Retinoic acid and BMP4 cooperate with p63 to alter chromatin dynamics during surface epithelial commitment

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Human embryonic stem cell (hESC) differentiation promises advances in regenerative medicine1–3, yet conversion of hESCs into transplantable cells or tissues remains poorly understood. Using our keratinocyte differentiation system, we employ a multi-dimensional genomics approach to interrogate the contributions of inductive morphogens retinoic acid and bone morphogenetic protein 4 (BMP4) and the epidermal master regulator p63 (encoded by TP63)4,5 during surface ectoderm commitment. In contrast to other master regulators6–9, p63 effects major transcriptional changes only after morphogens alter chromatin accessibility, establishing an epigenetic landscape for p63 to modify. p63 distally closes chromatin accessibility and promotes accumulation of H3K27me3 (trimethylated histone H3 lysine 27). Cohesin HiChIP10 visualizations of chromosome conformation show that p63 and the morphogens contribute to dynamic long-range chromatin interactions, as illustrated by TFAP2C regulation11. Our study demonstrates the unexpected dependency of p63 on morphogenetic signaling and provides novel insights into how a master regulator can specify diverse transcriptional programs based on the chromatin landscape induced by exposure to specific morphogens.

As published protocols of human embryonic stem cell (hESC)-derived keratinocytes suffer from excessive heterogeneity12–15, we developed a xeno-free, chemically defined differentiation system using E6 medium16 supplemented with two morphogens, retinoic acid (RA) and BMP4 (Fig. 1a). Treatment with RA and BMP4 (RA/BMP4 treatment) produced functional keratinocytes that behaved similarly to those described previously16 (Supplementary Fig. 1). Differentiating cells progressed through a simple epithelial state as indicated by immunofluorescence analysis of epithelial markers keratin 18 (K18)17 and epidermal master regulator p63 (encoded by TP63) (refs 18,19) at day 7, followed by high levels of p63 and keratinocyte maturation marker keratin 14 (K14)20 at day 45 (Fig. 1a). Robust p63 expression occurred only when both morphogens were present, indicating a synergistic role for p63 accumulation (Fig. 1b,c and Supplementary Fig. 1). As morphogenetic exposure for 7 days induced both uniform p63 expression and subsequent keratinocyte development21–24, we interrogated this key 7-day period with a multi-dimensional genomics approach to understand the functional interaction between p63 and the morphogens.

To assess the individual contributions to chromatin dynamics, we created p63 gain- and loss-of-function hESCs using CRISPR–Cas9 (clustered, regularly interspaced palindromic repeat–CRISPR-associated protein 9) technology (Fig. 1d,f and Supplementary Fig. 2a,b) to yield four cell types: d0 (wild-type hESCs), d0 p63GOF (hESCs ectopically expressing p63), d7 p63WT (morphogen-treated wild-type hESCs with endogenous p63), and d7 p63KO (morphogen-treated hESCs with no p63 expression). We used the ΔNp63α isoform for our p63GOF cell line because it is predominantly expressed in our system, consistent with published reports of developing keratinocytes21–23 (Supplementary Fig. 2c,d). Furthermore, we designed the p63-knockout (p63KO) to mimic the alleles of the p63-null mouse24 (Supplementary Fig. 2d), in which the DNA-binding domain is deleted. We verified loss of p63 protein in these cell lines through immunofluorescence, western blot, and sequencing (Fig. 1e,g and Supplementary Fig. 2).

Previous studies indicate that p63 overexpression can drive surface ectoderm maturation24, yet remarkably, expression of p63 in hESCs was insufficient to induce an exit from pluripotency and a switch towards epidermal differentiation (Fig. 1e and Supplementary Fig. 2e). Consistent with this observation, transcriptome analysis using RNA sequencing (RNA-seq) showed minimal expression changes between d0 and d0 p63GOF (only 320 genes changing), compared with the expression changes between d7 p63WT and d7 p63KO (more than 2,400 genes) (Fig. 1h). Furthermore, morphogen exposure resulted in a p63-independent exit from pluripotency and was required for p63 regulation of key transcription factors associated with epithelial development (Fig. 1h and Supplementary Fig. 2f). These transcription factors, including TFAP2C and KLF4, were all repressed by p63 on morphogen treatment. Notably, other key epidermal and surface ectoderm-promoting developmental transcription factors, such as JUN27,28 and MSX1 (ref. 29), were p63-independent at this stage (Supplementary Fig. 2f). The morphogen–dependence of these factors together with their p63-independence suggests that these regulators work in parallel.

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Fig. 1 | Morphogens and p63 cooperate to drive early epithelial differentiation. a, RA/BMP4 treatment of hESCs for 7 days induces K18 and p63 expression. Functional keratinocytes expressing K14 and p63 grow out in keratinocyte selection media (n = 3). Scale bar, 50 μm. FGF, fibroblast growth factor; TGFβ, transforming growth factor-β. b, c, hESCs need exposure to both RA and BMP4 to achieve high p63 expression. Error bars represent s.d., n = 3. P values (Tukey HSD post-hoc test): BMP4 versus RA/BMP4 (P = 0.0011), BMP versus RA (NS, not significant; P=0.7591), RA/BMP4 versus RA (***P=0.0022). d, Strategy for generating the d0 p63GOF cell line. Numbered black boxes denote exons. Dox, doxycycline; PPP1R12C, protein phosphatase 1 regulatory subunit 12C; rtTA, reverse tetracycline-controlled transactivator. EF1α1, elongation factor 1α1. e, Expression of p63 in the d0 p63GOF line (n = 3). Scale bar, 50 μm. f, Strategy for generating the p63KO line. g, Immunofluorescence validation of the p63KO line (n = 3). Scale bar, 50 μm. h, Differential expression analysis from RNA-seq (measured by DESeq2) between d0 and d0 p63GOF (upper panel), and d7 p63WT and p63KO (lower panel). Genes have no change in expression (gray), increased expression (>2-fold change) in the d0 or d7 p63WT (red), or decreased expression (<−2-fold change) in the d0 or d7 p63WT (blue). Key transcription factors associated with epithelial development are induced by the morphogens and repressed by p63. FPKM, fragments per kilobase of transcript per million. i, Using HOMER analysis, the p63 motif was the most significantly recovered motif under p63 ChIP−seq peaks. j, p63 binds distal to TSSs, as depicted at the HES1 locus, and to the same sites in d0 p63GOF and d7 p63WT. Tracks represent n = 2. k, p63 binds to similar sites genome-wide with and without morphogen presence.
Fig. 2 | The morphogens establish an epigenetic landscape that p63 modifies at a distance. a, Differential accessible regions between d0 and d7 p63WT as analyzed using DESeq2 on ATAC-seq signal. Heatmaps represent the signal at these ATAC regions within the various cell types and assays: p63 ChIP-seq signal (red, left panel), ATAC-seq signal (blue, middle panel), and H3K27me3 ChIP-seq signal (purple, right panel). Upon morphogen treatment, 14,191 differential regions become more accessible (morphogen-dependent). b, Differential H3K27me3 regions between d7 p63WT and p63KO as analyzed by DESeq2. Heatmaps represent the same datasets as in a only signal is shown at the 3,793 differential H3K27me3 sites. c, ATAC-seq (blue) and H3K27me3 (purple) signal at p63 binding sites (red). d, Signal intensities of p63 ChIP-seq, ATAC-seq, and H3K27me3 ChIP-seq shown at the differential p63 peaks in both data sets (Fig. 1i). Remarkably, over 70% of the sites showed significant differences in p63 occupancy cannot explain the dramatic morphogen-modulated p63 activity.

with p63 to specify the surface ectoderm fate, further highlighting the importance of the morphogen contributions. d7 p63 over-expression (d7 p63GOF) resulted in no significant changes in expression of p63-dependent genes, indicating the existence of maximal endogenous p63 repression at this timepoint (Supplementary Fig. 2g). Thus, we conclude that morphogenetic signaling promotes a simple epithelial state, while enabling p63 to modify the morphogen-induced transcriptome to drive epidermal fates.

The striking influence of morphogens on p63 activity led us to investigate whether differences in p63 genomic occupancy accounted for the altered transcriptional activity. p63 chromatin immunoprecipitation followed by sequencing (ChIP–seq) in d0 p63GOF and d7 p63WT identified 7,960 and 6,097 p63 binding sites, respectively, with the p63 motif significantly enriched under peaks in both data sets (Fig. 1l). Remarkably, over 70% of the sites were identical between data sets (Fig. 1j,k), while 17% of peaks were gained in the d0 p63GOF (Supplementary Fig. 3a). Thus differences in p63 occupancy cannot explain the dramatic morphogen-regulated p63 activity.

We next characterized how the morphogens and p63 affected chromatin accessibility and deposition of four histone modifications (histone H3 lysine 27 acetylation (H3K27ac), H3K27me3, histone H3 lysine 4 trimethylation (H3K4me3) and histone H3 lysine 4 monomethylation (H3K4me1)) using the assay for transposase-accessible chromatin followed by sequencing (ATAC–seq). More than one-third of the morphogen-dependent accessible sites became more accessible on p63 loss (Fig. 2a,d), Comparison of established histone modifications in d7 p63WT and p63KO showed significant differences in H3K27me3, yet no observable differences on activating promoter or enhancer marks (Supplementary Fig. 3a–c). p63 absence resulted in a significant decrease in signal of the H3K27me3 mark, whereas H3K27me3 increased in d0 p63GOF (Fig. 2b,d). Interestingly, unlike what might be expected based on previous keratinocyte studies30,31, global loss of H3K27me3 in the d7 p63KO did not coincide with increased expression of histone-lysine
Fig. 3 | p63−TSS connections are associated with negative regulation genome-wide. a, Number of p63 binding sites (BS), p63-dependent (p63-dep) H3K27me3 sites, morphogen-dependent (morph-dep) ATAC sites, and p63-dep TSSs participating in chromatin looping (Anchored, red) versus those that are not (Not Anchored, blue). b, Percentage of p63-independent (p63-indep) genes (blue) and p63-dep genes (red), whose TSS is connected to p63 by direct binding (0°), direct contact (1°), or via one (2°) or two (3°) morph-dep ATAC and/or p63-dep H3K27me3 elements. p63-dep genes and p63-indep genes are connected to p63 via one or more of these chromatin conformations. FDR by Monte Carlo simulation *FDR < 0.05, ***FDR < 0.001 (Supplementary Fig. 6a). c, Gene ontological terms associated with p63-connected, p63-indep genes (blue) and p63-dep genes (red). d, Empirical cumulative distribution function (ecdf) of the log2[fold change] in gene expression between d7 p63WT versus d7 p63KO cells for all p63-connected genes (red) and 1° p63-connected genes (blue) compared to all genes (black). n, number of genes. Two-sided t test.

g, ecdf of the expression level changes (d7 p63WT versus d7 p63KO) of genes whose TSS is connected to a p63BS and morph-dep ATAC site, which are connected to each other (green) compared to all genes (black). n, number of genes. Two-sided t test.
Fig. 4 | p63 negatively regulates TFAP2C expression through morphogen-induced and p63-dependent distal elements and connectivity. a, Cohesin HiChIP identifies complex looping interactions at the TFAP2C locus. Schematic of morphogen- and p63-dependent interactions (upper panels) with virtual 4C plots of the normalized HiChIP data (bottom panel). In the virtual 4C plots, the dotted line and eye denote the viewpoint from which the interactions are drawn for the graph: the top plot depicts all interactions from the TFAP2C TSS; the second plot depicts all interactions from the p63 binding site; the third plot depicts all interactions from the ATAC d7 peak; the fourth plot depicts all interactions from the distal H3K27me3 peak. Together, these plots represent the full connectivity at the TFAP2C locus (n = 3). b, A 520-bp deletion surrounding the p63 binding site (p63BSKO) was generated using CRISPR/Cas9. c, Deletion of the p63 binding site leads to an increase in TFAP2C expression similar to the levels seen in the d7 p63KO cells. Loss of TFAP2C expression leads to a dramatic increase in p63 expression. Relative pixel intensity was calculated from three independent images (*P < 0.05; ***P < 0.005; NS, not significant). Error bars represent s.e.m. Scale bar, 20 μm. d, ChIP–qPCR for H3K27me3 at the TFAP2C locus shows a decrease in the histone mark in the d7 p63BSKO, similar to the d7 p63KO (***P < 0.005), n = 2. ATAC–qPCR shows an increase in accessibility at the ATAC d7 peak in d7 p63BSKO, again similar to the d7 p63KO cells (*P = 0.04, ***P = 0.002), n = 4. Graphs depict signal relative to input and error bars represent s.e.m. Ctrl, control; IgG, immunoglobulin G.
N-methyltransferase EZH2 (Supplementary Fig. 3d). Furthermore, H3K27me3 ChIP-seq signal decreased during differentiation: 1,396 of 1,433 differential regions had higher signal in d0 than in d7 p63WT, and 1,283 of these differential regions were p63-dependent. Gene ontology terms for the genes associated with morphogen-dependent H3K27me3 regions related to cell and neuron fate commitment (Supplementary Fig. 3e). ChromHMM (v.1.10) analysis indicated that most of the accessibility changes and p63 binding sites occur in enhancers (Supplementary Fig. 3f). We conclude that p63 edits a subset of the morphogen-induced accessibility changes and regulates the accumulation of H3K27me3.

Lineage selectors can act either directly or at a distance on the epigenetic landscape to alter accessibility or histone modification deposition24. To determine how p63 acts, we intersected the p63-dependent H3K27me3 regions and morphogen-dependent accessible sites with p63 binding sites, showing that few of the p63 binding sites overlapped with either of these changing elements (Fig. 2c,e). These data indicate that most of the p63 epigenetic regulatory action occurs distal to p63 binding. Interestingly, when we assigned p63 binding sites, morphogen-dependent accessible sites, and differential H3K27me3 regions to the nearest genes through GREAT (v.3.0.0), we found that these elements converge on a common gene set, despite each being in distinct genomic regions (Fig. 2f and Supplementary Fig. 3g,h).

To assess the connectivity and dynamics of the three-dimensional architecture between these distinct genomic regions, we employed cohesin HiChIP, a method analogous to high-throughput chromosome conformation capture (Hi-C)19, in all four cell types. We identified high-confidence chromatin contacts with 10-kb resolution using FitHiC (v.1.0.1)18 (Supplementary Fig. 4) and demonstrated that 54% of p63 ChIP-seq peaks in d7 p63WT participate in these chromatin connections (Fig. 3a). In addition, most morphogen- and p63-dependent dynamic elements participate in looping connections. Notably, only 34% of genes identified by GREAT as having transcriptional start sites (TSSs) connected to p63 binding sites were verified by cohesin HiChIP, reinforcing the non-uniformity of the existing chromatin landscape (Supplementary Figs. 3 and 5).

For the 4,412 protein-coding, p63-dependent genes (≥1.5-fold change in gene expression between d7 p63WT and d7 p63KO), we determined the connectivity of their TSSs to a p63 binding site, indicating that 13% of these genes were in direct contact with p63 via chromatin looping (1st) and 11% were in indirect contact via a morphogen-dependent accessible site or p63-dependent H3K27me3 region (2nd) (Fig. 3b and Supplementary Table 1). Although more complex conformations through multiple elements (3rd) were detected, random simulation demonstrated that p63 was not connected to p63-dependent genes by 3rd connections at a frequency above random chance (false discovery rate (FDR) = 0.173); therefore, we focused on the 0th, 1st, and 2nd p63 connections (Fig. 3b and Supplementary Fig. 6). Analysis of p63 connectivity to p63-independent genes (<1.5-fold change in gene expression between d7 p63WT and d7 p63KO) was also conducted (Fig. 3b and Supplementary Table 1).

By interrogating the correlation between p63 connection to any protein-coding TSS and transcriptional regulation, we found that p63 connectivity was insufficient to regulate gene expression (Fig. 3b). Both p63-dependent and p63-independent genes connected to a p63 site were involved in organ development and cell differentiation, consistent with known p63 function (Fig. 3c)18. Although connection of the TSS to p63 was not a guarantee of p63 regulation, the probability of transcriptional repression was significantly higher at genes connected to p63 (Fig. 3d). Furthermore, d7 p63-independent genes connected to p63 include keratinocyte differentiation genes whose expression becomes p63-dependent later during keratinocyte maturation, including TP63 itself (Supplementary Table 2)22,34,35. These data suggest that p63 and morphogen-regulated chromatin connections foreshadow future gene action. Notably, specific chromatin conformation types were not indicative of positive or negative p63 gene regulation (Supplementary Table 3). In all, a large subset of the morphogen- and p63-dependent elements are physically connected at d7 (Fig. 3e), accounting for the ability of p63 to regulate the epigenetic landscape at a distance.

Next, we determined the extent to which p63 and the morphogens influenced connectivity (Fig. 3f). In 1st and 2nd connections, contacts between morphogen-dependent accessible sites and p63 binding sites were regulated by both the morphogens and p63, with loss of p63 abolishing the connections, and overexpression of p63 failing to enhance them. Conversely, p63–H3K27me3 and p63–TSS interactions were enhanced by the morphogens and p63 overexpression, and weakened by p63 loss (Supplementary Fig. 7). Finally, we found greater repression at TSSs connected to both p63 and morphogen-dependent accessible sites (Fig. 3g) than at TSSs connected to both p63 and an H3K27me3 peak (Supplementary Fig. 7b). These findings indicate that morphogen- and p63-dependent conformational changes drive optimal p63-regulated transcription.

From our global analyses, we identified TFAP2C, which encodes a critical epithelial regulator25, as a gene induced by morphogens and repressed by p63 that exhibits a complex chromatin architecture driving its regulation. To illustrate p63–morphogen interactions, we dissected the p63 negative feedback regulation of this key developmental regulator (Fig. 4). Cohesin HiChIP analysis (Fig. 4a, Supplementary Fig. 8a–c) identified a distal p63 binding site with three d7 p63WT connections to the TSS; through a direct contact, the adjacent morphogen-dependent accessible site, and the distal H3K27me3 peak, all within 400 kb. We confirmed our cohesin HiChIP with UM1-4C (a targeted chromosome conformation capture (4C) approach that uses unique molecular identifiers (UMIs))23 using primer viewpoints around the three connections (Supplementary Fig. 9).

Comparison of the chromosome conformation among the different cell lines indicated that p63 presence enhances connectivity to all three of the main loops at d7, and in the absence of p63, the connections and transcriptional output collapse. Morphogen exposure connects p63 to the induced neighboring morphogen-dependent accessible site, but the connection relies on ongoing p63 expression to maintain it, as loss of p63 fails to uphold it despite morphogen presence.

To validate the importance of the morphogen-dependent accessible site, we removed the region using CRISPR/Cas9 and demonstrated a loss of morphogen-induced TFAP2C expression (Supplementary Fig. 8d). Furthermore, we hypothesized that removal of the p63 binding site should drive both TFAP2C and p63 expression, given our observation that TFAP2C induces p63 expression in hESCs (L.L. and A.E.O., unpublished observations) and that p63 provides important early negative regulation of TFAP2C. To test this, we deleted the p63 binding site (p63BSKO) and found dramatically elevated levels of TFAP2C at d7, consistent with the predicted negative feedback modulation of TFAP2C by p63 (Fig. 4b,c and in p63BSPM line, Supplementary Fig. 10). Moreover, d7 p63BSKO showed increased expression of p63, demonstrating the need for tight p63–morphogen regulation to control the levels of key developmental factors. Given that high levels of p63 in d7 p63GOF do not further alter gene expression (Supplementary Fig. 2g), these data confirm that p63 tightly modulates TFAP2C expression through its binding and connections at the locus. Histone ChIP coupled with quantitative PCR (ChIP–qPCR) showed a loss of H3K27me3 accumulation at both the TSS and the distal H3K27me3 site in d7 p63BSKO, whereas other non-p63-connected sites remained unaffected (Fig. 4d). Similarly, the morphogen-dependent accessible site became more accessible in d7 p63BSKO, to levels found in d7 p63KO (Fig. 4d), confirming the connectivity of these distal elements.
Here, we deepen our understanding of the interplay between morphogens and lineage selectors and find that morphogens provide the powerful driving force for cell state change by inducing expression of the lineage factor while also altering chromatin accessibility, histone modifications, and chromosome conformation. We speculate that transcription factors and epigenetic regulators, whose expression are morphogen induced, control these landscape alterations to poised the chromatin for p63 action. p63 then modifies the morphogen-dependent landscape to drive surface ectoderm differentiation. We demonstrate that p63 cannot function without morphogens, indicating the importance of these downstream factors in helping p63-mediated transcriptional regulation. Sequence analysis of morphogen-dependent accessibility sites implicates combinatorial regulation by multiple transcription factors with p63 as the driver of this process.

Our results illustrate how chromatin connections to the lineage selector p63 are necessary for and likely to induce gene expression changes, but alone are not sufficient to induce these changes. Our finding that p63 at d7 is poised to act on later keratinocyte differentiation genes (Supplementary Table 2) suggests the existence of additional inductive influences after addition of RA and BMP4 that enable broader p63-dependent transcription. This is functionally similar to ‘poised’ histone modifications and provides a structural explanation of how the order of morphogen exposure can determine downstream transcriptional programs.

Previous studies have highlighted the importance of p63 interactions with chromatin remodelers such as DNMT3A and PRC2, suggesting a mechanism of action for p63 at these distal enhancer regions. Interestingly, as protein levels of EZH2 are unaltered in our p63KO cells (Supplementary Fig. 3d), the reduction in H3K27me3 that we observe is likely to be dependent upon p63 interactions with a variety of other transcription factors and chromatin remodelers depending on the stage of differentiation.

This study has important implications for the apparent autonomy of lineage selectors and for the basis of morphogenesis. Our work suggests that small changes in morphogen activity can dramatically alter the induced chromosomal landscape and connectivity, explaining how a single lineage selector like p63 can direct a panoply of transcriptional programs depending on specific morphogen exposure.

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Author contributions
S.P.M. and J.M.P. designed and executed experiments, analyzed data, and wrote the manuscript. S.N.P. analyzed data and wrote the manuscript. J.L.T., E.B., M.R.M., C.R., H.H.Z., L.L., and X.B. executed experiments and contributed to experimental design. E.L., D.A., A.J.R., and G.S. contributed to data analysis. H.Y.C. and P.A.K. contributed to experimental design. A.E.O. designed experiments, analyzed data, wrote the manuscript, and conceived the project with S.P.M.

Competing interests
The authors declare no competing interests.

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Methods
CRISPR/Cas9-guided genome editing. Guide RNAs (gRNAs) were designed using the tool available on the CRISPR Design web page (see URLs)40, selected based on the highest scores and the least off-targets, and incorporated into a DNA fragment bearing all of the necessary components for gRNA expression. Donor sequences were designed by selecting 700-bp arms flanking the region to be modified. Both gRNAs and donor sequences were synthesized as 5'-phosphorylated gene blocks (IDT) and cloned into a blunt-end plasmid with pmc0523, except for gRNAs targeting the AAVS1 locus, which were acquired through Addgene (Plasmid 72833). The d16-p6GOF line was generated by integrating the humanized ΔNp63 mouse cDNA under the control of a tetracycline responsive element (TRE) to the AAVS1 locus. Doxycycline (Sigma) was added to the media to titrate the Np63 mouse cDNA. For differentiation, 6.2 × 103 H9 human embryonic stem cells were cultured on Vitronectin Recombinant Human Protein (Life Technologies) in Essential 8 (Life Technologies) and in a Stratagene real-time PCR machine. Primer sequences for qPCR are provided as described previously. 

Cohesin HiChIP. In situ chromosome conformation capture (3C) was performed as described previously. In brief, 25 × 10^6 cells were crosslinked and digested with Mbol (NEB). After digestion, biotin was incorporated into the sticky ends of fragments before ligation. Cohesin HiChIP was performed to enrich for proximity ligations bound to cohesin, using an Smc1 antibody (Bethyl, A300-055A). The library quality was assessed on a MiSeq sequencer before sequencing on an Illumina HiSeq 4000. Three replicates were pooled and sequenced across two HiSeq lanes for a total of 1,200 million reads per sample.

UMI-4C. UMI-4C was performed as described previously. In brief, 1 × 10^6 cells were crosslinked in suspension with 1% formaldehyde then quenched with glycine, and pelleted cells were lysed in 1 ml fresh cold lysis buffer (50 mM Tris–HCl, pH 7.5, 150 mM NaCl, 5 mM EDTA, 0.5% SDS, 0.1% Triton X-100, 1% protease inhibitors) for 30 minutes on ice. The nuclei were extracted and resuspended in water. DpnII buffer, and 10% SDS for DpnII digestion. Three rounds of DpnII digestion were performed, adding 200 U of HC DpnII (NEB) for 2 h, incubating overnight, and then 2 hours more, at 37°C with rotation. After inactivation of DpnII, the 3C reactions were incubated with 40 μl of Ecoli T4 ligase (NEB) for 1 h at 16°C. Crosslinks were reversed overnight at 65°C with Protease K (Qiagen) and treated with RNase A (Qiagen) for 45 minutes at 37°C. The DNA was then purified with one phenol-chloroform extraction (Thermo Scientific) and ethanol precipitation, and resuspended in 150 μl of 10 mM Tris–HCl, pH 8.0. DNA was quantified using Qubit before proceeding with library preparation. Two independent, biological replicates were sequenced per sample.

Next-generation sequencing processing of ChIP-seq and ATAC-seq data. Quality control of fastq files was done with FASTQC (v.0.11.3). Sequence alignment was performed using TopHat (TopHat configuration: -p 10 --library-type fr-firststrand -p 100 --mate-std-dev 100, or bowtie2 (ref. 47) for ChIP-seq (parameters: -p 24 -S -m 1 -X 2000). Aligned reads were processed to remove PCR duplicates using samtools (see ATAC-seq datasets). Peak calling was carried out with MACS2 (ref. 42) using default settings with a P-value of 0.05. To filter out non-reproducible peaks, called peaks from biological replicates were adapted through the IRDR framework implemented in R5.
Differential counting, heatmaps, and average profiles. For ChIP−seq and ATAC−seq, a union list of the MACS2 called peaks per sample not filtered by IDR was generated using bedtools merge, and raw reads covering each region were recovered from bam files using bedtools mappability. For RNA-seq, raw reads on reference genes were recovered using HOMER (version 4.8 (ref. 13)) analyzeRepeats.pl command. To test for differential counting, raw reads were compared using the DESeq2 package implemented in R (v.3.4.3)33, and filtered based on an adjusted P value of <0.05 and 2-fold change. For heatmaps and average profiles, tag counts were recovered ±5 kb from the peak summit using HOMER annotatePeaks.pl command with -hist 25 -ghist or -hist 25 parameters. Heatmap images were generated using Java TreeView. Average profiles and scatter plots were plotted using Python (v.3.5.2) matplotlib.

Motif discovery and gene ontology. De novo motif discovery was performed using the HOMER findMotsGenome function with size 200 as a parameter. Gene ontology analysis was performed using DAVID (v.6.7)57 for RNA-seq data and GREAT58 for ChIP−seq and ATAC−seq data.

Chromatin state determination. ChromHMM software27 was used to learn and identify chromatin states as instructed in the manual. Encode chromatin segmentation using ChromHMM was used as a reference to label each state using a custom script using bedtools intersect. The enrichment of each state for a set of peaks was calculated using the NeighborhoodEnrichment command and compared among samples using a custom script. Enrichments were plotted using Python matplotlib library.

Analysis of UMI-4C data. UMI-4C data were aligned and analyzed using HiC-Pro58 and the DpnII segmented genome annotation file. Interaction matrices of 5-kb resolution were generated and used to create Virtual 4C profiles through a custom python script and the matplotlib library.

Analysis of cohesin HiChIP data. HiChIP paired-end reads were aligned to hg19 using HiC-Pro. Duplicate reads were removed, assigned to MboI restriction fragments, filtered for valid interactions, and then used to generate binned interaction matrices of both 5-kb and 10-kb resolution. The 5-kb interaction matrices were used to visualize contacts by Virtual 4C, similar to the UMI-4C analysis. The 10-kb interaction matrix was used to call high-confidence contacts (defined as contacts ≥10, FDR <0.001) using the contact caller, FitHiC. Default FitHiC settings were used to generate an FDR for each bin pair. These high confidence cohesin contacts were used in the subsequent analyses.

Contact connection analyses. An element was considered participating, or anchored, in cohesin connections, if it possessed at least one high-confidence contact bin in a given cell type. When considering ways in which p63 was connected to a TSS (defined as TSS ±5 kb), four chromatin conformations were considered. 0° connections were defined as two elements overlapping in physical space (for example, p63BS contained within the TSS). 1° connections were defined as one element anchored in one bin of a cohesin contact and the second element anchored in the other bin. More complicated connections between elements were also considered: 2° connections were defined as two elements in distinct physical space both forming 1° connections to the same third element. Finally, 3° connections were when one element formed a 1° connection to a second element, which also formed a 1° connection to a third element, which also had a 1° connection to the fourth (target) element.

Empirical cumulative distribution function analyses. Empirical cumulative distribution function (ECDF) was performed to determine whether the cumulative levels of log, fold change in a subset of genes was differential compared to all protein-coding genes using a combination of custom Unix and Python scripts. Only protein-coding genes for which there was a fold change value calculated using DESeq2 on the RNA-seq data were used in constructing the ecdfs.

Differential contact analysis. The Bioconductor package edgeR (v.3.18.1)34 was used to perform multiple-comparison differential analysis of high-confidence FitHiC contacts in d0, d0 p63GOF, d7 p63WT, and d7 p63KO cells. The Anderson–Darling two-sample test, a modification of the K-S test, which gives greater weight to the tails, was used to calculate statistical significance between populations of the fold change in contact connectivity.

Statistics and reproducibility. All data represent similar results from three independent, biological experiments and cell cultures (n = 3), unless otherwise stated. Number of independent, biological experiments for deep sequencing replicates are indicated in the appropriate Methods subsections (n ≥2 in all cases, except for HiChIP for which n = 3). The center values of all graphs depict the mean, unless otherwise stated. Significance values were calculated using a Student's two-sided t test, unless otherwise indicated. The following notation applies for all figures: *P<0.05, **P<0.01, ***P<0.005, ****P<1×10−6. Statistical methods for specific analyses are detailed above in the corresponding Methods subsections. The two-tailed Anderson–Darling test, which calculates the probability that two datasets come from the same population, was used to calculate P values comparing populations in Fig. 3f and Supplementary Fig. 7a.

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

Data availability

All sequencing data are available through Gene Expression Omnibus (GEO) accession number: GSE119997.

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Experimental design

1. Sample size
   Describe how sample size was determined.

   For cellular assays, all sample sizes were based on at least 3 independent, biological cell culture experiments and differentiations. Technical replicates were assayed simultaneously yet not included in the total n value. Replicates for deep sequencing experiments represent 2 independent, biological cultures. All n values denoted in the figure legends represent independent biological experiments.

2. Data exclusions
   Describe any data exclusions.

   No data were excluded from the analyses.

3. Replication
   Describe whether the experimental findings were reliably reproduced.

   All attempts to reproduce our experimental findings were successful.

4. Randomization
   Describe how samples/organisms/participants were allocated into experimental groups.

   No randomization was used in this study, as all experiments were conducted on cell cultures and pools of cells.

5. Blinding
   Describe whether the investigators were blinded to group allocation during data collection and/or analysis.

   No blinding was used in this study as experimental samples were cell cultures and pools of cell populations and did not require human subjectivity.

Note: all studies involving animals and/or human research participants must disclose whether blinding and randomization were used.

6. Statistical parameters
   For all figures and tables that use statistical methods, confirm that the following items are present in relevant figure legends (or in the Methods section if additional space is needed).

   | n/a | Confirmed |
   |-----|-----------|
   | ☒   | The exact sample size (n) for each experimental group/condition, given as a discrete number and unit of measurement (animals, litters, cultures, etc.) |
   | ☒   | A description of how samples were collected, noting whether measurements were taken from distinct samples or whether the same sample was measured repeatedly |
   | ☒   | A statement indicating how many times each experiment was replicated |
   | ☒   | The statistical test(s) used and whether they are one- or two-sided (note: only common tests should be described solely by name; more complex techniques should be described in the Methods section) |
   | ☒   | A description of any assumptions or corrections, such as an adjustment for multiple comparisons |
   | ☒   | The test results (e.g. P values) given as exact values whenever possible and with confidence intervals noted |
   | ☒   | A clear description of statistics including central tendency (e.g. median, mean) and variation (e.g. standard deviation, interquartile range) |
   | ☒   | Clearly defined error bars |

See the web collection on statistics for biologists for further resources and guidance.
7. Software

Describe the software used to analyze the data in this study.

The software used for all analysis is cited in the methods and all custom scripts are available on GitHub. In brief, we used the following programs: Bowtie, TOPHAT, HOMER, Bedtools, MACS2, DESeq2, IDR, ChromHMM, HiC-Pro, and FitHiC. We describe in detail how we used these programs in the Methods section.

For manuscripts utilizing custom algorithms or software that are central to the paper but not yet described in the published literature, software must be made available to editors and reviewers upon request. We strongly encourage code deposition in a community repository (e.g. GitHub). Nature Methods guidance for providing algorithms and software for publication provides further information on this topic.

8. Materials availability

Indicate whether there are restrictions on availability of unique materials or if these materials are only available for distribution by a for-profit company.

All materials are available upon request.

9. Antibodies

Describe the antibodies used and how they were validated for use in the system under study (i.e. assay and species).

Antibodies used in this study are also cited in the Methods:

For IF:
- AP-2gamma (Cell Signaling 2320S)
- p63 (Genetex GTX102425)
- KRT18 (R&D AF7619)
- KRT14 (BioLegend 906001)
- OCT4 (BioLegend 631902)

For ChIP/HiChIP:
- p63 (Active Motif 39739)
- H3K4me3 (Abcam ab8580)
- H3K4me1 (Abcam ab8895)
- H3K27ac (Abcam ab4729)
- H3K27me3 (Millipore 07-449)
- SMC1 (Bethyl A300-055A)

10. Eukaryotic cell lines

a. State the source of each eukaryotic cell line used.

The H9 human embryonic stem cell line was acquired through the Stanford Stem Cell Bank.

b. Describe the method of cell line authentication used.

The H9 cell line was validated through sequencing.

c. Report whether the cell lines were tested for mycoplasma contamination.

Cell lines are tested for mycoplasma contamination monthly and were negative for contamination at the time of the experiments.

d. If any of the cell lines used are listed in the database of commonly misidentified cell lines maintained by ICLAC, provide a scientific rationale for their use.

No commonly misidentified cell lines were used.

11. Description of research animals

Provide details on animals and/or animal-derived materials used in the study.

No animals were used.

12. Description of human research participants

Describe the covariate-relevant population characteristics of the human research participants.

The study did not involve human research participants.
ChIP-seq Reporting Summary

Form fields will expand as needed. Please do not leave fields blank.

Data deposition

1. For all ChIP-seq data:
   a. Confirm that both raw and final processed data have been deposited in a public database such as GEO.
   b. Confirm that you have deposited or provided access to graph files (e.g. BED files) for the called peaks.

2. Provide all necessary reviewer access links. The entry may remain private before publication.
   All data are available through GEO accession number GSE114846.

3. Provide a list of all files available in the database submission.
   All FastQ files and final BED files for called peaks are available.

4. If available, provide a link to an anonymized genome browser session (e.g. UCSC).

Methodological details

5. Describe the experimental replicates.
   Two biological replicates were sequenced for each experiment.

6. Describe the sequencing depth for each experiment.
   The PCR conditions were the following, as recommended in the NEBNext protocol:
   98C, 30s
   10-12 cycles:
   98C, 10s
   65C, 30s
   72C, 30s
   Final extension:
   72C, 5m
   Sequencing was single-end, with 50 bp reads.
   The sequencing depth for each experiment was between 30 and 60 million reads per replicate.

7. Describe the antibodies used for the ChIP-seq experiments.
   Antibodies used are described in the Methods section:
   p63 (Active Motif, 39739, 100 uL)
   Validated for ChIP-seq:
   Drier, Y., Cotton, M. J., et al. (2016), 'An oncogenic MYB feedback loop drives alternate cell fates in adenoid cystic carcinoma.', Nat Genet, 48 (3), pp. 265-72
   Katoh, I., Fukunishi, N., et al. (2016), 'Repression of Wnt/β-catenin response elements by p63 (TP63).', Cell Cycle, 15 (5), pp. 699-710
   Rhie, S. K., Hazelett, D. J., et al. (2014), 'Nucleosome positioning and histone modifications define relationships between regulatory elements and nearby gene expression in breast epithelial cells.', BMC Genomics, 15, pp. 331
   H3K4me3 (Abcam, ab8580)
   Validated for ChIP-seq (989 total citations):
Ye Y et al. Chromatin remodeling during in vivo neural stem cells differentiating to neurons in early Drosophila embryos. Cell Death Differ 24:409-420 (2017). ChIP; Drosophila melanogaster.

Pan G et al. PATZ1 down-regulates FADS1 by binding to rs174557 and is opposed by SP1/SREBP1c. Nucleic Acids Res 45:2408-2422 (2017). ChIP; Human.

H3K4me1 (Abcam, ab8895) Validated for ChIP-seq (464 total citations):
Park YK & Ge K Glucocorticoid Receptor Accelerates, but Is Dispensable for, Adipogenesis. Mol Cell Biol 37:N/A (2017). ChIPseq; Mouse
Ye Y et al. Chromatin remodeling during in vivo neural stem cells differentiating to neurons in early Drosophila embryos. Cell Death Differ 24:409-420 (2017). ChIP; Drosophila melanogaster.

H3K27Ac (Abcam, ab4729) Validated for ChIP-seq (457 total citations):
Park YK & Ge K Glucocorticoid Receptor Accelerates, but Is Dispensable for, Adipogenesis. Mol Cell Biol 37:N/A (2017). ChIPseq; Mouse
Ye Y et al. Chromatin remodeling during in vivo neural stem cells differentiating to neurons in early Drosophila embryos. Cell Death Differ 24:409-420 (2017). ChIP; Drosophila melanogaster.

H3K27me3 (Millipore, 07-449) Validated for ChIP-seq:
Bmi1 promotes erythroid development through regulating ribosome biogenesis. Gao, R; Chen, S; Kobayashi, M; Yu, H; Zhang, Y; Wan, Y; Young, SK; Soltis, A; Yu, M; Vemula, S; Fraenkel, E; Cantor, A; Antipin, Y; Xu, Y; Yoder, MC; Wek, RC; Ellis, SR; Kapur, R; Zhu, X; Liu, Y Stem cells (Dayton, Ohio) 33 925-38 2015
EZH2 modulates angiogenesis in vitro and in a mouse model of limb ischemia. Mitić, T; Caporali, A; Floris, I; Meloni, M; Marchetti, M; Urrutia, R; Angelini, GD; Emanuelli, C Molecular therapy : the journal of the American Society of Gene Therapy 23 32-42 2015

8. Describe the peak calling parameters.

Bowtie2 was used for read mapping and the parameters were as follows:
-p 24 -S -a -m 1 --best --strata
For peak calling using MACS2, default settings were specified with a p-value of 0.05.

9. Describe the methods used to ensure data quality.

Methods to ensure data quality are described in the Methods section. Fastq files were analyzed using FASTQC. IDR was run on all files, yielding final peak files of FDR 5% between 5,000 and 20,000, depending on the experiment.

10. Describe the software used to collect and analyze the ChIP-seq data.

Software used for data analysis is described in the Methods section, but includes: fastqc, bowtie2, MACS2, IDR, DESeq2, Bedtools, HOMER, and ChromHMM.