Published in final edited form as:

Science. 2022 July ; 377(6601): eabk2820. doi:10.1126/science.abk2820.

Synthetic regulatory reconstitution reveals principles of mammalian *Hox* cluster regulation

Sudarshan Pinglay[1,†], **Milica Bulajić**[2,†,‡], **Dylan P. Rahe**[2], **Emily Huang**[1], **Ran Brosh**[1], **Nicholas E. Mamrak**[1], **Benjamin R. King**[1,§], **Sergei German**[1], **John A. Cadley**[1], **Lila Rieber**[3,¶], **Nicole Easo**[1], **Timothée Lionnet**[1,4,5], **Shaun Mahony**[3], **Matthew T. Maurano**[1,6], **Liam J. Holt**[1,5,7], **Esteban O. Mazzoni**[2,*], **Jef D. Boeke**[1,5,7,*]

[1]Institute for Systems Genetics, NYU Langone Health, New York, NY 10016, USA.

[2]Department of Biology, New York University, New York, NY 10003, USA.

[3]Center for Eukaryotic Gene Regulation, Department of Biochemistry and Molecular Biology, Pennsylvania State University, University Park, PA 16802, USA.

[4]Department of Cell Biology, NYU Langone Health, New York, NY 10016, USA.

[5]Department of Biomedical Engineering, NYU Tandon School of Engineering, Brooklyn, NY 11201, USA.

[6]Department of Pathology, NYU Langone Health, New York, NY 10016, USA.

[7]Department of Biochemistry and Molecular Pharmacology, NYU Langone Health, New York, NY 10016, USA.

Abstract

[*]Corresponding author: eom204@nyu.edu (E.O.M.); jef.boeke@nyulangone.org (J.D.B.).
[‡]Present address: Biogen Inc., Cambridge, MA 02142, USA.
[§]Present address: Bristol-Myers Squibb Co., Lawrenceville, NJ 08648, USA.
[¶]Present address: Element Genomics Inc., Durham, NC 27701, USA.
[†]These authors contributed equally to this work.

Author contributions: S.P., M.B., E.O.M., and J.D.B. conceived of the project. S.P., M.B., D.P.R., E.H., R.B., N.E.M., B.R.K., and N.E. performed experiments. S.P., M.B., D.P.R., L.R., N.E.M., S.M., and M.T.M. analyzed data. S.G. and J.A.C. provided computational support. T.L., M.T.M., L.J.H., E.O.M., and J.D.B. supervised research. S.P., M.B., E.O.M., and J.D.B. wrote the manuscript with input from all authors.

Competing interests: J.D.B. is a founder and director of CDI Labs Inc.; a founder of and consultant to Neochromosome Inc.; a founder of, scientific advisory board member of, and consultant to ReOpen Diagnostics LLC; and past or present scientific advisory board member of Sangamo Inc., Modern Meadow Inc., Rome Therapeutics Inc., Sample6 Inc., Tessera Therapeutics Inc., and the Wyss Institute. The other authors declare no competing interests.

SUPPLEMENTARY MATERIALS
science.org/doi/10.1126/science.abk2820
Materials and Methods
Supplementary Text
Figs. S1 to S20
Tables S1 to S17
References (52–79)
MDAR Reproducibility Checklist

View/request a protocol for this paper from Bio-protocol.

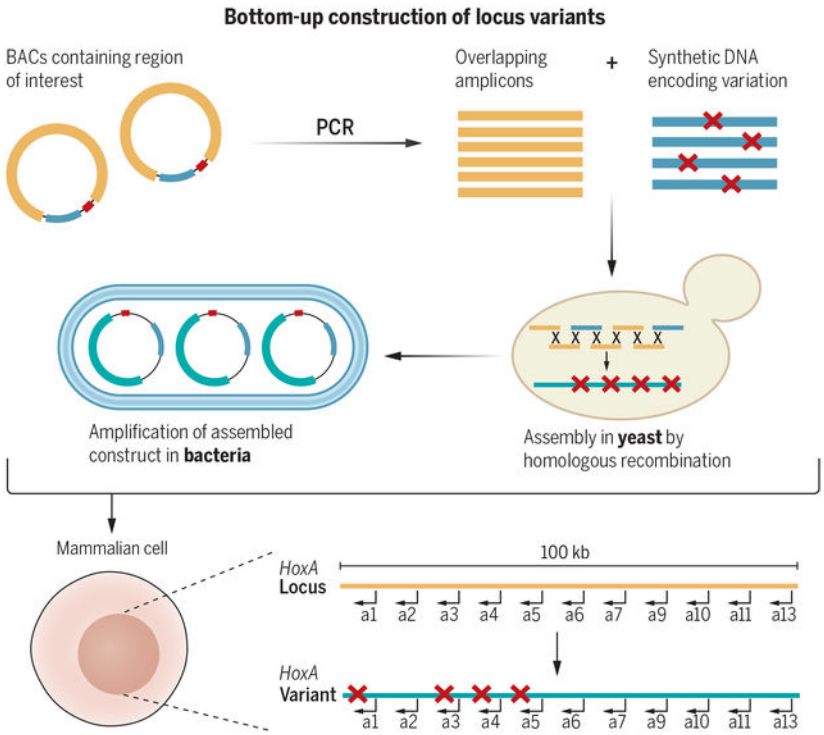
INTRODUCTION: Despite an ever-expanding catalog of noncoding elements that are implicated in the control of mammalian gene expression, how the regulatory input from multiple elements is integrated across a genomic neighborhood has remained largely unclear. This challenge is exemplified at *Hox* clusters (~100 to 200 kb), which contain genes that specify positional identity along the anterior-posterior axis of the developing embryo. In response to developmental morphogens such as retinoic acid (RA), the *HoxA* cluster splits into an active (*HoxA1–5*) and inactive domain (*HoxA6–13*) at the level of gene expression and chromatin. Although distal enhancers, intracluster transcription factor binding, and topological organization have emerged as the major regulatory modules in establishing this expression pattern, their relative contributions remain elusive.

RATIONALE: Despite the advent of a vast suite of genome editing tools, it has remained challenging to simultaneously manipulate multiple regulatory elements across large genomic windows to deconvolve their relative contributions. Taking inspiration from the bottom-up approaches of synthetic biology and biochemical reconstitution, we developed "synthetic regulatory reconstitution" as a framework for the study of gene regulation to address this gap. The synthesis of large DNA constructs (>100 kb) permits any combination of complex modifications to be made, at the scale required to probe regulation across a native genomic neighborhood. We fabricated *HoxA* cluster variants that encode various combinations of the previously identified regulatory modules and integrated them into an ectopic location in the mouse genome. This enabled us to directly test the independent ability of these variant ectopic clusters to reconstitute distinct aspects of *HoxA* regulation.

RESULTS: We harnessed the efficient homologous recombination machinery in yeast to construct four rat *HoxA* variants (130 to 170 kb) and delivered them at single copy to the housekeeping *Hprt1* locus of mouse embryonic stem cells. Upon RA-induced differentiation, an ectopic *HoxA* cluster lacking distal enhancers (*SynHoxA*) induced both the appropriate subset of *HoxA* genes and the corresponding chromatin boundary. The presence of distal enhancers (*Enhancers+SynHoxA*) increased transcription levels, especially at early time points. Further, both *SynHoxA* and *Enhancers+SynHoxA* reorganized into active and inactive topological domains upon differentiation, mirroring the endogenous organization. The mutation of just four retinoic acid response elements (RAREs) present in *SynHoxA* (*RARE*Δ) almost completely eliminated any response of the ectopic cluster to RA, at the levels of both gene expression and chromatin reorganization. The addition of distal enhancers to *RARE*Δ could not fully rescue this loss of gene expression phenotype.

CONCLUSION: Our data suggest that at *HoxA*, the primary module of active gene and chromatin boundary specification in response to RA is through the presence of internal transcription factor binding sites. Distal enhancers are dispensable for the specification of active genes but synergize with intracluster activator binding to boost the amount of transcription. Therefore, mammalian *Hox* clusters contain all the regulatory information that is necessary to convert a morphogenetic signal into a stable transcriptional, epigenetic, and topological state. This study showcases the power of synthetic regulatory reconstitution, a generalizable platform for the dissection of gene regulation at other loci in complex genomes.

Graphical Abstract



Bottom-up construction of variant mammalian genomic loci at the >100-kb scale. Making multiple edits on the same allele over large genomic windows has remained challenging in mammalian cells. Variant loci with an arbitrary number of changes can be constructed in yeast using synthetic DNA and site-specifically integrated into the genome of mammalian cells to study their behavior. BAC, bacterial artificial chromosome.

Abstract

Precise *Hox* gene expression is crucial for embryonic patterning. Intra-*Hox* transcription factor binding and distal enhancer elements have emerged as the major regulatory modules controlling *Hox* gene expression. However, quantifying their relative contributions has remained elusive. Here, we introduce “synthetic regulatory reconstitution,” a conceptual framework for studying gene regulation, and apply it to the *HoxA* cluster. We synthesized and delivered variant rat *HoxA* clusters (130 to 170 kilobases) to an ectopic location in the mouse genome. We found that a minimal *HoxA* cluster recapitulated correct patterns of chromatin remodeling and transcription in response to patterning signals, whereas the addition of distal enhancers was needed for full transcriptional output. Synthetic regulatory reconstitution could provide a generalizable strategy for deciphering the regulatory logic of gene expression in complex genomes.

Mammalian *Hox* genes encode deeply conserved transcription factors (TFs) that are organized into four dense clusters (*HoxA* to *HoxD*) lacking other coding genes (1). In response to developmental morphogens, *Hox* genes are expressed along the anterior-posterior axis of the developing embryo in a spatial and temporal pattern that mirrors the organization of the genes within the cluster (2–4). The alteration of this “colinear” *Hox* gene expression pattern results in gross developmental defects or diseases such as cancer (5, 6).

In undifferentiated cells, where no *Hox* genes are expressed, the entire *HoxA* cluster (~130 kb) is targeted by Polycomb repressive complex 2 (PRC2) and marked by heterochromatin (7). Patterning signals such as retinoic acid (RA) and Wnt activate transcription through their downstream TFs. In response to RA, activated RA receptors (RARs) are bound to retinoic acid response elements (RAREs) found within the *HoxA* cluster. This is correlated with the separation of the cluster into two domains that contain transcribed and repressed genes, respectively (8–12) (Fig. 1A). RARE mutations can lead to decreased or abolished expression of the neighboring *HoxA* genes in the central nervous system, solidifying the notion that RAREs directly control *Hox* gene expression (9, 13, 14). However, the relationship between RARE activity and chromatin domain formation is not yet well established.

A collection of intricate genetic manipulations has revealed a complex regulatory landscape surrounding the *Hox* clusters (15–19). For example, the *HoxA* cluster relies on several RA and Wnt responsive distal regulatory elements (enhancers) located in the gene-poor region between *HoxA1* and the next gene, *Skap2* (~300 kb away). Deleting some of these enhancers reduces, but never eliminates, *HoxA* expression in response to RA or Wnt (20–22). Distal enhancer access to the genes in the inactive domain is restricted by the formation of a strong topological boundary at CCCTC-binding factor (CTCF) sites within the cluster upon differentiation. Disruption of this boundary results in the misexpression of posterior *HoxA* genes in response to RA (15, 23, 24).

Thus, different regulatory modules are integrated to control *Hox* gene expression: local TF binding, distal enhancers, and topological DNA organization. However, a synergistic model describing their relative contribution and interactions has remained elusive (Fig. 1B).

The relative contribution of a cis-regulatory module could be measured by (i) generating a variant allele that only contains the elements that constitute the module, and (ii) isolating the variant allele from confounding factors, such as the compensatory effect of other regulatory elements in cis. Testing the necessity of individual elements by generating loss-of-function variants has received a lot of attention. However, tests of sufficiency have remained intractable owing to the inability to precisely manipulate DNA at a scale that accurately models the complexity and size of native loci (>100 kb) (25).

Short reporter constructs enable the study of variants only over a small genomic window (<10 kb) and also suffer from a lack of controlled genomic context, as they are largely randomly integrated or reside on episomal plasmids (26). Although they can test the ability of a sequence to drive expression, they lack the scale to study *Hox* cluster chromatin domain formation (>100 kb). Larger bacterial or yeast artificial chromosomes (BACs/YACs) can provide more genomic context and have been elegantly employed to understand regulation at *Hox* clusters (27–29). However, they are not easy to manipulate, making it challenging to generate a large number of variants that can be tested in vivo (26, 30, 31). Further, these large constructs are often randomly integrated into the genome, which confounds comparison across different constructs as a result of position effects and the integration of multiple copies. Methods for precise, single-copy integration of large DNA molecules in mammalian cells have not yet been applied to the study of *Hox* cluster regulation (32,

33). Despite the revolution in genome editing, it is still inefficient and time-consuming to make intricate structural rearrangements or multiple defined edits in cis, phased on a single homolog (34, 35).

We recently described a pipeline that harnesses the endogenous homologous recombination machinery in yeast to de novo assemble ~100-kb regions of mammalian genomes and integrate them into a defined location in mouse embryonic stem cells (mESCs) (36). This bottom-up assembly of genomic loci enables the introduction of an arbitrary number of variants in cis that are independent of any natural template.

Here, we apply and extend this technology to study the relative contributions of genomic context, distal enhancers, and intracluster regulatory elements to *HoxA* regulation. We constructed variants of the *HoxA* cluster (ranging from 130 to 170 kb) encoding combinations of the previously identified regulatory modules and integrated them into an ectopic locus, thereby isolating them from the native genomic neighborhood. We then asked whether the variant ectopic clusters were sufficient to reconstitute the transcriptional and epigenetic *HoxA* response to activating patterning signals (Fig. 1C).

Synthetic *HoxA* strategy and construction

All *HoxA* constructs described here were derived from rat (*Rattus norvegicus*) *HoxA* sequence, which shares ~90% sequence identity with the mouse *HoxA* sequence. This facilitated experiments in two distinct genetic backgrounds: (i) cells containing the endogenous *HoxA* cluster ($HoxA^{+/+}$), allowing direct comparisons of expression levels to an internal control, and (ii) cells lacking endogenous mouse *HoxA* to eliminate potential sequence-mapping challenges ($HoxA^{-/-}$).

We first constructed a 134-kb wild-type minimal rat *HoxA* cluster (*SynHoxA*) (Fig. 2 and fig. S1). *SynHoxA* contains all *HoxA* coding genes and encompasses the sequence corresponding to the contiguous domain repressed by H3K27me3 (trimethylated histone H3 Lys^{27}) in undifferentiated mESCs. We produced polymerase chain reaction (PCR) amplicons from BACs bearing the rat *HoxA* cluster, with overlap between adjacent segments to enable homologous recombination. These amplicons were recombined in yeast to produce the 134-kb *SynHoxA* construct, termed an “assemblon” (fig. S1B). Edits to the assemblon can be made by switching the wild-type amplicons with synthetic DNA bearing the desired changes or by editing the assemblons directly using highly efficient, marker-free CRISPR/Cas9-based engineering in yeast (37). Assemblons were recovered from yeast into bacteria to purify large amounts of DNA (Fig. 2A). To test the contribution of distal enhancers to *HoxA* regulation, we fused all experimentally verified distal regulatory elements directly upstream of the core *SynHoxA* assemblon, generating the 170-kb *Enhancers+SynHoxA* construct (fig. S2).

DNA sequencing at each step confirmed assemblon integrity (Fig. 2C and fig. S3). We detected mutations at a frequency of ~1 nucleotide per 6 kb, likely arising from PCR errors (fig. S3). We verified that none of these mutations were likely to affect the function of the clusters in our differentiation system described below (table S1).

All constructs were delivered to the *Hprt* locus on the X chromosome of both $HoxA^{+/+}$ and $HoxA^{-/-}$ mESCs using Cre-mediated recombination (38) (figs. S4 and S5). We reasoned that *Hprt* would be an appropriate site to attempt regulatory reconstitution because it is a housekeeping gene with little regulatory activity in the surrounding regions, and its use as a "safe harbor" site is well documented (39, 40). Furthermore, transposing the *HoxA* cluster to an open locus would be the most stringent test of its ability to recruit the repressive PRC2 complex in undifferentiated cells. We used capture sequencing to verify that mESC clones contained the entire assemblon, specifically at Hprt and in single copy (41) (Fig. 2B).

Induction of *SynHoxA* gene expression during motor neuron differentiation

We investigated the response of the ectopic clusters to RA in a widely used differentiation system that recapitulates key aspects of ventral spinal cord development, including the activation of the appropriate *HoxA* genes (fig. S6A) (42, 43). Endogenous *HoxA* genes are repressed before exposure to patterning signals, and *HoxA1–5* are induced in response to the "anterior" RA signal (10).

We performed RNA-seq on wild-type $HoxA^{+/+}$ cells containing ectopic *SynHoxA* variants over the course of the differentiation protocol. Comparison to previously published control datasets by principal components analysis (PCA) revealed that the samples grouped largely according to time since RA treatment (44, 45) (fig. S6B). Cells containing *SynHoxA* variants down-regulated the expression of pluripotency markers, up-regulated the expression of markers of motor neuron differentiation, and induced *HoxA1–5* from the endogenous cluster upon exposure to RA as expected (Fig. 3, A and C, and fig. S6, C and D). Thus, the presence of an ectopic synthetic cluster does not affect the ability of cells to differentiate appropriately after RA treatment.

The ectopic *Enhancers+SynHoxA* cluster induced *SynHoxA1–5* starting 24 hours after RA treatment (Fig. 3B and figs. S7 and S8, A and B), whereas *SynHoxA6* to *SynHoxA13* remained repressed throughout. Similarly, ectopic *SynHoxA* specifically induced *SynHoxA1–5* (Fig. 3D and figs. S7 and S8C) without misexpression of any posterior genes. Thus, neither endogenous genomic context nor the wide spacing of enhancer elements is strictly required for a *Hox* cluster to induce the appropriate genes.

We quantified gene induction from the ectopic clusters by comparing each gene to its endogenous mouse *HoxA* counterpart (Fig. 3, E and F). Both ectopic clusters induced lower *SynHoxA1* transcription, whereas *SynHoxA2* induction surpassed the endogenous gene. The induction kinetics of *SynHoxA3–5* were slower than *HoxA3–5*, but the mRNA levels became comparable after 96 hours, particularly in the *Enhancers+SynHoxA* construct. In general, *Enhancers+SynHoxA* induced higher levels of *SynHoxA1–5* expression than *SynHoxA*, especially at early time points (Fig. 3 and figs. S7 and S8, B and C).

In our differentiation model, temporal colinearity of genes from the endogenous *HoxA* cluster is exhibited by (i) an early induction of *HoxA1* (peaks at 24 hours) and its subsequent down-regulation and (ii) the sequential induction of *HoxA2–5* from 24 to 96 hours. At 96 hours, the expression level is *HoxA2* < *HoxA3* < *HoxA4* < *HoxA5* (figs. S7 to S9). The

minimal *SynHoxA* cluster does not recapitulate the temporal expression pattern of *HoxA1*. *SynHoxA1* is expressed at low levels throughout but increases steadily with time, mirroring the regulation of *SynHoxA2–5* genes. However, this is partially rescued with the addition of enhancers in *Enhancers+SynHoxA*. *SynHoxA1* expression in this context peaks at 48 hours and decreases at 96 hours. The temporal expression of *SynHoxA2–5* is retained in both constructs (fig. S9).

The observed bulk differences in gene expression between the ectopic clusters could be attributed either to a change in the number of cells that induce expression or to expression level changes in each cell. To address this, we investigated the response of the synthetic clusters to RA at the single-cell level using(i) *HoxA5* antibody staining and (ii) RNA single-molecule fluorescence in situ hybridization for anterior *SynHoxA* genes. We performed these experiments in the $HoxA^{-/-}$ background. Gene expression from the ectopic clusters was not influenced by the presence or absence of the endogenous *HoxA* cluster (fig. S10), and the cells differentiated appropriately (fig. S11). In both methods, the observed bulk differences in expression were not due to a difference in the number of cells that express anterior *SynHoxA* genes, but due to an increase in the amount of expression per cell in the presence of the enhancers (figs. S12 to S15).

We investigated whether these findings could be extended to other patterning signals beyond RA, such as the posteriorizing Wnt signal (10, 22). Both ectopic clusters induced more posterior genes upon treatment with Wnt, up to *SynHoxA11* (fig. S16). Intriguingly, the addition of distal enhancers does not seem to consistently modify the Wnt response across *SynHoxA* genes as much as it did for RA.

Together, these data support a model in which the *HoxA* cluster contains all the necessary information to decode patterning signals into the appropriate positional identity, independent of distal enhancers and the native genomic architecture. Distal enhancers are not required to specify active genes but have a critical role in modulating transcriptional output in response to RA. Whereas both ectopic clusters induce the correct subset of genes, additional elements may be required to fine-tune expression amplitude and timing.

Chromatin reorganization at *SynHoxA* clusters through differentiation

In undifferentiated cells, *Hox* clusters are carpeted with repressive H3K27me3 marks and recruit CTCF to potential boundary positions established in response to extracellular signals. The endogenous *HoxA* cluster responds to RA by separating into an active and inactive domain, forming a precise chromatin boundary between *HoxA5* and *HoxA6* (10, 23). We investigated the chromatin dynamics and CTCF recruitment of the relocated *HoxA* clusters by performing chromatin immunoprecipitation sequencing (ChIP-seq) in the $HoxA^{-/-}$ background to more reliably map reads to the ectopic clusters, thereby enhancing resolution.

Prior to differentiation, both *Enhancers+SynHoxA* and *SynHoxA* were entirely covered with high levels of H3K27me3. In addition, CTCF was recruited to the appropriate sites (Fig. 4, A and B). Thus, the ability to recruit all components for correct patterning is intrinsic to the *HoxA* cluster sequence and is independent of endogenous genomic context or distal

enhancers. Upon RA treatment, *Enhancers+SynHoxA* remodeled chromatin in a manner that is similar to the endogenous cluster. H3K27me3 decreased in the *SynHoxA1–5* domain and increased at *SynHoxA6–13* (Fig. 4C). An increase in acetylated H3K27 (H3K27ac) complemented H3K27me3 removal from *SynHoxA1–5*. H3K27me3 was entirely cleared by 48 hours, slightly slower than the endogenous locus, which clears by 24 hours (10). Thus, the endogenous genomic context at *HoxA* is not required to translate an extracellular signal into an accurate epigenetic chromatin state.

The minimal *SynHoxA* cluster also recruited H3K27ac to anterior genes upon RA activation (Fig. 4D). Unlike the endogenous *HoxA* cluster and *Enhancers+SynHoxA*, H3K27me3 was not entirely lost from the *SynHoxA1–5* domain during differentiation (Fig. 4D). Nonetheless, *SynHoxA* formed the appropriate, albeit weak, chromatin boundary at the *SynHoxA5–a6* CTCF binding site (Fig. 4E and fig. S17, A and B). Thus, the minimal *SynHoxA* cluster has the intrinsic ability to induce dynamic chromatin domains, independent of genomic context or enhancers.

Topological organization of ectopic *SynHoxA* clusters

The three-dimensional structure of the *HoxA* cluster changes during motor neuron differentiation, transitioning from a single association domain to two domains containing active or repressed chromatin (15, 23, 24). We investigated the topological organization of the ectopic clusters by performing Hi-C, a technique for detecting genome-wide chromatin interactions, during differentiation (0 and 48 hours after RA treatment). Both clusters formed self-associating domains in undifferentiated cells without generating a de novo topologically associating domain (TAD) boundary (fig. S18). Similar to the endogenous cluster, *Enhancers+SynHoxA* broke into two domains during differentiation, with enhancers and active genes in one domain and repressed genes in the other (fig. S18A). The minimal *SynHoxA* similarly transitioned from a compact self-associated state in undifferentiated cells into two domains during differentiation (fig. S18B). We did not observe strong evidence for trans-chromosomal interactions between the ectopic locus and its endogenous enhancers (tables S2 and S3). Thus, the ectopic *HoxA* clusters have the intrinsic ability to self-organize in three dimensions, mirroring the expression and chromatin changes that occur upon differentiation.

The RARE sites within the *HoxA* cluster are required for the RA response

The minimal *SynHoxA* transformed the RA signal into the correct transcriptional and chromatin programs. This behavior could theoretically depend on RAREs located within the *HoxA1–5* domain, other sequences within the cluster, or interactions with other regulatory elements at the ectopic locus. To distinguish between these possibilities, we built a third construct lacking both RAREs and enhancers (*RAREΔ SynHoxA*) and integrated it into wild-type and $HoxA^{-/-}$ mESCs (figs. S19 and S20) (9).

Cells carrying *RAREΔSynHoxA* differentiated and induced endogenous *HoxA* genes appropriately (Fig. 5A and fig. S20, D and E). The ectopic cluster was decorated with H3K27me3 in mESCs, indicating that RAREs are not required to recruit the repressive

chromatin machinery. However, unlike the previous constructs, *RARE*Δ *SynHoxA* failed to up-regulate the expression of *SynHoxA1–5* or to form a chromatin boundary in response to RA (Fig. 5, B to D, and figs. S8D and S17C). *SynHoxA5* was the only gene with any signal, potentially due to a poorly characterized RAR binding site located between *HoxA5* and *HoxA6* (9, 10). This null phenotype indicates that the behavior of ectopic *SynHoxA* clusters reflects their innate regulatory potential and not the effect of novel interactions formed at the *Hprt* locus.

This lack of RA response provided the ideal background to measure the independent contribution of distal enhancers to *HoxA* gene regulation. To that end, we built and integrated a fourth construct: *Enhancers*+*RARE*Δ *SynHoxA*, with the distal enhancers inserted upstream of *RARE*Δ *SynHoxA* (figs. S19 and S20). In *Enhancers*+*RARE*Δ *SynHoxA*, *SynHoxA2–5* were induced at low levels in response to RA (Fig. 6, A and B, and fig. S8E). A faint chromatin boundary formed at the appropriate location between *SynHoxA5* and *SynHoxA6* (Fig. 6, C and D, and fig. S17D). This suggests that the distal enhancers may have a weak ability to activate *HoxA* gene transcription without the driving force provided by internal RAREs, or that they synergize with the poorly characterized RARE still present in this construct.

Discussion

We developed a “synthetic regulatory reconstitution” approach to characterize the relative contributions of distal enhancers and intracluster TF binding in specifying distinct aspects of *HoxA* regulation. A minimal ectopic cluster lacking distal enhancers induced the correct subset of genes in response to two developmental signals (RA and Wnt). The presence of distal enhancers increased the transcriptional output from the ectopic cluster in response to RA, especially at earlier time points. These results are consistent with previous studies in which deletion of enhancers from the endogenous locus resulted in lower *Hox* gene transcription in response to morphogens (20, 22, 46). At the time point we investigated, the ectopic cluster with distal enhancers did not induce higher expression in response to Wnt. This may indicate that some Wnt-responsive enhancers are missing in these constructs, or that the distal enhancers are ineffective in this configuration. Alternatively, earlier time points may reveal differences in transcriptional dynamics or output between these constructs.

The importance of both RAREs and distal enhancers to *Hox* gene expression was firmly established before this work. However, it was difficult to quantify their relative contributions to the establishment of transcriptional and chromatin domains at the scale of an entire *Hox* cluster with previous techniques. Our results show that an ectopic cluster lacking all previously described internal RAREs (*RARE*Δ *SynHoxA*) failed to respond to RA both at the transcriptional and chromatin levels. This gene expression phenotype was not fully rescued by the addition of distal enhancers in *Enhancers*+*RARE*Δ *SynHoxA*. However, some chromatin remodeling was observed. Together, removal of internal RAREs led to virtually complete loss of gene expression, whereas removal of enhancers led to a reduction in expression at early time points, with almost complete rescue at later time points. Therefore, distal enhancers do not induce high levels of transcription or induce drastic

chromatin remodeling in the absence of intracluster RAREs, but synergize with the RAREs to play a critical role in fine-tuning expression levels (Fig. 6, E and F).

All ectopic clusters recruited CTCF and PRC2 in embryonic stem cells, implying that this property is *Hox* cluster–intrinsic. Therefore, precise CTCF positioning within the cluster does not depend on interactions with other elements at the endogenous TAD boundary. Together, our results imply that *Hox* clusters are discrete units with an intrinsic ability to respond to patterning signals, strengthening original observations made at the *HoxD* cluster using random BAC transgenesis (28, 29). This is also congruent with the idea that the evolution of novel distal enhancers is a source of morphological novelty in secondary structures such as limbs (16, 47).

The inability of the minimal *SynHoxA* to fully clear repressive marks in the anterior domain could have several causes. First, the boost in transcription provided by distal enhancers at early time points might facilitate the clearance of repressive chromatin. Second, the enhancers could serve as platforms to recruit additional chromatin modifiers. Finally, the vector backbone that is introduced as part of the delivery harbors repressive chromatin modifications throughout differentiation (Fig. 4). The spread of repressive chromatin from this region might be more effectively prevented by enhancer sequences that contain fewer CpG islands than the cluster itself. Future experiments using scarless delivery methods will enable us to distinguish between these hypotheses (41). Although we see no strong genetic or topological evidence for trans-chromosomal interactions, we cannot fully exclude the possibility that they may play some part in activating *SynHoxA* genes. Higher-resolution chromatin conformation data centered on the ectopic clusters may help to address this question.

This study represents a proof of principle for “synthetic regulatory reconstitution.” Targeting large, fully editable constructs to precise genomic locations enables quantitative comparisons between variants and promises to address critical questions in gene regulation and genome organization. Multiple elements required for the finer analysis of constructs through differentiation, such as live-cell imaging of transcription and chromatin mobility, could also be included via bottom-up synthesis (48). Testing different ectopic sites such as those marked with constitutive heterochromatin, cross-species transplants of regulatory landscapes, and phenotyping in richer systems such as living mice and gastruloids are all attractive avenues to explore (49).

Differences from endogenous gene expression dynamics were observed, even for the *Enhancers+SynHoxA* construct. Thus, even the largest construct does not contain all the regulatory information required for refining gene expression. The great value in pursuing the synthetic regulatory reconstitution strategy is realized in cases where endogenous regulation cannot be fully recapitulated. This points to gaps in knowledge that we can attempt to fill by building successively larger or more intricate ectopic constructs in the future until no differences are observed when compared to the endogenous cluster. Reconstitution is a powerful framework for dissecting complex biochemical processes because it allows for exquisite control over components of the system under study (50, 51). By analogy, our approach allows for the generation of locus-scale variant constructs with any combination

of desired changes. We expect synthetic regulatory reconstitution to be a fundamental component of the toolbox for studying transcriptional regulation.

Methods summary

A full description of the methods can be found in the supplementary materials. In brief, *SynHoxA* constructs were fabricated in yeast and integrated into mESCs as described (36, 41). *SynHoxA* mESCs were differentiated to motor neurons and characterized by RNA-seq, ChIP-seq, and Hi-C as described (10, 44, 45). Sequencing data were analyzed using custom pipelines.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

ACKNOWLEDGMENTS

We thank the Mazzoni, Boeke, and Holt labs as well as the Institute for Systems Genetics community for their support, M. Khalfan (Genomics Core Facility at NYU) for making the reform tool publicly available, B. Ragipani for preliminary analysis on motor neuron differentiation markers, N. Zesati and S. Arora for help with preliminary visualization of Hi-C data, the Experimental Pathology core at NYU Langone for help with sectioning, and J. Skok and D. Reinberg for their insights.

Funding:

Supported by NHGRI grant RM1HG009491 (J.B., M.T.M., and E.O.M.), NINDS grant R01NS100897 and NIGMS grant R01GM138876 (E.O.M.), New York State Stem Cell Science predoctoral training grant C322560GG (M.B.), NIH grants R01AG075272 and R01GM127538 and Melanoma Research Foundation Award 687306 (T.L.), and NIH grant F32CA239394 (B.R.K.).

Data and materials availability:

All sequencing data from this study are deposited in NCBI GEO under GSE190906.

REFERENCES AND NOTES

1. Duboule D, The rise and fall of Hox gene clusters. Development 134, 2549–2560 (2007). doi: 10.1242/dev.001065; [PubMed: 17553908]
2. Kmita M, Duboule D, Organizing axes in time and space;25 years of colinear tinkering. Science 301, 331–333 (2003). doi: 10.1126/science.1085753; [PubMed: 12869751]
3. McGinnis W, Krumlauf R, Homeobox genes and axial patterning. Cell 68, 283–302 (1992). doi: 10.1016/0092-8674(92)90471-N; [PubMed: 1346368]
4. Duboule D, Morata G, Colinearity and functional hierarchy among genes of the homeotic complexes. Trends Genet 10, 358–364 (1994). doi: 10.1016/0168-9525(94)90132-5; [PubMed: 7985240]
5. Lewis EB, A gene complex controlling segmentation in Drosophila. Nature 276, 565–570 (1978). doi: 10.1038/276565a0; [PubMed: 103000]
6. Shah N, Sukumar S, The Hox genes and their roles in oncogenesis. Nat. Rev. Cancer 10, 361–371 (2010). doi: 10.1038/nrc2826; [PubMed: 20357775]
7. Margueron R, Reinberg D, The Polycomb complex PRC2 and its mark in life. Nature 469, 343–349 (2011). doi: 10.1038/nature09784; [PubMed: 21248841]
8. Mahony S et al. , Ligand-dependent dynamics of retinoic acid receptor binding during early neurogenesis. Genome Biol 12, R2 (2011). doi: 10.1186/gb-2011-12-1-r2; [PubMed: 21232103]

9. Nolte C, De Kumar B, Krumlauf R, Hox genes: Downstream “effectors” of retinoic acid signaling in vertebrate embryogenesis. Genesis 57, e23306 (2019). doi: 10.1002/dvg.23306; [PubMed: 31111645]
10. Mazzoni EO et al. , Saltatory remodeling of Hox chromatin in response to rostrocaudal patterning signals. Nat. Neurosci 16, 1191–1198 (2013). doi: 10.1038/nn.3490; [PubMed: 23955559]
11. Noordermeer D et al. , The dynamic architecture of Hox gene clusters. Science 334, 222–225 (2011). doi: 10.1126/science.1207194; [PubMed: 21998387]
12. Soshnikova N, Duboule D, Epigenetic temporal control of mouse Hox genes in vivo. Science 324, 1320–1323 (2009). doi: 10.1126/science.1171468; [PubMed: 19498168]
13. Dupé V et al. , In vivo functional analysis of the HoxA-1 3′ retinoic acid response element (3’RARE). Development 124, 399–410 (1997). doi: 10.1242/dev.124.2.399; [PubMed: 9053316]
14. Frasch M, Chen X, Lufkin T, Evolutionary-conserved enhancers direct region-specific expression of the murine HoxA-1 and HoxA-2 loci in both mice and Drosophila. Development 121, 957–974 (1995). doi: 10.1242/dev.121.4.957; [PubMed: 7743939]
15. Lonfat N, Montavon T, Darbellay F, Gitto S, Duboule D, Convergent evolution of complex regulatory landscapes and pleiotropy at Hox loci. Science 346, 1004–1006 (2014). doi: 10.1126/science.1257493; [PubMed: 25414315]
16. Montavon T, Duboule D, Chromatin organization and global regulation of Hox gene clusters. Philos. Trans. R. Soc. Lond. B Biol. Sci 368, 20120367 (2013). doi: 10.1098/rstb.2012.0367 [PubMed: 23650639]
17. Montavon T, Soshnikova N, Hox gene regulation and timing in embryogenesis. Semin. Cell Dev. Biol 34, 76–84 (2014).doi: 10.1016/j.semcdb.2014.06.005; [PubMed: 24930771]
18. Montavon T et al. , A regulatory archipelago controls Hox genes transcription in digits. Cell 147, 1132–1145 (2011). doi: 10.1016/j.cell.2011.10.023; [PubMed: 22118467]
19. Berlivet S et al. , Clustering of tissue-specific sub-TADs accompanies the regulation of HoxA genes in developing limbs. PLOS Genet 9, e1004018 (2013). doi: 10.1371/journal.pgen.1004018; [PubMed: 24385922]
20. Cao K et al. , SET1A/COMPASS and shadow enhancers in the regulation of homeotic gene expression. Genes Dev 31, 787–801 (2017). doi: 10.1101/gad.294744.116; [PubMed: 28487406]
21. De Kumar B et al. , Analysis of dynamic changes in retinoid-induced transcription and epigenetic profiles of murine Hox clusters in ES cells. Genome Res 25, 1229–1243 (2015). doi: 10.1101/gr.184978.114; [PubMed: 26025802]
22. Neijts R et al. , Polarized regulatory landscape and Wnt responsiveness underlie Hox activation in embryos. Genes Dev 30, 1937–1942 (2016). doi: 10.1101/gad.285767.116; [PubMed: 27633012]
23. Narendra V et al. , CTCF establishes discrete functional chromatin domains at the Hox clusters during differentiation. Science 347, 1017–1021 (2015). doi: 10.1126/science.1262088; [PubMed: 25722416]
24. Narendra V, Bulajić M, Dekker J, Mazzoni EO, Reinberg D, CTCF-mediated topological boundaries during development foster appropriate gene regulation. Genes Dev 30, 2657–2662 (2016). doi: 10.1101/gad.288324.116; [PubMed: 28087711]
25. Ostrov N et al. , Technological challenges and milestones for writing genomes. Science 366, 310–312 (2019). doi: 10.1126/science.aay0339; [PubMed: 31624201]
26. Gasperini M, Starita L, Shendure J, The power of multiplexed functional analysis of genetic variants. Nat. Protoc 11, 1782–1787 (2016). doi: 10.1038/nprot.2016.135; [PubMed: 27583640]
27. Lehoczky JA, Innis JW, BAC transgenic analysis reveals enhancers sufficient for HoxA13 and neighborhood gene expression in mouse embryonic distal limbs and genital bud. Evol. Dev 10, 421–432 (2008). doi: 10.1111/j.1525-142X.2008.00253.x; [PubMed: 18638319]
28. Spitz F, Gonzalez F, Duboule D, A global control region defines a chromosomal regulatory landscape containing the HoxD cluster. Cell 113, 405–417 (2003). doi: 10.1016/S0092-8674(03)00310-6; [PubMed: 12732147]
29. Spitz F et al. , Large scale transgenic and cluster deletion analysis of the HoxD complex separate an ancestral regulatory module from evolutionary innovations. Genes Dev 15, 2209–2214 (2001). doi: 10.1101/gad.205701; [PubMed: 11544178]

30. Peterson KR et al. , Use of yeast artificial chromosomes (YACs) in studies of mammalian development: Production of beta-globin locus YAC mice carrying human globin developmental mutants. Proc. Natl. Acad. Sci. U.S.A 92, 5655–5659 (1995). doi: 10.1073/pnas.92.12.5655; [PubMed: 7539923]
31. Heintz N, BAC to the future: The use of bac transgenic mice for neuroscience research. Nat. Rev. Neurosci 2, 861–870 (2001). doi: 10.1038/35104049; [PubMed: 11733793]
32. Liberante FG, Ellis T, From kilobases to megabases: Design and delivery of large DNA constructs into mammalian genomes. Curr. Opin. Syst. Biol 25, 1–10 (2021). doi: 10.1016/j.coisb.2020.11.003
33. Wallace HA et al. , Manipulating the mouse genome to engineer precise functional syntenic replacements with human sequence. Cell 128, 197–209 (2007). doi: 10.1016/j.cell.2006.11.044; [PubMed: 17218265]
34. McCarty NS, Graham AE, Studená L, Ledesma-Amaro R, Multiplexed CRISPR technologies for gene editing and transcriptional regulation. Nat. Commun 11, 1281 (2020). doi: 10.1038/s41467-020-15053-x; [PubMed: 32152313]
35. Kraft K et al. , Deletions, Inversions, Duplications: Engineering of Structural Variants using CRISPR/Cas in Mice. Cell Rep 10, 833–839 (2015). doi: 10.1016/j.celrep.2015.01.016; [PubMed: 25660031]
36. Mitchell LA et al. , De novo assembly and delivery to mouse cells of a 101 kb functional human gene. Genetics 218, iyab038 (2021). doi: 10.1093/genetics/iyab038;
37. DiCarlo JE et al. , Genome engineering in Saccharomyces cerevisiae using CRISPR-Cas systems. Nucleic Acids Res 41, 4336–4343 (2013). doi: 10.1093/nar/gkt135; [PubMed: 23460208]
38. Iacovino M et al. , Inducible cassette exchange: A rapid and efficient system enabling conditional gene expression in embryonic stem and primary cells. Stem Cells 29, 1580–1588 (2011). doi: 10.1002/stem.715; [PubMed: 22039605]
39. Jasin M, Moynahan ME, Richardson C, Targeted transgenesis. Proc. Natl. Acad. Sci. U.S.A 93, 8804–8808 (1996). doi: 10.1073/pnas.93.17.8804; [PubMed: 8799106]
40. Gasperini M et al. , CRISPR/Cas9-Mediated Scanning for Regulatory Elements Required for HPRT1 Expression via Thousands of Large, Programmed Genomic Deletions. Am. J. Hum. Genet 101, 192–205 (2017). doi: 10.1016/j.ajhg.2017.06.010; [PubMed: 28712454]
41. Brosh R et al. , A versatile platform for locus-scale genome rewriting and verification. Proc. Natl. Acad. Sci. U.S.A 118, e2023952118 (2021). doi: 10.1073/pnas.2023952118; [PubMed: 33649239]
42. Wichterle H, Lieberam I, Porter JA, Jessell TM, Directed differentiation of embryonic stem cells into motor neurons. Cell 110, 385–397 (2002). doi: 10.1016/S0092-8674(02)00835-8; [PubMed: 12176325]
43. Peljto M, Wichterle H, Programming embryonic stem cells to neuronal subtypes. Curr. Opin. Neurobiol 21, 43–51 (2011). doi: 10.1016/j.conb.2010.09.012; [PubMed: 20970319]
44. Aydin B et al. , Proneural factors Ascl1 and Neurog2 contribute to neuronal subtype identities by establishing distinct chromatin landscapes. Nat. Neurosci 22, 897–908 (2019). doi: 10.1038/s41593-019-0399-y; [PubMed: 31086315]
45. Bulajić M et al. , Differential abilities to engage inaccessible chromatin diversify vertebrate Hox binding patterns. Development 147, dev.194761 (2020). doi: 10.1242/dev.194761;
46. Su G et al. , CTCF-binding element regulates ESC differentiation via orchestrating long-range chromatin interaction between enhancers and HoxA. J. Biol. Chem 296, 100413 (2021). doi: 10.1016/j.jbc.2021.100413; [PubMed: 33581110]
47. Freitas R, Gómez-Marín C, Wilson JM, Casares F, Gómez-Skarmeta JL, HoxD13 contribution to the evolution of vertebrate appendages. Dev. Cell 23, 1219–1229 (2012).doi: 10.1016/j.devcel.2012.10.015; [PubMed: 23237954]
48. Sato H, Das S, Singer RH, Vera M, Imaging of DNA and RNA in Living Eukaryotic Cells to Reveal Spatiotemporal Dynamics of Gene Expression. Annu. Rev. Biochem 89, 159–187 (2020). doi: 10.1146/annurev-biochem-011520-104955; [PubMed: 32176523]
49. Beccari L et al. , Multi-axial self-organization properties of mouse embryonic stem cells into gastruloids. Nature 562, 272–276 (2018). doi: 10.1038/s41586-018-0578-0; [PubMed: 30283134]

50. Ganzinger KA, Schwille P, More from less - bottom-up reconstitution of cell biology. J. Cell Sci 132, jcs227488 (2019). doi: 10.1242/jcs.227488;

51. Liu AP, Fletcher DA, Biology under construction: In vitro reconstitution of cellular function. Nat. Rev. Mol. Cell Biol 10, 644–650 (2009). doi: 10.1038/nrm2746; [PubMed: 19672276]

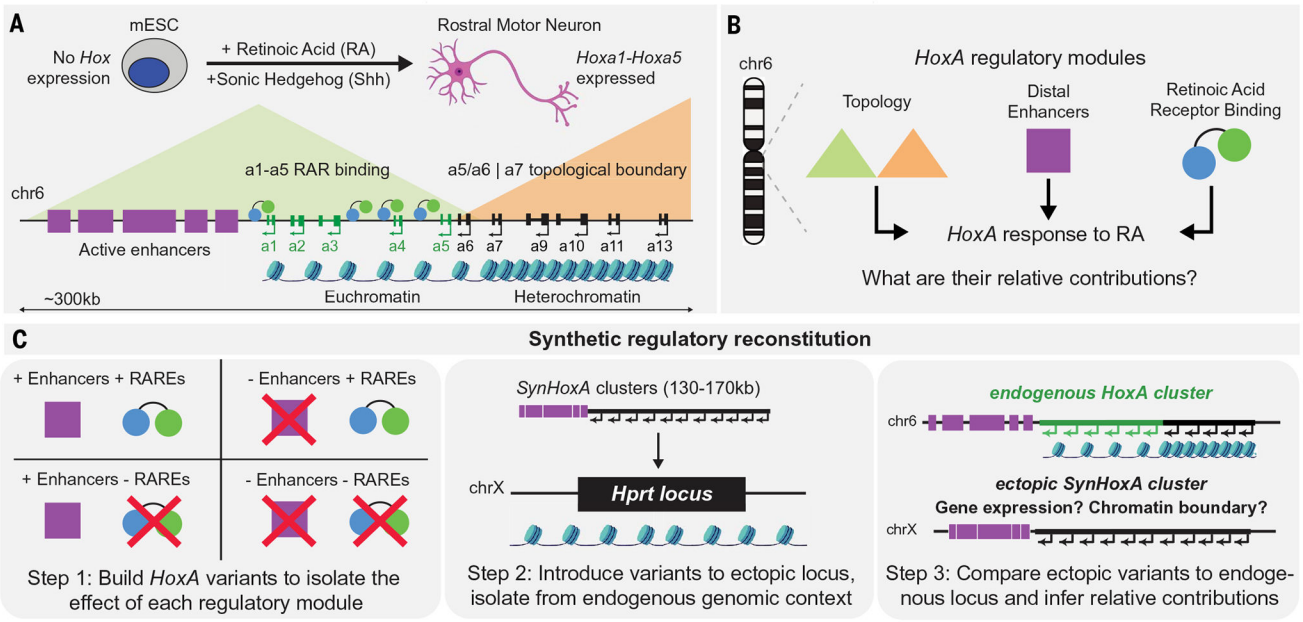


Fig. 1. *HoxA* regulation relies on the integration of multiple regulatory modules.

(**A**) Schematic of *HoxA* regulation in response to retinoic acid (RA) during in vitro mouse ES cell (mESC)–motor neuron differentiation. (**B**) Multiple regulatory modules, including enhancers, TF binding, and topology, are integrated to drive *HoxA* cluster response to RA. (**C**) Schematic of the synthetic regulatory reconstitution approach. Synthetic *HoxA* variants encoding various combinations of regulatory modules are built and integrated at an ectopic location in the genome. The response of these synthetic ectopic clusters to RA reveals their sufficiency and relative contribution in driving faithful *HoxA* expression.

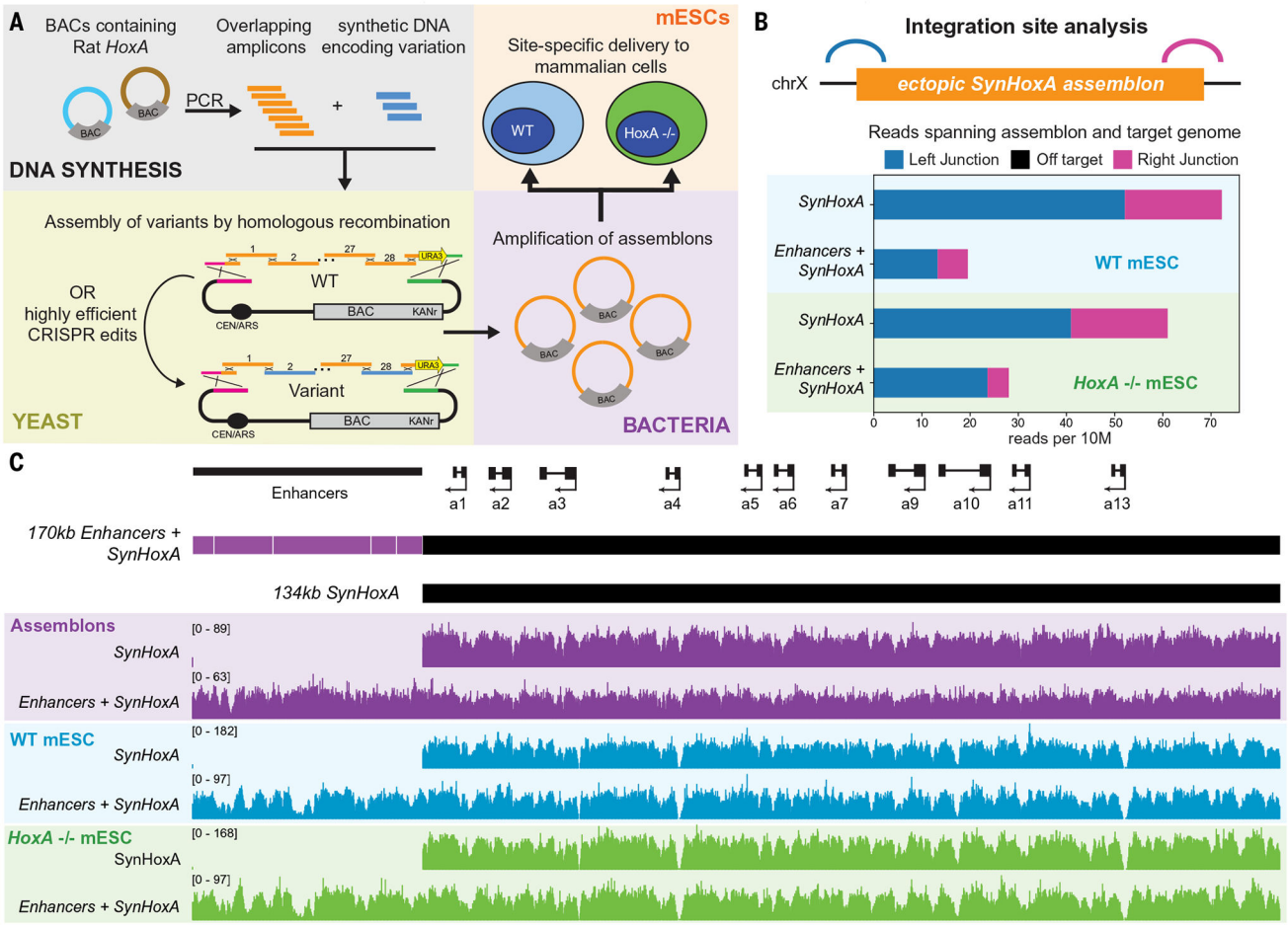


Fig. 2. Build and delivery of *SynHoxA* constructs.

(**A**) Schematic of the process to generate mESCs bearing ectopic synthetic *HoxA* clusters via homologous recombination-based assembly in yeast and amplification in bacteria. (**B**) Analysis of reads spanning the synthetic construct and the host genome by bamintersect revealed only the expected junctions with no off-target integrations. (**C**) Schematic of the 134-kb *SynHoxA* and 170-kb *Enhancers+SynHoxA* constructs. Sequencing data for assemblon DNA isolated from bacteria (purple) and from capture sequencing after integration into wild-type (blue) and *HoxA*$^{-/-}$ (green) mESCs.

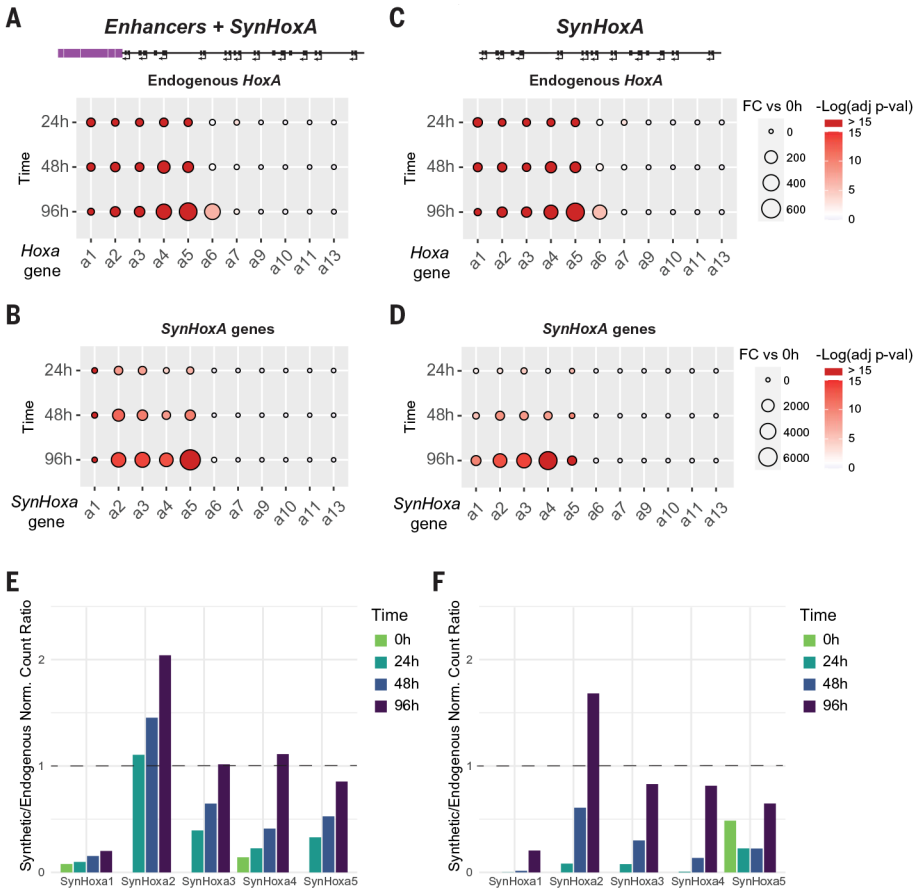


Fig. 3. *SynHoxA* variants up-regulate the correct subset of genes in response to RA patterning signal.

(**A** to **D**) Fold change (FC) of RNA-seq data for endogenous mouse *HoxA* [(A) and (C)] and *SynHoxA* [(B) and (D)] genes during RA differentiation, relative to expression before RA treatment ($n = 2$). *SynHoxA* variants induce the correct genes (*SynHoxA1–5*) in response to RA. (**E** and **F**) Ratios of gene expression for *SynHoxA* genes to endogenous mouse *HoxA* genes ($n = 2$). Counts for the endogenous *HoxA* genes were halved to normalize for two endogenous *HoxA* versus one ectopic *SynHoxA* copy.

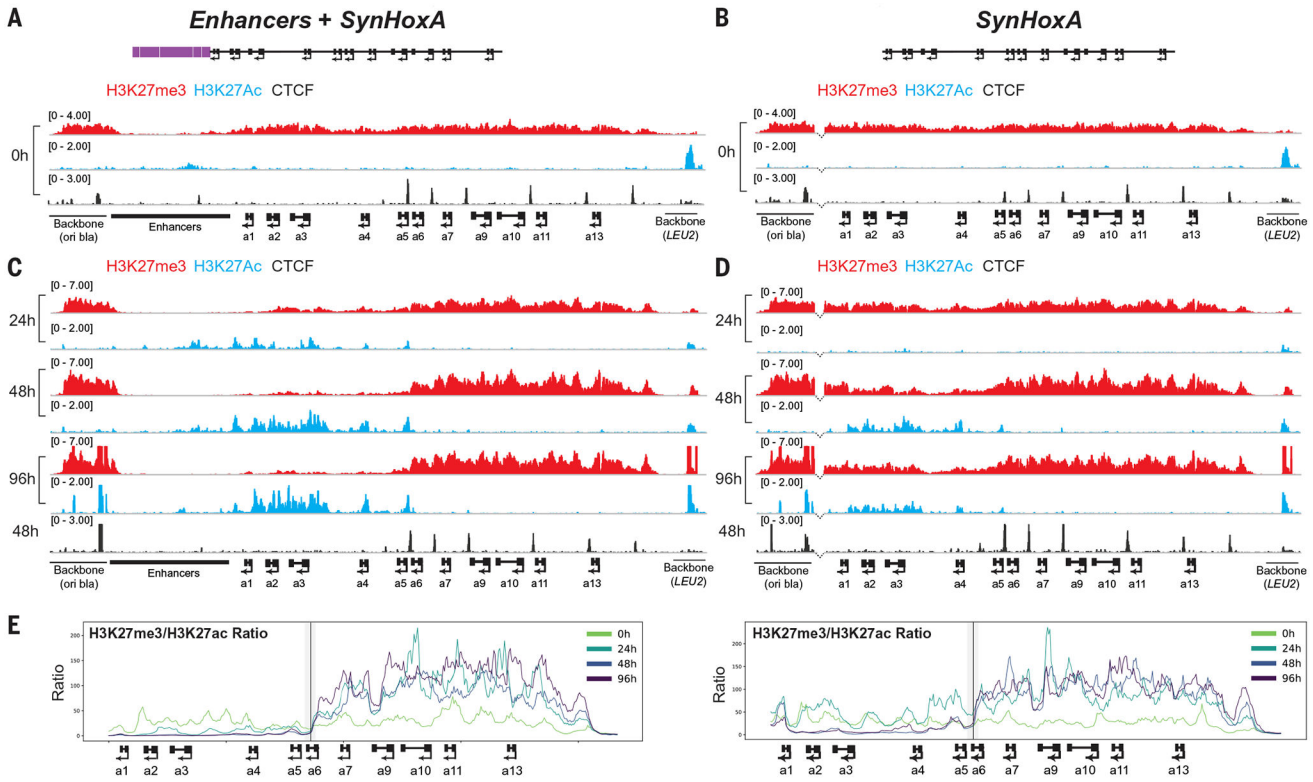


Fig. 4. Distal enhancers are required for full clearance of repressive chromatin and the formation of a sharp chromatin boundary.

ChIP-seq analysis ($n = 2$) of activating H3K27ac (blue), repressive H3K27me3 (red), and CTCF (black) at *SynHoxA* clusters. (**A** and **B**) In undifferentiated cells, *SynHoxA* clusters contained H3K27me3, lacked H3K27ac, and recruited CTCF to correct locations. (**C** and **D**) In response to RA, the *SynHoxA1–5* domain contained activating H3K27ac and cleared repressive H3K27me3. Dotted lines at the anterior breakpoint between the cluster and the vector sequences in (B) and (D) indicate presence of enhancer sequences in the reference genome to which no reads are mapped. (**E**) RPKM (reads per kilobase per million mapped reads) normalized ratios of repressive H3K27me3 to active H3K27ac across *SynHoxA*. The black line marks the *HoxA5-HoxA6* CTCF site; gray shading indicates the windows contributing to ChIP-seq signal at the site.

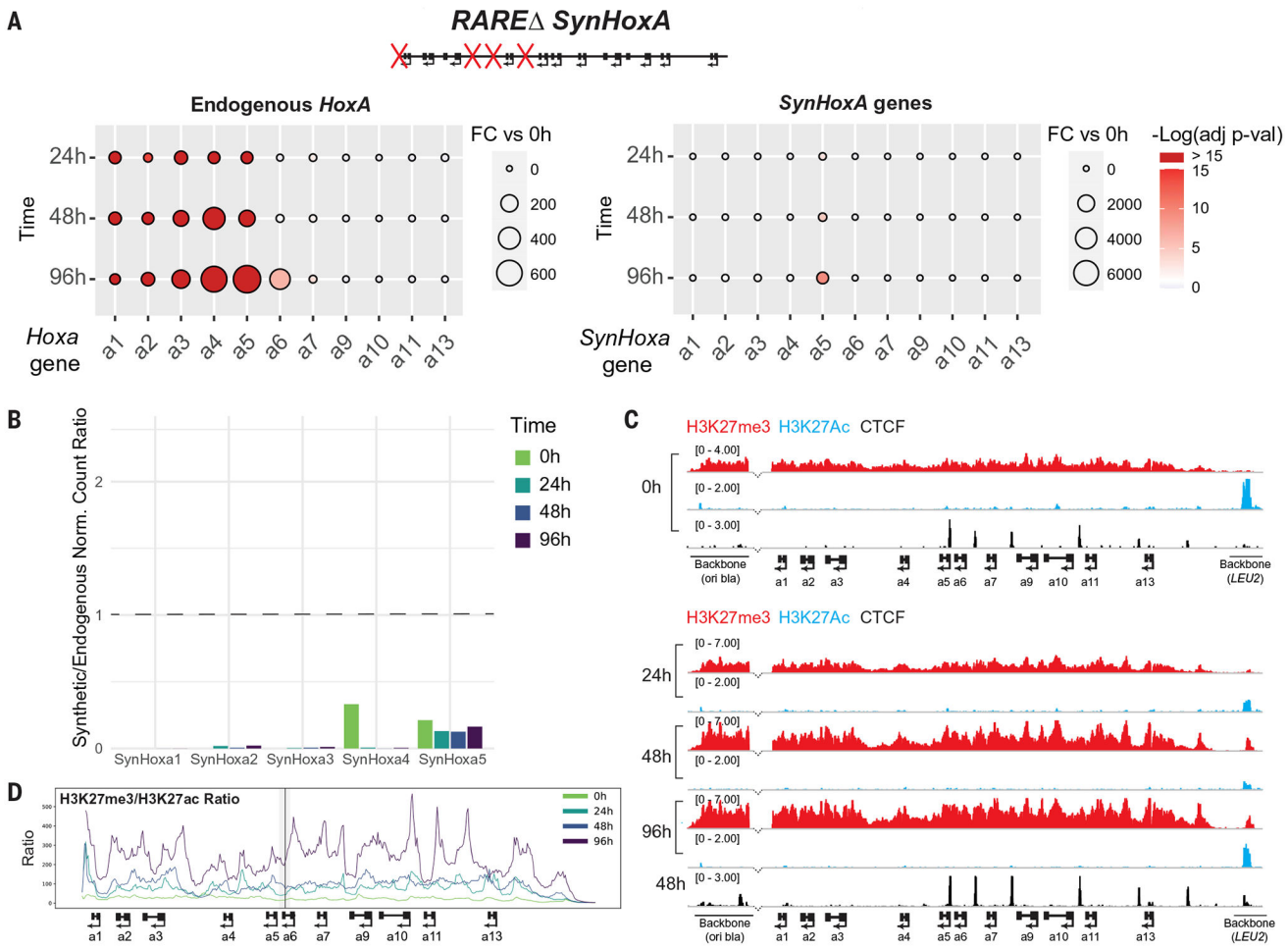


Fig. 5. Retinoic acid receptor response element (RARE) sites are required for the RA response.

(**A**) Fold change of RNA-seq data for endogenous *HoxA* and *SynHoxA* genes during differentiation ($n = 2$). (**B**) Ratios of gene expression for *SynHoxA* genes to endogenous mouse *HoxA* genes ($n = 2$). (**C**) ChIP-seq revealed no evidence of H3K27me3 (red) clearance and H3K27ac (blue) recruitment at *RARE*Δ *SynHoxA*. Dotted lines show the anterior breakpoint between the cluster and the vector sequences in (C) as in Fig. 4. (**D**) Ratio of repressive H3K27me3 to active H3K27ac chromatin across *SynHoxA* as in Fig. 4E.

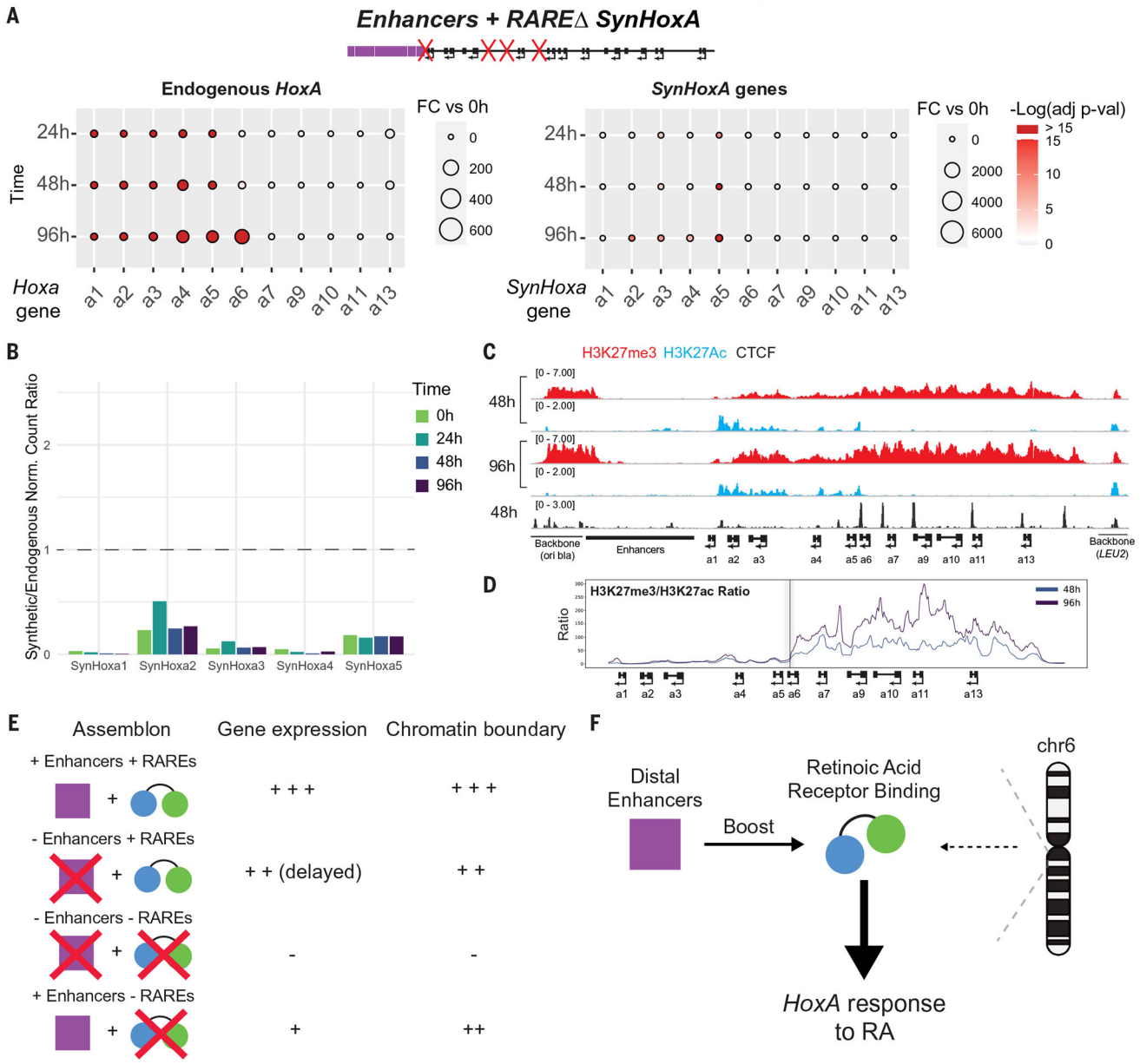


Fig. 6. The addition of enhancers to *SynHoxA RAREΔ* does not rescue gene expression.

(**A**) Fold change of RNA-seq data for endogenous *HoxA* and *SynHoxA* genes during differentiation ($n = 2$). (**B**) Ratios of gene expression for *SynHoxA* genes to endogenous mouse *HoxA* genes ($n = 2$). (**C**) ChIP-seq revealed the appropriate recruitment of CTCF (black) and the formation of a weak chromatin boundary at *Enhancers+RAREΔ SynHoxA* upon differentiation ($n = 2$). (**D**) Ratio of repressive H3K27me3 to active H3K27ac chromatin across *SynHoxA* as in Fig. 4E. (**E**) Summary of gene expression and chromatin boundary phenotypes across all *SynHoxA* clusters. (**F**) Model describing relative contributions of distal enhancers, intra-*Hox* binding, and genomic context to the RA response at *HoxA*.