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Epigenetic Control of Mammalian LINE-1 Retrotransposon by Retinoblastoma Proteins

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Abstract

Long interspersed nuclear elements (LINEs or L1 elements) are targeted for epigenetic silencing during early embryonic development and remain inactive in most cells and tissues. Here we show that E2F-Rb family complexes participate in L1 elements epigenetic regulation via nucleosomal histone modifications and recruitment of histone deacetylases (HDACs) HDAC1 and HDAC2. ChIP experiments demonstrated that (i) Rb and E2F interact with human and mouse L1 elements, (ii) L1 elements are deficient in both heterochromatin-associated histone marks H3 tri methyl K9 and H4 tri methyl K20 in Rb family triple knock out (Rb, p107, p130) fibroblasts (TKO), (iii) L1 promoter exhibits increased histone H3 acetylation in the absence of HDAC1 and HDAC2 recruitment, (iv) L1 expression in TKO fibroblasts is upregulated compared to wild type counterparts, (v) L1 expression increases in the presence of the HDAC inhibitor TSA. On the basis of these findings we propose a model in which L1 sequences throughout the genome serve as centers for heterochromatin formation in an Rb family-dependent manner. As such, Rb proteins and L1 elements may play key roles in heterochromatin formation beyond pericentromeric chromosomal regions. These findings describe a novel mechanism of L1 reactivation in mammalian cells mediated by failure of co-repressor protein recruitment by Rb, loss of histone epigenetic marks, heterochromatin formation, and increased histone H3 acetylation.

Keywords

5' untranslated region; Epigenetics; HDAC; Long interspersed nuclear elements; Pocket protein family

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CONFLICT OF INTEREST STATEMENT

The authors declare that there are no conflicts of interest in relation to the submitted paper.

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1. INTRODUCTION

Long Interspersed Nuclear Element-1 (L1) is a mammalian retrotransposon that comprises 15 to 30 percent of the human and murine chromosomal DNA content [1]. L1 is transcribed from its 5' UTR (5' untranslated region) and inserted into the host genome via a copy and paste mechanism that involves an RNA intermediate and reverse transcriptase activity [2]. A complete cycle of L1 retrotransposition can be associated with DNA inversions, duplications or insertions [3–8]. Genomic L1 insertions may positively or negatively regulate transcriptional control of gene expression, as shown for *apolipoprotein (a)* gene [9]; *Slc6a6* and *Chapsyn110/Psd-93* genes [10]. Several L1 insertion-associated diseases have been identified, most notably hemophilia and colon cancer [4,5,11]. Non-insertional mechanisms may also contribute to changes in gene expression via regulation of splicing events with other cellular genes leading to production of aberrant RNA products [12].

The molecular mechanisms responsible for transcriptional control of human L1s are not well understood, but the YY1, SRY, and RUNX3 transcription factors have been shown to bind the 5' regulatory region of the L1 element [13–15]. Analyses of human L1 transcripts indicate that several start sites upstream and downstream of the +1 nucleotide are used suggesting that a complex mechanism mediates transcriptional control. In contrast to humans, the 5' UTR sequences of L1 in mice consist of monomeric units that align in tandem and have an additive effect for promoter function [11,16]. Previously we identified a novel mouse L1 element, classified as L1MdA5, that contains two cis-acting regulatory elements (5'-TGACTCGAGC-3') involved in constitutive and stress-inducible control of transcriptional activity [16].

Patterns of DNA methylation at CpG islands alter chromatin structure by forming highly packed, inactive heterochromatic regions varying from localized fragments within a chromosome to a whole chromosome, as suggested for X-chromosome inactivation [17,18]; and are particularly relevant for silencing of L1 retroelements. Changes in the DNA methylation status have also been documented for ras [19], and cell-cycle related proteins p15, p16, and Rb [20], and linked to the oncogenic phenotype. Increased L1 expression is seen during development and transformation implicating changes in L1 methylation status during the course of oncogenesis [21]. L1s may regulate chromatin activity by changing methylation status and heterochromatin formation, by providing their own DNA sequences with promoter properties, or by activating cryptic promoters within target genes [22,23].

Global hypomethylation of 5-methylcytosine and CpG islands leads to L1 activation, chromosomal instability and elevated mutation rates [24,25], hence linking retrotransposition and tumorigenesis [26,27]. Interestingly, the E2F/Rb protein complex binds CpG islands to regulate a number of genes [28,29], and associates with histone methyltransferases (HMTases) and histone deacetylases (HDACs) [30,31]. Since L1 elements are regulated by their degree of methylation, and E2F/Rb proteins are potential mediators of DNA and histone methylation, we hypothesized that L1 expression is regulated by Rb family-E2F complexes in normal and transformed cells. Here we summarize the results of studies showing that Rb and E2F bind to the L1 promoter in both human and mouse cells. Mouse embryonic cells lacking Rb family members, Rb, p107, and p130 (also known as the pocket proteins family), show marked upregulation of L1 expression coupled to reductions in histone H3 and H4 trimethylation and H3 deacetylation. Based on these findings we conclude that epigenetic regulation of L1s involves not only conventional CpG island methylation mechanisms, but a novel mechanism involving the assembly of E2F/Rb/HDAC complexes and L1 in the regulation of global epigenomic heterochromatin formation and regulation of mammalian gene expression.

2. MATERIALS AND METHODS

Cell culture

Human cervical cancer cells (HeLa) and human bone osteosarcoma epithelial cells (U2OS) expressing both an IPTG-inducible p16INK4a (inhibitor of cyclin-dependent kinase 4A) and a chimeric E2F DNA binding domain fused to estrogen receptor, mER-DB-E2F proteins [32] were grown in DMEM media (Mediatech, Herndon, VA), supplemented with 10% FBS (Atlanta Biologicals, Norcross, GA), and 2 mM glutamine (JRH Biosciences, Lenexa, KS). p16INK4a and mER-DB E2F expression was achieved by culturing the cells in the presence of 1 mM IPTG (Sigma-Aldrich, St. Louis, MO) for 24 or 72 hours, or 100 nM Tamoxifen (Sigma-Aldrich, St. Louis, MO) for 24 hours. For p16INK4a and mER-DB E2F coexpression experiments cells were initially treated with 1 mM IPTG for 24 hours followed by a combined treatment with 1 mM IPTG and 100 nM Tamoxifen for another 24 hours. Control mouse primary embryo fibroblasts (MEFs) and Rb family triple knock out cells (TKO), a kind gift from Dr. J. Sage and Dr. T. Jacks [33] were grown in DMEM supplemented with 10% FBS heat inactivated and 2 mM glutamine. For genotoxic stress treatments, control MEFs and Rb TKO MEFs were grown in complete media, 0.06 % DMSO, or 3 μ M benzo-a-pyrene (B(a)P) for 18 hours. Primary aortic smooth muscle cells isolated from C57BL/6J mice were grown in 10-cm petri dishes in M199 media (Invitrogen Corporation, Carlsbad, CA) supplemented with 10% FBS; 2mM L-glutamine and antibiotic-antimycotic (Invitrogen Corporation, Carlsbad, CA). Unless indicated otherwise, all treatments were completed at 90% confluence and cells were kept in a humidified incubator at 37 °C and 5% CO₂.

RNA extractions and Real time PCR

Total RNA was extracted using Trizol (Invitrogen Corporation, Carlsbad, CA) following manufacturers' instructions. Primers against the ORF1 region in both the human and mouse L1 gene were obtained using Primer 3 software (<http://frodo.wi.mit.edu/cgi-bin/primer3/primer3.cgi>). To prepare cDNA, 1 μ g of total RNA was mixed with 500 ng of random hexamers, 10 mM dithiothreitol, 500 μ M dNTP mix, 40 U RNaseOUT™ ribonuclease inhibitor, and 200 U M-MLV reverse transcriptase. Samples were incubated at 37°C for 1 h according to manufacturer's instructions (Invitrogen Corporation, Carlsbad, CA). Real-time quantitative PCR was performed in 25- μ L reaction volumes containing 0.25- μ L aliquots of cDNA, gene-specific primer pairs, and SYBR Green fluorescent dye (Molecular Probes, Eugene, Oregon). PCR primer sequences and expected product sizes are described in supplementary table 1. Amplification and analysis were performed in an Mx3000P Real-Time PCR System according to the manufacturer's instructions (Stratagene, Cedar Park, TX). The PCR cycle parameters were set at 95°C for 20 sec, 60°C for 30 sec, and 72°C for 30 sec, for a total of not more than 45 cycles. The fluorescent intensity of SYBR Green was monitored at the end of each extension step; relative amounts of the target cDNA were estimated by the threshold cycle (Ct) number, and compared to two control genes, β -actin (ACTB) and glyceraldehyde-3-phosphate dehydrogenase (GAPDH). Three independent samples were analyzed for each condition and/or cell type, and each sample was compared in at least 3 independent RT-PCR amplifications.

ChIP Assays

Chromatin immunoprecipitation (ChIP) assays were performed as described previously [34], with minor modifications. Cells were grown in 10-cm petri dishes to 90% confluence, fixed with 1% formaldehyde for 5 minutes and washed twice with ice-cold PBS. Cells were lysed with modified RIPA buffer (50 mM Tris-Cl pH 7.4; 1 mM EDTA; 150 mM NaCl; 0.1% SDS; 1% TritonX-100; 0.1% Na-Deoxycholate). Chromatin was sheared to fragment sizes

below 3 Kb by sonication in a Sonifier® Model 450 ultrasonic cell disruptor (Branson Ultrasonics Corporation, Danbury, CT) with five cycles at 40% amplitude. Each sonication cycle was performed for 30 seconds and samples were kept on ice-ethanol bath at all the times. Immunoprecipitation reactions were performed using 30 to 60 µg of chromatin. Four micrograms of antisera for Stat-1 (AB16951, lot 0703054410), (Chemicon International, Temecula, CA); Rb (sc-50; C-15, lot C0205), E2F-1 (sc-193; C-20, lot G0505), E2F-4 (sc-866; C-20, lot C0504), HDAC1 (sc-7871; H-51, lot H3104), HDAC2 (sc-7899; H-54, lot C0905), (Santa Cruz Biotechnology, Santa Cruz, CA); histone H3 tri methyl K9 (ab8898-100, lot 100455), histone H4 tri methyl K20 (ab9053-100, lot 62688), pan histone H4 (ab10158-100, lot 168884), (Abcam, Cambridge, MA); histone H3 N-terminus (06-755, lot 31949), pan histone H3 C-Terminus (07-690, lot 30374); acetyl histone H3 (06-599, lot 29505), acetyl histone H4 (06-598, lot 29867) (Upstate Biotechnology, Lake Placid, NY) or normal rabbit IgG (sc-2027; lot A170, Santa Cruz Biotechnology, Santa Cruz, CA), were added and immunocomplexes allowed to form for different times, depending on the targeted proteins as specified. Immunoprecipitations against trimethylated histones and total histones were performed for 2 hours, while immunoprecipitations against transcription factors and histone deacetylases were performed overnight. Protein A-agarose beads (Invitrogen Corporation, Carlsbad, CA) were used to capture immunocomplexes and extensive washes were performed to disrupt non-specific interactions. Samples were treated overnight with proteinase K (Sigma-Aldrich, St. Louis, MO). Protein-DNA associations were reversed by incubation of the samples at 65°C for six hours and the DNA purified using a standard phenol-chloroform extraction. Upon resuspension, 0.5 to 2.0 µL of each sample was used in a PCR reaction to amplify the 5' UTR, the 3' UTR or the ORF1 region of the human L1 retrotransposon or the mouse L1Md-A2 gene. PCR primer sequences and expected product sizes are described in supplementary tables 2 and 3. Control PCR reactions for the human endogenous retrovirus type K (HERV-K) long terminal repeat (LTR) are described in supplementary table 3. PCR primers for GAPDH promoter have been described previously [35,36]. Measurement of the L1 promoter sequence enrichment within CHIP samples was performed according to ChampionCHIP™ qPCR Data Analysis manual (www.sabiosciences.com). PCR primer sequences and expected product sizes are described in supplementary table 3.

Western blot studies

Total cellular protein extracts were obtained using modified RIPA buffer (50 mM Tris-Cl pH 7.4; 1 mM EDTA; 150 mM NaCl; 0.1% SDS; 1% TritonX-100; 0.1% Na-Deoxycholate, 1X protease inhibitor cocktail). Thirty µg of total protein per sample were separated on 4–12% NuPAGE Bis-Tris gels under denaturing conditions following manufacturer's directions (Invitrogen Corporation, Carlsbad, CA). Proteins were transferred to PVDF membranes for 2 hr at 100 V using a PowerPac 3000 power supply unit (Bio-Rad Laboratories, Hercules, CA) and blocked in 5% milk in TBS-Tween 20 overnight. Antibodies against Rb (sc-50; C-15, lot C0205; goat polyclonal; 200 µg/mL), HDAC1 (H-51; sc-7872; rabbit polyclonal; lot H3104, 200 µg/mL), HDAC2 (H-54X; sc-7899; rabbit polyclonal; lot C0905, 200 µg/0.1 mL), LINE-1 (sc-67197 (H110); rabbit polyclonal; 200 µg/mL) and GAPDH (V-18; sc-20357; goat polyclonal; lot D1805, 200 µg/mL) were purchased from Santa Cruz Biotechnology (Santa Cruz, CA) were used at a 1:1000 dilution (0.2 µg/mL) in 5% milk in TBS-Tween 20. Membranes were incubated with the primary antibodies overnight, followed by 5 washes with TBS-Tween 20. Secondary antibodies (goat α-rabbit IgG-HRP; sc-2004; 200 µg/0.5 mL; lot F2705 and donkey α-goat IgG-HRP; sc-2056; 200 µg/0.5 mL; lot I3004) were added at a 1:5,000 dilution for 60 min followed by 5 washes with TBS-Tween 20. Detection was performed using ECL reagents (Amersham Biosciences, Buckinghamshire, England). Membranes were exposed to film for 1–5 min.

HDAC inhibitor treatments

Human cervical cancer cells (HeLa), wild type and Rb TKO MEFs were allowed to grow to 80% confluence in complete media with antibiotics. Cells were treated for 16 hours with vehicle (ethanol) or 3 μ M trichostatin A (TSA) (cat 194146, lot 7902F); (MP Biomedicals, Aurora OH). Total cell lysates were prepared with RIPA buffer and 20 μ g of protein were used to perform Western blot analysis.

Promoter analysis

To identify putative E2F transcription factor binding sites, promoter region DNA sequences from the human L1RP and the mouse L1Md-A2 genes (GeneBank accessions AF148856 and M13002 respectively; www.ncbi.nlm.nih.gov) were analyzed using MatInspector software (www.genomatrix.de).

3. RESULTS

Rb and E2F bind to L1 retrotransposon in vivo

L1 elements are highly repetitive sequences that contain CpG islands in their promoter regions [37,38]. Because the E2F/Rb complex binds CpG-rich regions in the genome with high affinity [29,36], we hypothesized that E2F/Rb complexes bind to L1 elements in vivo and play a role in regulation of L1 expression. MatInspector analysis showed that both human and mouse retrotransposons contain putative E2F binding sites within their 5' UTR or its vicinity (Fig. 1). Analysis of the human L1 promoter identified a putative E2F binding site (5'-gcaaGGCGgcaa-3') starting at base pair 413 of the promoter region. Likewise, analysis of mouse L1Md-A2 and L1Md-A5 retrotransposon promoters identified at least one conserved E2F binding site (5'-ttccGCGCgatt-3') at base pair 941. This sequence is related to the best known canonical sequence for E2F-1 (5'-TTTSSCGC-3') where S= C or G [39]. To determine if E2F/Rb complexes bind to the L1 promoter in vivo, HeLa cells, primary vascular (aortic) smooth muscle cells (vSMC), mouse embryo fibroblasts (MEFs), and Rb family null fibroblasts (TKO) were processed for ChIP analysis using antisera against E2F1, E2F4, and pRb.

Given that L1 is ubiquitous and locates to all human chromosomes, we scanned the human genome database for the colocalization of both L1 and human gene promoters to discard the presence of E2F sites on DNA regulatory regions of human genes adjacent to the L1 5' UTR. We found that of a total of 628 positive hits, only two sequences corresponding to putative genes possessed a regulatory region within 3 kb from the L1 5' UTR (see supplementary tables 4 and 5). Of note, MatInspector analysis did not identify E2F core sequences within the regions analyzed. To minimize non-specific precipitation of highly repetitive DNA sequences, chromatin was sheared to sizes below 1.0 Kb and immunoprecipitations were performed using decreasing amounts of chromatin (supplementary figure S1 A–C). In addition, Western blotting was completed using total cell lysates from multiple MEF and Rb null TKO cell lines to assess specificity of the Rb antisera. Supplementary figure 1E shows that Rb protein is detected in wild type, but not TKO MEFs. In addition, qPCR was performed in both wild type and TKO cells to confirm that mRNA for pRb, p107, and p130 was undetectable in TKO cells (not shown). As an additional control for non-specific DNA precipitation, the GAPDH gene promoter was PCR amplified in both mouse and human cells (supplementary figures S1 B and D). Upon selection of the optimum quantities of chromatin (40 μ g and 60 μ g for TKO and HeLa respectively), we performed ChIP assays followed by qPCR for HeLa cells (Figure 2A) and TKO cells (Figure 2B). As control for non specific enrichment of L1 sequences we amplified the 3' UTR region of the L1 element.

In keeping with these findings, ChIP assays for Rb in HeLa cells followed by qPCR show specific enrichment for L1 DNA (Fig 2A). This is consistent with experiments using a fixed amount of Rb antisera and increasing amounts of HeLa chromatin where a concentration-dependent increase in L1 amplification was detected (supplementary figure S1 A). When using low amounts of chromatin, the Rb immunoprecipitated sample did not amplify L1 DNA to levels above the background signal obtained using isotype-matched IgG control (supplementary figure S1-A bottom). In parallel measurements, IgG samples showed background amplification of L1 that did not change over a wide range of chromatin concentrations, indicating that amplification of L1 in HeLa cells was specific for Rb. To control for exogenous contamination with L1 DNA, mock samples incubated in the presence of buffers and reagents but lacking chromatin or PCR mix alone were included. In both mock and PCR control lanes, PCR amplification was not detected, thus confirming the specificity of the analysis. For all of our ChIP experiments we used a similar approach.

To further examine the specificity of molecular interactions, antisera against Stat-1 transcription factor, a non-target DNA binding protein, was used as an additional control for non-specific precipitation of L1 DNA. qPCR was also completed for both the 3' UTR region of LINE-1 and the long terminal repetitive sequence (LTR) of the human endogenous retrovirus type K (HERV-K) (Figure 2C). Amplicons were separated by electrophoresis and submitted for sequencing in both directions to confirm identity. Next, a similar assay was performed using chromatin from mouse embryo fibroblasts lacking Rb proteins p107, p130, and pRb (TKO) (supplementary figure S1-C). PCR amplification of L1 in TKO cells using Rb antisera did not amplify L1 above the IgG background level. Similar PCR amplification profiles for GAPDH were seen in both cell types for both Rb and IgG antibodies (supplementary figure S1 B and D respectively), indicating that non specific DNA in each sample was more or less constant. Thus, enrichment after the PCR reactions using primers directed against L1 was specific. A similar result was obtained after qPCR amplification of the L1 promoter and the 3' UTR region (Fig. 2B). The results shown in Figures 2A, 2B and 2C show that (i) L1 enrichment was only seen for E2F or Rb antisera, (ii) the precipitation of L1 DNA with Stat-1 antisera did not enrich for L1; (iii) amplification of L1 3'UTR region or HERV-K yielded comparable amplification for all antibodies, (iv) in cells lacking Rb protein there is not enrichment in L1 DNA after ChIP experiments using Rb antisera, and (v) the amplified sequence corresponded to the specific target (L1 5'UTR, L1 3'UTR or HERV-K LTR) (sequence data not shown). These data indicate that enrichment of L1 DNA only takes place when E2F and Rb antisera is used and that other repetitive sequences in the genome, either L1 3'UTR or HER-K LTR, are not enriched under the experimental conditions used. In silico mapping within the human genome for sequences that match the L1 amplicons generated was used to identify the approximate location of regulatory sequences in closest proximity. Only one sequence (gene locus PARP1P2) was identified in close proximity to the L1 5'UTR that could account for false positive amplification (supplementary Table 5). It should also be noted that although the nearest regulatory regions for human genes were found to be located as far as 6 Kb from PCR amplification targets, several batches of chromatin were sonicated to fragments smaller than 500 bp to minimize the risk of nonspecific L1 coprecipitation. Because new insertions of L1 elements are rare and cDNA are copied and inserted in reverse orientation, the number of copies of full length L1 within the genome is considerably low.

We next determined if Rb is bound in vivo to L1 elements in mouse primary vSMC and MEF. TKO MEFs and HeLa cells were also examined (Fig. 3A and 3B). We found that in all cell types positive for Rb expression, the Rb immunoprecipitated samples were enriched in L1 DNA above the IgG isotype background signal. The PCR control for GAPDH did not reveal differences between the Rb and the IgG lanes, confirming the specificity of the measurement (Fig. 3B). Consistent with these findings, a ZFX1A PCR control was used to

show that Rb antibody specifically enriches for an E2F/Rb target [40]. Overall, these data showed that i) E2F/Rb complexes bind in vivo to L1 elements in a human cancer cell line as well as two different mouse cell types, ii) detection of the L1 specific PCR product depends on the amount of starting material, iii) despite the presence of E7 viral oncoprotein in HeLa cells, Rb remains bound to L1.

Next, we performed ChIP assays with antisera against E2F1 and E2F4 on chromatin from MEFs, TKO, and HeLa cells (Fig. 3C). PCR reactions showed that compared to the IgG isotype control, both E2F1 and E2F4 immunoprecipitated samples were enriched in L1 DNA in mouse and human cells. Since ChIP analyses depend on the specificity of the antisera used, we also tested the samples not only for GAPDH amplification (Fig. 3D), but also for specific amplification of the zinc finger transcription factor ZFX1A, a novel E2F target gene and observed a specific enrichment of the PCR product in E2F, but not IgG or mock lanes [40]. These results showed that both E2F1 and E2F4 proteins bind L1 elements in vivo in human and mouse cells.

E2F/Rb complexes interact with AHR, and AHR regulates L1 expression in both mouse and human cells [41,42]. Following MatInspector analysis results suggesting the presence of putative E2F or AHR binding sites within the human L1 5'UTR and to further characterize the interaction between E2F/Rb complexes and L1 DNA, we wanted to know if E2F/Rb and AHR bind in vitro to the L1 5'UTR. To address this question, 50 bp oligonucleotide probes containing putative E2F and AHR binding sites within the human L1 5'-UTR were used in electrophoretic mobility shift assays (EMSA). Two different nuclear extract preparations from MEFs were screened for binding activity and yielded similar results. Multiple DNA-protein complexes were resolved with the E2F and AHR oligonucleotides (Fig. 6, lanes 2 and 7). The specificity of the interactions was confirmed using a 100-fold excess unlabeled oligonucleotide of the respective sequences (compare lanes 2 and 5 and lanes 7 and 10). To determine whether these complexes contained E2F or AHR, specific antisera to each of these proteins were tested for impact of DNA-protein binding. Figure 6, lanes 3 and 4 shows that when shifted using an E2F probe, E2F antibody preferentially neutralized complexes 2, 3 and 5, while AHR antibody preferentially inhibited 2 and 3. Lanes 8 and 9 show that using an AHR probe, E2F preferentially neutralized complexes 2 and 3, while AHR only supershifted complex 6 to give rise to complex 4. These data are consistent with ChIP assays in primary aortic vascular smooth muscle cells with antisera against E2F1 and AHR (not shown).

L1 elements contain epigenetic marks typical of pericentromeric heterochromatin

The DNA-bound protein complexes formed by E2F and the pocket protein family repress transcription through recruitment of co-repressor proteins that modify chromatin epigenetically. Specifically, Rb recruits histone deacetylases and histone methyltransferases that silence gene expression through nucleosomal histone modifications [30,31]. A hallmark of gene silencing and heterochromatin formation is the trimethylation of histone H3 at lysine 9 (H3K9 TriMe) by the Suv39h 1 and 2 histone methyltransferases (HMTases) and histone H4 at lysine 20 (H4K20 TriMe) by the Suv4-20h1 and Suv4-20h2 HMTases [43]. Since the absence of pocket proteins in TKO cells is associated with loss of histone H4 K20 trimethylation in the pericentromeric region of mouse chromosomes, we reasoned that a similar loss of epigenetic modifications would occur in L1. To determine if epigenetic silencing marks in the L1Md retrotransposon are dependent on the pocket protein family, antibodies against H3K9 TriMe and H4K20 TriMe were employed in ChIP assays using chromatin from both wild type and TKO MEFs (Fig. 4A). The specificity of antibodies used in these assays was established previously in experiments showing that Rb null cells exhibit aberrant histone methylation patterns in pericentromeric heterochromatic regions when compared to their wild type counterparts [30]. As an additional control, histone H3 and

histone H4 immunoprecipitation and PCR amplification of the mouse GAPDH promoter were performed (supplementary figure 2A bottom). The results showed that epigenetic marks for H3K9 TriMe and H4K20 TriMe are present in the L1 retrotransposon in wild type MEFs and allowed a marked enrichment of the L1 DNA (Fig. 4A and supplementary figure S2-A). The H3 control antisera rendered also strong amplification L1 PCR. These results indicate that the immunoprecipitation was specific. Overall, these data suggest that heterochromatic silencing marks are present in L1 elements in wild type MEFs.

We next determined if L1 elements in TKO cells show alterations in trimethylation marks as previously documented for other repetitive sequences found in pericentromeric regions [30]. Both H3K9 and H4K20 TriMe antisera in TKO cells showed lower enrichment for L1 than control MEFs. The H3 and IgG controls and the GAPDH PCR control showed similar results to the described for MEFs (Fig 4A and supplementary figure S2-A). Quantitative results using real time PCR for amplification of the ZFX1A gene promoter, an E2F/Rb target, showed similar results (36). These data indicate that in TKO cells, both trimethylation marks for H3K9 and H4K20 in L1 are reduced. Thus, L1Md retrotransposon carries the epigenetic marks that are a hallmark of constitutive heterochromatin [43], and these marks are partially, but not solely, dependent on the presence of the Rb family of proteins [30].

ChIP assays were completed for L1Md with antisera against total acetyl histones H3 and H4 in both wild type and TKO MEFs. Immunoprecipitation of both acetylated histones produced a small, but consistent amplification of L1 in wild type MEFs (Fig. 4B and supplementary figure S2-B). On the other hand, immunoprecipitation of acetylated histone H3 in Rb null cells yielded a higher PCR signal for L1Md, while the acetylated histone H4 generated a signal lower than that seen in wild type MEFs (Fig. 4B and supplementary figure S2-B). These amplification products were of higher intensity than background for both cell types. Since histone deacetylases 1 and 2 (HDAC 1 and HDAC2) are recruited to chromatin via Rb, studies were conducted to determine if these proteins bind L1 elements in vivo. We found that in wild type MEFs, both HDAC antisera immunoprecipitated L1 sequences (Fig. 4C). The IgG background signal was lower than any of the specific reactions and mock reactions did not show amplification (Fig 4C and supplementary figure S2-C). qPCR results showed the L1 amplification in TKO cells is lower than for wild type MEFs after HDAC antisera immunoprecipitation (Fig. 4C). To rule out differences in protein abundance that may differentially influence ChIP amplification, Western blot analysis was performed using lysates from the same cells used in ChIP assays. TKO MEFs from two different mouse lines (Δ and μ) expressed higher levels of HDAC1 and HDAC2 compared to wild type MEFs. The protein levels of GAPDH were similar in TKO and wild type cells, suggesting that Rb proteins regulate expression of HDAC1 and HDAC2, and that differential amplification was not accounted for by differences in protein recruitment to the L1 promoter. Finally, to assess the role of HDACs in L1 expression in vivo, HeLa or MEFs were treated with 3 μ M of the HDAC inhibitor trichostatin A (TSA). Western blot results showed that TSA increased expression of L1 ORF2p in both cell types implicating HDAC's repressive function in the regulation of L1 in vivo (supplementary figure S3). Interestingly, the L1 protein expression levels in TKO cells did not seem to change, suggesting that in Rb null cells the HDAC-dependent silencing mechanism for L1 gene is impaired.

Overall, these data indicate that i) L1Md nucleosomal histones are mostly in a deacetylated state in wild type MEFs, ii) the absence of Rb family of proteins increases acetylated histone H3 in L1 nucleosomal histones, iii) recruitment of HDAC1 and HDAC2 to L1 elements in vivo depends on the pocket protein family, (iv) HDAC-dependent mechanisms mediate L1 expression in vivo, and (v) pharmacological inhibition of HDAC function induces L1 expression.

Rb family null cells show increased levels of endogenous L1 expression

Since L1 showed both diminished marks for heterochromatin formation and destabilization of HDAC1 and HDAC2 recruitment to the promoter in TKO cells, we hypothesized that the degree of gene silencing for L1 would decrease. We therefore asked if the observed differences in nucleosomal histone modifications influence the expression of endogenous L1 at the RNA level. Thus, real time PCR was performed using wild type and TKO MEFs (Fig. 5A). Comparison of wild type and TKO MEFs showed that L1 is markedly upregulated in TKO cells, revealing a ten fold increase in L1 expression in these cells.

To further understand the mechanisms involved in E2F/Rb-mediated L1 expression, we used a human U2OS-derived cell line, DNE2F1 EH1 [32]. This line contains an IPTG-inducible cyclin-dependent kinase inhibitor p16INK4a gene, expresses endogenous Rb and E2F proteins, and a chimeric dominant-negative mutant of E2F1 (DNE2F1) E2F-1 DNA binding domain-Estrogen Receptor cytoplasmic protein (mER-E2F-1-DB) that can be inducibly activated by tamoxifen-estrogen receptor (Fig. 5B). In this system, Rb can be activated to bind E2F and to actively repress E2F target genes upon IPTG induction. Also, E2F responsive genes can be transcriptionally inactivated upon 4-OHT treatment and overexpression of the dominant negative DNA binding domain of E2F1 which displaces E2F from the promoters, hence preventing E2F-mediated transcription. The DN E2F1 cells were pretreated for 72 hours with vehicle, IPTG, tamoxifen, or both IPTG and tamoxifen followed by RNA isolation. Treatment of cells with IPTG leads to cdk4/6 inhibition and Rb activation through p16INK4a action. Active (hypophosphorylated) Rb then associates with DNA-bound E2F and represses gene expression. Conversely, tamoxifen treatment triggers the translocation of the chimeric mER-E2F-1-DB into the nucleus where it competes and displaces endogenous E2F from the target promoters. This cellular model has been previously characterized [32,40]. In the absence of IPTG (p16 is not expressed), these cells resemble the Rb null phenotype (Rb is hyperphosphorylated by cdk4/6 action). RT-PCR analysis for cells treated with tamoxifen showed little or no effect on L1 expression, while treatment with IPTG decreased the L1 expression levels (Fig. 5B). As a control for the response of the system to a known E2F target, changes in gene expression response for ribonucleotide reductase 1 control gene were studied [40]. Although treatment of cells with both IPTG and tamoxifen produced a small increase in L1 level, tamoxifen treatment alone did not alter L1 expression. It may be possible that IPTG and tamoxifen combined treatment activate cellular factor(s) and/or signaling pathways that affect L1 gene transcription and therefore the activation detected in our assays. Overall, the lack of tamoxifen effect on L1 expression regardless of Rb status, and the decreased expression levels of L1 upon IPTG treatment, support the interpretation that L1 is subject to Rb-mediated repression, but not an E2F activation target gene, hence implicating Rb proteins in L1 retrotransposon silencing.

4. DISCUSSION

Evidence is presented here showing that: i) E2F/Rb complexes bind L1 retrotransposons in both human and mouse cells in vivo and in vitro; ii) pocket protein family mediate and/or stabilize silencing epigenetic marks in L1 nucleosomal histones; iii) HDAC1 and HDAC2 are involved in L1 silencing and their recruitment to L1 is dependent on Rb; iv) TKO MEFs show marked upregulation of L1.

L1 is epigenetically modified in an Rb family-dependent manner

Epigenetic silencing of chromatin depends, among others things, on the enzymatic activity of histone deacetylases and histone methyltransferases. Histone H3 and H4 trimethylation at lysine 9 (H3K9 TriMe) and 20 (H4K20 TriMe) by the Suv39h 1 and 2, Suv4-20h1, and Suv4-20h2 HMTases are a hallmark for heterochromatin formation [43]. These marks are

associated with highly repetitive sequences in the pericentromeric regions in mammalian chromosomes. ChIP assays with antisera against Rb, E2F-1, and E2F-4 in human and mouse cells yielded specific enrichment in L1 sequences, indicating that these proteins specifically bind to mouse and human L1 elements in vivo. Recent studies have found that E2F proteins bind to a large set of poorly characterized CpG-containing genes, and that E2F/Rb complexes also bind to unconventional DNA sequences that diverge greatly from the well known E2F DNA consensus motif [39,44]. In addition, Rb mediates gene silencing and heterochromatin formation through its association with DNA and histone methyltransferases [31,40,45]. This activity has been characterized not only for known E2F target genes, but also in pericentromeric regions of chromosomes which are rich in repetitive sequences [30,46].

To determine if the pocket protein family mediates silencing of L1 via histone hypermethylation, ChIP assays were performed using anti H3K9 TriMe and anti H4K20 TriMe in wild type and Rb family null MEFs. We found that mouse retrotransposons of the L1 family contain these silencing marks. These findings show that epigenetic marks for heterochromatin formation are present in retrotransposons located at non-pericentromeric regions, and that they depend on the E2F/Rb complex repressor activity. Since the loss of the pocket protein family decreases the trimethylation status of L1 elements, but not completely abolishes signal detection, we suggest that Rb proteins function mostly in stabilization of epigenetic marks. HMTases may be recruited to chromatin by transcription factors other than the pocket protein family or yet uncovered trimethylases are responsible for the remaining modification of L1 elements. Additional Rb family-dependent epigenetic modifications include the deacetylation of histone tails by HDAC recruitment. Previous reports have shown that TKO MEFs contain a higher level of acetylated H3 than wild type MEFs [30]. Consistent with this report, we found that histone H3 is hyperacetylated in L1 elements from TKO cells, and loss of Rb proteins disrupt the presence of HDAC1 and HDAC2 in vivo in L1 elements. Interestingly, TKO MEFs contained higher levels of HDACs than wild type cells, suggesting that the pocket protein family regulate HDACs expression and that HDACs are not limiting factors in the regulation of LINE-1 promoter in TKO cells. Thus, L1 elements may be epigenetically modified at the histone level in an Rb family-dependent manner. The pocket protein family seems to be responsible for recruitment of HDACs and, at least partially, the recruitment of HMTases. Overall, the lack of HDAC recruitment to L1 elements increases acetylated histone H3, but not of H4, and may lead to L1 increased gene expression. Preliminary bisulfite genome sequencing studies show that TKOs exhibit a modest, but consistent change in DNA methylation of A-type monomers within the 5'UTR. This observation is consistent with our finding that modifications of epigenetic H3 and H4 silencing marks are decreased, but not completely ablated in TKOs compared to wild type counterparts. Thus, while Rb seems to play a role in recruiting corepressors with histone and DNA methylation activity, other transcription factors likely participate in recruitment and stabilization of molecular interactions. As such, the absence of Rb proteins impairs, but does not ablate methylation of CpG-rich regions within the L1 promoter region. To our knowledge, this is the first report showing that Rb binds in vivo to non-pericentromeric highly repetitive sequences.

L1 gene expression is upregulated in Rb family mutant cells

Since Rb family-mediated recruitment of corepressors to gene promoters leads to gene silencing [31,45,47], we reasoned that the pocket protein family could regulate L1 expression through modulation of chromatin repressive states. If so, absence or inactivation of Rb proteins should remove L1 repressive marks and increase transcription. In keeping with this hypothesis we found that L1 was upregulated in TKO cells. We also found that the fold activation as detected with primers against the promoter was greater than with primers

against ORF1 (data not shown). This is consistent with reports showing that full length L1 transcripts are scarce due to lack of processivity of the RNA PolII complex and premature polyadenylation signals within the L1 element [11,48]. In agreement with the increased expression of L1 in TKO cells, studies using a cell system where both the absence of p16INK4a and/or the overexpression of a dominant negative E2F-1 resemble the Rb null phenotype revealed similar results. First, the absence of p16INK4a removes the Rb-mediated L1 repression and accordingly L1 expression decreased in cells where p16INK4a was overexpressed (Rb active). Second, the lack of significant changes in L1 expression after tamoxifen treatment (E2F displaced from the promoters by a dominant negative E2F-1) indicates that L1 is not an E2F activation target. These data support the hypothesis that L1 is subject to Rb mediated repression (e.g. IPTG treatment, p16INK4a overexpression), but is not part of the E2F transactivation pathway. Based on these findings we conclude that E2F and Rb family protein complexes are required to regulate the expression of L1MdA genes and that L1 elements are regulated not only by DNA methylation, but also through histone modifications.

Implications for Cancer Biology

The data presented here has significant implications for the role of L1 reactivation in the regulation of normal and transformed phenotypes and oncogenesis. The differences in L1 regulation observed between MEF and TKO cells are likely accounted for by their Rb status since all 3 lines employed were derived from different donors and share identical genetic backgrounds. Previous studies comparing cell cycle kinetics has shown that MEF and TKO cells exhibit different cycling patterns [30]. Using sorted mouse embryonic stem cells (MES) D3 (ES D3) we recently found that L1 is differentially expressed at different stages of the cell cycle, suggesting that E2F/Rb and/or other cell cycle protein complexes might mediate the expression of L1 elements in a cell-cycle dependent manner (not shown). Ongoing studies are in progress to evaluate the relevance of Rb molecular interactions to functional regulation of L1 in mammalian cells. Within this context it should be noted that aryl hydrocarbon receptor (AHR) transcription factor, a PAS-bHLH transcription factor involved in cell cycle regulation, is known to interact directly with Rb [41], and AHR modulates the transcriptional activity of L1 in both mouse and human cells [42]. Disregulation of the E2F/Rb pathway may lead to disassembly of corepressor complexes, loss of HDAC recruitment, loss of epigenetic silencing marks in Rb genomic targets, and concomitant L1 reactivation. Due to the ubiquitous distribution of L1 in the mammalian genome, these changes may result in genomic instability and global rearrangements of the epigenetic environment in an L1 element-dependent fashion.

In conclusion, we have shown that L1 elements retain a conserved evolutionary epigenetic mechanism that relies on Rb proteins to regulate its expression. This mechanism involves the covalent modification of histones, as well as the recruitment of HDAC1 and HDAC2. Also, E2F transcription factors bind in a conserved manner to the L1 elements but its binding does not regulate the epigenetic signals associated to repression.

These molecular events implicate epigenetic control of L1s by at least two mechanisms: (i) DNA methylation, and (ii) nucleosomal hypermethylation and/or deacetylation [30,47]. Since LINEs are dispersed throughout the genome and can be frequently found within or near single copy genes, and E2F and Rb complexes potentially mediate the formation of heterochromatin-like domains, L1 elements may function as master regulators of chromatin structure through heterochromatic silencing of discrete chromosomal regions in genes that are regulated in a cell-cycle-dependent fashion.

Rb proteins may function as a regulator of epigenetic silencing of L1 elements in wild type cells via histone deacetylation/methylation and/or DNA methylation. We link the decrease

in histone epigenetic silencing marks and the loss of HDACs recruitment, to a gain in histone H3 acetylation of the L1 element, hence reflected in a more opened chromatin structure. This would explain the augmented L1 expression seen upon genotoxic stress. It is expected therefore that individuals with predisposition to Rb inactivation may have abnormal expression of LINE-1, and possibly L1-regulated putative genes, that would accelerate the process of tumorigenesis through loss of cell cycle regulatory control and increased retrotransposition rates along with genomic instability.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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References

- Allen E, Horvath S, Tong F, Kraft P, Spiteri E, Riggs AD, Marahrens Y. High concentrations of long interspersed nuclear element sequence distinguish monoallelically expressed genes. *Proc Natl Acad Sci U S A*. 2003; 100:9940–9945. [PubMed: 12909712]
- Lavie L, Maldener E, Brouha B, Meese EU, Mayer J. The human L1 promoter: variable transcription initiation sites and a major impact of upstream flanking sequence on promoter activity. *Genome Res*. 2004; 14:2253–2260. [PubMed: 15520289]
- Symer DE, Connelly C, Szak ST, Caputo EM, Cost GJ, Parmigiani G, Boeke JD. Human L1 retrotransposition is associated with genetic instability in vivo. *Cell*. 2002; 110:327–338. [PubMed: 12176320]
- Kazazian HH Jr. Mobile elements and disease. *Curr Opin Genet Dev*. 1998; 8:343–350. [PubMed: 9690999]
- Kazazian HH Jr, Wong C, Youssoufian H, Scott AF, Phillips DG, Antonarakis SE. Haemophilia A resulting from de novo insertion of L1 sequences represents a novel mechanism for mutation in man. *Nature*. 1988; 332:164–166. [PubMed: 2831458]
- Kazazian HH Jr, Goodier JL. LINE drive. retrotransposition and genome instability. *Cell*. 2002; 110:277–280. [PubMed: 12176313]
- Gilbert N, Lutz-Prigge S, Moran JV. Genomic deletions created upon LINE-1 retrotransposition. *Cell*. 2002; 110:315–325. [PubMed: 12176319]
- Bennett EA, Coleman LE, Tsui C, Pittard WS, Devine SE. Natural genetic variation caused by transposable elements in humans. *Genetics*. 2004; 168:933–951. [PubMed: 15514065]
- Yang Z, Boffelli D, Boonmark N, Schwartz K, Lawn R. Apolipoprotein(a) gene enhancer resides within a LINE element. *J Biol Chem*. 1998; 273:891–897. [PubMed: 9422746]
- Muotri AR, Chu VT, Marchetto MC, Deng W, Moran JV, Gage FH. Somatic mosaicism in neuronal precursor cells mediated by L1 retrotransposition. *Nature*. 2005; 435:903–910. [PubMed: 15959507]
- Ostertag EM, Kazazian HH Jr. Biology of mammalian L1 retrotransposons. *Annu Rev Genet*. 2001; 35:501–538. [PubMed: 11700292]
- Belancio VP, Hedges DJ, Deininger P. LINE-1 RNA splicing and influences on mammalian gene expression. *Nucleic Acids Res*. 2006; 34:1512–1521. [PubMed: 16554555]
- Athanikar JN, Badge RM, Moran JV. A YY1-binding site is required for accurate human LINE-1 transcription initiation. *Nucleic Acids Res*. 2004; 32:3846–3855. [PubMed: 15272086]

14. Tchenio T, Casella JF, Heidmann T. Members of the SRY family regulate the human LINE retrotransposons. *Nucleic Acids Res.* 2000; 28:411–415. [PubMed: 10606637]
15. Yang N, Zhang L, Zhang Y, Kazazian HH Jr. An important role for RUNX3 in human L1 transcription and retrotransposition. *Nucleic Acids Res.* 2003; 31:4929–4940. [PubMed: 12907736]
16. Lu KP, Ramos KS. Redox regulation of a novel L1Md-A2 retrotransposon in vascular smooth muscle cells. *J Biol Chem.* 2003; 278:28201–28209. [PubMed: 12714586]
17. Yoder JA, Walsh CP, Bestor TH. Cytosine methylation and the ecology of intragenomic parasites. *Trends Genet.* 1997; 13:335–340. [PubMed: 9260521]
18. Bailey JA, Carrel L, Chakravarti A, Eichler EE. Molecular evidence for a relationship between LINE-1 elements and X chromosome inactivation: the Lyon repeat hypothesis. *Proc Natl Acad Sci U S A.* 2000; 97:6634–6639. [PubMed: 10841562]
19. Feinberg AP, Vogelstein B. Hypomethylation of ras oncogenes in primary human cancers. *Biochem Biophys Res Commun.* 1983; 111:47–54. [PubMed: 6187346]
20. Guo SX, Taki T, Ohnishi H, Piao HY, Tabuchi K, Bessho F, Hanada R, Yanagisawa M, Hayashi Y. Hypermethylation of p16 and p15 genes and RB protein expression in acute leukemia. *Leuk Res.* 2000; 24:39–46. [PubMed: 10634644]
21. Schulz WA. L1 retrotransposons in human cancers. *J Biomed Biotechnol.* 2006; 2006:83672. [PubMed: 16877821]
22. Britten RJ. DNA sequence insertion and evolutionary variation in gene regulation. *Proc Natl Acad Sci U S A.* 1996; 93:9374–9377. [PubMed: 8790336]
23. Britten RJ. Cases of ancient mobile element DNA insertions that now affect gene regulation. *Mol Phylogenet Evol.* 1996; 5:13–17. [PubMed: 8673282]
24. Eden A, Gaudet F, Waghmare A, Jaenisch R. Chromosomal instability and tumors promoted by DNA hypomethylation. *Science.* 2003; 300:455. [PubMed: 12702868]
25. Gaudet F, Hodgson JG, Eden A, Jackson-Grusby L, Dausman J, Gray JW, Leonhardt H, Jaenisch R. Induction of tumors in mice by genomic hypomethylation. *Science.* 2003; 300:489–492. [PubMed: 12702876]
26. Jurgens B, Schmitz-Drager BJ, Schulz WA. Hypomethylation of L1 LINE sequences prevailing in human urothelial carcinoma. *Cancer Res.* 1996; 56:5698–5703. [PubMed: 8971178]
27. Roman-Gomez J, Jimenez-Velasco A, Agirre X, Cervantes F, Sanchez J, Garate L, Barrios M, Castillejo JA, Navarro G, Colomer D, Prosper F, Heiniger A, Torres A. Promoter hypomethylation of the LINE-1 retrotransposable elements activates sense/antisense transcription and marks the progression of chronic myeloid leukemia. *Oncogene.* 2005; 24:7213–7223. [PubMed: 16170379]
28. Weinmann AS, Yan PS, Oberley MJ, Huang TH, Farnham PJ. Isolating human transcription factor targets by coupling chromatin immunoprecipitation and CpG island microarray analysis. *Genes Dev.* 2002; 16:235–244. [PubMed: 11799066]
29. Weinmann AS, Bartley SM, Zhang T, Zhang MQ, Farnham PJ. Use of chromatin immunoprecipitation to clone novel E2F target promoters. *Mol Cell Biol.* 2001; 21:6820–6832. [PubMed: 11564866]
30. Gonzalo S, Garcia-Cao M, Fraga MF, Schotta G, Peters AH, Cotter SE, Eguia R, Dean DC, Esteller M, Jenuwein T, Blasco MA. Role of the RB1 family in stabilizing histone methylation at constitutive heterochromatin. *Nat Cell Biol.* 2005; 7:420–428. [PubMed: 15750587]
31. Robertson KD, Ait-Si-Ali S, Yokochi T, Wade PA, Jones PL, Wolffe AP. DNMT1 forms a complex with Rb, E2F1 and HDAC1 and represses transcription from E2F-responsive promoters. *Nat Genet.* 2000; 25:338–342. [PubMed: 10888886]
32. Young AP, Nagarajan R, Longmore GD. Mechanisms of transcriptional regulation by Rb-E2F segregate by biological pathway. *Oncogene.* 2003; 22:7209–7217. [PubMed: 14562049]
33. Sage J, Mulligan GJ, Attardi LD, Miller A, Chen S, Williams B, Theodorou E, Jacks T. Targeted disruption of the three Rb-related genes leads to loss of G(1) control and immortalization. *Genes Dev.* 2000; 14:3037–3050. [PubMed: 11114892]
34. Orlando V, Strutt H, Paro R. Analysis of chromatin structure by in vivo formaldehyde cross-linking. *Methods.* 1997; 11:205–214. [PubMed: 8993033]

35. Annicotte JS, Fayard E, Swift GH, Selander L, Edlund H, Tanaka T, Kodama T, Schoonjans K, Auwerx J. Pancreatic-duodenal homeobox 1 regulates expression of liver receptor homolog 1 during pancreas development. *Mol Cell Biol.* 2003; 23:6713–6724. [PubMed: 12972592]
36. Wells J, Graveel CR, Bartley SM, Madore SJ, Farnham PJ. The identification of E2F1-specific target genes. *Proc Natl Acad Sci U S A.* 2002; 99:3890–3895. [PubMed: 11904439]
37. Hata K, Sakaki Y. Identification of critical CpG sites for repression of L1 transcription by DNA methylation. *Gene.* 1997; 189:227–234. [PubMed: 9168132]
38. Woodcock DM, Lawler CB, Linsenmeyer ME, Doherty JP, Warren WD. Asymmetric methylation in the hypermethylated CpG promoter region of the human L1 retrotransposon. *J Biol Chem.* 1997; 272:7810–7816. [PubMed: 9065445]
39. Bieda M, Xu X, Singer MA, Green R, Farnham PJ. Unbiased location analysis of E2F1-binding sites suggests a widespread role for E2F1 in the human genome. *Genome Res.* 2006; 16:595–605. [PubMed: 16606705]
40. Liu Y, Costantino ME, Montoya-Durango D, Higashi Y, Darling DS, Dean DC. The zinc finger transcription factor ZFX1A is linked to cell proliferation by Rb-E2F1. *Biochem J.* 2007; 408:79–85. [PubMed: 17655524]
41. Puga A, Barnes SJ, Dalton TP, Chang C, Knudsen ES, Maier MA. Aromatic hydrocarbon receptor interaction with the retinoblastoma protein potentiates repression of E2F-dependent transcription and cell cycle arrest. *J Biol Chem.* 2000; 275:2943–2950. [PubMed: 10644764]
42. Teneng I, Stribinskis V, Ramos KS. Context-specific regulation of LINE-1. *Genes Cells.* 2007; 12:1101–1110. [PubMed: 17903170]
43. Schotta G, Lachner M, Sarma K, Ebert A, Sengupta R, Reuter G, Reinberg D, Jenuwein T. A silencing pathway to induce H3-K9 and H4-K20 trimethylation at constitutive heterochromatin. *Genes Dev.* 2004; 18:1251–1262. [PubMed: 15145825]
44. Oberley MJ, Tsao J, Yau P, Farnham PJ. High-throughput screening of chromatin immunoprecipitates using CpG-island microarrays. *Methods Enzymol.* 2004; 376:315–334. [PubMed: 14975315]
45. Nielsen SJ, Schneider R, Bauer UM, Bannister AJ, Morrison A, O'Carroll D, Firestein R, Cleary M, Jenuwein T, Herrera RE, Kouzarides T. Rb targets histone H3 methylation and HP1 to promoters. *Nature.* 2001; 412:561–565. [PubMed: 11484059]
46. Isaac CE, Francis SM, Martens AL, Julian LM, Seifried LA, Erdmann N, Binne UK, Harrington L, Sicinski P, Berube NG, Dyson NJ, Dick FA. The retinoblastoma protein regulates pericentric heterochromatin. *Mol Cell Biol.* 2006; 26:3659–3671. [PubMed: 16612004]
47. Pradhan S, Kim GD. The retinoblastoma gene product interacts with maintenance human DNA (cytosine-5) methyltransferase and modulates its activity. *EMBO J.* 2002; 21:779–788. [PubMed: 11847125]
48. Perepelitsa-Belancio V, Deininger P. RNA truncation by premature polyadenylation attenuates human mobile element activity. *Nat Genet.* 2003; 35:363–366. [PubMed: 14625551]

FIG. 1.

Schematic representation of human and mouse LINE-1 genes and location of known and putative transcription factor binding sites. Arrows indicate the directionality of the DNA sequence within the promoter region. Core DNA binding sequences are shown and numbers represent the location of the last nucleotide in the core sequence as obtained after MatInspector analysis.

FIG. 2.

Rb binds to human LINE-1 in vivo. (A) LINE-1 qPCR amplification results for HeLa chromatin titration after immunoprecipitation with Rb antisera. Results are presented as the % input. PCR was performed with primers against the 5'UTR or the 3'UTR region of the human L1 element. (B). LINE-1 qPCR amplification results after ChIP assays from Rb null family of proteins mouse embryo fibroblasts (Rb TKO MEFs) following Rb antisera immunoprecipitation. PCR primers were directed against the mouse L1MdA 5'UTR and 3'UTR regions respectively. (C) LINE-1 qPCR in HeLa cells were performed as described for A. In addition to the L1 primers, a primer set for amplification of the long terminal repeat region of the HERV-K type was used.

FIG. 3. E2F and Rb are bound to both human and mouse LINE-1 elements *in vivo*. (A) LINE-1 PCR amplification results for mouse primary aortic vascular smooth muscle cells, wild type control mouse primary embryo fibroblasts (MEFs), Rb TKO MEFs, and human HeLa cells. All assays were performed with 40 μ g of chromatin for each immunoprecipitation. (B) GAPDH PCR amplification results for each of the samples described above. (C) E2F-1 and E2F-4 antisera immunoprecipitations were made with 40 μ g of chromatin from MEFs, Rb TKO MEFs, or HeLa cells followed by LINE-1 PCR amplification. (D) GAPDH PCR amplification results for each of the samples described in (C).

FIG. 4.

Heterochromatin epigenetic marks are present in LINE-1 genes and depend on Rb proteins. Results were generated using PCR primers directed against the L1MdA 5'UTR region and expressed as the % input. (A) LINE-1 qPCR with primers against the 5'UTR region of the mouse L1MdA element after immunoprecipitation with antisera against histone H3 tri methyl K9, histone H4 tri methyl K20, and pan-histone H3. (B) LINE-1 PCR amplification results for Rb TKO and wild type MEFs cells with antisera against Pan acetyl histone H3, Pan acetyl histone H4, and Pan histone H3. (C) LINE-1 PCR amplification results for wild type and Rb TKO MEFs with antisera against HDAC1 and HDAC2. (D) Western blot analysis for HDAC1 and HDAC2 in total protein lysates from cells used in the ChIP assays.

FIG. 5. LINE-1 expression is augmented in cells lacking Rb family expression. To perform the RT-PCR we used a primer set for mouse L1Md-A2 promoter, L1-A2 (16) and a primer set for the human L1, ORF2 region. (A) Real time PCR results compared L1 expression in Rb TKO MEFs and wild type control MEFs with primers for the LINE-1 promoter. (B) U2OS cells expressing an IPTG-inducible p16INK4a and a Tamoxifen-regulated chimeric mER-E2F-DB were used to study the expression levels of LINE-1 upon treatment with IPTG, Tamoxifen, or both. Real time PCR was performed with primers against the ORF2 region of the human LINE-1 gene.

FIG. 6. E2F/Rb and AHR Complexes Bind Specifically to L1 Promoter DNA In Vitro. EMSA using biotin-labeled probes E2F1, (5'-tgagatcaaactgcaaGGCGgcaacgaggctgggggagggcgcccgcca-3'; bp 401-450) or AHR (5'-agagcagtgg ttctcccagCACGCagctggagatctgagaacgggcagac -3'; bp 631-680) of the human L1 5'UTR were run using nuclear extracts (NE) from wild type MEFs. Uppercase letters show the core sequence for the transcription factor binding sites as identified by MatInspector analysis. Antibody (Ab) against E2F-1 (E) or AHR (A) transcription factors or unlabeled probes (Comp) were used where indicated. Shifted and supershifted DNA/protein complexes and free probe are indicated by arrows.