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Multiple Epigenetic Modifiers Induce Aggressive Viral Extinction in Extraembryonic Endoderm Stem Cells

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SUMMARY

To prevent insertional mutagenesis arising from retroviral reactivation, cells of embryonic origin possess a unique capacity to silence retroviruses. Given the distinct modes of X chromosome inactivation between embryonic and extraembryonic lineages, we investigated paradigms of viral extinction. We show that trophectoderm stem cells do not silence retroviral transcription, whereas extraembryonic endoderm stem cells aggressively extinguish proviral transcription, even more rapidly than do embryonic stem cells. By using a short hairpin RNA library, we identified epigenetic modifiers of retroviral extinction in extraembryonic endoderm stem cells. Multiple chromatin remodeling and polycomb repressor complex proteins act to modulate integrated, as well as endogenous, retroviral element silencing, with a subset of factors displaying differential effects between stem cell types. Furthermore, our data suggest that small RNAs play a role in this process through interactions with the Argonaute family. Our results further the understanding of mechanisms regulating retroviral transcription in different stem cell lineages.

INTRODUCTION

To contend with the constant threat of retroviral infection, both from exogenous as well as endogenous elements, mammalian genomes have evolved complex defense mechanisms to impede the life cycle of invading parasitic nucleic acids. Historically, these defensive strategies have been separated into two broad categories: viral restriction and viral extinction (Niwa et al., 1983; Cherry et al., 2000).

Restriction is a process whereby protein factors encoded by the host genome interact directly with viral elements to block some aspect of the invading virus's life strategy. Proteins such as Friend virus susceptibility 1 (FV1), tripartite interaction motif 5a (TRIM5a), zinc finger antiviral protein (ZAP), TRIM19/PML (promyelocytic leukemia), and the TRIM28-ZFP809 (zinc finger antiviral protein 809) complex all restrict retroviral tropism via direct biochemical interactions (Best et al., 1996; Kaiser et al., 2007; Gao et al., 2002; Turelli et al., 2001; Wolf and Goff, 2009; Rowe et al., 2010).

Less well understood is the process of retroviral extinction, which is progressive silencing of proviral transcription that occurs over long-term cellular growth or differentiation (Cherry et al., 2000; Laker et al., 1998). This process is epigenetic in nature and has been associated with acquisition of DNA methylation and other transcriptionally repressive chromatin modifications at the viral integration site (Harbers et al., 1981; Jähner et al., 1982; Pannell et al., 2000; Poleshko et al., 2008; Matsui et al., 2010).

Cells of embryonic origin are unique in their capacity to silence retroviruses, which is understandable given the necessity of preventing insertional mutagenesis that could arise from retroviral reactivation. γ-retroviruses, like the mouse leukemia virus (MLV), can integrate into embryonic carcinoma and embryonic stem cells but are silenced by both restriction- and extinction-based mechanisms (Loh et al., 1990; Niwa et al., 1983; Teich et al., 1977). The early embryo possesses three stem cell lineages: embryonic stem (ES), trophectoderm stem (TS), and extraembryonic endoderm (XEN) stem cells that give rise to the embryo proper, placenta, and yolk sac, respectively. Although viral extinction has been well documented in cells of embryonic origin, the capacity of extraembryonic stem cells to epigenetically silence γ-retroviruses has not been examined. Given the distinct epigenetic mechanisms regulating X chromosome inactivation in embryonic and extraembryonic cells (Kunath et al., 2005; Takagi and Sasaki, 1975), we hypothesized that different modes of viral extinction may also exist between separate lineages of the preimplantation embryo. Specifically, we sought to determine whether stem cells derived from trophoblast and extraembryonic endoderm could epigenetically extinguish a MLV variant modified to escape retroviral restriction but which is susceptible to extinction (Cherry et al., 2000; Grez et al., 1990; Laker et al., 1998).

To examine viral extinction in stem cells, we assayed transcriptional activity of genetically marked mouse γ-retroviruses in primary ESCs, TSCs, and XEN stem cells. Unlike ESCs,

TSCs did not silence proviral transcription but rather maintained consistent and high expression levels over long-term culture. Surprisingly, integration of γ-retrovirus into XEN cells produced rapid transcriptional silencing, by comparison to ESCs. We present data that indicate XEN cell extinction is epigenetic in nature and mediated by multiple chromatin remodeling and polycomb repressor complexes. These complexes not only act to modulate the silent state of integrated retroviruses but also play significant roles in repressing endogenous retroelement transcription. Furthermore, our results suggest that these repressor complexes are recruited to sites of viral integration through interactions with the Argonaute family of proteins.

RESULTS

Distinct Patterns of Retroviral Extinction in Embryonic and Extraembryonic Stem Cells

For decades, scientists have intensively studied mechanisms of gene regulation in ESCs, including those involved in exogenous and endogenous retroviral silencing. However, only a few epigenetic factors employed in retroviral silencing have been identified. Furthermore, epigenetic mechanisms operating in cohabiting extraembryonic stem cells have been virtually ignored, the assumption being that all stem cells utilize similar mechanisms. To determine whether stem cells derived from trophoblast and extraembryonic endoderm possess similar or distinct capacities to silence retroviruses, we chose to examine transcriptional activity of a MLV variant that is closely related to the endogenous type C retroviral element. To achieve this, we constructed a series of genetically marked mouse embryonic stem cell viruses (MSCV) (Figures 1A and 2A) and assayed their transcriptional activity in infected primary ESCs, TSCs, and XEN cells.

Within 24 hr of infection, expression of virally delivered GFP could be detected within each of the three stem cell lineages (Figure 1B; Table S1 available online). To assay for viral extinction, cells were propagated for 6–15 weeks, with the percentage of GFP-positive cells monitored via flow cytometry at each passage (Figure 1C). Consistent with previous observations (Cherry et al., 2000), ESCs exhibited a progressive decline in the number of cells expressing GFP with near complete viral extinction occurring after 15 weeks in culture (passage 40) (Figure S1). Surprisingly, TSCs did not inactivate the γ-retroviral reporter but rather maintained a constant level of GFP-expressing cells. This trend was maintained through 12 weeks of observation (30 passages) and correlated with continued expression of TSC-specific markers (data not shown). By comparison, XEN cells exhibited aggressive silencing of viral transcription such that within 3 weeks (6–7 passages), GFP expression was virtually undetectable.

Retroviral Silencing in XEN Cells Is Epigenetic in Origin

Given the surprising speed with which the γ-retroviral reporter was silenced in the XEN cell lineage, we examined viral extinction in this relatively uncharacterized cell type. To ensure that the observed extinction was an epigenetic phenomenon, and not an artifact of cellular differentiation or GFP toxicity, three experiments were conducted. First, to determine whether loss of viral expression was a result of cellular differentiation, RNA was extracted from MSCV-GFP-infected XEN cells at passages 2, 7, and 10 and assayed for expression of XEN cell markers. Abundant expression of transcripts encoding GATA binding protein 4 (*Gata4*), Forkhead box protein A2 (*Foxa2*), Hepatocyte

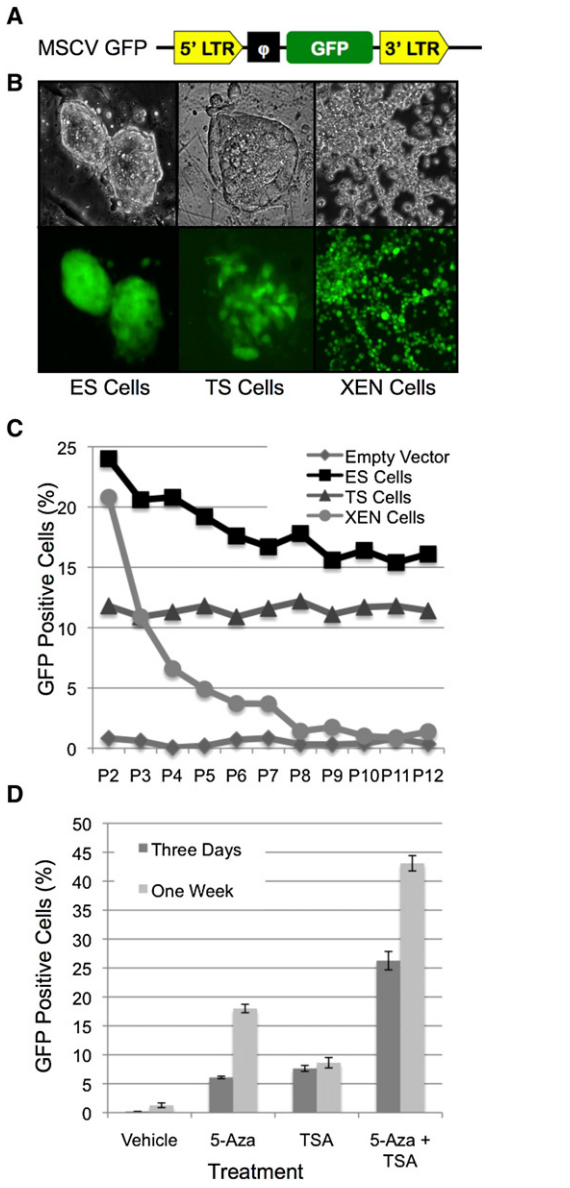

Figure 1. Distinct Patterns of Retroviral Extinction between Embryonic and Extraembryonic Stem Cells that Is Epigenetic in Origin

(A) Schematic representation of γ-retroviral reporters. MSCV GFP is a MSCV variant containing GFP.

(B) Light micrograph and fluorescent images showing expression of γ-retroviral delivered GFP in ESCs, TSCs, and XEN cells at passage three.

(C) Expression of viral-delivered GFP as measured by cytometry over a 6 week period. ESCs exhibited a gradual decline in GFP-positive cells, whereas TSCs displayed no measurable change in GFP-positive cell numbers. XEN cells rapidly silenced the γ-retroviral GFP reporter. No fluorescence was observed in cells infected with an empty vector.

(D) MSCV reporter extinction was an epigenetic phenomenon. Cytometry analysis of XEN cells containing the silenced MSCV GFP γ-retroviral reporter (passage 11) after treatment with vehicle, 5-Aza, and TSA alone or in combination for 3 days or 1 week. Data represent percentage of cells expressing GFP. Error bars, SEM.

nuclear factor 4 (*Hnf4*), and Sex determining region Y-box 7 (*Sox7*) was observed via reverse transcription polymerase chain reaction (RT-PCR) (Figure S1; Kunath et al., 2005). Maintenance of XEN cell marker expression, together with consistent XEN cell morphology throughout the culture period, indicated that silencing of virally delivered GFP was not associated with loss of stemness.

Second, to ensure that the observed extinction was not due to loss of integrated virus, nor to GFP toxicity, infected XEN cell DNA was examined for MSCV-GFP provirus at passage 2 through 10 by quantitative PCR (qPCR). No difference in the ratio of GFP to Glyceraldehyde 3-phosphate dehydrogenase (*Gapdh*) promoter amplification was detected throughout the experiment, indicating that the number of cells containing the integrated provirus was relatively unchanged (Figure S1).

Finally, to determine whether viral reporter silencing was epigenetic in nature, pharmacological inhibition of DNA methylation and histone deacetylation was employed. To this end, XEN cells were again infected with MSCV-GFP, cultured for 10 passages to allow γ-retroviral reporter silencing, then treated with 5-Aza-2′-deoxycytidine (5-Aza) or trichostatin-A (TSA) either alone or in combination. Treatment with 5-Aza or TSA alone induced a 5- to 15-fold increase in reactivation of proviral transcription compared to vehicle alone. Treatment with both drugs produced a synergistic response leading to transcriptional activation (Figure 1D). Thus, integration of γ-retrovirus into the XEN cell genome rapidly initiated an aggressive epigenetic-based antiviral response, resulting in complete silencing of proviral transcription.

RNA Interference Screen for Epigenetic Modifiers of Viral Extinction

To identify specific biochemical factors that mediate this epigenetic response, we conducted a loss-of-function, RNA interference (RNAi), positive selection screen. By sing a microRNA-based short hairpin RNA library (shRNA^{mir}) (Silva et al., 2005) that was designed to target 250 known protein-coding genes involved in epigenetic gene regulation (~3 shRNA^{mir}/gene; Table S2), we addressed the question of how γ-retroviruses are silenced in mouse XEN cells. To conduct our screen, we designed two separate γ-retroviral reporters with distinct fluorescent and drug-selectable markers (Figure 2A). These constructs were packaged into infectious retroviral particles and delivered into early-passage XEN cells. Cells were drug selected, withdrawn from drug selection, and passaged to allow for retroviral extinction. As previously observed, integrated virus was rapidly silenced (Figure S2). Additionally, cultures were verified for maintenance of stemness via XEN cell markers as described above (Figure S1).

At passage 11, replication-deficient lentiviral particles (Figure 2B) were used to deliver the shRNA^{mir} epigenetic library into XEN cells containing a silenced γ-retroviral reporter. These constructs were specifically designed to facilitate delivery into ESCs, TSCs, and XEN cells (unpublished data). shRNA^{mir} constructs were matched to contain distinct fluorescent and drug selection markers from the γ-retroviral reporters (Figure 2C). As a control, a shRNA^{mir} targeting the firefly *luciferase* (Luc) gene was stably transduced into XEN cells, and experiments were conducted in parallel. Upon delivery of the shRNA^{mir} library, cells were passaged for 1 week, then drug selected with either neomycin or puromycin to enrich for XEN cells with reactivated MSCV ChIN or MSCV PIG reporters, respectively. Surviving colonies were picked and DNA isolated, and perspective shRNA^{mir}s conferring resistance to silencing were amplified and sequenced (425 colonies in total). Results of the screen based on a comprehensive scoring system (Figure S3) are summarized in Table 1. Three of the top 25 identified factors have known interactions with viral elements, which validates our experimental design: SWI/SNF-related, matrix-associated, actin-dependent regulator of chromatin, subfamily a, member 5 (SMARCA5); DNA methyltransferase 1 (DNMT1); and Helicase, lymphoid-specific (HELLS) (Chong et al., 2007; De La Fuente et al., 2006). In contrast, the majority of the candidates identified in this screen are novel regulators of retroviral silencing.

Validation of mRNA Depletion of Individual Candidate Genes

To validate the most promising candidates that emerged from our screen, shRNA^{mir}s targeting the top ten candidates based on our scoring system were reintroduced into XEN cells. Target mRNA depletion was verified with a combination of quantitative RT-PCR (qRT-PCR) and western blot analysis (Figures S4 and S5). As controls, candidate shRNA^{mir}-targeted XEN cells were compared to wild-type XEN cells, XEN cells infected with MSCV ChIN or MSCV PIG, as well as Luc shRNA^{mir}-targeted XEN cells. For analysis, results were normalized to wild-type expression levels. All tested shRNA^{mir}s produced target mRNA depletion. Our scoring system gives strong preference to genes for which multiple independent shRNAs emerged. Although arguably we may be bypassing strong candidates, it minimized the potential for off-target shRNAs to be included. The observation that shRNA^{mir}s eliciting potent depletion scored higher in the screen then weaker ones validates the scoring system.

Differential Expression of Candidates between ESC, TSC, and XEN Cell Lineages

To determine whether differences in proviral silencing between ESCs, TSCs, and XEN cells could be explained by a lineage-specific absence of candidate factors or differences in expression levels, relative transcript abundance was investigated via qRT-PCR analysis. To ensure accurate quantitation of candidate transcript levels between cell types, measurements were normalized against the geometric mean of β-actin, 7SK, and Hexokinase transcript levels. Very little SWI/SNF-related, matrix-associated, actin-dependent regulator of chromatin, subfamily a, member 1 (*Smarca1*) expression was detected in TSCs; both ESCs and XEN cells possessed greater than 1000-fold higher transcript abundance than did TSCs (Figure 3). The remaining factors showed a 2- to 10-fold increase in transcript abundance in ESCs compared to TSCs and XEN cells, including SWI/SNF-related, matrix-associated, actin-dependent regulator of chromatin, subfamily a, member 5 (*Smarca5*), Histone deactylases *Hdac2, Hdac7*, and *Hdac9*, Polycomb group ring finger 6 (*Pcgf6*), DNA methyltransferase 3a (*Dnmt3a*), Argonaute1 (*Ago1*), and Embryonic ectoderm development (*Eed*). The lone exception was SWI/SNF-related, matrix-associated, actin-dependent regulator of chromatin, subfamily c, member 2 (*Smarcc2*), which was 3 times more abundantly expressed in XEN cells than in ESCs or TSCs.

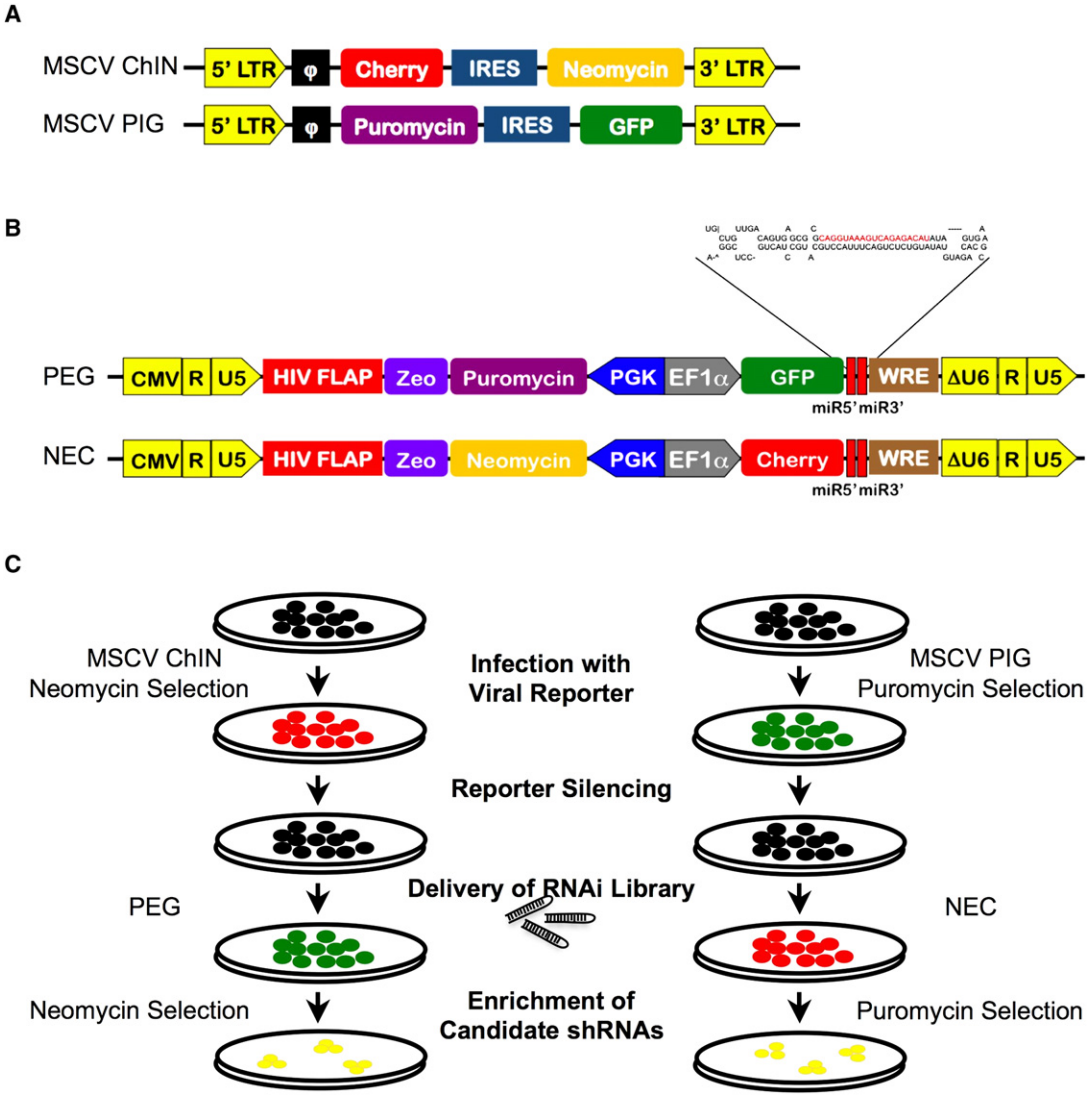

Figure 2. Viral Reporter Constructs and Screening Strategy

(A) Schematic representation of γ-retroviral reporters used in the screen. MSCV ChIN encodes mCherry and neomycin. MSCV PIG contains GFP and puromycin.

(B) Schematic representation of lentiviral constructs used to deliver the shRNA^{mir} library. Two versions of the library were used to induce RNAi in XEN cells, one containing puromycin and GFP (PEG) while the other contained neomycin and mCherry (NEC).

(C) Screening strategy for epigenetic modifiers of γ-retroviral silencing. Left, infectious MSCV ChIN γ-retroviral particles were delivered into XEN cells. Cells were selected in G418 for 3 days to obtain pure populations, withdrawn from drug selection, then cultured for ten passages to induce viral reporter silencing. Once silencing was confirmed, packaged, infectious lentiviral particles from the PEG shRNA^{mir} expression cassette were used to transduce XEN cells at low multiplicity to achieve ~1 shRNA^{mir} integrant/cell. After 1 week in culture, cells were selected with G418 to enrich for MSCV ChIN activation. Right, a similar experiment was conducted with the MSCV PIG γ-retroviral reporter, NEC shRNA^{mir} lentiviral construct, and puromycin drug selection. Surviving colonies from both experiments were isolated after another week in culture, and shRNAs targeting candidate genes were identified via PCR amplification.

Retroviral Extinction Correlates with a Switch from Transcriptionally Permissive to Repressed Chromatin

Given that SMARCA5 was identified in a previous genetic screen for modifiers of retroviral gene silencing (Chong et al., 2007), it is not surprising that other gene family members (SMARCA1 and SMARCC2) may be involved in proviral suppression. Chromatin remodeling is associated with numerous heterogeneous protein complexes and diverse changes in chromatin structure, including histone deacetylation (Xue et al., 1998). To characterize histone acetylation changes associated with proviral silencing, we conducted quantitative chromatin immunoprecipitation (ChIP) analysis of the MSCV 5′ long terminal repeat (LTR) and proximal packaging region (Psi) (Figure 4A) over the course of XEN cell viral extinction. A 1000-fold reduction in signal between passages 2 and 6 was observed when lysates were immunoprecipitated with antibodies recognizing histone 3 acetylation (H3Ac) (Figure 4B). This dramatic drop in acetylated histones strongly supports the involvement of histone deacetylases (HDAC2, HDAC7, and/or HDAC9) in proviral extinction. In further support of this data, Poleshko et al. (2008) and Keedy et al.

Table 1. Top 25 Candidate Genes Mediating γ-Retroviral Silencing in XEN Cells

Gene Name	Other Names	Score	Complex Association
Smarca1	*Snf2L, Nurf140*	637	NURF
Smarca5	*Snf2h, MommeD4*	349	PRC1, SIN3a-HDAC, SNF2h-COHESIN-NuRD CEN
Hdac7a		201	SIN3-HDAC
Hdac2	*Rpd3, Yaf1, Yyibp*	180	PRC2, SIN3a, NcOR
Hdac9	*Hdac7b, Hdrp, Mitr*	135	?
Pcgf6	*Mblr, Rnf134*	108	PRC1
Smarcc2	*Baf170*	99	BRG, BAF
Dnmt3a		90	PRC2, PRC3
Eif2c1	*Ago1*	80	RISC
Eed		77	PRC2, PRC3, PRC4
Myst4	*Morf, Moz2, Kat6b, Qkf*	63	?
Smarcd1	*Baf60a*	36	BRG, BAF
Smarcad1	*Etl1*	31	?
Sfmbt2		24	PHO-RC
Phc2	*Mph2, Edr2*	24	PRC1
SirT1	*Sir2, Sir2alpha*	24	PRC4
Myst2	*Hbo1, Hboa, Kat7*	21	?
Smyd3	*Zmynd1*	21	?
Dnmt1	*MommeD2*	19	PRC2, PRC3
Hells	*Lsh, Smarca6, Yfk8, Pasg*	18	DNMTs / HMTs
Suv39h1	*Kmt1a*	16	PRC1, PRC2
Myst1	*Mof, Kat8*	16	PRC1?
Ezh2	*Enx-1, Kmt6*	14	PRC2, PRC3, PRC4
Phc3	*Hph3, Edr3*	9	PRC1
Actl6b	*Actl6, Baf53b, ArpNa*	8	BAF

(2009) identified a role for the class 1 histone deacetylases HDAC1-3 in suppressing retroviral transcription. Here, we have potentially expanded the family of HDACs involved in retroviral extinction to include both class 1 (HDAC2) and class 2 (HDAC7 and HDAC9) histone deacetylases.

One interesting candidate to emerge from our screen is PCGF6. This protein, which was recently identified as a key component of the self-renewal process in ESCs (Hu et al., 2009), is a putative subunit of the Polycomb Repressive Complex 1 (PRC1) (Lee et al., 2007). PRC1 is a multimeric protein complex known to possess histone demethylating activity. Specifically, PCGF6 directly associates with members of the Jumonji AT-rich interactive domain (JARID) family of proteins, and thus the enzymatic removal of histone 3 lysine 4 trimethylation (H3K4me3) (Lee et al., 2007). When lysates were precipitated with an antibody recognizing H3K4me3, a 60-fold reduction in signal was observed between passages 2 and 6 (Figure 4B).

The third protein complex identified in our screen was PRC2, a repressive complex composed minimally of EED, Enhancer of zeste homolog 2 (EZH2), and Suppressor of zeste 12 homolog (SUZ12) (Schwartz and Pirrotta, 2007; Sparmann and van Lohuizen, 2006). Two of the three core components (EED and EZH2) were identified in our screen, strongly implicating this complex in proviral silencing. Furthermore, PRC2 interacts with suppressor of variegation 3-9 homolog 1 (SUV39H1), HDAC2, and the DNMT family of proteins, all of which were identified in our screen. Through these interactions, PRC2 mediates transcriptional repression via histone 3 lysine 9 (H3K9) and lysine 27 (H3K27) methylation, as well as by directing DNA methylation (Cao et al., 2002; Kuzmichev et al., 2002; Sewalt et al., 2002; Viré et al., 2006). To examine the role of PRC2 in viral extinction, we examined changes in H3K9 trimethylation (H3K9me3) and H3K27 trimethylation (H3K27me3) via ChIP. When lysates were immunoprecipitated with antibodies recognizing H3K9me3, a 300-fold increase in signal was detected between passages 2 and 6 (Figure 4B). By comparison, a slower, modest increase in H3K27me3 signal was observed over the experimental time course.

PRC2 associates with all three members of the DNA methyltransferase family and directs DNA methylation of EZH2 target promoters (Viré et al., 2006). Given the strong association between DNA methylation and retroviral transcriptional silencing (Cherry et al., 2000; Harbers et al., 1981) and the identification of both DNMT1 and DNMT3A in our screen, we examined the DNA methylation status of the viral LTR in XEN cells undergoing viral extinction by using the bisulphite mutagenesis and sequencing analysis. As a control, NIH 3T3 cells were infected and time points measured in parallel. De novo DNA methylation at the MSCV 5′ LTR was progressively acquired over time, such that by passage 7, DNA strands were hypermethylated (Figure 4C). In contrast, MSCV LTR remained hypomethylated in NIH 3T3 cells over the period assayed. Collectively, these data indicate that the significant change in local chromatin structure from a transcriptionally permissive to silent state was probably due to the identified repressive complexes.

Epigenetic Factors Localize to Retroviral Elements in Silenced XEN cells

To further examine a potential role for the identified factors in regulating γ-retroviral extinction, it was necessary to establish a link between the candidates and transcriptional regulation of integrated proviral elements. To determine whether candidate proteins were directly associated with 5′ MSCV viral LTR, ChIP analysis was conducted with proteins for which ChIP-grade antibodies were available: AGO1, HDAC2, HDAC7, SMARCA1, and SMARCA5. All assayed proteins produced enrichment of the γ-retroviral LTR at passage 3 (Figure 5A; Figure S6), as measured by qPCR, with the highest enrichment for SMARCA1 and AGO1.

Candidate Epigenetic Factors Are Required for Endogenous Retroviral Silencing

To further establish a link between candidate factors and transcriptional regulation of proviral elements, we turned our analysis to endogenous retroviral elements. Endogenous retroviruses are remnants of ancient viral infections that persist within the genome. Generally, endogenous retroviral elements are maintained in a silent state. To determine whether the identified epigenetic factors were involved in retroviral transcriptional repression, we conducted shRNA-mediated depletion of candidate factors in XEN cells and then assessed their effect on endogenous retroviral transcription. To this end, transcript levels

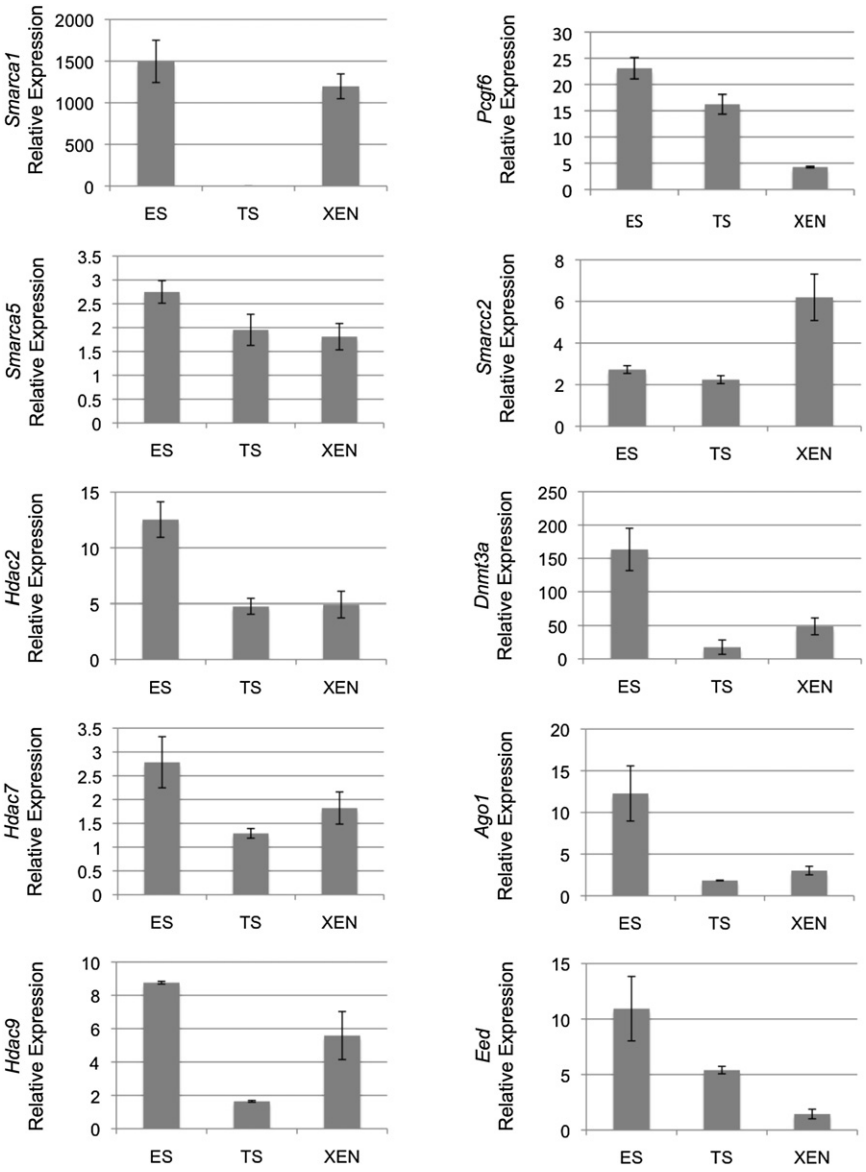

Figure 3. Comparison of Candidate Factor Transcript Levels between ESCs, TSCs, and XEN Cells

Quantitative RT-PCR analysis of mRNA transcripts encoding top ten candidate genes. Measurements normalized to the geometric mean of transcripts encoding housekeeping genes β-actin, 7SK RNA, and Hexokinase. Error bars, SEM.

of the Line1, IAP, and virus-like 30S (VL30) retro-elements were quantitated in cells containing shRNA^{mir}s targeting the top ten candidates, via qRT-PCR analysis. Comparisons were made to controls, wild-type XEN cells, MSCV ChIN-infected XEN cells, MCSV PIG-infected XEN cells, and Luc shRNA^{mir}-targeted XEN cells. Results were normalized to wild-type expression levels. All tested shRNA^{mir}s demonstrated transcriptional activation for at least one endogenous retroviral family (Figure 5B), with preferential activation of specific retro-elements by some candidates. For example, depletion of *Pcgf6* mRNA produced the strongest transcriptional activation of Line1 and VL30 elements, whereas RNAi directed against *Smarca5* produced the strongest activation of IAP elements (Figure 5B), consistent with the latter as an agouti yellow gene modifier (Chong et al., 2007).

To determine whether candidates identified in XEN cells were also involved in silencing retro-elements in the embryonic lineage, RNAi depletion of each top ten candidate was conducted in ESCs (Figure 5B). Similar to XEN cells, depletion of *Smarca5* induced strong expression of Line1, IAP, and VL30 retro-elements. However, unlike XEN cells, depletion of neither *Pcgf6* nor *Smarca1* induced retro-element reactivation. Furthermore, *Dnmt3a* RNA depletion in ESCs showed the strongest activation of retro-elements as opposed to the more modest reactivation seen in XEN cells. These data indicate that factors identified in this screen not only interact with and silence the MSCV γ-retrovirus but also play significant roles in repressing transcriptional activity of endogenous retroviruses.

DISCUSSION

In this study, we investigated viral extinction in ESCs, TSCs, and XEN cells. Our work shows that the three cell lineages of the early mammalian embryo have vastly different viral silencing strategies as well as different capacities to suppress retroviral activity. Cells derived from the inner cell mass have long been shown to repress retroviral activity (Harbers et al., 1981; Jähner et al., 1982; Stewart et al., 1982). This capacity was explained by the necessity to constrain endogenous retroviral activity, given that insertional mutagenesis during early embryonic time points could potentially create detrimental heritable mutations, reducing reproductive fitness (Loh et al., 1990; Niwa et al., 1983; Teich et al., 1977).

In comparison to embryonic stem cells, extraembryonic cells possess vastly different responses to retroviral integration. We observed that trophectoderm stem cells fail to silence retroviral transcription. Consistent with this, early studies of placental tissue via electron microscopy found an abundance of endogenous type C retroviral particles in syncytial trophoblasts, suggesting that robust retroviral activity occurs within this tissue type (Gross et al., 1975; Kalter et al., 1973). Later, it was discovered that long terminal repeats of active retroviral elements act as alternative placental-specific or primary promoters, and retroviral envelope genes confer fusogenic and immunosuppressive functions (Cohen et al., 2009; Mi et al., 2000; Rawn and Cross, 2008). One explanation for exaptation of retroviral elements is that relaxation of epigenetic control of specific retroviral

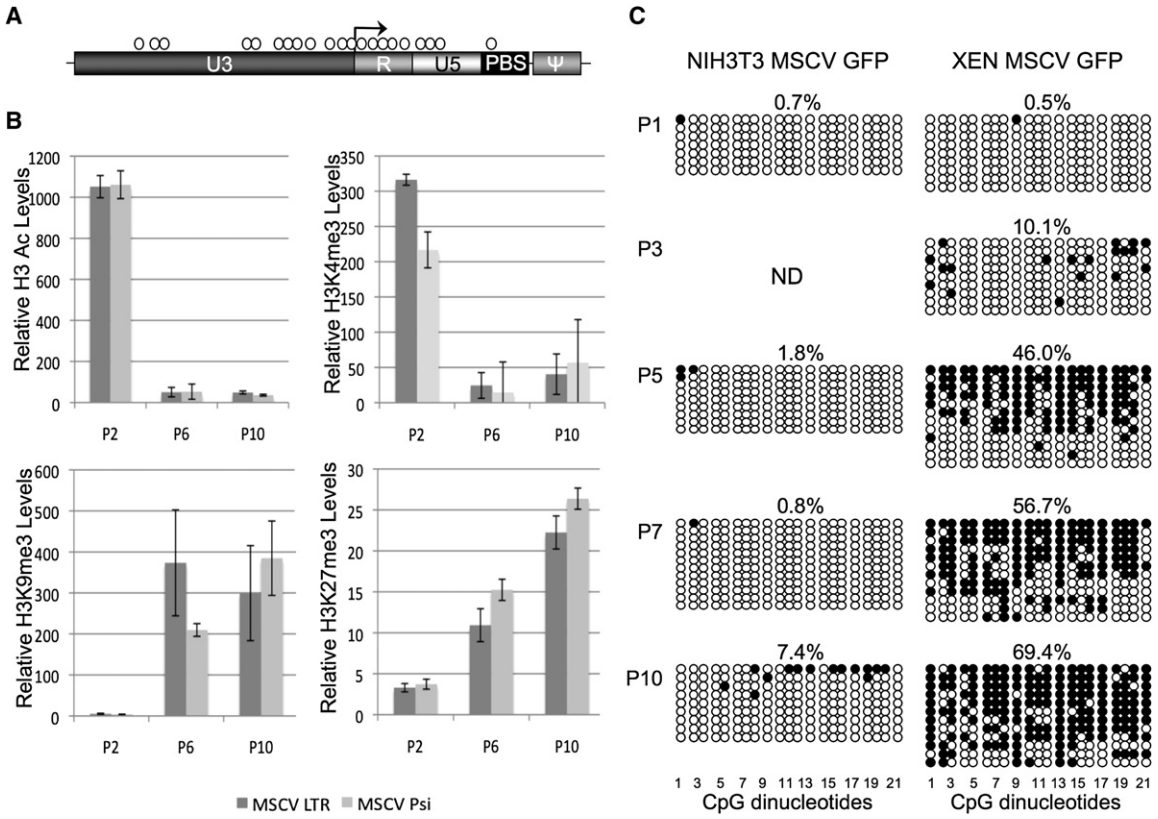

Figure 4. MSCV Extinction Correlated with a Switch from Transcriptionally Permissive to Constrained Chromatin

(A) Schematic representation of 5′ MSCV viral long terminal repeat (LTR) and packaging signal (ψ, Psi) regions. Open circle, CpG dinucleotides. Arrow, transcription start site.

(B) Loss of active and gain of repressive histone modification during retroviral silencing. ChIP analysis of integrated 5′ MSCV LTR and Psi regions. qPCR measured enrichment after immunoprecipitation via antibodies to H3Ac, H3K4me3, H3K27me3, and H3K9me3 at passages 2, 6, and 10. Measurements were normalized to the *Gata4* promoter. Error bars, SEM.

(C) MSCV LTR became hypermethylated in XEN cells. Methylation status of LTR in individual DNA strands of cultured XEN cells (passage 1, 3, 5, 7, 10) as determined by bisulfite mutagenesis and sequencing analysis. Unmethylated CpGs are represented as empty circles while methylated CpGs are depicted as filled circles. Each line denotes an individual DNA strand. Percent methylation was number of methylated CpGs over total number of CpGs. NIH 3T3 fibroblasts were infected as a negative control. ND, not determined.

elements may have coevolved with placental development, enabling expression of these essential retroviral factors.

Prior to this study, one might have assumed that cells of extraembryonic origin would have similar strategies for regulating retroviral elements. The unexpectedly aggressive retroviral extinction observed in XEN cells indicates that the permissive transcriptional environment seen in the trophectoderm lineage is not a shared feature of extraembryonic stem cell types. Our results indicate a potent antiviral response requiring the interaction of at least three repressive complexes to initiate, establish, and maintain retroviral silencing.

Despite the fact that viral extinction has been studied for decades, the precise epigenetic factors employed in silencing retroviruses had yet to be determined. To identify specific biochemical factors that mediate this epigenetic response, we utilized loss-of-function RNAi screens for epigenetic factors that mediate silencing of integrated retroviruses. Interestingly, 18 of the top 25 candidates associate with 1 of 3 epigenetic modifier complexes (Chromatin remodeling complex, Polycomb repressive complex 1, and Polycomb repressive complex 2), all of which have well-characterized roles in posttranslational modification of chromatin to a transcriptionally silent state (Roberts and Orkin, 2004; Schwartz and Pirrotta, 2007; Sparmann and van Lohuizen, 2006). Three of the identified factors have known interactions with viral elements. SMARCA5 and DNMT1 were identified as modifiers of intracisternal A particles within the agouti variable yellow locus (Chong et al., 2007). Moreover, HELLS was identified as a potent modulator of endogenous retroviral methylation and expression (De La Fuente et al., 2006). In contrast, the majority of candidates identified in this screen are novel regulators of retroviral silencing. This work represents the first step toward understanding how mammalian genomes recognize integrated parasitic nucleic acids and the biochemical responses initiated to silence these elements.

One striking observation from this study was that TSCs failed to silence the retroviral reporter. Given that very little *Smarca1* expression was detected in TSCs in comparison to ESCs and XEN cells, it is tempting to speculate that SMARCA1 may play

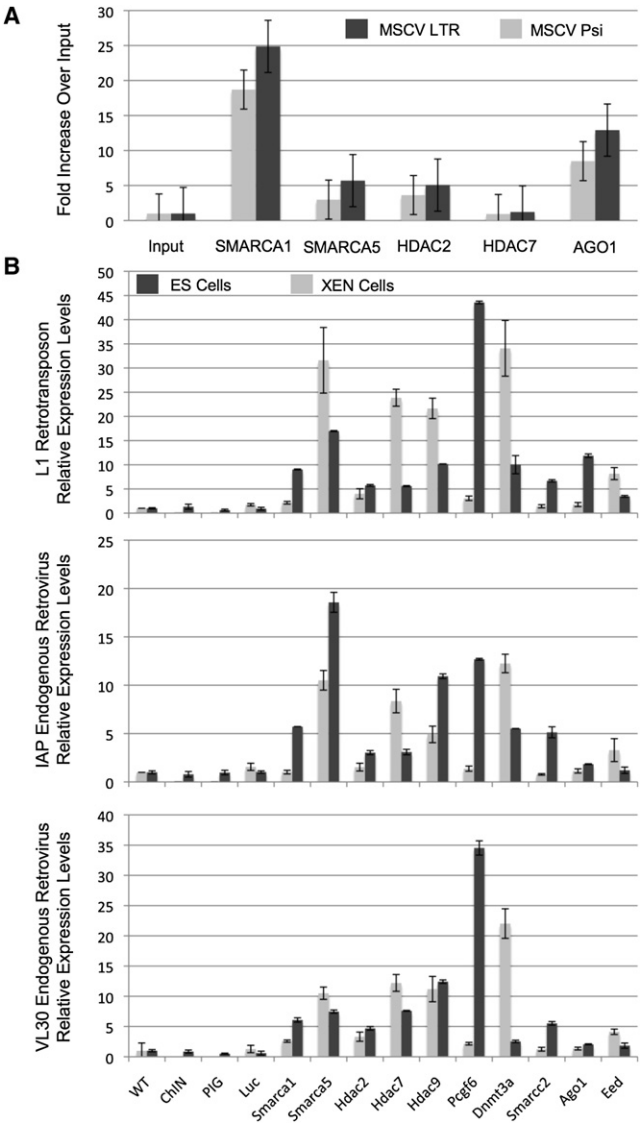

Figure 5. Epigenetic Factors Localized to MSCV LTR in Silenced XEN Cells and Were Required for Endogenous Retroviral Silencing

(A) Top candidate proteins localized to MSCV 5′ LTR and Psi elements. ChIP analysis of integrated LTR was performed with antibodies specific for AGO1, HDAC2, HDAC7, SMARCA1, and SMARCA5. All candidates demonstrated strong enrichment of the provirus compared to input via qPCR.

(B) Distinct complexes repress different families of endogenous retroviral elements. qRT-PCR analysis of Line1 (L1), IAP, and VL30 retro-element families in controls and candidate-depleted ESCs and XEN cells. Transcriptional activity of each retrovirus family via two independent primer sets was normalized to *Mrpl1*. Error bars, SEM.

a major role in retroviral silencing. Experiments generating ectopic *Smarca1* expression in TSCs would determine whether SMARCA1 confers the capacity to silence integrated virus. With regard to the aggressive versus the slow viral extinction of XEN cells and ESCs, respectively, the similar or greater expression of the remaining candidate factors in ESCs indicates that they are not likely to provide differential viral silencing capacities, with the exception of SMARCC2. More abundant expression of and greater silencing activity of endogenous retro-elements by SMARCC2 in XEN cells is suggestive of a role in rapid viral extinction. Thus, evidence from this study suggests that although many complexes are common between the three stem cell lineages, significant differences also exist, potentially explaining the varied capacities to extinguish proviral transcription. We and others postulate that multimeric epigenetic complexes have a core set of proteins that mediate their chromatin-modifying activity while possessing a subset of "exchangeable" proteins that confer target specificity (Wolf and Goff, 2009). In the case of XEN cells, SMARCC2 may provide specificity.

Another interesting finding to emerge from this study is that multiple silencing pathways can act to silence proviral transcription, while at the same time functioning to silence distinct classes of endogenous retro-elements. Because different retro-element promoter regions lack primary sequence homology, no single sequence or binding protein could be utilized to direct broad spectrum silencing (Mandal and Kazazian, 2008). This suggests that multiple repressive complexes capable of targeting distinct retro-elements exist, allowing common biochemical pathways to function for different retroviral elements. Furthermore, given the importance of restraining endogenous retro-element transcription to genome stability, it is likely that functional redundancy has also evolved (Kazazian and Goodier, 2002). Support for this hypothesis comes from the observation that depletion of most candidate factors generated only a 2- to 12-fold reactivation of endogenous retro-elements. These results align with those reported for *Dnmt1*$^{-/-}$ and *Np95*$^{-/-}$ as well as *Suv39h1/h2*$^{-}$ and *Dnmt3a/3b* double null ESCs (Martens et al., 2005; Dong et al., 2008; Sharif et al., 2007).

Although we and others have identified several candidate proteins, which modify proviral chromatin (Chong et al., 2007; De La Fuente et al., 2006), no mechanism has been identified by which epigenetic modifiers are recruited to viral integration sites. One candidate emerging from our screen, Eukaryotic translation initiation factor 2C1 (EIF2C1), better known AGO1, is suggestive of a potential recruitment mechanism. AGO1 is a member of a clade of RNA-binding proteins that form the catalytic core of the RNA-induced silencing complex (Carmell et al., 2002). Thus, one potential mechanism of recruitment is through an RNA interaction via homologous siRNAs processed from virally derived, double-stranded RNA. Recognition of parasitic nucleic acids through DNA:RNA or RNA:RNA pairing is a potential mechanism for the genome to distinguish "self" from parasitic "non-self" (Malone and Hannon, 2009; Tam et al., 2008; Watanabe et al., 2006; Aravin et al., 2007).

The capacity of retroviral elements to modify expression of protein coding genes and influence organism phenotype was first described by Barbara McClintock (McClintock, 1956). Clearly, eukaryotic cells encountered retroviral challenges very early in evolution and evolved complex strategies for suppressing these integrated elements. The subsequent molecular arms race that ensued between the host and pathogen allowed development of complex epigenetic mechanisms, which may have been adapted to other areas of transcriptional regulation (Cohen et al., 2009; Kazazian, 2004). Understanding the interplay between endogenous retroviral elements and the epigenome will aid our understanding of basic biology of transcriptional regulation and mammalian development.

EXPERIMENTAL PROCEDURES

Vector construction and oligonucleotide sequences can be found in Supplemental Information.

Cell Culture

C57BL/6 female mice (B6) (Jackson Laboratory) were superovulated by intraperitoneal injection of 6.25 IU pregnant mare's serum gonadotropin (Intervet Canada) followed by 6.25 IU human chorionic gonadotropin (Intervet Canada) 44–48 hr later. Females were mated with *Mus musculus castaneus* (CAST) males (Jackson Laboratory). Pregnancy was determined by a vaginal plug the following morning (day 0.5). F_1 hybrid embryos were flushed from genital tract of females at day 3.0 to recover morulae. Primary ESCs, TSCs, and XEN cells were derived from B6XCAST F_1 embryos as previously described (Kunath et al., 2005; Nagy et al., 1993; Tanaka et al., 1998). In brief, ESC cultures were maintained in DMEM (Sigma D5671) supplemented with 50 μg/ml penicillin/streptomycin (Sigma), 100 μm β-mercaptoethanol, 1× LIF (Sigma), 2 mM L-Glutamine (Sigma), 1× MEM nonessential amino acids (Sigma), and 15% hyclone ESC grade fetal bovine serum (FBS). TSC and XEN cell cultures were maintained as described (Kunath et al., 2005; Tanaka et al., 1998) via RPMI (Sigma R0883) supplemented with 50 μg/ml penicillin/streptomycin, 1 mM sodium pyruvate, 100 μm β-mercaptoethanol, 1 μg/ml heparin (Sigma), 2 mM L-glutamine, 1× FGF basic, 1× FGF4 (R&D Systems), and 20% hyclone ESC grade FBS. Cells were grown on a mitomycin C (Sigma)-treated feeder fibroblast layer. For experiments involving DNA methylation and histone deacetylation inhibitor treatment, XEN cell were cultured in media supplemented with 0.15 μm 5-Aza-2′-deoxycytidine (Sigma) and/or 200 nM Trichostain A (Sigma). Experiments were performed in compliance with the guidelines set by the Canadian Council for Animal Care, and the policies and procedures approved by the University of Western Ontario Council on Animal Care.

Production of Recombinant Virus

Recombinant gamma and lenti class retroviral particles were prepared as described (Lois et al., 2002). In brief, viral vectors described above, along with plasmids encoding a vesicular stomatitis virus glycoprotein pseudotype (plasmid pMDG) plus either Psi (pPsi) or Delta (pCMV-Delta 8.9) packaging elements (for gamma or lenti viruses, respectively), were transfected into 6-well dishes containing NIH HEK293 cells (ATCC) at 70% confluency, via calcium phosphate precipitation. Ratio of plasmids was 2.3 μg viral transfer vector, 1.0 μg pMDG, and 1.7 μg pPsi or pCMV-DeltaR 8.91. Media was replaced 24 hr posttransfection, and cells were cultured for an additional 48–72 hr. After which, virus-containing media was collected, syringe filtered through a 0.45 μm filter, and aliquoted into 2 ml snap cap tubes.

Somatic and Stem Cell Infection

NIH 3T3 (ATCC) cells, ESCs, TSCs, and XEN cells were seeded into 24-well plates to give a density of 50% confluence after 12–18 hr of growth. The next day, cells were infected by delivery of filtered virus directly into the culture media along with 1× polybrene solution (final concentration). Cells were spun at 1000 × g for 1 hr and cultured in viral media overnight. The following day, media was changed, and cells were incubated for 24–48 hr before assessing fluorescence transgene expression. Once GFP/mCherry expression was established, cells were subpassed via the standard protocol for each cell type, and selection with the appropriate antibiotic was initiated. Cells were infected with a dilution series of viral particles to ensure that less then 20% of cells became infected, as measured by GFP or mCherry expression. Low levels of infection were crucial to ensuring approximately a single viral integration event per cell. NIH 3T3 cells were selected with 2 μg/ml puromycin or 400 μg/ml neomycin, while XEN cells were selected with 1 μg/ml or 200 μg/ml puromycin and neomycin, respectively (Sigma Aldrich). Studies examining candidate suppression in ESCs utilized siRNAs (QIAGEN) (Table S2) transected into cells via Lipofectamine 2000 (Invitrogen) according to the manufacturer's recommendations.

Flow Cytometry

For cytometry analysis, cells were washed twice with warm PBS and trypsinized with 1× trypsin (Sigma) into a single-cell suspension. Cells were spun and resuspended in 10% FBS PBS solution. Cells were then analyzed on a Beckman Coulter Epics XL-MCL Flow Cytometer by normalizing readings first to nontransgenic wild-type cells, and then measuring fluorescence of 3 independent groups of 50,000 cells for each sample. Results from three independent measurements were averaged and standard error of the mean (SEM) determined.

RNA Isolation and Reverse Transcription

Cultured cells were grown to 80% confluence, washed twice in warm PBS, and dissociated with 1× trypsin (Sigma). Cells were spun down and washed once in cold PBS, then RNA was isolated with Trizol (Invitrogen) according to the manufacturer's protocol. 1 μg of purified total RNA was treated with amplification-grade DNaseI (Invitrogen) according to the manufacturer's protocol, and then reverse transcribed with the SuperScriptII system (Invitrogen) by combining 1 μl random hexamer oligonucleotides (Invitrogen), 1 μl 10 mM dNTP (Invitrogen), and 11 μl RNA plus water. This mixture was brought to 70°C for 5 min and then cooled to room temperature. SuperScriptII reaction buffer, DTT (Invitrogen), and SuperScriptII were then added according to manufacturer's protocol and the mixture was brought to 25°C for 5 min, 42°C for 50 min, 45°C for 50 min, 50°C for 30 min, then 70°C for 5 min.

Real-Time PCR Ampification

Real-time PCR analysis of mRNA levels was carried out with the iQ SYBR Green Supermix (BioRad) according to the manufacturer's instructions. Reactions were performed on a MJ Thermocycler Chromo4 Real Time PCR system (BioRad). Samples were normalized to the mouse ribosomal binding protein (*Mrpl1*) gene or the geometric mean of transcripts encoding β-actin, 7SK, and Hexokinase (Mamo et al., 2007).

PCR Analysis of Drug-Resistant Colonies

Cells undergoing selection during the screening process were grown in 20 cm dishes, emerging drug-resistant colonies were picked, and then subpassaged into 24-well plates under continued selection to maintain clonal populations. Upon confluence, colonies were harvested and DNA isolated via the QIAGEN DNeasy Blood & Tissue Kit, according to manufacturer's protocol. To identify $shRNA^{mir}$s present in surviving colonies, 100 ng genomic DNA was seeded into a PCR reaction (PCR hot start Ready-to-go beads, GE) containing a 5′ GFP or mCherry primer and a 3′ woodchuck response element (WRE) primer located within the lentiviral backbone. Amplicons were sequenced at the London Regional Genomic Centre with GFP or mCherry primers.

Chromatin Immunoprecipitation Analysis

Cultured cells were grown to 80% confluence, washed twice in warm PBS, trypsinized, and then resuspended in warm growth media containing 0.1 volume of crosslinking solution (Kondo et al., 2004). Subsequently, ChIP reactions were performed as described (Martens et al., 2005), which was followed by DNA purification with a QIAGEN PCR Cleanup kit. Antibodies used in the immunoprecipitation of modified histones and candidate proteins were anti-Rabbit IGG (Santa Cruz SC-2027), anti-Acetylated-Histone H3 (Millipore 06-599), anti-Trimethyl Histone H3 Lysine 4 (Millipore 04-745), anti-Trimethyl Histone H3 Lysine 9 (Abcam Ab8898), anti-Trimethyl Histone H3 Lysine 27 (Millipore 17-622), anti-AGO1, monocolonal (Millipore 04-083), anti-AGO1 polyclonal (Millipore 07-599) (Janowski et al., 2006; Kim et al., 2006), anti-HDAC2 (Abcam Ab7029), anti-HDAC7 (Abcam Ab50212), anti-SMARCA1 (Abcam Ab37003), and anti-SMARCA5 (SNF2H Abcam Ab3749). Antibodies for modified histones were used at 1 μg/ChIP reaction while antibodies to candidate proteins were used at 5 μg/ChIP reaction. The concentration of IGG was adjusted from 1 μg to 5 μg as appropriate. For quantitative analysis, real-time PCR was carried out with the iQ SYBR Green Supermix according to the recommended protocol. Reactions were performed on a MJ Thermocycler Chromo4 Real Time PCR system. Samples were normalized to measurements taken for the *Gata4* promoter, and data were analyzed with formula previously described (Mukhopadhyay et al., 2008).

Sodium Bisulfite Mutagenesis and Sequencing Assay

Bisulfite mutagenesis and sequencing with agarose embedding was performed (Market-Velker et al., 2010) with modification. Lysed cells (10 μL) were embedded in 20 μl 2% low melting point agarose (Sigma). After bisulfite

mutagenesis, 22 μl diluted agarose was added to Ready-to-go PCR Beads containing MSCV BIS F1 and R primers and 1 μl of 240 ng/mL tRNA. PCR reactions were halved, allowing for two independent PCR reactions. First round product (5 μL) was seeded into each second round PCR reaction with MSCV BIS F2 and R primers. Samples were sent to the Nanuq Sequencing Facility or BioBasic Inc. for sequencing. To obtain representative number of DNA strands, 15–20 clones were sequenced. Sequences with less than 95% conversion rates were not included. Identical clones (identical location and number of methylated CpGs and unconverted non-CpG cytosines) were included once.

SUPPLEMENTAL INFORMATION

Supplemental Information includes Supplemental Experimental Procedures, six figures, and three tables and can be found with this article online at doi:10.1016/j.stem.2010.03.014.

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