAC1 levels were only slightly reduced (Figure 6D). However, in parallel, we confirmed (51,57) that HDAC1 inhibition with a specific HDAC1 siRNA decreased DNMT1 protein levels (Figure 6D and Supplementary Figure S6D) similarly to SLU7 depletion. To exclude a role of HDAC1 reduction in SLU7 knockdown-mediated DNMT1 degradation, we tested the effects of HDAC1 overexpression. As shown in Figure 6E, the ectopic expression of HDAC1 did not impair DNMT1 loss upon SLU7 knockdown (Figure 6E and Supplementary Figure S6D). The fact that overexpression of HDAC1 in SLU7 depleted cells did not protect DNMT1 from degradation, led us to hypothesize that SLU7 may be necessary for the interaction between DNMT1 and HDAC1, facilitating HDAC1-mediated DNMT1 deacetylation and stabilization. In fact, co-immunoprecipitation assays with both, anti-SLU7 and anti-DNMT1 antibodies, revealed the interaction between endogenous SLU7, DNMT1 and HDAC1 proteins (Figure 6F). More importantly, downregulation of SLU7 significantly reduced the interaction between DNMT1 and HDAC1 (Figure 6G and Supplementary Figure S6E). All these data suggest that SLU7 is required for HDAC1 biding to DNMT1, preventing DNMT1 acetylation, and averting its proteasomal degradation.

SLU7 is required for DNMT1 stability *in vivo*

Having shown that SLU7 is required to maintain DNMT1 stability in multiple cell lines *in vitro*, we wondered whether SLU7 was also necessary to maintain DNMT1 protein stability *in vivo*. We took advantage of the *in vivo* proliferating scenario of liver regeneration after partial hepatectomy, where DNMT1 gains functional prominence (33). We carried out 2/3 partial hepatectomy (PH) in mice 21 days after knocking down SLU7 expression with an adenoassociated virus (AAV) encoding a SLU7-specific shRNA (AAV-shSLU7) (16,17) (Supplementary Figure S7A). As described (58), *Dnmt1* mRNA levels were similarly induced after 24 h, 48 h and 72 h of PH in both control AAV-Ren and AAV-shSLU7 mice (Figure 7A). However, the increase in DNMT1 protein levels was compromised in AAV-shSLU7 mice compared with control AAV-Ren mice both at 48 h and 72 h after PH (Figure 7B). Indicative of alterations in global DNA methylation in the context of SLU7 knockdown during hepatocellular regeneration, we observed the induction of expression of the *H19* imprinted gene in AAV-shSLU7 mice (Supplementary Figure S7B). To strengthen these observations, we studied DNMT1 SLU7 dependency in two other models of hepatic proliferation associated with acute and chronic liver injury (Supplementary Figure S7C and D). In both cases, the level of DNMT1 protein induced upon treatment with the hepatotoxin CCl_4 was lower in AAV-shSLU7 than in control AAV-Ren-infected mice (Figure 7C). To evaluate whether the mechanisms identified *in vitro* also operate *in vivo*, we checked whether liver DNMT1 and SLU7 interacted with each other after PH and CCl_4 treatment. *In vivo* co-immunoprecipitation assays revealed the interaction between DNMT1 and SLU7 and more importantly, their association with HDAC1 (Figure 7D). These results show for the first time that the presence of the splicing factor SLU7 is essential to ensure the high levels of cellular DNMT1 protein induced *in vivo* in a proliferating scenario.

DISCUSSION

Gene expression is a tightly controlled cell-type specific and dynamic process. Its regulation involves epigenetic modifications of chromatin to allow the access of the transcriptional machinery to the DNA, the subsequent processing of the pre-mRNAs and the translation of the specific mRNAs into proteins. These mechanisms are finely coordinated and interconnected, and involve the activity of epigenetic modulators, transcription and splicing factors and post-translational modifiers (59,60) which in many cases are essential to preserve cell identity and cell survival. In the last years, our laboratory has discovered that the splicing regulator SLU7 is required to preserve the mature and functional hepatic transcriptome (16) and we have uncovered its crucial role to secure correct cell division, preventing the induction of genome instability and cell cycle arrest

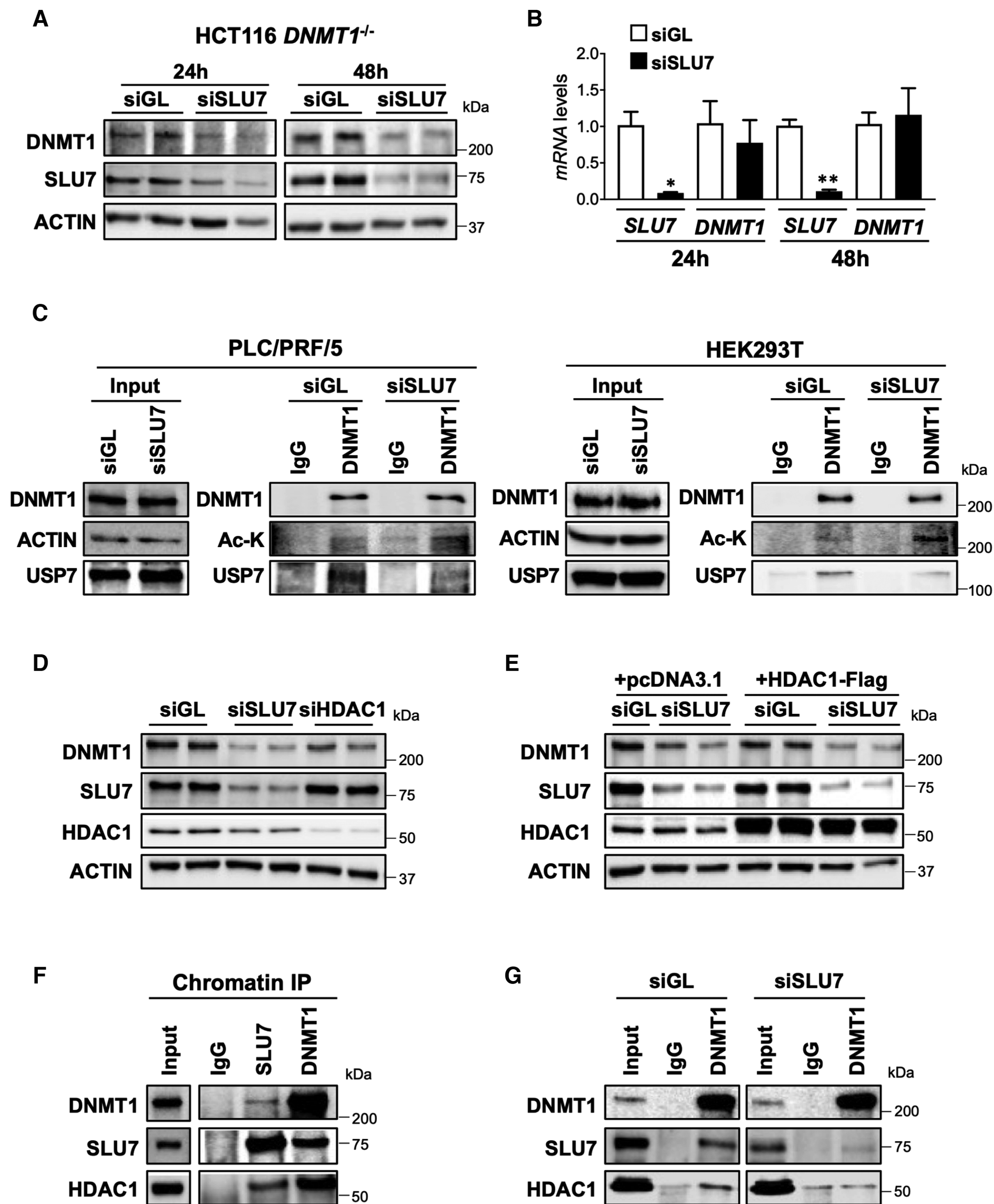


Figure 6. SLU7 knockdown induces DNMT1 acetylation and impairs HDAC1-DNMT1 interaction. (**A**) Representative Western blots of DNMT1 and SLU7 in HCT116 DNMT1$^{-/-}$ cells 24 and 48 h after transfection with siSLU7. ACTIN expression was shown as loading control. (**B**) Histograms reporting *SLU7* and *DNMT1* mRNA levels by real time qPCR from three independent experiments including those shown in panel A. *RPLP0* expression was used as housekeeping gene. *$P < 0.05$, **$P < 0.01$. (**C**) SLU7 knockdown leads to increase acetylation of endogenous DNMT1. Protein lysates from 36h control siGL and siSLU7 transfected PLC/PRF/5 and HEK293T cells treated with 10 μM MG-132 for the last 20 h were immunoprecipitated with anti-DNMT1 and blotted with anti-acetyl-lysine (Ac-K), DNMT1 and USP7 antibodies. DNMT1, USP7 and ACTIN are shown as control in the cell inputs. (**D**) DNMT1 protein decrease was detected by Western blot analysis in PLC/PRF/5 cells 48 h after SLU7 and HDAC1 knockdown. (**E**) DNMT1, SLU7 and HDAC1 protein levels were analyzed by Western blot in PLC/PRF/5 cells 36 h after transfection with control siGL or siSLU7 and pcDNA3.1 empty plasmid or the corresponding plasmid to express HDAC1-flag. ACTIN was used as loading control. (**F**) Chromatin-bound cell extracts from PLC/PRF/5 cells were immunoprecipitated with anti-SLU7 and anti-DNMT1 antibodies or pre-immune IgG and blotted with DNMT1, SLU7 and HDAC1 antibodies. (**G**) Extracts from PLC/PRF/5 cells 36 h after siGL and siSLU7 transfection were subjected to DNMT1 immunoprecipitation and tested by Western blot with antibodies against DNMT1, SLU7 and HDAC1. Immunoprecipitation with pre-immune IgG and inputs were used as controls.

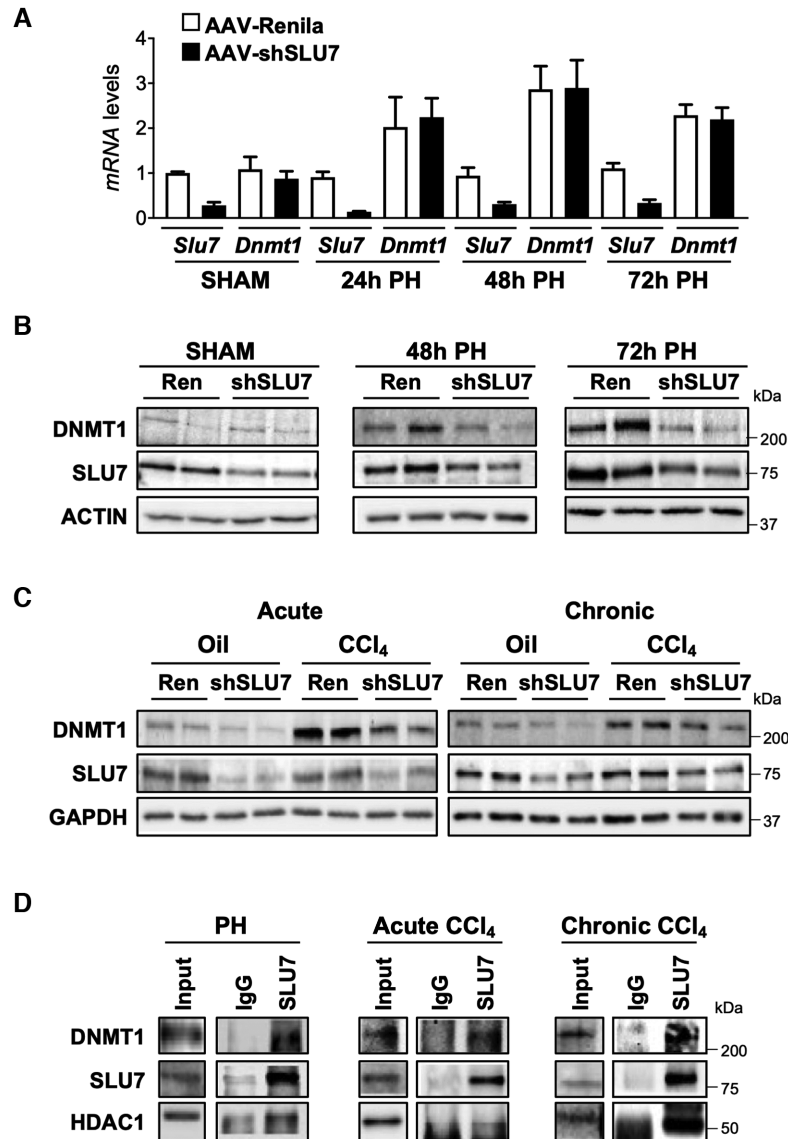


Figure 7. SLU7 is required to ensure DNMT1 stability *in vivo*. (**A**) *Slu7* and *Dnmt1* mRNA levels in the liver of mice 24, 48 and 72 h after 2/3 partial hepatectomy (PH) or control surgery (SHAM) 21 days after injection of adenoassociated virus to inhibit liver SLU7 expression (AAV-shSLU7) or control virus (AAV-Ren) (n = 5 mice per group). *RPLP0* expression was used as housekeeping gene. (**B**) Western blot detection of DNMT1 and SLU7 protein levels in the liver of AAV-shSLU7 and AAV-Ren mice Sham operated or 48 and 72 h after 2/3 partial hepatectomy (PH). ACTIN expression was shown as loading control. (**C**) Western blot detection of DNMT1 and SLU7 protein levels in the liver of AAV-shSLU7 and AAV-Ren mice after CCl4 induction of acute or chronic liver damage. GAPDH expression was shown as loading control. (**D**) SLU7 immunoprecipitation *in vivo* after 2/3 partial hepatectomy (PH) and CCl_4-mediated induction of acute or chronic liver damage. Protein lysates were immunoblotted with anti-SLU7, anti-DNMT1 and anti-HDAC1 antibodies. Immunoprecipitation with pre-immune IgG and inputs were shown as control.

(17). Moreover, our results show that SLU7 is essential for the survival of cancer cells (17,19). Mechanistically we have characterized that to perform these functions, SLU7 regulates not only the alternative splicing of multiple genes, but also gene transcription, being part of P-CREB/CBP transcriptional complexes activated by cAMP (16). Our present work provides solid evidence that SLU7 is also part of the epigenetic chromatome playing a pivotal role in the regulation of DNMT1 stability and the maintenance of DNA methylation. Therefore, altogether our findings identify SLU7 as a pleiotropic factor, and demonstrate that SLU7 may constitute an integrative hub of different levels of gene expression regulation, including epigenetic DNA remodeling, modulation of promoter activity and mRNA splicing.

The enzyme DNMT1 is required for maintaining DNA methylation after DNA replication (5,6). However, despite this doubtless dominant role in DNA methylation maintenance, independent studies using different methodologies to deplete DNMT1 have yielded discrepant results on how its effects on global DNA methylation status are exerted. Whereas DNMT1 knockout cells generated by genetic deletion of several exons in the N-terminal regulatory domain (56) or in the C-terminal catalytic domain (12) can sus-

tain most of DNA methylation, DNMT1 depletion using antisense or siRNAs results in significant loss of global DNA methylation (61). Apparently, there is an extremely low threshold level of DNMT1 protein below which loss of DNA methylation occurs, but above which no major changes are detected (47,62). In our experiments, we found that SLU7 knockdown results in a significant reduction of total cellular 5-mC content, and the number and distribution of demethylated CpGs mimic to some extent those obtained upon DNMT1 depletion. The relatively limited overlap at individual CpG and gene levels could be explained by different reasons: (i) the time at which DNA methylation was analyzed. DNA demethylation is a passive process that requires cell division and RRBS analyses were carried out 72 h after siRNA transfection given that at later times cell viability is compromised (19); (ii) the coexistence of a mixture of cells with different methylation content including, normal and SLU7/DNMT1 silenced cells, and cells at any phase of the cell cycle. In fact, demethylation heterogeneity was also observed between different clones upon stable DNMT1 silencing (63,64); (iii) the restrictive criterion for methylation differences with respect to the control siGL used, as we only considered beta-value differences greater than 0.6. Nevertheless, we found that both DNMT1 and SLU7 silencing upregulate the expression of (a) specific genes where promoter CGI hypomethylation was directly detected, (b) imprinted genes such as *H19*, and methylated (c) *MAGE* antigens and (d) retroviral genes. The parallelism in the functional consequences of SLU7 and DNMT1 knockdown is reinforced by the fact that in both cases we detected the emergence of hypermethylated CpGs. This surprising hypermethylation was also observed in previous studies in which DNMT1 activity was inhibited (32), the DNMT1/UHRF1 complex was disrupted (65) or even in *Dnmt1* knockout models (33). This intriguing and relevant finding deserves further investigation to be understood. Altogether, these data demonstrate that SLU7 is essential to maintain DNMT1 levels and DNA methylation, and that SLU7 is a splicing factor which controls gene expression through multiple mechanisms including epigenetic DNA modification.

DNMT1, like SLU7, (https://www.mousephenotype.org/data/genes/MGI:2385598#phenotypesTab), are essential for embryonic survival (66). Moreover, as we have demonstrated for SLU7 (16,17), DNMT1 is also required for maintaining genomic stability and functional maturity of hepatocytes during early postnatal liver growth and regeneration (33). Furthermore, DNMT1 disruption has also been associated with the induction of DNA damage, cell cycle arrest at G2 phase, mitotic defects, genomic instability and apoptosis (12). As mentioned above, all these events also occur upon SLU7 knockdown (16,17,19). Although apparently, SLU7 knockdown induces a faster and stronger phenotype compared to DNMT1 knockdown, we cannot exclude that some effects observed in SLU7 depleted cells may be mediated in part by the loss of DNMT1.

Given the interest in understanding the dynamics of DNA methylation, multiple works have demonstrated that different mechanisms and players participate in the regulation of DNMT1 expression, revealing a complex, tightly regulated and dynamic scenario (13,14). Our present data uncover a central and non-redundant role for SLU7. We demonstrate that SLU7 depletion results in a rapid and marked decrease of DNMT1 levels, not only in hepatic cells, but in cells of multiple origins. Unexpectedly, SLU7 is not necessary neither for *DNMT1* transcription nor for the correct *DNMT1* mRNA processing. Instead, SLU7 is essential to maintain DNMT1 protein stability. Interestingly, our results demonstrate that SLU7 is a new partner in the chromatome of the protein complex formed by DNMT1/UHRF1/G9a associated with the chromatin to secure DNA methylation (44–46) and that SLU7 is required to maintain the integrity of this protein complex. Moreover, we found that SLU7 is necessary to preserve the total levels of the different protein members of this complex, supporting its role in the maintenance of DNA methylation, as it has been demonstrated that disruption of the complex (67) results in a global DNA hypomethylation. This distinguishes SLU7 from the other members of the complex, as for instance depletion of G9a reduces DNA methylation activity, but it has no effect on DNMT1 protein location or levels (44). Moreover, knockdown of UHRF1 severely reduces the association of DNMT1 to chromatin (45) and DNA methylation (45) but it does not affect DNMT1 global levels. Altogether, our results demonstrate a unique position of SLU7 in maintaining DNMT1 stability, which apparently does not depend solely on the integrity of the complex. Although as discuss below, we have unraveled novel molecular mechanisms to explain how SLU7 is regulating DNMT1 stability, further studies are needed to understand whether mechanisms different from the integrity of the complex itself, such as unexplored splicing or post-translational alterations, are implicated in the stability of the other members of the complex. In any case, the role of SLU7 in maintaining not only DNMT1 stability, but the integrity of the DNA methylation complex, together with a possible role on other DNA methylation factors, such as DNMT3, would explain the huge impact of SLU7 depletion on DNA methylation.

Several post-translational modifications govern the steady-state levels of DNMT1 protein (68). Indeed, the increase of DNMT1 in cancers is largely due to alterations in DNMT1 protein turnover rather than to higher mRNA abundance (69,70). It has been shown that DNMT1 protein stability is regulated by dynamic phosphorylation (50), methylation (50,53,71) and acetylation (52,54,55). For instance, DNMT1 acetylation prevents its binding to USP7, promoting its degradation (51). In fact, we found that SLU7 knockdown promotes DNMT1 acetylation, preventing USP7 binding and inducing DNMT1 proteasomal degradation. We further demonstrated that SLU7 interacts with DNMT1 and allows its binding to the deacetylase HDAC1, securing DNMT1 deacetylation and stabilization (57). Therefore, we uncovered an unknown role of SLU7 in the regulation of post translational modifications of proteins such as DNMT1, more specifically as a regulator of the acetylation pathway. In this sense, it has been recently shown that the splicing factor SF2/ASF (SRSF1) directly interacts with the E2 conjugating enzyme Ubc9 promoting the sumoylation of specific substrates (72). These results further illustrate the pleiotropic functions of some proteins such as SLU7 and their relevance in the establishment of crosstalks between different cellular functions.

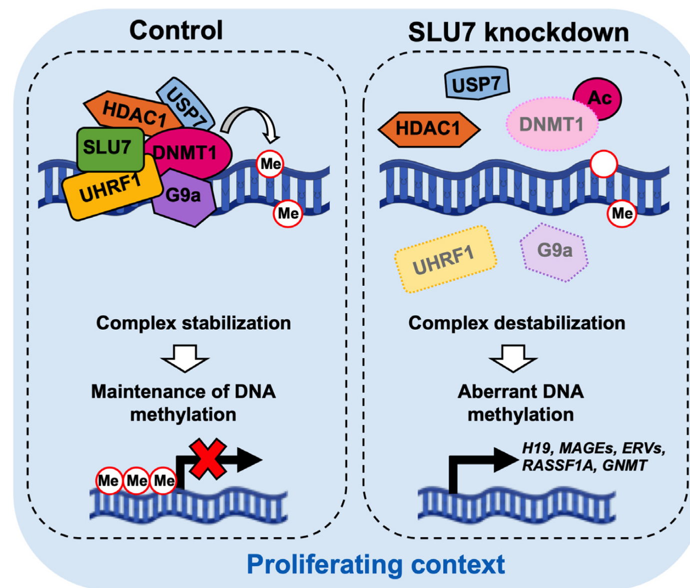


Figure 8. Schematic representation of the mechanisms regulated by SLU7 involved in DNA methylation maintenance. See text for details.

In non-dividing cells DNMT1 shows constitutive low levels of expression (42,43). In contrast, DNMT1 abundance is enhanced in cell proliferating scenarios when the CpGs of the newly synthetized DNA strands need to be methylated (42,43). Our data confirm this switch in DNMT1 abundance and identify SLU7 as essential to stabilize DNMT1 protein levels induced in proliferating cells in a cell cycle phase- and cell type-independent manner. In this regard, we consistently found that SLU7 stabilizes DNMT1 protein in a wide panel of human cancer cell lines of different origins and in the non-transformed HEK293T cell line. However, no significant changes were observed in DNMT1 levels upon SLU7 knockdown when both transformed and non-transformed cells were arrested by high confluency without serum at G0. Importantly, we demonstrate that this DNMT1 dependency on SLU7 also operates *in vivo*, using a mouse model of liver proliferation induced to recover liver mass after partial hepatectomy or hepatocyte acute and chronic damage. We found that *in vivo*, SLU7 also interacts with DNMT1 and HDAC1, and the downregulation of SLU7 using a specific shRNA expressed from an adenoassociated virus (AAV-shSLU7) prevents the induction of DNMT1 protein, without affecting the induction of its mRNA, after PH and liver damage.

All in all, in this study, we demonstrate for the first time that SLU7 is required to maintain DNA methylation. As summarized in Figure 8, we show that SLU7 is part in the chromatome of the protein complex implicated in DNA methylation. Moreover, we discover that SLU7 is essential to secure DNMT1 stability, facilitating its interaction with HDAC1, and thus preventing its acetylation. Importantly, and in contrast with other works, our data are based on interactions and mechanisms addressed by endogenous proteins. Furthermore, we also provide evidence that these mechanisms operate *in vivo*. Overall, our results uncover new and unexpected functions of the splicing factor SLU7 and demonstrate that it plays a holistic function in the regulation of gene expression.

DATA AVAILABILITY

Other additional methods and data will be available upon request.

SUPPLEMENTARY DATA

Supplementary Data are available at NAR Online.

ACKNOWLEDGEMENTS

We thank Dr Rob M. Ewing (Southampton, UK) and Dr Donald E. Ayer (University of Utah, EEUU) for kindly providing the HCT116 *DNMT1*$^{-/-}$ cell line and the pcDNA3.1-HDAC1-Flag plasmid respectively. We thank all members of the Hepatology Program at CIMA and Dr A. Gutierrez-Uzquiza from Complutense University of Madrid for helpful discussions and comments.

FUNDING

MINECO [PID2019-104265RB-I00/AEI/10.13039/501100011033, PID2019-104878RB-100AEI/10.13039/501100011033, SAF2016-75972-R]; Instituto de Salud Carlos III (ISCIII) co-financed by 'Fondo Europeo de Desarrollo Regional' (FEDER) 'Una manera de hacer Europa [PI19/00163]; Gobierno de Navarra [55/2018, PC082-083-084 EHGNA]; Eurorregion [2020/101]; CIBERehd; HEP-ACARE Project from Fundación La Caixa; AECC post-doctoral fellowship [POSTD18014AREC to M.A.]; Ministerio de Educación FPI fellowship [BES-2017-079883 to M.R.]; Ministerio de Educación FPU fellowship

[FPU18/01461 to M.G.R.]; Ramón y Cajal Program contract [RYC2018-024475-1 to M.G.F.B.]; Fundación Eugenio Rodríguez Pascual, Fundación Mario Losantos, Fundación M Torres and the generous support of Mr. Eduardo Avila. Funding for open access charge: MINECO [PID2019-104265RB-I00/AEI/10.13039/501100011033].
Conflict of interest statement. None declared.

REFERENCES

1. Baylin,S.B., Esteller,M., Rountree,M.R., Bachman,K.E., Schuebel,K. and Herman,J.G. (2001) Aberrant patterns of DNA methylation, chromatin formation and gene expression in cancer. *Hum. Mol. Genet.*, **10**, 687–692.
2. Strahl,B.D. and Allis,C.D. (2000) The language of covalent histone modifications. *Nature*, **403**, 41–45.
3. Hermann,A., Gowher,H. and Jeltsch,A. (2004) Biochemistry and biology of mammalian DNA methyltransferases. *Cell. Mol. Life Sci.*, **61**, 2571–2587.
4. Bird,A. (2002) DNA methylation patterns and epigenetic memory. *Gene Dev.*, **16**, 6–21.
5. Hervouet,E., Nadaradjane,A., Gueguen,M., Vallette,F.M. and Cartron,P.-F. (2012) Kinetics of DNA methylation inheritance by the Dnmt1-including complexes during the cell cycle. *Cell Div.*, **7**, 5.
6. Song,J., Teplova,M., Ishibe-Murakami,S. and Patel,D.J. (2012) Structure-based mechanistic insights into DNMT1-mediated maintenance DNA methylation. *Science*, **335**, 709–712.
7. Chen,R.Z., Pettersson,U., Beard,C., Jackson-Grusby,L. and Jaenisch,R. (1998) DNA hypomethylation leads to elevated mutation rates. *Nature*, **395**, 89–93.
8. Papamichos-Chronakis,M. and Peterson,C.L. (2013) Chromatin and the genome integrity network. *Nat. Rev. Genet.*, **14**, 62–75.
9. Gaudet,F., Hodgson,J.G., Eden,A., Jackson-Grusby,L., Dausman,J., Gray,J.W., Leonhardt,H. and Jaenisch,R. (2003) Induction of tumors in mice by genomic hypomethylation. *Science*, **300**, 489–492.
10. Baylin,S.B. and Ohm,J.E. (2006) Epigenetic gene silencing in cancer – a mechanism for early oncogenic pathway addiction? *Nat. Rev. Cancer*, **6**, 107–116.
11. Fernández-Barrena,M.G., Arechederra,M., Colyn,L., Berasain,C. and Avila,M.A. (2020) Epigenetics in hepatocellular carcinoma development and therapy: The tip of the iceberg. *Jhep Reports*, **2**, 100167.
12. Chen,T., Hevi,S., Gay,F., Tsujimoto,N., He,T., Zhang,B., Ueda,Y. and Li,E. (2007) Complete inactivation of DNMT1 leads to mitotic catastrophe in human cancer cells. *Nat. Genet.*, **39**, 391–396.
13. Kar,S., Deb,M., Sengupta,D., Shilpi,A., Parbin,S., Torrisani,J., Pradhan,S. and Patra,S. (2012) An insight into the various regulatory mechanisms modulating human DNA methyltransferase 1 stability and function. *Epigenetics*, **7**, 994–1007.
14. Lin,R.-K. and Wang,Y.-C. (2014) Dysregulated transcriptional and post-translational control of DNA methyltransferases in cancer. *Cell Biosci*, **4**, 46.
15. Jeltsch,A. and Jurkowska,R.Z. (2016) Allosteric control of mammalian DNA methyltransferases – a new regulatory paradigm. *Nucleic Acids Res.*, **44**, 8556–8575.
16. Elizalde,M., Urtasun,R., Azkona,M., Latasa,M.U., Goñi,S., García-Irigoyen,O., Uriarte,I., Segura,V., Collantes,M., Di Scala,M. *et al.* (2014) Splicing regulator SLU7 is essential for maintaining liver homeostasis. *J. Clin. Invest.*, **124**, 2909–2920.
17. Jiménez,M., Urtasun,R., Elizalde,M., Azkona,M., Latasa,U.M., Uriarte,I., Arechederra,M., Alignani,D., Bárcena-Varela,M., Álvarez-Sola,G. *et al.* (2019) Splicing events in the control of genome integrity: role of SLU7 and truncated SRSF3 proteins. *Nucleic Acids Res.*, **47**, 3450–3466.
18. Castillo,J., Goñi,S., Latasa,M.U., Perugorría,M.J., Calvo,A., Muntané,J., Bioulac-Sage,P., Balabaud,C., Prieto,J., Avila,M.A. *et al.* (2009) Amphiregulin induces the alternative splicing of p73 into its oncogenic isoform DeltaEx2p73 in human hepatocellular tumors. *Gastroenterology*, **137**, 1805–1815.
19. Urtasun,R., Elizalde,M., Azkona,M., Latasa,M.U., García-Irigoyen,O., Uriarte,I., Fernández-Barrena,M.G., Vicent,S., Alonso,M.M., Muntané,J. *et al.* (2016) Splicing regulator SLU7 preserves survival of hepatocellular carcinoma cells and other solid tumors via oncogenic miR-17-92 cluster expression. *Oncogene*, **35**, 4719–4729.
20. Maor,G., Yearim,A. and Ast,G. (2015) The alternative role of DNA methylation in splicing regulation. *Trends Genet.*, **31**, 274–280.
21. Zhang,J., Zhang,Y.-Z., Jiang,J. and Duan,C.-G. (2020) The crosstalk between epigenetic mechanisms and alternative RNA processing regulation. *Front. Genet.*, **11**, 998.
22. Bowler,E.H., Smith-Vidal,A., Lester,A., Bell,J., Wang,Z., Bell,C.G., Wang,Y., Divecha,N., Skipp,P.J. and Ewing,R.M. (2019) Deep proteomic analysis of Dnmt1 mutant/hypomorphic colorectal cancer cells reveals dysregulation of epithelial–mesenchymal transition and subcellular re-localization of Beta-Catenin. *Epigenetics*, **15**, 107–121.
23. Meissner,A., Gnirke,A., Bell,G.W., Ramsahoye,B., Lander,E.S. and Jaenisch,R. (2005) Reduced representation bisulfite sequencing for comparative high-resolution DNA methylation analysis. *Nucleic Acids Res.*, **33**, 5868–5877.
24. Stockwell,P.A., Chatterjee,A., Rodger,E.J. and Morison,I.M. (2014) DMAP: differential methylation analysis package for RRBS and WGBS data. *Bioinform. Oxf. Engl.*, **30**, 1814–1822.
25. Heinz,S., Benner,C., Spann,N., Bertolino,E., Lin,Y.C., Laslo,P., Cheng,J.X., Murre,C., Singh,H. and Glass,C.K. (2010) Simple combinations of lineage-determining transcription factors prime cis-regulatory elements required for macrophage and B cell identities. *Mol. Cell*, **38**, 576–589.
26. Perugorria,M.J., Castillo,J., Latasa,M.U., Goñi,S., Segura,V., Sangro,B., Prieto,J., Avila,M.A. and Berasain,C. (2009) Wilms' tumor 1 gene expression in hepatocellular carcinoma promotes cell dedifferentiation and resistance to chemotherapy. *Cancer Res.*, **69**, 1358–1367.
27. Castillo,J., Erroba,E., Perugorría,M.J., Santamaría,M., Lee,D.C., Prieto,J., Avila,M.A. and Berasain,C. (2006) Amphiregulin contributes to the transformed phenotype of human hepatocellular carcinoma cells. *Cancer Res.*, **66**, 6129–6138.
28. Berasain,C., García-Trevijano,E.R., Castillo,J., Erroba,E., Lee,D.C., Prieto,J. and Avila,M.A. (2005) Amphiregulin: an early trigger of liver regeneration in mice. *Gastroenterology*, **128**, 424–432.
29. Bárcena-Varela,M., Caruso,S., Llerena,S., Álvarez-Sola,G., Uriarte,I., Latasa,U.M., Urtasun,R., Rebouissou,S., Alvarez,L., Jimenez,M. *et al.* (2019) Dual targeting of histone methyltransferase G9a and DNA-methyltransferase 1 for the treatment of experimental hepatocellular carcinoma. *Hepatology*, **69**, 587–603.
30. Berasain,C., García-Trevijano,E.R., Castillo,J., Erroba,E., Santamaría,M., Lee,D.C., Lee,D.C., Prieto,J. and Avila,M.A. (2005) Novel role for amphiregulin in protection from liver injury. *J. Biol. Chem.*, **280**, 19012–19020.
31. Perugorria,M.J., Latasa,M.U., Nicou,A., Cartagena-Lirola,H., Castillo,J., Goñi,S., Vespasiani-Gentilucci,U., Zagami,M.G., Lotersztajn,S., Prieto,J. *et al.* (2008) The epidermal growth factor receptor ligand amphiregulin participates in the development of mouse liver fibrosis. *Hepatology*, **48**, 1251–1261.
32. Estève,P.-O., Zhang,G., Ponnaluri,C.V., Deepti,K., Chin,H., Dai,N., Sagum,C., Black,K., Corrêa,I.R. Jr, Bedford,M.T. *et al.* (2015) Binding of 14-3-3 reader proteins to phosphorylated DNMT1 facilitates aberrant DNA methylation and gene expression. *Nucleic Acids Res.*, **44**, 1642–1656.
33. Kaji,K., Factor,V.M., Andersen,J.B., Durkin,M.E., Tomokuni,A., Marquardt,J.U., Matter,M.S., Hoang,T., Conner,E.A. and Thorgeirsson,S.S. (2016) DNMT1 is a required genomic regulator for murine liver histogenesis and regeneration. *Hepatology*, **64**, 582–598.
34. Baylin,S.B. and Jones,P.A. (2011) A decade of exploring the cancer epigenome — biological and translational implications. *Nat. Rev. Cancer*, **11**, 726–734.
35. Hesson,L.B., Cooper,W.N. and Latif,F. (2007) The role of RASSF1A methylation in cancer. *Dis. Markers*, **23**, 73–87.
36. Huidobro,C., Toraño,E.G., Fernández,A.F., Urdinguio,R.G., Rodríguez,R.M., Ferrero,C., Martínez-Camblor,P., Boix,L., Bruix,J., García-Rodríguez,J.L. *et al.* (2013) A DNA methylation signature associated with the epigenetic repression of glycine N-methyltransferase in human hepatocellular carcinoma. *J. Mol. Med.*, **91**, 939–950.
37. Botezatu,A., Bleotu,C., Nastase,A., Anton,G., Bacalbasa,N., Duda,D., Dima,S.O. and Popescu,I. (2015) Epigenetic silencing of

GNMT gene in pancreatic adenocarcinoma. *Cancer Genom. Proteom*, **12**, 21–30.
38. Jones,P.A., Ohtani,H., Chakravarthy,A. and Carvalho,D.D. (2019) Epigenetic therapy in immune-oncology. *Nat. Rev. Cancer*, **19**, 151–161.
39. Chiappinelli,K.B., Strissel,P.L., Desrichard,A., Li,H., Henke,C., Akman,B., Hein,A., Rote,N.S., Cope,L.M., Snyder,A. *et al.* (2015) Inhibiting DNA methylation causes an interferon response in cancer via dsRNA including endogenous retroviruses. *Cell*, **162**, 974–986.
40. Smet,C.D. and Loriot,A. (2013) DNA hypomethylation and activation of germline-specific genes in cancer. *Adv. Exp. Med. Biol.*, **754**, 149–166.
41. Chua,K. and Reed,R. (1999) Human step II splicing factor hSlu7 functions in restructuring the spliceosome between the catalytic steps of splicing. *Gene Dev*, **13**, 841–850.
42. Kheir,T. and Lund,A.H. (2010) Epigenetic dynamics across the cell cycle. *Essays Biochem.*, **48**, 107–120.
43. Kinney,S.R.M. and Pradhan,S. (2011) Progress in molecular biology and translational science. *Prog. Mol. Biol. Transl.*, **101**, 311–333.
44. Esteve,P.-O., Chin,H.G., Smallwood,A., Feehery,G.R., Gangisetty,O., Karpf,A.R., Carey,M.F. and Pradhan,S. (2006) Direct interaction between DNMT1 and G9a coordinates DNA and histone methylation during replication. *Gene Dev.*, **20**, 3089–3103.
45. Bostick,M., Kim,J., Estève,P.-O., Clark,A., Pradhan,S. and Jacobsen,S.E. (2007) UHRF1 plays a role in maintaining DNA methylation in mammalian cells. *Science*, **317**, 1760–1764.
46. Schermelleh,L., Haemmer,A., Spada,F., Rösing,N., Meilinger,D., Rothbauer,U., Cardoso,M.C. and Leonhardt,H. (2007) Dynamics of Dnmt1 interaction with the replication machinery and its role in postreplicative maintenance of DNA methylation. *Nucleic Acids Res.*, **35**, 4301–4312.
47. Cai,Y., Tsai,H.-C., Yen,R.-W.C., Zhang,Y.W., Kong,X., Wang,W., Xia,L. and Baylin,S.B. (2017) Critical threshold levels of DNA methyltransferase 1 are required to maintain DNA methylation across the genome in human cancer cells. *Genome Res.*, **27**, 533–544.
48. Denis,H., Ndlovu,M.N. and Fuks,F. (2011) Regulation of mammalian DNA methyltransferases: a route to new mechanisms. *EMBO Rep.*, **12**, 647–656.
49. Qin,W., Leonhardt,H. and Pichler,G. (2011) Regulation of DNA methyltransferase 1 by interactions and modifications. *Nucleus*, **2**, 392–402.
50. Estève,P.-O., Chang,Y., Samaranayake,M., Upadhyay,A.K., Horton,J.R., Feehery,G.R., Cheng,X. and Pradhan,S. (2010) A methylation and phosphorylation switch between an adjacent lysine and serine determines human DNMT1 stability. *Nat. Struct. Mol. Biol.*, **18**, 42–48.
51. Du,Z., Song,J., Wang,Y., Zhao,Y., Guda,K., Yang,S., Kao,H.Y., Xu,Y., Willis,J., Markowitz,S.D. *et al.* (2010) DNMT1 stability is regulated by proteins coordinating deubiquitination and acetylation-driven ubiquitination. *Sci. Signal*, **3**, ra80.
52. Peng,L., Yuan,Z., Ling,H., Fukasawa,K., Robertson,K., Olashaw,N., Koomen,J., Chen,J., Lane,W.S. and Seto,E. (2011) SIRT1 deacetylates the DNA methyltransferase 1 (DNMT1) protein and alters its activities. *Mol. Cell. Biol.*, **31**, 4720–4734.
53. Esteve,P.-O., Chin,H.G., Benner,J., Feehery,G.R., Samaranayake,M., Horwitz,G.A., Jacobsen,S.E. and Pradhan,S. (2009) Regulation of DNMT1 stability through SET7-mediated lysine methylation in mammalian cells. *Proc National Acad Sci*, **106**, 5076–5081.
54. Cheng,J., Yang,H., Fang,J., Ma,L., Gong,R., Wang,P., Li,Z. and Xu,Y. (2015) Molecular mechanism for USP7-mediated DNMT1 stabilization by acetylation. *Nat. Commun.*, **6**, 7023.
55. Hong,Q. and Shao,Z. (2011) Ubiquitination/deubiquitination and acetylation/deacetylation: making DNMT1 stability more coordinated. *Acta Pharmacol. Sin.*, **32**, 139–140.
56. Rhee,I., Jair,K.-.W., Yen,R.-.W., Lengauer,C., Herman,J.G., Kinzler,K.W., Vogelstein,B., Baylin,S.B. and Schuebel,K.E. (2000) CpG methylation is maintained in human cancer cells lacking DNMT1. *Nature*, **404**, 1003–1007.
57. Zhou,Q., Agoston,A.T., Atadja,P., Nelson,W.G. and Davidson,N.E. (2008) Inhibition of histone deacetylases promotes ubiquitin-dependent proteasomal degradation of DNA methyltransferase 1 in human breast cancer cells. *Mol. Cancer Res.*, **6**, 873–883.
58. Wang,S., Zhang,C., Hasson,D., Desai,A., SenBanerjee,S., Magnani,E., Ukomadu,C., Lujambio,A., Bernstein,E. and Sadler,K.C. (2019) Epigenetic compensation promotes liver regeneration. *Dev. Cell*, **50**, 43–56.
59. Braunschweig,U., Gueroussov,S., Plocik,A.M., Graveley,B.R. and Blencowe,B.J. (2013) Dynamic integration of splicing within gene regulatory pathways. *Cell*, **152**, 1252–1269.
60. André,K.M., Sipos,E.H. and Soutourina,J. (2020) Mediator roles going beyond transcription. *Trends Genet.*, **37**, 224–234.
61. Robert,M.-F., Morin,S., Beaulieu,N., Gauthier,F., Chute,I.C., Barsalou,A. and MacLeod,A.R. (2002) DNMT1 is required to maintain CpG methylation and aberrant gene silencing in human cancer cells. *Nat. Genet.*, **33**, 61–65.
62. Patel,K., Dickson,J., Din,S., Macleod,K., Jodrell,D. and Ramsahoye,B. (2010) Targeting of 5-aza-2′-deoxycytidine residues by chromatin-associated DNMT1 induces proteasomal degradation of the free enzyme. *Nucleic Acids Res.*, **38**, 4313–4324.
63. Minoguchi,S. and Iba,H. (2008) Instability of retroviral DNA methylation in embryonic stem cells. *Stem Cells*, **26**, 1166–1173.
64. Bai,J., Zhang,X., Hu,K., Liu,B., Wang,H., Li,A., Lin,F., Zhang,L., Sun,X., Du,Z. *et al.* (2015) Silencing DNA methyltransferase 1 (DNMT1) inhibits proliferation, metastasis and invasion in ESCC by suppressing methylation of RASSF1A and DAPK. *Oncotarget*, **7**, 44129–44141.
65. Chin,H.G., Ponnaluri,Vk.C, Zhang,G., Estève,P.-O., Schaus,S.E., Hansen,U. and Pradhan,S. (2014) Transcription factor LSF-DNMT1 complex dissociation by FQI1 leads to aberrant DNA methylation and gene expression. *Oncotarget*, **5**, 83627–83640.
66. Li,E., Bestor,T.H. and Jaenisch,R. (1992) Targeted mutation of the DNA methyltransferase gene results in embryonic lethality. *Cell*, **69**, 915–926.
67. Pacaud,R., Brocard,E., Lalier,L., Hervouet,E., Vallette,F.M. and Cartron,P.-F. (2014) The DNMT1/PCNA/UHRF1 disruption induces tumorigenesis characterized by similar genetic and epigenetic signatures. *Sci Rep-uk*, **4**, 4230.
68. Scott,A., Song,J., Ewing,R. and Wang,Z. (2014) Regulation of protein stability of DNA methyltransferase 1 by post-translational modifications. *Acta Bioch Bioph Sin*, **46**, 199–203.
69. Agoston,A.T., Argani,P., Yegnasubramanian,S., Marzo,A.M., Ansari-Lari,M., Hicks,J.L., Davidson,N.E. and Nelson,W.G. (2005) Increased protein stability causes DNA methyltransferase 1 dysregulation in breast cancer. *J. Biol. Chem.*, **280**, 18302–18310.
70. Marzo,D.A., Marchi,V., Yang,E., Veeraswamy,R., Lin,X. and Nelson,W. (1999) Abnormal regulation of DNA methyltransferase expression during colorectal carcinogenesis. *Cancer Res.*, **59**, 3855–3860.
71. Zhang,H., Gao,Q., Tan,S., You,J., Lyu,C., Zhang,Y., Han,M., Chen,Z., Li,J., Wang,H. *et al.* (2019) SET8 prevents excessive DNA methylation by methylation-mediated degradation of UHRF1 and DNMT1. *Nucleic Acids Res.*, **47**, 9053–9068
72. Pelisch,F., Gerez,J., Druker,J., Schor,I.E., Muñoz,M.J., Risso,G., Petrillo,E., Westman,B.J., Lamond,A.I., Arzt,E. *et al.* (2010) The serine/arginine-rich protein SF2/ASF regulates protein sumoylation. *Proc. Natl. Acad. Sci. U.S.A.*, **107**, 16119–16124.