Epigenetic inheritance is gated by naïve pluripotency and Dppa2

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Abstract

Environmental factors can trigger cellular responses that propagate across mitosis or even generations. Perturbations to the epigenome could underpin such acquired changes, however, the extent and contexts in which modified chromatin states confer heritable memory in mammals is unclear. Here, we exploit a precision epigenetic editing strategy and forced Xist activity to programme de novo heterochromatin domains (epialleles) at endogenous loci and track their inheritance in a developmental model. We find that naïve pluripotent phases systematically erase ectopic domains of heterochromatin via active mechanisms, which likely acts as an intergenerational safeguard against transmission of epialleles. Upon lineage specification, however, acquired chromatin states can be probabilistically inherited under selectively favourable conditions, including propagation of p53 silencing through in vivo development. Using genome-wide CRISPR screening, we identify molecular factors that restrict heritable memory of epialleles in naïve pluripotent cells, and demonstrate that removal of chromatin factor Dppa2 unlocks the potential for epigenetic inheritance uncoupled from DNA sequence. Our study outlines a mechanistic basis for how epigenetic inheritance is constrained in mammals, and reveals genomic and developmental contexts in which heritable memory is feasible.

Keywords heterochromatin; DCas9; epigenetic editing; p53; X-inactivation

Subject Categories Chromatin, Transcription & Genomics; Development; Stem Cells & Regenerative Medicine

DOI 10.15252/embj.2021108677 | Received 8 May 2021 | Revised 12 January 2022 | Accepted 17 January 2022 | Published online 24 February 2022

The EMBO Journal (2022) 41: e108677

Introduction

Cellular identity is maintained by the constellation of trans- and cis-acting factors that regulate gene expression programmes. Among these, epigenetic mechanisms, including histone modifications and DNA methylation, play a key role in establishing and perpetuating transcription states during development (Atiasi & Stunnenberg, 2017; Grosswendt et al., 2020). For example, heterochromatin domains facilitate stable transcriptional silencing, and are characterised by repressive H3K9me3 and DNA methylation or by H3K27me3 (Allshire & Madhani, 2018). Once established, DNA methylation patterns propagate through cell divisions via the maintenance methylase DNMT1, while histone modifications, such as H3K27me3 and H3K9me3, utilise self-reinforcing feedback loops (Smith & Meissner, 2013; Reinberg & Vales, 2018). These entail “read-write” modules that associate with the replication fork to reinstate modification patterns and mutual cross-talk between epigenetic systems, which together are thought to promote stable “epigenetic” inheritance. Nevertheless, chromatin marks are also subject to active reversal mechanisms and imperfect maintenance during replication, and are consequently rendered in a dynamic equilibrium of opposing influences (Stewart-Morgan et al., 2020). Thus, while chromatin states can convey a degree of heritable memory through reinforcing loops, they also exhibit plasticity in response to extrinsic cues.

These dual properties have implicated epigenetic systems as potential mechanisms that underlie genome–environment interactions (Cavalli & Heard, 2019). Indeed, across phylogeny and model organisms, environmental changes can induce specific epigenetic alterations—known as epialleles—that drive major phenotypic responses and adaptations (Seong et al., 2011; Simola et al., 2016; Jiang & Berger, 2017; Yang et al., 2017; Ge et al., 2018; Duempelmann et al., 2019; Torres-Garcia et al., 2020). In mammals, emergent phenotypes have also been linked with chromatin changes as a response to environmental contexts, for example, hypoxia (Batie et al., 2019; Chakraborty et al., 2019) or availability of metabolic intermediates (Haws et al., 2020). Chromatin perturbations more generally are additionally associated with human disease susceptibility (Feinberg, 2018; Panzera & Pospisilik, 2018). Understanding the potential prevalence and heritability of epialleles (or epimutations) in mammals is therefore of great interest.

Early embryogenesis is considered a susceptibility window for induction of epialleles (Cavalli & Heard, 2019; Bertozzi & Ferguson-Smith, 2020). Importantly, if epigenetic perturbations occur during development they have the potential to be inherited throughout adult tissues, possibly influencing disease risk (Walker & Shuk-mei, 2012; Hitchins, 2015). Furthermore, evidence is accruing across model organisms that adverse environments even prior to conception can induce epigenetic perturbations that are intergenerationally

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inherited, and influence offspring phenotype (Carone et al., 2010; Ost et al., 2014; Rechavi et al., 2014; Huypens et al., 2016; Ciabrilli et al., 2017; Klosin et al., 2017). However, in mammals, pre-implantation development entails reprogramming of parentally inherited epigenomes, including rewiring of chromatin and global DNA demethylation (Hackett & Surani, 2013). While this reprogramming is often believed to be directly linked with—or even prerequisite for—emergence of naïve pluripotency, epigenome reprogramming could alternatively function as a barrier to inheritance of acquired or ectopic chromatin states (epialleles) (Kazachkova et al., 2018). In any case, the potential for heritable epialleles in mammals and the underlying mechanisms that enable or antagonise this development are relatively uncharacterised.

The advent of epigenome editing tools has provided a means to programme precise epigenetic perturbations at regulatory loci that can model environmentally induced epialleles. Previous reports have shown that targeted H3K9me3 and DNA methylation, or Polynise this during development are relatively uncharacterised.

To investigate the potential for memory of epigenetic states, we first developed an optimised CRISPR-based epigenetic programming tool. Here, we employed a catalytically dead (d)Cas9 fused with an array of five optimally spaced GCN4 repeats (dCas9 GCN4) (Morita et al., 2016). These serve as docking sites to recruit up to five “effectors” to a specific genomic locus via their single-chain antibody (scFv) domain (Fig 1A). This modular system amplifies both the quantitative level and domain size of ON-target epigenome editing, relative to dCas9 effector fusions, while minimising OFF-target effects (Pflueger et al., 2018). To target de novo heterochromatin, we generated KRABGFP-scFv and DNMT3A/3L GFP-scFv effectors, which promote direct deposition of H3K9me3 and DNA methylation respectively (Quenneville et al., 2012).

We placed all components under a DOX-inducible promoter and destabilised dCas9GCN4 protein and effectors with d2 domains, which together facilitate precise temporal control over epigenome editing activity. This is important to assess subsequent epigenetic memory without confounding reiterative targeting. To track the temporal ON-OFF dynamics in real-time, all effectors are tagged with superfolder GFP, which also enables cell isolation via flow cytometry (Fig EV1A–C). Finally, we used an “enhanced” gRNA scaffold (AT-flip, extended stem loop) linked with a tagBFP (Chen et al., 2013), which further amplifies ON-target activity and facilitates tracking respectively. In summary, dCas9GCN4 and KRABGFP-scFv expression can be induced by DOX treatment and traced in real-time via GFP, while BFP is constitutively expressed. Reciprocally, the system is destabilised and can be rapidly switched back OFF by removal of DOX.

**Programmed heterochromatin epialleles fully silence gene activity in single cells**

To follow programmed epialleles at endogenous loci, we initially used a naïve pluripotent ESC line wherein the endogenous Esg1 gene carries a knock-in tdTomato (Fig 1A) (Hackett et al., 2018). We introduced dCas9GCN4::KRABGFP-scFv and a single gRNA BFP that targets the Esg1 promoter (~87 bp of TSS) via piggyBac, and assessed the extent of de novo programmed epigenetic states after 7 days (7 d) induction with DOX. Quantitative CUT&RUN qPCR demonstrated a highly significant deposition of heterochromatic H3K9me3 (P = 0.014) marks across the Esg1 promoter specifically with KRABGFP-scFv, relative to either untransfected or control GFP-scFv targeting (Fig 1B). This was paralleled by enrichment of another heterochromatin mark, H4K20me3 (P = 0.002), which often co-localises with H3K9me3 (Schotta et al., 2004), and complete loss of endogenous H3K4me3 modification (P = 0.0013). Moreover, bisulphite pyrosequencing revealed a highly significant increase in DNA methylation (P = 0.0005) across the entire Esg1 promoter region (Fig 1C). These results indicate that upon single-gRNA tethering of a flexible array of five KRABGFP-scFv effectors, a de novo domain of heterochromatic modifications is established.

To further investigate the extent and specificity of programmed heterochromatin we performed CUT&RUN-seq. We observed that our epigenetic editing system deposits a broad domain encompassing ~12 kb of H3K9me3 and H4K20me3 around the endogenous Esg1 locus, while previously abundant H3K4me3 is undetectable (Fig 1D). Importantly the de novo peaks of H3K9me3 and H4K20me3 are of a magnitude comparable to the strongest peaks throughout the genome, suggesting they recapitulate robust physiological heterochromatin status (Figs 1E and EV1D). Moreover, targeting was highly specific, since we observed minimal OFF-target changes in H3K9me3, H4K20me3 and H3K4me3 (Figs 1E and EV1D). Taken together, these data reveal that a substantial epigenomic domain (~ 10 kb), which bears the key hallmarks of repressive heterochromatin, is specifically programmed at an endogenous genomic locus.

We next asked whether this de novo heterochromatin domain is associated with induction of transcriptional silencing. Esg1 is highly active in pluripotent cells, and the endogenous reporter facilitates dynamic single-cell analysis over time. As expected, addition of DOX led most cells to become GFP positive, indicative of activation...
Figure 1. Programming an epiallele promotes a robust heterochromatin domain and gene silencing.

A Schematic showing recruitment of the modular epigenetic editing system to an endogenous promoter upon addition of DOX. A knock-in reporter downstream facilitates single-cell analysis.

B The relative abundance of indicated histone modifications assayed by CUT&RUN-qPCR after KRABGFP-scFv (blue) or control GFPscFv (light brown) targeting, relative to untransfected (grey). Shown are independent quantifications at two genomic positions on the Esg1 promoter, indicated relative to TSS (−300 bp, −700 bp). Data are mean of two or three independent biological replicates.

C Histograms showing the average DNA methylation across three genomic regions of the Esg1 promoter determined by bisulphite pyrosequencing in two biological replicates.

D CUT&RUN genome tracks for H3K4me3, H4K20me3 and H3K4me3 in untransfected (grey), control GFPscFv (light brown) or KRABGFP-scFv (blue) targeted ESC after 7 days of DOX treatment. Grey box highlights the region of heterochromatin spreading induced by epigenetic editing.

E Scatterplots demonstrating specificity and magnitude of programmed modifications at Esg1 by CUT&RUN-seq. Shown are all promoters genome wide.

F Epifluorescence images of Esg1-tdTomato ESC in −DOX (top) or −DOX conditions (bottom), where targeted heterochromatin is deposited.

G Single-cell expression of Esg1-tdTomato in control (GFPscFv) or upon heterochromatin induction (KRABGFP-scFv) using a gRNA targeting close to the TSS (+87 bp) or further upstream on the promoter (−475 bp). Each data point indicates a cell, percentage indicates the proportion of fully silenced cells (mean of four biological replicates) and bars represent median. P-values calculated by comparing +DOX with −DOX conditions.

Data information: In all panels, asterisks indicate P-values by unpaired t-test over biological replicates, *P < 0.05, **P < 0.01, ***P < 0.001. Error bars ± SD.
of the epigenetic editing system (Fig 1F). Strikingly, this concomitantly led to complete loss of Esg1tdTomato-positive cells, which was additive with time (Figs 1F and EV1C). Quantitative single-cell expression revealed >99% cells exhibited transcriptional repression, with >85% reaching a complete OFF state, indistinguishable from ESC that do not carry the tdTomato reporter (Neg), and corresponding to >500-fold transcriptional silencing (Fig 1G). In contrast, the GFPscFv control exhibited only modest repression, which likely reflected steric hindrance due to TSS binding, since a gRNA targeting upstream (−475 bp) elicited full silencing with KRABGFP-scFv (>500 fold) but its control GFPscFv exhibited no effect on transcription (Fig 1G). In summary, these data indicate that our system is able to ectopically programme heterochromatin states at Esg1, which is directly linked with powerful transcriptional silencing at the single-cell level, implying high penetrance of deposition across a broad domain. We refer to this enhanced genetic tool as inducible CRISPR unleashing of silencing by heterochromatin (iCRUSH) (Fig 1A).

**Induced heterochromatin is progressively erased in ESC**

Extant paradigms suggest that large domains of heterochromatic H3K9me3, H4K20me3 and DNA methylation are heritable, and self-propagate via “read-write” feedback machinery (Hathaway et al., 2012; Reinberg & Vales, 2018). To understand this in a developmental context, we next investigated the potential for propagation of induced heterochromatin epialleles in naïve ESC, which faithfully recapitulate in vivo epiblast when maintained in 2i/L (Hackett & Surani, 2014). Withdrawal of DOX resulted in a rapid switch off of the iCRUSH epigenetic editing system as determined by quantitative cytometry for GFP, and consistent with dCas9GCN4 and KRABGFP-scFv being destabilised, therefore fully releasing the inducing signal (Fig 2A). In parallel, we observed a progressive loss of Esg1tdTomato silencing (Fig 2B). Interestingly, 4 days after DOX washout (D-wo (4 days)), we observed a graded distribution of Esg1 expression among single cells, indicative of probabilistic reactivation dynamics. By 7 days after release (D-wo (7 days)), however, all cells reverted to the ON state, reflecting >500-fold increase in Esg1 expression (Fig 2B).

To determine if transcriptional re-expression corresponds to loss of programmed epigenetic states, we used bisulphite pyrosequencing and CUT&RUN. Consistent with the reactivation dynamics, we found that DNA methylation is partially maintained at the Esg1 promoter at the early time point (D-wo (4 days)) but is almost completely erased by 7 days washout (Fig 2C). However, we found that the high levels of deposited H3K9me3 and H4K20me3 are largely erased by 4 days after DOX withdrawal (Fig 2D and E).

Following 7 days release of the epigenetic editing trigger, the Esg1 chromatin state completely reverts to the initial configuration, including erasure of H3K9me3, H4K20me3 and DNA methylation, and reacquisition of the endogenous H3K4me3 mark (Fig 2D and E). While our system deposits high levels of DNA methylation, we additionally checked whether co-targeting KRABGFP-scFv together with the catalytic domain of Dnm3ta and its cofactor Dnm3tL (3a3L-GFP-scFv) would enhance epigenetic inheritance, since such effects have been reported in cancer and primed cell lines (Fig EV2A) (Amabile et al., 2016; Nunez et al., 2021). Although we found a slight further increase in DNA methylation by compound recruitment (Fig EV2B), we observed equivalent or faster erasure of epigenetic memory (Fig EV2C). Taken together, our data argue that induction of a robust heterochromatin domain, and consequently extensive epigenetic silencing, is readily reversible from OFF→ON in naïve pluripotent cells.

**Epigenetic inheritance is restricted by naïve ESC**

To confirm that failure to propagate robust heterochromatin in ESC is not a phenotype specific to Esg1, we generated a second endogenous reporter ESC line by inserting tdTomato downstream of the p53 gene, separated by a T2A self-cleavable domain (Fig 2F). Targeting KRABGFP-scFv to the p53tdTomato promoter recapitated the same extensive heterochromatin deposition including DNA methylation, H3K9me3, H4K20me3, and loss of H3K4me3, and robust (>100-fold) single-cell silencing, as achieved at Esg1tdTomato (Fig 2F–H). Upon 7 days DOX withdrawal, we found that p53 expression becomes fully reactivated in ESC (Fig 2F–H). This is paralleled by erasure of targeted DNA methylation and H3K9me3, and reacquisition of endogenous H3K4me3, consistent with heterochromatin failing to confer epigenetic memory in naïve ESC.

To examine this across further genomic locations, we programmed heterochromatin to additional endogenous loci, selected to represent different regulatory features (e.g. imprinting control regions, promoters). We imposed strong epigenetic silencing with iCRUSH, yet most loci (Pten, Cdh1, Greb1, Adams7, Smoc1 and Jade1) reverted to their original expression status within 7 days DOX withdrawal (Fig 2I). Nevertheless, we did observe that imprinted genes (Peg3, Mest and Plag1) are exceptions and, uniquely, maintain memory of de novo silencing in naïve ESC (Fig 2I). This suggests that heterochromatin domains at ectopic sites can be epigenetically inherited in a genomic context-dependent manner, with imprinted loci providing the necessary sequence substrate for propagation. However, in general, we find de novo chromatin states at endogenous single-copy loci are not heritable over extended periods in naïve ESC. This supports a dynamic competition of opposing activities that generally disfavour epigenetic inheritance during the phase of naïve pluripotency, potentially as a safeguard to restrict intergenerational transmission of aberrant epialleles.

We next asked if this principle in naïve ESC holds for other epigenetic silencing pathways by exploiting a hybrid female ESC line carrying a DOX-inducible Xist allele on the BL6-derived X-chromosome (TX1072) (Schulz et al., 2014) (Fig 3A). Activation of Xist leads to programmed silencing of X-linked genes in cis via recruitment of repressive epigenetic systems, with a principal role for polycomb (Zylicz et al., 2019). In differentiated cells, cis repression propagates independently, resulting in stable silencing memory (X-Chromosome inactivation (XCI)), even after withdrawal of Xist (Loda & Heard, 2019). However, using transcriptomics, we observed that while strong epigenetic silencing is initially imposed in naïve ESC, withdrawal of DOX led to an almost complete reactivation of X-linked genes after 3 days (Fig 3B), extending a previous finding based on two marker genes (Wutz & Jaenisch, 2000). Hierarchical clustering revealed the majority of genes (81%) exhibit fast reactivation dynamics (<3 days) in ESC (Fig 3C). A second cluster (8% of genes) also reactivated but with slower dynamics (<7 days), and these overlapped with X-linked loci that reactivate late in vivo, for example, Fmr1b and Prm5 (Borensztein et al., 2017). A third cluster (4%) was resistant to initial silencing in naïve cells (ESC escapes), while the final cluster (7% of genes) did exhibit memory of silencing following
Figure 2. Induced heterochromatin epialleles are progressively erased in naive pluripotent cells.

A  Representative flow cytometry density plots showing gRNA<sup>BFP</sup> (+87 bp of TSS) and KRAB<sup> GFP-ScFv</sup> expression after DOX treatment (7 days) and DOX washout (4 days).

B  Esg<sup>1</sup>-tdTomato expression at single-cell resolution during DOX washout in control (GFP<sup>ScFv</sup>) or induced heterochromatin (KRAB<sup>GFP-ScFv</sup>) cells. Horizontal bars indicate median, each data point a single cell.

C  Histograms of mean DNA methylation levels across the Esg<sup>1</sup> promoter (6 CpG sites) during DOX washout in two biological replicates.

D  CUT&RUN tracks at +DOX, and 4 and 7 days of DOX washout in control GFP<sup>ScFv</sup> or KRAB<sup>GFP-ScFv</sup> for indicated histone marks. Grey boxes highlight the domain of heterochromatin spreading in +DOX.

E  CUT&RUN-qPCR quantification of the relative abundance of each mark at Esg<sup>1</sup> promoter in two or three independent biological replicates, normalised to a positive control region and untransfected cells.

F  p53-tdTomato expression in single cells during DOX washout in control (GFP<sup>ScFv</sup>) or induced heterochromatin (KRAB<sup>GFP-ScFv</sup>) cells. Each data point indicates a cell, and horizontal lines represent the median.

G  Bisulphite pyrosequencing quantification of DNA methylation at the p53 promoter (4 CpG sites) at indicated time point in two biological replicates.

H  CUT&RUN-qPCR quantification of the relative abundance of H3K9me3 and H3K4me3 at p53 promoter in biological replicates.

I  Heatmap representing relative expression by qRT-PCR of each indicated gene upon heterochromatin targeting (+DOX) or after DOX washout (D-wo (7 days)), normalised to the untransfected control in three biological replicates. Statistics are measured between KRAB<sup>GFP-ScFv</sup> and control (GFP<sup>ScFv</sup>) at DOX-washout time point.

Data information: in all panels, asterisks indicate P-values by unpaired t-test, *P < 0.05, **P < 0.01, ***P < 0.001. Error bars ± SD.
DOX withdrawal (Fig 3C). This “memory” cluster was enriched for tandem gene families such as the Rhox, Mage and Xlr clusters. Overall, however, these data suggest that the vast bulk of X-linked genes cannot propagate programmed epigenetic silencing in ESC, which is in contrast to differentiated cells, and supports the principle that naïve pluripotency specifically antagonises epiallele memory.

**CRISPR screen reveals key factors that antagonise epigenetic memory in ESC**

To investigate whether the reversal of repressive epialleles in naïve cells is driven by passive dilution during cell divisions, or by active erasure, we transiently transfected iCRUSH to epigenetically silence the Esg1 reporter for 3 days with DOX. This led to ~100-fold silencing, deposition of significant levels of DNA methylation, H3K9me3 and H4K20me3, and loss of H3K4me3 (Fig EV2D and E). We then released the epigenetic editing system by DOX washout and concomitantly treated the cells with or without the cell cycle inhibitor RO3306 (Fig EV2F), which blocks cells at the G2/M phase boundary. We observed Esg1 reactivation is only weakly impaired by cell cycle inhibition (Fig EV2G), spanning at least 60 h (Fig EV2H). Thus, while passive dilution may partially contribute to reversion of epigenetic memory, active mechanisms play a key role in erasing de novo epialleles in naïve ESC.
We therefore sought to identify the putative factors that actively counteract epigenetic memory in pluripotent phases by designing a genome-wide loss-of-function CRISPR screen (Fig 4A). We introduced a single copy of Cas9 nuclease tagged with GFP (Cas9Δ2A-GFP) into Esg1tdTomato ESC that also carry dCas9GCN4 in the OFF state, and infected these cells with a pooled lentiviral library of exon-targeting gRNA covering 19,674 genes (Doench et al., 2016). We subsequently induced self-inactivation of the Cas9Δ2A-GFP with a pair of specific gRNAs, which we confirmed by flow sorting cells according to loss of GFP (GFPΔ0) (Fig 4A). This cell population is now composed of a heterogeneous pool of knockout cells, to which we could apply our epigenetic editing system to identify the factors that antagonize epigenetic inheritance.

To achieve this, we targeted heterochromatin to Esg1tdTomato and isolated cells that subsequently retained sensing memory (TOM Δ0) following release of dCas9GCN4::KRAB-GFP-scFv using a gating strategy to distinguish between cells remaining fully silenced (bottom 2.5% (TOM Δ0-wide)) (Fig EV3A) and those that retain a degree of repression memory (TOM Δ0-wide) (Fig EV3B). We then used model-based analysis of genome-wide CRISPR-Cas9 knockout (MAGECK) to identify the gene knockouts enriched in the TOM Δ0 populations that retained epigenetic memory relative to the complementary TOMΔ0 population over short (3 days) and extended (7 days) timescales (Li et al., 2014). As expected, top hits across both gates were associated with roles in transcriptional or translational regulation, and comprised many candidates with established or predicted epigenetic functions. This included the SWI/SNF histone remodeler Smarcc1 (FDR 0.03), the H3K79 methyltransferase Dot1L (FDR 0.16) and the H3K4 histone methyltransferase Kmt2d (FDR 0.13), although this latter was enriched only at the shorter time point (Figs 4B and C and EV3B). Additionally, we noted cells that propagated silencing memory also exhibited significant enrichment for knockouts of the X-linked zinc-finger protein Kansl2 (FDR 0.03) and the H3K4 histone methyltransferase Kmt2d (FDR 0.16) and the H3K4 histone methyltransferase Kmt2d (FDR 0.13), although this latter was enriched only at the shorter time point (Figs 4B and C and EV3B). Additionally, we noted cells that propagated silencing memory also exhibited significant enrichment for knockouts of the X-linked zinc-finger protein Zmym3 (FDR 0.01), the NSL complex subunit Kansl2 (FDR 0.05) and Dppa2 (FDR 0.03) (Figs 4B and C, and EV3B and C), which is a pluripotency-specific gene recently identified as being critical for the maintenance of pluripotency (Eckersley-Maslin et al., 2019, 2022 The Authors The EMBO Journal 41:e108677 | 2022 7 of 20). Moreover, ATAC-seq revealed that Dppa2 facilitated a stochastic memory function (Fig EV4C–E). We next investigated transmission of programmed chromatin states in Dppa2−/− cells. After DOX induction of iCRUSH, we observed that DNA methylation and H3K9me3 are deposited comparably in both WT and Dppa2−/− cells, and endogenous H3K4me3 is equivalently erasable (Fig 5C and D). Upon release of dCas9GCN4::KRAB-GFP-scFv (DOX washout), however, while WT cells underwent a complete recovery of the epigenetic landscape, Dppa2−/− exhibited significant inheritance of DNA methylation (Fig 5C), and also propagated the H3K4me3-depleted state at Esg1 (Fig 5D). Moreover, ATAC-seq revealed that induced chromatin inaccessibility status was transmitted mitotically in Dppa2−/− ESC but not WT (Figs 5E and EV4F).

To examine the role of Dppa2 further, we traced the single-cell dynamics of transcriptional memory in multiple-knockout ESC lines (Fig EV4A). While WT cells rapidly lose silencing memory after 7 days DOX washout, the majority of Dppa2−/− cells remain fully silenced at this stage (Fig 5A). Importantly, inheritance of this silenced state in the absence of Dppa2 is subsequently maintained across a consistent fraction of cells for at least 43 days after DOX withdrawal (> 100 cell replications), with the population therefore exhibiting a bimodal distribution (Fig 5B). Importantly, population doubling time was similar between wild-type and knockout cells (Fig EV4B). This implies that abrogation of Dppa2 facilitates heritability of ectopic silencing in a probabilistic manner, potentially by shifting the odds against reversion, and thus promoting steady-state inheritance. Notably, flow sorting TOM Δ0 and TOMΔ0 fractions after 26 days of DOX washout revealed that, while the TOMΔ0 remained positive, the TOMΔ0 reacquired a bimodal distribution, supporting a stochastic memory function (Fig EV4C–E).

Epigenetic inheritance is permitted by deletion of DPPA2

To determine the generality of epiallele propagation in Dppa2−/− ESC, we targeted heterochromatic silencing to additional loci, which we previously showed do not exhibit memory in wild-type ESC (Fig 2I). Here, a trend of memory was propagated at Pten, Cdh1, Greb1 and Adamis7 in the absence of Dppa2 (Fig EV4G). While these did not reach significance, this could potentially reflect an incompletely penetrant memory (bimodality) at the single-cell level similar to Esg1, which we cannot resolve at the population level by qRT-PCR. Importantly, however, we did observe significant inheritance of an induced repressed state at Jade1 (aka Phf17) and Smoc1, specifically in Dppa2−/− ESC.

To expand this analysis to a larger unbiased scale, we generated Dppa2 knockout ESC in the inducible Xist background (TX1072) (Fig EV4A) and assayed the transcriptome following DOX withdrawal, which releases Xist-mediated epigenetic silencing. Analysis of all X-linked loci that are reactivated in wild-type ESC revealed Dppa2−/− cells exhibited a significant (P = 0.041), albeit modest, block in gene re-expression following release of Xist (Fig 5F). Hierarchical clustering deconvolved two broad groupings. The first gene cluster (92.9%) reactivated in Dppa2−/− ESC similarly with wild-type kinetics. In contrast, the second cluster (7.1%) identified a broad set of genes that exhibit epigenetic memory of prior silencing specifically in the absence of Dppa2, including Flna, Shroom2, Kctd12b and Rnf128 (Fig 5G and H). Of note, epigenetic silencing induced by Xist is preferentially linked with polycomb pathways (H3K27me3 and H2AK119ub) and histone deacetylation (Zylicz et al., 2019), whereas targeting with iCRUSH programmes
repression with H3K9me3, H4K20me2 and DNA methylation epialleles. This suggests absence of DPPA2 unlocks the potential for propagating at least two distinct modes of heterochromatin-based silencing in naïve ESC.

Taken together these data suggest that once an aberrant heterochromatic state occurs, pluripotent cells rely, at least partly, on DPPA2 to re-establish the original epigenetic configuration. Indeed, using CUT&RUN-seq to chart DPPA2 occupancy in ESC, we...
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Figure 5. Epigenetic inheritance is unlocked by deletion of Dppa2.

A Time course showing percentage cells that propagate heritable epigenetic silencing of Esg1-tdTomato in wild-type or Dppa2−/− cells. Each data point represents average of three independent clonal lines.

B Distribution of quantitative Esg1-tdTomato expression in Dppa2−/− (red) or wild type (blue) after 7 or 43 days DOX washout showing bimodal epigenetic memory only upon Dppa2 abrogation, in one representative example. Numbers indicate percentage of Esg1-tdTomato-negative cells.

C Bisulphite pyrosequencing quantification of DNA methylation at Esg1 promoter in WT (blue) or Dppa2−/− (red) ESC, each assayed in two or three independent lines.

D CUT&RUN-qPCR quantification of H3K9me3 and H3K4me3 relative to a positive control region and to the −DOX control in one (∼DOX) or three independent clonal lines.

E Heatmap indicating expression dynamics of X-linked genes that reactivate in WT. Shown are average profiles from independent three WT and two Dppa2−/− clonal lines at the indicated time points, which form two unsupervised hierarchical clusters: genes that reactivate in both WT and Dppa2−/− (upper) and genes which maintain memory only in Dppa2−/− (lower).

F Scatterplot of genome accessibility across all promoters after washout (7 days D-wo) time points. Bars indicate median from independent clonal lines of Dppa2−/− or WT. Boxes indicate quartiles, and whiskers represent the 5th to 95th percentiles.

G X-linked reactivation dynamics (naive ESC).

H Expression dynamics (log2 RPM) of four representative genes from the memory cluster. Statistics calculated between two or three in independent clonal lines in Dppa2−/− or WT, respectively, at 7 days DOX washout.

Data information: In all panels, asterisks indicate P-values by unpaired t-test; *P < 0.05, **P < 0.01, ***P < 0.001. In panels (A, C, D and H), error bars are ± SD.
observed that DPPA2 binds strongly to all CpG-dense gene promoters (Fig EV4H), and > 70% of all transcriptional start sites (TSS), including all responsive targets tested above (Fig EV4I and J). This implies DPPA2 acts as an epigenome “surveyor” in naïve pluripotent cells by sampling most promoters and promoting probabilistic reversion of epimutations. While parallel erasure mechanisms must also operate, since epialleles at many genes revert independent of Dppa2, DPPA2 activity underlies the capacity of naïve ESC to reset aberrant epigenetic states across a significant cohort of sensitive loci.

**Aberrant epialleles can be propagated upon exit from pluripotency**

*Dppa2* is only expressed during pluripotent phases suggesting epialleles acquired during or after this may confer heritable mitotic memory through subsequent lineage commitment when DPPA2 is absent. To investigate this, we differentiated wild-type ESC towards definitive endoderm as an *in vitro* model of development (Fig 6A). Because *Esg1* is repressed during differentiation as part of the normal developmental programme, we initially focused on *p53*, wherein heterochromatin and transcriptional silencing are also robustly erased in ESC (Fig 2F–H).

In contrast to ESC, upon differentiation to definitive endoderm, we observed a highly penetrant memory of *p53* silencing among single cells (Fig 6B), whereas no memory effects were observed upon control targeting with GFP<sup>sert</sup> (Fig EV5A). Analysis of chromatin revealed inheritance of targeted DNA methylation (> 85%) at *p53* specifically in differentiating endoderm cells (Fig 6C), while there is also heritable memory of the H3K4me3 depletion (Fig 6D). Interestingly, deposited H3K9me3 is erased in endoderm (Fig 6D), implying it does not self-reinforce or drive silencing in this context. To investigate this epigenetic memory further, we used ATAC-seq and observed highly significant loss of accessibility specifically at targeted *p53* upon *de novo* heterochromatin formation (Figs 6E and EV5B). Following 7 days DOX withdrawal, this inaccessible chromatin state exhibited robust memory during endoderm differentiation. In contrast, chromatin accessibility is restored in ESC upon DOX withdrawal (Fig 6E). These data imply that differentiated cells, but not naïve pluripotent ESC, are competent for epigenetic inheritance of ectopic heterochromatin.

To extend this we next targeted heterochromatin to five additional endogenous loci and tracked their memory in wild-type endoderm. We found that *Jade1* and *Greb1* exhibit robust inheritance (*P < 0.05*) of a prior silenced state specifically in endoderm (Fig EV5C), whereas *Cdh1, Adamts7* and *Pten* reinitiate their original activity, implying a degree of context dependency. Notably of all targets, *p53* exhibited the most striking propagation of silencing, which we reasoned may reflect a confluence of epigenetic memory and a selective advantage, given the role of *p53* in restricting proliferation. Indeed, by mixing equal (1:1) proportions of silenced *p53* cells with untargeted controls and withdrawing DOX, we found epigenetically repressed cells became dominant in the endoderm population, comprising > 95% by d8, but not in naïve ESC, where memory of prior silencing is rapidly erased (Fig 6F). Moreover, endoderm cells with prior *p53* silencing replicate faster and with greater viability (Fig EV5D). Taken together, these data suggest that the potential for epiallele inheritance of *de novo* heterochromatin is influenced by multiple cell type- and genomic context-dependent factors. In the case of *p53*, augmenting weak-acting heterochromatin inheritance in differentiated cells with a favourable advantage may tip the balance of dynamic forces to enable robust propagation of ectopic chromatin states within the population.

To investigate whether the difference in epiallele propagation between naïve and committed cells is functionally linked with global DNA hypomethylation in naïve ESC (Leitch *et al.*, 2013), we switched ESC to serum/LIF culture (S/L). S/L maintains ESC populations as functionally naïve (they contribute to blastocyst chimeras), but promotes a more developmentally advanced epigenetic state, including DNA hypermethylation, thereby enabling us to parse the influence of global DNA methylation status on memory. We observed that programmed heterochromatic silencing at *p53* is readily erased in S/L ESC, albeit with modestly slower dynamics than 2i/L ESC (Fig 6G). In contrast, differentiated cells maintain silencing memory (Fig 6B). The delayed dynamics in S/L relative to 2i/L could indicate a contributory, but non-essential role of global DNA hypomethylation for erasure, or alternatively may reflect the metastable status of ESC in S/L, with some subpopulations not in “naïve” status. In any case, these data argue that global hypomethylation *per se* is not requisite, and that additional properties of naïve cells underlie their unique capacity to reset acquired heterochromatin states (including *Dppa2* activity).

**Epigenetic inheritance during mammalian development in vivo**

To more closely model the developmental process that occurs *in vivo* when pluripotent cells differentiate into all lineages, we differentiated ESC towards fates representative of multiple germ layers: ectoderm, endoderm and epiblast-like cells (EpiLC) (as a model of primed pluripotency). Upon release of the iCRUSH heterochromatic trigger (Fig EV5E), we observed maintenance of *p53* silencing in all three differentiation programmes (Fig EV5F). Notably this included primed pluripotent EpiLC, emphasising the preferential capacity of naïve pluripotency to reset epialleles. All differentiating cells replicated with comparable kinetics (Fig EV5G) and activated master lineage regulators, while repressing naïve markers, indicating successful differentiation (Fig EV5H). These data suggest that epigenetic aberrations acquired during the pluripotency window can potentially be inherited in all tissues.

To determine whether perturbed epigenetic states acquired at loci such as *p53* can self-propagate during *in vitro* development, potentially affecting disease risk, we tested epigenetic inheritance during embryogenesis. We introduced *p53* epigenetically-silenced ESC (KRAB<sup>GFP<sup>sert</sup></sup>) into E3.5 blastocysts and traced the memory during post-implantation development (−DOX), as compared to a control (GFP<sup>sert</sup>) (Fig 7A). By epifluorescence microscopy, we observed a strong contribution of ESC to all tissues of the E10.5 embryo (Fig 7B), and a consistent fraction of the cells carried BFP expression, as analysed by flow cytometry (Fig 7C). Analysis of tdTomato within the BFP<sup>pos</sup> cells revealed that *p53* is fully activated in controls, as expected (Fig 7C). In contrast, embryos with prior *p53* epigenetic silencing had a significant tendency (*P = 0.03*) to propagate memory of this through development (Fig 7D). Indeed, up to 7% of foetal cells inherited epigenetic silencing memory (Fig 7D and E). Given the central role of *p53* as a tumour suppressor, this has the potential to have a major impact on disease susceptibility.
Figure 6. Aberrant epialleles can be propagated in wild-type cells upon exit from pluripotency.

A Schematic of experimental timeline: heterochromatin epialleles are induced at p53-tdTomato in ESC followed by endoderm differentiation (day 0), with DOX removed after 24 h (day 0). In parallel, cells are maintained as naive ESC. Chromatin and expression analysis to record memory in endoderm and ESC is performed at 4 and 7 days of DOX washout.

B Single-cell expression of p53-tdTomato during DOX washout in endoderm or naive ESC, following induction of heterochromatic silencing. Each data point indicates a cell, and horizontal lines represent the median.

C Bisulphite pyrosequencing quantification of DNA methylation at the p53 promoter in ESC or endoderm following DOX withdrawal in two or three biological replicates for KRAB-GFP-scFv. Dashed line indicates levels of DNA methylation after targeting KRAB-GFP-scFv (+DOX) for 7 days.

D CUT&RUN qPCR quantification of the relative memory of induced H3K9me3 and H3K4me3 at the p53 promoter in independent biological replicates of endoderm or ESC.

E ATAC-seq scatterplots showing genome accessibility at all promoters comparing GFP-scFv and KRAB-GFP-scFv in +DOX or DOX washout conditions in ESC or endoderm cells. Shown below are relevant genome tracks of the p53 promoter (highlighted in grey).

F Cell growth competition assay following 1:1 mixing of cells bearing control GFP or KRAB-GFP-scFv epigenetic silencing of p53, in ESC or endoderm.

G Line plot showing the percentage of p53-tdTomato epigenetically silenced cells in 2i/L and serum/Lif (S/L) culturing conditions at +DOX and 2, 4 or 7 days of DOX washout. Error bars ± SD measured over two biological replicates.

Data information: In all panels, asterisks indicate P-values by unpaired t-test; *P < 0.05, **P < 0.01, ***P < 0.001. Error bars ± SD measured over two biological replicates.
Overall, these data suggest that an epiallele acquired during or after naïve pluripotent phases can be inherited through subsequent development. Importantly, this effect is highly context dependent and relies on supporting activities or influences that reinforce or promote propagation, either directly or indirectly.

**Discussion**

Here, we used a precision epigenetic editing strategy (iCRUSH) to define the transcriptional function and memory of heterochromatin epialleles at endogenous loci. Our method of compound recruitment of multiple “effector” modules using dCas9GCN4 facilitated programing of major (> 10 kb) heterochromatin domains, sufficient to drive robust epigenetic silencing. These *de novo* domains comprised H3K9me3, H4K20me3 and DNA methylation, and concomitant loss of H3K4me3, with modification levels comparable or greater than endogenous heterochromatic regions, which are thought to self-propagate via “read-write” reinforcement (Reinberg & Vales, 2018).

Nevertheless, we found that naïve pluripotent cells act as a fundamental roadblock to inheritance of heterochromatin domains occurring outside of normal genomic contexts, even when providing a selective advantage such as silencing p53. This supports the concept of an epigenetic “tabula rasa” during early mammalian development that acts to prevent intergenerational transmission of inherited or acquired chromatin epialleles. An exception to this principle is imprinted regions, wherein programmed chromatin was stably maintained, highlighting the role of underlying DNA sequence context for epigenetic memory. This contextual influence is further exemplified by the effects of cell identity, with our data revealing the potential for epigenetic inheritance in mammals during normal *in vivo* development. Thus, we propose a unique and defining feature of naïve pluripotency is to reset aberrant chromatin states at endogenous loci to establish a pristine epigenome for development. Indeed, the functional properties of pluripotency *per se* are relatively unaffected by impairing global DNA demethylation (McLaughlin et al, 2019), and thus purging otherwise heritable epialleles could be a key operative function of epigenome reorganisation during pluripotent phases (Festuccia et al, 2016).

To decipher the underlying mechanisms that restrict epigenetic inheritance in naïve pluripotent cells, we designed a genome-wide CRISPR screening strategy. We found that loss of *Kmt2d* enables prolonged memory of epigenetic silencing in naïve ESC, presumably because of reduced H3K4me3 re-deposition, yet the original...
epigenetic state is eventually restored, suggesting an important but non-critical role of Kmt2d in opposing heritable silencing. In contrast, we show that deletion of Dppa2 enables robust long-term epigenetic inheritance of programmed epigenetic silencing in naïve cells. Interestingly, this was a probabilistic effect, with most cells potentiating memory, but with a fraction delaminating to reactivate expression over time. This suggests that removing Dppa2 shifts the balance of opposing factors to favour propagation of epigenetic silencing without fully saturating the odds against reversion.

Importantly, in Dppa2 mutants, the majority of loci remain in their erstwhile epigenetic configuration prior to acquiring a forced epimutation. This argues that loss of Dppa2 sensitises some loci to stably inherit any stochastic or programmed epigenetic changes, and implies DPPA2 acts as an epigenome surveyor to counteract epigenetic inheritance during pluripotent phases. This extends recent observations which showed a subset of developmental loci and LINE1 directly acquire silencing in Dppa2 mutants (Gretarsson & Hackett, 2020), by revealing that loss of Dppa2 renders a further fraction of the genome predisposed to inherit prospective epigenetic perturbations, potentially induced by external exposures. Mechanistically, DPPA2 is thought to target H3K4me3 through interactions with the COMPASS complex (Eckersley-Maslin et al, 2020), which emphasises that the molecular pathways that impair heterochromatin inheritance in ESC could converge on promoting antagonistic H3K4me3. Indeed, we found that, in the absence of DPPA2, memory is favoured on non-canonically imprinted H3K4me3 genes (Jade1 and Smoc1) (Hanna et al, 2019). DPPA2 is also a putative mitotic bookmarking factor, which may be functionally relevant for restricting aberrant epigenetic memory through cell division (Djeighoul et al, 2020). Nevertheless, it is important to note that additional or redundant factors are at play, since we observed epigenetic reversion of many loci in Dppa2 mutants, such as p53 and X-linked genes. This suggests there is a broad network of mechanisms that precludes epigenetic inheritance specifically in naïve cells, with DPPA2 playing a key role within these regulatory systems.

In contrast to naïve cells, heritable epigenetic silencing has been reported in differentiated cells (Amabile et al, 2016; Bintu et al, 2016; Nuñez et al, 2021). This has, however, typically been in cancer-derived cell lines and the potential for mitotic propagation of de novo epigenetic states in a normal developmental context is relatively unexplored. We reasoned that if an epimutation occurred during or after early pluripotent phases in vivo, it could heritably influence subsequent cellular/organismal phenotype, via clonal inheritance in neighbouring cells. Indeed, we found that while epigenetic silencing at some loci is reset, others demonstrate robust mitotic transmission of heterochromatin-mediated silencing, including p53. This appears to reflect the confluence of weak-acting probabilistic inheritance and a selective advantage conferred by stable p53 repression. Such a phenomenon of ‘Darwinian’ epigenetic inheritance was recently shown in yeast (Catania et al, 2020; Torres-Garcia et al, 2020). Importantly, we demonstrate this effect in vivo in mice, with up to 7% of cells within a whole embryo heritably maintaining the legacy of prior p53 silencing. This is relevant as even a small fraction of organismal cells correspond to a large absolute number (order of 10 billion in adult mouse) that carry epigenetic silencing, and such constitutional epimutations of tumour suppressors have been linked to cancer risk (Hitchins, 2015; Sanderson et al, 2017). Moreover, the principle of probabilistic inheritance of epigenetic silencing in vivo shown here could have implications for other aspects of health and disease linked with early life environmental exposures that can promote epigenome changes.

In summary, we find the window of naïve pluripotency robustly counteracts induced epigenetic memory, in part through DPPA2 activity, implying an intrinsic role of naïve status could be to erase epimutations during early mammalian development. Upon differentiation, however, when Dppa2 is downregulated, we find acquired chromatin states can self-propagate through development, particularly when providing a selective advantage. This highlights a previously unappreciated but crucial developmental function of naïve pluripotency, and contributes to understanding the complex inputs that feed into epiallele propagation in mammals.

Materials and Methods

Routine cell culture

Naïve murine embryonic stem cells (mESC) were derived freshly (mixed 129/B6, XY), or obtained from (Hackett et al, 2018), and were routinely cultured on gelatin-coated plates in t2i/L media: ND1ff (N2B27) (Takara #40002) supplemented with titrated 2i (0.2 μM PD0325901 and 3 μM CHIR 99021), 1,000 U/ml leukaemia inhibitory factor (LIF), 1% FBS, 1% penicillin streptavidin and maintained in humidified atmosphere at 37°C and 5% CO2. Cells were passaged every 2–3 days by dissociation with TrypLE and medium was changed daily. Mycoplasma contamination checks were performed routinely by ultrasensitive qPCR assay (Eurofins).

For X-chromosome reactivation experiments, TX1072 cells (mixed Cast/B6, XX) were a gift from Edith Heath, and were cultured as described before in 2i+Lif media: DMEM (Sigma), 15% FBS (Gibco), 1% penicillin streptavidin, 0.1 mM β-mercaptoethanol, 1,000 U/ml (LIF), CHIR99021 (3 μM) and PD0325901 (1 μM).

DNA transfection

DNA transfection was accomplished with Lipofectamine 3000 (Thermo Fisher Scientific #L3000001) unless otherwise stated. ES cells were seeded at least 24 h in advance to be ~50% confluent on the day of transfection. Appropriate amounts of DNA were calculated according to manufacturer’s instructions. Media were changed after 6 h, and replaced with antibiotic selection containing medium where appropriate.

Flow cytometry

For fluorescence-activated cell sorting (FACS) or flow analysis, cells were gently dissociated in cell suspension by TrypLE, resuspended in PBS plus FBS 1% (FACS media) and filtered (BD, cup-Filcons #340632). A FACS Aria III (Becton Dickinson) or Attune NxT Flow Cytometer (Thermo Fisher Scientific) were used for sorting or analysis respectively. Data analysis was performed with FlowJo v10.5.3 (Tree Star, Inc.).

Epigenetic editing tool constructs

Epigenetic editing tools comprising dCas9-SCN4, KRAB-GFP-icFv, 3a3L-GFP-icFv, Mut3a3L-GFP-icFv and GFPicFv were cloned into PiggyBac...
recipient plasmids, properly linearised with restriction enzymes, by homology arm recombination using In-fusion HD-Cloning (Takara #639650) according to manufacturer’s instructions (Fig EV1A). For the pPB_TRE3G::dCas9-GCN4_EF1α::TetOn-Hygro, the Streptococcus pyogenes dCas9-GCN4 was PCR amplified from the PlatTET-gRNA2 plasmid (Moria et al, 2016) (Addgene #82559), and cloned together with a d2 destabilisation domain under control of the TRE3G promoter in a PiggyBac backbone vector also containing the TET-ON3G transactivator and the hygromycin resistance gene separated by an IRES sequence and under control of the CAG promoter. For the effector plasmids (pPB_TRE3G::ScFv-GFP-KRAB_EF1α::Neo and pPB_TRE3G::ScFv-GFP-3a3L_EF1α::Neo), the GCN4-specific scFv domain and the sfGFP gene were amplified from PlatTET-gRNA2 pPB_TRE3G::ScFv-GFP-3a3L_EF1α::Neo, the GCN4-specific scFv domain and the sfGFP gene were amplified from PlatTET-gRNA2 plasmid (Addgene #82559) and fused in frame with the human ZNF10 KRAB domain (amplified from the pAAVS1-NdI-CrispRi (Addgene #73498)) or the catalytic domain (CD) of mouse Dnmt3a and the C-terminal part of mouse Dnmt3a and the C-terminal part of mouse Dnmt3a (amplified from pET28-Dnmt3a3L-sc27 (Addgene #71827)) and cloned in PiggyBac plasmids under control of the TRE3G promoter. The effector is also destabilised with a d2 domain and the vector also carries constitutive expression of the Neomycin resistance gene. The control effector gRNA<sub>scFv</sub> was cloned as described above but without any epigenetic domain. Finally, abolition of the cytosine methyltransferase catalytic activity in Dnmt3a CD (Mutha3a3L) was achieved by a single replacement of a cysteine at position 296 by a serine (Hsieh, 1999).

Similarly, the U6::gRNA_EF1α::BFP-Puro, carrying an enhanced gRNA scaffold, was amplified from Addgene plasmid #60955 and cloned into a PiggyBac recipient plasmid (pPB_U6::gRNA_EF1α::BFP-Puro).

To design all sgRNA for targeting the epigenetic editing system, the GPP web portal (Broad Institute) was used. Reverse complement gRNA sequences (Table EV1) with appropriate overhangs were amplified by PCR from the pSpCas9-B2a2-S2a2 (Addgene #60955) according to manufacturer’s instructions (Fig EV1A). For generating the reporter cell lines (pPB_TRE3G::dCas9-GCN4_EF1α::TetOn-Hygro, pPB_TRE3G::ScFv-KRAB_GFP_EF1α::Neo and pPB_U6::gRNA_EF1α::BFP-Puro containing appropriate gRNA sequence and gRNA_EF1α::BFP-Puro digested with BlpI (NEB #R0585S) and BstXI (NEB #R0113S). All ligated assembled plasmids were amplified by bacteria transformation and purified by endotoxin-free mini-preparations (ZymoResearch #D4200). Correct assembly and sequences were confirmed by Sanger sequencing (Genewiz).

**Generation of reporter cell lines**

The heterozygous Esg<sub>1</sub><sup>tdTomato</sup> reporter cell line was derived from the Stella-GFP::Esg<sub>1</sub><sup>tdTomato</sup> (SGET) compound reporter line (Hackett et al, 2018) by CRISPR inactivation of the Stella-GFP reporter. For generating the p53<sup>tdTomato</sup> reporter cell line, we obtained T2A-tdTomato dsDNA sequence by PCR amplification from a donor vector with ultramers carrying 180 bp overlaps complementary to the 3‘ end of the p53 gene. We introduced this into cells by transfection of 129/B6 XY ESC together with the spCas9 plasmid pX459 (Addgene #62988), carrying a single gRNA sequence complementary for the p53 3‘ end. After antibiotic selection for transient px459, TOM<sup>tdTomato</sup> single cells were sorted by at FACS. Single cells were expanded clonally and correct monoallelic integration to generate p53<sup>tdTomato</sup> was validated by PCR genotyping and Sanger sequencing (Genewiz). Normal levels of p53 mRNA expression were verified by qPCR.

**Epigenetic editing and memory assay**

For stable integration of the epigenetic editing system, Esg<sub>1</sub><sup>tdTomato</sup> or p53<sup>tdTomato</sup> WT or KO reporter ESC lines were co-transfected with the Piggybac plasmids: pPB_TRE3G::dCas9-GCN4_EF1α::TetOn-Hygro, pPB_TRE3G::ScFv-KRAB_GFP_EF1α::Neo and pPB_U6::gRNA_EF1α::BFP-Puro containing appropriate gRNA sequence and gRNA_EF1α::BFP-Puro digested with BlpI (NEB #R0585S) and BstXI (NEB #R0113S). All ligated assembled plasmids were amplified by bacteria transformation and purified by endotoxin-free mini-preparations (ZymoResearch #D4200). Correct assembly and sequences were confirmed by Sanger sequencing (Genewiz).

**Cell cycle inhibition**

For cell cycle inhibition, to test active versus passive epigenetic erasure, the Esg<sub>1</sub><sup>tdTomato</sup> cell line, already carrying the dCas9<sup>GXXCN4</sup> and KRAB<sup>GFP-scFv</sup> gRNA<sub>scFv</sub>, was transiently transfected with a pPB_U6::gRNA_EF1α::BFP-Puro containing gRNA against Esg<sub>1</sub> TSS (gRNA<sub>6750v</sub>). 1.2 ng/ml of puromycin selection was added after 6 h together with DOX (100 μg/ml). Cells were cultured for 3 days and then sorted for TOM<sup>top</sup> status. TOM<sup>top</sup> cells were then cultured in absence of DOX for a total of 4 days, with the cell cycle inhibitor RO3306 (9 μM) added after 24 h (when GFP had just switched off), and removed after 48 h. Cells were analysed by flow cytometry at 24 h intervals and gated for absence of expression of GFP and BFP.

**ESC differentiation**

To induce endodermal, ectodermal or epiblast-like cell (EpiLC) differentiation, naïve ESCs were cultured for 5 days in presence of DOX (100 ng/ml) seeded at a confluence of 6 × 10<sup>3</sup>/cm<sup>2</sup> on gelatin-coated plates (for endoderm and ectoderm) or fibronectin-coated plates (for EpiLC) and maintained in 12/i/L media for 24 h in presence of doxycycline (100 ng/ml). After removal of ESC medium and 5× washes with PBS, differentiation was induced as follows: (i) endodermal differentiation was induced with IDE1 (STEMCELL Technologies #72512)-containing medium (Borowiak et al, 2009) (RPML (Thermo Fisher Scientific #12-633-012) supplemented with 0.02% FBS, 2 mM L-glutamine, 5 μM IDE1 and 1% penicillin streptavidin); (ii) ectoderm differentiation was induced with ND1ff (NB27 Takara #40002) supplemented with 0.25 μM Retinoic Acid (Sigma Aldrich#R2625), 0.02% FBS and 1% penicillin streptavidin and (iii) EpiLC differentiation was induced with ND1ff supplemented with 20 ng/ml ActivinA (PeproTech #120-14P), 12 ng/ml bFGF.
(PeproTech #450-33), 1% knockout serum replacement (Thermo Fisher #10828010) and 1% penicillin streptavidin. In all cases, DOX treatment was maintained for the first 24 h of differentiation and then cells were washed five times and cultured for a maximum of 8 days without DOX, with media change every other day. Flow cytometry was performed at 5 and 8 days of differentiation (corresponding to 4 and 7 days of DOX washout, respectively) and, at the same time, cells harvested for bisulphite pyrosequencing and CUT&RUN.

**Growth competition assay**

For assaying the competition advantage of the p53 epigenetically silenced cells, p53<sup>GFP<sup>-tdTomato</sup></sup> reporter line, carrying dCas9<sup>GFP<sup>-cDNA</sup></sup>, KRAB<sup>GFP</sup>-scFv and a gRNA against p53 (gRNA<sup>345up</sup>), was induced with DOX (100 ng/ml). In parallel, p53<sup>tdTomato</sup> reporter line without the epigenetic editing tool from a similar passage number was transfected with a PiggyBac plasmid driving constitutive GFP expression (CAG: GFP) and subjected to two subsequent rounds of sorting to enrich GFP<sup>pos</sup> cells. After 7 days of DOX induction, KRAB-induced p53<sup>tdTomato</sup>-negative (TOM<sup>-neg</sup>) cells were enriched by flow cytometry and equally mixed (1:1) with cells expressing constitutive GFP and subjected to DOX washout. After 5 or 8 days from mixing, cells were analysed by flow cytometry to measure the proportion of GFP<sup>pos</sup> and GFP<sup>neg</sup> cells.

**Generation of knockout ESC lines**

Knockouts (KO) cell lines were generated by transiently transfecting two spCas9 plasmids (pX459) carrying one gRNA each targeting exon junctions to minimise amplification from contaminant DNA (Table EV2). The reaction was performed using SYGreen Blue Mix (PCRbio # PB20.15-20) and a QuantStudio 5 (Applied Biosystems) thermal cycler.

**Bisulphite pyrosequencing**

DNA bisulphite conversion was performed directly starting from cell pellets (a maximum of 1 × 10<sup>5</sup> cells per sample) using the EZ DNA Methylation-Direct kit (Zymo Research #D5021) following the manufacturer’s instructions. Target genomic regions were PCR amplified using 1 µl of converted DNA with biotin-conjugated bisulphite primers (Table EV2), using the PyroMark PCR kit (Qiagen, #978703). Pyrosequencing assay conditions were generated using the PyroMark Q24 Advanced 3.0 software and the sequencing reaction was performed with PyroMark Q24 advanced reagents (Qiagen, #970902) according to manufacturer’s instructions. Briefly, 10 µl of the PCR reaction was mixed with streptavidin beads (GE Healthcare #17-5113-01) by shaking for 5 min at room temperature and, after separation of DNA strands and release of samples into the Q4 plate (Qiagen) using PyroMark workstation (Qiagen), sequencing primers were annealed to DNA by heating at 80°C for 2 min and cooling down at RT for 5 min. Pyrosequencing was run on PyroMark Q24 advanced pyrosequencer (Qiagen) with target-specific dispensation order (Table EV4). Results were analysed with PyroMark Q24 Advanced 3.0 software.

**CUT&RUN**

The CUT&RUN (Cleaveage Under Targets and Release Using Nuclease) protocol (Skene & Henikoff, 2017) was used to detect protein–DNA interaction and histone modifications. Briefly, a total of 3 × 10<sup>5</sup> cells per sample were pelleted and washed twice with wash buffer (20 mM HEPES pH 7.5, 150 mM NaCl and 0.5 mM spermidine containing protease inhibitor) and incubated with conacavalin A magnetic beads (Sigma Aldrich C7555) by rotating for 10 min at room temperature. After placing samples on a magnet stand, the supernatant was removed. Cells were resuspended with HRP-linked secondary antibody incubation was carried on in 0.5% milk/PBS/0.05% tween for 1 h at room temperature. After washing thrice with 0.5% milk/PBS/0.05% tween, detection was performed by incubating the membrane with Pierce ECL western blot solution (ThermoFisher #32132) for 5 min prior imaging with ChemiDoc XRS+ system (BioRad).

**RNA preparation and real-time qPCR**

Total RNA was extracted using the PicoPure RNA Isolation kit (Applied Biosystems #KIT0204) for less than 1 × 10<sup>4</sup> cells or the RNeasy kit (Qiagen #74004) otherwise, following manufacturer instructions. One microgram of RNA was used as input to generate complementary DNA (cDNA), with a mixture of random hexamers and reverse transcriptase, following DNAase treatment (TAKARA PrimeScript RT Reagent Kit with gDNA Eraser #RR047A). A control reaction in which the RNA was incubated with all the other components except the reverse transcriptase enzyme mix (-RT control) was performed. cDNA was diluted 1:10 and specific targets quantified by real-time quantitative qPCR using primers designed at exon–exon junctions to minimise amplification from contaminant DNA (Table EV2). The reaction was performed using SYGreen Blue Mix (PCRBio # PB20.15-20) and a QuantStudio 5 (Applied Biosystems) thermal cycler.
antibody buffer (wash buffer with 0.02% digitonin and 2 mM EDTA) containing 0.5 µg of target-specific antibody (Table EV3), and left rotating overnight at 4°C.

Samples were then placed on a magnet stand to remove antibody buffer, washed thrice with wash buffer containing 0.02% digitonin (Dig-wash buffer), and incubated with 700 ng/ml of purified protein-A–:MNase fusion (pA-MNase) on a rotator at 4°C for 1 h followed by two more washes. MNase reaction was thus activated by adding 4 mM CaCl2 and incubating at 0°C for 30 min and immediately stopped with 1× final concentration of STOP buffer (340 mM NaCl, 20 mM EDTA, 200 mM EGTA, 0.02% digitonin, 250 µg washogen and 250 µg RNaseA). Target chromatin was released by incubating at 37°C for 10 min, centrifuiging at full speed for 5 min at 4°C and the supernatant collected after incubation on magnet stand. DNA was finally released from chromatin by incubation with 0.4% SDS (Promega #V6551) and 0.5 mg/ml Proteinase K (Thermo Fisher Scientific #AM2546) at 70°C for 10 min. Purification and size selection of DNA were performed using SPRI beads (Beckman Coulter #B23318) following the instruction for double size selection with 0.5× and 1.3× bead volume-to-sample volume ratio. CUT&RUN DNA fragments were either subjected to quantitative PCR to amplify selected targets or to next-generation sequencing to evaluate chromatin marks genome-wide.

For CUT&RUN-qPCR, DNA fragments were diluted 10 times with H2O and 2 µl amplified with SYGreen Blue Mix (PCRBio) and primers specific for target and control regions (in which the mark is expected to be enriched (positive controls) or depleted (negative controls)) (Table EV2) using the QuantStudio 5 (Applied Biosystems) thermal cycler. Note that primers were designed to amplify minimum amplicon sizes as CUT&RUN produces small fragments. Relative abundance of histone marks was estimated comparing Ct values of target regions to positive control regions.

For CUT&RUN sequencing, libraries were made starting from 10 ng of CUT&RUN DNA fragments using the NEBNext Ultra II DNA Library Prep Kit for Illumina (NEB #E7645S) using the following PCR programme: 98°C 30 s, 98°C 10 s, 65°C 10 s and 65°C 5 min, steps 2 and 3 repeated for 12–14 cycles depending on input DNA. After quantification and quality check with an automated electrophoresis system (Agilent Tape Station system), library samples were sequenced on the Nextseq Illumina sequencing system (paired-end 40 sequencing). Raw Fastq sequences were trimmed to remove adapters with TrimGalore (v0.4.3.1, -phred33 --quality 20 --stringency 1 -e 0.1 --length 20), quality checked and aligned to the custom mouse mm10 genome with the inserted tdTomato reporter using Bowtie2 (v2.3.4.2, -I 50 -X 800 --fr -N 0 -L 22 --n-score 'S,1,1.15' --n-ceil 'L,0.0,0.15' --dpad 15 --dbar 4 --end-to-end --score-min 'L,-0.6,-0.6'). Analysis of the mapped sequences was performed using seqmonk software (v1.46.0) to generate log2 reads per million (RPM) using the RNA-seq quantification pipeline for directional libraries.

**ATAC-seq**

Prior to harvesting, cells were initially treated in culture medium with 200 U/ml of DNase for 30 min at 37°C to digest degraded DNA released from dead cells. After 5× washes with 1xPBS, cells were detached with TrypLE, 5× 10^5, counted and pelleted at 500 RCF at 4°C for 5 min. Supernatant was removed and cells resuspended in 50 µl of cold ATAC resuspension BUFFER (10 mM Tris-HCl pH7.4, 10 mM NaCl and 3 mM MgCl2) with 0.1% NP40, 0.1% Tween20 and 0.01% digitonin and incubated on ice for 3 min. Lysis was washed out using 1 ml of cold ATAC resuspension buffer with 0.1% Tween20 and mixed. Nuclei were pelleted at 500 RCF for 10 min at 4°C. After removal of supernatant, nuclei were resuspended in 50 µl of transposition mixture (25 µl 2xTD buffer, 2.5 µl transposase (Illumina Tagment DNA Enzyme and Buffer Kit #20034197), 16.5 µl PBS1x, 0.5 µl 1% digitonin, 0.5 µl 10% tween20 and 5 µl H2O) and incubated at 37°C for 30 min in a thermomixer with 1,000 RPM shaking. Reaction product was cleaned-up with DNA clean and concentration kit (Zymo Research #D4014) following manufacturer instructions and eluted in 21 µl of elution buffer. Twenty microlitre of this product was used for PCR amplification using Q5 hot start high-fidelity polymerase (NEB #M0494S) and a unique combination of the dual-barcoded primers P5 and P7 Nextera XT Index kit (Illumina #15055293) following the cycling conditions: 1. 98°C for 30 s; 2. 98°C for 10 s; 3. 63°C for 30 s; 4. 72°C for 1 min; 5. 72°C for 5 min and repeat from 2 to 4 for five cycles. After the first five cycles, 5 µl of the pre-amplified mixture was used to determine additional cycles by qPCR amplification using SYGreen Blue Mix (PCRBio) and the above used P5 and P7 primers in a QuantiStudio 5 (Applied Biosystems) thermal cycler. After qPCR amplification, profiles were manually assessed plotting linear RN versus cycle and the number of the additional PCR cycles to be performed equal to one-third of the maximum fluorescent intensity in this plot (Buenrostro et al, 2015). The identified number of extra PCR cycles were performed by placing the pre-amplified reaction back in the thermal cycler. Final clean-up of the amplified library was performed using the DNA clean and concentration kit (Zymo #D4014) and DNA
amplicons eluted in 20 µl of H2O. After quantification and quality check with an automated electrophoresis system (Agilent Tape Station system), library samples were pooled together and sequenced on the NextSeq Illumina sequencing system (paired-end 40 sequencing).

For sequencing, raw reads were first trimmed with TrimGalore (v0.4.3.1, reads > 20 bp and quality > 30) and then quality checked with FastQC (v0.72). Output files were aligned to custom mouse mm10 genome with the inserted tdTomato reporter using Bowtie2 (v2.3.4.3, paired-end settings, fragment size 0-1,000, --fr, allow mate dovetailing). Uninformative reads were removed with Filter BAM (v2.4.1, mapQuality ≥ 30, isProperPair, IchrM) and duplicated reads were filtered with MarkDuplicates tool (v2.18.2.2). The mapped and filtered sequences were then analysed with seqmonk (Babraham bioinformatics, v1.46.0) by performing enrichment quantification of the normalised reads. Correlation plots were generated by comparing enrichment of reads at promoters in sample versus control conditions.

**Genome-wide CRISPR screen**

For the CRISPR screening, stable integration of spCas9-T2A-GFP was achieved in Esg1tetTomato reporter ESC by insertion into the Rosa26 safe harbour locus by CRISPR targeting with a pair of Rosa26-specific gRNAs. After antibiotic selection and single-cell GFPpos FACS sorting, integrity of the construct was verified by PCR genotyping and Sanger sequencing. The PiggyBac dCas9GCN4 construct was subsequently introduced in these cells as described before and after single colony picking and expansion, successful integration of dCas9GCN4 was functionally tested.

To introduce the genome-wide perturbation, we used lentiviral vectors carrying the Brie gRNA library which contains 76,637 different gRNA that target 19,674 genes (Doench, 2016), produced by Pharmakey. The PiggyBac dCas9GCN4 line already carrying dCas9-GCN4 was functionally tested. After 3 days of DOX washout, 3 × 10^7 cells were sorted with pPB_TRE3G::pPY_CAG_Pbase and pPB_U6::gRNA_EF1a::BFP-Puro containing a gRNA against Esg1 and pPY_CAG_Phase using Xfect mESC transfection reagent (Takara #631320) and selection (neomycin (300 µg/ml) and DOX (100 ng/ml) induction was started after 24 h. After 4 days of DOX washout, 3 × 10^7 cells were sorted in parallel from the pPB_TRE3G and pPB_U6 for genomic DNA extraction as an early time point (D-w (3 days)), using a gating strategy to separate fully silenced cells (pPB_TRE3G) or cells ranging from fully to mildly silenced (pPB_U6). At the same time, 3 × 10^7 unsorted cells were passaged up to a total of 7 days of DOX washout and sorting have been repeated as before to separate TOMneg-wide and TOMpos for the final time point (D-wo (7 days)). Genomic DNA was isolated from purified populations by using DNeasy blood and tissue kit (Qiagen #69504) following manufacturer instruction including RNAsenase step.

DNA libraries were prepared from TOMneg-wide, TOMpos-wide and TOMpos at D-w (3 days) and D-wo (7 days) time points in multiple parallel reactions, each containing 500 ng of gDNA, with custom primers containing the P7 flow cell overhangs (5'-CAAGCAGAGACCGATACCGATNNNNNNNTGACTGGAGTTCAGACGTG TGTCCTTCGGATCTTCAATGTGTTTCCCCCTGACCTGT-3'), including 8 bp barcode and P5 overhang (5'-AATGATACGGCGACACCGAGATCTACACTCTTTCCCTACACGCTCCTTCCTTGT GGAAGACGAAACAGCGG-3') using the Q5 hot start high-fidelity polymerase (NEB #M0494S) for 22–24 cycles. sgRNA amplicons were purified using SPRI beads (Beckman Coulter #B23318) following the instruction for double size selection with 0.5× and 1.2× bead volume-to-sample volume ratio. Purified fragments were checked and quantified with a tape station automated electrophoresis system (Agilent). Equal amplified library amounts were pooled together into a multiplexed library and sequenced for single-end 50 bp (SE-50).

**Statistical analyses**

For analysis of CRISPR screens, counting of sgRNA representation in the isolated subpopulation of cells was performed using the Model-based Analysis of Genome-wide CRISPR-Cas9 Knockout (MAGEK, v0.5.9) tool (Li et al., 2014). Reads were first trimmed using cutadapt (v1.15) (cutadapt -g TTGTGAAAAAGCAGGA-CACCC) and quality checked using FastQC and then, the gRNAs counts were normalised to total reads within the sample (MAGEK - count -norm-method total). Last, the TOMneg-wide and TOMpos-wide were compared to the TOMpos for each time point to identify significantly enriched/depleted gRNAs/gens with a false discovery rate (FDR) < 0.2, using the -test command in MAGEK. Statistical analysis of replicate data including Cut&Run-qPCR and RT-PCR was performed using appropriate strategies in Prism GraphPad statistical software (v8.4.3).

**Embryo manipulation**

All experiments involving mice were carried out in accordance with the approved protocol and guidelines by the laboratory animal management and ethics committee of the European Molecular Biology Laboratory (EMBL) under license. Prior to microinjection,
p53-ΔTomato reporter ESCs were transfected with dCas9GCN4, p53-gRNA4sup and KRAB-GFP-wFPI or alternatively GFP-wFPI and treated with DOX for 7 days. ESC microinjection was performed by the Gene Editing & Embryology Facility of EMBL Rome, using E3.5 embryos derived from natural mating of C57BL/6j mice. Injected embryos were implanted back into pseudo-pregnant foster mothers. All animals employed and procedures were in accordance with the gold standard Italian and European Union regulation guidelines and approved by the local ethical committee.

After 7 days from the injection, at the embryonic development day 10.5, the decidua was collected from the uterus and put into a 6 cm dish with cold PBS+10% FBS. Embryos were then extracted from the decidua and moved to fresh PBS+10% FBS, placenta removed and cleaned from debris and tissue fragments. Individual embryos were moved in one well of a round-bottom 96-well plates (Corning #CLS3367) containing 50 μl of TrypLE and incubated at 37°C for 20 min and pipetted until the embryo is entirely dissociated in single cell. The single-cell suspension was then diluted with 100 μl of PBS+1%FBS and spun down at 1,200 rpm for 5 min. Cell pellet was then resuspended in 300 μl of FACS medium (1xPBS+1%FBS) and filtered (BD, cup-Filcons #340632) for quantitative flow cytometry analysis with Attune Nxt.

Data availability

All data derived from CRISPR screening, RNA-seq, Cut&Run-seq and ATAC-seq have been deposited in publicly available ArrayExpress database under the accession codes E-MTAB-10522 (http://www.ebi.ac.uk/arrayexpress/experiments/E-MTAB-10522/), E-MTAB-10523 (http://www.ebi.ac.uk/arrayexpress/experiments/E-MTAB-10523/), E-MTAB-10524 (http://www.ebi.ac.uk/arrayexpress/experiments/E-MTAB-10524/), E-MTAB-10525 (http://www.ebi.ac.uk/arrayexpress/experiments/E-MTAB-10525/), E-MTAB-11182 (http://www.ebi.ac.uk/arrayexpress/experiments/E-MTAB-11182/) and E-MTAB-11183 (http://www.ebi.ac.uk/arrayexpress/experiments/E-MTAB-11183/).

Expanded View for this article is available online.

Acknowledgements

We thank Agnese Loda and Edith Heard for sharing the TX1072 ESC line and experimental advice, and Pablo Navarro for scientific inspiration. We are grateful to Catrin Lloyd for help with bioinformatics analysis, Marzia Munafò for critical reading of the manuscript and Monica Di Giacomo, Unpnishad Sharma and Tafsut Tala-igihl for experimental assistance. We are also grateful to all EMBL core facilities, in particular to Neil Humphreys (GEF), Jim Sawitzke (GEVF), FCF and GBCS for key experimental support. We thank Claire Rougeulle, Sylvia Erhardt, Eileen Furlong and Mathieu Boulard for their contributions to thesis advisory committee discussions (for V.C.). This study was funded by a European Molecular Biology Laboratory (EMBL) programme grant to J.A.H. Open access funding enabled and organized by ProjektDEAL.

Author contributions

Jamie A Hackett: Conceptualization; Formal analysis; Supervision; Funding acquisition; Writing—original draft; Project administration; Writing—review & editing. Valentina Carlini: Data curation; Formal analysis; Investigation; Writing—review & editing. Cristina Policarp: Methodology.

In addition to the CReDiT author contributions listed above, the contributions in detail are:

VC performed experiments, data analysis and co-wrote the manuscript. CP provided experimental support. JAH performed data analysis, designed and supervised the study, and wrote the manuscript.

Disclosure and competing interests statement

The authors declare that they have no conflict of interest.

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