Orphan CpG islands amplify poised enhancer regulatory activity and determine target gene responsiveness

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CpG islands (CGIs) represent a widespread feature of vertebrate genomes, being associated with ~70% of all gene promoters. CGIs control transcription initiation by conferring nearby promoters with unique chromatin properties. In addition, there are thousands of distal or orphan CGIs (oCGIs) whose functional relevance is barely known. Here we show that oCGIs are an essential component of poised enhancers that augment their long-range regulatory activity and control the responsiveness of their target genes. Using a knock-in strategy in mouse embryonic stem cells, we introduced poised enhancers with or without oCGIs within topologically associating domains harboring genes with different types of promoters. Analysis of the resulting cell lines revealed that oCGIs act as tethering elements that promote the physical and functional communication between poised enhancers and distally located genes, particularly those with large CGI clusters in their promoters. Therefore, by acting as genetic determinants of gene–enhancer compatibility, CGIs can contribute to gene expression control under both physiological and potentially pathological conditions.

Enhancers are a heterogeneous group of distal cis-regulatory elements containing clusters of transcription factor binding sites (TFBSs) that control gene expression in a distance- and orientation-independent manner. The regulatory properties of enhancers have been mostly investigated using transgenic reporter assays in which enhancer activity is evaluated by measuring the capacity to activate transcription of a reporter gene from a minimal promoter. In these assays, the investigated sequences are placed at short distances from the reporter genes, using a limited set of minimal promoters. On the other hand, insulators prevent enhancers from ectopically activating nontarget genes. In vertebrates, insulators are typically bound by CTCF, which, together with Cohesin, can form long-range chromatin loops that demarcate the boundaries of regulatory domains and limit enhancer activity. Current models of enhancer function implicitly assume that enhancers and genes can effectively communicate with each other, regardless of distance or sequence composition, as far as they are located within the same regulatory domain. However, recent studies show that the disruption of regulatory domains does not always lead to changes in gene expression or enhancer–gene communication. Similarly, enhancers and their developmental target genes can reside within the same regulatory domain together with ‘bystander’ genes that are not responsive to the enhancers. Therefore, additional factors, such as the type of core-promoter elements, contribute to enhancer responsiveness. However, it is currently unknown whether other genetic factors (for example, distance or enhancer sequence composition) can also contribute to such responsiveness, which is essential to understand the pathological consequences of human structural variation.

We previously showed that poised enhancers (PEs) control the induction of major neural genes upon mouse embryonic stem cell (ESC) differentiation. Before becoming active in anterior neural progenitors (AntNPCs), PEs are already bookmarked in ESCs with unique chromatin and topological features, including binding by polycomb-group protein complexes (PcG) and preformed contacts with their target genes. PEs have a distinctive genetic composition that includes not only clusters of TFBSs but also nearby CGIs. CGIs are a prevalent feature of vertebrate gene promoters, providing them with a permissive chromatin state that facilitates transcription initiation. However, only half of the CGIs found in the mouse and human genomes are associated with promoters (pCGIs), while the other half, known as oCGIs, remain poorly studied. oCGIs have been proposed to act as alternative gene promoters or highly active enhancers with limited tissue specificity. Nevertheless, the mechanisms whereby oCGIs might contribute to transcriptional regulation remain unknown. Here we show that oCGIs act as long-range potentiators of PEs, enabling the functional communication between PEs and developmental genes with CpG-rich promoters. Therefore, our work uncovers CGIs as major determinants of enhancer–gene compatibility and provides important insights into how gene expression programs are specifically and precisely deployed during development.
Fig. 1 | Genetic properties and functional relevance of oCGIs associated with PEs. a, Percentage of PEs within the indicated maximum distances (0.25 kb or 3.00 kb) of a CGI identified by CAP-seq (left), an NMI (middle) or a computationally defined CGI (right). b, Comparison of the CpG%, observed/expected CpG ratio, GC% and sequence length between random regions (n = 436,000), CAP-CGIs associated with PE-distal (PE-CAP-CGI; n = 276) and CAP-CGIs associated with the TSS of developmental genes (devTSS-CAP-CGI; n = 1,926) (Methods). P values were calculated using unpaired two-sided Wilcoxon tests with Bonferroni correction for multiple testing; black numbers indicate median fold-changes; green numbers indicate non-negligible Cliff’s delta effect sizes. The center line of the violin plot represents the median, the boxes encompass the interquartile range and the whiskers extend to the minimum and maximum. c, Percentage of CGI block sizes (1, 2 or ≥3 CAP-CGIs) associated with PE-distal (n = 253) or the TSS of developmental genes (devTSS; n = 1,522) with at least one CAP-CGI in <3 kb. The devTSSs were classified in two groups based on the length of the H3K27me3 domains associated with them (>6 kb (n = 1,522) and >10 kb (n = 599)). d, Left panel, ChiP–seq data from ESCs (p300 and H3K27me3) and AntNPCs (H3K27ac) at the Sox1 locus. The PE Sox1(+)35 is highlighted in yellow. Right panel, close-up view of the PE Sox1(+35) with additional epigenomic and genomic data, including a computationally defined CGI. Vert. Cons, vertebrate PhastCons. e, Sox1 expression was investigated by RT-qPCR in cells that were WT, homozygous for a deletion of the PE Sox1(+35) CGI (PE Sox1 CGI−/−) or homozygous for a deletion of the complete PE Sox1(+35) (PE Sox1−/−). N = 2 independent PE Sox1 CGI−/− ESC clones (circles and diamonds) and n = 1 PE Sox1−/− clone were studied. For each ESC clonal line, n = 2 replicates of the AntNPC differentiation were performed. Expression values were normalized to two housekeeping genes (Eef1a and Hprt) and are presented as fold-changes with respect to WT ESCs. The colored area of the violin plot represents the expression values distribution and the center line represents the median. n = 1 independent biological replicate of this experiment is shown in Extended Data Fig. 1f. ObsExp, observed to expected CpG ratio.

Results

Genetic properties of PE-associated oCGIs. PEs identified in mouse ESCs are commonly located in proximity to computationally predicted CGIs44. However, computational models underestimate the abundance of CGIs, especially those distally located from transcription start sites (TSSs)45. Using biochemically identified CGIs (11) CGIs identified by CXXC affinity purification and deep sequencing (CAP–seq) (that is, CAP-CGIs)43 and (2) nonmethylated islands (NMIs)253, we found that ~60–80% of PEs are located within 3 kilobases (kb) of a CAP- CGI or an NMI, respectively (Fig. 1a). In comparison with the CAP-CGIs located in proximity of the TSSs of developmental genes, those associated with PEs were shorter and had lower CpG density (Fig. 1b). Moreover, PEs tend to be associated with single CAP-CGIs, whereas developmental gene promoters frequently contain clusters of two or more CAP-CGIs52 (Fig. 1c and Extended Data Fig. 1a). Here we use the term oCGI regardless of whether these sequences are identified computationally or experimentally, although many of the PE oCGIs display lower GC content and CpG ratios than the classically defined CGIs (Fig. 1b).

pCGIs serve as recruitment platforms for proteins that can modify chromatin (for example, PcG, TET1)23,24. Consequently, pCGIs are hypomethylated and enriched in H3K27me3 (refs. 14,15). Analysis of publicly available data14,25–28 showed that PEs associated with CAP-CGIs are also hypomethylated and more enriched in H3K27me3 than PEs or active enhancers (AE) not linked to CAP-CGIs (Extended Data Fig. 1b,c). Therefore, PEs are pervasively found in proximity of CGIs, which in turn might endow them with unique chromatin features.
oCGIs are necessary for PE regulatory function. To start evaluating the regulatory role of oCGIs in the context of PEs, we generated mouse ESC lines with a homozygous deletion of the oCGI associated with PE Sox1(+35), a PE that controls the expression of Sox1 in neural progenitors (Fig. 1d and Extended Data Fig. 1d). The oCGI deletion severely reduced H3K27me3 levels around PE Sox1(+35) in ESCs (Extended Data Fig. 1e). Next, we measured Sox1 expression in wild-type (WT), PE Sox1(+35)/CGI−/− and PE Sox1(+35)−/− ESCs as well as upon their differentiation into AntNPCs. In ESCs, neither the deletion of the oCGI nor of the whole PE Sox1(+35) affected Sox1 expression (Fig. 1e and Extended Data Fig. 1f). However, in AntNPCs the oCGI deletion reduced Sox1 expression by >2-fold (Fig. 1e and Extended Data Fig. 1f), thus suggesting that oCGIs might positively influence the cis-activation capacity of PEs.6

Dissection of PE regulatory logic by genetic engineering. The functional assessment of PE oCGIs using loss-of-function approaches has certain limitations: (1) oCGIs can be difficult to delete individually, as they frequently overlap with nearby TFBSs (Extended Data Fig. 2a); (2) PE target genes typically display complex regulatory landscapes in which multiple enhancers control gene expression,20, thus potentially masking the regulatory function of individual oCGIs; and (3) the loss of CGI-bound proteins (for example, PcG) can elicit global molecular changes that indirectly alter PE loci.

To systematically dissect the contribution of oCGIs to the regulatory function of PEs, we designed a genetic engineering approach to generate ESCs in which the components of selected PEs are modularly inserted (that is, TFBS, oCGI or TFBS+oCGI) into a fixed genomic location (Fig. 2a). We reasoned that by selecting insertion sites located within topologically associating domains (TADs) containing developmental genes not expressed in ESCs or AntNPCs and, thus, without AEs in these cell types, any changes in the expression of the selected genes could be attributed solely to the inserted PE sequences. To implement this approach, we initially inserted the PE Sox1(+35) components (that is, PE Sox1(+35)TFBS, PE Sox1(+35)CGI or PE Sox1(+35)TFBS+CGI) approximately 100 kb downstream of Gata6 (Fig. 2a), a gene with multiple CGIs around its promoter region and weakly expressed in both ESCs and AntNPCs. The selected insertion site was not conserved and was not close to any CTCF binding site (CBS), thus minimizing the risk of disrupting any regulatory element. Using this strategy, we established two homozygous ESC clones for each of the PE Sox1(+35) inserts described above (Extended Data Fig. 2b). Next, we measured Gata6 expression in the previous ESC lines and upon their differentiation into AntNPCs. In ESCs none of the engineered PE Sox1(+35) combinations affected Gata6 expression (Fig. 2b and Extended Data Fig. 2c). Strikingly, upon differentiation into AntNPCs, Gata6 was strongly induced in cells with the TFBS+CGI insertion (~50-fold versus WT). In contrast, cells with the TFBS displayed considerably milder Gata6 induction (~7-fold versus WT), while the CGI had no effect on Gata6 expression (Fig. 2b and Extended Data Fig. 2c).

oCGIs amplify PE regulatory activity. To evaluate whether the previous observations could be generalized, we generated two additional groups of transgenic ESC lines: (1) PE Sox1(+35) components were inserted within the Foxa2-TAD (~100 kb downstream of Foxa2 TSS, which contains several CGIs and is inactive in ESCs and AntNPCs); (2) PE Wnt8b(+21) (ref. 14) components were inserted within the Gata6-TAD (~100 kb downstream of Gata6-TSS) (Extended Data Fig. 2d–g). Importantly, the TFBS+CGI inserts were able to strongly induce gene expression in AntNPCs (Fig. 2c,d and Extended Data Fig. 2h,i), while the TFBS or the oCGI alone led to either no or minor gene inductions, respectively (Fig. 2c,d and Extended Data Fig. 2h,i).

Next, we investigated whether the boosting capacity of the oCGIs could be attributed to other types of regulatory information beyond their CpG-richness (for example, TFBSs). In silico motif analyses using as input either the CAP-CGIs or the TFBSs/p300 peaks from PEs in which these elements do not overlap (Fig. 2c) showed that p300 peaks, but not CAP-CGIs, were strongly enriched in binding motifs for pluripotency transcription factors (TFs)14 (for example, OCT4, SOX2, NANOG). Similarly, differential motif analyses revealed that CpG-rich motifs were strongly overrepresented among PEs compared with AEs (Extended Data Fig. 3a). CGIs can serve as recruitment platforms for proteins containing ZF-CxxC domains (for example, KDM2B, TET1)21, which could contribute to the unique chromatin features of PEs (Extended Data Fig. 1b,c). Analysis of KDM2B and TET1 chromatin immunoprecipitation sequencing (ChIP-seq) data generated in ESCs22,23 showed that the binding of these proteins to PEs was positively correlated with the presence of nearby CAP-CGIs (Extended Data Fig. 3b). Next, we designed an artificial CGI (aCGI; Methods) and inserted it alone or together with the PE Sox1(+35)/TFBS at the Gata6-TAD (Extended Data Fig. 4a,b,d). Notably, the TFBS+aCGI considerably increased Gata6 expression in AntNPCs compared with the TFBS (Fig. 2f and Extended Data Fig. 4e), whereas the insertion of the aCGI alone did not alter Gata6 expression (Extended Data Fig. 4e). Although we cannot completely dismiss that some oCGIs contain relevant binding sites for tissue-specific TFs, our results indicate that the CpG-richness of the oCGIs is important to increase the regulatory activity of PEs.

The boosting properties of oCGIs might be attributed to a premature induction of the target gene, an increase in the number of cells in which the target gene becomes induced and/or an increase in the expression levels within individual cells. To address this, we focused on those cell lines containing the different PE Sox1(+35) components inserted within the Gata6-TAD. Upon differentiation...
of the TFBS+CGI ESCs into AntNPCs, Gata6 did not become induced until day 4, thus matching the expression dynamics of Sox1 (the endogenous target of PE Sox1(+)35)) and arguing against premature gene induction due to the presence of the CGI (Extended Data Fig. 5a). Next, we performed immunofluorescence assays to visualize GATA6 and SOX1 proteins in WT and Gata6-TAD cells. SOX1 became strongly and homogeneously induced in AntNPCs derived from all the evaluated cell lines33 (Fig. 2g and Extended Data Fig. 5b–d). Notably, GATA6 was also induced in ~50% and ~60% of the AntNPCs derived from TFBS+CGI or TFBS+aCGI ESCs, respectively (Fig. 2g and Extended Data Fig. 5c,d). In contrast, the TFBS resulted in noisier and more heterogeneous GATA6 expression, while no GATA6 could be detected in cells having the CGI or aCGI alone. These results suggest that oCGIs increase the number

![Diagram](image)

**Figure 2**. TFBS and CGI modulate gene expression. **a**. Venn diagram showing the overlap of TFBS and CGI in TFBS+CGI, TFBS, and aCGI groups. **b**. Fold change in Sox1 and Gata6 expression in WT and AntNPCs derived from ESCs with PE Sox1 insertion in Gata6-TAD. **c**. Fold change in Sox1 and Foxa2 expression in WT and AntNPCs derived from ESCs with PE Sox1 insertion in Foxa2-TAD. **d**. Fold change in Wntlb expression in WT and AntNPCs derived from ESCs with PE Wntlb insertion in Gata6-TAD. **e**. Motif analyses (SeqPos, Homer) for TFBS and CGI. **f**. Fold change in Sox1 and Gata6 expression in WT and AntNPCs derived from ESCs with PE Sox1 and aCGI insertion in Gata6-TAD. **g**. Immunofluorescence staining of GATA6 and SOX1 proteins in WT, TFBS, CGI, and TFBS+CGI ESCs.
of cells in which the PE target genes get induced, thus potentially leading to high gene expression precision\(^44\).

**oCGIs do not increase the local activation of PEs.** To investigate the mechanisms whereby oCGIs potentiate the regulatory function of PEs, we focused on the ESC lines in which the **PE Sox1**\((+35)\) components were inserted within the **Gata6-TAD**. pCGIs are typically devoid of CpG methylation and display low nucleosomal density, which might provide a chromatin environment permissive for TF binding and transcription initiation\(^45,46\). Bisulfite sequencing experiments in **TFBS + CGI and TFBS ESCs** showed that the TFBS sequences acquired intermediate CpG methylation levels when inserted alone, while becoming completely unmethylated when combined with the oCGI (Fig. 3a and Extended Data Fig. 6a). In contrast, formaldehyde-assisted isolation of regulatory elements (FAIRE) assays showed that the oCGI only moderately increased chromatin accessibility whether inserted alone or in conjunction with the TFBS (Extended Data Fig. 6b). To simultaneously measure nucleosome occupancy and CpG methylation at the inserted TFBS with single-DNA-molecule resolution\(^47\), we also performed nucleosome occupancy and methylcyne PCR (Nome-PCR) assays. These experiments confirmed that oCGIs protect nearby TFBSs from CpG methylation without a major impact on chromatin accessibility (Extended Data Fig. 6c,d). Furthermore, upon differentiation into AntNPCs, the TFBSs got progressively demethylated in the TFBS cells (Fig. 3a and Extended Data Fig. 6a), suggesting that, even in the absence of an oCGI, TFs can access and activate PEs in AntNPCs\(^48\).

To test this prediction, we performed chromatin immunoprecipitation coupled to quantitative PCR (ChIP-qPCR) experiments to measure p300 binding and H3K27ac levels, two major hallmarks of PE transcription initiation\(^49\). In contrast, the recruitment of RNAP2 and Mediator to the TFBS insert was either considerably weaker or undetectable, respectively (Fig. 3c). Moreover, upon differentiation of PEs, we focused on the ESC lines in which the oCGI associated with the PE became strongly and similarly enriched in H3K27ac and p300 (Fig. 3d). However, the binding of these proteins to the TFBS + CGI insert when compared with the oCGIs alone (Extended Data Fig. 6f). Intriguingly, PR1 recruitment (that is, CBX7, PHC1 and RING1B) was considerably stronger for the TFBS + oCGI insert than for the oCGI alone (Fig. 3f).

Since pCGIs can mediate long-range homotypic interactions between distal pCG-bound loci\(^50,51\), we investigated the three-dimensional organization of the **Gata6** locus in our engineered ESC lines. Circular chromosome conformation capture coupled to sequencing (4C-seq) experiments using either the **Gata6** promoter or the PE Sox1\((+35)\) insertion site as viewpoints revealed strong PE-Gata6 contacts only in the TFBS + CGI cells (Fig. 3g). The lack of PE-gene contacts in cells with the **Gata6** alone could be attributed to the weaker recruitment of PR1 to the insert in these cells\(^51\) (Extended Data Fig. 6f). Furthermore, the strong interactions between the TFBS + CGI insert and the Gata6 promoter were also observed upon differentiation into AntNPCs (Extended Data Fig. 6g). Although the TFBS insert alone did not substantially contact with the **Gata6** promoter in AntNPCs (Extended Data Fig. 6g), **Gata6** was induced, albeit weakly, in these cells (Fig. 2b). This could be explained by the more transient and/or heterogeneous interactions between **Gata6** and the PE in the absence of the oCGI and/or by the capability of enhancers to induce gene expression without getting into close proximity of their target genes\(^50,51\). Next, to evaluate whether oCGIs are important for PE-gene contacts in an endogenous genomic context, we performed 4C-seq experiments in the ESCs in which the oCGI associated with **PE Sox1**\((+35)\) was deleted. Importantly, the deletion of this oCGI reduced the interactions between Sox1 and PE Sox1\((+35)\) (Fig. 3b).

Overall, our data suggest that oCGIs increase the functional communication between PEs and their target genes by bringing them into close spatial proximity.

**CpG-poor promoters do not show responsiveness to distal PEs.** Developmental genes, such as those regulated by PEs\(^52,53\), contain...
large CGI clusters around their promoters, whereas tissue-specific genes tend to have CpG-poor promoters\textsuperscript{22}. PEs and their target genes could spatially segregate from genes with CpG-poor promoters by engaging into active (that is, transcription factories) or inactive (that is, polycomb bodies) homotypic chromatin interactions depending on their transcriptional state\textsuperscript{46,51}. Therefore, the responsiveness of developmental genes to PEs could depend not only on the presence of PE oCGIs, but also on CGIs located at the target gene promoters. In agreement with this hypothesis, analysis of Hi-C data generated in ESCs\textsuperscript{7,52} showed that PEs strongly interact with developmental
genes with CGI-rich promoters located in the same TADs, but not with genes with CGI-poor promoters (Fig. 4a and Extended Data Fig. 6h). To test whether CpG-poor promoters are responsive to PEs, we inserted the PE Sox1(+35) components into the Gria1-TAD, approximately 100 kb upstream of the Gria1-TSS (Fig. 4b and Extended Data Fig. 7a). Similarly to Gata6 and Foxa2, Gria1 is not expressed in either ESCs or AntNPCs. However, the Gria1 promoter does not contain CGIs and is not bound by PcG but fully DNA methylated instead (Fig. 4b). Remarkably, upon differentiation of the Gria1-TAD cell lines, none of the PE Sox1(+35) inserts were able to induce Gria1 expression (Fig. 4c and Extended Data Fig. 7b).

To gain mechanistic insights into this lack of responsiveness, we measured DNA methylation, H3K27ac, p300, RNAP2, MED1 and eRNA levels around the inserted PE Sox1(+35) constructs. Similarly to what we observed within the Gata6-TAD, the TFBS became demethylated in ESCs, albeit partially, when combined with the oCGI (Extended Data Fig. 7c). Furthermore, upon differentiation into AntNPCs, the TFBS + CGI and TFBS inserts became strongly similar and strongly enriched in H3K27ac and p300 (Fig. 4d). However, in contrast to what we observed in the Gata6-TAD, we did not detect eRNAs around any of the PE Sox1(+35) inserts (Fig. 4e).

Concurrently, the recruitment of RNAP2 and MED1 to the PE Sox1(+35) was weak regardless of whether the TFBS was alone or with the oCGI (Fig. 4f). In addition, RNAP2 and MED1 were not recruited to the Gria1 promoter, thus in agreement with the lack of Gria1 induction observed upon differentiation of the Gria1-TAD ESC lines (Fig. 4f).

Our results indicate that H3K27ac and eRNA production can be uncoupled from each other and represent different steps during PE activation (Figs. 3b, 4c, and 4d). Namely, the accumulation of H3K27ac might occur as PEs become locally activated, while the production of eRNAs, which is coupled with gene transcription, could signify the functional activation of the PEs50,51. To assess if these observations could be generalized, we compared eRNA production between three classes of AEs using nascent transcriptomic and epigenomic data generated in ESCs52,54: (1) Class I enhancers located in TADs containing only poorly expressed genes; (2) Class II enhancers located in TADs with at least one highly expressed gene; and (3) Class III enhancers whose closest gene within the same TAD is highly expressed (Methods). Interestingly, Class I enhancers showed ~2- and 2.5-fold lower eRNA levels than Class II and Class III enhancers, respectively, while H3K27ac levels were similar among the three enhancer groups (Fig. 4g and Extended Data Fig. 7d). These results suggest that enhancer and gene transcription are frequently coupled and mutually dependent on each other52,57.

Promoters with large CGI clusters are responsive to PEs. The experiments within the Gata6-TAD suggest that the responsiveness to PEs involves the physical proximity between PEs and their target genes, which in ESCs is likely to be mediated by PcGs present at both PEs and promoters14,58. ChiP experiments in the Gria1-TAD ESC lines revealed that PcGs were recruited to the PE Sox1(+35) inserts containing an oCGI (Fig. 5a and Extended Data Fig. 8a), albeit not as strongly as for the Gata6-TAD (Fig. 3e, f). Furthermore, the Gria1 promoter, which does not contain pCGIs, was not bound by PcG (Fig. 5a and Extended Data Fig. 8a).

Accordingly, 4C–seq analyses showed that none of the inserted PE Sox1(+35) constructs were able to interact with the Gria1 promoter (Fig. 5b). In principle, the addition of pCGIs to the Gria1 promoter could increase PcG recruitment and, consequently, the physical and functional communication with the distal PE Sox1(+35) constructs. To test this prediction, we introduced one of the Gata6 pCGIs into the Gria1 promoter in those ESC lines containing either the TFBS + CGI or TFBS inserts 100 kb away from the Gria1-TSS (Fig. 5c and Extended Data Fig. 8b). Upon differentiation into AntNPCs, the addition of the pCGI did not result in detectable Gria1 messenger RNA or eRNAs around the PE inserts, suggesting that a single CGI is not sufficient to trigger the long-range responsiveness to PEs (Fig. 5c and Extended Data Fig. 8c). Interestingly, in comparison with the PcG levels observed for promoters with large CGI clusters (for example, Gata6), the insertion of a single pCGI into the Gria1 promoter led to relatively mild PcG recruitment (Fig. 5d). This could explain, at least partly, the lack of Gria1 responsiveness to the distal PE Sox1(+35). Alternatively, the Gria1 promoter might contain core-promoter elements that are not responsive to developmental enhancers11,12. To evaluate this possibility, we generated ESC lines in which the PE Sox1(+35)TFBS or PE Sox1(+35) TFBS + CGI constructs were integrated 380 base pairs (bp) upstream of the Gria1-TSS (Fig. 5e and Extended Data Fig. 8d,e). Remarkably, both the TFBS + CGI and TFBS inserts were able to strongly induce Gria1 expression upon differentiation into AntNPCs (Fig. 5e and Extended Data Fig. 8f). These results show that the Gria1 promoter can respond to the PE Sox1(+35) and suggest that the boosting effect of the oCGI might be lost when the PEs are located close to gene promoters.

When inserted into the Gria1-TAD, the PE Sox1(+35) TFBS + CGI did not acquire the same chromatin state as within the Gata6-TAD (that is, lower PRC1 levels and higher DNA methylation (Fig. 5a and Extended Data Fig. 7c)). Therefore, the constitutive heterochromatin environment of the Gria1-TAD might result in chromatin and/or topological properties at the PE insertion site that somehow compromise the regulatory function of the oCGI. To investigate this and further assess whether developmental genes with large CGI clusters in their promoters are particularly responsive to PEs, we inserted the PE Sox1(+35) constructs into the Sox7/Rp111-TAD, right between Sox7 and Rp111 (24 kb from Sox7 and Rp111 TSSs) (Fig. 5f and Extended Data Fig. 8g). Sox7 and Rp111 are both inactive in ESCs and AntNPCs, but differ in their type of
promoter (Fig. 5f): Sox7 is an endodermal regulator whose promoter contains a large CGI cluster and is strongly bound by PcG, while Rp1l1 is specifically expressed in mature rod cells and its promoter does not contain CGIs and is not bound by PcG. Remarkably, upon AntNPC differentiation, none of the PE Sox1(+35) inserts were able to induce Rp1l1 expression (Fig. 5g and Extended Data Fig. 8h). In stark contrast, Sox7 was strongly induced by the TFBS + CGI, while the TFBS alone led to a milder gene induction (Fig. 5g and Extended Data Fig. 8h). Together with our experiments in other TADs, these results strongly indicate that developmental genes with large CGI clusters in their promoters are particularly responsive to distal PEs.

CGIs and TAD boundaries control gene expression specificity. Our data suggest that, in addition to TAD boundaries, the interactions
between PE-associated oCGIs and pCGI clusters proximal to developmental genes might contribute to gene expression specificity during embryogenesis. To test this prediction, we genetically engineered two different loci: Six3/Six2 and Lmx1a/Lrrc52/Mgst3 (Fig. 6a,d).

We first focused on the Six3/Six2 locus (Fig. 6a): (1) Six3 and Six2 are contained within two neighboring TADs separated by a conserved TAD boundary; (2) Six3 and Six2 display mutually exclusive expression patterns during embryogenesis (for example,
Six3 in brain; Six2 in facial mesenchyme); (3) the Six3-TAD contains a PE (that is, PE Six3(−133)) that controls the induction of Six3 in AntNPCs without any effects on Six2 (Ref. 14); and (4) in ESCs, the PE Six3(−133) strongly interacts with Six3 but not with Six2 (Ref. 14), although both genes contain multiple pCGIs. Next, we generated ESCs with either a 36-kb deletion spanning the Six3/Six2-TAD boundary (del36) or a 110-kb inversion that places Six3 within the Six2-TAD and vice versa (inv110) (Fig. 6a and Extended Data Fig. 9a,b). Upon differentiation into AntNPCs, Six2 was strongly induced in del36 and inv110 cells (−12- and −35-fold versus WT, respectively), while Six3 expression was dramatically reduced in inv110 cells (−77-fold versus WT) and mildly affected in del36 cells (−2.5-fold versus WT) (Fig. 6b). In agreement with these gene expression changes, 4C-seq experiments in WT, del36 and inv110 ESCs showed that both the boundary deletion and the inversion resulted in increased interactions between Six2 and PE Six3(−133) (Fig. 6c). Furthermore, a CBS immediately upstream of the PE Six3(−133) could also contribute to the long-range communication (Fig. 6a). However, the deletion of this CBS did not have any major impact on Six3 or Six2 expression in either WT or inv110 AntNPCs, respectively (Extended Data Fig. 9c–e). Altogether, these results indicate that Six3 and Six2, whose promoters have large CGI clusters, are responsive to the PE Six3(−133) and, potentially, to other enhancers located within the Six3-TAD (Ref. 14).

Next, we focused on the Lmx1a/Lrrc52/Mgst3 locus (Fig. 6d). Lmx1a and Lrrc52/Mgst3 are located in neighboring TADs separated by a strong TAD boundary. The three genes have different types of promoters and expression patterns in ESCs and AntNPCs. Lmx1a, a developmental gene with a large CGI cluster in its promoter, is bound by PcG in ESCs and induced in AntNPCs. Lrrc52, a tissue-specific gene without CGIs, is not bound by PcG in ESCs and is inactive in ESCs and AntNPCs. Mgst3, a ubiquitously expressed gene with a single and short CGI centered on its TSS, is not bound by PcG and is active in both ESCs and AntNPCs. The Lmx1a-TAD contains a PE (that is, PE Lmx1a(+/113)) that becomes active in AntNPCs and that presumably contributes to Lmx1a induction in these cells. Considering all this, we generated two ESC lines with a 260-kb inversion that places Lmx1a and PE Lmx1a(+113) within the Lrrc52/Mgst3-TAD (inv260) (Fig. 6d and Extended Data Fig. 9f). Notably, neither Lrrc52 nor Mgst3 was induced upon differentiation of the inv260 ESCs into AntNPCs (Fig. 6e). These results indicate that tissue-specific and housekeeping genes without large CGI clusters in their promoters are not responsive to distal PEs.

Overall, our data suggest that PEs specifically execute their regulatory functions due to the combined effects of TAD boundaries, which provide insulation, and homotypic interactions between oCGIs and pCGIs, which confer enhancer responsiveness (Fig. 7).

**Discussion**

Deciphering the factors that control enhancer–promoter compatibility is a major challenge in the enhancer field. According to current models, insulator proteins demarcate TAD boundaries and restrict enhancers to act upon genes located within their same TADs. Nonetheless, enhancers do not promiscuously activate all the genes present within a TAD, suggesting that additional factors control enhancer responsiveness. Massively parallel reporter assays in *Drosophila* showed that enhancer responsiveness is determined by the sequence composition of core promoters. We now show that, in the context of PE loci, such responsiveness is also dependent on distal genetic elements, namely oCGIs, which serve as tethering elements that allow PEs to preferentially activate promoters containing large CGI clusters (Fig. 7). Although CGIs are considered a vertebrate-specific genomic feature, regulatory sequences with similar tethering functions have been also described in invertebrates.

Our data suggest that the role of oCGIs as potentiators of PE regulatory functions does not involve the local activation of PEs.
but rather the establishment of long-range interactions with developmental genes (Fig. 7). In pluripotent cells, these PE–gene interactions are likely to be mediated by PRC1 complexes recruited to both oCGIs and pCGIs\cite{2,4,6,7,21}. Intriguingly, our data suggest that the binding of PRC1 to the PEs is increased by the combination of TFBS and oCGIs. While the importance of CGIs as PcG recruitment
target genes (Fig. 3d), suggesting that oCGIs might favor the formation of phase-separated transcriptional condensates74. Once PEs are active, multivalent interactions occurring within these condensates could robustly maintain PE–gene communication4. According to our analyses, the regulatory function of the PE-associated oCGIs could be primarily attributed to their CpG-richness. Namely, oCGIs can serve as recruitment platforms for ZF-CXXC proteins that, as part of major complexes (for example, PcG, TrxG, Mediator), can facilitate the physical and functional communication between PEs and their target genes4,75,76. In addition, TFs with CG-rich binding sites (for example, Sp1)77,78 might be also recruited to oCGIs and thereby contribute to PE–gene communication. Lastly, some oCGIs might contain binding sites for tissue-specific TFs that are important for the regulatory activity of PEs5.

We propose a model whereby the precise and specific induction of certain developmental genes is achieved through the combination of CGI-mediated long-range chromatin interactions and the insulation provided by TAD boundaries (Fig. 7). As illustrated by the TFAP2A and EPHA4 loci, the function of CGIs as determinants of enhancer–gene compatibility can help in understanding why only some structural variants that disrupt TAD organization lead to enhancer adoption and major changes in gene expression (Extended Data Fig. 10)5,63. Therefore, our findings may have important medical implications, as they could improve our ability to predict the pathological consequences of human structural variation5 (Fig. 7 and Extended Data Fig. 10).

Online content
Any methods, additional references, Nature Research reporting summaries, source data, extended data, supplementary information, acknowledgements, peer review information; details of author contributions and competing interests; and statements of data and code availability are available at https://doi.org/10.1038/s41588-021-00888-x.

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platforms is well established14,71, how the TFBS can contribute to PRC1 recruitment is still an open question. Furthermore, our experiments in the Gria1-TAD suggest that a single pCGI is not sufficient to enable the long-range communication with PEs. This could be explained, at least partly, by the low levels of PRC1 recruitment that a single CGI can confer in comparison with the large CGI clusters associated with developmental gene promoters. Genetic engineering experiments whereby multiple and long CGIs are inserted in CpG-poor promoters will be required to assess if these genetic features are sufficient to increase the long-range responsiveness to PEs. Regardless, once recruited, Pc complexes might keep PEs and their target genes close together during pluripotent cell differentiation, ensuring that PEs uniformly induce their target genes as they become active. Then, once RNAs are produced at both PEs and their target genes, this would result in PRC eviction72. Although PRC1 recruitment is still an open question. Furthermore, our experiments in the Gria1-TAD suggest that a single pCGI is not sufficient to enable the long-range communication with PEs. This could be explained, at least partly, by the low levels of PRC1 recruitment that a single CGI can confer in comparison with the large CGI clusters associated with developmental gene promoters. Genetic engineering experiments whereby multiple and long CGIs are inserted in CpG-poor promoters will be required to assess if these genetic features are sufficient to increase the long-range responsiveness to PEs. Regardless, once recruited, Pc complexes might keep PEs and their target genes close together during pluripotent cell differentiation, ensuring that PEs uniformly induce their target genes as they become active. Then, once RNAs are produced at both PEs and their target genes, this would result in PRC eviction72. Although PRC1 might also contribute to PE–gene communication once PEs become active67, additional proteins are likely to be involved in the maintenance of such contacts7. Interestingly, upon PE activation the oCGIs increase the loading of Mediator and RNAP2 to both PEs and their target genes (Fig. 3d), suggesting that oCGIs might favor the formation of phase-separated transcriptional condensates61. Once PEs are active, multivalent interactions occurring within these condensates could robustly maintain PE–gene communication4. According to our analyses, the regulatory function of the PE-associated oCGIs could be primarily attributed to their CpG-richness. Namely, oCGIs can serve as recruitment platforms for ZF-CXXC proteins that, as part of major complexes (for example, PcG, TrxG, Mediator), can facilitate the physical and functional communication between PEs and their target genes4,75,76. In addition, TFs with CG-rich binding sites (for example, Sp1)77,78 might be also recruited to oCGIs and thereby contribute to PE–gene communication. Lastly, some oCGIs might contain binding sites for tissue-specific TFs that are important for the regulatory activity of PEs5.

We propose a model whereby the precise and specific induction of certain developmental genes is achieved through the combination of CGI-mediated long-range chromatin interactions and the insulation provided by TAD boundaries (Fig. 7). As illustrated by the TFAP2A and EPHA4 loci, the function of CGIs as determinants of enhancer–gene compatibility can help in understanding why only some structural variants that disrupt TAD organization lead to enhancer adoption and major changes in gene expression (Extended Data Fig. 10)5,63. Therefore, our findings may have important medical implications, as they could improve our ability to predict the pathological consequences of human structural variation5 (Fig. 7 and Extended Data Fig. 10).

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Methods

Cell lines and differentiation protocol. E14Tg2a/E14 (E14) mouse ESCs were cultured on gelatin-coated plates with Knock-out DMEM (Life Technologies, 10829018) supplemented with 13% FBS (Life Technologies, 10082147) and leukemia inhibitory factor (LIF). For the AntNPC differentiation19, ESCs were plated at 12,000 cells per cm² on gelatin-coated plates and grown for 3 d in N2B27 medium supplemented with 10 ng/ml bFGF (Life Technologies, PHG0368) without serum or LIF. Subsequently, cells were grown for another 2 d in N2B27 medium without bFGF (days 3–5). From day 2 to day 5, the N2B27 medium was supplemented with 5 mM Xav939 (ref. 85) (Sigma, 284028-89-3). N2B27 medium: Advanced DMEM F12 (Life Technologies, 21040125) and Neurobasal medium (Life Technologies, 12348017) (1:1), supplemented with 1× F12 (Life Technologies, 21041025) and Neurobasal medium (Life Technologies, 12088074), 20 mM l-glutamine (Life Technologies, 25300024), 40 mM sodium pyruvate (Life Technologies, 15260037) and 0.1 mM 2-mercaptoethanol (Life Technologies, 31350010).

RNA isolation, complementary DNA synthesis and quantitative PCR with reverse transcription. Total RNA was isolated using InnuPrep RNA Mini Kit (Analytik Jena, 845-KS-2040250). cDNA was generated using ProtoScript II First Strand cDNA Synthesis Kit (New England Biolabs, E6560L). Quantitative PCR with reverse transcription (qPCR) analyses were performed on the Light Cycler 48001 (Roche) using Lef1al and Hprt as housekeeping genes. For each sample, RT–qPCRs were performed as technical triplicates using primers listed in Supplementary Data 1.

ChIP. First, 5 × 10⁶ (p300/RNA2P2/MEDI/PcG ChIPs) or 1 × 10⁶ (histone ChIPs) cells were crosslinked with 1% formaldehyde for 10 min at room temperature and quenched with 0.125 M glycine for 10 min. Cells were washed and resuspended sequentially in three lysis buffers (Buffer 1: 50 mM HEPES, 140 mM NaCl, 1 mM EDTA, 10% glycerol, 0.5% NP-40, 0.25% Triton X-100; Buffer 2: 10 mM Tris, 200 mM NaCl, 1 mM EDTA, 0.5 mM EGTA; Buffer 3: 10 mM Tris, 100 mM NaCl, 1 mM EDTA, 0.5 mM EGTA, 0.1% Na-deoxycholate, 0.8% Na-lauroylsarcosine) to isolate chromatin. Chromatin was sonicated for 15 cycles (20 s on, 30 s off, 25% amplitude) using an EpiShear probe sonicator (Active Motif). Sonicated chromatin was incubated overnight at 4 °C with 3 g of antibody for histones or 10 µg of antibody for other proteins. Next, 50 µl of Protein G magnetic beads (Invitrogen) were added and incubated for 4 h at 4 °C. Magnetic beads were washed and the chromatin eluted, followed by de-crosslinking and DNA purification. The ChIP and input DNA samples were analyzed by quantitative PCR (qPCR) using two mm10 intergenic regions as negative controls (chr2:73,030,265-73,030,373, chr6:52,339,345-52,339,505). The qPCR samples for each sample were performed as technical triplicates. All antibodies and primers used in ChIP–qPCR experiments are listed in Supplementary Data 1.

Bisulfite sequencing. Bisulfite conversion of 400 ng of genomic DNA was performed using the EZ DNA Methylation Kit (Zymo Research, D5001). The investigated sequences were amplified by PCR using EpTaq polymerase (Takara Bio, R1042B) and primers described in Supplementary Data 1. PCR products were cloned into the pGEM-T vector (Promega, A1360) and sequenced with the M13 reverse primer.

Immunofluorescence. Cells were fixed for 10 min in 3.7% paraformaldehyde at room temperature and incubated with 0.1% Triton X-100 for 15 min at room temperature and blocked in PBS with 5% BSA for 1 h at room temperature. Cells were incubated with primary antibodies (anti-GATA6 (AF1700, R&D Systems, 852;339,505) or anti-SOX1 (AF3369, R&D Systems, 852;339,505) in blocking solution for 30 min at room temperature. Nuclei were stained with 5 mM Xav939 (ref. 85) (Sigma, 284028-89-3). N2B27 medium: Advanced DMEM F12 (Life Technologies, 21040125) and Neurobasal medium (Life Technologies, 12348017) (1:1), supplemented with 1× N2 (Life Technologies, 1752048X), 1× B27 (Life Technologies, 12587010), 20 mM l-glutamine (Life Technologies, 25300024), 40 mM sodium pyruvate (Life Technologies, 15260037) and 0.1 mM 2-mercaptoethanol (Life Technologies, 31350010).

oCGI deletion using CRISPR-Cas9. To generate the deletion of the PE Sox1(+35) CGI, a pair of single guide RNAs (sgRNAs) flanking the oCGI were designed with Benchmark’s CRISPR toolkit (www.benchling.com) (Supplementary Data 1). For each sgRNA, two oligonucleotides were synthesized (IDT), annealed and cloned into a CRISPR-Cas9 expression vector (pX330-hCas9-long-chimeric-grna-2p; Leo Kurian's laboratory). EScs were transfected with the pair of sgRNAs–Cas9-expressing vectors using Lipofectamine 2000 (Thermo Scientific, Thermofisher, L3000001). After 16 h, puromycin selection was performed for 48 h. Surviving cells were isolated in 96-well plates by serial dilution and clones with the deletion were identified by PCR using primers listed in Supplementary Data 1. The presence of the deletion was confirmed by Sanger sequencing.

Homology-dependent knock-in. Knock-In of PE modules was performed as previously described in ref. 86. Briefly, an sgRNA was designed for the insertion site of interest and cloned in the CRISPR-Cas9 expression described above. Then, the cassette-vector was generated by ligation: (1) 300-bp homology arms flanking the insertion site; (2) construct of interest; and (3) cloning vector. The resulting construct was used as a template to amplify the full-length product for (left homology arm + construct + right homology arm) by PCR (Supplementary Data 2). The resulting PCR product was column-purified (28104, QIAgenes). ESCs were transfected with the sgRNA–Cas9-expressing vector and the knock-in donor using Lipofectamine (Thermo Scientific, L3000001). After 16 h, puromycin selection was performed for 48 h. Surviving cells were isolated in 96-well plates by serial dilution and clones with insertions were identified by PCR using the primers listed in Supplementary Data 1. The PE insertions were confirmed by Sanger sequencing.

FAIRE. Chromatin was sonicated as described for ChIP and then subjected to three rounds of phenol/chloroform extraction/chloroform precipitation followed by ethanol precipitation87. The FAIRE and input DNA was analyzed by qPCR using two mm10 intergenic regions as negative controls (chr2:73,030,265-73,030,373, chr6:52,339,345-52,339,505) and the primers listed in Supplementary Data 1.

NOME-PCR. Nuclear extraction and M.CvIP1 treatment were performed as described previously88. Briefly, isolated nuclei were incubated with 200 U of M.CvIP1 (NEB, M0227L) for 15 min at 37 °C. Then, bisulfite conversion was performed using the EZ DNA Methylation Kit (Zymo Research, D5001) and the converted DNA was amplified by PCR. PCR products were cloned into the pGEM-T vector (Promega, A1360) and sequenced with the M13 reverse primer. NOME-PCR data were analyzed with the NOMEPlot web tool (http://www.landeiralab.ugr.es/software).89

Computational and statistical analyses. Statistics and reproducibility. Immunofluorescence assays and genotyping of all the ESC lines were independently performed twice with similar results.

For RT–qPCR measurements in transgenic cell lines, expression levels were measured in two independent biological replicates. In each of these biological replicate experiments, two different clonal cell lines for each of the investigated genotypes were generally studied (unless stated otherwise), and for each clonal cell line two replicates of the AntNPC differentiation were measured. The statistical significance of the expression differences was calculated between AntNPCs with the TFRS + CGI module and AntNPCs with the other PE modules whenever the number of biological replicates was n ≥ 3.

Analyzes of qPCR data. For RT–qPCR, relative gene expression levels were calculated with the comparative delta Ct method (2⁻ΔΔCt), whereby the differences in Ct values between the genes of interest and two housekeeping genes (Lef1al and Hprt) were considered. Primers were found in Supplementary Data 1.

For ChIP–qPCR, for each sample, signals were calculated as percentage of input using technical triplicates and normalized to the average signals obtained in the same sample for two negative control regions (Chr2_neg and Chr6_neg; Supplementary Data 1).

aCGI design. The aCGI was designed by randomly incorporating nucleotides into an 800-bp sequence with a 50% higher chance of incorporating C or G rather than A or T. These GC-rich sequences were filtered to fulfill the Gardner–Garden criteria (that is, observed/expected ratio of CpGps > 0.6 and G+C percentage...
For Fig. 4g and Extended Data Fig. 7d (left), PRO-seq fastq files were obtained from GEO (GSE115713; sample IDs: SRR7300121, SRR7300122) and the two replicates were combined and preprocessed as described above. For Extended Data Fig. 7d (middle and right), two PRO-seq bigWig files (one for each DNA strand) were obtained from GEO (GEO136091).

TAD maps from ESCs were retrieved from refs. 18,90. For Fig. 4g and Extended Data Fig. 7d (left): mESC_Dixon2012-raw_TADs.txt. For Extended Data Fig. 7d (middle and right): mESC_Bonev_2017-raw.domains.

**H3K27ac and PRO-seq enhancer levels quantification.** Figure 4g and Extended Data Fig. 7d (left): H3K27ac and PRO-seq reads with a mapping quality <10 were discarded using SAMtools94. Next, bigwig files were generated with deepTools95 using bamCoverage (reads per genomic content normalization) and then used to calculate the H3K27ac and PRO-seq enhancer mean scores with computeMatrix from deepTools. For H3K27ac and PRO-seq, the signals were calculated using a ±1 kb or ±0.5 kb window from the enhancer midpoints, respectively.

Extended Data Fig. 7d (middle and right): H3K27ac and PRO-seq mean signals for the enhancers were calculated with the bigWigAverageOverBed UCSC binary tool. PRO-seq signals for each enhancer from the two different strands were averaged and the same was done for the signals coming from different H3K27ac replicates.

**AEs classification.** Three groups of AEs were defined: (1) Class I enhancers located in TADs only containing poorly expressed genes (<0.5 FPKM); (2) Class II enhancers located in TADs with at least one gene with >10 FPKM; (3) Class III enhancers whose closest gene within the same TAD has >10 FPKM.

**Balancing of H3K27ac levels within enhancer classes.** Enhancers with similar H3K27ac levels belonging to the three enhancer classes were selected by applying the nearest neighbor matching method (without replacement and ratio = 1) using MatchIt (https://cran.r-project.org/web/packages/MatchIt/MatchIt.pdf) and considering the enhancer group 1 as the treatment condition.

Cliff’s delta effect size estimator. Cliff’s delta102,103 was used to quantify the differences between groups of genomic regions. This measure is robust to skewed signal distributions104. Cliff’s delta was estimated using the cliff.delta() function from the R package effsize (https://cran.r-project.org/web/packages/effsize/index.html). Differences between groups with [delta] < 0.147 can be considered as negligible and [delta] ≥ 0.147 as non-negligible.

**Hi-C analyses.** Preprocessing. GSE130723: the .hic files for two Hi-C replicates (GSM3752487, GSM3752488) generated in ESCs were downloaded. The hic format was converted to .cool format using a 5-kb matrix resolution with the hic2cool software (https://github.com/4dn-dcc hic2cool). GSE98671: the .cool format files for two untreated ESC Hi-C replicates (GSM2644945, GSM2644946) at a 20-kb matrix resolution were downloaded.

For both datasets the corresponding replicates in .cool format were merged with cooler merge96 and normalized with cooler balance97.

**Definition of PE–gene pairs.** Group A. When a PE–distal was found in a TAD with a devTSS, both coordinates were selected to define a PE–gene pair. Only devTSS with a CAP-CGI in <3 kb were considered.

Group B. CGI-poor TSSs do not have a CAP-CGI in <3 kb and are not enriched in H3K27me3 (H3K27me3 ChIP–seq peaks described above). When a PE–distal was found in a TAD with a CGI-poor TSS, both coordinates were selected to define a PE–gene pair.

Two additional filters were applied: (1) PE–gene pairs were balanced to compare groups of PE–gene pairs without significant differences in their linear genomic sizes. PE–gene pairs with similar lengths were selected by applying the nearest neighbor matching method (without replacement and ratio = 1) using MatchIt (https://cran.r-project.org/web/packages/MatchIt/MatchIt.pdf) and considering Group A as the treatment condition; (2) only TSSs of genes with expression <1 FPKM were considered.

We used TADs defined in mESC_Dixon2012-raw_TADs.txt.

**Pile-up plot generation.** The pile-up plots for the GSE130723 and GSE98671 Hi-C datasets were generated with coolplot.py98 using a padding of ±50 kb or ±100 kb, respectively.

**TF motif analyses.** The genomic coordinates of PEs and AEs were defined by p300 peaks identified in ESCs19 and located ≥2.5 kb away from any ResSeq TSS.

**CAP-CGI versus p300 peaks.** Among the previously reported PEs13, we considered only those with a CAP-CGI in <3 kb and that did not overlap with the p300 peaks defining the PEs. Then, motif analyses were performed separately for the CAP-CGIs and the p300 peaks associated with the selected PEs using Homer10 and SeqPos10.
ChIP-seq profile plots. PE classification. PE-distal were separated into four groups:

1. PEs with overlapping TFBS/p300 and CAP-CGIs; 2. PEs with TFBS/p300 separated by 1–3kb from a CAP-CGI; and 3. PEs without CAP-CGIs in 3kb. The coordinates of AEs without CAP-CGI in <3kb were also considered.

Datasets used. H3K27me3: GSE157748 (Extended Data Fig. 1b, left) and GSE89209 (Extended Data Fig. 1b, right). H3K27me3 ChIP–seq datasets from ESCs were used. For GSE89209, the fastq files SRR443529 was processed as described in previous sections. For GSE157748, bigwig files (GSM4775418, GSM4775419) were downloaded and combined using bigWigMerge and bedGraphToBigWig UCSC tools106.

TET1: GSE104067 was used. The bigwig files of three untreated ESC replicates (GSM2788888, GSM2788889, GSM2788890) were downloaded and combined using bigWigmerge and bedGraphToBigWig UCSC tools106.

KDM2B: GSE126862 was used. The bigwig file with all the merged untreated ESC replicates (GSE126862 KDM2B_CXXCgi_30%_KDM2B_Unt_mm10_downsampled_merged.bed) was downloaded. Bigwig coordinates were converted from mm10 to mm9 with CrossMap111.

Plots generation. Profiles plots were generated using computeMatrix and plotProfile from deepTools112.

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

Data availability
All the 4c-seq data generated in this study are available through the GEO (GSE136465). All the generated transgenic ESC lines are available upon request.

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Author contributions
T.P and A.R.-I. conceptualized the project. Experimental investigations were performed by T.P, T.E., M.M.-F, M.M.-S. and E.H. T.P. and A.R.-I. wrote, reviewed and edited the manuscript. S.C.-M., V.S.-G. and T.B. performed the experimental investigations. T.P. and A.R.-I. conceptualized the project. Experimental investigations were performed by T.P, T.E., M.M.-F, M.M.-S. and E.H. T.P., V.S.-G. and T.B. were responsible for obtaining resources. A.R.-I. was responsible for supervision and funding acquisition.

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Extended Data Fig. 1 | See next page for caption.
Extended Data Fig. 1 | Genetic and epigenetic features of the oCGIs associated with PEs. a, Comparison of CpG%, observed/expected CpG ratio, GC% and sequence length between random regions (n = 436000), NMIs associated to PE-distal (PE-NMIs; n = 345) and NMIs associated to the devTSS (devTSS-NMIs; n = 1476) (Methods). The p-values were calculated using two-sided unpaired Wilcoxon tests with Bonferroni correction for multiple testing; black numbers indicate median fold-changes; green numbers indicate non-negligible Cliff Delta effect sizes. The coloured area of the violin plot represents the expression values distribution and the center line represents the median.
b, H3K27me3 ChIP-seq levels around: PE-distal with overlapping TFBS/p300 peaks and CAP-CGIs (n = 135), PE-distal with TFBS/p300 peaks separated by 1bp-1kb from CAP-CGIs (n = 65), PE-distal with TFBS/p300 peaks separated by 1-3kb from CAP-CGIs (n = 53), PE-distal without CAP-CGIs within 3kb (n = 254) and AES without CAP-CGI within 3kb (n = 8115).
c, % of CpG methylation at CAP-CGI associated with PE-distal (PE-CAP-CGI; n = 276) and CAP-CGI associated with the TSS of developmental genes (devTSS-CAP-CGI; n = 1926) in the indicated cell types (Methods).
d, For the identification of the PE Sox1(+35)CGI deletion, primer pairs flanking each of the deletion breakpoints (1+3 and 4+2), located within the deleted region (5+6) or amplifying a large or small fragment depending on the absence or presence of the deletion (1+2) were used.
e, H3K27me3 levels at PE Sox1(+35) were measured by ChIP-qPCR in WT ESCs and n = 2 independent PE Sox1(+35) CGI−/− ESC clones using primers adjacent to the deleted region. The bars display the mean of n = 3 technical replicates (black dots).
f, Independent biological replicate for the data presented in Fig. 1d. Sox1 expression was investigated by RT-qPCR in ESCs and AntNPC with the indicated genotypes. n = 2 independent PE Sox1 CGI−/− ESC clones (circles and diamonds) and n = 1 PE Sox1−/− clone were studied. For each cell line, n = 2 replicates of the AntNPC differentiation were performed. Expression values were normalized to two housekeeping genes (Eyf1a and Hprt) and are presented as fold-changes with respect to WT ESCs. The coloured area of the violin plot represents the expression values distribution and the center line represents the median.
Extended Data Fig. 2 | See next page for caption.
Extended Data Fig. 2 | Engineering of PEs modules within the Gata6-TAD and FoxA2-TAD. a, Epigenomic and genomic features of two previously characterized PEs in which the oCGIs overlap with conserved sequences bound by p300 and, thus, likely to contain relevant TFBSs. b, The different PE Sox1(+35) insertions were identified using primer pairs flanking the insertion borders (1+3 and 4+2; 1+5 and 6+2; 1+3 and 6+2), amplifying potential duplications (4+3, 3+2 and 4+1; 6+5, 5+2 and 6+1) and amplifying a large or small fragment depending on the absence or presence of the insertion (1+2), respectively. The PCR results obtained for WT ESCs and for two ESC clonal lines with homozygous insertions of the PE Sox1(+35) modules in the Gata6-TAD are shown. c, Independent biological replicate for the data presented in Fig. 2b. d-e, Strategy used to insert the PE Wnt8b(+21) (d) or the PE Sox1(+35) (e) components into the Gata6-TAD (d) or Foxa2-TAD (e), respectively. The right panels shows the TADs in which Gata6 (d) or Foxa2 (e) are included according to publicly available Hi-C data, with the red triangle indicating the integration site of the PE modules, approximately 100 Kb downstream of Gata6 (d) or Foxa2 (e). f-g, For identifying the successful insertion of the different PE Sox1(+35) (f) or PE Wnt8b(+21) (g) modules, primer pairs flanking the insertion borders (1+3 and 4+2; 1+5 and 6+2; 1+3 and 6+2), amplifying potential duplications (4+3, 3+2 and 4+1; 6+5, 5+2 and 6+1) and amplifying a large or small fragment depending on the absence or presence of the insertion (1+2), respectively, were used. The PCR results obtained for two ESC clonal lines with homozygous insertions of the indicated PE modules in the Foxa2-TAD (f) or Gata6-TAD (g), respectively, are shown. h-i, Independent biological replicates for the data shown in Fig. 2c (h) and Fig. 2d (i). In (c), (h) and (i), the expression differences between AntNPCs with the TFBS + CGI module and AntNPCs with the other PE modules were calculated using two-sided non-paired t-tests (**: foldchange > 2 & p < 0.001; *: foldchange > 2 & p < 0.05; ns: not significant; fold-change < 2 or p > 0.05).
Extended Data Fig. 3 | PEs are enriched in CpG-rich motifs and are bound by CxxC-domain containing proteins. a, Comparison of the TF motifs enriched in either PEs with a CAP-CGI in <3kb and active enhancers without CAP-CGIs in <3kb. Motif enrichment analyses were performed with Homer (left) and AME (right). b, ChIP-seq signals for KDM2B (upper panel) and TET1 (lower panel) are shown around: PE-distal with overlapping TFBS/p300 peaks and CAP-CGIs (n=135), PE-distal with TFBS/p300 peaks separated by 1bp-1kb from CAP-CGIs (n=65), PE-distal without CAP-CGIs within 3kb (n=254). ChIP-seq profile plots were generated using either the p300 peaks (left) or the CAP-CGIs (right) associated with the PEs as midpoints.
Extended Data Fig. 4 | See next page for caption.
Engineering of ESC lines containing the PE Sox1(+35) TFBS and an artificial CGI within the Gata6-TAD.

**a.** Strategy used to insert the PE Sox1(+35)TFBS alone or together with an aCGI into the Gata6-TAD. The upper left panel shows the epigenomic and genetic features of the PE Sox1(+35). The lower left panel shows the PE Sox1(+35) modules inserted into the Gata6-TAD. The right panel shows the Gata6-TAD according to publically available Hi-C data. The red triangle indicates the integration site of the PE Sox1(+35) modules approximately 100 Kb downstream of Gata6.

**b.** For the identification of the PE Sox1(+35)TFBS+aCGI insertion, primer pairs flanking the insertion borders (1+3 and 4+2), amplifying potential duplications (4+3 and 4+4) and amplifying a large or small fragment depending on the absence or presence of the insertion (1+2), respectively, were used. The PCR results obtained for two ESC clonal lines with homozygous insertions of PE Sox1(+35)TFBS+aCGI in the Gata6-TAD are shown.

**c.** Independent biological replicate for the data presented in Fig. 2f. The expression differences between AntNPCs with the TFBS+CGI module and AntNPCs with the other PE modules were calculated using two-sided non-paired t-tests (*: fold-change > 2 & p<0.05; ns: not significant; fold-change<2 or p>0.05). The PCR results obtained from two ESC clonal lines with heterozygous insertions of aCGI in the Gata6-TAD are shown.

**d.** For the identification of the aCGI insertion alone, primer pairs flanking the insertion borders (1+3 and 4+2), amplifying potential duplications (4+3 and 4+4) and amplifying a large or small fragment depending on the absence or presence of the insertion (1+2), respectively, were used. The PCR results obtained from two ESC clonal lines with heterozygous insertions of aCGI in the Gata6-TAD are shown.

**e.** The expression of Gata6 and Sox1 was measured by RT-qPCR in cells that were either WT or heterozygous for the aCGI insertion in the Gata6-TAD (two different clones; circles and diamonds). For each cell line, n = 2 replicates of the AntNPC differentiation were performed. The results obtained in n = 2 independent biological replicates are presented in each panel (Rep1 and Rep2).
Extended Data Fig. 5 | Gata6 expression patterns in cell lines with the PE Sox1(+35) modules inserted within the Gata6-TAD. a, Gata6 and Sox1 expression was measured by RT-qPCR in ESCs and at intermediate stages of AntNPC differentiation (Day 3 and Day 4). The analysed cells were either WT or homozygous for the insertions of the different PE Sox1(+35) modules within the Gata6-TAD. For the cells with the PE module insertions, n=1 clonal cell line was studied. For each cell line, n=2 replicates of the AntNPC differentiation were performed. Expression values were normalized to two housekeeping genes (Eef1a and Hprt) and are presented as fold-changes with respect to WT ESCs. b, Quantification of cells expressing GATA6 or SOX1 according to immunofluorescence assays as the ones shown in Fig. 2g. The analysed cells were either WT or homozygous for the insertions of the different PE Sox1(+35) modules within the Gata6-TAD. c, The expression patterns of GATA6 (upper panel) and SOX1 (lower panel) were investigated by immunofluorescence in WT ESCs or AntNPCs that were either WT, homozygous for the insertion of the PE Sox1(+35)TFBS+aCGI in the Gata6-TAD or heterozygous for the insertion of the aCGI alone in the Gata6-TAD. Nuclei were stained with DAPI. Scale bar=100µm. d, Quantification of cells expressing GATA6 or SOX1 according to the immunofluorescence assays described in (c). In (b) and (d), the bars display the mean of n=3 technical replicates (black dots).
Extended Data Fig. 6 | See next page for caption.
Extended Data Fig. 6 | Epigenetic and topological characterization of the Gata6-TAD cell lines. a, Bisulfite sequencing data presented in Fig. 3a for the indicated Gata6-TAD cell lines. The circles correspond to individual CpG dinucleotides located within the TFBS module. Unmethylated CpGs are shown in white, methylated CpGs in black and not-covered CpGs in gray. b, Chromatin accessibility at the endogenous PE Sox1(+35) and the Gata6-TAD insertion site (P1 and P2) were measured by FAIRE-qPCR in cells with the indicated genotypes. c, DNA methylation and nucleosome occupancy at the TFBS were simultaneously analyzed by NOMe-PCR in the indicated Gata6-TAD ESC lines. In the upper panels, the black and white circles represent methylated or unmethylated CpG sites, respectively. In the lower panels, the blue or white circles represent accessible or inaccessible GpC sites for the GpC methyltransferase, respectively. Red bars represent inaccessible regions large enough to accommodate a nucleosome. The dotted line indicates where the TFBS starts. The grey shaded area represents a nucleosome-depleted region. d, Scatter plots showing population-averaged nucleosome occupancy (red) and DNA methylation (black) levels within the TFBS in the indicated Gata6-TAD ESC lines. The grey shaded area represents a nucleosome depleted region. e-f, H3K4me1, H3K4me3, H2AK119ub, CBX7 and PHC1 levels at the endogenous PE Sox1(+35) and the Gata6-TAD insertion site (P1 and P2) were measured by ChIP-qPCR in cells with the indicated genotypes. ChIP-qPCR signals were calculated as described in Fig. 3. g, 4C-seq experiments were performed using the Gata6 promoter as a viewpoint in AntNPC with the indicated genotypes. h, Pile-up plots showing average Hi-C signals in ESC between two groups of PE-gene pairs: PEs and developmental genes with CGI-rich promoters; PEs and genes with CGI-poor promoters. For each PE-gene pair, both the PE and the gene were located within the same TAD. Left panels include all the considered PE-gene pairs (n = 401 pairs for developmental genes; n = 900 for CGI-poor promoters; middle panels include PE-gene pairs with the same genomic size in the two groups (n = 401 pairs); right panels consist of PE-gene pairs with the same genomic size and genes with expression levels <1 FPKM (n = 290 pairs) (Methods).
Extended Data Fig. 7 | See next page for caption.
Extended Data Fig. 7 | Generation of cell lines with engineered PE Sox1(+35) modules within the Gria1-TAD and global characterization of H3K27ac and eRNA levels at active enhancers. a, ESC clonal lines with insertions of the different PE Sox1(+35) modules were identified using primer pairs flanking the insertion borders (1 + 3 and 4 + 2; 1 + 5 and 6 + 2; 1 + 3 and 6 + 2), amplifying potential duplications (4 + 3, 3 + 2 and 4 + 1; 6 + 5, 5 + 2 and 6 + 1) and amplifying a large or small fragment depending on the absence or presence of the insertion (1 + 2), respectively. The PCR results obtained for WT ESCs or two ESC clonal lines with homozygous insertions of the different PE Sox1(+35) modules in the Gria1-TAD are shown. b, Independent biological replicate for the data presented in Fig. 4b. The expression differences between AntNPCs with the TFBS + CGI module and AntNPCs with the other PE modules were calculated using two-sided non-paired t-tests (ns: not significant; fold-change < 2 or p > 0.05). c, Bisulfite sequencing analyses of ESC lines with the indicated PE Sox1(+35) modules inserted in the Gria1-TAD. The circles correspond to individual CpG dinucleotides located within the TFBS: unmethylated CpGs (white), methylated CpGs (black) and not-covered CpGs (gray) are shown. The plot on the right summarizes the DNA methylation levels measured within the TFBS in the indicated ESC lines. d, Active enhancers (AEs) identified in ESCs based on the presence of distal H3K27ac peaks were classified into three categories (Methods): Class I (AEs in TADs containing only poorly expressed genes; n = 271 (left); n = 340 (middle, right)); Class II (AEs in TADs with at least one highly expressed gene; n = 271 (left); n = 2353 (middle); n = 340 (right)); Class III (AEs whose closest genes in the same TAD is highly expressed; n = 271 (left); n = 1262 (middle); n = 340 (right)). The violin plots show the H3K27ac and eRNA levels in ESC for each AE category. P-values were calculated using unpaired Wilcoxon tests with Bonferroni correction for multiple testing; the numbers in black indicate the median fold-changes between the indicated groups; the coloured numbers correspond to Cliff Delta effect sizes: negligible (red) and non-negligible (green). In the left and right panels, eRNA levels for the three enhancers classes are compared after correcting for H3K27ac differences (Methods).
Extended Data Fig. 8 | See next page for caption.
Extended Data Fig. 8 | Generation and characterization of cell lines with PE insertions at the Gria1 and Sox7/Rp11 TADs. a, H2AK229ub and SUZ12 levels at the endogenous PE Sox1(+35), the Gria1 promoter and the Gria1-TAD insertion site (P1 and P2; Fig. 4d) were measured by ChIP-qPCR in ESCs with the indicated genotypes. ChIP-qPCR signals were calculated as in Fig. 3. b, ESC clonal lines in which a pCGI was inserted 380bp upstream of the Gria1-TSS in cells with the indicated PE Sox1(+35) modules 100Kb upstream from Gria1 were identified using the indicated primer pairs. PCR results for clonal ESC lines with the indicated double homozygous insertions are shown. c, eRNA levels at the endogenous PE Sox1(+35) and the Gria1-TAD insertion site (P1 and P2) were measured by RT-qPCR in cells with the indicated genotypes. Expression values were calculated as in Fig. 3. d, Strategy to insert the indicated PE Sox1(+35) modules 380bp upstream (red triangle) of the Gria1-TSS. e, ESC clonal lines with the PE Sox1(+35) modules 380bp upstream of the Gria1-TSS were identified using the indicated primer pairs. PCR for ESC clonal lines with homozygous insertions of the indicated PE Sox1(+35) modules are shown. f, Independent biological replicate for the data presented in Fig. 5e. g, ESC clonal lines with the PE Sox1(+35) modules within the Sox7/Rp11-TAD were identified using primers flanking the insertion borders (1+3 and 4+2; 1+3 and 6+2), amplifying potential duplications (4+3, 3+2 and 4+1) and amplifying a large or small fragment depending on the absence or presence of the insertion (1+2), respectively. PCR results for ESC clonal lines with homozygous insertions of the indicated PE Sox1(+35) modules are shown. h, Independent biological replicate for the data presented in Fig. 5g. In (a) and (c), the bars display the mean of n = 3 technical replicates (black dots). In (f) and (h), the expression differences between AntNPCs with the TFBS + CGI module or the other PE modules were calculated using two-sided non-paired t-tests (**: fold-change > 2 & p<0.0001; ns: not significant; fold-change<2 or p>0.05).
Extended Data Fig. 9 | See next page for caption.
Extended Data Fig. 9 | Generation of ESC lines with structural variants. a, ESC lines with the Six3/Six2 TAD boundary deletion were identified using primers flanking the deleted region (1+3 and 4+2), amplifying the deleted fragment (5+6) and amplifying a large or small fragment depending on the absence or presence of the deletion (1+2), respectively. The PCR results for two ESC clonal lines with 36Kb homozygous deletions (del36) are shown. b, ESC lines with the Six3/Six2 inversion were identified using primer pairs flanking the inverted region (1+3, 4+2, 1+4 and 3+2) and amplifying potential duplications (4+3, 3+3 and 4+4). The PCR results for two ESC clonal lines with 110Kb homozygous inversions (inv110) are shown. c, Epigenomic and genetic features of a CTCF binding site (CBS; highlighted in grey) located upstream of the PE Six1(−133) (highlighted in yellow). d, ESC lines with the CBS deletion were identified using primers flanking the deleted region (1+2) or located in the CBS (3+4). The PCR results for two ESC clonal lines with homozygous CBS deletions are shown. e, The expression of Six3 and Six2 was measured by RT-qPCR in cells with the indicated genotypes. For each of the engineered structural variants, n=2 independent clonal cell lines were generated (circles and diamonds). In each plot, the number of circles and/or diamonds corresponds to the number of AntNPC differentiations performed. The results obtained in n=2 independent biological replicates are presented in each panel (Rep1 and Rep2). Expression values are presented as fold-changes with respect to WT ESCs. f, ESC lines with the Lmx1a-TAD boundary inversion were identified using primers flanking the inverted region (1+3, 4+2, 1+4 and 3+2) and amplifying potential deletions (1+4) or duplications (4+3, 3+3 and 4+4). The PCR results for three ESC clonal lines with 260Kb homozygous inversions (inv260) are shown.
Extended Data Fig. 10 | See next page for caption.
Extended Data Fig. 10 | Examples of human congenital diseases caused by structural variants that disrupt developmental loci with PE-associated oCGIs. a, Upper panel: heterozygous inversion in a patient with Branchio-oculo-facial syndrome (BOFS)\textsuperscript{5}. Lower panel: epigenomic and genetic features of TFAP2A neural crest (NC) cognate enhancers (left), 6q16.2 genes (middle) and TFAP2A (right). In the lower left panel, enhancer reporter assays in chicken embryos are shown for two representative TFAP2A enhancers\textsuperscript{5}. Computational CGI and NMI are represented as green rectangles. The inversion places one TFAP2A allele into a novel TAD and impairs its normal expression in NC cells due to the physical disconnection from its enhancers. TFAP2A has a promoter with a large CGI cluster and marked with a broad H3K27me3 domain in ESCs. Some TFAP2A NC enhancers are associated with oCGIs and marked with H3K27me3 in ESCs. Moreover, this inversion places genes originally found within the 6q16.2 locus in proximity of the TFAP2A NC enhancers within a shuffled domain. The promoters of these 6q16.2 genes (i.e GPR63 and NDUFAF4) contain a short CGI centered on their TSSs. In agreement with our findings, none of the 6q16.2 genes is responsive to the TFAP2A NC enhancers\textsuperscript{5}. b, Upper panel: deletion found in families with brachydactyly involving a TAD boundary located between the EPHA4 and the PAX3 loci\textsuperscript{63}. Lower panel: epigenomic and genetic features of the EphA4 cognate enhancers in the mouse E11.5 limb (left) and in human ESCs (right). Representative reporter assay in E11.5 mouse embryos for the hs1507 element is shown in the middle\textsuperscript{63}. The deletion includes EPHA4, a gene highly expressed in the developing limb, and the TAD boundary separating the EPHA4 and PAX3 TADs. As a result, enhancers that control EPHA4 expression in the limb establish ectopic interactions with PAX3 (that is enhancer adoption) and strongly induce its expression in the limb. The PAX3 promoter contains a large CGI cluster and is marked with H3K27me3 in ESCs, while one of the major EPHA4 enhancers (hs1507) is associated with an oCGI and is marked with H3K27me3 in ESCs. The high responsiveness of PAX3 to the EPHA4 enhancers is in agreement with our findings.
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Sample size For all the transgenic ESC lines we generated at least two clonal lines with the same genotype. Taking into account the amount of different cell lines generated and that the clones from the same cell line were always responding in a very similar manner, we decided to keep a minimum of two clones per investigated genotype.

Data exclusions No data was excluded

Replication All experiments were performed independently at least twice and all the attempts at data replication were successful. The exact number of replicates is described in the corresponding figure legends.

Randomization Randomization was not relevant for our study as the sample sizes of the different experiments were too small for randomization.

Blinding The investigators were not blinded to allocation during experiments and outcome assessment. There was one person in charge of generating and characterizing all the transgenic cell lines and we did not have additional personal that could be solely in charge of analyzing the data. Blinding is typically used with randomization and large sample sizes, which, as stated before, does not apply to our experiments.

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| ☒ ☒ Dual use research of concern | |

Antibodies used

| Antibody Source | ID | Application | Dilution |
|-----------------|----|-------------|----------|
| H3K4me1 Active Motif 39297 | ChiP-qPCR | 3 μl |
H3K4me3 Active Motif 39159 ChiP-qPCR 3 μl
H3K27ac Active Motif 39133 ChiP-qPCR 3 μl
H3K27me3 Active Motif 39155 ChiP-qPCR 3 μl
H2AK119ub Cell Signaling Technology B2405 ChiP-qPCR 3 μl
P300 Active Motif 61401 ChiP-qPCR ChiP-qPCR 10 ul
RBP2 Active Motif 39097 ChiP-qPCR 10 ul
MED1 Bethyl Laboratories A300-793A ChiP-qPCR 10 ul
RING18 Active Motif 39663 ChiP-qPCR 10 ul
SUV39H2 Abcam ab12073 ChiP-qPCR 10 ul
CBX7 Abcam ab21873 ChiP-qPCR 10 ul
PHC1 Cell Signaling Technology 13768S ChiP-qPCR 10 ul
GATA6 R&D systems AF1700 Immunofluorescence 8μl/ml
SOX1 R&D systems AF3369 Immunofluorescence 8μl/ml
Donkey anti-Goat IgG Alexa Fluor Plus 488 Invitrogen A32814 1 ul/ml
Donkey anti-Goat IgG Alexa Fluor Plus 594 Invitrogen A32758 1 ul/ml

Validation
H3K4me3, H3K27ac and H3K27me3 antibodies were validated in: Cruz-Molina, S. et al. PRC2 Facilitates the Regulatory Topology Required for Poised Enhancer Function during Pluripotent Stem Cell Differentiation. Cell Stem Cell 20, 1−17 (2017).

H2AK119ub antibody was validated in: Gao Z et al., PCGF homologs, CBX proteins, and RYBP define functionally distinct PRC1 family complexes. Mol Cell. 3. 34-56 (2012).
P300 antibody was validated by the manufacturer: "ChiP was performed using the ChiP-IT High Sensitivity Kit (Cat. No. 53040) with chromatin from 4.5 million LNCaP cells and 5 μl of p300 antibody. ChiP DNA was sequenced on the Illumina HiSeq and 15 million sequence tags were mapped to identify p300 binding sites. p300 along with H3K4me1 are markers of active enhancer elements and are therefore expected to co-localize. A sampling of the p300 ChiP-Seq data shows the expected co-localization of p300 and H3K4me1." [https://www.activemotif.com/catalog/details/61401].

RNA2 antibody was validated by the manufacturer: "RNA pol II antibody (mAb) tested by ChiP-Seq. ChiP was performed using the ChiP-IT High Sensitivity Kit (Cat. No. S3040) with chromatin from 2.3 million HL-60 cells and 20 μl of antibody. ChiP DNA was sequenced on the Illumina HiSeq and 30 million sequence tags were mapped to identify Pol II binding. ChiP-Seq data from three specific genes is shown as an example. Pol II binding is detected at the 5' end of the genes, throughout the gene bodies and at the 3' end of the genes." [https://www.activemotif.com/catalog/details/39097/ma-pol-ii-antibody-mab]

SUV39H2 antibody was validated in: Zhang W et al. The BAF and PRC2 Complex Subunits Dpf2 and Eed Antagonistically Converge on Tbx3 to Control ESC Differentiation. Cell Stem Cell 24:138-152.e8 (2019).

CBX7 antibody was validated in: Fursova NA et al. Synergy between Variant PRC1 Complexes Defines Polycomb-Mediated Gene Repression. Mol Cell 74:1020-1036.e8 (2019).

PHC1 antibody was validated in: Blackledge NP et al. PRC1 Catalytic Activity is Central to Polycomb System Function. Mol Cell. (4)857-874 (2020).

GATA6 antibody was validated in: Molotkov et al. Distinct Requirements for FGFR1 and FGFR2 in Primitive Endoderm Development and Exit from Pluripotency. Dev. Cell, 2017;5(0).

SOX1 antibody was validated in: Lager et al. Rapid functional genetics of the oligodendrocyte lineage using pluripotent stem cells. Nat Commun, 2018;9(1):3708.

Donkey anti-Goat IgG Alexa Fluor Plus 488 and Donkey anti-Goat IgG Alexa Fluor Plus 594 were validated in: Cruz-Molina, S. et al. PRC2 Facilitates the Regulatory Topology Required for Poised Enhancer Function during Pluripotent Stem Cell Differentiation. Cell Stem Cell 20, 1−17 (2017).

Eukaryotic cell lines

Policy information about cell lines

Cell line source(s)
Male mouse ESC line E14Tg2a was used for all experiments. This cell line was a kind gift from Joanna Wysocka’s lab (Stanford University).

Authentication
The insertions, deletions and inversions were authenticated by PCR genotyping and confirmed by Sanger-sequencing.

Mycoplasma contamination
Both WT E14 mESC and all the transgenic ESC lines tested negative for mycoplasma contamination.

Commonly misidentified lines
None

(See ICLAC register)