Systematic analysis of intrinsic enhancer-promoter compatibility in the mouse genome

Graphical abstract

Highlights
- Intrinsic compatibility of thousands of enhancer-promoter combinations was tested
- Compatibilities exhibit a broad spectrum, from promiscuous to highly specific
- Enhancer-promoter compatibility and chromatin looping appear to be independent

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In brief
A major question in genome biology is how enhancers “choose” their target promoter. Using high-throughput reporter assays, Martinez-Ara and colleagues surveyed the intrinsic compatibilities of thousands of enhancer-promoter pairs in mouse cells. They found a wide diversity of specificities, mostly likely driven by a complex grammar of sequence motifs.
SUMMARY

Gene expression is in part controlled by cis-regulatory elements (CREs) such as enhancers and repressive elements. Anecdotal evidence has indicated that a CRE and a promoter need to be biochemically compatible for promoter regulation to occur, but this compatibility has remained poorly characterized in mammalian cells. We used high-throughput combinatorial reporter assays to test thousands of CRE-promoter pairs from three Mb-sized genomic regions in mouse cells. This revealed that CREs vary substantially in their promoter compatibility, ranging from striking specificity to broad promiscuity. More than half of the tested CREs exhibit significant promoter selectivity. Housekeeping promoters tend to have similar CRE preferences, but other promoters exhibit a wide diversity of compatibilities. Higher-order transcription factors (TF) motif combinations may account for compatibility. CRE-promoter selectivity does not correlate with looping interactions in the native genomic context, suggesting that chromatin folding and compatibility are two orthogonal mechanisms that confer specificity to gene regulation.

INTRODUCTION

How genes are regulated by cis-regulatory elements (CREs) such as enhancers and repressor elements is a long-standing topic in molecular biology (Banerji et al., 1981; Tuan et al., 1985; Fiering et al., 1995; Lettice et al., 2003; ENCODE Project Consortium, 2012; van Arensbergen et al., 2014; Zabidi and Stark, 2016; Farley et al., 2015; Robson et al., 2019; Segert et al., 2021). One conundrum is how CREs “choose” their target promoters. Some enhancers can activate multiple promoters in cis over short and long genomic distances (Shlyueva et al., 2014; Schoenfelder and Fraser, 2019; Furlong and Levine, 2018), while others show remarkable specificity, regulating only one of its neighboring promoters or even skipping one or more promoters to activate more distal ones. In part, 3D folding and compartmentalization of the chromatin fiber help to establish this specificity, by facilitating certain enhancer-promoter contacts and curbing others (Lupiániz et al., 2015; Schoenfelder and Fraser, 2019; Furlong and Levine, 2018).

However, there is also substantial evidence that biochemical (in)compatibility between CREs and promoters contributes to the specificity of their regulatory interactions. This is akin to a lock-and-key mechanism: proteins bound to the CRE and the promoter must be compatible to form a productive complex. Examples of such intrinsic selectivity have been documented particularly in Drosophila and in some instances could be attributed to a specific sequence motif in the promoter (Li and Noll, 1994; Merli et al., 1996; Butler and Kadonaga, 2001; Juven-Gershon et al., 2008; Kwon et al., 2009). Data obtained with massively parallel reporter assays (MPRAs) in Drosophila cells have suggested a general separation of enhancer-promoter compatibility into housekeeping and tissue-specific classes (Arnold et al., 2017). Some of this specificity may be determined by the recruitment of co-factors (Haberle et al., 2019). However, a thorough understanding of the underlying mechanisms is still lacking.

While several studies of individual enhancer-promoter combinations indicate that biochemical compatibility also plays a role in mammals (e.g., Bertolino and Singh, 2002; Vakoc et al., 2005; Jing et al., 2008; Deng et al., 2012; Chang et al., 2004), systematic studies of this mechanism have so far been lacking in mouse or human cells. Thus, it is still unknown how widespread such intrinsic compatibility is in mammalian cells, and what drives this compatibility.

In order to address this issue, we systematically tested the compatibility of thousands of combinations of candidate CREs (cCREs) and promoters using MPRAs. We used plasmid-based MPRAs because they are highly scalable (Inoue and Ahituv,
et al., 2020; Davis et al., 2020; King et al., 2020; Sahu et al., 2021). We considered this to be important because the extra regulatory information contained in those additional sequences may be relevant for interactions of the promoters with CREs.

Compared with promoters, the annotation of cCREs is much less accurate. However, most cCREs are centered around DNase I hypersensitive sites (DHSs) (Groudine et al., 1983; Joshi et al., 2015; ENCODE Project Consortium, 2012). We therefore selected fragments of ~400 bp centered around all detected DHS peaks in each locus (Figures 1A–1C). This definition of cCREs within the range of typical enhancer definitions (Long et al., 2016). Some authors consider enhancers combinations of multiple DHSs or longer stretches of DNA sequences. However, other studies have shown that the activity of these long enhancers can be reproduced by shorter versions of ~500 bp (Barakat et al., 2018; Agrawal et al., 2021). Coordinates of all tested genomic fragments are provided in Data S1.

We designed two MPRA variants to test many cCRE-P combinations (Figures 1D and 1E). In the first variant, which we will refer to as Upstream assay, we obtained 82–192 individual cCREs and 18–25 Ps per locus by PCR amplification (Table S1). We pooled all of these fragments and randomly ligated them to form dimer fragments, which we then cloned en masse into a reporter vector, “upstream” of a randomlybarcode transcribed unit that lacked a promoter itself. This resulted into highly complex libraries of cCRE-P, cCRE-cCRE, P-P, and P-cCRE pairs, with each individual element in two possible orientations. We then sequenced the libraries to identify the paired fragments, their orientations in the reporter vector, and their linked barcodes. Owing to the simple random ligation step, libraries with tens of thousands of cCRE-P combinations can be obtained with this approach (Tables S1 and S2). Here, we focus on the analysis of cCRE-P pairs, but data from all other configurations are also provided as Data S2.

In a second and complementary approach, we constructed a library in which the cCREs are placed “downstream” of the reporter gene, i.e., separated ~1 kb from the promoter (Figure 1E). This was done in two steps: we first cloned a selection of 10 promoters upstream of the barcoded transcription unit, resulting in a set of reporters with different promoters. Next, we inserted a pool of cCREs into this set, downstream of the barcoded reporter unit and in both possible orientations. We will refer to the assays done with the resulting library as Downstream assay. Due to the two-step cloning protocol, the Downstream assay is less scalable than the Upstream assay but nevertheless allows for testing of hundreds of cCRE-P combinations (Table S1).

We used all P and cCRE DNA fragments from each of the three loci in separate Upstream assays, whereas we focused on ten promoters and all cCREs from the Klf2 locus in the Downstream

Figure 1. Regulatory element selection and library construction

(A–C) Representations of Nanog, Tcfp21, and Klf2 loci, respectively. In (C), the zoom-in displays a DNase I sensitivity track (Joshi et al., 2015) where peaks overlap with cCREs.

(D) Cloning strategy for the Upstream assay. cCREs and promoters were amplified by PCR from genomic DNA and pooled. Fragments in this pool were then randomly ligated to generate duplets. Singlets and duplets were cloned into the same barcoded vector to generate two libraries per locus, a singlet library and a combinatorial library.

(E) Cloning strategy for the Downstream assay. The singlet pool from the Klf2 locus was cloned into ten vectors, each of them carrying a different promoter. The resulting ten sub-libraries were combined into one Downstream assay library.
assay. Table S1 provides summary statistics of the individual library compositions. Due to the random nature of the combinatorial cloning, we did not recover all possible pairs. Nevertheless, in the three Upstream assays combined we tested a total of 10,678 cCRE-P pairs, or 3,747 pairs if we do not take orientations into account. For the Downstream assay these numbers were 1,364 and 752, respectively. From the Klf2 locus 847 and 676 pairs, respectively, overlapped between the Upstream and Downstream assay. As references, we also inserted each P and cCRE individually (i.e., unpaired) in the upstream position.

**Boost indices estimate promoter-specific activity of cCREs**

We then transiently transfected each of these libraries into mESCs. 24 h after transfection, we collected mRNA from the cells and counted the transcribed barcodes by reverse transcription followed by PCR amplification and high-throughput sequencing. In parallel, barcodes were counted in the plasmid libraries. For each barcode, we then normalized the counts in cDNA over the counts detected in the plasmid DNA. Further data processing is described in the STAR Methods. We performed 3 biological replicates per library, which correlated with an average Pearson $r = 0.87$ (0.83–0.90) for the Upstream assay and $r = 0.98$ (0.98–0.99) for the Downstream assay. (Figures S1A–S1C)

We first analyzed the transcriptional activities of all singlet (unpaired) P and cCREs in the upstream position. For promoters, these basal activities varied over a ~100-fold dynamic range (Figures 2A and S2A). Of all cCREs, 40.4% showed detectable transcriptional activity in the upstream position without any P (Figures 2A and S2A). Such autonomous transcriptional activity is a frequently observed property of enhancers (Djebali et al., 2012; Andersson et al., 2014; van Arensbergen et al., 2017), and hence, these elements are likely to be enhancers. For a few cCREs this activity was as high as some of the strongest promoters, suggesting that they may in fact be unannotated promoters or very strong enhancers.

We then determined the ability of each cCRE to alter the activity of each linked P. For this, we calculated a “boost index” for each cCRE-P pair, defined as the log$_2$-fold change in activity of the cCRE-P pair compared with the promoter element alone. Unexpectedly, 20 negative controls that we included in the Klf2 libraries, consisting of randomly generated DNA sequences of similar size and G/C content as the cCREs, showed a modestly negative boost index (median value $-0.45$ when inserted upstream) (Figure S1D). This is possibly because lengthening of the reporter constructs alters the topology, supercoiling, transfection efficiency, or a combination of these parameters. We therefore corrected all cCRE-P boost indices for this non-specific negative bias (see STAR Methods). After this correction the negative controls had a marginal residual bias (median log$_2$ value $-0.19$), which we deemed acceptable (Figure S1D).

**Identification of activating and repressive cCREs**

For each of the three genomic loci, the matrix of corrected boost indices shows a wide diversity of patterns across the cCREs. We observed this both in the Upstream and Downstream assays (Figures 2B–2D and S2B–S2D). For example, in the Klf2 locus Upstream assay, cCRE E097 activates most of the tested promoters, while E046 (Figure 2B) and E057 (arrow in Figure 2C) only activate a distinct subset of promoters. Several elements are primarily acting as repressors (e.g., E030 [Figure 2B] and E040, [arrow in Figure 2C]), and some seem neither activating nor repressive (e.g., E070 [Figure 2B] and E085 [arrow in Figure 2C]).

We broadly classified the cCREs according to their overall effects on the linked promoters (Figure S3A). In the Upstream assays, 21% of cCREs showed positive boost indices that were significantly higher than the rest of cCREs across all tested promoters, indicating that they can act as enhancer elements. About 17% of the cCREs showed negative boost indices significantly below the rest of cCREs and hence are putative repressor elements. For the remaining 62% of cCREs, the boost indices across their linked promoters were not significantly higher or lower than the rest; these “ambiguous” elements either have no regulatory effects at all, or they have a mixed repressive/activating/inactive effect that depends on the linked P (see below).

We were somewhat surprised to identify similar numbers of putative enhancers and repressors because most annotated cCREs in mammalian genomes are predicted to be enhancers rather than repressive elements (ENCOD Project Consortium et al., 2020; ENCODE Project Consortium, 2012). In some cases, this repression may be underestimated in our analysis, as the estimates of negative boost indices for lowly active promoters are less reliable due to the higher noise-to-mean ratios at low expression levels (Figure S3B).

For activating elements, the boost indices varied in part according to the basal activities of the cCRE and promoters. Strong boosting occurred primarily at promoters with low basal activities, while highly active promoters were more difficult to boost (FigureS3C). This suggests a saturation effect, or it could indicate that promoters with high basal activity are less dependent on distal enhancers. For cCREs, their basal activity is generally a strong positive predictor of their enhancer potency (Figure S3D). However, exceptions to this rule occur, as some

Figure 2. Singlet and combinatorial activities of cCREs and promoters from the Klf2 locus

(A) Transcription activities of singlet cCREs and promoters. Each dot represents the mean activity of one singlet. Horizontal lines represent the average background activity of empty vectors (black line) plus or minus two standard deviations (gray lines). Elements with activities more than two standard deviations above the average background signal are defined as active. (B) Examples of Upstream assay cCRE-P combinations for cCREs E097, E046, E030, and E070 of the Klf2 locus. Bar plots represent the mean boost index of each combination, vertical lines represent the standard deviations. Crosses mark missing data. (C and D) Boost index matrices of cCRE-P combinations from the Klf2 locus according to Upstream (C) and Downstream (D) assays. White tiles indicate missing data. Bar plots on the right and top of each panel show basal activities of each tested P or cCRE, respectively, with the black line indicating the background activity of the empty vector. All data are averages of 3 independent biological replicates.

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cCRE-P pairs show high boost indices even though the basal activity of the cCRE is low (Figure S3D, upper left quadrant).

**cCRE effects are partially orientation and position independent**

Next, we asked whether the ability of cCREs to regulate the linked promoters was generally independent of their orientation and position. This was originally posited for enhancers (Banerji et al., 1981) and in some cases also reported for repressive elements (Segert et al., 2021). Indeed, in the Upstream assays, we found a general positive correlation of the boost indices between the two orientations of the cCREs (Pearson’s r = 0.68) (Figure S4A). These results are similar to those recently obtained with a minimal core promoter (Klein et al., 2020). In the Downstream assay, the correlation between orientations was somewhat lower (Pearson’s r = 0.47) (Figure S4B). This may be due to the lower dynamic range of the Downstream assay data (Figure S1C). To simplify, for all other analyses we combined the boost indices of + and − orientations of the cCREs by averaging.

We then investigated the degree of position-independence, by comparing the overlapping P-cCRE pairs from the Klf2 locus Downstream and Upstream assays. This showed an overall Pearson correlation of 0.64 (Figure S4C). We conclude that repressive and activating effects of cCREs are substantially but not completely position independent, at least for the ten tested promoters from the Klf2 locus.

**Extensive selectivity of cCREs for promoters**

Visual inspection of the boost index matrices suggested that some cCREs alter the expression of most promoters to similar degrees, while others selectively alter the expression of a subset of promoters. In addition to the examples in Figure 2B from the Klf2 locus, strikingly specific promoter responses to some cCREs are illustrated for the Tftp21 locus in Figure 3. For example, E060, which forms part of an annotated super-enhancer (Khan and Zhang, 2016), activates most of the tested promoters, but with boost indices that can vary >50-fold between promoters. Two other remarkable examples from the Tftp21 locus are E032 and E0125, which each show different degrees of specificity, between low or no activation of some promoters and very strong activation of others. Much broader specificity is observed for E064, E073, E074, and E090 from the Nanog locus, which are part of previously identified super-enhancers (Blinka et al., 2016) (Figure S2D).

We investigated the degrees of selectivity more systematically. Figures 4A and 4B depicts the distribution of the boost indices for each cCRE. Clearly, some cCREs have a much broader range of boost indices than others. We used an ANOVA approach with Welch F test to systematically identify cCREs for which the variance of boost indices was larger than could be explained by experimental noise (see STAR Methods). Strikingly, out of 233 cCREs with more than 5 tested cCRE-P combinations, a total of 139 (59.9%) (Figures 4B and 4C) showed significant unexplained variance at an estimated false discovery rate (FDR) cutoff of 5%. Thus, at least throughout the three loci that we tested, cCRE-P selectivity is widespread, ranging from strong specificity for a few promoters to low specificity as seen in quantitative differences in the regulation of a broad set of promoters.

Intersection of the ANOVA-based classification of selective/unselective cCREs with the above broad classification into enhancers and repressors indicates that almost all (94%) general enhancer elements exhibit significant P selectivity. In contrast,
only 34% of the repressors are detectably biased toward a subset of promoters (Figure 4D). However, we note that this percentage may be underestimated because at low expression levels the noise levels are higher (Figure S3B). Interestingly, among the “ambiguous” cCREs, 55% are in fact selective. Such elements mostly activate or repress only very few promoters and leave most other promoters unaffected. The remainder of the ambiguous cCREs are probably not functional (e.g., E70 from the Klf2 locus, Figure 2B). In summary, these results indicate that more than half of all tested cCREs exhibits significant preference for specific promoters.

Promoters of housekeeping and developmental genes in Drosophila were reported to have distinct specificities toward cCREs (Zabidi et al., 2015). To investigate whether such a dichotomy could also be observed in our data, we focused on the Klf2 locus, which has roughly equal numbers of housekeeping and non-housekeeping promoters (Hounkpe et al., 2021) (the Tfcp2l1 and Nanog loci only have three and zero housekeeping genes, respectively). Indeed, hierarchical clustering of the boost index matrix showed a rough separation of the two classes of promoters (Figure 5A). However, this is largely due to the highly similar cCRE specificities among the housekeeping promoters, whereas the cCRE specificities of the non-housekeeping promoters are much more diverse and generally as distinct from each other as from the housekeeping promoters (Figure 5B). To test whether a housekeeping versus non-housekeeping dichotomy may largely explain our identification of cCRE selectivity (Figures 4B and 4C), we repeated this analysis after removing all housekeeping promoters. This yielded highly similar results (123 of 221 cCREs are significantly selective at 5% FDR cutoff, Figure S5). We conclude that housekeeping promoters may be similarly regulated, but cCRE selectivity goes beyond a simple distinction between housekeeping and non-housekeeping promoters.

Similar results with an independent MPRA dataset from human cells
In a parallel study, Bergman et al. (2022) conducted similar large-scale cCRE-P combinatorial MPRA in human K562 cells. Based on mathematical modeling of their data, the authors proposed that intrinsic cCRE and P activities generally combine multiplicatively to determine reporter activity. To test whether cCRE-P selectivity could also be detected in these K562 data, we subjected these data to our ANOVA with Welch F test. Indeed, for the majority of cCREs (90.46% at 0.1% FDR cutoff) the variance of boost indices was significantly larger than could be explained by experimental noise (Figures S6A and S6B). We conclude that the K562 data also point to a significant layer of cCRE-P selectivity.

Selectivity may be mediated by combinations of multiple TF motifs
Taken together, these results point to a broad spectrum of cCRE specificities for promoters, ranging from largely indiscriminate to highly selective. We searched in our dataset for sequence motifs that may account for these effects, focusing on binding motifs of transcription factors (TFs) that are expressed in mESCs.

We first searched for TF motifs in the cCREs that correlate with boost indices across all promoters. This yielded several dozens of TFs that are candidate activators or repressors (Figure S7A). Several of these, such as Sox2, Nanog, ETV4, and GABPA are...
known key regulators in mESC cells (Kim et al., 2008; Akagi et al., 2015; Kinoshita et al., 2007). These TFs may broadly contribute to enhancer activity.

Next, we searched for motifs associated with cCRE-P selectivity. We reasoned that selectivity may be due to certain combinations of TFs bound to cCRE and P. First, we asked whether for any TF the simultaneous presence of its motif at cCRE and P correlated with boost indices (Figure S7B). This only yielded a weak association of FOXO motifs (at a 5% FDR cutoff). Possibly this is due to FOXO1, a known regulator in mESCs (Zhang et al., 2011). We then asked if selectivity may be mediated by multiple TFs rather than single TFs. For this purpose, we took the TF motifs associated with enhancer activity with effect sizes >0.1 (n = 66) and searched for combinations of motifs that would be associated with higher boost indices if present at both the cCRE and the P (Figures 6A and 6B). This yielded a few dozen stronger associations (at a 1% FDR cutoff). Some of these associations may be redundant either because of motif similarity or because of motif co-occurrence. For example, the 5 associations between Sox2 and Klf motifs may represent the Klf4-Sox2 pair (Figure 6B), which are known to cooperate in mESCs (Wei et al., 2009). These results indicate that selectivity may be mediated by combinations of multiple TF motifs. Our dataset does not provide sufficient statistical power for an exhaustive search of such combinations.

Chromatin looping is independent of compatibility

Finally, we considered that certain pairs of cCREs and promoters frequently contact each other in the nucleus, as is indicated by focal or stripe-like enrichment patterns in high-resolution Hi-C maps (Bonev et al., 2017; Hsieh et al., 2020). While long-range contacts are irrelevant in our MPRAs because the tested elements are directly linked, we asked whether such physical contacts in the native genomic context are related to the selectivity of cCREs for certain promoters according to our MPRAs. We considered two models. In one model, the biochemical interactions that underlie cCRE-P selectivity may promote or stabilize cCRE-P looping interactions. Alternatively, looping interactions and cCRE-P selectivity may be independent aspects of cCRE-P interplay that each work by different mechanisms.

To discriminate between these two models, we investigated whether the boost indices of cCRE-P pairs correlate with their contact frequencies in micro-C, a high-resolution variant of Hi-C (Hsieh et al., 2020). Remarkably, we found no correlation between these two quantities (Figure 7A). We also found an extremely weak, although statistically significant, correlation between higher boost indices and longer linear distances of cCRE-P pairs along the genome (Figure 7B).

We conclude that cCRE-P contacts in the nucleus may be independent of their functional compatibility as detected in our
reporter assays, raising the interesting possibility that chromatin looping and compatibility are two orthogonal mechanisms of gene regulation.

**DISCUSSION**

Only a few other studies have so far attempted to analyze cCRE-P compatibility systematically. An early survey of 27 cCRE-P combinations in human cells did not find evidence for specificity (Kermekchiev et al., 1991), but the assay employed may have been insufficiently quantitative, and the choice of tested elements may have been biased. In contrast, testing of ~200 cCRE-P pairs in zebrafish pointed to extensive specificity (Gehrig et al., 2009). An MPRA study in Drosophila cells using seven different promoters and genome-wide cCREs suggested that cCRE-P specificity broadly separates between housekeeping and tissue-specific promoters (Zabidi et al., 2015). Parallel to our work, a MPRA study was reported of thousands of cCRE-P combinations in a human cell line (Bergman et al., 2022).

Our results reveal a broad spectrum of specificities: some cCREs are promiscuous, others are highly specific for certain promoters, and in many instances the specificity is quantitative rather than qualitative. By statistical analysis, we found that more than half of the cCREs exhibit a degree of specificity that cannot be explained by experimental noise. We also found evidence for such specificity in the MPRA data of Bergman et al. (2022). Although this study focused on the observation that a substantial fraction of the variance in reporter activity can be explained by a multiplicative combination of enhancer and promoter activities, both datasets indicate that CRE-P selectivity as well as intrinsic enhancer and promoter activities contribute to reporter expression (see also Bergman et al. [2022]). We suggest that such a general multiplicative rule and a more complex grammar of enhancer-promoter specificities are two sides of the same coin of gene regulation.

It is likely that cCRE-P compatibility is governed by a complex grammar of TF combinations. Underlying this grammar may be a diversity of molecular mechanisms, including direct and indirect TF-TF interactions (e.g., Wei et al. [2009], local concentration of activating factors [Davis et al., 2020; Tak et al., 2021], or functional bridging by co-factors [El Khattabi et al., 2019; Haberle et al., 2019]). Due to the complexity of this grammar, its elucidation may require much larger cCRE-P combinatorial datasets than generated here, as well as systematic mutational analysis (Fuqua et al., 2020; Kircher et al., 2019) of individual cCRE-P combinations. Nevertheless, our statistical analysis highlights several candidate combinations of TF motifs that may contribute to the compatibility of some cCRE-P pairs.

Our data indicate that some of the cCREs tested may be repressive elements rather than enhancers even though they were selected from DHSs. This is similar to a recent screen of cCREs in human cells, which identified a large set of candidate repressive elements (Pang and Snyder, 2020) and to another screen in Drosophila (Gisselbrecht et al., 2020). It will be interesting to further explore the physiological regulatory role of these elements.

Surprisingly, we found that the boost indices of cCRE-P pairs generally do not correlate with their contact frequencies in the native chromatin context. This suggests that 3D genome organization and compatibility are regulated by different mechanisms. We envision that compatibility and 3D organization may be two independent layers necessary for correct selective gene regulation: 3D organization such as the formation of chromatin loops and compartments may determine whether CREs and promoters are able to interact, while compatibility may determine whether such an interaction is functional, i.e., gives rise to a change in P activity.

**Limitations of the study**

Our current data were generated with transiently transfected plasmids. Advantages of this “reductionist” approach are that it largely eliminates possible confounding effects of chromatin packaging and 3D folding and that thousands of cCRE-P combinations could be tested. However, MPRAs are intrinsically artificial, as the tested elements are taken out of their natural sequence context, may be too small or incorrectly spaced, or require a natural chromatin context to function properly. Thus, further studies are needed to verify and analyze the impact of the observed specificities in the native genomic context.

**STAR+METHODS**

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Supplemental information can be found online at https://doi.org/10.1016/j.molcel.2022.04.009.

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AUTHOR CONTRIBUTIONS
M.M.-A., F.C., and B.v.S. designed the study. M.M.A. and F.C. developed computational methods and performed analyses. M.M.A. and J.v.A. developed experimental methods. M.M.A. performed experiments. B.v.S. and M.M.A. wrote the manuscript, with input from F.C. and J.v.A. B.v.S. supervised the study.

DECLARATION OF INTERESTS
J.v.A. is founder of Gen-X B.V. and Annogen B.V. F.C. is a co-founder of enGene Statistics GmbH. B.v.S. is member of the advisory board of Molecular Cell.

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# STAR METHODS

## KEY RESOURCES TABLE

| REAGENT or RESOURCE | SOURCE | IDENTIFIER |
|---------------------|--------|------------|
| **Bacterial and virus strains** | | |
| e. cloni 10G supreme electrocompetent E. coli | Lucigen | Cat# 60081-1 |
| 5-alpha competent bacteria | NEB | Cat# C2987 |
| **Chemicals, peptides, and recombinant proteins** | | |
| My-Taq Red mix | Bioline | Cat# BIO-25044 |
| Fast-link ligase | Lucigen | Cat# LX7050H |
| Klenow HC 3’→5’exo− | NEB | Cat# M0212L |
| CleanPCR magnetic beads | CleanNA | Cat# CPCR-0050 |
| Gibson Assembly® Master Mix | NEB | Cat# E2611S |
| XmaII (AvrII) restriction enzyme | Thermo Fischer | Cat# ER1561 |
| XcmI restriction enzyme | NEB | Cat# R0533 |
| Nhel restriction enzyme | NEB | Cat# R0533 |
| T4 DNA ligase | Roche | Cat# 1079009001 |
| I-Ceul restriction enzyme | NEB | Cat# R699S |
| I-SceI restriction enzyme | NEB | Cat# R694S |
| TRIsure | Bioline | Cat# BIO-38302 |
| Dnase I recombinant, RNase-free | Roche | Cat# 04716728001 |
| Maxima reverse transcriptase | Thermo Fischer | Cat# EP0743 |
| Neurobasal medium | Gibco | Cat# 21103-049 |
| DMEM-F12 medium | Gibco | Cat# 11320-033 |
| N27 | Gibco | Cat# 17504-044 |
| B2 | Gibco | Cat# 17502-048 |
| LIF | Sigma-Aldrich | Cat# ESG1107 |
| CHIR-99021 | MedChemExpress | Cat# HY-10182 |
| PD0325910 | MedChemExpress | Cat# HY-10254 |
| monothioglycerol | Sigma | Cat# M6145-25ML |
| **Critical commercial assays** | | |
| ISOLATE II PCR and Gel Kit | Bioline | Cat# BIO-52059 |
| End-It DNA End-Repair Kit | Epicentre | Cat# ER0720 |
| Takara ligation kit version 2.1 | Takara | Cat# 6022 |
| PureLink HiPure Plasmid Filter Maxiprep Kit | Invitrogen | Cat# K210016 |
| Mouse Embryonic Stem Cell Nucleofector Kit | Lonza | Cat# VPH-1001 |
| GeneJET RNA Purification Kit | Thermo Fischer | Cat# K0732 |
| MycoAlert Mycoplasma Detection Kit | Lonza | Cat# LT07-318 |
| **Deposited data** | | |
| Raw and Processed data | This study | GEO: GSE186265 |
| Code | This Study | GitHub: https://github.com/vansteensellab/EPCombinations https://doi.org/10.5281/zenodo.6406791 |
| Annotated mouse TSSs | GENCODE | Release vM16: https://www.gencodegenes.org/mouse/release_M16.html |
| mESC TAD coordinates | Bonev et al., 2017 | Supplementary Table: https://www.ncbi.nlm.nih.gov/pmc/articles/PMC5651218/bin/mmc2.xlsx |
| Mouse reference genome mm10 genome build (GRCh38) | Genome Reference Consortium | https://www.ncbi.nlm.nih.gov/grc/mouse |

(Continued on next page)
**RESOURCE AVAILABILITY**

**Lead contact**
Further information and requests for resources and reagents should be directed to and will be fulfilled by the Lead Contact Bas van Steensel (b.v.steensel@nki.nl).

**Materials availability**
Plasmids generated in this study are available without restriction upon request from the Lead Contact.

**Data and code availability**
- Raw sequencing data and processed data are available at Gene expression Omnibus (GEO): GSE186265. This paper also analyzes existing, publicly available data. The accession numbers for these datasets are listed in the key resources table. Lab journal records are available at https://osf.io/5a7h6/.
- Code of data processing pipelines and analysis scripts are available at https://github.com/vansteensellab/EPCombinations (DOI: 10.5281/zenodo.6406791).
- Any additional information required to reanalyze the data reported in this paper is available from the lead contact upon request.

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### REAGENT or RESOURCE SOURCE IDENTIFIER

| REAGENT or RESOURCE | SOURCE | IDENTIFIER |
|---------------------|--------|------------|
| DNAse Hypersensitivity mESCs 2i+1if | Joshi et al., 2015 | GEO: GSE72164 |
| DNAse Hypersensitivity mESCs serum | ENCODE | GEO: GSE37074 |
| TF motif database | Diaferia et al., 2016 | GitHub: https://github.com/vansteensellab/EPCombinations https://doi.org/10.5281/zenodo.6406791 |
| RNA-seq mESCs 2i+1if | Joshi et al., 2015 | GEO: GSE72164 |
| ExP K562 count data | Bergman et al., 2022 | GEO: GSE184426 |
| MicroC mESCs | Hsieh et al., 2020 | GEO: GSE130275 |

### Experimental models: Cell lines

| E14TG2a mESCs | ATCC | Cat# CRL-1821 |

### Table S3

| Oligonucleotides | This Study | N/A |

### Recombinant DNA

| SuRE vector (JvAp101) | van Arensbergen et al., 2017 | N/A |
| Downstream Assay vector (JvAp102) | This Study | N/A |

### Software and algorithms

| Homer v4.10 | Heinz et al., 2010 | http://homer.ucsd.edu/homer/data/software/homer.v4.10.zip |
| BatchPrimer3 v1.0 | You et al., 2008 | https://wheat.pw.usda.gov/demos/BatchPrimer3/ |
| R version 4.0.5 (2021-03-31) | R Core Team, 2021 | https://www.r-project.org/ |
| ggplot2 | Wickham, 2016 | https://ggplot2.tidyverse.org/ |
| Bowtie2 version 2.3.4 | Langmead and Salzberg, 2012 | http://bowtie-bio.sourceforge.net/bowtie2/index.shtml |
| Python version 3.6.2 | Rossum and Drake, 2009 | https://www.python.org/downloads/release/python-362/ |
| Snakemake version 4.4.0 | Koster and Rahmann, 2012 | https://anaconda.org/bioconda/snakemake/files?version=4.4.0 |
| Starcode version 1.1 | Zorita et al., 2015 | https://github.com/guil11aume/starcode |
| MEME suite version 5.0.2 | Bailey et al., 2009 | https://meme-suite.org/meme/doc/download.html |
| HRT Atlas v1.0 | Hounkpe et al., 2021 | www.housekeeping.unicamp.br |

### Other

| Hamilton Microlab® STAR | Hamilton | https://www.hamiltoncompany.com/automated-liquid-handling/platforms/microlab-star |
EXPERIMENTAL MODEL AND SUBJECT DETAILS

Cell culture
All experiments were conducted in E14TG2a male mouse embryonic stem cells (mESC) (ATCC CRL-1821) cultured in 2i+LIF culturing media. 2i+LIF was made according to the 4DN nucleome protocol for culturing mESCs (https://data.4dnucleome.org/protocols/cb03c0c6-4ba6-4bbe-9210-c430ee4fadb2/c). The reagents used were Neurobasal medium (#21103-049, Gibco), DMEM-F12 medium (#12300-033, Gibco), BSA (#15280-037; Gibco), N27 (#17504-044; Gibco), B2 (#17502-048; Gibco), LIF (#ESG1107; Sigma-Aldrich), CHIR-99021 (#HY-10182; MedChemExpress) and PD0325901 (#HY-10254; MedChemExpress), monothioglycerol (#M6145-25ML; Sigma) and glutamine (#25030-081, Gibco). Monthly tests (#LT07-318; Lonza) confirmed that the cells were not contaminated by mycoplasma.

METHOD DETAILS

Selection of cCREs and promoters
For the design of the libraries we selected the cCREs and promoters from three TADs centered around each of the Klf2, Nanog and Tcf21 genes, using TAD coordinates from (Bonev et al., 2017). cCREs were selected based on DNase I hypersensitivity mapping data from mESCs in both 2i+LIF (Joshi et al., 2015) and serum (ENCODE Project Consortium, 2012) culturing conditions, which we reprocessed and aligned to the mm10 genome build. DNAse I hypersensitivity sites (DHSs) were called using Homer v4.10 (Heinz et al., 2010) with default parameters and peak style “factor”. We defined cCREs as 450 bp windows centered on each peak. For promoters we used the Gencode mouse TSS annotation (Frankish et al., 2019). From each TSS we defined as promoters the -375 +75 bp region. If the promoter regions overlapped with any cCRE then the promoter was redefined as the 450 bp region surrounding the center of the intersection of both elements. PCR primers were designed for each cCRE and promoter using the batch version of Primer3 (BatchPrimer3 v1.0) (You et al., 2008) allowing for primers to be designed on the 50 bps of each end. This yielded PCR products of ~400 bp for each element. Coordinates and sequences of all cCREs and Ps used as templates for primer design are included in Data S1.

Upstream assay library generation
For each locus, cCREs and promoters were amplified from mouse genomic DNA (extracted from E14TG2a mESCs, ATCC CRL-1821) by PCR using My-Taq Red mix (#BIO-25044; Bioline) in 384 well plates using automated liquid handling (Hamilton Microlab® STAR). PCRs were checked on gel and had a success rate between 60 and 90% depending on the locus. Equal volumes (10 ul) of the resulting PCR products were mixed, and the resulting pool was purified by phenol-chloroform extraction followed by gel purification (BIO-52059; Bioline). The purified DNA fragments were then blunted and phosphorylated using End-It DNA End-Repair Kit (#ER0720; Epicentre). Part of the repaired pool was set apart for cloning of singlet libraries. The remainder was self-ligated using Fast-link ligase (LK0750H; Lucigen), after which duplets of ~800 bp were excised from agarose gel and purified (BIO-52059; Bioline). Singlet and duplet pools were A-tailed using using Klenow HC 3'→5' exo– (#M0212L; NEB). The SuRE barcoded vector was prepared as previously described (van Arensbergen et al., 2017). First, we digested 10 μg of the SuRE vector with Nhel (#R0131S; NEB) and XcmI (#R0533; NEB) and performed a gel-purification. Barcodes were generated by performing 10 PCR reactions of 100 μl each containing 5 μl of 10 μM primer 256JvA, 5 μl of 10 μM primer 264JvA and 1 μl of 0.1 μM template 254JvA (see Table S3 for oligonucleotide sequences). A total of 14 PCR cycles were performed using MyTaq Red Mix (#BIO-25043; Bioline), yielding ~30 μg barcodes. Barcodes were purified by phenol-chloroform extraction and isopropanol precipitation after which they were digested overnight with 80 units of AvrII (#ER1561; Thermo Fischer) and purified using magnetic bead purification (#CPCR-0050; CleanNA). The linear vector and the barcodes were then ligated in multiple 100 μl reactions containing 3 μg digested vector and 2.7 μg digested barcodes, 20 units Nhel (#R0131S; NEB), 20 units XcmI, 10 μl of 10 mM ATP, 10 units T4 DNA ligase (#10799009001 Roche). A cycle-ligation of six cycles was performed (10 min at 22 °C and 10 min at 37 °C), followed by 20 min heat-inactivation at 80 °C. The ligations were purified by magnetic beads and digested with 40 units of XcmI (#R0533S; NEB) for 3 h, and size-selected by gel-purification.

Then singlet and duplet pools were separately ligated overnight into the SuRE barcoded vector using Takara ligation kit version 2.1 (#6022; Takara). Ligation products were purified using magnetic bead purification (#CPCR-0050; CleanNA). Next, 2 μl of the purified ligation products were electroporated into 20 μl of electrocompetent e. coli 10G supreme (#60081-1; Lucigen). Each library was grown overnight in 500 ml of standard Luria Broth (LB) with 50 μl/ml of kanamycin and purified using a maxiprep kit (#210016, Invitrogen).

Downstream assay library generation
The Downstream assay vector was based on a pSMART backbone (Addgene plasmid # 49157; a gift from James Thomson). It was constructed using standard molecular biology techniques and contains a green fluorescent protein (GFP) open reading frame followed by a barcode, and a psiCheck polyadenylation signal (PAS) introduced during barcoding, followed by the cloning site for inserts and a triple polyadenylation site (SV40+bGH+psiCheckPAS).
The 10 highest expressing promoters of the Kit2 Upstream library were selected to be cloned into the Downstream assay vector at the promoter position. These Promoters were amplified by PCR and individually inserted by Gibson assembly (#E2611S; NEB) into the Downstream assay vector. Then each of the 10 constructs were transformed into standard 5-alpha competent bacteria (#C2987; NEB) grown overnight in 500 ml of standard Luria Broth (LB) with 50 μl/ml of kanamycin and purified.

Each of these promoter-containing vectors was then barcoded similarly as the SuRE vector (van Arensbergen et al., 2017). For this, we digested 10 μg of each vector with AvrII (#ER1561; Thermo Fischer) and XcmI (#R0533; NEB) and performed a gel-purification. Barcodes were generated by performing 10 PCR reactions of 100 μl each containing 5 μl of 10 μM primer 275JvA, 5 μl of 10 μM primer 465JvA and 1 μl of 0.1 μM template 274JvA (see Table S3 for oligonucleotide sequences). A total of 14 PCR cycles were performed using MyTaq Red Mix (#BIO-25043; Bioline), yielding ~30 μg barcodes. Barcodes were purified by phenol-chloroform extraction and isopropanol precipitation after which they were digested overnight with 80 units of Nhel (#R0131S; NEB) and purified using magnetic bead purification (#CPCR-0050; CleanNA). Each vector variant and the barcodes were then ligated in one 100 μl reaction containing 3 μg digested vector and 2.7 μg digested barcodes, 20 units Nhel (#R0131S; NEB), 20 units AvrII, 10 μl of 10× CutSmart buffer, 10 μl of 10 mM ATP, 10 units T4 DNA ligase (#10799009001 Roche). A cycle-ligation of six cycles was performed (10 min at 22 °C and 10 min at 37 °C), followed by 20 min heat-inactivation at 80 °C. The ligation reaction was purified by magnetic beads and digested with 40 units of XcmI (#R0533S; NEB) for 3 h, and size-selected by gel-purification, yielding ~1 μg barcode vector for each variant.

The singlets were cloned into the enhancer position of the downstream barcoded vector variants by overnight ligation using Takara ligation kit version 2.1 (#6022; Takara). Ligation products were purified using magnetic bead purification (#CPCR-0050; CleanNA). Next, 2 μl of the purified ligation products were electroporated into 20 μl of electrocompetent e. coli 10G supreme (#60081-1; Lucigen). Each library was grown overnight in 500 ml of standard Luria Broth (LB) with 50 μl/ml of kanamycin and purified using a max-iprep kit (K210016, Invitrogen).

Inverse PCR and sequencing to link inserted elements to barcodes
We identified barcode-insert combinations in the plasmid libraries by inverse-PCR followed by sequencing as described (van Arensbergen et al., 2017). In brief, the combination of barcode and element(s) was excised from the plasmid by digestion with I-CeuI(#R0699S; NEB); this fragment was circularised; remaining linear fragments were destroyed; and circular fragments were linearised again with I-SceI (#R0694S; NEB). These linear fragments were amplified by PCR with sequencing adaptors. The final product was sequenced on an Illumina MiSeq platform using 150-bp paired-end reads. This process was done separately for each of the libraries. In the singlet libraries the barcodes should be associated to only one insert and in the combinatorial libraries the barcodes should be associated with duplets.

Libraries transfection
E14TG2a mouse embryonic stem cells were transiently transfected using Amaza nucleofector II, program A-30, and Mouse Embryonic Stem Cell Nucleofector™ Kit (#VFH-1001, Lonza). Kit2 and Nanog loci Upstream assay libraries were mixed and transfected together, Tfcp2l1 to together, the Downstream assay vector. Then each of the 10 constructs were transformed into standard 5-alpha competent bacteria (#C2987; Bioline) and frozen at -80 °C. RNA was extracted and processed for sequencing as described (van Arensbergen et al., 2017) with a few modifications. Cells were transfected as a mix. Three independent biological replicates were done for each library. For the Upstream Assay the cDNA was produced using Maxima reverse transcriptase (#EP0743; Thermo Fisher Scientific) in 20 μl reactions and amplified by PCR as described (van Arensbergen et al., 2017). Per biological replicate 8 to 10 reactions were carried out in parallel in order to cover enough barcode complexity of the library. For the Downstream Assay the RNA was extracted and processed the same way until cDNA production. Here, cDNA was produced using a specific primer (304JvA sequence inTable S3 for oligonucleotide sequences) using Maxima reverse transcriptase (#EP0743; Thermo Fisher Scientific) in 20μl reactions using the same conditions previously described (van Arensbergen et al., 2017). Primer 304JvA introduces an adaptor sequence 5’ to the primer sequence which is targeted in the first PCR (see below) to ensure strand specific amplification of barcodes. Then cDNA was amplified in 2 steps (nested PCRs) in order to make the reaction strand-specific. The first PCR reaction was run for 10 cycles (1 min 96 °C, 10 times (15 s 96 °C, 15 s 60 °C), 15 s 72 °C) using (index variants of) primers 285JvA (containing the S2, index and p5 adaptor) and 305JvA (targeting the adapter introduced by 304JvA). Each 20 μl RT reaction was amplified in a 100-μl PCR reaction with MyTaq Red mix. The second PCR reaction was performed using 10μl of the product of the previous reaction in 100 μl reactions (1 min 96 °C, 8×(15 s 96 °C, 15 s 60 °C) using the same index variant primer and primer 437JvA (containing the S1, and p5 adaptor). For both Upstream and Downstream assays, the resulting PCR products were sequenced on an Illumina 2500 HiSeq platform with 65bp single end reads.
Plasmid DNA (pDNA) barcode sequencing

For normalisation purposes, barcodes in the plasmid pools were counted as follows. For both assays the process was the same. For each library 1 μg of plasmid was digested with I-SceI in order to linearise the plasmid. Then, barcodes were amplified by PCR from 50 ng of material using the same primers and reaction conditions as in the amplification of cDNA in the Upstream assay, but only 9 cycles of amplification were used (1 min 96 °C, 9 times (15 s 96 °C, 15 s 60 °C, 15 s 72 °C)). For each library, two technical replicates were carried out by using different index primers for each replicate. Samples were sequenced on an Illumina 2500 HiSeq platform with 65bp single end reads.

Calculation of boost indices

We initially calculated raw boost indices simply as a log₂ ratio of the activity of each cCRE–P pair over the activity of the corresponding P alone. However, 20 negative controls that we included in the Klf2 libraries, consisting of randomly generated DNA sequences of similar size and G/C content as the cCREs (Data S1), generally showed a negative boost index by this measure (median value -0.45 when inserted upstream) (Figure S1D). We therefore calculated corrected boost indices as the log₂ ratio of cCRE-P activity over the median cCRE-P activity per promoter (Figure S1D). Importantly, in the Klf2 library data this largely removed the negative bias that we observed with the negative controls; we thus assume that this correction is adequate and therefore also applied it to the boost indices obtained with the other libraries. For the analyses in Figures 2, 3, 4, 5, 6, 7, S2–S5, and S7 except Figures S4A and S4B the boost indices of cCREs were averaged over both orientations of the cCREs as boost indices correlated between orientations (Figure S4). Boost indices of all cCRE-P pairs are included in Data S2.

Identification of activating and repressive cCREs

To classify cCREs according to their general effect on all promoters we performed a Wilcoxon test on the boost indices of each cCRE combined with every promoter against the rest of the population. P-values were corrected for multiple hypothesis testing using the Snakemake (Koster and Rahmann, 2012) pipeline. Statistical details for individual experiments have been provided in the main text, figure legends, and Method Details.

Pre-Processing of cDNA and pDNA reads

For each replicate of each library pool transfection barcodes were extracted from the single end reads by using a custom python script that identifies from read 1 the barcode and cCRE or P element, and from read 2 the cCRE or P element. In case of singlet libraries both reads should identify the same element, whereas in combinatorial libraries read 1 is derived from the barcode-proximal element and read 2 from the barcode distal element. In the combinatorial libraries we cannot distinguish between a combination of one element with itself in the same orientation or a single element, therefore these were removed from combinatorial libraries. In the Downstream Assay both reads identify the only element cloned in the downstream position. If no element was found, the barcode was assigned as empty vector. The resulting barcode-to-element(s) lists were clustered using Starcode (version 1.1) (Zorita et al., 2015) to remove errors from barcode sequencing. Finally, barcodes present in multiple libraries or matched with multiple element combinations were removed from the data.

Post processing of cDNA and pDNA counts

For each transfection, barcodes identified in the cDNA were matched to the barcodes in the iPCR data, and all barcodes were counted in cDNA and pDNA replicates. Barcode counts were normalised to the total number of barcode reads from each sample. Activity per barcode was then calculated as a cDNA:pDNA ratio of normalised counts. Next, activities from multiple barcodes belonging to the same element singlet or combination were averaged, requiring a minimum of 5 barcodes per singlet or combination and at least 8 pDNA counts per barcode. The mean activity of each singlet or combination across replicates was calculated as the geometric mean of the three replicates. Activities for all pairs including cCRE-cCRE, P-P and P-cCRE pairs are included in Data S2.
Benjamini-Hochberg method and an FDR cutoff of 5% was chosen. cCREs significantly activating or repressing at the 5% FDR cutoff were classified as activators or repressors respectively. The rest of cCREs were classified as ambiguous.

**Analysis of selectivity**
We performed a Welch’s ANOVA (or Welch F-test) on the calculated Boost indices to assess the selectivity of each cCRE with more than 5 cCRE-P combinations. For this purpose, each replicate of each orientation of the cCRE-P was used as a datapoint and each cCRE-P combination was used as a group. P-values were corrected for multiple hypothesis testing using the Benjamini-Hochberg method and an FDR cutoff of 5% was chosen. The Welch F-test was chosen over the classic ANOVA due to heteroscedasticity of the data.

**Analysis of Houskeeping and non-housekeeping promoter selectivity**
We classified the promoters from the Klf2 locus as Housekeeping and non-housekeeping according to the publicly available HRT Atlas v1.0 database (Hounkpe et al., 2021). We used this classification to overlay it with a clustered and reduced version of the Klf2 locus boost index matrix. This matrix was clustered using hierarchical clustering. The complete Klf2 locus boost index matrix was used to assess the similarities between promoters by correlating each promoter using pearson correlation. We then separated correlations in within Housekeeping promoters, within Non-Housekeeping promoters and between Housekeeping and Non-Housekeeping.

**Analysis of selectivity on K562 ExP data**
The cDNA and pDNA count data from (Bergman et al., 2022) was processed the same way as our data and boost indices were calculated using the same procedure. Because of the higher number of promoters available in the data we randomly down-sampled the dataset 10 times to have 10 subsets of 230 Enhancers and 20 promoters. This was done in order to limit the degrees of freedom to be able to apply the Welch F-test.

On each of the processed down-sampled subset we performed a Welch’s ANOVA (or Welch F-test) on the calculated Boost indices to assess the selectivity of each E with more than 5 E-P combinations. For this purpose, each replicate of E-P combination was used as a datapoint and each E-P combination was used as a group. P-values were corrected for multiple hypothesis testing using the Benjamini-Hochberg method and an FDR cutoff of 0.1% was chosen. This cutoff was more stringent than on the analysis performed on our dataset as the number of tests performed was one order of magnitude higher. Again, the Welch F-test was chosen over the classic ANOVA due to heteroscedasticity of the data.

**TF motif Survey**
We used a custom TF motif database provided by the lab of Gioacchino Natoli containing 2,448 TF motifs which was built on top of a previously published version (Diaferia et al., 2016) (Dataset composition and sources available at https://github.com/vansteensellab/EPCombinations). TF motifs were filtered for expression of TFs in mESCs cultured in 2i+LIF according to published RNA-seq (higher expression than 1RPM) (Joshi et al., 2015). We scored presence or absence of a TF motif in each cCRE using FIMO (MEME suite, version 5.0.2) (Bailey et al., 2009). We then searched for motifs associated with (1) general enhancer activity, (2) self-compatibility and (3) duplets of self-compatible motifs. In (1), for each TF motif we compared the general cCRE-P population to combinations where the TF motif was present at the cCRE. In (2), for each TF motif we compared the cCRE-P combinations where the TF motif was present at the cCRE to the combinations where it was present at both the cCRE and the promoter. In (3), we took all the significant TF motifs at a 1% FDR and an effect size higher than 0.1 (n=66). Then we tested all pairwise non-repeated TF motif duplets. Per TF motif duplet we compared the cCRE-promoters where both TF motif were present at the cCRE to the combinations where both were present at both the cCRE and the promoter. In all comparisons a Wilcoxon test was applied to the boost indices of each group and the effect size was calculated a difference of median boost indices. In each analysis p-values were corrected for multiple hypothesis testing using the Benjamini-Hochberg method. We required a minimum of 50 cCRE-promoter combinations per group.

**Micro-C data correlation**
Processed publicly available Micro-C data was obtained from (Hsieh et al., 2020). Contact scores between cCRE-P pairs were averaged across bins overlapping a ±500 bp window from the location of each element using 400 bp bins. Pearson correlation was calculated on the relationship between Boost indices and contact frequencies of cCRE-P pairs.