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In vivo characterization of *Linc-p21* reveals functional *cis*-regulatory DNA elements

Abigail F Groff[a,b,c], **Diana B Sanchez-Gomez**[a], **Marcela ML Soruco**[a,d], **Chiara Gerhardinger**[a], **A. Rasim Barutcu**[a,c], **Eric Li**[a], **Lara Elcavage**[a], **Olivia Plana**[a], **Lluvia V Sanchez**[a], **James C Lee**[a,e], **Martin Sauvageau**[a,c,d], and **John L Rinn**[a,c,d,f]

[a]Department of Stem Cell and Regenerative Biology, Harvard University, Cambridge, MA, 02138

[b]Department of Systems Biology, Harvard Medical School, Boston, MA, 02115

[c]The Broad Institute of MIT and Harvard, Cambridge, MA, 02142

[d]Department of Pathology, Beth Israel Deaconess Medical Center, Boston, MA, 02215

[e]Department of Medicine, University of Cambridge School of Clinical Medicine, Addenbrooke's Hospital, Cambridge, UK

Summary

The *Linc-p21* locus, encoding a long non-coding RNA, plays an important role in p53 signalling, cell cycle regulation, and tumour suppression. However, despite extensive study, confusion exists regarding its mechanism of action: is activity driven by the transcript acting *in trans*, *in cis*, or by an underlying functional enhancer? Here, using a knockout mouse model and a massively parallel enhancer assay, we delineate the functional elements at this locus. We observe that even in tissues with no detectable *Linc-p21* transcript, deletion of the locus significantly affects local gene expression, including of the cell cycle regulator *Cdkn1a*. To characterize this RNA-independent regulatory effect, we systematically interrogated the underlying DNA sequence for enhancer activity at nucleotide resolution, and confirmed the existence of multiple enhancer elements. Together, these data suggest that, *in vivo,* the *cis*-regulatory effects mediated by *Linc-p21*, in the presence or absence of transcription, are due to DNA enhancer elements.

eTOC blurb

[f]Corresponding Author: john_rinn@harvard.edu.

For extended experimental procedures, please see supplemental materials online.

Accession Numbers

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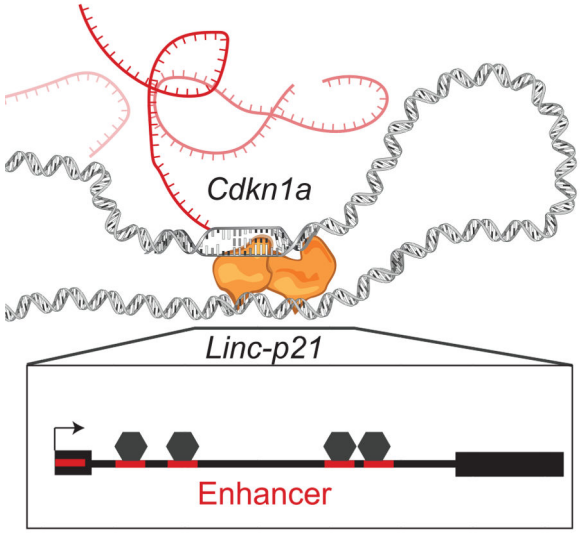
Author Contributions

A.F.G. and J.L.R. conceived the experiments. A.F.G., D.B.S.G., M.M.L.S., C.G., E.L., L.E., O.P., and L.V.S. performed the experiments. A.F.G., A.R.B., and J.C.L. analyzed the data. A.F.G., J.C.L., and J.L.R. wrote the manuscript with input from M.S., C.G., and A.R.B.. All authors approved the final version of the manuscript.

Using a knockout mouse model and massively parallel reporter assay, Groff *et al.* show that the *lincp21* gene, which was previously thought to produce a *cis*-acting lincRNA, actually contains multiple enhancer elements that are directly responsible for regulating transcription of nearby genes, including *Cdkn1a*.



Introduction

It has long been known that transcription occurs at many more sites in the genome than encode proteins. Among the main constituents of the resulting non-coding transcriptome are long non-coding (lnc)RNAs, which are more than 200 nucleotides in length and exhibit tissue-specific expression (ENCODE Project Consortium et al., 2007, Mercer et al 2009, Cabili et al., 2011, St Laurent et al., 2015, Quinn and Chang, 2016). Although originally dismissed as transcriptional noise, it is now clear that several lncRNAs have important biological functions (Wang and Chang, 2011, Guttman and Rinn, 2012). However, the rapid creation of entire catalogs of lncRNAs – made possible by RNA sequencing – has meant that our knowledge of where lncRNA genes are located far exceeds our understanding of their functions. Indeed, it is even unclear as to whether the functional element at these loci is the RNA transcript itself or the underlying DNA sequence, which could have enhancer activity. For example, while the *Lockd* locus fulfils all of the requirements of a lncRNA, the phenotype associated with deletion of this locus is actually due to loss of the underlying DNA element and not the RNA transcript (Paralkar et al., 2016).

Linc-p21 is one of the most-studied lncRNAs due to its role in p53 signalling and relevance to human disease (Huarte et al., 2010, Dimitrova et al., 2014, Yoon et al., 2012, Tang et al., 2015). Indeed, since its discovery in 2010, dozens of studies have examined *Linc-p21* in human and/or mouse cell-based assays, and have collectively identified roles in a range of biological processes including cell cycle control, reprogramming, apoptosis, and energy metabolism (Dimitrova et al., 2014, Bao et al., 2015, Huarte et al., 2010, Yang et al., 2014). However, despite being the subject of extensive study, this locus has not yet been examined in tissues or *in vivo*. Moreover, confusion exists regarding the mechanism by which the

Linc-p21 locus functions in any context. For example, at different times this locus has been thought to produce a *trans*-acting lncRNA (Huarte et al., 2010), a *cis*-acting lncRNA (Dimitrova et al., 2014), or an enhancer-derived RNA (Allen et al., 2014). Here, we aimed to resolve this confusion by characterizing the functional elements at the *Linc-p21* locus *in vivo.* Using a *Linc-p21* knockout mouse model (Sauvageau et al., 2013, Goff et al., 2015), we demonstrate that deletion of *Linc-p21* results in the *cis*-dysregulation of several genes, including *Cdkn1a*. Interestingly, this dysregulation was observed across multiple tissues, even those in which *Linc-p21* RNA was not expressed, and thus cannot be due to an RNA-dependent mechanism. To better understand how a DNA-dependent effect might be mediated, we comprehensively surveyed the entire *Linc-p21* locus for enhancer activity using a massively parallel reporter assay, and identified multiple enhancer elements including a conserved p53-binding site. Collectively, we show that the *Linc-p21* locus harbours DNA enhancer elements that are directly responsible for the *cis*-regulation of multiple genes *in vivo*.

Results

Deletion of Linc-p21 results in quantifiable effects on whole organ gene expression

The mouse *Linc-p21* gene resides in a 21.6 kb locus on chromosome 17, approximately 15 kb upstream of the cell cycle regulator *Cdkn1a*. We previously targeted this locus for deletion using homologous recombination in which the entire gene body and a portion of the promoter, including the known p53-binding motif, were replaced with a *lacZ* reporter that maintains transcription at the locus (Figure 1A, Sauvageau et al., 2013). Using this reporter mouse, we initially investigated the spatial and temporal expression of *Linc-p21* in organ development in order to identify relevant tissues for downstream experiments (Figure S1). Importantly, because deletion of the p53 motif meant that endogenous *Linc-p21* expression may not have been faithfully reported by the model, we validated the observed expression pattern in wildtype mice, confirming that *Linc-p21* expression was detectable in the tissues where *lacZ* was present in the reporter mouse.

Overall, we detected differential expression of the *Linc-p21* locus in different tissues, with expression being highest in striated muscle – an observation that may relate to the role that *Cdkn1a* is known to play in muscle development (Halevy et al., 1995, Guo et al., 1995). Interestingly, we also found that transcription at this locus was strongly decreased in adulthood (Figure S1).

Based on these results, we selected a range of embryonic tissues for high throughput RNA sequencing analyses (Figure 1B, File S1), including some in which *Linc-p21* was expressed in wildtype mice (e.g. hindlimb and heart) and others in which there was no detectable expression (e.g. liver and lung) (Figure 1C, S1). We also included adult and embryonic brain samples from our previous study of whole brain sequencing (Goff et al., 2015). For each tissue, we conducted a comprehensive analysis of whole-genome transcription between wildtype and *Linc-p21* knockout (File S2). We then performed pathway analysis using the list of genes that were differentially expressed in one or more tissues and observed enrichment for genes involved in cell cycle and muscle-related processes, consistent with the observed expression pattern of *Linc-p21* (Figure S2A,B).

Identification of DNA regulatory elements within the *Linc-p21* locus *in vivo*

To better characterize the transcriptional perturbations that occurred in the absence of *Linc-p21*, we next identified the genes that were significantly differentially expressed in all of the tissues examined. Strikingly, 3 of the 4 genes that met these criteria (*Glo1*, *Rnps1*, and *Cdkn1a*) are located on chromosome 17 – the same chromosome as *Linc-p21* (Figure 1D). *Cdkn1a* is a well-known regulator of the cell cycle, and its dysregulation is thus in keeping with the observation that the genes involved in cell cycle were consistently upregulated in the knockout (Figure S2D).

To further investigate this regulatory effect upon local gene expression, we examined whether other nearby genes within a 4Mb region centred on *Linc-p21* were similarly dysregulated in different tissues. Of the 84 genes within this window, we observed that 8 were significantly dysregulated in one or more of the tissues examined (4 upregulated and 4 downregulated) and that a substantial proportion of the remainder (34 of 76 genes) showed non-significant expression effects of a similar direction in all tissues in response to *Linc-p21* deletion (Figure 2A). Notably, in each of the tissues examined, the number of significantly dysregulated genes in proximity to the *Linc-p21* locus was higher than would have been expected by chance (based on permutation testing using 10,000 randomly selected size-matched regions in each tissue; Figure 2B-E and File S3). To provide a genome-wide context, we identified those genes whose expression changed in the same direction across all tissues (irrespective of statistical significance) and observed that a highly significant fraction of these were located on chromosome 17 (91 of 698, $p<2.1\times10^{-19}$; Figure S2C,E). Such a chromosomal bias for gene expression effects is consistent with multiple *cis*-regulatory effects arising from this locus.

To delineate the relative roles of the DNA element and the RNA transcript in mediating these *cis*-regulatory effects, we next examined the expression of *Linc-p21* and *Cdkn1a* in wildtype tissues and detected a positive correlation between their RNA abundances (Figure 3A, $R^2=0.42$, $p=2.4\times10^{-6}$). For each tissue, we then compared the reduction in *Cdkn1a* expression that was observed in the knockout mouse with the expression of *Linc-p21* in that tissue in the wildtype. We hypothesized that if *Linc-p21* RNA was responsible for activating *Cdkn1a,* then the reduction in *Cdkn1a* expression should be proportional to the endogenous abundance of *Linc-p21* RNA. However, this relationship was not observed. In fact, in all of the tissues examined, the magnitude of the change in expression of *Cdkn1a* was wholly unrelated to the wildtype expression level of *Linc-p21* in that tissue (Figure 3B, p=0.96). To consider whether this phenomenon was limited to *Cdkn1a* or more widespread, we performed pathway analysis between individual wildtype and knockout tissues. This demonstrated similar enrichment for cell cycle processes and muscle-related processes across all tissues, even those in which *Linc-p21* was not endogenously expressed (Figure S2D, File S3). Together this suggested that the regulatory effects mediated by this locus were not due to either transcription of *Linc-p21* or the mature RNA transcript, and implied that another, RNA-independent regulatory mechanism must be present.

The *Linc-p21* locus contains multiple enhancer elements

Based on these results, we investigated whether the *Linc-p21* DNA sequence might contain functional enhancer elements that could explain the observed *cis*-regulatory effects. We first examined histone modifications and transcription factor binding sites across the locus using publically available datasets derived from murine heart tissue. We found that the *Linc-p21* gene body and promoter have multiple features typically associated with enhancer activity, including monomethylation of histone 3 at lysine 4 (H3K4me) and acetylation of histone 3 at lysine 27 (H3K27ac, Figure 3C, data from Rosenbloom et al., 2013). Moreover, chromatin contact data from a genome-wide promoter capture method, HiCap, indicated that the *Linc-p21* locus and *Cdkn1a* promoter physically interact through intra-chromosomal looping (upper panel, Figure 3C, Sahlén et al., 2015). To assess if *Linc-p21* interacts with other nearby loci, we analysed all of the interactions within a 4Mb region using available data from a complementary method, Hi-C (Dixon et al., 2012). We found that genes which physically interact with the *Linc-p21* locus were significantly enriched for those which were dysregulated following *Linc-p21* deletion, compared to the background interaction rate (Figure S3A). Similar interactions were also identified in human capture Hi-C data (Mifsud et al., 2015) between *LINC-p21* and the promoters of genes whose orthologs were significantly dysregulated in the knockout mouse (including *CDKN1A*, *PPIL1* and *CPNE5*; Figure S3B). These findings are consistent with the *Linc-p21* locus containing regulatory DNA elements that mediate conserved intra-chromosomal *cis*-regulatory contacts.

To test for functional DNA regulatory elements, we assessed whether the following 1kb regions could promote transcription of luciferase from a reporter vector in C2C12 cells, a mouse myoblast cell line: (1) a region surrounding the promoter and first exon, including the conserved p53 motif, (2) a region from intron 1, and (3) a negative control from an upstream intergenic region that has neither enhancer histone marks nor evidence of transcription (Figure 4A). We observed strong activation of luciferase activity from the *Linc-p21* promoter but not the intronic region (Figure 4B). Much of this signal was shown to be due to the p53-binding site, as a strong decrease in luciferase activity was observed if the 16-bp p53 motif was disrupted by site-directed mutagenesis. Moreover, if this motif was inserted into intron 1 (the region that previously lacked enhancer activity), a 29-fold increase in luciferase activity was observed. Notably, however, this increased activity level was still much lower than that of the native promoter (Figure 4B). Together these results suggest that the p53 motif in the *Linc-p21* promoter is a major factor in driving enhancer activity. However, because ectopic insertion of this motif into an intronic site did not fully recapitulate the enhancer activity of the promoter, and because presence of enhancer-related histone marks are present throughout the gene body, we hypothesized that other as-yet-unidentified DNA regulatory elements might be present. To investigate this possibility, we used a massively parallel reporter assay (MPRA; Melnikov et al., 2012) to systematically and comprehensively interrogate the *Linc-p21* locus – at nucleotide resolution – and establish whether additional enhancer elements were present. To do this, we synthesized a library of 2,225 individually-tagged 140-bp oligos that redundantly tiled the entire *Linc-p21* locus (including the gene body and promoter; Figure 4C). This oligo library was cloned into a GFP^+ reporter vector (Melnikov et al., 2012, Melnikov et al., 2014), and the coverage in the final pooled library was checked by high throughput sequencing (Figure 4D). The plasmid

library was then transfected into C2C12 cells and after 24 hours RNA was extracted and indexed libraries were constructed and sequenced.

In each sample, the number of tags (indicative of a transcriptional event) was quantified, normalized for sequencing depth, and used to calculate the signal for each base-pair of the region (Melnikov et al., 2012, Kheradpour et al., 2013, Figure S4). Using this approach, we confirmed the enhancer activity of the p53-binding site across multiple independent oligos. Strikingly, we also observed 4 other regions of enhancer activity within the *Linc-p21* locus, two of which displayed stronger enhancer activity than that of the known p53-motif (Figure 4E). Using available data from the same cell line, we observed that the first of these regions overlapped with an experimentally confirmed CCAAT/Enhancer Binding Protein (CEBPB) binding site and the second was located proximal to a MyoD ChIP peak (Figure 4E, Rosenbloom, et al., 2013) – a finding that connects the known role of MyoD in muscle development with our finding that the *Linc-p21* locus is most highly expressed in muscle (Figures 1C, S1). Collectively, these data indicate that the *Linc-p21* locus is a complex genomic environment containing several functional DNA elements that interact with, and regulate the transcription of, multiple local genes including *Cdkn1a*.

Discussion

Since its discovery in 2010, *Linc-p21 has* been the subject of intense study due to its reported roles in important biological processes (Huarte et al., 2010, Dimitrova et al., 2014, Yang et al., 2014, Hall et al., 2015, Bao et al., 2015, Yoon et al., 2012, Wu et al., 2014, Wang et al., 2014, Tran et al., 2015, Tang et al., 2015). However, despite extensive investigation, the nature of transcription at the *Linc-p21* locus and the mechanism by which the gene functions have not been tested outside of cell-based assays. Here we present, to our knowledge, the first study of the *Linc-p21* locus *in vivo*, and by implementing a whole gene deletion and reporter knock-in, are able to disentangle the relative contributions of DNA and RNA to the observed *cis*-regulatory effects (Bassett et al., 2014, Goff and Rinn, 2015). Moreover, by combining this approach with MPRA, we demonstrate that, *in vivo,* the *Linc-p21* locus is a complex DNA enhancer element that regulates the expression of multiple genes in a range of tissues in *cis*.

Several lines of evidence come together to support this conclusion. First, we observed that *Linc-p21* deletion consistently led to changes in the expression of local genes irrespective of whether the *Linc-p21* locus itself was transcribed – thereby excluding an RNA-dependent regulatory mechanism. Second, MPRA data revealed that multiple enhancer elements are present within the *Linc-p21* locus, including a known p53 motif which was confirmed to have strong enhancer activity. Third, the *Linc-p21* locus physically interacts in 3D space with the promoters of local genes that are dysregulated following *Linc-p21* deletion, including *Cdkn1a* – an observation that is consistent with studies that have shown that p53-bound DNA elements can interact with local genes via DNA looping (Link et al., 2013, Melo et al., 2013, Younger et al., 2015). Accordingly, we believe that *Linc-p21* represents an example of a primed p53 enhancer, in which the structural contact and even the enhancer activity is established independent of p53, but is further activated upon p53 binding. In keeping with this conclusion, global run-on sequencing analysis has previously identified

transcription at the *Linc-p21* locus in p53-null cells, suggesting that the enhancer is functional even without p53 binding (Allen et al., 2014).

It is important to consider how these data fit with other studies in which *Linc-p21* has been proposed to be a *trans*- or *cis*-acting lincRNA. While several of the initial studies of *Linc-p21* reported *trans*-regulatory effects, many of these are now thought to be mediated indirectly via genes that *Linc-p21* regulates in *cis*- (Dimitrova et al., 2014). Indeed, Dimitrova and colleagues demonstrated that *Cdkn1a* expression was reduced by ~50% following deletion of the *Linc-p21* promoter and concluded that *Linc-p21* is a *cis*-regulatory lncRNA (Dimitrova et al., 2014). However, this deletion included the p53-binding site that we have shown has important enhancer activity. These data would therefore also be consistent with a functional DNA element, and further highlight both the specific role of this element in the control of *Cdkn1a* expression and the general importance of genetic strategies that can definitively delineate the relative contributions of DNA and RNA to any observed function.

The *Linc-p21* locus exemplifies the difficulties in elucidating the relative functions of DNA, RNA, and the act of transcription at non-coding loci. While there are clear examples of lncRNAs that function through their RNA transcript (Brown et al. 1992, Rinn et al., 2007), there are also examples where the RNA is dispensable and either transcription itself or elements within the DNA sequence are responsible for observed functions (Paralkar et al., 2016). These potential mechanisms cannot be resolved through deletion of the entire locus (Basset et al. 2014, Goff and Rinn, 2015) and thus other methods are needed to dissect their relative contributions.

To date, most of these methods have focused upon disrupting the RNA transcript by reducing transcription or truncating the transcript using terminator sequences (Paralkar et al., 2016, Basset et al. 2014). Here we show that MPRA can be used to detect functional DNA elements within non-coding loci, and thus provides an important complementary technique. Indeed, using this approach, we discover several previously uncharacterized regions within the *Linc-p21* locus that have enhancer activity and overlap histone and protein ChIP peaks – consistent with DNA enhancer elements. Moreover, these regions of enhancer activity lie within the vicinity of structural chromosome contact points that would bring them to the promoter of *Cdkn1a* and other nearby genes. Interestingly, many direct targets of p53 are known to associate with primed enhancers that are further activated by p53 binding (Melo et al., 2013, Allen et al., 2014, Younger et al., 2015). It could therefore be hypothesized that these novel enhancer regions might be pioneer-factor binding sites that could organize local chromatin contacts and bring the *Linc-p21* p53 motif into the vicinity of the nearby promoters, where it can simultaneously activate transcription in both regions.

The *Linc-p21* promoter region (but not the transcript) is well conserved between human and mouse, both in terms of sequence homology and physical interactions with orthologous genes (Figure S3B). Intriguingly, this suggests that enhancer contacts between this locus and nearby genes may be evolutionarily conserved, which might also explain why genetic variation in this region has been associated with human disease, including colorectal cancer and cardiac defects (Sotoodehnia et al., 2010, Dunlop et al., 2012, Ritchie et al., 2013, Hong

et al., 2014). Collectively, these data provide a starting point to better understand the exact nature of the regulatory interactions at this locus, which could ultimately provide important insights into the pathogenesis of several human diseases.

In summary, our data demonstrate that the *cis*-regulatory effects mediated by *Linc-p21* are due to a functional DNA element rather than the RNA transcript. As such, we would support the reclassification of the *Linc-p21* RNA as an eRNA, and note that although this term usually implies some *cis*-regulatory function, in this example neither the transcript nor transcription are required for local gene regulation by the locus. It is important to note, however, that the RNA transcript may still have other functions that are not related to *cis*-regulation, and which have not been identified in this study. For example, we did not seek to explore the mechanism underlying any potential *trans*-regulatory effects, and we also cannot exclude the possibility that the RNA has a function at an earlier developmental stage which is subsequently lost as tissues differentiate. Moreover, we suspect that many lncRNA loci probably contain both functional DNA elements and RNA transcripts, and that these possibilities should not be considered mutually exclusive. Indeed, just as coding gene loci can contain functional intragenic enhancers (Li et al., 2012, Zhang et al., 2013) and still produce translated mRNA transcripts, we expect that a similarly complex situation will exist at many non-coding loci. Unravelling the respective functions of DNA and RNA at these loci is likely to be an on-going challenge for the field, and one that may ultimately lead to a revision of our classification of non-coding transcripts, along with the catalogs of transcripts themselves.

Experimental Procedures

Mice

Mice were housed under pathogen-free conditions in Harvard University's Biological Research Infrastructure. All procedures were approved by the Harvard University Committee on the Use of Animals in Research and Teaching and performed in accordance with the National Institutes of Health guidelines.

RNA isolation and RNA-Seq library preparation and sequencing

Global gene expression was assessed by RNA sequencing (RNAseq) of different organs and tissues from at least 3 *Linc-p21* knockout and 3 wildtype embryos. Hindlimbs, liver, lungs, and heart were harvested from E14.5 embryos and immediately homogenized in TRIzol (Life Technologies). Total RNA was extracted by chloroform extraction followed by spin-column purification (RNeasy mini kit, Qiagen). RNA-Seq libraries (TruSeq RNA Sample Preparation Kit v2; Illumina) were prepared as previously described using 500ng of total RNA and a 10-cycle PCR enrichment to minimize PCR artifacts (Sauvageau et al., 2013, Goff et al., 2015). Knockout and wildtype samples from different litters were processed within each library preparation. The indexed libraries were sequenced in pools of six (Illumina HiSeq 2000, 101-bp paired-end reads).

RNA-Seq analysis

Reads from fastq samples were aligned to the mouse genome (mm10) using Tophat2 with non-standard options "--no-coverage-search --max-multihits 10 -p 8" (Kim et al. 2013). Each sample was quantified using Cuffquant with nonstandard options "-p 8 --no-update-check", and differential analysis was performed for each wildtype-vs-knockout tissue comparison using Cuffdiff2 with nonstandard option "-p 8". We also performed a Cuffdiff2 analysis in which all wildtype-vs-knockout samples were assessed together using nonstandard option "-p 8" (Trapnell et al. 2012). All analysis scripts are available as Files S2 and S4, all code is available on Github, and we frequently used Cummerbund for analysis and to generate figures (Goff et al 2013, https://github.com/rinnlab/lincp21).

Cloning and mutagenesis

The *Linc-p21* locus was cloned using a BAC plasmid (RP24-248L4) obtained from Children's Hospital Oakland Research Institute. Acc651 and Xho1 restriction sites were added to amplification primers to enable ligation into the multiple cloning site of the pGL4.23 vector (Promega) containing the luciferase gene. The p53 binding site was perturbed using inverse PCR and 5′ phosphorylated primers containing mutations amplified off of the Exon 1 clone in pGL4.23 similar to the Quick Change protocol (Agilent Technologies). The pGL4.73 vector was used in co-transfection as a transformation control expressing Renilla luciferase.

Cell culture and Transfection

The C2C12 cell line was obtained from ATCC (CRL-1772) and maintained according to the recommended guidelines. Transfections were performed using TransfeX reagent (ATCC ACS-4005) for luciferase assays and Lipofectamine 3000 (Thermo Fisher) for the MPRA experiment. All experiments were performed in triplicate.

Massively Parallel Reporter Assay (MPRA)

We designed 140bp oligos to redundantly tile the genomic region spanning the *Linc-p21* locus and 500bp of its promoter, as previously described (Melnikov et al., 2012, Melnikov et al., 2014, Kheradpour et al., 2013). Our pool consisted of 90bp genomic regions starting every 50bp. Each genomic region was represented by 5 unique barcodes. Oligos were synthesized by the Broad Institute Technology Core and cloned into final GFP^+ constructs as previously described (Melnikov et al., 2012, Melnikov et al., 2014, Kheradpour et al., 2013). 10ug of GFP^+ pooled construct was used to transfect C2C12 cells in 6-well plates. After 24 hours, cells were harvested in Trizol and total RNA was extracted as described and treated with DNase. MPRA libraries were constructed from 1ug input as previously described RNA (Melnikov et al., 2014). Libraries from the pooled vector construct were used as a control. All libraries were purified by a triple SPRI bead cleanup (0.65×, 0.8×, 0.8×, Agencourt AMPure XP, Beckman Coulter), quantified by Qubit, and size-checked on a BioAnalyzer before deep sequencing (HiSeq 2500). Experiments were performed in triplicate.

MPRA Analysis

We counted tags originating from reads containing GFP sequence and with a perfect match to the barcodes we designed, and then normalized each sample to the total number of counts from that sample. We calculated the median ratio of RNA library signal to vector library signal for each base-pair and calculated rolling signal means across the locus with different windows and slides (File S5). For all analysis reported in this paper, we used a window of 500bp and a slide of 50bp. To generate p-values for significance of any given region, we permuted the signal ratio values across the entire locus 1000 times and repeated the sliding window analysis for each permutation, generating a randomized permutation p-value for each 500bp window.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Highlights

- *Linc-p21* regulates transcription of multiple local genes *in vivo*, including *Cdkn1a*
- *Linc-p21* RNA is entirely dispenable for this *cis*-regulatory function
- Massively parallel reporter assay identifies several enhancer elements at this locus
- The *Linc-p21* locus physically interacts with *cis*-regulated genes in human and mouse

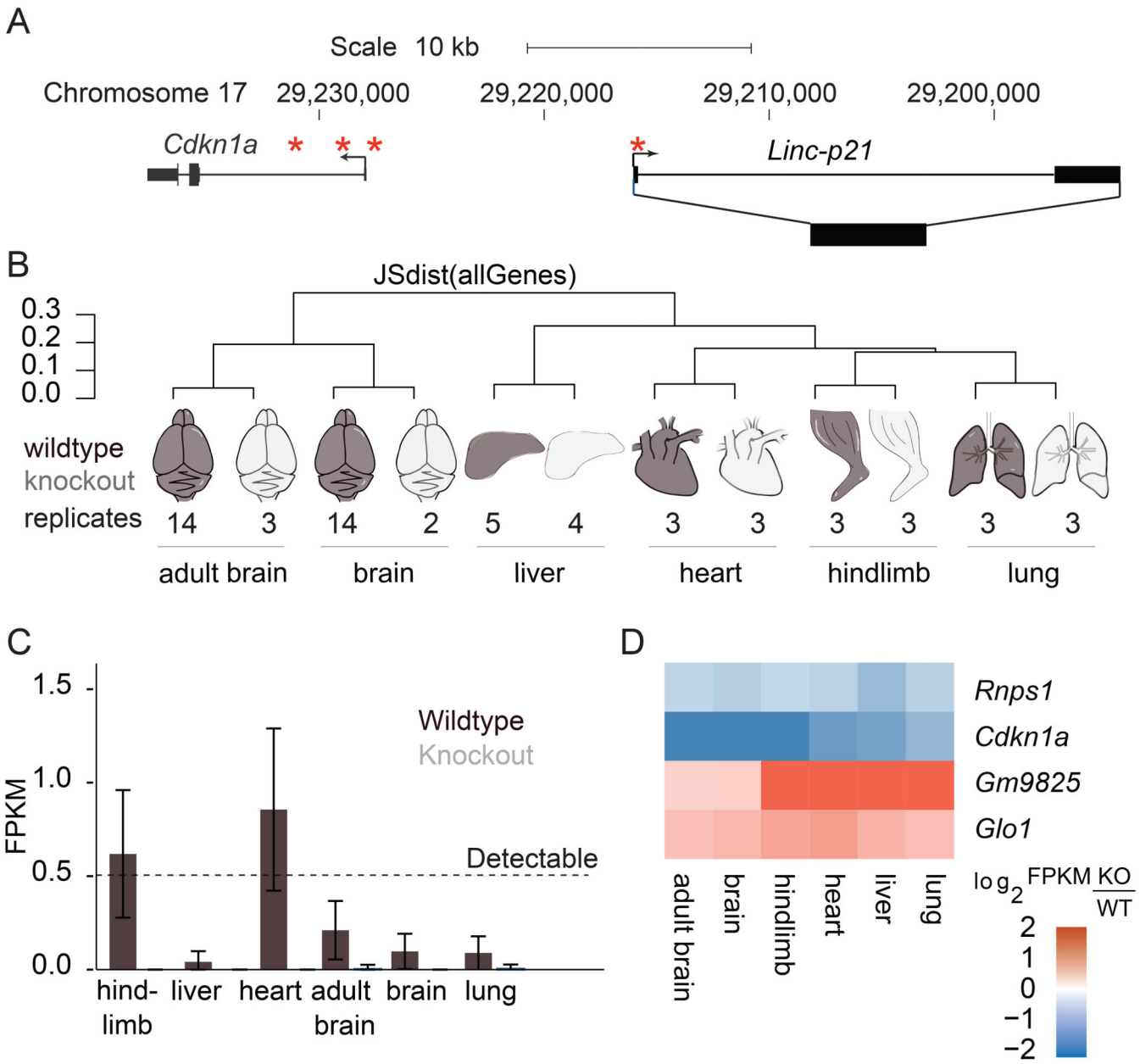

Figure 1. ***Linc-p21 in vivo*** **deletion overview**

(A) *Linc-p21/Cdkn1a* locus on mouse chromosome 17. Asterisks indicate known p53 binding sites. (B) Dendrogram showing the type and number of samples sequenced and the Jenson-Shanon distance, a measure of total transcriptome similarity, between their expression profiles. Wildtype shown in black, knockout shown in grey. (C) Average *Linc-p21* expression profile in each tissue. Error bars represent 95% confidence interval. (D) Heatmap depicting expression of genes significantly differentially expressed in every tissue. Log_2 fold-change was calculated using the average FPKMs for all replicates (KO/WT). See also Figure S1.

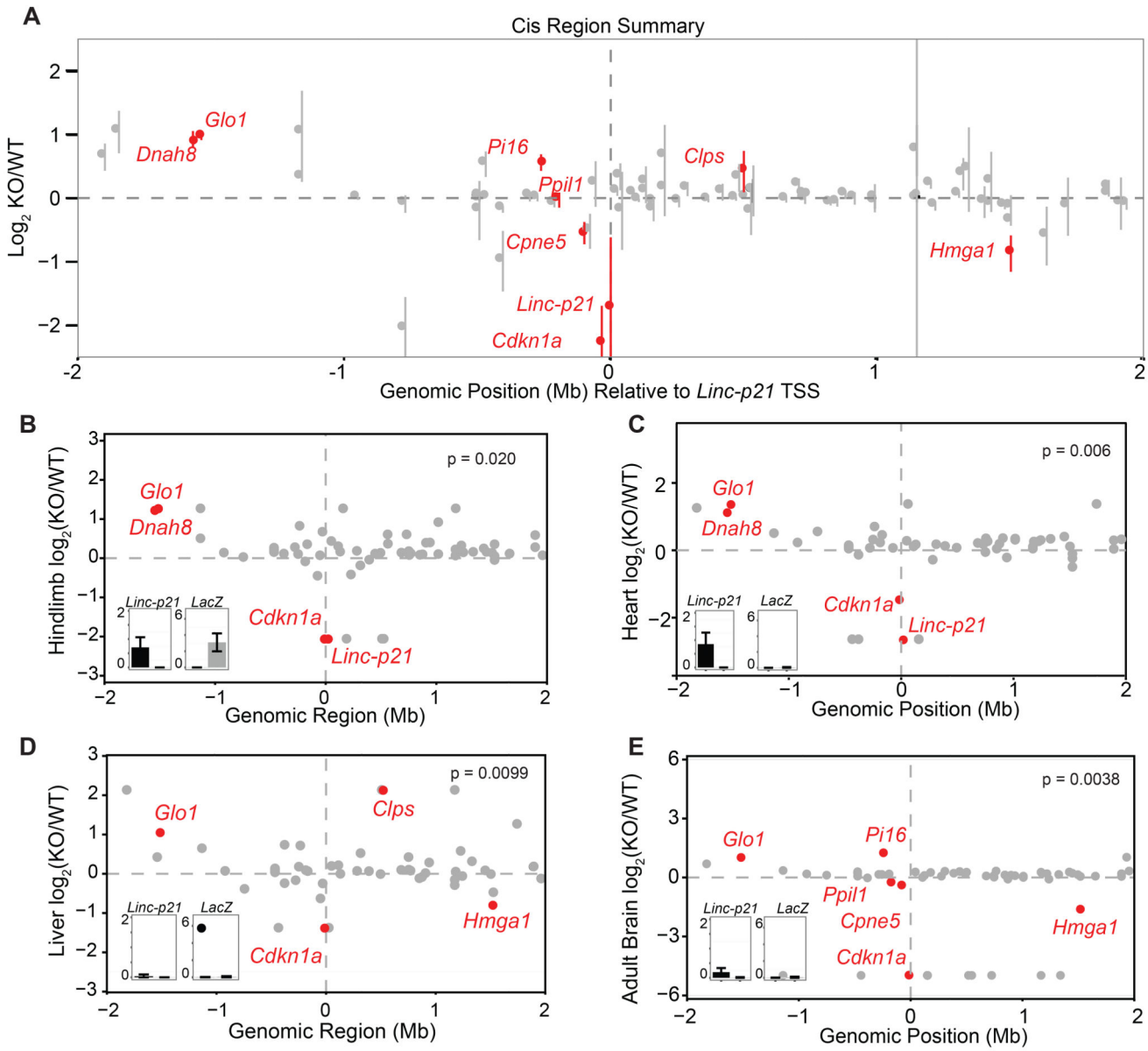

Figure 2. *Linc-p21* transcript is not required for local gene regulation

(A) Summary of local transcriptional changes upon *Linc-p21* deletion (+/− 2Mb of *Linc-p21*). Dots represent average $\log_2$ fold change across all 6 tissues and error bars represent standard error. Red indicates significant differential expression in at least one tissue. (B-E) Expression of genes in local region (+/− 2Mb of *Linc-p21*) for E14.5 hindlimbs (B), heart (C), liver (D), and adult brain (E). In each plot, the Y-axis represents the $\log_2$ FPKM fold change and genes marked in red were significantly differentially expressed. The p value represents the probability that this number of genes would be differentially expressed within a region of this size. Insets show *Linc-p21* and *lacZ* expression (FPKM) in wildtype (black) and knockout (gray). See also Figure S2.

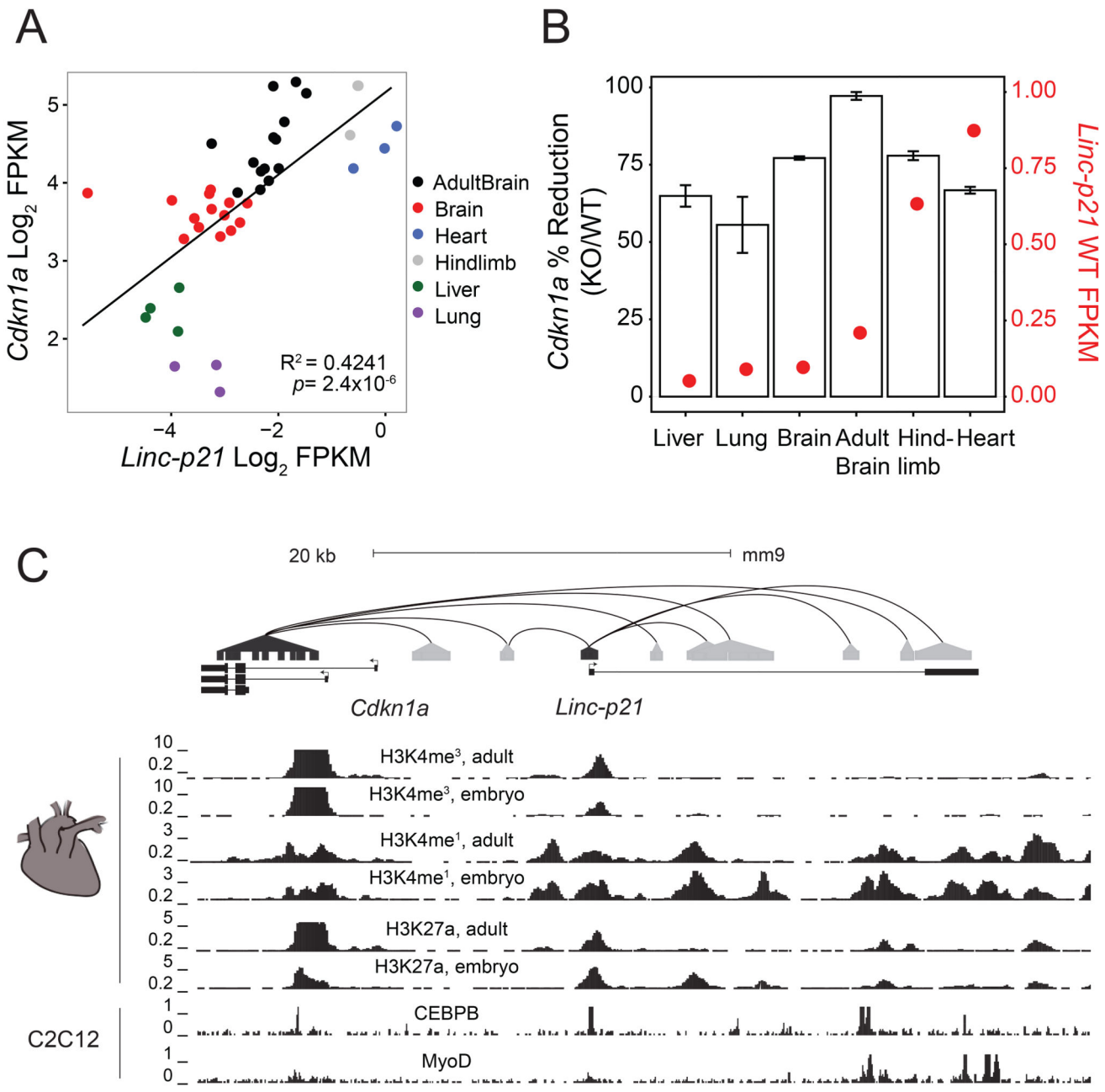

Figure 3. In vivo evidence of enhancer activity in the *Linc-p21* locus

(A) Correlation of *Cdkn1a* expression with *Linc-p21* transcript expression. X-axis represents *Linc-p21* expression and Y-axis represents *Cdkn1a* expression in each wildtype replicate. Color indicates the tissue of origin, and the linear regression line is shown. (B) Average change in *Cdkn1a* expression in knockout tissues (1-KO/WT, bars, left y-axis) plotted against average wildtype *Linc-p21* expression levels (red dots, right y-axis); error bars represent standard error. (C) Publically available chromatin interaction data between *Linc-p21* and *Cdkn1a* from HiCap (plotted as black lines between capture probes and gray distal regions). Publically available histone mark and transcription factor binding data at this locus.

All panels are from embryonic or adult heart tissue, except CEBPB and MyoD binding which are from C2C12 cells. See also Figure S3.

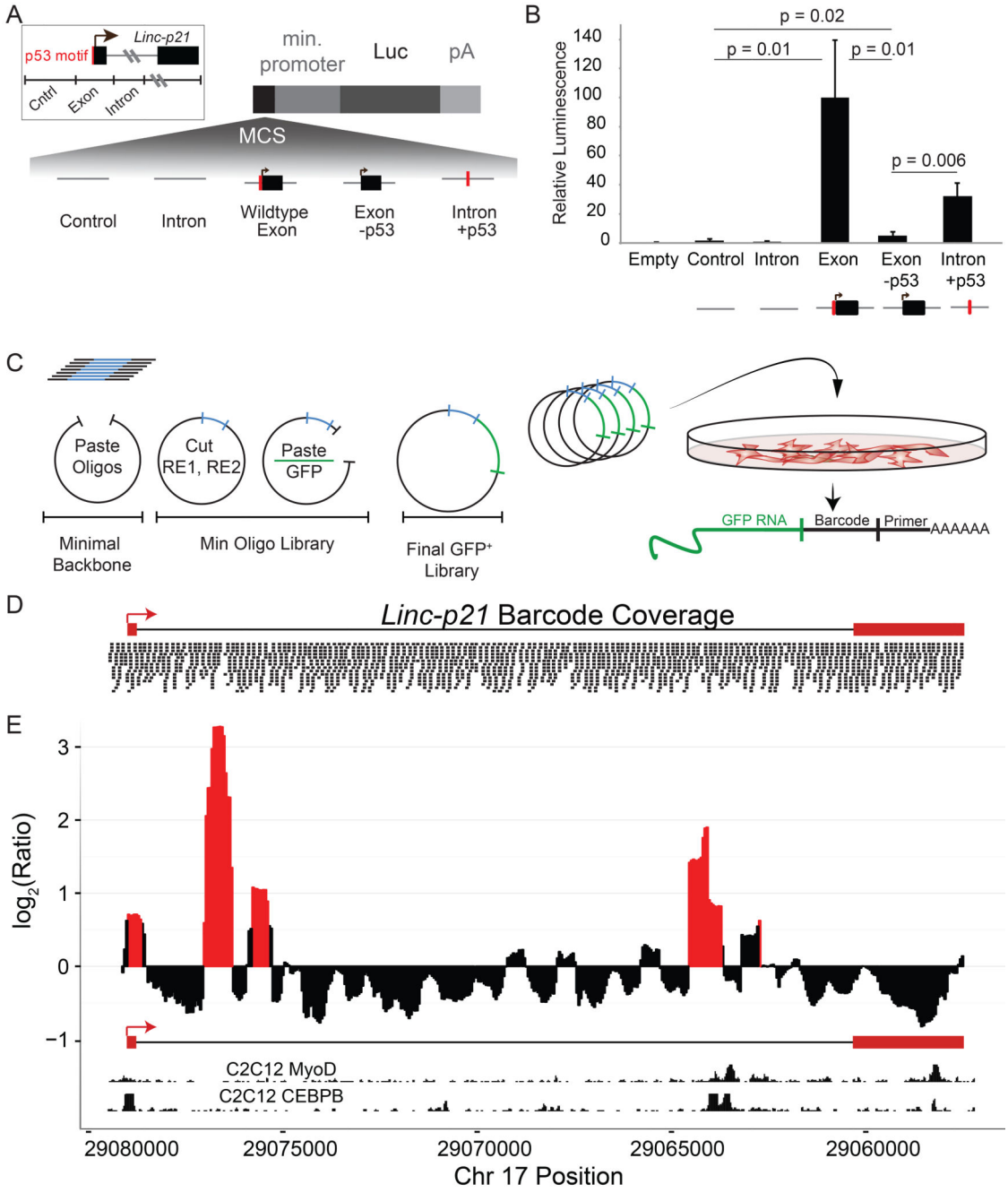

Figure 4. MPRA of the entire *Linc-p21* locus reveals enhancer activity

(A) Experimental design for luciferase reporter assay using an intergenic control and regions from the *Linc-p21* promoter and exon 1 (“exon”), intron 1 (“intron”), exon with mutated p53 binding sequence (“exon -p53”) and intron with p53 binding sequence (“intron+p53”). Each region was cloned into a luciferase reporter construct. P53 motif indicated in red. (B) Relative luminescence for each construct, normalized against the signal from exon fragment and averaged across triplicate samples. P values were calculated using unpaired one-tailed t-tests. Error bars represent standard error of the mean. (C) Massively Parallel Reporter Assay

(MPRA) experimental design: oligos were synthesized, subcloned into a minimal backbone, opened by enzymatic digestion and re-ligated with a GFP cDNA insert. Pooled constructs were transfected into C2C12 cells in triplicate and libraries were made from GFP+ RNA. (D) Coverage of final pooled GFP+ vector library across the *Linc-p21* locus and promoter (assessed by high throughput sequencing). (E) MPRA signal across the *Linc-p21* locus. Y-axis represents the $\log_2$ ratio of normalized RNA to control signal per base (averaged in 500bp sliding windows every 50bp). Significance ($p<0.01$) is calculated by comparing this signal to 1000 random shuffles of the input data. Significant peaks are shown in red. Inlayed tracks are CEBPB and MyoD ChIP-seq signals from UCSC genome browser. See also Figure S4.