Epigenetic reactivation of LINE-1 retrotransposon disrupts NuRD corepressor functions and induces oncogenic transformation in human bronchial epithelial cells

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1. Introduction

Human L1 is ~6 kb and consists of an internal promoter located within the 5’translated region (5’UTR), two open reading frames (ORFs) encoding ORF1p and ORF2p, and a 3’UTR with a poly(A) tail and signal (Dombroski et al., 1991; Scott et al., 1987). L1 elements constitute a large family of mammalian retrotransposons that have continuously replicated for over 100 Myr (Boissinot et al., 2000). Approximately 100 retrotransposition-competent L1s remain in the human genome that function as autonomous elements through a copy-and-paste mechanism (Brouha et al., 2003). The human L1 promoter is highly conserved among active L1s, except for a single nucleotide deletion at position 74, and a single nucleotide polymorphism (SNP) at 711 (t/c), both of which differentiate Ta-1d from the Ta-Ind, Ta-0, and Pre-Ta families (Boissinot et al., 2000; Swergold, 1990). The active human Ta family arose ~4MYA and subsequently differentiated into two major subfamilies, Ta-0 and Ta-1, each of which contains additional members. Ta-1 is younger than Ta-0 and now accounts for at least 50% of the Ta family (Boissinot et al., 2000).

Abbreviations
BaP, benzo(a)pyrene; HBECs, human bronchial epithelial cells; Line-1/L1, long interspersed nuclear element-1 (LINE-1 or L1); MBD, methyl-CpG-binding domain proteins; MTA, metastasis-associated proteins; NuRD, nucleosomal and remodeling deacetylase; RNPS, ribonucleoprotein particles (RNPs).
A cycle of retrotransposition involves transcription of both ORFs from a common 5′UTR by RNA PolII, followed by translation in the cytoplasm (Swergold, 1990). The L1-encoded proteins exhibit either cis- (major) or trans- (minor) preference to bind L1 mRNA or SINEs and other cellular mRNAs, to form ribonucleoprotein particles (RNPs) (Hohjoh and Singer, 1996; Wei et al., 2001). The RNP-containing L1 mRNA, or other mRNAs, mainly localizes to the cytoplasm, but can translocate into the nucleus where the endonuclease domain of ORF2p nicks a single strand of genomic DNA to expose a 3′-OH group that can then be used by the reverse transcriptase domain of ORF2p to prime and reverse synthesize L1 cDNA from the 3′ end (Feng et al., 1996; Hattori et al., 1986). This process can lead to full-length or truncated insertions of L1 sequences throughout the genome (Moran et al., 1996).

Because uncontrolled retrotransposition can be genotoxic and detrimental to the host, active L1 elements are silenced in most somatic cells via DNA methylation and histone covalent modifications (Montoya-Durango et al., 2009; Teneng et al., 2011). In addition, mammalian cells evolved several mechanisms against retrotransposition, including the deamination by APOBEC proteins, L1 mRNA degradation, and preferential targeting of L1 sequences to gene-poor regions (Bojang et al., 2014; Esnault et al., 2006). L1 is reactivated by tobacco carcinogens such as benzo(a)pyrene (BaP) through a process that is orchestrated by the aryl hydrocarbon receptor (AHR) and retinoblastoma (RB) family of proteins to influence the epigenetic machinery (Lu and Ramos, 2003; Montoya-Durango et al., 2009; Stribinskis and Ramos, 2006; Teneng et al., 2007). L1 reactivation can reshuffle the genome to afford a survival advantage or mediate acquisition of stem cell-like properties and epithelial-to-mesenchymal transition (EMT), as seen in non-small-cell lung cancers (NSCLCs) (Bojang et al., 2013; Ikeda et al., 2013; McClintock, 1984; Saito et al., 2010). We recently showed that L1 couples EMT with oncogenesis in human bronchial epithelial cells (HBECs) through a signaling cascade initiated by TGF-β1 (Reyes-Reyes et al., 2017).

Given the possible roles of L1 in lung pathogenesis, elucidation of the molecular mechanisms of L1 regulation is of interest. In this context, we have shown that the nucleosomal and remodeling deacetylase (NuRD) multiprotein complex is enriched in the 5′untranslated DNA sequence of L1 in human and murine cells. Further, genetic ablation of RB proteins destabilizes interactions within the NuRD complex, and mediates nuclear rearrangement of Mi2β, an ATP-dependent helicase subunit with nucleosome remodeling activity (Montoya-Durango et al., 2016). The NuRD multiprotein complex consists of six core components, including the chromodomain-helicase-DNA-binding proteins 3 or 4 (also known as Mi2α and Mi2β, respectively); methyl-CpG-binding domain proteins 1/2/3 (MBD1/2/3); histone-asociated proteins 1/2/3 (MTA1/2/3); and histone-binding proteins (Rbhp4/7, RbAp46/48), histone deacetylases 1/2 (HDAC1/2), and nuclear zinc finger proteins (Gata2α/2b/p66α/β)) (Xue et al., 1998; Zhang et al., 1999). HDAC1/2 and Rbhp4/7 are not exclusive to the NuRD complex and are also present in the SIN3 and CoREST multiprotein complexes, while Mi2αβ, MBDs, and MTAs are relatively specific to the NuRD complex, and their interactions with core components and other accessory proteins mediate recruitment and functionally of the NuRD complex (Humphrey et al., 2001).

The present studies were conducted to elucidate structural and functional determinants of NuRD corepressor expression within the L1 promoter in HBECs following reactivation by the tobacco carcinogen, BaP. Evidence is presented here that L1 reactivation in HBECs by BaP shifts the L1 promoter from a heterochromatic euchromatic state through disassembly and delocalization of the NuRD multiprotein complex. This response leads to cellular accumulation of ORF1 and ORF2 mRNA and proteins. The corepressor function of NuRD is dependent upon the DNA and ATPase domains of Mi2β, working together with MBD2/3 to regulate the state of L1 chromatin. Expression of a constitutively active L1 in HBECs was tumorigenic in a SCID mouse xenograft model, a finding that directly implicates L1 reactivation in neoplasia and malignant transformation of lung epithelial cells.

2. Materials and methods

2.1. Plasmids and cloning

Mi2β wild-type (WT) and a biologically inactive W508A chromodom state mutant were kind gifts from J. Hagman at the University of Colorado (Ramírez et al., 2012). DNA (E874A-) and ATPase (W879A)-binding domain mutants were created as described previously using PCR primers as specified in Table 1 (Ramírez et al., 2012). Mutant fragments were joined by nested PCR and cloned into a Age I and pSHA I linearized MSCI vector by infusion cloning (Clontech). L1-5′UTR promoter was cloned into the BglII site of pGL4.15 (luc2P/Hygro) vector using infusion cloning and primers listed in Table 1. Cloned fragments were sequenced in reverse and forward directions to confirm identity and orientation. HBECs were transfected with
pB001CTR or pB015WT using Lipofectamine, as previously described. The pB015WT and pB001CTR (i.e., pGL4.15 backbone) vectors were used to create stably transfected HBECs constitutively expressing L1-ORF1 and ORF2 proteins, as confirmed by indirect immunofluorescence.

2.2. Real-Time (RT) PCR, siRNA treatment, western analysis, and indirect immunofluorescence

BEAS-2B cells (CRL#: 9609, ATCC) is a nontransformed HBEC line immortalized with a replication-defective SV-40/adenovirus 12 hybrid (Cat#: 15338100, Thermo Scientific). Despite the presence of T-antigen in replication-defective lines (Butel and Rapp, 1966), BEAS-2B cells remain RB/p53 competent (Pan et al., 2011), and are representative of squamous epithelial cells within the proximal airway. The integrity of RB and p53 signaling was confirmed in studies showing intact RB phosphorylation and p53 acetylation in BEAS-2B cells (data not shown). Cells were grown to 70% confluence and treated with scrambled siRNA or siRNAs directed against Mi2b, MBD2/3, and MTA1/2/3 for 48 h. Total RNA was extracted and 0.5–1 μg used for cDNA synthesis (Invitrogen Superscript II) with OligoDT primers (Tables 2 and 3). The homogeneity of PCR products was confirmed using an RT-PCR melting curve for SYBR probes. For TaqMan probes, DDCT was used to calculate the relative quantity of L1. For SYBR probes, a standard curve method was used to calculate relative quantity. For western blot analysis, HBECs were lysed with RIPA buffer and 15–30 μg total protein loaded onto 4–12% gradient gels (Invitrogen). A polyclonal antibody against L1-ORF1 generated by New England Peptide and validated in previous studies (Ramos et al., 2011; Reyes-Reyes et al., 2016) was used in all experiments (Bojang et al., 2013). The optimization of L1-ORF1 detection by Western analysis in HBECs is presented in Fig. S1. The overexpression of L1 proteins in cells transfected with pB015WT was confirmed by Western and documented previously (Reyes-Reyes et al., 2017). Mi2β (Cat#: SAB5300324). MBD2/3 (Cat #: SAB2101140/SAB1300141), and MTA2/3 (Cat#: M7569, WH0057504MI) antibodies were obtained from Sigma or Cell Signaling. Primary antibodies against NuRD proteins were diluted 1/1000, while the ORF1 antibody was diluted 1/500 and incubated overnight. The antibody against GAPDH was obtained from Santa-Cruz (Cat#: sc-47724). Indirect immunofluorescence was conducted as described in detail previously (Bojang et al., 2013).

2.3. Chromatin immunoprecipitation assay followed by RT-PCR

Approximately 1 × 10⁶ HBECs were grown to 70% confluence and treated with 1% formaldehyde for

### Table 1. Cloning of Mi2β and L1-5'UTR.

| Sequence name | Sequence |
|---------------|----------|
| DNA (E874AI)-IF | 5'-GAATTCCATGTGCTGCTGACAGCCTATGAATTGATC-3' |
| DNA (E874AI)-IR | 5'-AGAAATTGCCATGTGCCATCTGTCAATGATGTTGATC-3' |
| ATPase (W879A)-1F | 5'-CTGAGCCCTATGCAAGAAGAATACAGGGTCTGAGCTGTCAGCAG-3' |
| ATPase (W879A)-1R | 5'-GTCACAAACCCTAGATCATCATTACCTTTTTATTTTG-3' |
| pGL4.15Luc-1F | 5'-GGAGATATACAGATGTCGGGAGGAGAGGAGATGGGCCAAGC-3' |
| pGL4.15Luc-1R | 5'-GCCAGAGCCAGATCTCCTTTGTTATCTACTTCTGAGGC-3' |

### Table 2. L1-ORF1/2 TaqMan primers.

| Sequence name | Sequence |
|---------------|----------|
| L1-ORF1 Taqm -1F | ATGTTAGGGAGGAGCAGCAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGA |
10 min at 37 °C and washed 2x with ice-cold PBS supplemented with protease and phosphatase inhibitor cocktails (Cat#: 539-134-1SET). Chromatin immunoprecipitation assay (ChIP) was conducted as described before (Montoya-Durango et al., 2009; Teneng et al., 2011). After incubation with respective antibodies for NuRD components, complexes were pelleted, the supernatant removed, and samples kept on ice. Immune complexes were eluted by addition of 250 μL elution buffer (1% SDS 0.1 M NaHCO₃), vortexed briefly, and incubated at room temperature for 15 min with gentle rotation. The eluate was combined with 20 μL of 5M NaCl and incubated at 65 °C for 4 h to reverse formaldehyde cross-linking of DNA/protein complexes. DNA was recovered by phenol/chloroform extraction and ethanol precipitation. DNA was resuspended in nuclease-free H₂O and quantified by Cytochrome C. RT-PCR was used to determine enrichment for LI-5'UTR sequences using tiling primers (P1-P4) that span the 5'UTR promoter region of LI. The specificity of NuRD target recognition was examined in previous studies (Montoya-Durango et al., 2009) and validated by DNA sequencing (Table 4 and Fig. S3). ChIP measurements in BaP- and DMSO-treated samples used the P3 primer to focus on the RB1/E2F recognition site within the promoter.

### 2.4. Formaldehyde-assisted isolation of regulatory elements

Formaldehyde-assisted isolation of regulatory elements (FAIRE) was used to study LI sequences associated with regulatory activity based on preferential formaldehyde cross-linking of nucleosome-bound DNA relative to nucleosome-depleted regions of the genome (Giresi et al., 2007). HBECs were grown to 70% confluence and treated with 0.1 μM BaP or equivalent volume of DMSO for 12 h. The medium was removed and the cells cross-linked with 1% formaldehyde for 10 min at 37 °C. Cells were washed 2x with ice-cold DPBS (Dulbecco’s phosphate-buffered saline pH 7.4) supplemented with protease and phosphatase inhibitor cocktails (Cat#: 539-134-1SET) before being scraped and pelleted for 5 min at 800 g at 4 °C and resuspended in DPBS. Two 500-μL aliquots of lysate were set aside for RNA analysis and input control. A total of 500 μL of lysate was resuspended in FAIRE lysis buffer (1% SDS, 100 mM NaCL, 50 mM Tris pH 8, 2 mM EDTA, and 2 mM EGTA supplemented with protease and phosphatase inhibitor cocktails) and sonicated as described for 30 sec and cooled in ice for ~5 min. Unbound DNA and DNA cross-linked to proteins were separated by phenol/chloroform extraction. Unbound DNA in the aqueous phase was ethanol precipitated with 1/10 of 3M sodium acetate (NaAc) and 2.5X of 95% ethanol (EtoH) overnight followed by centrifugation at 14000 g for 20 min. DNA was washed with 70% ETOH once, air-dried, and resuspended in 50 μL of sterile nuclease-free H₂O. Total DNA was quantified and serial dilutions of 2.5, 5, and 10 ng used for RT-PCR analysis using a primer set (P4) that spans the CpG shore region of the LI promoter (Table 5). For LI transcript analysis, RNA was isolated as previously described using RNeasy Plus Mini Kit (Cat#: 74134). Total gDNA for use as input control was isolated using Purelink genomic DNA isolation kit (Cat#: K1820-01).

### 2.5. Viral transfection and luciferase analysis

HEK293T-17 cells were used to generate virus titers for subsequent transduction of the virus containing Mi2β and mutant plasmids into BEAS-2B cells. HEK293T-17 cells were grown to 90% confluence and transfected with retrovirus packaging cells and supernatants were harvested 48 h later, filtered and concentrated.

### Table 4. PRIMERS FOR NuRD component used in RT-PCR.

| Sequence name | Sequence |
|---------------|----------|
| HCHD4-1F      | CTC GGG CCA GTG TAG AGG TC |
| HCHD4-1R      | TCT TCA TTT TCT GGG TGG GTT |
| HMTA2-1F      | GCC ATT ATG GGA CGG GC |
| HMTA2-1R      | GGC CAT CAG TGT GGT GTA GT |
| HMTA3-1F      | AGT TGA GCC TGA CTT GAC CG |
| HMTA3-1R      | TCC CCT GAT ATG TGT TGC GG |
| HMBD2-1F      | CAA CCG GTA ACC AAA GTC ACA |
| HMBD2-1R      | GCT GAC GTG GCT GGT CAT TC |
| HMBD3-1F      | AAG ATG AAC AAG AGC CGC CA |
| HMBD3-1R      | TGT TAA TCT TGG TCA CCG GC |
| ACTIN-1F      | CCGGACGCTAGTATGTTCA |
| ACTIN-1R      | TCTTCTTGTCTCTGAGACCACCTC |
| 185-1F        | AGTCCAGGGGCTCCACTAAAC |
| 185-1R        | GATCCAGGGGCTCCACTAAAC |

### Table 5. Mi2β ChIP RT-PCR primers.

| Sequence name | Sequence |
|---------------|----------|
| HL1UTR-1F     | GGAACAGCTCCGCTCTACAG |
| HL1UTR-1R     | TACCCCTTTCTTTGACTCG |
| HL1UTR-2f     | GTGTTCTAATCCGACTGAA |
| HL1UTR-2R     | TGTGCTAATCCGACTGAA |
| HL1UTR-3f     | CACGGAATCTCAGCTATTG |
| HL1UTR-3R     | CCGAGGTTGGAGCCTACAGA |
| HL1UTR-4f     | CAGGGAAGCTCAGCTTTG |
| HL1UTR-4R     | AGTCCAGGGGCTCCACTAAAC |
with Mi2βWT/mutants retroviral vectors (6 μg), combined with pSPAX-2 packaging vector (3 μg) and pVSV-G envelope vector (1.5 μg) diluted in 500 μL of dilution media (10 mM HEPES/150 mM NaCl, pH 7.05). Retroviral packaging vectors (pSPAX-2 and pVSV-G) were a kind gift from Schenten at the University of Arizona (Addgene plasmid # 12260 and Addgene plasmid # 36399, respectively). An aliquot of 42 μL of PEI (1.0 μg/μL) was added to each transfection and incubated at room temperature for 15 min. DNA/PEI complexes were added to the cells and incubated for 18 h. Transfection media were removed and new media containing 10% FBS added. The viral supernatant was collected the next day and filtered through 0.45-μm PES filter tubes. Viral titer was incubated with one volume of X-concentrator (Clontech) overnight and centrifuged at 1500 g for 45 min. The supernatant was removed and virus resuspended in 100 μL PBS to make 10X viral concentrate. A total of 10 μL of viral titer was used to transiently transduce transfected cells. HBECs were grown to 70% confluence and transiently transfected with Mi2 (pB001ctr) or wild-type (WT) L1-Ta plasmids. A 1 : 50 dilution of ORF1p antibody was used to monitor L1-ORF1 protein in tumors derived from grafted cells or human tissues by immunohistochemistry (IHC). Images were taken using an Axiovision inverted microscope at 40 × magnification.

### 2.7. Statistics

The differences between groups were evaluated using the Mann–Whitney U-test to compare samples drawn from the same distribution. Mann–Whitney U-test is a nonparametric alternative test often used to evaluate normalized data because it does not require the assumption of normal distribution when trying to determine differences between two independent samples. The number of replicates and independent measures for each of experiment are included in the figure legends.

### 3. Results

#### 3.1. L1 reactivation by BaP disrupts NuRD promoter occupancy and partially shifts constituent proteins from the nuclear to cytoplasmic compartment

We have previously shown that the silenced L1-Ta promoter is enriched in repressive heterochromatin marks, namely histone-3 lysine-9 trimethylation (H3K9Me3) and histone-4 lysine-20 trimethylation (H4K20Me3) (Teneng et al., 2011). We also showed that RB proteins stabilize the NuRD multiprotein complex to effect L1 silencing and that BaP interferes with RB functions via AHR-dependent mechanisms (Montoya-Durango et al., 2009, 2016; Teneng et al., 2007). AHR is present in the repressor complex assembled by RB on the L1 promoter in human and murine cells (Montoya-Durango et al., 2009). However, the structural and functional determinants of NuRD corepression during L1 reactivation by tobacco carcinogens in HBECs have not been elucidated. To fill this gap, experiments were initiated to study L1 reactivation in HBECs challenged with BaP, a carcinogenic aromatic hydrocarbon present in tobacco smoke.

The retention of NuRD constituent proteins on the L1-5'UTR promoter in HBECs challenged with 0.1 μM BaP is presented in Fig. 1A. This concentration was chosen based on dose–response and time–response experiments in BEAS 2B cells showing significant induction of L1 in the range of 0.1 to 3 μM (data not shown). Marked reductions in enrichment for Mi2β, MBD2/3, and MTA2 were seen 12 h after carcinogen challenge, indicating that BaP displaces NuRD macromolecular complex constituents from the L1 promoter. Next, FAIRE was employed to separate unbound and bound DNA sequences based on their relative affinities for the aqueous and organic phases, respectively (Giresi et al., 2007). BaP treatment reduced protein trapping at genomic regions containing L1-5'UTR DNA sequence (Fig. 1B), indicating a shift of L1 chromatin from a heterochromatic to euchromatic state. These changes were associated with transcriptional activation and accumulation of L1 ORF1 and ORF2 mRNA (Fig. 1C). The lower levels
of ORF2 mRNA compared to ORF1 mRNA are consistent with the presence of cryptic polyadenylation sites in ORF2 that cause early termination of transcription (Kazazian et al., 1988).

L1 reactivation in HBECs following carcinogen challenge showed time-dependent profiles (Fig. S2), with induction of L1 mRNA peaking at 12 h and returning to baseline by 48 h. Western analysis at 12 h showed accumulation of L1 ORF1 and ORF2 proteins in cells challenged with BaP (Fig. S2), confirming effective translation of mRNA into proteins. CYP1A1 protein was detected by immunofluorescence in BEAS 2B cells (Fig. S4), suggesting that BaP metabolites may contribute to the L1 induction response. We have previously shown that CYP-derived metabolites of BaP readily activate L1 and are significantly more potent than the parent compound in vascular cells (Lu and Ramos, 2003). To further evaluate the disruption of NuRD corepressor complex interactions by BaP, the localization of NuRD constituent proteins was examined by indirect immunofluorescence. In these studies, HP1γ staining was measured as an index of the structural integrity of heterochromatin domains (Lechner et al., 2000; Mansfield et al., 2011). HP1γ staining in vehicle-treated HBECs showed distinct focal accumulations, while staining in BaP-treated cells was diffuse and considerably less pronounced (Fig. 2A,D, column 1). Mi2β and MTA2 staining strongly colocalized with DAPI staining, and was visibly nuclear in DMSO-treated cells, indicating that under basal conditions these proteins preferentially reside within the nuclear compartment (Fig. 2A,C, columns ii and iv). In keeping with previous reports (Takeshita et al., 2016), the nuclei of BaP-treated cells appeared swollen at times. Staining for Mi2β and MTA2 in HBECs treated with BaP was distributed
**Fig. 2.** Benzo(a)pyrene disassembles the NuRD complex and shifts constituent proteins from the nuclear to cytoplasmic compartment. (A, B) Mi2β and (C, D) MTA2 immunofluorescence staining in HBECs treated with DMSO or 0.1 μM BaP for 12 h. (E, F) Densitometric measurement of fluorescence intensity for Mi2β and MTA2 in the cytoplasmic compartment of control and BaP-treated HBECs, respectively. HP1γ was used as an index of structural integrity of heterochromatin domains. DAPI was used to stain the nuclei, with merged signals denoting colocalization of proteins. The staining for HP1γ was switched from red to green and vice versa to control for nonspecific staining by the secondary antibody. Similar results were seen in three independent experiments. The differences between groups were evaluated by the Mann–Whitney U-test to compare samples drawn from the same distribution (n = 32 cells/experiment). Scale bars are 50 μm. Statistical significance is denoted by (*).
between the nuclear and cytoplasmic compartments (Fig. 2B,D, columns ii and iv), suggesting a partial shift to the cytoplasmic compartment. To confirm this interpretation, nuclear fluorescence intensity was quantified and subtracted from total fluorescence intensity using IMAGEJ software (imagej.nih.gov). Increased fluorescence intensity for cytoplasmic Mi2β and MTA2 was observed in cells treated with BaP compared to DMSO controls (Fig. 2E,F, respectively). Thus, challenge of HBECs with BaP is associated with disintegration of heterochromatin leading to disassembly and detachment of NuRD components from the L1 promoter. These changes in turn were associated with a partial shift of constituent proteins from the nuclear to cytoplasmic compartment. The exact mechanism responsible for translocation of NuRD constituent proteins is not known, but may involve nuclear export signals and exportins, as shown previously for MBD2-interacting proteins (Lechner et al., 2000).

### 3.2. Mi2β binding to the L1 promoter in HBECs effects L1 retroelement silencing

Mi2β is an ATP-dependent helicase subunit with nucleosome remodeling activity that is specific to the NuRD complex. Therefore, the next set of experiments was completed to characterize the influence of Mi2β binding to the L1 promoter in HBECs by ChIP using tiling primers (P1–P4) that spanned the complete L1-Ta 5’UTR promoter sequence coupled with antibodies specific to Mi2β (Fig. 3A). L1-Ta 5’UTR sequences were highly enriched in samples treated with Mi2β antibody compared to IgG control for all primer sets used to amplify promoter sequences (Fig. 3B). DNA electrophoresis and sequencing were completed to confirm the identity and size (P1: 224 bp, P2: 175 bp, P3: 188 bp, and P4: 315 bp) of amplified sequences (Fig. S3). Thus, chromatin-bound Mi2β is present across the entire length of the L1 promoter.

Genetic ablation of Mi2α/β mRNA and protein using target-specific siRNA (Fig. 4A, panels i and iii, respectively) increased the levels of L1 ORF1 mRNA and protein (Fig. 4A, panels ii and iv, respectively). L1 ORF1 mRNA and protein were detectable under unstimulated conditions (See Fig. S1) and significantly upregulated by siRNA treatment. The functional role of MBD2/3 and MTA2/3 was examined next using gene-specific siRNA directed against these proteins (Fig. 5A,B). L1-ORF1 mRNA and protein were markedly upregulated by MBD2/3 knockdown (Fig. 5A, panel ii), while ablation of MTAs was without effect (Fig. 5B, panel ii). These findings identify Mi2β and MBDs as the key architectural elements that effect L1 silencing. The negative regulatory function of Mi2β likely reflects its activity within the NuRD macromolecular complex, while MBDs may exert regulatory control through binding to methyl groups in DNA. Collectively, these findings show that functional modulation of Mi2β and MBDs is a fundamental molecular event in the activation of L1 in HBECs.

**Fig. 3.** Mi2β Interacts with L1 Promoter Sequences. (A) Schematic representation of the location of tiling primers used to characterize Mi2β binding along the full-length L1-5’UTR (Table 4). CpG islands and shores within the promoter are also shown. (B) Solubilized chromatin was immunoprecipitated with antibodies against Mi2β or mouse IgG. After isolation and normalization of genomic DNA, RT-PCR was performed using tiling primers specific to L1-5’UTR promoter. Similar results were seen in three independent experiments. Statistical significance is denoted by (*).
3.3. Mutations in the DNA- and ATPase-binding domains, but not the chromodomain of Mi2β negatively regulate NuRD corepressor activity

To further confirm the role Mi2β in L1 suppression, the suppressor activity of Mi2βWT was compared to that of mutants within the DNA-binding domain, ATPase-binding domain, and chromodomain (Ramírez et al., 2012). L1-Ta S′UTR promoter-driven luciferase and Renilla vector (internal control) were transiently transfected into HBECs. Cells recovered from transfection for 24 h before treatment with retroviral vectors encoding Mi2βWT or mutants for an additional 24 h. Transduction efficiency was optimal 24 h after exposure (data not shown). A significant, but modest, reduction in L1 reporter activity was seen in Mi2β WT-transfected cells (Fig. 6A). The modest response likely implicates significant contributions by other accessory proteins to basal activation of the L1 promoter. Importantly, mutants in the DNA- and ATPase-binding domains of Mi2β lost their ability to suppress luciferase reporter gene expression, while the chromodomain mutant was without effect (Fig. 6A).

Cotreatment of cells transfected with Mi2βWT retroviral vectors significantly reduced both basal- and BaP-driven luciferase expression (Fig. 6B), confirming that disruption of Mi2β activity by BaP plays an important role in regulating the repressive functions of the NuRD complex on L1. The partial reversal seen upon reconstitution with Mi2β confirms that combinatorial functions with other members of the repressor complex are essential for L1 repression.

3.4. Expression of a constitutively active synthetic L1 is oncogenic in a SCID xenograft mouse model

The final set of experiments examined the oncogenic potential of L1 in a SCID mouse xenograft model. Given that L1 activation in HBECs by BaP peaks at 12 h and returns to baseline by 48 h (Fig. S2), an in vitro model of constitutive L1 activation that replicates the smoking behavior of habitual users who smoke ≥12 cigarettes per day was examined. Our estimation is consistent with previous reports showing regular patterns of smoking frequency of smokers...
SCID mice were injected subcutaneously with $6 \times 10^6$ HBECs stably expressing empty pB001ctr vector ($n=6$) or a constitutively active wild-type $L1$ construct (pB015WT) ($n=15$) (Bojang et al., 2013). pB015WT consists of $L1$RP sequence cloned into a pGL4.15 vector backbone with $L1$ ORF1 and ORF2 proteins tagged with streptavidin–hemagglutinin (Strep–HA) and flag-myc double tags, respectively (Fig. 7A). A cytomegalovirus (CMV) promoter was placed upstream of the $L1$-5'UTR for constitutive expression. No differences in animal weights were seen between the two groups (data not shown). $L1$ was tumorigenic in SCID mice, with tumor burden first detected one week after inoculation and sustained for up to eight weeks (Fig. 7B). No tumors were seen in control mice at any time during the experiment, confirming the nontumorigenic phenotype of HBECs (i.e., BEAS-2B cells), and the innocuous nature of the control vector. Peak tumor incidence occurred four weeks after inoculation (12/15 mice), with significant tumor growths ranging in size from 1.47 to 42.0 mm$^2$ in 6 of 15 mice during week five. Variable degrees of tumor regression and recurrence were seen over the course of the eight-week experiment (Fig. 7B). The expression of $L1$-ORF1 protein was examined in the grafted tumors by IHC to confirm molecular phenotype (Fig. 7C). $L1$-ORF1 protein expression was positive in all mouse tumors examined ($n=6$). Representative samples are shown in Fig. 7C, panels i and ii. Normal human lung tissue and p53 null lung tissue were used as negative and positive controls, respectively (Fig. 7C, panels iii and iv). High levels of $L1$-ORF1 protein were detected in p53 null tissue, but not in normal lung. These findings demonstrate that activation of $L1$ in HBECs creates a microenvironment that supports oncogenic transformation and implicate molecular interactions between $L1$ and p53 as critical players in lung cancer.

4. Discussion

Evidence is presented here establishing a functional linkage between NuRD macromolecular complex disassembly, $L1$-Ta reactivation, and malignant transformation in HBECs challenged with the lung carcinogen BaP. These findings build upon earlier reports by our group showing that $L1$ reactivation involves the following: 1) enrichment of activating H3K4Me3 and H3K9Ac marks; 2) increased histone H3 acetylation of the 5'UTR; 3) reduced DNMT1 recruitment; and 4) CpG hypomethylation in both transformed and non-transformed epithelial cells (Montoya-Durango et al., 2017). SCID mice were injected subcutaneously with $6 \times 10^6$ HBECs stably expressing empty pB001ctr vector ($n=6$) or a constitutively active wild-type $L1$ construct (pB015WT) ($n=15$) (Bojang et al., 2013). pB015WT consists of $L1$RP sequence cloned into a pGL4.15 vector backbone with $L1$ ORF1 and ORF2 proteins tagged with streptavidin–hemagglutinin (Strep–HA) and flag-myc double tags, respectively (Fig. 7A). A cytomegalovirus (CMV) promoter was placed upstream of the $L1$-5'UTR for constitutive expression. No differences in animal weights were seen between the two groups (data not shown). $L1$ was tumorigenic in SCID mice, with tumor burden first detected one week after inoculation and sustained for up to eight weeks (Fig. 7B). No tumors were seen in control mice at any time during the experiment, confirming the nontumorigenic phenotype of HBECs (i.e., BEAS-2B cells), and the innocuous nature of the control vector. Peak tumor incidence occurred four weeks after inoculation (12/15 mice), with significant tumor growths ranging in size from 1.47 to 42.0 mm$^2$ in 6 of 15 mice during week five. Variable degrees of tumor regression and recurrence were seen over the course of the eight-week experiment (Fig. 7B). The expression of $L1$-ORF1 protein was examined in the grafted tumors by IHC to confirm molecular phenotype (Fig. 7C). $L1$-ORF1 protein expression was positive in all mouse tumors examined ($n=6$). Representative samples are shown in Fig. 7C, panels i and ii. Normal human lung tissue and p53 null lung tissue were used as negative and positive controls, respectively (Fig. 7C, panels iii and iv). High levels of $L1$-ORF1 protein were detected in p53 null tissue, but not in normal lung. These findings demonstrate that activation of $L1$ in HBECs creates a microenvironment that supports oncogenic transformation and implicate molecular interactions between $L1$ and p53 as critical players in lung cancer.

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2009; Teneng et al., 2011). Thus, the association of NuRD complexes with the L1 promoter may serve as a key regulatory signal for the formation of regional domains of heterochromatin that can be disrupted by the lung carcinogen BaP during the course of malignant progression. NuRD subunits are known to associate with oncogenic transcription factors, and the loss of NuRD function has been linked to various types of malignancies (Lai and Wade, 2011). Furthermore, Mi2b binds to H3K9Me3, a modification characteristic of the silenced L1 promoter in transformed cells (Mansfield et al., 2011; Teneng et al., 2011), and activating histone marks delocalize Mi2b and MDBs and disrupt architectural anchoring of the NuRD complex to DNA (Allen et al., 2013; Baubec et al., 2013).

The ability of BaP to interfere with the assembly of NuRD subunits and their function likely involves AHR, a ligand-activated transcription factor regulated by BaP during the course of L1 reactivation (Teneng et al., 2007). AHR was first identified as the nuclear receptor responsible for transcriptional regulation of cytochromes P450 by dioxin and related aromatic hydrocarbons, and later shown to bind a variety of endogenous and exogenous ligands to effect molecular control of proliferation, apoptosis, differentiation, adhesion, and pluripotency. We have previously shown that AHR is present in the repressor complex assembled by RB on the L1 promoter in human and murine cells (Montoya-Durango et al., 2009). Others have shown that AHR interacts with NuRD complex subunits to regulate differentiation-specific genes that interfere with early embryonic programming (Gialitakis et al., 2017). Thus, AHR may be the critical effector used by BaP to interfere with NuRD functions in HBECs. This suggestion is supported by our own studies showing that genetic ablation of AHR under basal conditions in BEAS-2B cells dramatically increases L1 mRNA levels (Bojang and Ramos, unpublished data). As such, constitutive AHR may support critical protein–protein interactions that are
disrupted in the presence of BaP to facilitate disassembly of the NuRD repressor complex. Furthermore, AHR interacts with TGF-β1, an upstream regulator of L1 and inducer of EMT in HBECs (Chang et al., 2007; Ramos et al., 2011; Reyes-Reyes et al., 2017). Therefore, structural and functional interactions between

Fig. 7. Oncogenicity of Constitutively Active L1 in Human Bronchial Epithelial Cells. (A) Schematic representation of the construct utilized for stable expression of wild-type L1 or empty vector. (B) Tumor burden in SCID mice injected once subcutaneously with control (n = 6) or wild-type L1 (n = 15) vectors. (C) Immunohistochemistry of L1-ORF1p staining in (i) tumors isolated from 00LM-18 and (ii) 00LN-17P, SCID mice (iii) p53-null lung tissue, (iv) normal lung tissue. Scaled bars represent 10 μm.
AHR and NuRD complex subunits may cooperate with TGF-β1 in a highly contextual manner to regulate L1. Wang et al. (2009) reported that NuRD complexes regulate TGF-β1 signaling by enlisting LSD1, a lysine-specific demethylase that affords histone demethylase activity to the chromatin remodeling capacity of the NuRD complex (Wang et al., 2009). AHR may also participate in the regulation of CYP genes involved in BaP metabolism, a suggestion consistent with the expression of CYP1A1 protein in BEAS-2B cells and our previous report that oxidative BaP metabolites activate L1 expression. Although the relative contributions of unmetabolized versus metabolized BaP to the tumorigenic response in BEAS-2B cells were not examined, the 12-h lag time for induction of L1 in BEAS-2B cells suggests that metabolism of the parent hydrocarbon may contribute to the L1 reactivation response.

Based on our collective findings, we conclude that combinatorial interactions between AHR, RB, p53, and NuRD subunits effect epigenetic silencing of L1 and are direct targets of the lung carcinogen BaP during the course of L1 reactivation and malignant transformation (Lai and Wade, 2011; Montoya-Durango et al., 2009; Teneng et al., 2007). The immortalization of BEAS-2B cells with SV40 large T-antigen likely sensitizes RB and p53 to create a permissive environment for L1 reactivation by the carcinogen. This is consistent with the appearance of L1 protein in p53-null lung cancer, but not normal tissue, and the exquisite sensitivity of BEAS-2B cells to BaP. However, despite SV40 immortalization, the BEAS-2B cell line is not tumorigenic in the absence of L1 or carcinogen, remains RB and p53 competent during serial culture, and retains intact Akt signaling (Pan et al., 2011). We previously showed that forced expression of L1 in embryonic kidney cells disrupts differentiation programming and alters growth factor responsiveness (Ramos et al., 2011), suggesting that L1 alone is sufficient to drive the appearance of oncogenic phenotypes. NuRD complex disassembly has been linked to malignant progression through disruption of cellular differentiation and transformation (Kaur et al., 2007), while L1 reactivation has been linked with genomewide reprogramming, EMT, malignant transformation, and poor prognosis in NSCLC patients (Bojang et al., 2013; Ikeda et al., 2013; Saito et al., 2010). Further, disassembly of the NuRD complex from promoters is known to recruit SW1/SNF complexes that regulate EMT (Mohd-Sarip et al., 2017). The reactivation and mobilization of L1 can predispose lung cancer genomes to aberrant epigenetic control by creating sloppy DNA methylation shores that influence the methylation status and expression of surrounding loci (Grandi et al., 2015). In accord with this view, de novo somatic L1 retrotransposition occurs at higher frequencies in NSCLCs than in other tumors, and site-specific hypomethylation of L1s is associated with pathological features of NSCLC patients (Ikeda et al., 2013; Saito et al., 2010). Thus, the carcinogenicity of BaP and its impact on NSCLC evolution are likely tied to the disruption of NuRD complex functions and L1 reactivation.

While the repressive role of the NuRD complex has been extensively studied, it is not yet known how the loss of one or more of its components affects overall function. Some of the components have been shown to be mutually exclusive, while others exert opposing functions (Denslow and Wade, 2007). The results of our siRNA analyses showed that loss of Mi2β and MBD2/3 increases L1 mRNA, while loss of MTAs was without effect. These results are in line with the organization of the NuRD complex in a repressive state, where proteins are anchored to the DNA through Mi2β and MBD2/3, and MTAs do not directly contact DNA (Lai and Wade 2011). MTAs have been shown to interact with RBBP4/7 and GATAD2α/β, which in turn interact with MBD2/3 to anchor MTAs to the complex (Lai and Wade 2011; Allen et al., 2013). Therefore, promoter-specific analysis would be required to determine the functional impact of NuRD-related functions for other genetic targets, as well as its functionality in different cellular contexts.

Transcriptional regulation of L1 is highly contextual, cell type specific, and dependent upon the activation stimulus (Teneng et al., 2007). Given the functional linkage between NuRD core proteins and L1 retroelement reactivation, regulatory control of L1 is likely coupled to the expression of accessory proteins involved in the regulation of NuRD core protein functions (Lai and Wade 2011; Allen et al., 2013). For instance, the interaction of HIF1α with NuRD activates transcription, while interactions between NuRD and p53 are repressive (Lai and Wade 2011; Allen et al., 2013). This is in keeping with our finding that functional interactions between RB and AHR proteins repress L1 through interactions with the NuRD complex under basal conditions (Montoya-Durango et al., 2009, 2016). Mi2β plays a prominent role in the regulation of L1 expression upon carcinogen activation, with the DNA-binding and ATPase enzymatic activities, but not the chromo protein–protein interaction domain, serving to interfere with ectopic L1 expression. The minimal role of the chromodomain is consistent with the function and architecture of the NuRD complex, where NuRD core proteins mediate not only protein–protein interactions, but also remodeling of
nucleosomes, histone deacetylation and demethylation, and interactions with methylated DNA (Baubec et al., 2013; Xue et al., 1998). Our data suggest that DNA methylation is a prerequisite for L1 suppression, especially in the context of MBD functions (Jiang et al., 2004; Teneng et al., 2011). This assertion is consistent with the finding that cotreatment of HBEcs with Mi2β and BaP did not completely abolish BaP-driven luciferase activity.

Elevated expression of L1 retrotransposon has been associated with oncogenic transformation and L1 hypomethylation is known to induce stem cell-like properties and EMT in NSCLCs (Ikeda et al., 2013; Saito et al., 2010). However, the contextual determinants that define L1 functions as a driver or passenger event in tumorigenesis are not fully understood. Our in vivo studies examined these relationships using a constitutive model of L1 activation in HBEcs that mimics the repeated cycles of exposure seen in smokers who consume 12 or more cigarettes per day (Chandra et al., 2007; Nanez et al., 2011). Evidence was obtained that expression of constitutively active L1 in HBEcs is oncogenic in a SCID mouse xenograft model, confirming that L1 can function as an initiating event during malignant transformation of lung epithelial cells. The oncogenic activity of L1 may be exerted via retrotransposition-dependent and retrotransposition-independent mechanisms, with the former involving insertions, deletions, and structural damage, and the latter involving oncogenic signaling pathways that couple with EMT (Reyes-Reyes et al., 2017).

Interestingly, tumor growth in mice grafted with cells expressing L1 was restricted over time, suggesting that the oncogenic response initiated by L1 cooperates with other pathways that sustain the malignant response. In this regard, the ability of BaP to damage DNA and to mutate both p53 and KRAS in HBEcs may amplify the DNA damage response, propagate waves of genomic instability, and disrupt genetic regulatory networks of growth and differentiation. In fact, p53 is known to restrict retrotransposon activity through interaction with components of the piRNA (PIWI Interacting RNA) pathway (Wylie et al., 2016). Our own evidence supports this as L1-encoded ORF-1p was increased in human lung tumors null for p53, a finding consistent with previous reports (Wylie et al., 2016). We recently showed that a reverse transcriptase mutant of L1 can induce tumors in athymic mice that outgrow those induced by wild-type L1 (Reyes-Reyes et al., 2017), suggesting that retrotransposition and genotoxicity from the unregulated endonuclease activity of wild-type L1 pose significant restrictions on the tumorigenic process. This is an area worthy of exploration as novel chemotherapeutic agents can be designed to exploit this property in the clinical management of lung tumors.

In conclusion, evidence is presented here showing that challenge of HBEcs with the lung carcinogen BaP shifts the L1-Ta promoter from a heterochromatic to euchromatic state through disassembly of the NuRD complex and partial displacement of constituent proteins from the nucleus to the cytoplasm. Disruption of NuRD corepression correlates with accumulation of L1 mRNA and proteins and tumorigenesis.

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Author contributions

KSR conceived the study, KSR and PBJr designed the study, PBJr acquired the data, KSR and PBJr analyzed and interpreted the data, and KSR and PBJr wrote the manuscript.

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**Supporting information**

Additional Supporting Information may be found online in the supporting information section at the end of the article:

**Fig. S1.** Optimization of L1-ORF1 in HBECs by Western analysis.

**Fig. S2.** Time-dependent analysis of L1 mRNA accumulation in HBECs challenged with 0.1 μM BaP.

**Fig. S3.** DNA and Sequence analysis of L1-5′UTR sequences.

**Fig. S4.** Analysis of CYP1A1 expression after 12 h of BaP treatment by indirect immunofluorescence.