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Atrx promotes heterochromatin formation at retrotransposons

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Abstract

More than 50% of mammalian genomes consist of retrotransposon sequences. Silencing of retrotransposons by heterochromatin is essential to ensure genomic stability and transcriptional integrity. Here, we identified a short sequence element in intracisternal A particle (IAP) retrotransposons that is sufficient to trigger heterochromatin formation. We used this sequence in a genome-wide shRNA screen and identified the chromatin remodeler *Atrx* as a novel regulator of IAP silencing. *Atrx* binds to IAP elements and is necessary for efficient heterochromatin formation. In addition, *Atrx* facilitates a robust and largely inaccessible heterochromatin structure as *Atrx* knockout cells display increased chromatin accessibility at retrotransposons and non-repetitive heterochromatic loci. In summary, we demonstrate a direct role of *Atrx* in the establishment and robust maintenance of heterochromatin.

Keywords Atrx; Daxx; heterochromatin; histone H3.3; IAP retrotransposons

Subject Category Chromatin, Epigenetics, Genomics & Functional Genomics

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Introduction

Silencing of retrotransposons is essential for embryonic development. Firstly, novel retrotransposon insertions can impair genome stability [1], and secondly, aberrant activation of retrotransposon-based enhancers can confuse the transcriptome by affecting the expression of nearby genes [2,3]. In light of these findings, recent data have implicated retrotransposons in the development of human diseases, such as cancer [4]. Hence, it is important to understand how mammalian retrotransposons are targeted for silencing.

Silencing of retrotransposons involves the establishment of a heterochromatic domain covering the important regulatory regions of these elements. This heterochromatic structure shows a very similar modification signature to pericentric heterochromatin and

features high levels of DNA methylation as well as H3K9me3 and H4K20me3 [5]. Chromatin modifications on retrotransposons are established by the same molecular machineries that act on pericentric heterochromatin, except for H3K9me3 which is not predominantly mediated by Suv39h enzymes but rather depends on the histone methyltransferase Setdb1 [6]. Targeting of the repressive machineries to retrotransposons is incompletely understood. In part, DNA methylation imprints of germ cells are used to re-establish heterochromatin during early embryonic development [7,8]. In addition, there is strong evidence for *de novo* pathways that recognize specific retrotransposon sequences [9–11]. These pathways are active in embryonic stem (ES) cells and can be utilized to study *de novo* heterochromatin establishment.

To identify novel players in heterochromatin establishment and maintenance on retrotransposons, we chose the abundant class of IAP retrotransposons as model system. In ES cells, IAP silencing depends on the transcriptional repressor Trim28 that interacts with Setdb1 and thus mediates the recruitment of H3K9me3. Trim28 and Setdb1 also regulate the establishment and turnover of DNA methylation at IAP sequences in ES cells [12]. Interestingly, in differentiated cells IAP silencing does not require the Trim28 pathway anymore and largely depends on DNA methylation, suggesting that Trim28 is mainly an important factor in the *de novo* pathway for heterochromatin establishment [12,13]. How IAP sequences specifically recruit Trim28 and Setdb1 in ES cells is largely unclear. Likely candidates for targeting Trim28 to IAPs are sequence-specific KRAB zinc finger proteins. Recruitment of Trim28 by KRAB zinc finger proteins is well described *in vitro* [14–17] and on few endogenous targets including MLV proviruses and imprinted regions [9,18,19]. This hypothesis predicts that retrotransposons contain nucleation sites for silencing, bound by KRAB zinc fingers. In accordance with this model, novel IAP insertions into the genome of mouse ES cells recruit Trim28-dependent silencing [10,13].

Here, we systematically tested sequence elements of IAP retrotransposons for their ability to induce heterochromatin formation and identified a small region of 160 bp (SHIN) that is sufficient to trigger silencing. Based on this sequence, we developed a shRNA screen and identified the SNF2-type chromatin remodeler *Atrx* as

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strong modifier of IAP silencing. Atrx was initially identified as the gene responsible for the X-linked alpha thalassemia/mental retardation (ATR-X) syndrome [20]. ATRX-deficient cells display numerous phenotypes connected to heterochromatic regions, such as defective sister chromatid cohesion and telomere dysfunction [21–23]. ATRX loss is further connected to alterations in DNA methylation patterns at imprinted regions, rDNA loci, telomeric repeats, and pericentromeric heterochromatin [24,25]. Recruitment to heterochromatic domains may be due to direct binding of the Atrx ADD domain to H3K9me3 in the context of unmethylated H3K4 [26,27]. Interaction with HP1, which is highly enriched at heterochromatin, has been shown to also contribute to stable recruitment of Atrx [26,27].

Recently, Atrx was found to interact with the histone H3.3-specific chaperone Daxx to facilitate H3.3 deposition in telomeric and pericentromeric regions [28–30]. Notably, this H3.3 deposition pathway is frequently mutated in pancreatic neuroendocrine tumors, glioblastoma, and pediatric neuroendocrine tumors [31–35]. These mutations are generally associated with an activation of the alternative lengthening of telomeres (ALT) pathway, a telomerase-independent mechanism to extend telomere length by recombination [32,36]. It is currently unclear if H3.3 deposition by Atrx/Daxx is really necessary to establish proper heterochromatin domains across telomeres and pericentromeric heterochromatin. Furthermore, it is not known if Atrx affects heterochromatin in other regions of the genome.

In this study, we show that Atrx is crucial for heterochromatin formation at IAP retrotransposons. Interestingly, heterochromatin establishment does not involve Daxx-mediated histone H3.3 incorporation. Instead, Atrx is important for generating an inaccessible chromatin structure on retrotransposons and non-repetitive regions in the genome which is necessary for robust maintenance of heterochromatin. Thus, our data reveal a novel role of Atrx in heterochromatin organization and may help to explain pathological phenotypes associated with ATRX syndrome or ATRX-deficient tumors.

Results

Identification of a short heterochromatin inducing sequence (SHIN)

To monitor the silencing potential of specific DNA sequence elements of mouse IAP retrotransposons, we developed a lentiviral hEF1 α -EGFP reporter system (Fig 1A). When we combined the reporter with the well-characterized repressor binding site (RBS) of the MLV retrovirus [9,37], strong silencing of EGFP expression was observed 2 days post-transduction (Fig 1A). Similar effects were observed with the GAG region of IAP retrotransposons (Fig 1A). In order to more systematically test silencing potential of IAP sequence elements, we analyzed different IAP regions in our reporter assay (Fig 1B). A previously characterized region containing the UTR of IAP retrotransposons [10] displayed reporter silencing; however, the strongest effect was exerted by the ~2,000-bp-long IAP-GAG region (Fig 1B). As *de novo* silencing of IAP retrotransposons is restricted to ES cells [38], we did not detect silencing in mouse embryonic fibroblasts (MEF). To refine potential silencing initiating elements

within the GAG region, we further dissected this element into 400-bp fragments (Supplementary Fig S1A) and could finally determine a region of 160 bp which was sufficient to induce strong reporter silencing (Fig 1C). This sequence could not be further shortened without compromising silencing potential (Fig 1C) and is highly conserved in more than 600 IAP elements, predominantly of the IAP-Ez subclass (Supplementary Fig S1C, Supplementary Table S1). As will be outlined below, this short sequence element is sufficient to trigger heterochromatin formation; thus, we termed this region SHIN for short heterochromatin inducing sequence. Notably, SHIN silencing is not restricted to the hEF1 α promoter (Supplementary Fig S1B) and is orientation independent (Supplementary Fig S1B), suggesting that silencing is initiated by sequence-specific DNA binding factors.

The SHIN sequence induces heterochromatin

In ES cells, IAP silencing is mediated by heterochromatin that depends on Trim28 which, in turn, recruits the H3K9me3 methyltransferase Setdb1 [6,10,39]. Similar to pericentric heterochromatin, the H3K9me3-rich domains across IAP elements coincide with H4K20me3 and DNA methylation. To test if SHIN silencing is dependent on any of these chromatin modifications, we assessed the extent of SHIN repression in ES cell lines that are depleted for the major silencing machineries. Here, we measured the number of EGFP-positive cells relative to the control reporter that does not contain the SHIN sequence. Interestingly, cells deficient for Suv39h, Suv4-20h, Dnmt1, or Dnmt3ab can still effectively silence the SHIN reporter (Fig 2A). In contrast, Setdb1 knockout cells are severely compromised in SHIN silencing (Fig 2A). Also, lentiviral knock-down of Trim28, which did not even completely abolish Trim28 expression (Supplementary Fig S1D and E), resulted in strong silencing defects. These data demonstrate that SHIN silencing requires the major players that also regulate silencing of endogenous IAP elements.

Next, we asked if the SHIN sequence can directly trigger Trim28/Setdb1-dependent heterochromatin formation. To address this question, we used a well-established recombinase-mediated cassette exchange (RMCE) cell line to integrate the SHIN sequence into a defined position of the ES cell genome (Supplementary Fig S1F) [40,41]. We combined the SHIN sequence with a Tet-inducible EGFP-T2A-zeocin reporter which is not transcribed in the absence of doxycycline (Fig 2B). We also generated a control reporter lacking the SHIN sequence (Fig 2B). RMCE of the two reporter constructs resulted in the HA36::SHIN and HA36::control cell lines (Fig 2B). ChIP-qPCR analysis revealed high levels of H3K9me3 across the RMCE locus in HA36::SHIN cells, comparable to endogenous IAP elements (Fig 2B). In contrast, only residual occupancy for H3K9me3 was detected in HA36::control cells (Fig 2B). To test if H3K9me3 across the SHIN reporter depends on Setdb1, we performed CrispR/Cas knockout of Setdb1 in HA36::SHIN cells. Prolonged knockout of Setdb1 is lethal for ES cells. Thus, we could not select for Setdb1 knockout cells and used a mixed population of Setdb1-deficient/Setdb1-proficient cells for ChIP analyses 4 days post-sgRNA transduction (Fig 2C, Supplementary Fig S1G). H3K9me3 levels at major satellite repeats are dependent on Suv39h enzymes and therefore remained almost unchanged upon Setdb1 depletion (Fig 2C). Levels of H3K9me3 at

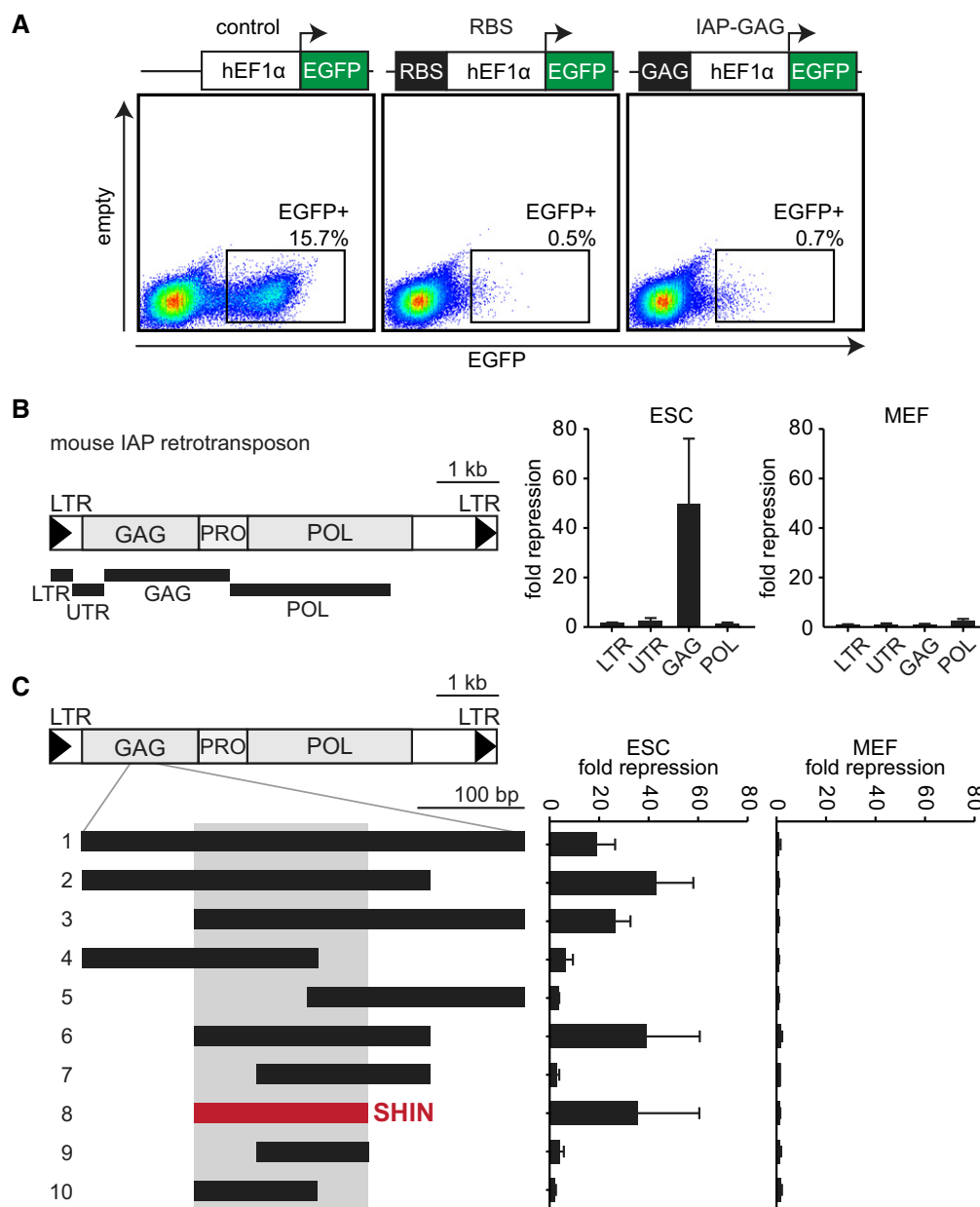


Figure 1. Identification of a short heterochromatin inducing sequence (SHIN) in mouse IAP retrotransposons.

A Reporter assay for retrotransposon silencing. Mouse ES cells were transduced with indicated lentiviral reporter vectors, and EGFP fluorescence was monitored 2 days later by FACS. PE was used as empty channel. RBS, repressor binding site of the MLV virus; IAP-GAG, GAG coding sequence of IAP retrotransposons.

B The GAG coding region of IAP retrotransposons leads to strong reporter silencing in mouse ES cells. Structure of IAP elements: LTR, long terminal repeats; UTR, 5' untranslated region; GAG, GAG protein coding sequence; PRO, coding sequence of retroviral protease; and POL, coding sequence of retroviral polymerase. Indicated sequence elements of IAP retrotransposons were cloned upstream of the EGFP reporter cassette and tested for silencing activity in mouse ES cells and MEFs. Fold repression was calculated relative to the control EGFP vector at the same multiplicity of infection (MOI). Bar plots indicate the mean of four to seven biological replicates. Error bars indicate the standard deviation.

C Identification of the 160-bp SHIN sequence. Sub-fragments of the GAG coding region were tested for silencing activity as in (B). The smallest fragment which was still able to induce full repression was designated SHIN. Bar plots indicate the mean of two to six biological replicates. Error bars indicate the standard deviation.

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endogenous IAP sequences were only mildly affected, indicating that this modification may have a low turnover on these sequences. Importantly, H3K9me3 was clearly reduced on a unique Setdb1 target region (pos control) and across the RMCE

locus containing the SHIN sequence (Fig 2C). In summary, our data show that the SHIN sequence represents an initiation site for heterochromatin formation from which H3K9me3 can spread over the entire locus.

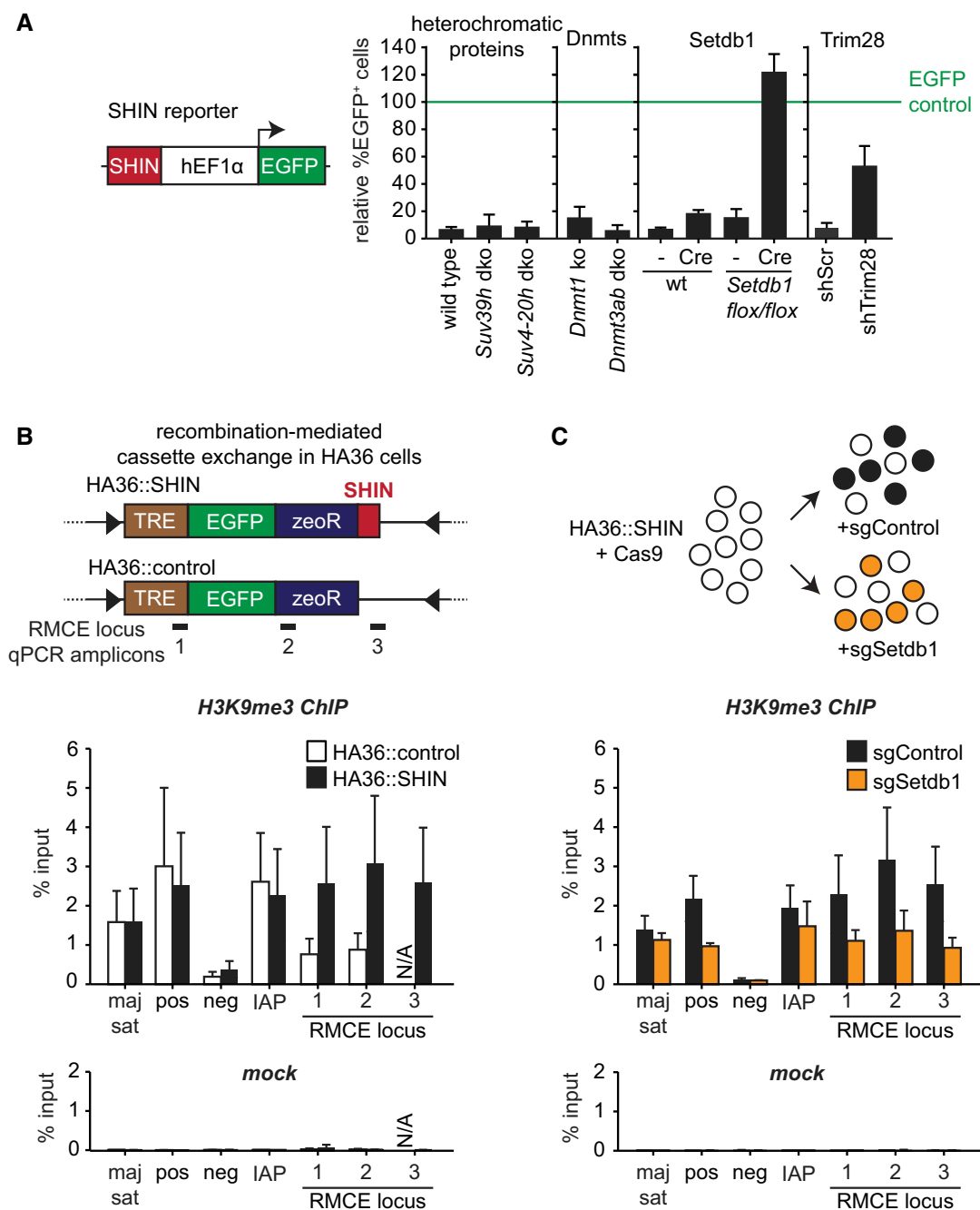


Figure 2. The SHIN sequence induces Trim28/Setdb1-dependent heterochromatin formation.

- A** SHIN silencing depends on the Trim28/Setdb1 pathway. Mouse ES cells were transduced with the SHIN reporter or control vector, and EGFP fluorescence was monitored 2 days later by FACS. The percentage of EGFP-positive cells after SHIN reporter transduction is shown relative to the control transduction (relative % EGFP⁺ cells). Setdb1 knockout cells were generated by the transduction of Setdb1^{flox/flox} ES cells with Cre-expressing virus (Cre). Trim28 knockdown was performed by the transduction of a lentiviral shRNA vector (shTrim28 #1). Bar plots indicate the mean of three to six biological replicates. Error bars indicate the standard deviation.
- B** The SHIN sequence recruits H3K9me3. Recombination-mediated cassette exchange of the indicated SHIN reporter and control vector resulted in ES cell lines HA36::SHIN and HA36::control, respectively. ChIP-qPCR analysis of H3K9me3 across the RMCE locus in HA36::SHIN and HA36::control cells. RMCE PCR amplicons (1–3) are indicated. Control regions: maj sat, major satellite repeats; pos, Polrmt; neg, Tia1; and IAP, endogenous IAP elements. N/A = sequence not present in the HA36::control construct. Bar plots indicate the mean of three biological replicates. Error bars indicate the standard deviation.
- C** Setdb1 mediates SHIN-induced H3K9me3. HA36::SHIN cells were stably transduced with a Cas9 expression vector and then transduced with sgRNAs against Setdb1 or a control sequence, respectively. ChIP-qPCR for H3K9me3 was performed 4 days after sgRNA transduction. Since we could not select for Setdb1 knockout, the chromatin isolate for ChIP is composed of deleted and non-deleted cells. RMCE PCR amplicons (1–3) are indicated. Control regions: maj sat, major satellite repeats; pos, Polrmt; neg, Tia1; and IAP, endogenous IAP elements. Bar plots indicate the mean of three biological replicates. Error bars indicate the standard deviation.

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SHIN silencing requires the Snf2-type chromatin remodeler Atrx

Heterochromatin formation across SHIN strictly depends on the Trim28/Setdb1 pathway and recapitulates silencing of endogenous IAP retrotransposons. Thus, we utilized our reporter assay to screen for additional factors that regulate retrotransposon silencing using a genome-wide shRNA screen (Fig 3A). We transduced wild-type ES cells with a pooled genome-wide shRNA library, selected shRNA expressing cells with puromycin and then transduced the SHIN reporter. Cells that escaped SHIN silencing were isolated based on their EGFP fluorescence, and shRNA sequences from these escaper cells were cloned into a plasmid library (Fig 3A). We tested 71 individual shRNA plasmids in a secondary screen. One shRNA which most strongly inhibited SHIN repression was found to target the SNF2-type chromatin remodeler Atrx (Fig 3B, Supplementary Table S2). This shRNA clearly reduces Atrx mRNA and protein levels in mouse ES cells (Supplementary Fig S2A and B). However, to rule out off-target effects, we generated four independent knockout ES cell lines (Supplementary Fig S2C) with loss of Atrx protein (Fig 3C). *Atrx* knockout (ko) cells grow normally and show no obvious alterations in their cell cycle profile (Supplementary Fig S2D). Importantly, SHIN silencing was defective in all *Atrx* knockout ES cell lines (Fig 3D), which confirmed the shRNA screening result.

We then asked if Atrx is important for the Trim28/Setdb1 pathway and performed SHIN silencing assays in wild-type versus *Atrx* ko cells, depleted for Setdb1 and Trim28, respectively (Supplementary Fig S2E and F). Although Setdb1 could be depleted by around 70% (Supplementary Fig S2E), SHIN silencing was only mildly impaired (Fig 3E). Setdb1 depletion in *Atrx* ko cells, in contrast, resulted in strong silencing defects (Fig 3E). This suggests that Atrx enhances the efficiency of Setdb1-dependent repression, although we cannot exclude the possibility that Atrx may act independently of Setdb1 in repressing the SHIN reporter, leading to synergistic effects when both Setdb1 and Atrx are depleted. Similar effects were observed for shRNA knockdown of Trim28 (Fig 3E).

To test if Atrx is required for Trim28/Setdb1-dependent repression outside of IAP retrotransposons, we performed silencing assays using a heterologous reporter system. The well-established repressor binding site of MLV retrotransposons (RBS) is recognized by the KRAB zinc finger protein Zip809, which in turn recruits Trim28 and Setdb1 to induce silencing in a variety of cell types. In wild-type cells, the RBS-EGFP reporter was efficiently silenced (Fig 3F, Supplementary Fig S2G). However, RBS silencing was compromised upon knockdown of Atrx in MEFs and in *Atrx* ko ES cells (Fig 3F, Supplementary Fig S2G). Taken together, our data demonstrate Atrx as a crucial component of the Setdb1/Trim28 silencing pathway.

SHIN silencing is independent of Daxx-mediated H3.3 deposition

On telomeres and pericentric heterochromatin, Atrx recruits Daxx to mediate H3.3 deposition [28–30]. In order to test if the Daxx/H3.3 pathway plays a role in SHIN repression, we generated *Daxx* ko cells (Supplementary Fig S3A). We found that SHIN silencing is defective in *Daxx* ko cells (Supplementary Fig S3B), suggesting that Daxx is an important component for retrotransposon silencing. To address the question whether H3.3 deposition by Daxx is critical for SHIN silencing, we generated rescue cell lines expressing wild-type and mutant Daxx proteins (Supplementary Fig S3C). Re-expression

of full-length Daxx protein in *Daxx* ko cells leads to a rescue in SHIN silencing (Supplementary Fig S3D). However, the expression of Daxx-mutant proteins with relaxed binding specificity for histone H3.3 (E231A) or impaired H3.3 interaction (R257A) also resulted in rescued SHIN silencing (Supplementary Fig S3D and E). In contrast, the expression of Daxx with a deletion of the C-terminal repressor domain [42] does not rescue SHIN silencing (Supplementary Fig S3D). These data point toward a H3.3-independent function of Daxx in SHIN repression. To confirm that H3.3 is really dispensable for retrotransposon silencing, we generated *H3.3* ko ES cell lines (Supplementary Fig S4A). Histone H3.3 is mainly transcribed from two loci in the mouse genome, H3f3a and H3f3b. Consequently, H3f3a/H3f3b double knockout cells have essentially lost H3.3 protein (Supplementary Fig S4B). Notably, SHIN silencing is completely intact in *H3.3* ko cells (Supplementary Fig S4C). Due to H3f3a duplications in the mouse genome that could eventually give rise to functional H3.3 transcripts (see blat search and alignment report in Supplementary Table S3), we performed H3f3a knockdown in H3f3b ko cells. These cells have lost H3.3 (Supplementary Fig S4D) but display robust SHIN silencing (Supplementary Fig S4E). Finally, we re-analyzed published H3.3 ChIP-seq datasets in control and *Atrx* ko ES cells [30]. We found only low enrichment of H3.3 in control ES cells (Supplementary Fig S4F). Surprisingly, upon deletion of Atrx, H3.3 becomes strongly enriched at IAP elements (Supplementary Fig S4F), suggesting that Atrx does not promote H3.3 incorporation at endogenous IAP elements, but rather inhibits excessive H3.3 deposition. Alternatively, higher chromatin turnover at IAP elements in the absence of Atrx may indirectly lead to more H3.3 incorporation. Together with our finding that H3.3 interaction mutants of Daxx can repress retrotransposon sequences and H3.3-depleted cells are not impaired in SHIN repression, our data demonstrate that SHIN silencing is independent of Daxx-mediated histone H3.3 incorporation.

Atrx is required for efficient heterochromatin formation

Next, we sought to investigate how Atrx mediates SHIN silencing on the molecular level. One possibility was that Atrx could mediate efficient heterochromatin formation across the SHIN locus. To test this hypothesis, we performed a re-silencing assay in HA36::SHIN ES cells in which we depleted Atrx by shRNA-mediated knockdown (Fig 4A). Strong induction of the Tet-inducible promoter using doxycycline alleviates SHIN silencing in a small population of cells, and constant selection pressure with zeocin allowed us to isolate a homogeneous population of cells with strong EGFP expression. Lack of promoter stimulation by doxycycline removal resulted in the rapid loss of EGFP expression within 4 days in both control (shScr) and Atrx-depleted (shAtrx) cells (Fig 4B, left panel). Loss of EGFP expression coincided with presence of H3K9me3 across the RMCE locus in shScr cells (Fig 4C, left panel, Supplementary Fig S5A). H3K9me3 could also be established in shAtrx cells; however, abundance of H3K9me3 appeared reduced in the test region 1 most distal to the SHIN sequence (Fig 4C, left panel), indicating reduced heterochromatin spreading. Surprisingly, re-silencing was very efficient in shScr cells despite continuous doxycycline induction (Fig 4B, right panel, Supplementary Fig S5A). A large proportion of these cells displayed strongly reduced EGFP expression and the establishment of H3K9me3 across the RMCE locus (Fig 4B and C, right panels).

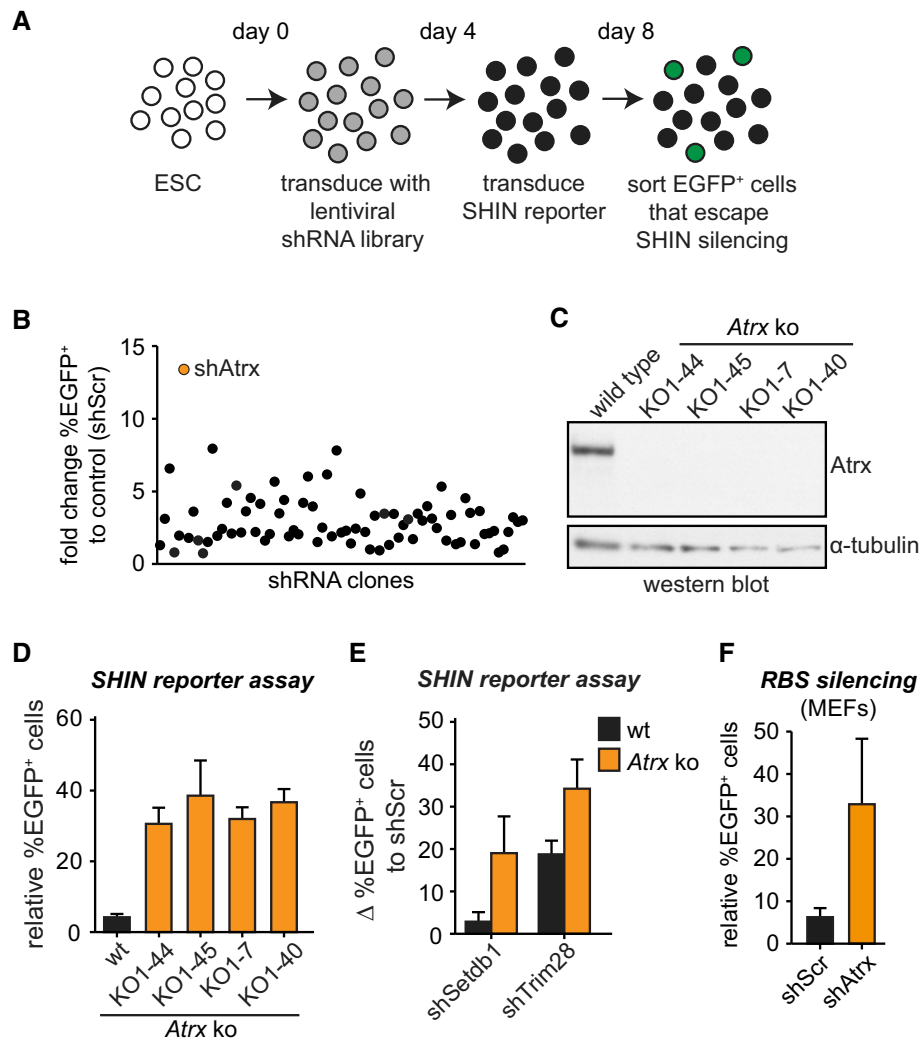


Figure 3. A shRNA screen identifies the chromatin remodeler Atrx as regulator of retrotransposon silencing.

- A Scheme of the shRNA screen. ES cells were transduced with a genome-wide pooled shRNA library at a low MOI and then transduced with the SHIN reporter. Cells that escaped SHIN silencing were isolated by FACS sorting based on their high EGFP fluorescence. shRNA sequences of the EGFP⁺ cells were amplified by PCR and cloned into a lentiviral vector.
- B SHIN reporter assay in ES cells that were transduced with 71 random shRNA clones from the primary screen and scrambled control shRNAs. Dot plot displays the ratio of EGFP⁺ cells in test shRNA versus control shRNA transductions. The test shRNA with strongest reduction in SHIN silencing was directed against Atrx (yellow dot). The top-scoring shRNAs of the secondary screen are listed in Supplementary Table S2.
- C Western blot for Atrx in wild-type and four independent Atrx ko cell lines. Tubulin serves as a loading control.
- D SHIN reporter silencing depends on Atrx. Wild-type and four independent Atrx ko ES cell lines were transduced with the SHIN reporter or control vector, and EGFP fluorescence was monitored 2 days later by FACS. The percentage of EGFP-positive cells after SHIN reporter transduction is shown relative to the control transduction (relative % EGFP⁺ cells). The bar plot indicates the mean of three biological replicates. Error bars indicate the standard deviation.
- E Atrx ko cells are sensitive to perturbations in the Setdb1/Trim28 silencing pathway. SHIN reporter silencing in wild-type and Atrx ko ES cells that were transduced with control (shScr), Setdb1, and Trim28 shRNAs. Bar diagram shows the difference in EGFP-positive cells upon knockdown of Setdb1 and Trim28 compared to control-treated cells. Bar plots indicate the mean of three to six biological replicates. Error bars indicate the standard deviation.
- F Atrx is generally important for Setdb1/Trim28-dependent silencing. Mouse embryonic fibroblasts were transduced with control and Atrx shRNAs. Silencing of an EGFP reporter construct containing the repressor binding site of the MLV retrovirus was monitored. The percentage of EGFP-positive cells after reporter transduction is shown relative to the control transduction (relative % EGFP⁺ cells). The bar plot indicates the mean of three biological replicates. Error bars indicate the standard deviation.

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In contrast, shAtrx cells are completely unable to induce heterochromatin formation across the RMCE locus in continuous presence of doxycycline (Fig 4B and C, right panels). In summary, these data indicate that Atrx is necessary for efficient establishment of

heterochromatin. On transcriptionally inactive loci, heterochromatin can be formed, but spreading appears reduced. In addition, Atrx is strictly required when heterochromatin formation is challenged by transcriptional activity.

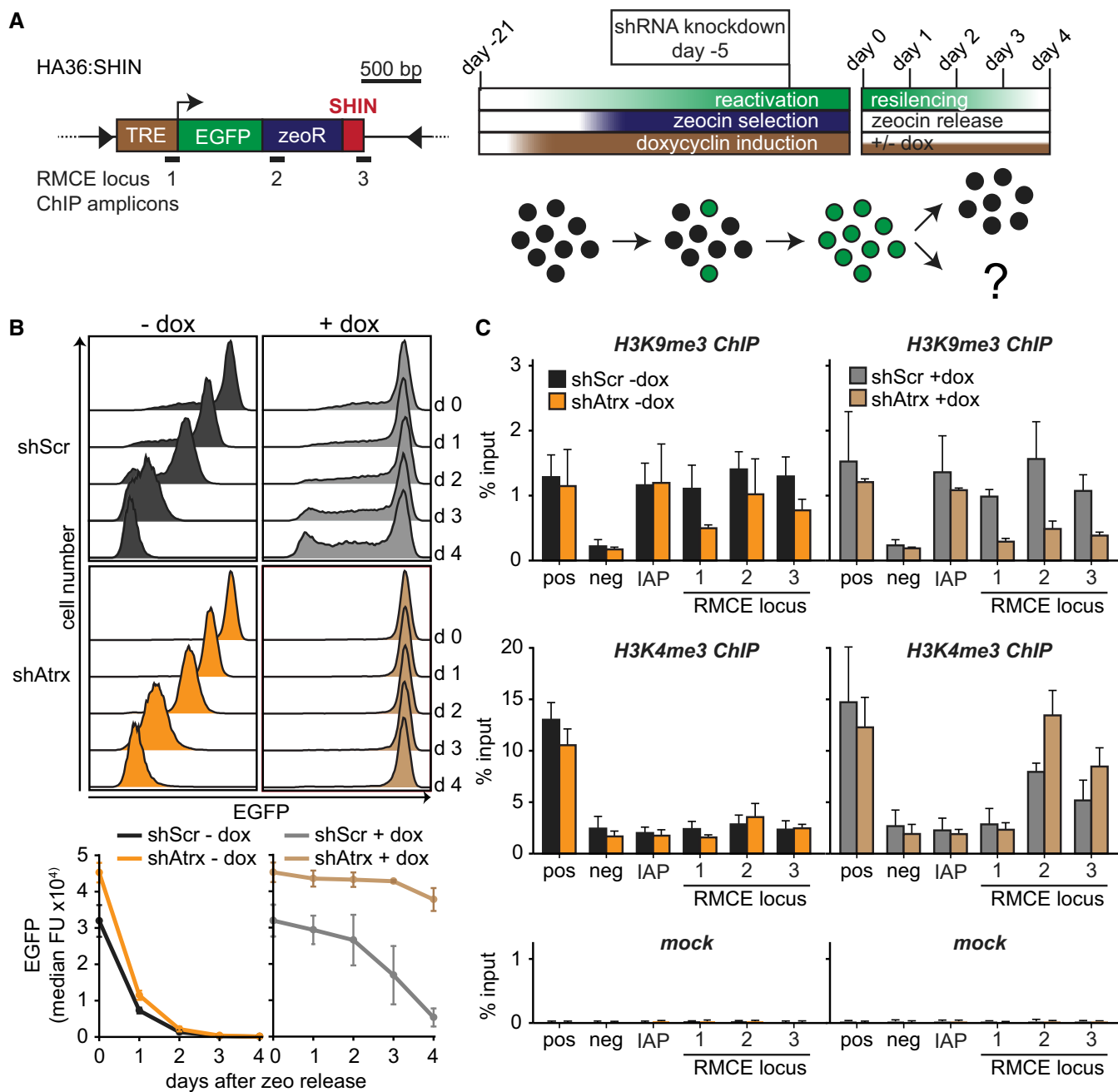


Figure 4. Atrx is required for efficient heterochromatin formation.

A Scheme of the re-silencing assay in HA36::SHIN cells. The silenced transgene in HA36::SHIN cells was reactivated by the addition of doxycycline which activates the Tet-responsive promoter (TRE). Cells in which the reporter was immediately activated (approx. 2–3%) were selected with zeocin. The EGFP⁺ cells were then transduced with control or Atrx shRNAs. Five days later, re-silencing was triggered by the removal of the zeocin selection pressure (day 0), and EGFP expression was monitored in the presence or absence of doxycycline during the next 4 days (day 1–day 4).

B Atrx is necessary for efficient silencing in competition with transcriptional activity. Re-silencing experiments as outlined in (A) were performed. Top panel: histograms displaying EGFP fluorescence during the course of re-silencing. Removal of doxycycline (–dox) inactivates the TRE promoter, resulting in loss of EGFP expression. Despite maintained TRE promoter stimulation (+dox), a large number of control cells (shScr) can silence the reporter, while Atrx-depleted cells (shAtrx) are unable to induce silencing. Lower panel: Line plots indicating the average of the median EGFP fluorescence in three to four biological replicates. Error bars indicate the standard deviation.

C Atrx knockdown leads to impaired heterochromatin establishment. ChIP-qPCR analysis of H3K9me3 and H3K4me3 across the RMCE locus in HA36::SHIN cells at day 4 of the re-silencing assays as outlined in (A). Positions of the primer pairs at the RMCE locus are indicated in (A). IAP, endogenous IAP elements. H3K9me3 ChIP: pos, Polrmt; neg, Tia1; IAP, global endogenous IAPs. H3K4me3 ChIP: pos, Tia1; neg, Polrmt; IAP, global endogenous IAPs. Mock (beads only control): pos, Polrmt; neg, Tia1. Bar plots indicate the mean of three biological replicates. Error bars indicate the standard deviation.

Source data are available online for this figure.

Atrx is important for heterochromatin maintenance

Thus far, our data showed that *Atrx* is necessary for efficient *de novo* establishment of heterochromatin. However, in embryonic stem cells endogenous IAP elements are already covered by a heterochromatic structure which needs to be maintained. We thus wondered if *Atrx* is important for heterochromatin maintenance. When we analyzed published ChIP-seq datasets for *Atrx*, *Setdb1*, *Trim28*, and *H3K9me3* in ES cells [43–45], we found strong enrichment of *Atrx* across IAP retrotransposons with highest occupancy over the SHIN region (Fig 5A). These data suggest that *Atrx* has a permanent role on these elements. ChIP-qPCR analyses for *Atrx* could confirm the enrichment on IAP elements and a non-repetitive control region (Fig 5B, top panel). However, heterochromatin marks, such as *H3K9me3* and DNA methylation, were not altered in *Atrx* ko cells (Fig 5B, Supplementary Fig S5B and C).

The strong enrichment of *Atrx*, *H3K9me3*, *Trim28*, and *Setdb1* on IAP sequences close to the SHIN region (Fig 5A) suggests that *Atrx* is recruited by *H3K9me3* and/or *Trim28/Setdb1*. Thus, we tested if *Atrx* is *de novo* recruited to a *Setdb1*-dependent heterochromatin locus by analyzing HA36::SHIN cells. Enrichment of *Atrx* at the SHIN region within the reporter locus demonstrates that the recruitment of *Atrx* occurs in the context of *Setdb1* and *Trim28* (Fig 5C). Thus, we investigated whether *Atrx* generally associates with *Setdb1/Trim28* target region in repetitive and non-repetitive regions of the genome. The overlap between *Atrx*, *Trim28*, and *Setdb1* in non-repetitive regions is relatively small (Supplementary Fig S7A). Prominent enrichment was mainly observed on imprinted genes and few intragenic loci (Supplementary Fig S7B–D, Supplementary Tables S5 and S6). In contrast, our analyses revealed that *Atrx* co-occupies many ERV classes which are also enriched for *Setdb1*, *Trim28*, and *H3K9me3*, for example, *IAP-Ez*, *MusD/ETn*, and *MMERVK10* elements (Fig 5D, Supplementary Fig S6, Supplementary Table S4). Thus, we sought to investigate if some of these ERV sequences become de-repressed in *Atrx* ko cells. Out of the three tested ERV classes with the enrichment of *Setdb1/Trim28/Atrx*, we detected the de-repression of *MusD/ETn* retrotransposons (Fig 5E), suggesting that *Atrx* function is rate limiting for the silencing of distinct ERV classes.

Challenges like DNA replication or transcription through intronic IAP repeats require heterochromatin to be constantly re-established. Based on our finding that *Atrx* is crucial for efficient establishment of heterochromatin, we wondered if heterochromatin maintenance is perturbed in *Atrx* ko cells when the *Setdb1/Trim28* pathway has reduced activity. Moderate knockdown of *Trim28* in wild-type cells resulted in slight up-regulation of endogenous IAP elements, demonstrating that heterochromatin maintenance is not fully ensured. However, when we performed *Trim28* knockdown in *Atrx* ko cells, we observed severe derepression of IAP retrotransposons (Fig 5F). These findings indicate that *Atrx* is required and rate limiting for the repression of endogenous IAP elements when heterochromatin maintenance is challenged.

Another way of challenging heterochromatin is to provoke transcription across a heterochromatinized locus. In HA36::SHIN cells, the EGFP reporter gene is not transcribed and covered with *H3K9me3* (Fig 2C). However, transcription can be induced by doxycycline that allows the recruitment of the reverse tetracycline transactivator (rtTA) to the Tet-responsive promoter of the reporter gene. Due to the heterochromatic state of the reporter gene in control cells (shScr + dox), doxycycline induction leads to a very low percentage of EGFP-positive cells (Fig 5G, Supplementary Fig S5D). In contrast, doxycycline induction upon *Atrx* knockdown (sh*Atrx* + dox) results in a much higher percentage of EGFP-expressing cells (Fig 5G, Supplementary Fig S5D). These data demonstrate that heterochromatin is more vulnerable in *Atrx* ko cells.

Atrx regulates heterochromatin accessibility

Heterochromatin is generally characterized by a high compaction grade which makes it largely inaccessible to transcriptional activators and refractory to challenges. Our finding that transcription of a heterochromatic locus can be more easily stimulated in the absence of *Atrx* suggests that structural properties of heterochromatin like local nucleosome density or higher order folding are compromised when *Atrx* is lost. To test this hypothesis, we performed MNase accessibility assays in wild-type versus *Atrx* ko ES cells. *Atrx* is only binding to a small fraction of the mouse genome, and thus, we did not detect global alterations in MNase accessibility [43] (Fig 6A and B). To investigate *Atrx*

Figure 5. Atrx is important for heterochromatin maintenance.

- Atrx* binds to IAP retrotransposons. Cumulative ChIP-seq coverage profiles across IAP elements for *Atrx*, *Setdb1*, *Trim28*, and *H3K9me3*. The structure of IAP elements is shown schematically; the position of the SHIN sequence is marked in dark gray. rpkm, reads per kilobase per million of reads.
- H3K9me3* is not altered in *Atrx* ko cells. ChIP-qPCR analysis for *Atrx* and *H3K9me3* in wild-type (wt) and *Atrx* ko ES cells. pos, Polrmt; neg, Tia1; IAP, global endogenous IAPs; and IAP SHIN, SHIN sequence of IAP elements. Bar plots indicate the mean of three biological replicates. Error bars indicate the standard error of the mean.
- Atrx* is recruited to newly formed heterochromatic sites. *Atrx* ChIP was performed in HA36::SHIN cells, in which the SHIN reporter locus was newly integrated into a defined locus by RMCE (see Fig 2B). Bar plots indicate the mean of three biological replicates. Error bars indicate the standard deviation.
- Setdb1*, *Atrx*, *H3K9me3*, and *Trim28* co-occupy distinct ERV elements. Binary heatmap showing ChIP-seq enrichment over input (> 1.5-fold) on all mouse ERV repeat classes. Selected ERV classes are indicated. Complete information is provided in Supplementary Fig S6 and Supplementary Table S4.
- MusD/ETn* retrotransposons are de-repressed in *Atrx* ko cells. RT-qPCR analysis of selected retrotransposon classes in wild-type (wt) and *Atrx* ko ES cells. Bar plots indicate the mean of three to eleven biological replicates. Error bars indicate the standard deviation.
- Heterochromatin maintenance is compromised in *Atrx* ko ES cells when core heterochromatin proteins are depleted. Wild-type and *Atrx* ko ES cells were transduced with control or *Trim28* shRNAs, and the expression of endogenous IAP elements was measured by qRT-PCR. Bar plots indicate the mean of three biological replicates. Error bars indicate the standard deviation.
- Heterochromatin is more vulnerable in *Atrx* ko cells. Addition of doxycycline to HA36::SHIN cells results in binding of the reverse Tet transactivator (rtTA) to the Tet-responsive promoter (TRE) in the SHIN reporter locus. Percentage of EGFP-expressing cells was monitored before and after the addition of doxycycline. Bar plots indicate the mean of three biological replicates. Error bars indicate the standard deviation.

Source data are available online for this figure.

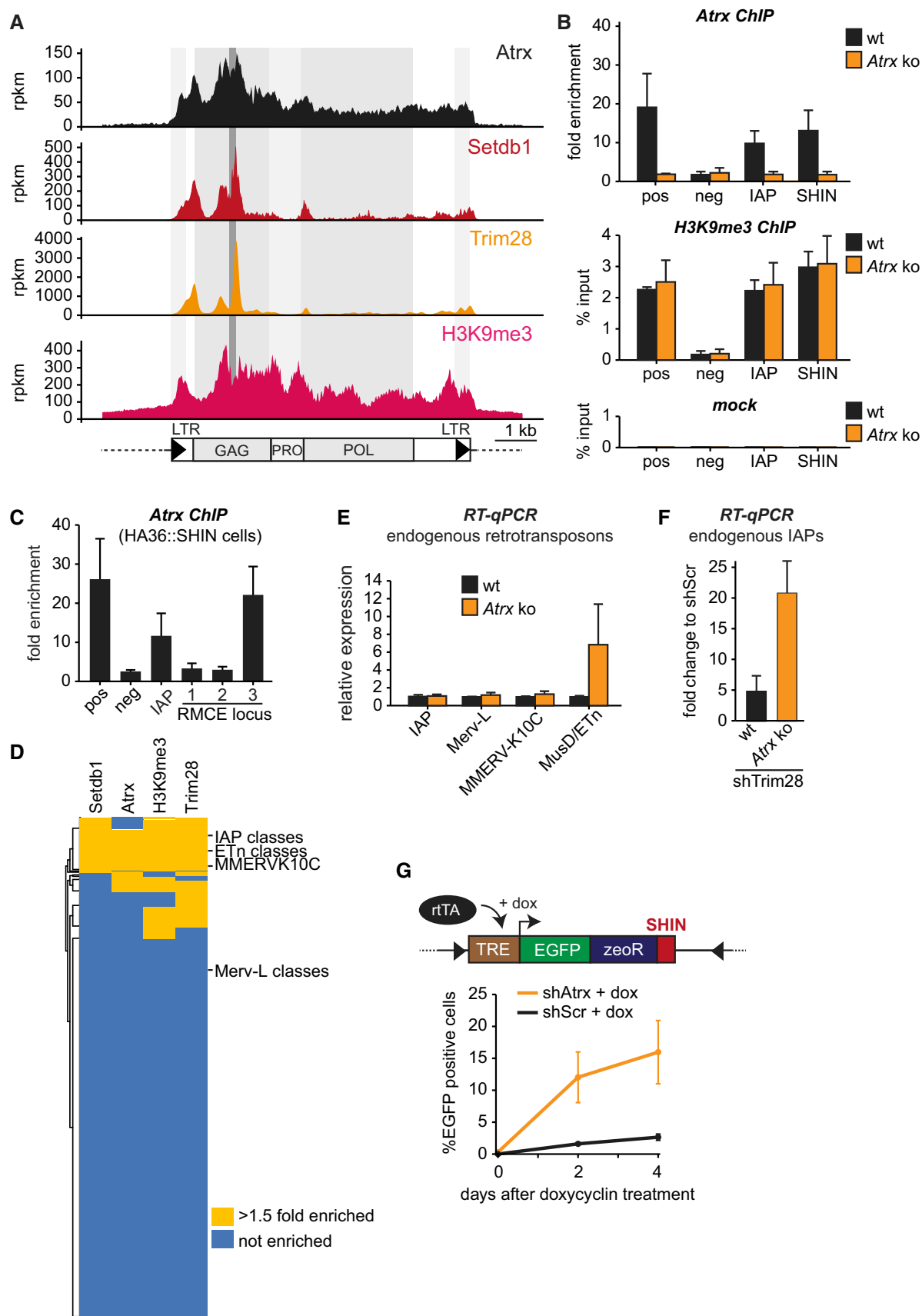


Figure 5.

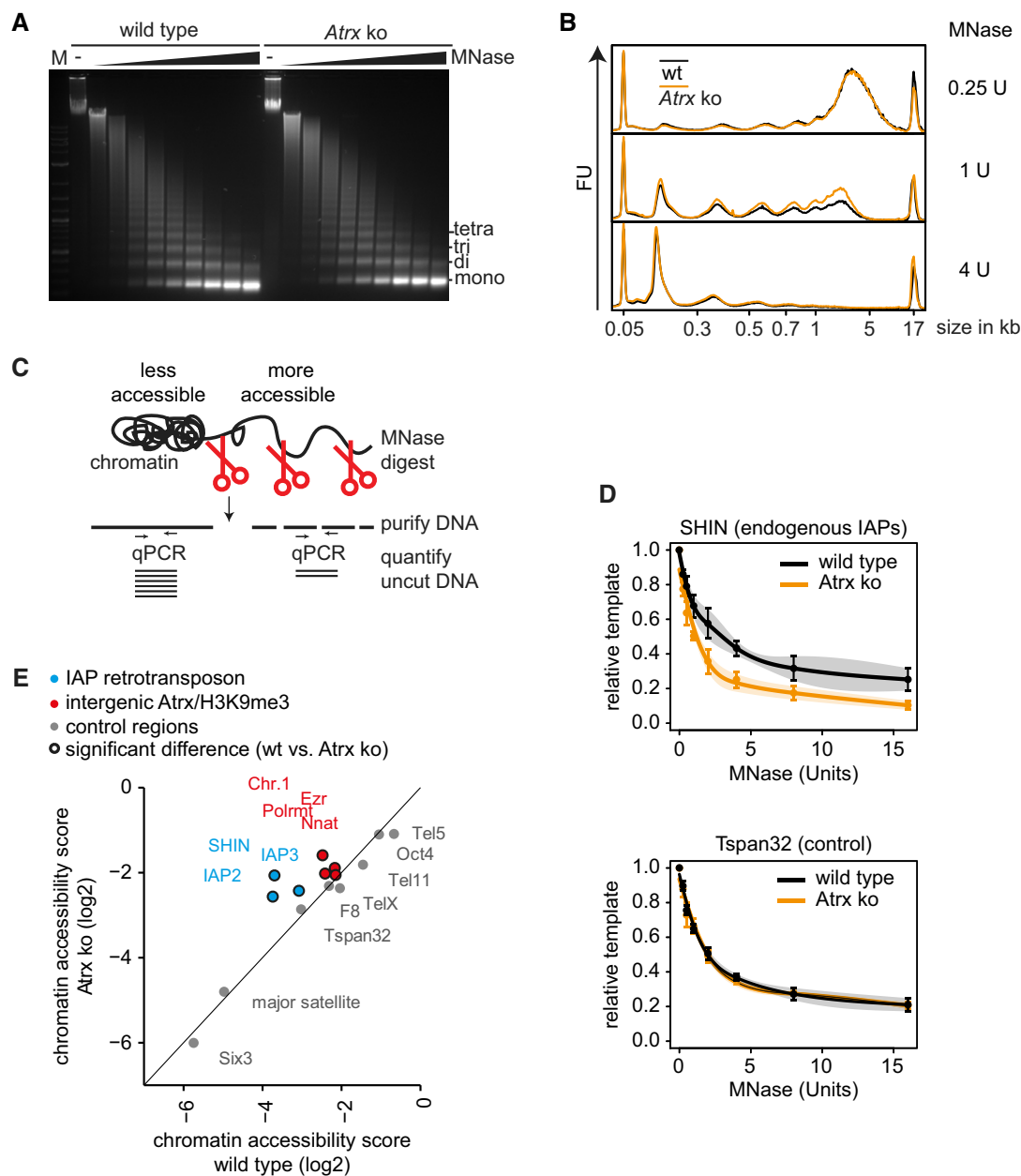


Figure 6. Atrx regulates heterochromatin compaction.

A Chromatin accessibility is not globally changed in *Atrx* ko cells. Nuclei of wild-type and *Atrx* ko ES cells were digested with increasing amounts of MNase (0–16 U), and DNA was analyzed on an agarose gel stained with ethidium bromide. M, size marker; “–”, no MNase; size of mono-, di-, tri-, and tetra-nucleosomes is indicated.

B Overlay of DNA electropherograms obtained from Bioanalyzer runs of the MNase-digested DNA from wild-type and *Atrx* ko ES cells. The first and the last sharp peak represent the markers of the Agilent DNA 12000 kit.

C Schematic of the locus-specific chromatin accessibility assay. MNase cuts open chromatin faster than more compact, less accessible regions. The amount of uncut DNA of a specific locus thus correlates with accessibility of this region and can be measured by qPCR.

D Heterochromatin on IAP elements is less compact in *Atrx* ko cells. Locus-specific chromatin accessibility assays were performed in wild-type and *Atrx* ko ES cells using a range of MNase concentrations. Curves represent an example of a smoothing fit to the data points. Shaded areas demarcate a confidence interval based on $\pm$ one functional standard deviation.

E Generally increased heterochromatin accessibility in *Atrx* ko cells. Locus-specific chromatin accessibility assays were performed in wild-type and *Atrx* ko ES cells. Curve fitting to the data points resulted in a chromatin accessibility score which positively correlates with chromatin accessibility (see Supplementary Materials and Methods). The average chromatin accessibility score for indicated regions in wild-type and *Atrx* ko cells was plotted as dots from three biological replicates. The entire calculated curve fits from three biological replicates were used to assess statistically significant differences in digestion behavior (shown as dots with black border). Since the chromatin accessibility score only reflects the slope of the curve fit at 50% digestion degree, this score does not directly correlate with statistically significant differences in overall digestion behavior (also see Supplementary Materials and Methods).

Source data are available online for this figure.

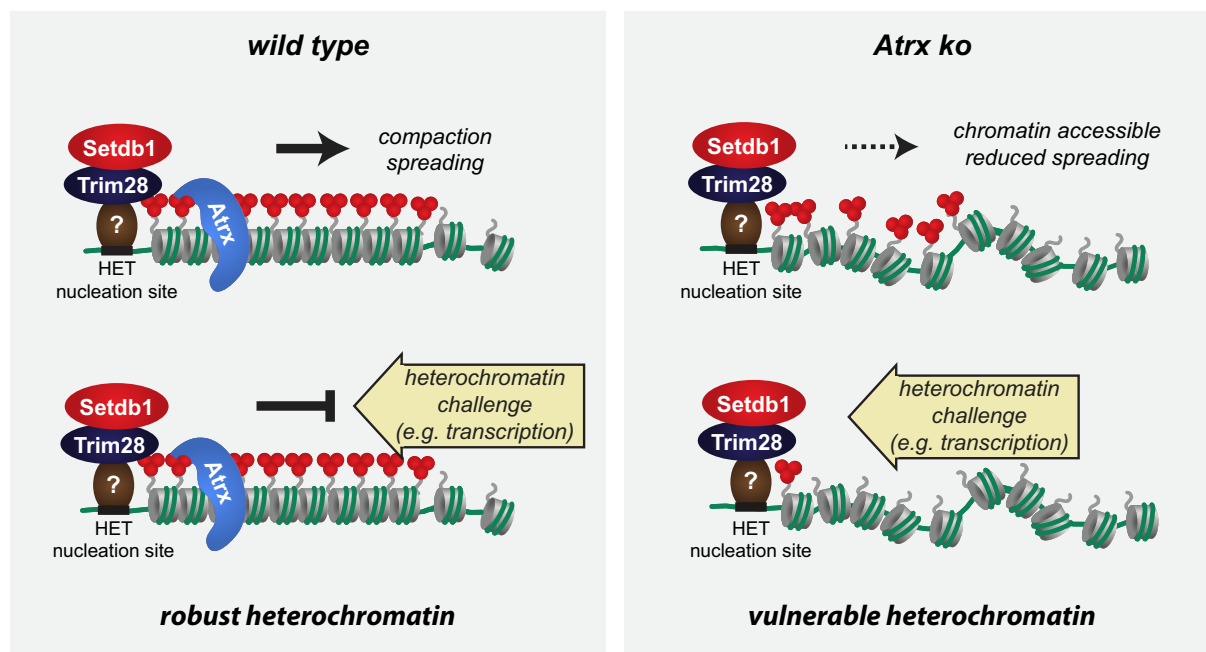


Figure 7. Atrx is important for the establishment and robust maintenance of heterochromatin.

Heterochromatin formation is thought to initiate on specific nucleation sites from which it can spread over large distances. Proteins that recognize these initiation sites and recruit Trim28/Setdb1 in the context of retrotransposons are currently unknown. Atrx is then necessary for the establishment and spreading of heterochromatin and mediates heterochromatin compaction. Thus, Atrx-deficient cells are characterized by more vulnerable heterochromatin which cannot easily be maintained upon challenges, such as transcription.

target regions, we employed a qPCR strategy to measure the MNase digestion degree on specific genomic loci with increasing MNase concentrations (Fig 6C). Curve fitting through these data points allows statistical assessment of the digestion degree between wild-type and Atrx-mutant cells (Supplementary Fig S7E). Further, we derive a chromatin accessibility score which represents the digestion rate at which 50% of the target locus is digested (Supplementary Fig S7E). MNase accessibility at endogenous IAP retrotransposons is significantly increased, whereas control regions, such as promoters of active and inactive genes, show no alterations (Fig 6D and E, Supplementary Fig S7F). Interestingly, chromatin accessibility was also increased on non-repetitive Setdb1/Trim28 targets (Fig 6E, Supplementary Fig S7E). These data demonstrate that Atrx renders Setdb1-dependent heterochromatin more inaccessible.

Discussion

In the mammalian genome, heterochromatin is very abundant across repetitive elements. Pericentric heterochromatin is mainly organized by the Suv39h-HP1-Suv4-20h pathway [46–48], while repetitive elements outside the pericentric compartment are controlled by the Trim28/Setdb1 pathway, with support by Suv39h enzymes [6,10,39,49]. A major characteristic of the heterochromatic state is its modification pattern of H3K9me3/H4K20me3/DNA methylation and the buildup of an inaccessible chromatin structure which does not allow strong transcriptional activity. Heterochromatin formation is thought to initiate on specific nucleation sites from which it can spread over large distances [50]. Initiation sites in

pericentric heterochromatin may be binding sites for specific transcription factors [51] or involve other recruitment mechanisms for Suv39h/HP1 complexes [52]. In the context of retrotransposons, recruitment mechanisms may involve KRAB zinc finger proteins which could serve as targeting platform for Trim28/Setdb1 [9]. Our work identified a novel nucleation site in IAP retrotransposons (SHIN) that is sufficient to induce heterochromatin and transcriptional repression of strong promoters (Fig 7). SHIN silencing requires the Trim28/Setdb1 pathway for heterochromatin formation across an integrated reporter gene (Fig 2C). Cumulative coverage maps across IAP elements revealed strong enrichment of Setdb1 and Trim28 across the SHIN sequence; however, additional coverage was detected at the LTR/UTR region and in the beginning of the POL coding sequence (Fig 5A). These findings suggest that endogenous IAP elements may feature several independent nucleation sites for heterochromatin formation. In support of this hypothesis, previous analyses of the LTR/UTR sequence revealed significant silencing potential [10], although to a lower extent as compared to the SHIN sequence (Fig 1). Which proteins recognize the SHIN sequence is currently unclear. As silencing can only be initiated in ES cells, KRAB zinc finger proteins with selective expression in ES cells are likely candidates. As we could not further shorten the SHIN sequence to less than 160 bp without compromising silencing potential, it is quite likely that multiple DNA binding proteins are involved in Trim28/Setdb1 recruitment.

Identification of the SHIN sequence as a strong heterochromatin nucleation site allowed a pilot screen for regulators of heterochromatin establishment through which we identified the chromatin remodeler Atrx. Atrx co-localizes with Trim28/Setdb1 and

H3K9me3 on IAP elements, other ERV classes, and several single copy loci (Fig 5A, Supplementary Fig S7A–D). We found that *Atrx* is *de novo* recruited to Setdb1-dependent heterochromatin (Fig 5C), suggesting that *Atrx* could be directly recruited by Setdb1 or Trim28. However, as *Atrx* features domains which bind H3K9me2/3 and HP1 [26,27], a complex combinatorial nature of interactions leading to *Atrx* recruitment is likely. This may be further complicated by potential interactions with non-coding RNAs [53] that may affect heterochromatin formation on IAP elements [54].

We found that in the absence of *Atrx* reporter silencing is delayed and spreading of heterochromatin is reduced (Fig 4), which could be due to reduced activity of Setdb1 in establishing H3K9me3. Being a putative chromatin remodeler, we hypothesize that *Atrx* assists in generating a proper nucleosomal array which can then serve as efficient substrate for Setdb1. Consistent with this hypothesis, we found that reduced Setdb1 activity leads to defects in the establishment of silencing when *Atrx* is not present (Fig 3E). Secondly, heterochromatin spreading and stability rely on the formation of properly spaced nucleosomal arrays by HP1 molecules binding to H3K9me3 [55]. *Atrx* activity may be required to establish such arrays, and, as a consequence, loss of *Atrx* would lead to compromised spreading. Alternatively, *Atrx* may also promote heterochromatin spreading by supporting Suv39h activity at retrotransposons [49]. A third function of *Atrx* in heterochromatin establishment may be the recruitment of additional factors to assist in this process. On telomeres, *Atrx* interacts with Daxx to facilitate histone H3.3 deposition [28–30]. We found that H3.3 is dispensable for silencing retrotransposon sequences in reporter assays, suggesting that incorporation of this histone variant is not important for Trim28/Setdb1-dependent heterochromatin formation. Instead, we find that Daxx is important for this process (Supplementary Fig S3). Recently, Daxx was found to be involved in silencing of exogenous ASV retroviruses by H3K9me3 and DNA methylation [56]. Although it is unclear how silencing is triggered in this context and if *Atrx*, Trim28, or Setdb1 are involved in this process, these data suggest Daxx as a global regulator of retrovirus repression. Interestingly, we find that Daxx requires a functional SUMO interaction motif to contribute to Setdb1-/Trim28-dependent repression (Supplementary Fig S3), suggesting that the interaction with sumoylated proteins is important in this context [42]. This is consistent with the repressive activity of Daxx when recruited by sumoylated transcription factors [42]. As Setdb1 recruitment to Trim28 has been shown to be SUMO dependent, and because several heterochromatin proteins, like HP1, are sumoylated [57,58], it is likely that Daxx recruitment might rely on the sumoylation of Trim28 or other factors. More thorough genome-wide screens for retrotransposon silencing are necessary to identify such interaction partners and additional components of this silencing pathway.

Heterochromatin formation is the net result of a dynamic balance between factors that build up heterochromatic structures and processes which act antagonistically [59]. Thus, the efficiency of the heterochromatin buildup machinery is particularly critical in genomic regions with prominent activity of antagonistic processes, for example, transcription. Examples for such regions are retrotransposons which reside in intronic regions of highly expressed genes. Unfortunately, due to the lack of polymorphisms, it is not possible to directly examine heterochromatin formation on these elements. However, we could study heterochromatin establishment in competition with transcriptional activity on the RMCE reporter locus. In wild-type cells,

this reporter locus could be efficiently silenced despite strong promoter activity. *Atrx* knockdown leads to impaired silencing. These data clearly demonstrate that in the absence of *Atrx*, the activity of the Trim28/Setdb1 pathway is strongly reduced. Importantly, the de-repression of MusD/ETn elements in *Atrx* ko cells represents a physiological situation in which the full activity of the Setdb1/Trim28 pathway is necessary for silencing (Fig 5E). The establishment of new heterochromatin domains, for example, in the context of differentiation, may also require *Atrx* for the full activity of the Setdb1/Trim28 pathway. The strong developmental phenotype of *Atrx* ko embryos [60] supports this hypothesis, although more analyses are necessary to identify *Atrx* target loci during developmental transitions.

Heterochromatin is generally characterized by low transcriptional activity. A likely explanation for this property is that binding of transcription factors and RNA polymerase is restricted by the largely inaccessible chromatin structure. We tested chromatin accessibility of different genomic regions in mouse ES cells and found that pericentric heterochromatin is highly inaccessible to MNase. A similarly low accessibility was only observed for a promoter region which is under control of the polycomb system (*Six3*, Fig 6E). IAP retrotransposons display higher chromatin accessibility as compared to pericentric heterochromatin, which may be due to a different organization of these regions. Pericentric heterochromatin is composed of large domains of major satellite repeats which cluster into higher order structures to form so-called chromocenters. As retrotransposons form much smaller domains of only several kilobases, higher order folding of the chromatin structure is probably limited. However, chromatin accessibility of IAP elements is still much lower as compared to active promoters and may protect these regions from various challenges. Thus, it is currently unclear if the observed differences in chromatin accessibility result from changes in large-scale chromatin compaction or from an altered local nucleosomal organization. In this study, we found that chromatin accessibility in *Atrx* ko cells is significantly increased on IAP elements, demonstrating that *Atrx* is important for proper heterochromatin organization. Interestingly, this more accessible heterochromatin architecture is indeed vulnerable to challenges. For example, strong transcriptional activators can more easily access their binding sites and lead to stronger transcriptional activation in *Atrx* ko cells. If *Atrx*-deficient heterochromatin is vulnerable to other challenges, for example, DNA damage, remains to be tested. Interestingly, chromatin architecture is not only altered on IAP retrotransposons. We also tested non-repetitive genomic regions with the enrichment of *Atrx* and H3K9me3 and consistently detected increased chromatin accessibility. Thus, our data provide strong evidence for a general role of *Atrx* in the establishment and robust maintenance of heterochromatin domains.

Materials and Methods

Reporter gene silencing assay

Reporter constructs were stably integrated into cells by lentiviral transduction, and the percentage of EGFP⁺ cells was measured by FACS after 2–4 days. Lentiviral particles were generated using standard protocols, and virus titers were determined by titration in HeLa cells. Mouse ES cells were transduced on gelatinized multi-well dishes using spinoculation at a low multiplicity of

infection to ensure a linear relationship between virus titer and transduction rate. The ratio of the percentage of EGFP⁺ cells generated by the reporter relative to the percentage of EGFP⁺ cells generated by a control EGFP vector of the same virus titer was used to quantify reporter silencing (relative % EGFP⁺ cells). In Fig 1 and Supplementary Fig S1, the reciprocal ratio (fold repression) is plotted.

shRNA screen

Feeder-independent ES cells were transduced with a genome-wide pooled shRNA library (Lentiplex, Sigma-Aldrich) at a low MOI, selected for shRNA expression using 0.5 µg/ml puromycin, and then transduced with the SHIN reporter. Cells that escaped SHIN silencing were isolated by FACS sorting based on their high EGFP fluorescence. shRNA sequences of the EGFP⁺ cells were amplified by PCR and cloned into the pLKO1 backbone. For validation of hits, 71 random shRNA sequences of the primary screen were separately transduced into ES cells and the SHIN reporter assay was performed as indicated above. The number of EGFP-positive cells was compared to a non-silencing scrambled control. Details are given in the Supplementary Materials and Methods.

Resilencing assay

T86 cells (HA36::SHIN cells harboring a reverse Tet transactivator) were treated with 2 µg/ml doxycycline for 2 days, and the re-expression of the silenced reporter locus was selected with 50 µg/ml zeocin and 2 µg/ml doxycycline for at least 2 weeks. Reactivated reporter cells were then transduced with a lentiviral shRNA targeting either Atrx or a scrambled control sequence, and knockdown cells were selected by the addition of 1 µg/ml puromycin 2 days after transduction. After additional 3 days, zeocin selection was released and resilencing of the EGFP reporter was monitored daily in the presence or absence of doxycycline by FACS. The median EGFP fluorescence was calculated using FlowJo™ (TreeStar). For ChIP experiments, cells were harvested 4 days after zeocin release. Details are given in the Supplementary Materials and Methods.

Supplementary information for this article is available online: <http://embor.embopress.org>

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Author contributions

GS and DS designed the experimental approach; DS, KS and SG carried out the bench experiments; JE performed the FACS sorting; IK, CF and FJT developed and carried out the statistical analyses; and GS and DS wrote the manuscript.

Conflict of interest

The authors declare that they have no conflict of interest.

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