Enhancers generate bidirectional noncoding enhancer RNAs (eRNAs) that may regulate gene expression. At present, the eRNA function remains enigmatic. Here, we report a 5′ capped antisense eRNA PEARL (Pcdh eRNA associated with R-loop formation) that is transcribed from the protocadherin (Pcdh) a HS5-1 enhancer region. Through loss- and gain-of-function experiments with CRISPR/Cas9 DNA fragment editing, CRISPRi, and CRISPRa, as well as locked nucleic acid strategies, in conjunction with ChiRIP, MeDIP, DRIP, QHR-4C, and HiChIP experiments, we found that PEARL regulates Pcdha gene expression by forming local RNA–DNA duplexes (R-loops) in situ within the HS5-1 enhancer region to promote long-distance chromatin interactions between distal enhancers and target promoters. In particular, increased levels of eRNA PEARL via perturbing transcription elongation factor SPT6 lead to strengthened local three-dimensional chromatin organization within the Pcdh superTAD. These findings have important implications regarding molecular mechanisms by which the HS5-1 enhancer regulates stochastic Pcdha promoter choice in single cells in the brain.

[Keywords: enhancer RNA PEARL; protocadherin gene expression; 3D chromatin structure; R-loop formation; SPT6; RNase H1; long-distance enhancer-promoter looping; superenhancer]

Supplemental material is available for this article.

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receptor, and UDP-glucuronosyltransferase gene clusters (Wu and Maniatis 1999; Zhang et al. 2004). The variable region of the human Pcdha gene cluster contains 13 highly similar alternate variable exons \( [\alpha_1 - \alpha_{13}] \) and two C-type variable exons \( [\alpha C1 \text{ and } \alpha C2] \), each of which is separately spliced to a single set of three downstream constant exons to generate diverse mRNAs (Wu and Maniatis 1999). Each Pcdha alternate variable exon is preceded by a promoter that is flanked by two forward-oriented CTCF sites (Fig. 1A; Guo et al. 2012, 2015; Monahan et al. 2012, Canzio et al. 2019; Jia et al. 2020).

The Pcdha cluster is regulated by a downstream super-enhancer composed of two composite enhancers of HS7 and HS5-1 (hypersensitive sites 5–1) (Ribich et al. 2006). In particular, the HS5-1 enhancer, flanked by two reverse-oriented CTCF sites, is located at \( \sim 30 \) kb downstream from the last constant exon (Fig. 1A; Guo et al. 2012, 2015; Monahan et al. 2012, Canzio et al. 2019; Jia et al. 2020).

Specifically, antisense transcription of lncRNA from specific variable antisense promoters, which leads to DNA demethylation by TET enzymes and subsequent recruitment of CTCF proteins, is the key determinant of the Pcdha promoter choice (Canzio et al. 2019). However, the mechanism by which long-distance chromatin interactions between the HS5-1 enhancer and its target promoters regulate Pcdha

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**Figure 1.** Protocadherin HS5-1 enhancer produces a prominent antisense eRNA PEARL. |A] Schematic representation of the human Pcdha gene cluster. The alternative and C-type isoforms are represented by blue and yellow boxes, respectively. HS7 and HS5-1 enhancers are indicated with black arrows. The HS5-1 eRNA transcription start site (TSS) was mapped to the HS5-1 enhancer region between the two CTCF-binding sites (CBSa and CBSb). |B] Shown are total RNA-seq, RNAPII, H3K4me3, H3K27ac, CTCF, and Rad21 ChIP-seq in the human Pcdha cluster. Total RNA-seq shows transcribed sense (red) and antisense (blue) transcripts. |C] Detailed features of the HS5-1 region are shown. The HS5-1 enhancer transcribes weak sense and strong antisense transcripts. H3K4me3 and H3K27ac mark active promoters and enhancers, respectively. CTCF and Rad21 have two binding sites in the HS5-1 region. |D] Schematic of the 5′RACE experiment. |E] Agarose gels show HS5-1 eRNA 5′RACE PCR products. Note that the smear below the prominent band PEARL represents several alternative TSSs [Supplemental Fig. S1B]. |Lanes 2–4] Negative controls. |M] 1.5-kb DNA ladder. |F] Mouse cortical total RNA-seq. |G] Magnification of the mouse HS5-1 enhancer region. The mouse Pcdha HS5-1 enhancer transcribes weak sense and strong antisense transcripts that are indicated in red and blue, respectively. |H,I] Quantitative RT-PCR analyses of the bidirectional eRNAs in HEC-1-B cells and the mouse cerebral cortex. |M] 1.5-kb DNA marker, |E] eRNA products, |NC] negative control. See also Supplemental Figure S1.
Results

Pedha HS5-1 antisense eRNA revealed by RACE experiments

Using the model cell line of HEC-1-B [Guo et al. 2015], we first performed strand-specific total RNA-seq experiments, which remove the abundant ribosomal RNAs [Amur et al. 2011], and found prominent antisense transcripts from the HS5-1 enhancer region [Fig. 1B,C]. These transcripts map to a position enriched of RNAPII [RNA polymerase II], H3K4me3 [histone 3 lysine 4 trimethylation], and H3K27ac [histone 3 lysine 27 acetylation], which are located between the two reverse-oriented CTCF sites [Fig. 1C]. These hallmarks of active enhancers are consistent with recent findings of rich depositions of CTCF sites (Fig. 1C). These hallmarks of active enhancers are consistent with recent findings of rich depositions of CTCF sites (Fig. 1C). These hallmarks of active enhancers are consistent with recent findings of rich depositions of CTCF sites (Fig. 1C).

To map the exact transcription start site (TSS), we carried out cap-dependent 5′ RACE [rapid amplification of cDNA ends] experiments and found a major 804-nt capped eRNA molecule that we named PEARL (Pedha eRNA associated with R-loop formation), although several minor 5′ capped transcripts with different TSSs were also detected, suggesting considerable heterogeneity in its start site positions [Fig. 1D,E; Supplemental Tables S1, S2, Supplemental Fig. S1A,B]. Despite repeated attempts of 3′ RACE, we were unable to determine the exact transcription termination site (TTS). Total RNA-seq with mouse cortical tissues also revealed a prominent HS5-1 antisense transcript [Fig. 1F,G]. In addition, sequence analyses showed that this HS5-1 eRNA transcript contains no conserved ORF [open reading frame], suggesting that it is noncoding. Moreover, in both human HEC-1-B cells and mouse cortical brain tissues, total RNA-seq experiments revealed weaker but detectable HS5-1 sense transcripts [Fig. 1C,G]. To confirm the bidirectional eRNA transcription, we performed quantitative RT-PCR experiments using either a sense or antisense primer as a reverse transcriptional primer and found that both can produce cDNA [Fig. 1H,I], suggesting that the HS5-1 enhancer transcription is bidirectional. In addition, we used oligo d[T] as the reverse transcriptional primer and found that it can produce cDNA, suggesting that the HS5-1 eRNAs are polyadenylated [Fig. 1H,I]. Finally, we quantified eRNA expression levels in model cell lines of HEC-1-B, SK-N-SH, HepG2, and HEK293T and found that HS5-1 eRNA expression levels correlate with those of Pedha expression [Supplemental Fig. S1C,D].

HS5-1 eRNA PEARL TSS deletion affects Pedha expression

To investigate the potential function of the eRNA PEARL in regulating Pedha gene expression, we specifically deleted the TSS region by CRISPR DNA fragment editing [Li et al. 2015; Shou et al. 2018] to avoid the perturbation of the two CBS [CTCF-binding site] elements [Fig. 2A,B], which are known to be essential for Pedha gene regulation [Guo et al. 2012; Jia et al. 2020]. We isolated two independent homozygous single-cell CRISPR deletion clones [Supplemental Fig. S2]. RNA-seq experiments revealed a significant decrease of Pedha a6, a12, and aC1 expression levels upon TSS deletion [Fig. 2C]; note that PedhaC2 contains no CBS [Guo et al. 2012, 2015] and is not regulated by HS5-1 [Ribich et al. 2006]. Next, we examined histone modifications of the HS5-1 enhancer and Pedha alternative promoters. We examined enrichment levels of H3K4me3 occupancy at strong enhancers (Henriques et al. 2018). These hallmarks of active enhancers are consistent with recent findings of rich depositions of CTCF sites (Fig. 1C). These hallmarks of active enhancers are consistent with recent findings of rich depositions of CTCF sites (Fig. 1C). These hallmarks of active enhancers are consistent with recent findings of rich depositions of CTCF sites (Fig. 1C). These hallmarks of active enhancers are consistent with recent findings of rich depositions of CTCF sites (Fig. 1C).

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eRNA expression levels in both cases [Supplemental Fig. S3A]. In addition, we inserted a pAS [polyadenylation signal] by homologous recombination to cause a premature termination of eRNA transcription [Fig. 3C; Supplemental S3B,C]. Two homozygous single-cell CRISPR clones were obtained by screening CRISPR insertion clones and their genotypes were confirmed by PCR and Sanger sequencing (Supplemental Fig. S3D,E).

RNA-seq experiments revealed a significant decrease of expression levels of $Pcdh\alpha_6$, $\alpha_{12}$, and $\alpha_C1$ in all three methods of perturbing eRNA transcription (Fig. 3D–F). We then examined H3K4me3 levels in the $Pcdh$ cluster after interfering with PEARL transcription and found that H3K4me3 enrichments are decreased at the $\alpha_6$, $\alpha_{12}$, and $\alpha_C1$ promoters as well as at the $HSS-1$ enhancer [Fig. 3G], consistent with the RNA-seq results [Fig. 3D–F]. In addition, RNAPII occupancy and H3K27ac enrichment at the $HSS-1$ enhancer were also reduced (Fig. 3H, I). We noted that, compared with perturbing eRNA

PEARL transcription initiation, the RNAPII occupancy and H3K27ac enrichment in the $HSS-1$ enhancer region were almost abolished upon blocking eRNA elongation or inserting a premature termination signal (Fig. 3J,K).

Next, we designed two sgRNAs to program dCas9 to the locations further downstream from the eRNA PEARL TSS (Supplemental Fig. S3F): one at 1158 bp downstream from the TSS and the other at 1959 bp downstream from the TSS. We found that the former reduced the expression levels of PEARL and $Pcdh\alpha$ as well as the $HSS-1$ enhancer activity, but the latter had no effect, presumably because it is located after $HSS-1$ eRNA termination (Supplemental Fig. S3G–I). These data suggested that the eRNA PEARL is required for $Pcdh\alpha$ gene expression.

Previous studies have shown that eRNAs play a role in gene activation by mediating the formation of chromatin loops [Lai et al. 2013; Li et al. 2013; Hsieh et al. 2014; Xiang et al. 2014]. In the $Pcdh$ cluster, the stochastic expression of $Pcdh$ depends on the long-distance chromatin
interactions between distal enhancer and target variable promoters mediated by CTCF and its associated cohesin complex (Guo et al. 2012; Canzio et al. 2019; Jia et al. 2020). Therefore, we asked whether the eRNA PEARL plays a role in CTCF/cohesin-mediated chromatin looping between the distal enhancer and its target variable promoters. To this end, we first measured the enrichments of CTCF and Rad21 by ChIP-seq and found that they were reduced at the HS5-1 enhancer region (Fig. 3L,M). In addition, they were also reduced at the Pcdhα6, α12, and αC1 variable promoters (Fig. 3L,M). We then performed QHR-4C and found that there is a decrease of long-distance chromatin interactions between the HS5-1 enhancer and its target variable promoters (Fig. 3N–P). Although these CRISPR experiments have caveats for interfering with the HS5-1 enhancer function, all in all, these data suggest that the eRNA PEARL may be necessary for chromatin interactions between the Pcdha HS5-1 enhancer and its target variable promoters.

Locally transcribed but not globally overexpressed PEARL regulates Pcdha expression

To understand the function of eRNA PEARL, we activated eRNA transcription in cis locally by using the CRISPR activation (CRISPRa) system with a dCas9-VP160 protein programmed to a region upstream of the eRNA TSS (Fig. 4A). We first confirmed the eRNA CRISPR activation by quantitative RT-PCR (Fig. 4B). We then performed RNA-seq and found that eRNA PEARL transcriptional activation results in a significant increase of expression levels of the Pcdhα6, α12, and αC1 genes (Fig. 4C). Consistently,
there is a significant increase of long-distance chromatin interactions between the HS5-1 enhancer and its target variable promoters (Fig. 4D–F).

We next overexpressed the eRNA PEARL in trans globally by a U6 promoter and found, surprisingly, that its overexpression has no effect on Pcdha expression (Fig. 4G–I). In addition, its overexpression also has no effect on long-distance chromatin interactions between the HS5-1 enhancer and its target variable promoters (Fig. 4J–L). Finally, we overexpressed the eRNA PEARL in the TSS deletion CRISPR clones and found that it rescues neither Pcdha gene expression (Fig. 4M–O) nor long-distance chromatin looping (Fig. 4P–R). Collectively, we concluded that locally transcribed, but not globally overexpressed, eRNA PEARL or its transcriptional process per se regulates Pcdha gene expression.

Local PEARL transcripts function in cis within hypomethylated HS5-1 enhancers

To investigate why locally transcribed, but not globally overexpressed, PEARL regulates Pcdha gene expression, we performed chromatin isolation by RNA purification followed by sequencing (ChIRP-seq) experiments (Chu et al. 2011). We synthesized 24 specific 3′-biotin DNA probes directed against the eRNA PEARL transcripts and divided them into odd and even pools to capture with streptavidin magnetic beads (Fig. 5A). We first confirmed that both odd and even probes enrich the eRNA PEARL transcripts (Fig. 5B). We found that there exist unique signals in the HS5-1 enhancer region with both odd and even probe pools, suggesting that the eRNA PEARL is located in the HS5-1 enhancer region (Fig. 5C, D). As a positive control, the noncoding RNA NEAT1 is specifically enriched in the MALAT1 locus in trans (Supplemental Fig. S4A; West et al. 2014). We also performed ChIRP followed by SDS-PAGE with silver staining and did not find any prominent protein in comparison with the known protein PSF association of NEAT1 (Supplemental Fig. S4B,C; West et al. 2014; Jiang et al. 2017a).

We next performed methylated DNA immunoprecipitation and sequencing experiments (MeDIP-seq) and found that, in contrast to hypermethylation in the Pcdha variable exons (Supplemental Fig. S4D,E), the eRNA
promoter within the H5S-1 enhancer is hypomethylated in both human HEC-1-B cells and mouse cortical tissues (Fig. 5E–H). Given that the R-loop structure is often formed by local RNAs in the hypomethylated region (Ginno et al. 2012; Arab et al. 2019; Niehrs and Luke 2020), these MeDIP-seq and ChIRP-seq data suggest that local eRNA PEARL transcripts function in cis within the hypomethylated H5S-1 enhancer region.

PEARL transcripts form local R-loops in situ in the enhancer region

R-loops or DNA–RNA hybrids are special three-stranded nucleic acid structures that form locally in vivo to perform physiological or pathological functions (Skourti-Stathaki and Proudfoot 2014; Crossley et al. 2019; Garcia-Muse and Aguilera 2019; Niehrs and Luke 2020), but whether eRNA in the distal enhancer region regulates activation of target promoters via local R-loop formation in situ is not known. To this end, we performed DRIP (DNA–RNA immunoprecipitation) assays with the S9.6 antibody, which specifically recognizes DNA–RNA hybrids (Fig. 5I; Boguslawski et al. 1986; Ginno et al. 2012; Tan-Wong et al. 2019). We found that the eRNA PEARL is significantly enriched in the S9.6 immunoprecipitate, suggesting that the eRNA PEARL forms R-loops in the enhancer region (Fig. 5J).

LNA-mediated knockdown of PEARL

We next used locked nucleic acid antisense oligonucleotides (LNAs) to specifically block and/or degrade [by endogenous RNase H] eRNA PEARL transcripts (Fig. 6A; Bennett and Swayne 2010; Li et al. 2013). RNA-seq experiments showed that there are significant decreases of expression levels of Pcdha genes (Fig. 6B). In addition, DRIP-seq with the S9.6 antibody showed that the H5S-1 R-loop is almost abolished upon LNA treatments (Fig. 6C) compared with no effect on the control NEAT1 R-loop [Supplemental Fig. S4F]. Finally, QHR-4C experiments showed that there is a reduction of long-distance chromatin interactions between the H5S-1 enhancer and its target promoters (Fig. 6D–F). These data suggest that eRNA PEARL transcripts form local R-loops in the H5S-1 enhancer and affect Pcdha gene expression through long-distance chromatin interactions.
Knockdown of R-loops by RNase H1 overexpression

RNase H1 enzyme hydrolyzes the RNA strand of RNA/DNA hybrids (Stein and Hausen 1969; Skourti-Stathaki and Proudfoot 2014; Nojima et al. 2018; Tan-Wong et al. 2019). We constructed a plasmid overexpressing V5-tagged RNase H1 lacking mitochondrial localization signal (Fig. 6G) and confirmed its overexpression by Western blot with a specific antibody against the V5 tag (Fig. 6H). We found that RNase H1 overexpression results in a significant decrease of the eRNA PEARL levels, suggesting that the eRNA PEARL forms DNA–RNA hybrids in vivo (Fig. 6I). In addition, RNase H1 overexpression leads to a significant decrease of expression levels of Pcdhα6, Pcdhα12, and PcdhαC1 (Fig. 6J). Finally, the long-distance chromatin interactions between the HS5-1 enhancer and its target variable promoters are also decreased upon RNase H1 overexpression (Fig. 6K–M). In conjunction with the ChIRP-seq data (Fig. 5), this suggests that the eRNA PEARL forms local RNA/DNA hybrids that may participate in long-distance chromatin interactions.

SPT6 knockdown leads to increased intradomain interactions within the Pcdh superTAD

Recent studies found that transcription elongation factor SPT6 (suppressor of Ty 6) restricts eRNA transcription and R-loop formation in enhancer regions (Nojima et al. 2018). Consistent with this, we found that SPT6 depletion by sequence-specific antisense LNA (Fig. 7A) results in a significant increase of eRNA PEARL expression (Fig. 7B). Interestingly, SPT6 depletion also leads to increased long-distance chromatin interactions between the HS5-1 enhancer and its target promoters (Fig. 7C–E), suggesting that the eRNA PEARL may participate in higher-order chromatin organization.

To further explore 3D chromatin organization mediated by PEARL, we constructed a stable cell line expressing a catalytically dead RNase H1 mutant (D210N) (Supplemental Fig. S5A), which lacks the RNA hydrolyzing catalytic activity but still binds RNA/DNA hybrids. This RNase H1 mutant can bind R-loops but cannot resolve them (Chen et al. 2017). We then performed HiChIP experiments, which are similar to ChIA-PET or in situ
HiC and detect protein-centric genome architecture [Mumbach et al. 2016], with an RNase H1-specific antibody. The overall heat map of HiChIP revealed RNase H1(D210N)-centric (presumably R-loop-mediated) chromatin conformation genome-wide (Supplemental Fig. S5B–D). Interestingly, PEARL depletion results in decreased chromatin interactions in the Pcdh clusters, whereas SPT6 depletion results in increased chromatin interactions (Fig. 7F–H).

Previous studies showed that the three Pcdh clusters are organized into one superTAD with two subTADs [Guo et al. 2015; Jiang et al. 2017b]. Our HiChIP data showed that chromatin interactions are abundant within the Pcdh superTAD [Fig. 7I]. Importantly, PEARL depletion by LNAs leads to significant decreases of intradomain interactions; in contrast, SPT6 depletion results in significant increases of local chromatin interactions within the Pcdh superTAD [Fig. 7J,K]. Given that SPT6 ensures proper eRNA transcription and R-loop formation [Nojima et al. 2018], these data suggest that eRNA PEARL and the formed R-loops participate in the Pcdh higher-order chromatin organization.

**Discussion**

Giving the ongoing debate about whether eRNA transcripts are functional [Kaikkonen and Adelman 2018], our data support that eRNA PEARL is an important regulator for Pcdha gene expression. Owing to the fact that eRNA is transcribed from enhancer DNA sequences, CRISPR genetic methods [deletion or insertion] also perturb the DNA sequences of the enhancer region. To minimize the effects of CRISPR genetic editing, we next used a CRISPR epigenetic method [CRISPR interference or CRISPR activation] to study the function of eRNA transcription and transcripts. Although it remains challenging to distinguish eRNA transcription and transcripts, we attempted to use various strategies to explore the function of eRNA PEARL. In particular, antisense LNAs caused

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**Figure 7.** Enhancing the eRNA PEARL by perturbing SPT6 alters higher-order chromatin organization. (A,B) The SPT6 and eRNA PEARL expression levels after LNA-mediated SPT6 depletion (n = 3). (C–E) QHR-4C interaction profiles with the HS5-1, Pcdha6, or Pcdha12 as a viewpoint after SPT6 depletion (n = 2). (F–H) The interaction heat map of the Pcdh clusters from HiChIP experiments at the 10-kb resolution. (I–K) Catalytic-dead RNase H1(D210N)-centric higher-order chromatin organization within the Pcdh superTAD after perturbing PEARL or SPT6. (L) A model for local R-loop formation of eRNA PEARL regulating Pcdha gene expression through long-distance chromatin looping. The eRNA PEARL expression and R-loop formation maintained by transcription elongation factor SPT6 facilitate proper long-distance chromatin contacts via CTCF/cohesin "loop extrusion." See also Supplemental Figure S5.
specific degradation of local eRNA PEARL transcripts by endogenous RNase H without altering DNA sequences or the spacing between CTCF sites. These data support that the local eRNA PEARL transcripts are functional and are not merely by-products of the HS5-1 enhancer. All in all, the combined data from various experiments suggest that eRNA PEARL regulates Pcdha expression via R-loop formation and promotes long-distance chromatin interactions between distal enhancers and target promoters.

The ~60 clustered Pcdh genes encode large numbers of cadherin-like cell adhesion proteins that function as neural identity tags in individual cells in the brain (Canzio et al. 2019; Wu and Jia 2021). The enormous cell surface repertoire for neuronal identities are achieved by combinatorial expression of ~15 members of the clustered Pcdh genes [two alternate α isoforms, four β isoforms, four alternate γ isoforms, and five C-type Pcdhs] (Guo et al. 2015; Canzio et al. 2019; Jia et al. 2020). Whereas expression of members of the Pcdhβ clusters is regulated by a superenhancer downstream from the Pcdha cluster, members of the Pcdha cluster are regulated by a superenhancer composed of HS5-1 and HS7. We show here, in mouse and cellular models, that HS5-1 transcribes a prominent antisense eRNA [Fig. 1; Supplemental Fig. S1] that forms R-loops locally in the HS5-1 enhancer region [Figs. 5, 6] and is required for the regulation of distal target promoters through modifying higher-order chromatin architecture [Figs. 6, 7]. We recently found that REST/NRSF binds to the location between the PEARL TSS and CBSb and that releasing REST/NRSF initiates the derepression of Pcdha expression [Tang et al. 2021], probably via PEARL transcription because of close location. Our data are consistent with a model that the R-loop formed by eRNA PEARL in the HS5-1 enhancer region participates in 3D chromatin organization to activate the chosen promoters of Pcdh α6, α12, and αC1 genes [Figs. 6, 7]. It is tempting to speculate that R-loop formation at the HS5-1 enhancer might stall the "loop extrusion" of the CTCF/cohesin complex, thus bringing the distal enhancer in close contact with variable promoters. The stochastically chosen Pcdha genes, in conjunction with balanced expression of members of the Pcdh β and γ clusters, determine individual neuron identity in the brain.

Materials and methods

Total RNA-seq

Total RNA-seq, which removes the abundant ribosomal RNAs, was performed as previously described [Ameer et al. 2011] with modifications. For more detailed procedures, see the Supplemental Material.

QHR-4C

Quantitative high-resolution chromosome conformation capture copy (QHR-4C) was performed as recently described [Jia et al. 2020] with modifications. For more experimental details, see the Supplemental Material.

Competing interest statement

The authors declare that they have no competing interests.

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Author contributions: Q.W. conceived and supervised the project. Y.Z. performed most experiments and analyzed data. S.X. helped with the experiments. M.Z. performed the HiChIP experiments. Y.Z. and Q.W. wrote the manuscript.

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