MacroH2A histone variants act as a barrier upon reprogramming towards pluripotency

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The chromatin template imposes an epigenetic barrier during the process of somatic cell reprogramming. Using fibroblasts derived from macroH2A double knockout (dKO) mice, here we show that these histone variants act cooperatively as a barrier to induced pluripotency. Through manipulation of macroH2A isoforms, we further demonstrate that macroH2A2 is the predominant barrier to reprogramming. Genomic analyses reveal that macroH2A1 and macroH2A2, together with H3K27me3, co-occupy pluripotency genes in wild-type (wt) fibroblasts. In particular, we find macroH2A isoforms to be highly enriched at target genes of the K27me3 demethylase, Utx, which are reactivated early in iPSC reprogramming. Finally, while macroH2A dKO-induced pluripotent cells are able to differentiate properly in vitro and in vivo, such differentiated cells retain the ability to return to a stem-like state. Therefore, we propose that macroH2A isoforms provide a redundant silencing layer or terminal differentiation ‘lock’ at critical pluripotency genes that presents as an epigenetic barrier when differentiated cells are challenged to reprogram.
MacroH2A isoforms are unique H2A histone variants due to the presence of a 30-kDa non-histone domain (macro domain) at their C-termini. MacroH2A variants are generally considered transcriptionally repressive in nature due to their association with forms of condensed chromatin such as the inactive X chromosome (Xi) and inactive genes. MacroH2A1 and macroH2A2 isoforms are encoded by two distinct genes (H2AFY and H2AFY2, respectively), and macroH2A1 is alternatively spliced, resulting in two macroH2A1 isoforms, macroH2A1.1 and macroH2A1.2, that differ by only one exon in the macro domain.

The pluripotent stem cell state is under the control of a highly regulated transcriptional circuitry, that is complemented by chromatin regulation. Embryonic stem cell (ESC) chromatin is considered to be more ‘open’ than that of its differentiated progeny, with robust chromatin remodelling activities to allow for efficient chromatin reorganization that occurs during lineage specification. In keeping with this, deposition of macroH2A1 is globally enriched in differentiated cells as compared with their pluripotent counterparts.

Two recent studies have probed macroH2A isoforms in the context of ESC pluripotency via RNA interference. Both demonstrated that macroH2A isoforms are dispensable for self-renewal, however they conflicted on the role of macroH2A during differentiation. While one study showed that loss of macroH2A isoforms inhibits proper differentiation, another reported that macroH2A-deficient ESCs executed X inactivation efficiently and were able to effectively differentiate towards multiple lineages. Thus, the role of macroH2A in ESC differentiation remains ambiguous, as it has yet to be examined in the context of genetically deficient mice.

MacroH2A isoforms have also been studied in the context of reprogramming via somatic cell nuclear transfer (SCNT). Intriguingly, macroH2A is rapidly removed from the mammalian somatic cell nucleus upon transplantation into mouse oocytes. Using an alternative system of SCNT, Gurdon and colleagues implicated macroH2A as a factor conferring resistance to Xi reactivation of differentiated mammalian nuclei when transferred into Xenopus oocytes.

Pluripotent cells can also be generated via ectopic expression of key pluripotency-related transcription factors (TFs) in somatic cells in order to generate induced pluripotent stem cells (iPSCs). However, the epigenome imparts a barrier during the reprogramming process towards pluripotency. We previously hypothesized that histone variants may act as an epigenetic barrier during somatic cell reprogramming because they are generally incorporated into chromatin in a replication-independent manner, and thus may mark particular genomic regions in fully differentiated cells.

Here, we have examined the contribution of macroH2A isoforms via induced pluripotency using genetically engineered mouse models deficient for both macroH2A1 and macroH2A2. We find that while macroH2A isoforms act cooperatively, macroH2A2 acts as the predominant epigenetic barrier when somatic cells are challenged to reprogram. During normal ESC differentiation and in development, macroH2A isoforms are globally incorporated into chromatin, and deposited at pluripotency genes, such as the Oct4 locus, a master regulator of pluripotency.

Results

MacroH2A is dynamic during differentiation and reprogramming. We investigated the levels of both macroH2A1 and macroH2A2 isoforms in the histone and chromatin fractions of ESCs induced to differentiate by multiple methods. We observed...
increased macroH2A1 and macroH2A2 in the histone fraction of ESCs differentiated by retinoic acid (RA) (Fig. 1a), which was corroborated by quantitative mass spectrometry (qMS)29 (Fig. 1b). Using embryo body (EB) formation assays, we detected similar global histone changes in the chromatin fraction (Fig. 1c). We also observed similar results by comparing ESCs with distinct differentiated cell types such as mouse embryonic fibroblasts (MEFs) and dermal fibroblasts (DFs) (Fig. 1d). Collectively, these data suggest that macroH2A isoforms are specifically deposited into chromatin upon differentiation, as well as during mouse development. Of note, we also observed a decrease in H2A.Z levels in these studies, suggesting that macroH2A and H2A.Z histone variants might have distinct roles during ESC differentiation.

Next we questioned whether macroH2A isoforms are removed from the chromatin fraction upon somatic cell reprogramming. Therefore, we used the Cre-excisable Stemcca polycistronic lentivirus encoding Oct4, Sox2, Klf4 and Myc (OSKM)30 to reprogram multiple batches of DFs isolated from wt 5v/129 mice. We observed that macroH2A1 and macroH2A2 levels are lower in the chromatin fraction of iPSCs when compared with the DFs, while H2A.Z levels are increased (Fig. 1e), and qMS analysis confirmed our immunoblot results (Fig. 1f). Together, these results suggest that low levels of macroH2A contribute to the pluripotent state and that macroH2A isoforms might act as a barrier to iPS reprogramming in somatic cells.

Characterization of macroH2A knockout DFs. In order to address this hypothesis, we isolated DFs from both wt and macroH2A1- and macroH2A2-deficient newborn mice (dko mice) (Fig. 2a; Supplementary Fig. S1a). These dKO mice are viable and free of obvious developmental defects (J.R.P., manuscript in preparation). Both male and female DFs were isolated in order to account for any potential sex differences that might be revealed during iPS reprogramming, as macroH2A coats the Xi in differentiated female cells (Fig. 2a).

Next, we examined the staining pattern of macroH2A1 in the DFs. As expected, wt cells have macroH2A staining throughout the nucleus and an obvious Xi was observed in female cells. In contrast, no detectable nuclear staining was observed in the dKO cells of either sex (Fig. 2b). We also examined the staining pattern of H3K27me3, a facultative heterochromatic mark that tracks with macroH2A genomic binding and is enriched on the Xi6,7,9. Both female cells of wt and dKO origin retain an Xi as evidenced by H3K27me3 (Fig. 2b). These results are consistent with studies showing that loss of macroH2A1 does not reactivate the Xi31. As the efficiency of reprogramming can be affected by the proliferation rates of the starting somatic cells32, we examined the growth properties of wt and dKO DFs. Using proliferation assays, as well as 5-ethyl-2'-deoxyuridine incorporation studies to detect cells in S phase, we did not find significant differences between wt and dKO DFs (Supplementary Fig. S1b,c). Thus, any potential differences observed upon reprogramming are not due to the initial proliferative state of the DFs.

iPS reprogramming is enhanced in the absence of macroH2A. Using these well-characterized DFs, we performed iPS studies using Stemcca polycistronic lentivirus encoding OSKM (Fig. 2c). By performing fluorescence-activated cell sorting (FACS) analysis for stage-specific embryonic antigen-1 (SSEA1), we observed a notable increase in the SSEA1 + population in macroH2A dKO cells early in the reprogramming process (4–8 days) (Fig. 2d). This suggests that loss of macroH2A isoforms facilitates the early stages of reprogramming. Alkaline phosphate (AP) staining, which marks undifferentiated ES-like cells, demonstrated enhanced reprogramming in macroH2A dKO cells (Fig. 2e,f). These results were corroborated by an independent reprogramming protocol via 4 factor (4F) OSKM reprogramming with individual retroviral plasmids33, or through the expression of a Stemcca lentiviral vector encoding 3F OSK (excluding cMyc) (Supplementary Fig. S2).

Next, reprogrammed colonies were stained for markers of pluripotency, including Oct4 and Nanog (Supplementary Fig. S3). Nanog staining demonstrates bona fide pluripotency of the iPSC colonies, as it is not encoded by Stemcca. By counting Nanog-positive colonies, we confirmed enhanced reprogramming in the absence of macroH2A isoforms (Fig. 2f). Of note, a tandem staining protocol of AP staining followed by Nanog immunofluorescence demonstrated that the majority of AP-positive colonies were fully reprogrammed (Fig. 2f).

Collectively, these studies suggest that loss of macroH2A isoforms enhances iPS reprogramming. We note here that although macroH2A was reported to be a barrier to Xi reactivation in SCNT studies by Gurdon and colleagues34, we show here that macroH2A acts as a barrier to iPS reprogramming independent of its role in X inactivation, as male and female cells reprogram with similar efficiencies (Fig. 2d–f).

macroH2A2 is the predominant barrier to reprogramming. We next inquired which macroH2A variants have a role in the reprogramming process. In order to address this question, we utilized DFs derived from both single (macroH2A1- or macroH2A2-deficient) and dKO mice (Fig. 3a). While both single KOs showed a trend toward increased reprogramming, we only observed a significant increase in reprogramming in the dKO DFs (Fig. 3b). This suggests that macroH2A1 and macroH2A2 act cooperatively in the reprogramming process.

In order to tease apart the role of the macroH2A1 splice variants, macroH2A1.1 and macroH2A1.2, and investigate barrier function by all individual macroH2A isoforms, we ectopically expressed macroH2A1.1, macroH2A1.2 and macroH2A2 as green fluorescent protein (GFP)-fusion proteins in dKO DFs (Fig. 3c and Supplementary Fig. S4a–c). Compared with canonical histone H2B, macroH2A2 significantly suppressed the enhanced reprogramming phenotype. While macroH2A1.1 had no effect, macroH2A1.2 showed a slight decrease in reprogramming efficiency (Fig. 3d). These data suggest that macroH2A2 acts as the predominant epigenetic barrier, with a potential contribution from macroH2A1.2. Intriguingly, the expression of multiple macroH2A variants simultaneously in dKO DFs (mH2A1.1 + mH2A1.2, mH2A1.2 + macroH2A2, all three isoforms) did not show significant barrier function, although mH2A1.2 + macroH2A2 displayed a slight decrease in AP-positive colonies (Supplementary Fig. S4d,e). This might be due to competition between variants or to deregulation of incorporation into chromatin when variants are simultaneously overexpressed. In addition, because only macroH2A1.1 has the ability to bind ADP-ribose34, this function may prevent macroH2A1.1 from being involved in creating a barrier for iPS reprogramming and, in turn, inhibit macroH2A1.2 or macroH2A2 function.

macroH2A co-localizes genomically with H3K27me3 domains. In order to gain insights into the mechanism by which macroH2A isoforms inhibit reprogramming, we hypothesized that their absence might alter the histone post-translational modification (PTM) landscape. By qMS analysis we show that iPS reprogramming affects wt and dKO PTM changes similarly, including a striking increase of H3K27ac and decrease of H3K27me3 levels (Fig. 4a; Supplementary Fig. S5). H3K27me3 and macroH2A occupancy have similar patterns by ChIP-chip analysis35, and recently, H3K27me3 demethylation (via Utx) was
shown to be a key step in iPS reprogramming\textsuperscript{35}. Therefore, we further dissected the interplay between macroH2A and H3K27 PTMs.

We next investigated macroH2A1, macroH2A2, H3K27me3 and H3K27ac genomic occupancy by native chromatin immunoprecipitation followed by deep sequencing (ChIP-seq) in wt DFs (Supplementary Fig. S6). We also performed ChIP-seq for K27 PTMs in dKO DFs in order to examine any potential differences in their patterns in the absence of macroH2A (see below; Supplementary Fig. S6). Consistent with our previous macroH2A1 ChIP-seq studies in K562 cells, we observed that transcription start sites (TSSs) lack macroH2A isoforms, while macroH2A-containing nucleosomes are present at upstream regulatory regions and/or gene bodies, and that macroH2A1 and macroH2A2 form large domains\textsuperscript{10} (Fig. 4b; Supplementary Fig. S6b). We also observed that macroH2A1, macroH2A2 and H3K27me3 followed a similar occupancy pattern around TSSs, while H3K27ac was enriched at TSSs\textsuperscript{36} (Fig. 4b). Of note, macroH2A1 ChIP in macroH2A dKO cells was performed as a control, generating a very low number of unique alignments and an enrichment pattern similar to Input sample (Supplementary Fig. S6).

Based on the ChIP-seq signal around the TSS of the six different data sets (of all annotated autosomal genes), we were able to identify four distinct classes of genes (Fig. 4c). Class I in particular, consists of genes bound by macroH2A1 and...
macroH2A that are enriched for H3K27me3, and devoid of H3K27ac (Fig. 4c and Supplementary Fig. S7a; see below). In this class of genes, there is strong macroH2A enrichment both upstream and downstream of the TSS, which we have previously shown to associate with transcriptionally repressed genes10. Next, by comparing all ChIP-seq reads using a pair-wise correlation analysis, we observed a striking correlation between macroH2A1 and macroH2A2 (Pearson correlation \( R = 0.92 \) for TSS; \( R = 0.94 \) for genome-wide) (Fig. 4d and Supplementary Fig. S7b). We therefore combined macroH2A1 and macroH2A2 target genes (referred to as macroH2A-bound genes), for further analysis. Hierarchical clustering also confirmed a correlation between both macroH2A isoforms and H3K27me3, while none of these repressive marks positively correlate with H3K27ac (Supplementary Fig. S7b). Interestingly, we did not observe a significant difference in enrichment of H3K27me3 and H3K27ac in the absence of macroH2A, as calculated using a Pearson correlation for the samples in wt and macroH2A dKO DFs (Fig. 4c, Supplementary Fig. S7c).

Collectively, global analysis of our ChIP-seq data demonstrates the following: (i) a striking genome-wide correlation between macroH2A1 and macroH2A2, which correlates with H3K27me3 and (ii) the absence of macroH2A isoforms does not globally alter H3K27 PMTs in the ground state of DFs.

**Figure 3 | macroH2A2 is the predominant epigenetic barrier in reprogramming.** (a) Chromatin extracts from wt, macroH2A1 /−/− (m1KO), macroH2A2 /−/− (m2KO) and dKO DFs probed with macroH2A1 and macroH2A2 antibodies. Amido black of core histones used for loading. (b) Representative wells of AP-positive iPS colonies at 12 days post infection, bright field image of single colonies shown; Scale bar, 100 μm. Quantitation of fold-change in AP-positive colonies over wt (shown right); mean ± s.d. (\( n = 4 \)); P-value (asterisk): \( P = 0.002 \) (wt versus dKO). (c) Chromatin extracts from dKO DFs expressing GFP-tagged macroH2A isoforms (top arrow), H2B-GFP (bottom arrow) and vector alone blotted for GFP. Amido black of core histones used for loading. (d) Representative wells of AP-positive iPS colonies at 12 days post infection, bright field and fluorescence image of individual dKO colonies expressing H2B-GFP and macroH2A GFP-tagged isoforms; Scale bar, 100 μm. Quantitation of fold-change in AP-positive colonies over wt (shown right); (o) represents uninfected; mean ± s.d. (\( n = 3 \)); unpaired Student’s test (two tailed) P-value (asterisk): \( P = 0.007 \) (wt versus dKO + H2B); \( P = 0.036 \) (dKO + H2B versus dKO + m2).

In order to probe the role of macroH2A as a barrier to reprogramming, we queried its presence at genes bound by at least one of the 4F (OSKM)37. These factors activate the ESC-specific transcriptional network through their binding mostly at promoter regions.37 We found that macroH2A is present at ~24% (810/3309) of OSKM-bound genes, while H3K27me3 occupies ~18% (613/3309) in DFs (Fig. 4e).

Next, we investigated the profiles of the master regulators of pluripotency. By examining the Oct4 (Pou5f1) and Nanog loci, using the UCSC browser, a macroH2A/H3K27me3 domain is evident (Fig. 4f). Such domains often span large regions of up to 100–200 kb, encompassing multiple genes and their regulatory regions. Next, we confirmed the macroH2A landscape at the Pou5f1 locus (which encodes Oct4) by ChIP-quantitative PCR (qPCR) analysis. We observed a peak of macroH2A1 enrichment upstream of the transcriptional start site (TSS) at the enhancer elements of Pou5f1 (Supplementary Fig. S8a). The distal enhancer (−2 Kb) is a site where the pluripotency TFs bind and regulate Oct4 expression in ESCs.38 ChIP analysis of Oct4 itself, Nanog and Sox2 demonstrates distinct binding at the −2 kb position in ESCs (Supplementary Fig. S8b). Furthermore, we performed ChIP analysis for macroH2A1 at the distal enhancer element in ESCs undergoing differentiation, which shows macroH2A1 deposition upon RA differentiation (Supplementary Fig. S8c), and is consistent with its global chromatin deposition (Fig. 1).
in reinforcing silent states of pluripotency genes in differentiated cells. These data, combined with an SSEA1-positive population early in the reprogramming process in macroH2A dKO cells (Fig. 2d), led us to query published data sets describing genes induced early in reprogramming. Recently the H3K27me3 histone demethylase, Utx, was described as a critical enzyme in the induction of iPS reprogramming35. The findings of Hanna and colleagues35, we find marked enrichment of a class of ~1400 genes that are transcriptionally upregulated in either normal or aberrant H3K27me3 levels. In keeping with the findings of Hanna and colleagues35, we find marked enrichment of H3K27me3 (88%) at the aberrantly methylated genes in wt DFs (Fig. 5b). Our analysis also revealed a striking enrichment of macroH2A-bound genes at these Utx target genes (75%), which contains TFs such as Sall and Sall4 (Fig. 5b). Using the UCSC browser, we observed distinct macroH2A/H3K27me3 domains at these pluripotency promoting factors (Fig. 5c).

To address the role of macroH2A occupancy at these genes, we probed the kinetics of pluripotency gene activation during reprogramming. Using quantitative reverse transcription–PCR (qRT–PCR) analysis, we compared the activation of multiple Utx target genes that are also bound by macroH2A in both wt and mH2A dKO DFs. Indeed, the absence of macroH2A leads to a more robust, and in some cases, an earlier transcriptional activation of such genes (Fig. 5d). Collectively, our data suggests that macroH2A and H3K27me3 cooperate in repressing pluripotency...
Absence of macroH2A does not impair pluripotency. Next, we queried whether macroH2A isoforms have a significant role in differentiation. Therefore, as depicted in Fig. 2c, iPSC colonies were picked and expanded in order to generate both wt and dKO iPSC lines. These cells were treated with a Cre recombinase-expressing adenovirus to remove the Stemcca cassette (Supplementary Fig. S9a). Using these lines, we investigated pluripotency potential in the absence of macroH2A isoforms.

Both wt and dKO iPSCs grew similarly to ESCs, without signs of spontaneous differentiation (Fig. 6a; bright field). In addition, AP and Nanog-positive staining was observed in both dKO and wt iPSCs (Supplementary Fig. S9b). Next, we examined the reactivation of the Xi, a hallmark of fully reprogrammed iPSCs, through H3K27me3 staining. Neither the wt nor the dKO iPSCs (two female lines of each examined) showed evidence of an Xi (Supplementary Fig. S9b). Therefore, the dKO cells displayed ESC-like features such as self-renewal, expression of pluripotency markers, and showed no evidence of Xi chromosomes. These results are consistent with recently published studies whereby loss of macroH2A isoforms in ESCs (via shRNAs) did not show evidence of a compromised ESC state.

Next, the pluripotency of wt and macroH2A dKO iPSCs was challenged. Differentiation was induced via EB formation assays, and similar morphology was observed between the two genotypes (Fig. 6a). To further examine the EB differentiation, we dissected the EB differentiation, we dissected the EB differentiation, we dissected the EB differentiation, we dissected the EB differentiation, we dissected the EB differentiation, we dissected the EB differentiation. qRT–PCR demonstrated that loss of macroH2A does not affect silencing of markers, and showed no evidence of Xi chromosomes. These results are consistent with recently published studies whereby loss of macroH2A isoforms in ESCs (via shRNAs) did not show evidence of a compromised ESC state.

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these genes (Fig. 6b). Although macroH2A is deposited at pluripotency genes (such as Pou5f1) during differentiation (Supplementary Fig. 58c), they are also silenced by additional mechanisms such as H3K27me3, as well as H3K9me3 and DNA methylation40,41. This suggests that macroH2A is not required for initial silencing of pluripotency genes, but may act to maintain silencing in coordination with other silencing mechanisms, akin to its role at the Xi4.

We also examined the kinetics of lineage marker expression towards the three germ layers upon EB differentiation. This analysis displayed remarkable similarity between wt and dKO iPSCs (Fig. 6c). Moreover, wt iPSCs show deposition of macroH2A1 and macroH2A2 in the chromatin fraction upon differentiation, with similar kinetics to ESCs (Fig. 1c), while macroH2A dKO cells display no signals, as expected (Supplementary Fig. S10d). Chromatin-bound levels of both Oct4 and H2A.Z decreased in a similar fashion in wt, dKO cells, and ESCs (Supplementary Fig. S10d; Fig. 1c).
ips-derived teratomas (Fig. 6d). No significant differences were observed between wt and macroH2A dKO cells for any of the lineage markers (Fig. 6d). Collectively, our pluripotency studies indicate that the absence of macroH2A isoforms does not compromise the self-renewal or differentiation potential of dKO iPSCs.

To assess the capacity of such differentiated cells to return back to an ESC-like state, differentiated EBs (day 20) were then challenged in ESC media in the presence of leukemia inhibitory factor (LIF). After one week, the number of colonies formed per cell plated was determined (Fig. 6e). Consistent with our iPSC reprogramming studies, we find that EB differentiated cells lacking macroH2A showed an increased ability to form AP-positive colonies, even after a prolonged period of differentiation, suggesting they retain stem-like plasticity.

Discussion

In recent years, there has been an overwhelming interest in pluripotency because of their potential to understand developmental processes and treat human disease42. Therefore, the ability to reprogram somatic cells to a pluripotent state, particularly by iPSC methodologies, has generated much excitement43. Yet despite its potential impact, the molecular mechanisms of reprogramming, especially as they relate to chromatin biology, remain unclear.

Recent studies have begun to address the role of chromatin factors during reprogramming, such as Chd144, the BAF45 complex and Wdr546. Interestingly, many of the global PTM changes that we found by our qMS analysis of iPSCs versus DFs (Fig. 4a), have recently been described to be necessary for iPSC reprogramming. These include loss of K79me2/3 (via antagonizing Dot1L methyltransferase)47, loss of K36me2/3 (mediated by Kdm2b)48, loss of H3K9me2/3 (via antagonizing Suv39H1 or G9a methyltransferases)47,49 and relevant to this study, loss of H3K27me3 (mediated by Utx)35. Moreover, increased global levels of acetylation such as H3K27ac and H3K9ac were also detected by qMS, and are modulated through inhibition of HDACs27 (Fig. 4a; Supplementary Fig. S5).

However, little is known of the role of histone variants in the context of iPSC reprogramming. Here we demonstrate that macroH2A acts as a barrier to somatic cell reprogramming. macroH2A is enriched in somatic cells enabling pluripotency genes to be maintained in a repressed state in differentiated cells. This deposition is correlated with enrichment of the repressive mark H3K27me3. Upon overexpression of reprogramming factors in differentiated cells, macroH2A provides an extra layer of silencing at pluripotency genes, and therefore acts as a barrier to reprogramming. The histone demethylase Utx is required to remove H3K27me3 at a subset of genes, also bound by macroH2A, that need to be reactivated upon reprogramming. Differentiation of pluripotent stem cells is not impaired in the absence of macroH2A, as it likely acts as a final ‘lock’ upon other silencing mechanisms. However, differentiated cells (for example, EBs) lacking macroH2A are more amenable to reactivation of the pluripotent state, further suggesting that its presence induces a barrier to reprogramming in differentiated cells.

Here we have explored the genomic landscape of macroH2A and demonstrated that macroH2A1 and macroH2A2, along with H3K27me3, are physically present at pluripotency genes in differentiated cells. Notably, macroH2A deposition is significantly enriched at Utx target genes, which are critical during the early
stages of reprogramming. In fact, in macroH2A dKO cells, the expression of SSEA1 (an early marker of reprogramming) was significantly increased when compared with wt fibroblasts (Fig. 2d). This suggests that loss of macroH2A isoforms facilitates the early stages of reprogramming upon ectopic OSKM (and OSK) expression, possibly by allowing for more efficient chromatin remodelling or facilitating demethylation of H3K27me3. Consistent with this, our kinetic studies during reprogramming suggest that Utx target genes are more efficiently reactivated in macroH2A dKO DFs than in wt cells (Fig. 5d).

Despite the strong correlation between macroH2A-bound genes and H3K27me3, the absence of macroH2A isoforms does not affect H3K27me3 localization in DFs, suggesting a redundancy between the two repressive modifications. Although the mechanism by which macroH2A gets deposited in chromatin is unknown (ATRX negatively regulates macroH2A deposition10), its chromatin incorporation may be important to stabilize H3K27me3 at pluripotency genes during reprogramming, thus acting as a barrier. Alternatively, since aberrantly methylated Utx target genes are also bound by macroH2A1 and macroH2A2, it is possible that H3K27me3-containing nucleosomes are a flag for macroH2A deposition.

Here we propose that macroH2A isoforms provide a redundant silencing layer at pluripotency genes that, in turn, presents as an epigenetic barrier when differentiated cells are challenged to reprogram (Fig. 7). Our data shows that macroH2A is not required for the initial silencing of pluripotency genes during differentiation, but is incorporated into pluripotency gene regulatory sites during the process. This is similar to the late deposition of macroH2A at the Xi, and suggests that formation of a multi-layered barrier prevents reactivation of unwanted genes in a somatic cell that might trigger alternative cell fates. In turn, we find that while cells derived from the genetically deficient macroH2A mouse model display enhanced reprogramming, they do not showed impaired differentiation. This is in contrast with a recent study where deletion of macroH2A isoforms in ESCs via RNA interference showed differentiation defects18. These differences may be attributed to the approaches used (knockout versus knockdown) or technicalities of the differentiation methods used.

In closing, deciphering the regulation of transcriptional programs during development by incorporation of histone variants may broaden our perspectives on cell identity, that is, by restricting cellular plasticity in the case of macroH2A, or by modulating cellular memory, as has been suggested for the H3 variant H3.3 (ref. S1). As macroH2A has been implicated as a tumour suppressor52,53, these new avenues may also enhance our understanding of cancer biology.

**Methods**

**Cell culture.** DFs, MEFs, and HEK293 cells were grown in DMEM (CellGro) with 10% FBS and 1% Penicillin/Streptomycin CCE ESCs (ES cell line derived from a male 129/Ola mouse strain), E14 (ES cell line derived from a male 129/Ola mouse strain) and iPSCs were maintained in standard ES media (see Supplementary Materials and Methods).

**Plasmids.** The 4F (Oct4, Sox2, Klf4 and Myc) used for iPSC reprogramming are encoded in a polynuclear lentiviral vector (Stemca, kindly provided by Gustavo Mostoslavsky, Boston University). Human H2B-GFP is encoded in pLKO.1, and GFP-tagged rat macroH2A1.1, rat macroH2A1.2 and human macroH2A2 constructs were cloned into this same plasmid.

**Differentiation procedures.** For RA differentiation, ESCs were plated on 1.0 g of Matrigel per cm² at a density of 5 × 104. The next day, LIF was removed and 2 µM of RA was administered. EBs were formed by plating 1 × 106 cells into low attachment condition dishes (suspension culture) in ES media without LIF.

**DF Isolation.** Pregnant females of known genotype were individually caged on day E15.5. Pups were killed following the Institutional Animal Care and Use Committee (IACUC) guidelines (protocol # 003255, University of Pennsylvania), and skins were carefully removed and placed in sterile PBS. Pups were sexed by checking the presence of the male or female gonads and were grouped according to their sex. Skins were placed with the dermal side down into a sterile 35 mm petri dish and floated in 0.25% trypsin-EDTA overnight in 4°C. The following day, the skin was washed and removed and the plated cells incubated in DMEM for 1 h at 37°C. The dermis was shaken to release the fibroblasts, and this mixed cell population was pelleted and plated in DMEM with 10% FBS, 1% P/S, 2 mM ATP, 1 mM GTP, and 2 mM -Glutamine. Calcium was raised to 2 mM to induce calcium-dependent differentiation and detachment of contaminating keratinocytes.

**iPS reprogramming.** iPS reprogramming was performed as described13 with slight modifications. Early passage DFs were plated on DMEM with 10% FBS and 1% Penicillin/Streptomycin 1 day before iPS reprogramming to the well surface area (10 000 cells on a 24-well plate, 50 000 cells on a 6-well plate or 500 000 on a 100-mm dish). Cells were infected using virus concentrated in fresh media and 8 ng ml⁻¹ Polybrene (Millipore) overnight. For overexpression of the GFP-tagged histones, cells were plated as described above, and infected the following day. Two days later they were passaged and infected with 4F the following day. ESC media was added 2 days after infection, and 4-5 days post infection the cells were trypsinized and re-plated onto 0.3% gelatin-coated plates with reactivated MEFs. AP staining was performed according to the manufacturer’s protocol (Stemgent). Immunofluorescence for Nanog was performed on 6-well plates after AP staining, as described below. SSEA1 staining was performed using a mouse monoclonal anti-SSEA-1 antibody conjugated to Phycoerythrin, and was performed according to the manufacturer’s protocol (BD). Staining was analysed by FACs on a LSRII machine and data was analysed with Flowjo.

**Chromatin fractionation and histone acid extraction.** Procedures performed as described10.

**Quantitative Mass Spectrometry.** qMS was performed as described53,54.

**Immunofluorescence and immunoblot.** Immunoblot were performed as described10. For immunofluorescence, ESCs, iPSCs or DFs were plated on chamber glass slides precoated with Matrigel (BD Biosciences). Immunofluorescence was performed as described41. Fluorescently conjugated secondary antibodies were subsequently used Alexa-488/594 (Molecular Probes). Nuclei were counterstained with 4’-diamidino-2-phenylindole (DAPI) and slides were mounted in vectashield (Vector Laboratories). Images presented were acquired on a Zeiss Imager Z1 microscope via deconvolution of 20–30 Z-stack projections using the AxioVision 40 Version 4.8.1.0 software, or were taken from a single projection. For a full list of antibodies used, please see Supplementary Materials and Methods.

**complementary DNA isolation and qPCR.** RNA was extracted with the QIAGEN RNeasy Mini Kit. A total of 1 µg of RNA was used to synthesize complementary DNA (cDNA) using Superscript II and Oligo d(T) primers (Invitrogen). qPCR and mRNA analysis was carried out as described41. cDNA expression was normalized to L7. Primer sequences are provided in Supplementary Materials and Methods.

**Native Chromatin Immunoprecipitation and ChIP-seq.** Native miH2A1 ChIP (Abcam, ab37264), H3K27me3 (Millipore, 07-449) and H3K27ac (Abcam 4729) ChIP-seq data analysis was performed as described10. ChIP-seq data was performed above. Primer sequences are provided in Supplementary Materials and Methods.

**ChIP-seq data analysis.** Sequence reads were aligned to the mouse genome NCBI build 37 (UCSC, mm9) using Bowtie short read aligner57 (v 0.12.7), with the following parameters: seed of 50 bp, maximum two mismatches, suppression (m) = 20, and reported alignments (e) = 20. Wiggle files (HAFZ2, unpublished pipeline D.H.), were generated using a 500-bp window sliding 250 bp, counting the number of aligned reads (5’-end of each aligned read), for both ChIP and Input samples. The number of alignments from each window was normalized to the total number of alignments and scaled by factor of 10⁷, to allow comparison between different samples. MAE software59 (v 1.4.1) was used to identify peaks (P-value cutoff = 1.00e-06, 5e-5 for K27me3 and K27ac; 5e-3 for mH2A1; 5e-4 for mH2A2), (bw = 300). Genes bound by either macroH2A1, macroH2A2, H3K27me3 or H3K27ac were found by Peak2Gene57 software tool (www.cistrome.org), allowing a span of 5 kb upstream or downstream of the peak. Association of histone variants/chromatin modifications with enrichment in broad domains was performed using Sicer60 (Window 200 bp, Gap 200 bp, fragment size 150 bp, P-value cutoff = 0.01).
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**Author contributions**

A.G.M, K.R. and E.B. conceived of this study. A.G.M performed DF isolations, all iPS experiments, and ChIP. Z.A.Q. performed immunoblots, ESC differentiation, and qRT–PCR. A.G.M. and D.H. performed native ChIP-seq. Chip-seq analysis performed by A.G.M., D.H. and D.V.-G. K.R. performed immunoblots, ESC differentiation, and ChIP. N.A.I. performed mouse husbandry and skin isolations, and C.C. generated KO mice with the support of J.R.P. C.S. performed teratoma experiments with support of I.R.L. G.L., S.L., and B.A.G. performed and analysed qMS data. J.R.P. provided mice for these studies. A.G.M., K.R. Z.Q., and E.B. designed experiments and interpreted results. E.B. wrote the manuscript with contributions from A.G.M. and J.R.P., with input from all other coauthors.

**Additional information**

Supplementary Information accompanies this paper at http://www.nature.com/naturecommunications

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An image in Supplementary Fig. S9b in this Article, reporting αH3K27me3/DAPI immunofluorescence in iPS cells from the ‘dKO’ group, was inadvertently duplicated from the ‘wt’ group. The correct version of the figure appears below.