Maternal SMCHD1 regulates Hox gene expression and patterning in the mouse embryo

Parents transmit genetic and epigenetic information to their offspring. Maternal effect genes regulate the offspring epigenome to ensure normal development. Here we report that the epigenetic regulator SMCHD1 has a maternal effect on Hox gene expression and skeletal patterning. Maternal SMCHD1, present in the oocyte and preimplantation embryo, prevents precocious activation of Hox genes post-implantation. Without maternal SMCHD1, highly penetrant posterior homeotic transformations occur in the embryo. Hox genes are decorated with Polycomb marks H2AK119ub and H3K27me3 from the oocyte throughout early embryonic development; however, loss of maternal SMCHD1 does not deplete these marks. Therefore, we propose maternal SMCHD1 acts downstream of Polycomb marks to establish a chromatin state necessary for persistent epigenetic silencing and appropriate Hox gene expression later in the developing embryo. This is a striking role for maternal SMCHD1 in long-lived epigenetic effects impacting offspring phenotype.

It is now clear that epigenetic information can be passed from generation to generation via the germ line, changes which can have long-lasting effects in the offspring. One of the most notable of these effects is the transmission of epigenetic information from the oocyte to the zygote. The oocyte supplies the entire cytoplasm containing all expressed mRNA and proteins to the zygote, sustaining it through its initial cell divisions until its own zygotic genome is transcribed, at embryonic day (E) 2.0 in mice. Genes whose expression is required in the oocyte for normal development of the offspring are known as maternal effect genes.

A classic example of the role of maternal effect genes in passing long-lived epigenetic information from parent to offspring is genomic imprinting, where genes are monoallelically expressed in a parent-of-origin-specific manner. Epigenetic imprints are imparted by germ cell-derived DNA methylation or trimethylation of lysine 7 on histone 3 (H3K27me3)²⁴. Maternal effect genes important for imprinting generally have a role in establishing and maintaining these germline marks⁵⁶.

Structural maintenance of chromosomes hinge domain containing 1 (Smchd1) is a recently defined maternal effect gene that is expressed in the oocyte and is required for genomic imprinting in the mouse placenta⁷⁸. In its zygotic form, SMCHD1 plays a key role in the epigenetic silencing of imprinted loci, along with other clustered gene families and the inactive X chromosome¹⁰⁻¹⁵. Heterozygous variants in SMCHD1 are also associated with the human diseases Facioscapulohumeral muscular dystrophy (FSHD) and Bosma arhinia microphthalmia (BAMS)¹⁶⁻¹⁹, demonstrating the important role SMCHD1 plays in normal development.

SMCHD1 is a member of the SMC family of proteins, large chromosomal ATPases important for chromosome structure²⁰. SMCHD1

---

1The Epigenetics and Development Division, WEHI, Parkville, VIC, Australia. 2The Department of Medical Biology, The University of Melbourne, Parkville, VIC, Australia. 3EMBL Australia, Monash University, Clayton, VIC, Australia. 4Australian Regenerative Medicine Institute, Monash University, Clayton, VIC, Australia. e-mail: edwina.mcglinn@monash.edu; blewitt@wehi.edu.au
also plays a role in chromatin architecture, mediating long-range interactions at its targets\textsuperscript{21,22}. Recruitment to at least one of its targets, the inactive X chromosome, is dependent on the polycomb repressive complex 1 (PRC1) mark ubiquitination of lysine 12 of histone H2A (H2A-K119ub)\textsuperscript{22,23}. For imprinted genes we have proposed that Smc1D1 is recruited downstream of PRC2's mark H3K27me\textsuperscript{3}. Precisely how zygotic or maternal Smc1D1 enables gene silencing is not yet clear.

One of the clustered gene families zygotic Smc1D1 binds and silences is the Hox genes\textsuperscript{24,25}, a highly conserved set of transcription factors that are responsible for correct patterning of body segments along the anterior-posterior (A-P) axis during embryonic development\textsuperscript{26-29}. Hox genes are only expressed at specific times and in specific tissues during post-implantation embryonic development\textsuperscript{30,31}. At all other times they are silent and marked by H2A-K119ub and H3K27me\textsuperscript{3,32} including in the oocyte and through pre-implantation development\textsuperscript{33-35}, opening the exciting possibility of maternal effects on Hox gene expression. Based on these data and Smc1D1's role as a maternal effect gene, we investigated whether maternal Smc1D1 has long-lasting effects at its targets in the embryo, specifically on the Hox genes. This was made possible because, unlike many maternal effect genes, deletion of maternal Smc1D1 does not result in embryonic lethality\textsuperscript{36}.

In this study we showed that maternal Smc1D1, found in the pre-implantation embryo, is required to prevent premature Hox gene activation in the early post-implantation embryo. Interestingly, these changes occurred without disruption of H2A-K119ub or H3K27me\textsuperscript{3} marks over Hox genes in the pluripotent state, and without significant loss of these Polycomb marks during differentiation, suggesting that maternal Smc1D1 acts downstream of Polycomb to regulate gene expression and normal skeletal patterning post-implantation.

**Results**

**Maternal Smc1D1 is required for normal skeletal patterning**

Given that previous work in our lab has shown that Smc1D1 mutants exhibit homeotic transformations\textsuperscript{19,20}, we first assessed whether Smc1D1 maternal knockout embryos also show abnormal skeletal patterning. We set up two crosses between wild-type Smc1D1 (Cast) parents, using MMTV-Cre or Zp3-Cre to knock out Smc1D1 in the oocyte (Fig. 1a–c) as we have previously\textsuperscript{3}. We set up three types of F1 crosses. The first was a control cross yielding embryos with wild-type Smc1D1 function (Smc1D1\textsuperscript{+/-}). This established a baseline of skeletal patterning in the F1 embryos (Fig. 1a). Almost all of these embryos had normal skeletal patterning, with 97% and 86% of control mice from the MMTV-Cre and Zp3-Cre colonies respectively having the expected 7 cervical vertebrae, 13 thoracic vertebrae, 6 lumbar vertebrae and 4 sacral vertebrae (Fig. 1d). In the second cross, Smc1D1 was deleted in the oocyte with either MMTV-Cre or Zp3-Cre, yielding Smc1D1 heterozygous embryos which lacked maternal Smc1D1 (Smc1D1\textsuperscript{ +/- }, Fig. 1b). We analysed skeletal patterning in embryos derived from both Cre models to ensure any phenotype observed was robust. The third cross, performed with the MMTV-Cre only, was reciprocal to the maternal deletion cross and generated both Smc1D1\textsuperscript{ +/- } (Smc1D1\textsuperscript{ +/- } and Smc1D1\textsuperscript{ +/- }) embryos, with the oocytes from which they were generated having wild-type levels of Smc1D1 (Fig. 1c). This latter cross tested whether any phenotype observed in the Smc1D1\textsuperscript{ +/- } embryos was due to haploinsufficiency for Smc1D1 after zygotic genome activation rather than lack of maternal Smc1D1, and controlled for the direction of the interstrain cross.

Smc1D1\textsuperscript{ +/- } embryos exhibited a highly penetrant addition of a rib on the seventh cervical element (C7), suggesting that C7 adopts the identity of T1 (Fig. 1b, e). We observed several morphological variations of this additional rib including a short ectopic rib, a rib which fused with T1 with and without subsequent bifurcation before joining the sternum, and a full rib which joined the sternum independently of T1 (Supplementary Data 1). Grouped together, any indication of C7 transformation was observed in the MMTV-Cre and Zp3-Cre models at a penetrance of 97% and 91%, respectively (Fig. 1d). Additional posteriorizing transformations were observed in a subset of Smc1D1\textsuperscript{ +/- } embryos when Smc1D1 was deleted with MMTV-Cre. These included (i) the loss of ribs on T13 leading to a complete T13-to-L1 transformation or severely hypomorphic ribs on T13, and (ii) a L6-to-S1 transformation. These phenotypes were observed at a lower penetrance than the additional C7 rib; 63% and 52% for the transformation altering T13 and L6 respectively (Fig. 1d). All three of these phenotypes had significantly higher penetrance following the maternal deletion of Smc1D1 compared to both the control and reciprocal cross (p < 0.001, chi-square test), and there was no sex-specificity in the phenotypes observed (Supplementary Data 1). Of note, when a lumbar transformation was observed, it was almost exclusively coincident with transformations at cervicothoracic and thoracolumbar transitions. This suggests serial homeotic transformation in these embryos, supported further by examples of transformation of vertebra surrounding these transition points where specific morphology can be delineated (e.g. C5-to-C6 and T1-to-T2; Supplementary Data 1). It is unclear why we observed fewer abnormalities and a lower penetrance of the common C7 transformation in the Zp3-Cre model. Potentially the heterozygosity for Smc1D1 (Smc1D1\textsuperscript{ +/- }) in the MMTV-Cre model mothers before Cre ablation, or the higher genetic heterogeneity (+/- genetic background) in the MMTV-Cre animals than the Zp3-Cre animals may play a part. Nonetheless, taken together, deletion of maternal Smc1D1 results in a highly penetrant posterior homeotic transformations that can encompass multiple axial regions, implying a potential global shift in patterning effectors. Given there were no abnormalities observed in the Smc1D1 heterozygous skeletons (Fig. 1c, d), these data support the view that maternal Smc1D1 is required for appropriate axial patterning.

We further examined the Smc1D1\textsuperscript{ +/- } embryonic skeletons and did not observe any striking abnormalities beyond those in the axial skeleton. This is consistent with no major abnormalities being observed in Smc1D1 null skeletons previously\textsuperscript{3}. Our previous work showed no significant effect of loss of maternal Smc1D1 on viability at mid-gestation or at weaning\textsuperscript{1}. Therefore, we chose to focus our attention here on maternal Smc1D1's regulation of Hox genes and axial patterning.

**Maternal Smc1D1 prevents precocious Hox gene activation**

We investigated whether there were changes in Hox gene expression in Smc1D1\textsuperscript{ +/- } embryos that may explain the homeotic transformation phenotype. We first re-analysed our published RNA-seq (RNA-seq) data from control and Smc1D1 maternal null E2.75 morula. Hox genes were not readily detectable, and there was no change observed in the maternal null compared with control morulae for the single detectable Hox gene (Hoxb13, Supplementary Figure 1). Given the challenge with detecting low level expression in low input samples, we went on to examine post-implantation developmental stages. The tissue we chose for RNA-sequencing was tailbud tissue of the E8.0-E8.5 embryo, dissected just anterior to the level of the node. This tissue contains the caudal end of the presomitic mesoderm (PSM) and the region harbouring progenitors of the vertebral column, the neuro-mesodermal progenitors (NMPs)\textsuperscript{39,40}. It is the Hox expression signatures within cells prior to somite formation that are known to instuct vertebral morphology later in development\textsuperscript{41}.

We conducted RNA-seq in tailbud tissue from Smc1D1\textsuperscript{ +/- } and Smc1D1\textsuperscript{ +/- } embryos from the MMTV-Cre model, with somite-matched replicates (Fig. 2a, b, Supplementary Fig. 1). We chose 6–11 somites for two reasons. Firstly, we wanted to focus on our most penetrant and robust change which was observed in the C7 vertebral element. Secondly, we theorised that loss of maternal Smc1D1 may lead to precocious Hox gene activation. As we focused on the most anterior and most penetrant change in the absence of maternal Smc1D1, we have
used timepoints most appropriate for analysis of anterior Hox gene expression. There was very limited differential expression genome-wide in these somite-matched samples. Indeed, what limited differential expression was present can be explained by the sex disparity in samples at each somite number (Supplementary Data 2). Moreover, there was no difference in somite range between control and Smchd1 maternal null embryos (Supplementary Data 2), suggesting there was no striking developmental delay following loss of maternal SMCHD1. Consistent with this, Hox gene expression was approximately normal in Smchd1wt and Smchd1Δ tailbud samples (Fig. 2c, d). As expected, most posterior Hox genes are not appreciably expressed at this time (Hox10–13). When we compared these two somite series, we saw a collective, albeit modest downregulation of Hox genes from the Hox1 to Hox9 paralogues in 6 somite tissue, followed by the modest upregulation of these genes in somite 8–11 tissue, specifically a trend toward precocious activation of the Hox2 to 7 paralogues in somite 10 and 11 tissue (Fig. 2e, f). We also observed a concomitant downregulation of posterior Hox genes that were detectably expressed (Hox10 and 11), particularly at the earlier somite stages (Fig. 2e, f).

To confirm and complement these genomic data, we performed whole-mount in situ hybridisation in E10.5 embryos from the MMTV-Cre model for Hoxc6 (Fig. 2g), one of the upregulated Hox genes in the tailbud of 9–11 somite embryos whose expression visually demarcates and functionally patterns the cervico-thoracic transition. Three of four Smchd1mat Δ embryos showed appreciable staining of Hoxc6 anterior to the normal expression boundary, which was not observed in the four Smchd1wt controls. These data are consistent with an anterior shift in Hoxc6 expression boundary in the absence of maternal SMCHD1, the axial skeleton phenotype and tailbud RNA-seq data. To further explore the precocious activation of Hox genes in the absence of maternal SMCHD1, we opted for an in vitro approach by differentiating murine embryonic stem cells (mESCs) into neuromesodermal progenitors (NMPs). We derived Smchd1wt and Smchd1Δ mESCs, deleting in the oocyte with Zp3-Cre and performed RNA-seq in

Fig. 1 | Maternal SMCHD1 is required for normal skeletal patterning. a–c Upper panels: the genetic crosses used to create control (a), maternal null (b) and reciprocal cross heterozygous control (c) embryos for skeletal analysis. The black mouse represents C57BL/6 strain, the brown mouse Castaneus strain. Middle and lower panels: E17.5 skeletons stained with alizarin red (bone) and alcian blue (cartilage), showing a sagittal view of the cervicothoracic region and dorsal view of the thoraco-lumbar-sacral region. The asterisks in b indicate abnormalities compared with the standard axial formulae found in the controls. The skeletons shown are from MMTV-Cre crosses. Scale bar is 5 mm. d The summarised data for all skeletons are provided for MMTV and Zp3-Cre. The asterisks indicate statistical significance in comparing to control (two-tailed chi-square test, degrees of freedom = 1, *** p < 0.001, n = 29 and 28 for the MMTV-Cre and Zp3-Cre control crosses, respectively, n = 33 and 23 for the MMTV-Cre and Zp3-Cre maternal deletion crosses respectively, n = 10 and 21 for Smchd1wt and Smchd1het skeletons respectively from the reciprocal cross using MMTV-Cre). Source data are provided in Supplementary Data 1. e Cartoon depiction of normal skeletal patterning and the Smchd1Δ phenotype are shown in e for the cervico-thoracic region.
the mESCs, where we observed no significant differentially expressed genes (n = 3, Supplementary Data 3, Supplementary Fig. 2). Next, we differentiated the Smchd1mat and Smchd1matΔ mESCs, harvesting RNA from differentiating cells every 12 to 24 h from their pluripotent to NMP-like state (n = 4, Fig. 3a). Just as observed in vivo, there was no differential expression when analysed genome-wide (Supplementary Fig. 2). The differentiation progressed as expected with the loss of pluripotency factor expression and increase in differentiation factors (Supplementary Fig. 2). At day 2 of differentiation, we observed a subtle decrease in Hox gene expression, although these genes were barely expressed above the detection limit of RNA-seq. 12 h after Wnt activation (day 2.5), we observed precocious activation of several anterior Hox genes from the Hox1 to 9 paralogues in the Smchd1mat cells (Fig. 3b, Supplementary Fig. 3), which corresponds to approximately E8.5 in vivo. These combined effects were more noticeable as a larger log fold change between day 2 and day 2.5 of differentiation in the maternal null compared with control cells for anterior Hox genes (Fig. 3c, d, p < 0.001). At day 3 (NMPs and mesodermal progenitors) and day 4 (24 h after GDF11 addition) corresponding to E9.5 in vivo, we observed a general downregulation of Hox gene expression, consistent with the Hox gene activation we observe at day 2.5 being precocious but not sustained (Fig. 3b).

To test whether the changes in Hox gene expression were due to loss of maternal SMCHD1 or haploinsufficiency for SMCHD1, we generated Smchd1mat and matched control mESC using the MMTV-Cre model with the deleted Smchd1 allele inherited from the father, as we had for the assessment of axial patterning (Fig. 1a). Using RNA-seq in an identical differentiation series as for the Smchd1wt and Smchd1mat mESCs, we found that Smchd1wt cells differentiated normally and had no significant differential expression when compared with their matched SMCHD1-replete controls (Supplementary Fig. 2). When Hox gene expression was examined more specifically, heterozygosity for Smchd1 did not influence Hox gene expression (Supplementary Fig. 3). These data suggest that absence of maternal SMCHD1, rather than haploinsufficiency for SMCHD1, causes precocious Hox gene expression during differentiation.

**Maternal SMCHD1 does not act upstream of Polycomb marks in mESCs**

To investigate the mechanism by which loss of maternal SMCHD1 caused upregulation of anterior Hox genes, we assessed whether the histone marks H2A.K119ub and H3K27me3 were perturbed in Smchd1mat mESCs. These histone marks are laid down by Polycomb repressive complex 1 (PRC1) and 2 (PRC2) respectively and induce a heterochromatic gene-silencing state at the Hox clusters in mESCs. Moreover, both marks are laid down on the maternal chromatin at Hox clusters and elsewhere in the genome. For non-canonical-imprinted genes, maternal H3K27me3 and H2A.K119ub are required for their silent state. Based on recent work from our lab on the role of maternal SMCHD1 at non-canonical imprinted genes, we hypothesised that maternal SMCHD1 acts downstream of PRC1 and PRC2. If this were the case, H3K27me3 and H2A.K119ub would be unperturbed over Hox clusters in Smchd1mat mESCs. We carried out CUT&RUN for H3K27me3 and H2A.K119ub in biological triplicate samples of Smchd1wt and Smchd1mat mESCs, and compared our data to publicly available Chip-seq datasets for these histone marks in mESCs (Supplementary Data 4). We observed enrichment of H3K27me3 and H2A.K119ub at the expected regions for mESCs, including the four Hox clusters (Fig. 4a, b, Supplementary Fig. 4); however, we found no change in
H3K27me3 or H2AK119ub enrichment between Smchd1wt and Smchd1mat Δ mESCs over the Hox clusters (Fig. 4a, b, Supplementary Fig. 4), and a very high positive correlation between the two genotypes genome-wide (Fig. 4c, d, $R^2 = 0.9266$ and $0.8721$, respectively). Moreover, there was no significant difference when considering just the maternal or paternal allele of each cluster (Supplementary Fig. 5).

Next, we tested whether loss of maternal SMCHD1 destabilised Polycomb marks during differentiation, which might account for the upregulation of Hox genes in its absence. We collected samples for CUT&RUN for H3K27me3 and H2AK119ub at days 2, 2.5 and 3 of mESC differentiation to NMPs, as this is when we observed changes in Hox gene expression. It has been shown that H3K27me3 marks are eroded from the anterior end of the HoxD cluster as these Hox genes are switched on in tailbud tissue43, so we expect a similar anticorrelation between gene expression and Polycomb coverage in our in vitro differentiation. We observed a subtle decrease in H3K27me3 and H2AK119ub enrichment at the anterior end of each Hox cluster during differentiation compared with the pluripotent state (Fig. 4e, f, Supplementary Fig. 6) although with some background signal still present, likely owing to the heterogenous population of cells present in the differentiation culture. To statistically test the differentiation-induced changes in the Polycomb marks and those induced by loss of maternal SMCHD1, we used the csaw package44 to call differential peaks both between Smchd1wt and Smchd1mat Δ samples and between each day of differentiation. This genome-wide method called very few differential peaks between days, none of which were over Hox clusters (Supplementary Data 6). Csaw peaks were also called between Smchd1wt and Smchd1mat Δ samples within each timepoint, again with few significant genome-wide peaks called and none over Hox clusters.

To look more closely at changes in Polycomb coverage over each Hox gene, FPKM values over Hox genes (normalised to genome-wide MACS peaks) were compared as a representation of the expected change in anterior to posterior Hox gene marking. In the pluripotent mESC we observed no significant difference between the Polycomb mark enrichment at Hox1 and Hox13 genes (Fig. 4g, h). At each stage of differentiation, we observed significantly lower enrichment of Polycomb marks at Hox1 compared with Hox13 genes, as expected due to activation of the anterior genes during this period (Fig. 4i–n). Interestingly, when comparing each set of Hox genes at each timepoint we observed significantly higher coverage of H2AK119ub (but not H3K27me3) at each set of Hox genes in Smchd1mat Δ samples compared to Smchd1wt at days 2 and 2.5 of differentiation (Fig. 4i, j, Supplementary Data 6). This increase in H2AK119ub coverage is not what we would expect given the upregulation of Hox genes seen at day 2.5 of differentiation.

**Fig. 3 | Precocious activation of anterior Hox genes in differentiating Smchd1 maternal null mESCs upon Wnt activation.** a mESC differentiation to NMP experimental setup, with media components indicated, along with timing of samples taken for RNA-seq. b Heatmap of the average log2 fold change for the expressed Hox genes between the Smchd1 maternal null and control samples. $n = 4$ from two technical replicates of two mESC lines derived from separate blastocysts for all days except Smchd1mat Δ day 2.5 where $n = 3$ from technical replicates of two independent mESC lines. c Heatmap of the average log2 fold change of Hox gene expression between the day 2.5 and day 2 samples, for each genotype. d The log2 fold change for the Hox1–9 genes between day 2.5 and day 2 of differentiation ($n = 27$ genes), for the average of the 4 replicates at day 2 and 3 replicates at day 2.5 for each genotype (Student’s t-test, two-tailed, equal variance). Source data are provided in Supplementary Data 3.
Fig. 4 Maternal SMCHD1 does not act upstream of H3K27me3 and H2AK119ub in mESCs. a H3K27me3 CUT&RUN in Smchd1<sup>wt</sup> and Smchd1<sup>matΔ</sup> ESCs cultured in 2i + LIF medium over the HoxA cluster as marked. n = 3 independent mESC lines per genotype, the average of which is shown. Genes are shown in grey above the CUT&RUN enrichment tracks and genome coordinates are shown below. Green indicates H3K27me3, dark green for Smchd1<sup>wt</sup> and light green for Smchd1<sup>matΔ</sup>. Y-axis is FPM. b as in a but for H2AK119ub. Blue represents H2AK119ub, dark blue for Smchd1<sup>wt</sup> and light blue for Smchd1<sup>matΔ</sup>. c Scatter plot of log2 transformed normalised counts for Smchd1<sup>wt</sup> and Smchd1<sup>matΔ</sup> over 43,798 H3K27me3 MACS2 peaks in mESCs called from<sup>41</sup>. The Pearson coefficient indicates very high correlation between the two genotypes (R<sup>2</sup> = 0.9266). d As in c but for H2AK119ub, 23,680 peaks called from<sup>42</sup>. Pearson coefficient again indicates very high correlation between the genotypes (R<sup>2</sup> = 0.8721). e as in a but sampled throughout the mESC to NMP differentiation series at days 2, 2.5 and 3. n = 4 from technical replicates of two independent mESC lines, derived from separate blastocysts for all days except Smchd1<sup>matΔ</sup> days 2.5 and 3 where n = 3 from technical replicates of two independent mESC lines, the average for which is shown. f as in e but for H2AK119ub, n = 4 from two technical replicates of two mESC lines derived from separate blastocysts for all days except Smchd1<sup>matΔ</sup> day 2 and Smchd1<sup>matΔ</sup> day 2.5 where n = 3 from technical replicates of two independent mESC lines, the average for which is shown. g, i, k, m. Comparison of H3K27me3 CUT&RUN FPKM values over grouped Hox1 and Hox13 genes, normalised to FPKM reads over genome-wide MACS peaks within each replicate to correct for efficiency of immunoprecipitation, each for cells grown in 2i + LIF (i), harvested at day 2 (k), day 2.5 (m) and day 3 (o). Individual Hox genes from each replicate are plotted with mean ± SEM. P-values were generated with unpaired, two-tailed t-tests, corrected for multiple testing with the Benjamini-Hochberg method<sup>46</sup> (ns = not significant, exact P-values given on graphs for significant results). h, j, l, n As in g, i, k, m but for H2AK119ub. n = 3 per genotype for day 2 and 2.5 and n = 4 for day 3 samples, in each case Hox 1 is 3 genes, Hox 13 is 4 genes. Source data are provided in Supplementary Data 4, 5 and 6.
differentiation, and is inconsistent with destabilisation of Polycomb marks following loss of maternal SMCHD1, but is suggestive of an interplay between SMCHD1 and Polycomb post-implantation.

We next asked whether alterations to DNA methylation may account for the effects of maternal SMCHD1. First, we re-analysed our previously published whole genome bisulphite sequencing data of E2.75 Smchd1 maternal null embryos, and found very low methylation over all FoxC3 Hox clusters and no difference in methylation between Smchd1mat and Smchd1mut embryos (Supplementary Fig. 7, Supplementary Data 7). Next, we performed reduced representation bisulphite sequencing at days 2 and 2.5 of differentiation in Smchd1mat and Smchd1mut differentiating mESCs. Again, we observed very low levels of methylation at the CpG islands at the Hox clusters, with no statistically significant difference in DNA methylation between Smchd1mat and Smchd1mut samples (Supplementary Fig. 7, Supplementary Data 7). These data suggest that maternal SMCHD1 does not influence Hox gene expression via modulation of DNA methylation.

Given that the Smchd1 maternal null mESCs retained their maternal effect on Hox gene expression and that loss of maternal SMCHD1 does not disrupt acquisition and/or maintenance of the Polycomb repressive complex marks or CpG island methylation throughout differentiation, within the limitations of our experiments, we conclude that maternal SMCHD1 does not act upstream of Polycomb and DNA methylation in its regulation of Hox clusters and other areas of the genome.

Discussion

In this study we have shown that maternal SMCHD1 is required for appropriate patterning of the axial skeleton, linked to its role in silencing Hox genes. Deletion of Smchd1 in the oocyte results in the highly penetrant posteriorising homeotic transformations of C7-to-T1, T13-to-T1 and L6-to-S1. This phenotype is similar to what is observed in zygotic PRC1 subunit knockouts and is attributable to Hox gene overexpression. We too observed a modest but consistent upregulation of anterior Hox genes both in vivo in the developing tailbud at E8.5 and in vitro soon after induction of Wnt signalling in mESC differentiating into NMPs. Hox gene silencing was restored later in differentiation, consistent with the relatively subtle axial patterning defects observed in the Smchd1 maternal null embryos. Interestingly, maternal SMCHD1 was not required to maintain appropriate Hox gene silencing earlier in development, either in the pluripotent state or in the morula. These data suggest that maternal SMCHD1, which controls the embryo in the preimplantation period, is required to ensure Hox genes are not prematurely activated in the post-implantation period. This is a long-lived effect of maternal SMCHD1 at the Hox clusters from approximately E2.75 when zygotic SMCHD1 is activated to around E8.5 when precocious Hox gene activation is observed.

Given the important role of PRC1 and PRC2 in silencing the Hox genes, their role in long-lived mitotic epigenetic memory, and the fact that they mark the Hox genes with H2AK119ub and H3K27me3 from the oocyte stage onwards, we asked whether maternal SMCHD1 may function together with the PRCs to silence Hox genes, using our mESC model. In undifferentiated mESCs we observed no change in H3K27me3 or H2AK119ub marks genome-wide in Smchd1 maternally deleted cells compared to control. These data suggest that maternal SMCHD1 acts downstream of Polycomb marks in this system, consistent with previous work showing that zygotic SMCHD1 acts downstream of H2AK119ub on the inactive X chromosome, that H3K27me3 is unchanged over SMCHD1 targets in zygotic Smchd1 null NSCs, and the role for maternal SMCHD1 at non-canonical imprinted genes controlled by H3K27me3 and H2AK119ub. Potentially, the retention of H2AK119ub and H3K27me3 in the absence of maternal SMCHD1 explains why Hox genes are not aberrantly expressed prior to E8.5.

In our embryos we observe a fairly restricted set of posterior homeotic transformations, with the highest penetrance observed for transformations at the anterior end of the skeleton and upregulation of anterior Hox genes pertinent to these transformations. Recent studies have reported the dynamic changes in H3K27me3 and H2AK119ub through the preimplantation period, relevant to this consideration. Zheng et al. showed that although H3K27me3 decorates the Hox clusters in the oocyte and sperm, paternal H3K27me3 is erased and is regained post-implantation, while maternal H3K27me3 remains constant at Hox loci. Mei et al. showed that the same is true for H2AK119ub except it is regained on the paternal allele by the early two-cell stage, faster than paternal H3K27me3 is regained. Considering Polycomb coverage over both alleles, Chen et al. showed both H3K27me3 and H2AK119ub were unchanged from the oocyte to morula stage for the posterior 3’ end of the HoxC cluster. Meanwhile, H3K27me3 was erased over the anterior end of the cluster, from the 1-cell to morula stages. H2AK119ub coverage was also reduced, but only at the 1-cell stage. Seeing as this is the window of time when exclusively maternal SMCHD1 protein is present in the embryo, maternal SMCHD1 may affect anterior Hox genes because lower levels of H3K27me3 and H2AK119ub create a higher dependence on maternal SMCHD1 for an appropriate chromatin state at the Hox genes. However, based on the timepoints sampled in our study, we cannot exclude a role for maternal SMCHD1 in also regulating more posterior Hox genes. These studies also show that neither the dynamic changes in Polycomb marks as the early embryo develops, nor the allele-specificity of them, is captured in mESC, as we also find in our CUT&RUN data from mESCs. Hence, although our Smchd1 maternal null mESCs appear to retain the maternal effect of SMCHD1 as they exhibit anterior Hox upregulation, in the future it will be important to study the effects of maternal SMCHD1 in the preimplantation period to fully elucidate the role of maternal SMCHD1 in regulating the Hox genes.

Maternal SMCHD1 acting downstream of Polycomb does not fully answer the question of how deletion of Smchd1 in the oocyte has such a long-lasting effect on the embryo, days after activation of zygotic SMCHD1. Given that zygotic SMCHD1 has a role in maintaining chromatin architecture, specifically long range chromatin interactions, including at Hox clusters, it is possible that this long-lasting epigenetic memory exists in the form of a particular chromatin conformation at Hox clusters that is put in place early in development by maternal SMCHD1. Without maternal SMCHD1, we propose that the chromatin state of the Hox clusters is destabilised, leaving Hox genes prone to inappropriate activation over time. While the Polycomb marks remain, zygotic SMCHD1 activation at the late morula stage appears to be insufficient to ensure appropriate Hox gene silencing later in development. Potentially this is because zygotic SMCHD1 cannot restore the chromatin architecture required for Hox silencing at the late morula stage or afterwards, as the establishment of such a chromatin state needs to occur within the context of the dynamic epigenetic reprogramming that happens earlier in pre-implantation development. If the Polycomb marks are sufficient for silencing in the short term, why would a maternal SMCHD1-mediated chromatin state be required to prevent premature Hox gene activation post-implantation? The early post-implantation period is another time of wholesale epigenome remodelling as the embryo undergoes germ-layer specification and gastrulation. Potentially a destabilised chromatin state created by the absence of maternal SMCHD1 is liable to disruption in the context of such genome-wide remodelling. Our data using CUT&RUN for H3K27me3 and H2AK119ub and reduced representation bisulphite sequencing to analyse DNA methylation at CpG islands during mESC differentiation suggest that these marks are not destabilised in the absence of maternal SMCHD1, at least not to the extent that would allow us to quantitate such a difference in bulk cell populations using our in vitro differentiation system. Therefore, we have not yet been able to identify the specific features of any such destabilised chromatin state. We did observe an enrichment of
H2AK19ub, although not H3K27me3, at day 2 and 2.5 of differentiation over Hox genes in the absence of maternal SMCHD1. This cannot explain the upregulation of Hox genes at this time, but may instead reflect an altered chromatin state, similar to our previously identified enrichment of H3K27me3 on the inactive X chromosome in the absence of zygotic SMCHD1 concomitant with major changes to the architecture of this chromosome. Since we have not examined the Polycomb marks in Smchd1 heterozygous samples, it remains possible that the subtle changes in H2AK19ub are due to haploinsufficiency for SMCHD1 rather than maternal effects, unlike those on Hox gene expression.

Xue et al. recently reported skeletal abnormalities and over-expressed Hox genes in the oocyte of a zebrafish model of maternal Smchd1 knockout. They also showed evidence that zygotic knockout of Lrf1, a known binding partner of SMCHD1, phenocopies the Smchd1 maternal knockout phenotype, suggesting that LRF1 could be involved in the same regulatory pathway of Hox expression as SMCHD1. These complementary results indicate a striking evolutionary conservation of Hox genes by maternal SMCHD1 in both mouse and zebrafish, although the mechanism of regulation in each model organism remains unknown.

Although further work is required to elucidate how maternal SMCHD1 has a long-lasting epigenetic memory in the developing embryo, this study shows that maternal SMCHD1 is required for appropriate Hox gene expression and, consequently, is also required for normal skeletal patterning in the mouse embryo. This work is relevant to our understanding of how maternal proteins influence offspring phenotypes, and may be relevant to humans considering the pathogenic variants in SMCHD1 observed in several human diseases.

Methods

Mouse strains and genotyping

Mice were bred, housed and maintained in accordance with standard animal husbandry procedures and experiments performed were approved by the WEHI Animal Ethics Committee under the animal ethics numbers 2018.004, 2020.048 and 2020.50. Mice experienced temperature in the range of 20–22 °C, 30–45% humidity and a 14 h light/10 h dark cycle. Smchd1fl/+ mice carrying the MMTV-Cre transgene, and Smchd1floxed mice carrying the Zp3 Cre transgene were created as previously described and maintained on the C57BL/6 background.

In this study three crosses were carried out to assess the effect of deleting Smchd1 in the oocyte on the developing embryo. The control cross involved Smchd1 WT MMTV-Cre+/+ or Zp3 Cre+/+ females and Castaneus (Cast) Smchd1+/+ male mice to generate Smchd1+/+ (Smchd1+/−) embryos. The maternal deletion test cross was carried out with Smchd1+/− MMTV-Cre+/+ or Zp3 Cre+/+ females and Cast Smchd1+/+ male mice to generate Smchd1+/− (Smchd1−/−) embryos which developed from Smchd1 homozygous-null oocytes. The third cross was a reciprocal cross between a Cast Smchd1+/− female and a Smchd1+/−, MMTV-Cre−/− male to generate Smchd1−/− and Smchd1+/− (Smchd1+/−) embryos. The reciprocal cross was only conducted with MMTV-Cre, and not Zp3-Cre, with the purpose of controlling for the heterozygosity of Smchd1−/− embryos. The FI nature of the embryos allows for allele-specific genomic analysis due to differential SNPs between the C57BL/6 and Cast genomes.

Genotypes were determined by PCR using GoTaq Green (Promega) for Smchd1, the X and Y chromosomes via OtC and Zfy respectively, and for the Cre transgene, TCAGGTGTGCTCCAGGCC, CCATGGAAGCAATGTGGGA and GGACGCTAAGCTTCGACAG were the oligonucleotides used to detect the Smchd1 deleted, Smchd1 floxed and Smchd1 wild-type alleles. CTGACGTTACACAAAT TTCCCTG and GATATAAGGAGCATTCCAGTTC were the oligonucleotides used to detect the Cre transgene. GTTCCTTGCTTTCCCCCTCTC and GCCATTACATGAGGACATC were used to detect OtC, GACTGACATGTCTAACATCTGTCG and CCTATTGCAGACAGCTTATG were used to detect Zfy.

Skeletal preparations

Whole-mount skeletal staining was performed on E17.5 embryos as previously described. Skin and organs were removed, embryos dehydrated and remaining tissue dissolved in acetone. After staining, skeletons were cleared in KOH, washed through a glycerol/water series and imaged in 100% glycerol. Images were acquired with a Vision Dynamic BK Lab System at the Monash University Paleontology Lab. Images were taken with a Canon 5D MkII with a 100 mm Macro lens (focus stop 1:3/11). Multiple images were taken to extend the focal depth, and stacked in ZereneStacker using the PMax algorithm. Two people independently scored vertebral formulae of each skeleton, blind to genotype and sex.

Whole-mount in situ hybridisation

Whole-mount in situ hybridisation was performed as previously described with some modifications. E10.5 embryos from the MMTV-Cre model were dissected in ice-cold DEPC-treated PBS and fixed in 4% paraformaldehyde (PFA), rotating overnight at 4 °C. All steps were performed by keeping at room temperature (RT) unless otherwise specified. Embryos were dehydrated by washing through a graded methanol/DEPC-treated PBS with 1% Tween-20 (PBT) series (25%, 50%, 75%, 100%) for 10 min in each solution. Embryos were then stored at −20 °C until in situ hybridisation, at which point all embryos (both genotypes) were processed in parallel to limit inter-experiment variation. To begin in situ hybridisation, embryos were rehydrated by washing through a reversed graded methanol/PBT series for 10 min in each solution, then washed twice for 5 min in PBT. Embryos were then treated with 10 µg/mL proteinase K for 15 min before being washed twice for 5 min each in PBT, then post-fixed with 4% PFA and 0.2% glutaraldehyde for 20 min and washed twice for 5 min each in PBT. Embryos were subsequently put in pre-warmed hybridisation solution (50% formamide; 5 x SSC (pH 4.5), 1% SDS; 50 µg/mL heparin; 50 µg/mL yeast tRNA (Sigma, R6750)) at 65 °C for 1 h, then 1 µg/mL DIG-labelled riboprobe was added and embryos were incubated at 65 °C, rocking overnight. The following day, embryos were washed in Solution I (50% formamide; 5 x SSC (pH 4.5); 1% SDS) three times for 30 min each, rocking at 65 °C. Embryos were then washed in Solution II (50% formamide; 2 x SSC (pH 4.5); 0.1% Tween-20) three times for 30 min each, rocking at 65 °C, then were washed in TBS with 1% Tween-20 (TBST) three times for 5 min. Embryos were put in blocking solution (TBST with 10% heat-inactivated sheep serum) for 2 h at RT, then blocking solution was removed and embryos were transferred to blocking solution containing 1:2000 anti-DIG antibody (Roche, 11093274910) then were incubated rocking overnight at 4 °C. The next day embryos were washed in TBST three times for 5 min at RT, five times for 1 h at RT, then overnight at 4 °C. The following day, embryos were washed in NNT (100 mM NaCl, 100 mM Tris-HCl (pH 9.5); 1% Tween-20) 3 times for 10 min before colour development in BM purple (Roche, 11442074001), rocking at room temperature, protected from light for approximately 4 h. Colour development was stopped, at the same time across all samples, by washing in PBT three times for 5 min, then embryos were post-fixed in 4% PFA overnight at 4 °C. Embryos were imaged as described above for skeletal preparations. The plasmid for Hoxc6 riboprobe generation was a kind gift from P. Sharpe.

Tailbud dissection

Tailbud dissection and somite counting was performed as previously described. In brief, embryos from the MMTV-Cre model were dissected in ice-cold DEPC-treated PBS. Tailbud tissue was horizontally dissected at a distance of 1.5 somites below the last segmented somite.
to ensure no contaminating somite tissue was included. Tailbud tissue was snap frozen on dry ice and stored at −80 °C for later RNA extraction. The yolk sac was used for genotyping. Somites were counted before fixing each embryo in 4% DEPC-treated paraformaldehyde at 4 °C overnight. Embryos were washed through a graded methanol/PBT (DEPC-treated PBS with 1% Tween-20 (v/v)) series as described above for whole-mount in situ hybridisation, before brief staining in dilute ethidium bromide solution and imaging under a fluorescence dissection microscope to confirm somite counting.

### mESC derivation and culture

mESCs were derived and cultured as previously described. Females were superovulated with five IU Folligon (MSD Animal Health Australia) 2 days before mating and 5 IU chorulon (MSD Animal Health Australia) on the day of mating. E3.5 blastocysts were flushed from the uterine horns of these females with M2 medium (Sigma-Aldrich) and were washed twice in 2i + LIF medium [KnockOut DMEM (Life Technologies), 1 x Glutamax (Life Technologies), 1 x MEM Non-Essential Amino Acids (Life Technologies), 1 x 2i Supplement (Life Technologies), 1 x B27 Supplement (Life Technologies), 1 x Beta-mercaptoethanol (Life Technologies), 1 x N2 Supplement (Life Technologies), 1 x Glutamax (Life Technologies), 1 x MEM Non-Essential Amino Acids (Life Technologies), 1 x N2 supplement (Life Technologies), 0.5 × N2 supplement, 0.5 × B27 supplement, 1 × Glutamax (Sigma-Aldrich) and were seeded on 6-well plates and on 13 mm circular glass coverslips (Hecht, cat no. 6.071.724) were washed 3 times for 5 min each in PBS before fixation in 4% paraformaldehyde for exactly 10 min. Fixed cells were then stored in 0.02% sodium azide in PBS at 4 °C for up to a week until all samples from differentiation were ready for processing. Cells were then washed 3 times for 5 min each again in PBS before permeabilisation in 0.5% TritonX in PBS for exactly 5 min on ice. Cells were washed again 3 times for 5 min each in PBS then non-specific binding sites were blocked in 1% bovine serum albumin (Sigma-Aldrich, A9418) in PBS for approximately 1 h at room temperature. Primary antibodies against T/Brachyury (Abcam, #ab209665) and Sox2 (ThermoFisher Scientific, cat #A19811-82) were then added in 1% BSA solution at a dilution of 1:100 and incubated with cells at 4 °C overnight in a humidified chamber. Cells were then washed 3 times for 5 min each in PBS before incubation with secondary goat anti-rabbit 647 (ThermoFisher Scientific, cat #A21244) and goat anti-rat 568 (Invitrogen, cat #A10177) antibodies each at a dilution of 1:500 in a dark humidified chamber for one hour at room temperature, before washing 3 times for 5 min each again in PBS and counterstaining with DAPI Flouresce for approximately 1 h at room temperature. Cells were washed 3 times for 5 min each again in PBS before mounting on Polyline microscope slides (LabServ, cat #LBS24981) with Vectashield Vibrance Antifade mounting medium (Vector Laboratories, cat #H-1700). Cells were imaged on an LSM 880 (Zeiss) confocal microscope at 40 x magnification and z-stacks were merged and composite images generated using the ImageJ distribution package FIJI.

### Immunofluorescence

Immunofluorescence was performed on differentiating mESCs to NMPs as previously described. In brief, cells grown on coverslips were 13 mm circular glass coverslips (Hecht, cat no. 6.071.724) were washed 3 times for 5 min each in PBS before fixation in 4% paraformaldehyde for exactly 10 min. Fixed cells were then stored in 0.02% sodium azide in PBS at 4 °C for up to a week until all samples from differentiation were ready for processing. Cells were then washed 3 times for 5 min each again in PBS before permeabilisation in 0.5% TritonX in PBS for exactly 5 min on ice. Cells were washed again 3 times for 5 min each in PBS then non-specific binding sites were blocked in 1% bovine serum albumin (Sigma-Aldrich, A9418) in PBS for approximately 1 h at room temperature. Primary antibodies against T/Brachyury (Abcam, #ab209665) and Sox2 (ThermoFisher Scientific, cat #A19811-82) were then added in 1% BSA solution at a dilution of 1:100 and incubated with cells at 4 °C overnight in a humidified chamber. Cells were then washed 3 times for 5 min each in PBS before incubation with secondary goat anti-rabbit 647 (ThermoFisher Scientific, cat #A21244) and goat anti-rat 568 (Invitrogen, cat #A10177) antibodies each at a dilution of 1:500 in a dark humidified chamber for one hour at room temperature, before washing 3 times for 5 min each again in PBS and counterstaining with DAPI Flouresce for approximately 1 h at room temperature. Cells were washed 3 times for 5 min each again in PBS before mounting on Polyline microscope slides (LabServ, cat #LBS24981) with Vectashield Vibrance Antifade mounting medium (Vector Laboratories, cat #H-1700). Cells were imaged on an LSM 880 (Zeiss) confocal microscope at 40 x magnification and z-stacks were merged and composite images generated using the ImageJ distribution package FIJI.

### CUT&RUN

CUT&RUN was performed as previously described. mESCs grown in 2i + LIF medium or sampled at days 2, 2.5 and 3 during differentiation were taken out of culture and counted using a haemocytometer before harvesting by centrifugation at 600 g for 5 min and room temperature then resuspending in wash buffer (20 mM HEPES pH 7.5, 150 mM NaCl, 0.5 mM spermidine and 1 x complete protease inhibitor (Roche)) three times. Approximately 200,000 cells were used for each antibody for replicate 1, and 500,000 each for replicates 2 and 3. 10 ul of canavalin A-coated beads (Banges Laboratories, #BPFS31) per sample were washed in binding buffer (20 mM HEPES pH 7.5, 10 mM KC1, 1 mM CaCl2, 1 mM MnCl2) then were resuspended in the original volume of beads. 10 ul of beads per sample were then bound to the cells in 1 ml wash buffer, mutating for 10 min at room temperature. H3K27me3 (Cell Signalling Technologies, C36B11) and H2AK119ub (Cell Signalling Technologies, D27C40) antibodies were added at a concentration of 1:100 in antibody binding buffer (2 mM EDTA in digitonin wash buffer (wash buffer with 0.025% digitonin) and antibody binding was conducted overnight at 4 °C with both H3K27me3, rotating on a nutator.

---

**Nature Communications** | (2022) 13:4295

https://doi.org/10.1038/s41467-022-32057-x
Samples were then washed three times with digitonin wash buffer before resuspending in digitonin wash buffer with PAG-MNase (Epicypher, 15–116) at a concentration of 1:20 and mutating at 4 °C for 1 h to allow PAG-MNase and epitope binding. Samples were then washed twice with digitonin wash buffer then once with low-salt rinse buffer (20 mM HEPES pH 7.5, 0.5 mM spermidine, 0.05% digitonin) before resuspending in 200 ul ice-cold incubation buffer (3.5 mM HEPES pH 7.5, 10 mM CaCl2, 0.05% digitonin). Samples were then incubated at 0 °C for exactly 30 min to allow MNase cleavage at antibody bound sites, before resuspending in 200 ul STOP buffer (170 mM NaCl, 20 mM EGTA, 0.05% digitonin, 50 μg/mL RNase A, 25 μg/mL glycogen) then incubation at 37 °C for 30 min. The supernatant containing the cleaved chromatin was separated from the ConA beads. DNA was extracted and purified using phenol/chloroform/isoamylalcohol followed by ethanol and glycogen precipitation, purified DNA was resuspended in 0.1% TE buffer (1 mM Tris-HCl pH 8.0, 0.1 mM EDTA). DNA was quantified with a Qubit dsDNA HS Assay Kit and 6 ng or total DNA if less than 6 ng was used as the input for sequencing library preparation.

Libraries were prepared using the NEBNext Ultra II DNA Library Prep Kit for Illumina (New England Biolabs, E7645), with adapters diluted 1:5 from the supplied concentration. Libraries were quantified on a HS D1000 tape on a 4200 Tapestation (Agilent Technologies) before pooling and sequencing on the Illumina NextSeq platform, with 75 bp single-end reads.

**RNA-sequencing**

RNA-sequencing was carried out on tailbud tissue, 2i mESCs and differentiating cells by first extracting RNA using a Quick-RNA Miniprep Kit (Zymo) with DNase I treatment according to the manufacturer’s instructions. 100 ng total RNA (or less if <100 ng was yielded from tailbud tissue) was used to prepare libraries using either a TrueSeq RNA Library Prep Kit v2 (Illumina) or a TrueSeq Stranded mRNA kit (Illumina) according to the manufacturer’s instructions. Libraries were size-selected for 200–600 bp and primer dimers cleaned up using Ampure XP beads (Beckman Coulter Life Sciences) and were quantified using a D1000 tape on a 4200 Tapestation (Agilent Technologies). Libraries were then pooled and sequenced on the Illumina NextSeq platform, with 75 bp single-end reads, except for 2i mESC RNA-seq libraries which had paired-end sequencing.

**RNA-sequencing analysis**

For tailbud, mESC and NMP differentiation RNA-seq, adapter trimming of Fastq files was performed using TrimGalore! v0.4.4 with Cutadapt v1.157 then QC was carried out using FastQC v0.11.8. Reads were mapped to the GRCm38.p6 version of the mouse reference genome using Bowtie2 v2.3.4.162 and Samtools v1.763. FPM was calculated over genes ± 10 kb using the feature probe generator and transcription start site, before normalisation against the average of all genes. FPM was calculated over genes ± 10 kb using the feature probe generator and transcription start site, before normalisation against the average of all genes.

**Differential gene expression analysis**

For E2.5 male embryo RNA-seq, data was obtained from and analysed as per 7. Read were trimmed using TrimGalore! v0.4.4 and mapped using hisat2 v2.0.5.3 and Samtools v1.7.8. Libraries were quantified using SeqMonk’s RNA-seq quantitation pipeline, correcting for total library size and transcript length to generate log2 RPKM values. Differences in Hox gene expression were quantified by subtracting Smchd1β from Smchd1α using FPKM values, then taking the log2 RPKM counts. Heatmaps were generated using GraphPad Prism v9.0.0. Differential gene expression analysis was carried out using the built-in edgeR analysis package66,67,68,69.

For F2.5 male embryo RNA-seq, data was obtained from and analysed as per 7. Read were trimmed using TrimGalore! v0.4.4 and mapped using hisat2 v2.0.5.3 and Samtools v1.7.8. Libraries were quantified using SeqMonk’s RNA-seq quantitation pipeline, correcting for total library size and transcript length to generate log2 RPKM values. Differences in Hox gene expression were quantified by subtracting Smchd1β from Smchd1α using FPKM values, then taking the log2 RPKM counts. Heatmaps were generated using GraphPad Prism v9.0.0. Differential gene expression analysis was carried out using the built-in edgeR analysis package66,67,68,69.

**Reduced representation bisulfite sequencing**

Cells were harvested at days 2 and 2.5 of differentiation by dissociation with Accutase (Sigma-Aldrich) for 5 min at 37 °C, washing with mESC wash media [KnockOut DMEM (Life Technologies), 10% KnockOut Serum Replacement (Life Technologies), 100 IU/mL penicillin/100 μg/mL streptomycin (Life Technologies)] then pelleting at 500 g for 5 min. Supernatant was removed and the cell pellet was frozen at −80 °C until extraction. DNA was extracted using a Quick-DNA Miniprep kit, cleaned using a Zymo research DNA Clean and concentrator 5 kit then quantified with a Qubit dsDNA assay kit (Thermo Fisher Scientific Q32853). 100 ng DNA was used as the input for library preparation with the NuGEN Ovation RRBS methyl-seq system (Integrated sciences). Bisulfite conversion was carried out using a QIAGEN EpiTect Fast DNA Bisulfite Kit. Libraries were quantified using a DS1000 tape on TapeStation 2200 (Agilent Technologies). Samples were sequenced on the Illumina NextSeq platform using 75 bp single-end reads.

**DNA methylation analysis**

For Reduced Representation Bisulfite Sequencing (RRBS), adapter trimming of Fastq files was performed using TrimGalore! v0.4.4 with Cutadapt v1.151 then trimming of the diversity bases introduced by library preparation with the NuGEN Ovation RRBS methyl-seq kit was carried out using the trimRRBSdiversityAdaptCustomers.py script.
provided by NuGEN\textsuperscript{7}. Reads were mapped to the GRCm38.p6 version of the mouse reference genome using Bismark v0.20.0\textsuperscript{8} and methylation calls were extracted using Bismark’s bismark_methylation_extractor function. Bismark.cov files were opened in SeqMonk and % methylation was calculated using SeqMonk’s Bisulphite methylation over features pipeline, filtering probes with over 10 reads for all libraries. % methylation files were exported and graphs were generated using GraphPad Prism v9.0.0. P-values were corrected with the Benjamini-Hochberg method\textsuperscript{9}. Whole Genome Bisulftite Sequencing (WGBS) data from E2.75 male and female Smchd1\textsuperscript{wt} and Smchd1\textsuperscript{mat} embryos (\(n = 6\) Smchd1\textsuperscript{mat} females, \(n = 5\) Smchd1\textsuperscript{wt} males, \(n = 4\) Smchd1\textsuperscript{mat} females, \(n = 8\) Smchd1\textsuperscript{mat} males) was obtained from BioProject accession PRJNA530651 for male data\textsuperscript{10}, GEO accession number GSE186315 for female data. Reads from male and female embryos of each genotype were added and Bismark files were generated and analysed for % DNA methylation over the Hox clusters as described above for RRBS data. P-values were generated with unpaired, two-tailed t-tests, corrected with the Benjamini-Hochberg method\textsuperscript{9}.

Statistics and reproducibility

No statistical method was used to predetermine sample size. No data were excluded from analyses. The experiments were not randomized. Investigators were blinded to allocation during experiments and outcome assessment for the skeletal scoring. All experiments were replicated at least twice but usually at least three times.

Reporting summary

Further information on research design is available in the Nature Research Reporting Summary linked to this article.

Data availability

All genomic data is available on the Gene Expression Omnibus under number GSE193740. Source data associated with each graph are provided in the Supplementary Data.

References

1. Schulz, K. N. & Harrison, M. M. Mechanisms regulating zygotic genome activation. Nat. Rev. Genet. 20, 221–234 (2018).
2. Ferguson-Smith, A. C. Genomic imprinting: the emergence of an epigenetic paradigm. Nat. Rev. Genet. 12, 565–575 (2011).
3. Reik, W. & Walter, J. Genomic imprinting: parental influence on the genome. Nat. Rev. Genet. 2, 21–32 (2001).
4. Inoue, A., Jiang, L., Lu, F., Suzuki, T. & Zhang, Y. Maternal H3K27me3 antagonises TAD formation and compartmentalisation on the inactive X chromosome. Dev. Cell. 25, 265–279 (2012).
5. Shimada, H. et al. SMCHD1 has separable roles in chromatin architecture and gene silencing that could be targeted in disease. BioRxiv, 2021.05.20.443934 (2021).
6. Mallo, M. & Alonso, C. R. The regulation of Hox gene expression during animal development. Dev. (Camb., Engl.) 140, 3951–3963 (2013).
7. Mallo, M., Wellik, D. M. & Deschamps, J. Hox genes and regional patterning of the vertebrate body plan. Developmental Biol. 344, 7–15 (2010).
8. Pearson, J. C., Lemons, D. & McGinnis, W. Modulating Hox gene functions during animal body patterning. Nat. Rev. Genet. 6, 893–904 (2005).
9. Burke, A. C., Nelson, C. E., Morgan, B. A. & Tabin, C. Hox genes and the evolution of vertebrate axial morphology. Dev. (Camb., Engl.) 121, 333–346 (1995).
10. Deschamps, J. & van Nes, J. Developmental regulation of the Hox genes during axial morphogenesis in the mouse. Dev. (Camb., Engl.) 132, 2931–2942 (2005).
11. Soshnikova, N. & Duboule, D. Epigenetic regulation of vertebrate Hox genes: A dynamic equilibrium. Epigenetics 4, 537–540 (2009).
32. Schuettengruber, B., Chourrout, D., Vervoort, M., Leblanc, B. & Cavalli, G. Genome Regulation by Polycomb and Trithorax Proteins. Cell 128, 735–747 (2007).
33. Boyer, L. A. et al. Polycomb complexes repress developmental regulators in murine embryonic stem cells. Nature 441, 349–353 (2006).
34. Zheng, H. et al. Resetting Epigenetic Memory by Reprogramming of Histone Modifications in Mammals. Mol. Cell 63, 1066–1079 (2016).
35. Chen, Z., Djekidel, M. N. & Zhang, Y. Distinct dynamics and functions of H2AK119ub1 and H3K27me3 in mouse preimplantation embryos. Nat. Genet. 53, 551–563 (2021).
36. Mei, H. et al. H2AK119ub1 guides maternal inheritance and zygotic deposition of H3K27me3 in mouse embryos. Nat. Genet. 53, 539–550 (2021).
37. McGlenn, E. et al. In ovo application of antagonistRNAs indicates a role for mir-196 in patterning the chick axial skeleton through Hox gene regulation. Proc. Natl Acad. Sci. 106, 18610–18615 (2009).
38. Wymeersch, F. J. et al. Position-dependent plasticity of distinct progenitor types in the primitive streak. eLife 5, e10042 (2016).
39. Kessel, M. & Gruss, P. Homeotic transformations of murine vertebral and concomitant alteration of Hox codes induced by retinoic acid. Cell 70, 89–104 (1992).
40. Goult, M. et al. A Gene Regulatory Network Balances Neural and Mesoderm Specification during Vertebrate Trunk Development. Developmental Cell 41, 243–261.e247 (2017).
41. Liu, X. et al. Distinct features of H3K4me3 and H3K27me3 chromatin domains in pre-implantation embryos. Nature 537, 558–562 (2016).
42. Furuova, N. A. et al. BAPI constrains pervasive H2AK119ub1 to control the transcriptional potential of the genome. Genes Dev. 35, 749–770 (2021).
43. Soshnikova, N. & Duboule, D. Epigenetic temporal control of mouse Hox genes in vivo. Science 324, 1230–1233 (2009).
44. Lun, A. T. & Smyth, G. K. csaw: A Bioconductor package for differential binding analysis of ChiP-seq data using sliding windows. Nucleic Acids Res. 44, e45 (2016).
45. Wanigasuriya I, et al. Maternal SMCHD1 controls both imprinted Xist expression and imprinted X chromosome inactivation. Epigenetics & Chromatin 15, 26 (2022).
46. van der Lugt, N. M. T., Alkema, M., Berns, A. & Deschamps, J. The Polycomb-group homolog Bmi-1 is a regulator of murine Hox gene expression. Mechanisms Dev. 58, 153–164 (1996).
47. Suzuki, M. et al. Involvement of the Polycomb-group gene Ring1b in the specification of the anterior-posterior axis in mice. Dev. (Camb., Engl.) 129, 4171–4183 (2002).
48. Steffen, P. A. & Ringrose, L. What are memories made of? How Polycomb and Trithorax proteins mediate epigenetic memory. Nat. Rev. Mol. Cell Biol. 15, 340–356 (2014).
49. Xue, S. et al. HOX epimutations driven by maternal SMCHD1/LRIF1 haploinsufficiency trigger homeotic transformations in genetically wildtype offspring. Nat. Commun. 13, 3583 (2022).
50. Nozawa, R. S. et al. Human inactive X chromosome is compacted through a PRC2-independent SMCHD1-H3K27me3 pathway. Nat. Struct. Mol. Biol. 20, 566–573 (2013).
51. Wagner, K. U. et al. Cre-mediated gene deletion in the mammary gland. Nucleic Acids Res. 25, 4233–4230 (1997).
52. Lewandoski, M., Wassarman, K. M. & Martin, G. R. Zp3−cre, a transgenic mouse line for the activation or inactivation of loxP-flanked target genes specifically in the female germ line. Curr. Biol. 7, 148–151 (1997).
53. Leong, H. S. et al. Epigenetic Regulator Smchd1 Functions as a Tumor Suppressor. Cancer Res. 73, 1591–1599 (2012).
54. Rigueur, D. & Lyons, K. M. Whole-mount skeletal staining. Methods Mol. Biol. 1130, 113–121 (2014).
55. Hauswirth, G. M. et al. Breaking constraint of mammalian axial formulae. Nat. Commun. 13, 243 (2022).
56. Wong, S. F. L. et al. Independent regulation of vertebral number and vertebral identity by microRNA-196 paralogs. Proc. Natl. Acad. Sci. 112, E4884–93 (2015).
57. Keniry, A. et al. Setdb1-mediated H3K9 methylation is enriched on the inactive X and plays a role in its epigenetic silencing. Epigenetics. Chromatin 9, 16 (2016).
58. Keniry, A. et al. BAF complex-mediated chromatin relaxation is required for establishment of X chromosome inactivation. Nat. Commun. 13, 1658 (2022).
59. Schindelin, J. et al. Fiji: an open-source platform for biological-image analysis. Nat. Methods 9, 676–682 (2012).
60. Skene, P. J., Henikoff S. An efficient targeted nuclease strategy for high-resolution mapping of DNA binding sites. eLife 6, e21856 (2017).
61. Martin, M. Cutadapt removes adapter sequences from high-throughput sequencing reads. EMBO journal 17, 10 (2011).
62. Kim, D., Langmead, B. & Salzberg, S. L. HISAT: a fast spliced aligner with low memory requirements. Nat. Methods 12, 357–360 (2015).
63. Li, H. et al. The Sequence Alignment/Map format and SAMtools. Bioinformatics 25, 2078–2079 (2009).
64. Andrews, S. SeqMonk: a tool to visualise and analyse high throughput mapped sequence. 1.48.0 edn (2007).
65. Robinson, M. D., McCarthy, D. J. & Smyth, G. K. edgeR: a Bioconductor package for differential expression analysis of digital gene expression data. Bioinformatics 26, 139–140 (2009).
66. Benjamin, Y. & Hochberg, Y. Controlling the False Discovery Rate: A Practical and Powerful Approach to Multiple Testing. J. R. Stat. Soc. Ser. B (Methodol.) 57, 289–300 (1995).
67. McCarthy, D. J., Chen, Y. & Smyth, G. K. Differential expression analysis of multifactor RNA-Seq experiments with respect to biological variation. Nucleic Acids Res. 40, 4288–4297 (2012).
68. Krueger, F. & Andrews, S. R. SNPsplit:Allele-specific splitting of alignments between genomes with known SNP genotypes. F1000Research 5, 1479 (2016).
69. Team RDC. R: A Language and Environment for Statistical Computing.). 2.6.2 edn. R Foundation for Statistical Computing (2019).
70. Liao, Y., Smyth, G. K. & Shi, W. The R package Subread is faster, cheaper and better for alignment and quantification of RNA sequencing reads. Nucleic Acids Res. 47, e47–e47 (2019).
71. Liao, Y., Smyth, G. K. & Shi, W. featureCounts: an efficient general purpose program for assigning sequence reads to genomic features. Bioinformatics 30, 923–930 (2013).
72. Robinson, M. D. & Oshlack, A. A scaling normalization method for differential expression analysis of RNA-seq data. Genome Biol. 11, R25 (2010).
73. Su, S. et al. Glimma: interactive graphics for gene expression analysis. Bioinformatics 33, 2050–2052 (2017).
74. Karayarawasam, H., Su, S., Voogd, O., Ritchie Matthew, E. & Law Charity, W. Dashboard-style interactive plots for RNA-seq analysis are R Markdown ready with Glimma 2.0. NAR Genomics Bioinformatics 3, iqab116 (2021).
75. M. L. Analysis Guide for NuGEN Ovation RRBS Methyl-Seq. (2018).
76. Krueger, F. & Andrews, S. R. Bismark: a flexible aligner and methylation caller for Bisulfite-Seq applications. Bioinformatics 27, 1571–1572 (2011).

Acknowledgements
We thank Jessica Martin (WEHI) for her valuable assistance with mouse lines. We thank Dr Andrew Langedam (Monash) for imaging assistance. This work was supported by grants and fellowships from the Australian National Health and Medical Research Council (GNT1098290 to MEB, fellowship GNT1194345 to MEB). NB was supported by an Australian
Research Training Program scholarship. MEB was supported by the Bellberry-Viertel Senior Medical Research fellowship. Additional support was provided by the Victorian State Government Operational Infrastructure Support, Australian National Health and Medical Research Council IRISS grant (9000653). The Australian Regenerative Medicine Institute is supported by grants from the State Government of Victoria and the Australian Government.

Author contributions
N.B. acquired, analysed and interpreted the data and drafted the paper. Q.G., A.T.d.F. and A.K. analysed and interpreted data. T.B. and K.B. acquired data. E.M. contributed to design of the work, data acquisition, analysis and interpretation. M.B. conceived and designed the work, analysed and interpreted the data. All authors edited the manuscript.

Competing interests
The Authors declare no competing interests.

Additional information
Supplementary information The online version contains supplementary material available at https://doi.org/10.1038/s41467-022-32057-x.

Correspondence and requests for materials should be addressed to Edwina McGlinn or Marnie E. Blewitt.

Peer review information Nature Communications thanks Gavin Kelsey, Robb Krumlauf and the other, anonymous, reviewer(s) for their contribution to the peer review of this work.

Reprints and permission information is available at http://www.nature.com/reprints

Publisher’s note Springer Nature remains neutral with regard to jurisdictional claims in published maps and institutional affiliations.

Open Access This article is licensed under a Creative Commons Attribution 4.0 International License, which permits use, sharing, adaptation, distribution and reproduction in any medium or format, as long as you give appropriate credit to the original author(s) and the source, provide a link to the Creative Commons license, and indicate if changes were made. The images or other third party material in this article are included in the article’s Creative Commons license, unless indicated otherwise in a credit line to the material. If material is not included in the article’s Creative Commons license and your intended use is not permitted by statutory regulation or exceeds the permitted use, you will need to obtain permission directly from the copyright holder. To view a copy of this license, visit http://creativecommons.org/licenses/by/4.0/.

© The Author(s) 2022