

# Identification of a novel epigenetic regulatory region within the pluripotency associated microRNA cluster, EEmiRC

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## ABSTRACT

**The miR-290 cluster is expressed in embryonic stem cells (ESCs) and is important for the maintenance of pluripotency, but little is known about the mechanisms regulating the early embryonic microRNA cluster (EEmiRC) expression. Here we report the identification of a 332-bp intragenic enhancer (IE) able to modulate the transcription of the mouse EEmiRC locus, presumably through binding of transcription modulators like Oct3/4, Sox2 and CTCF. This IE also contains a CpG island showing a differential pattern of DNA and histone methylation marks during differentiation of ESCs, which places EEmiRC in a novel regulatory feedback loop with DNA methylases. Deletion of IE significantly reduced the transcription of the EEmiRC, further proving the importance of this region in regulating the expression of EEmiRC.**

## INTRODUCTION

Embryonic stem cells (ESCs) have the unique potential to self renew and differentiate into all three germ layers, which involves coordinated changes in the balances between multiple signaling pathways (1,2). This exquisitely tight coordination involves regulation at the transcriptional level as well as at the post-transcriptional level through the action of small non-coding microRNAs (miRs) (2,3). MicroRNAs are 22–25-nt-long genome-encoded small RNAs able to post-transcriptionally modulate gene expression by base-pairing to target sites in the 3'-UTR of mRNAs. Biogenesis of mature microRNAs involves the obligatory generation of two intermediates: pri-miRNA (long, polyadenylated, capped transcript produced by PolII), which is further cleaved by a microprocessor complex formed by DGCR8 and Drosha to pre-microRNAs, short (60–70 nt) stem-looped

transcripts exported into the cytoplasm by exportin-5. Pre-microRNAs are recognized by a Dicer/TRBP/Ago complex and further cleaved to mature 22–25-nt-long microRNA duplexes. The leading strand of the mature microRNA duplex is loaded onto the RNA-induced silencing complex (RISC or miRISC) and heterodimerize with complementary sequences in the 3'-UTRs of target mRNAs, thus serving as a guide for RISC repression (4).

The vast majority of the miRNAs are located within introns (mostly in antisense orientation), in intergenic/non-protein-coding regions or can be organized in gene-like units, equipped with dedicated promoters driving transcription of capped and poly-adenylated pri-miRNAs (5). MicroRNA clusters are particularly interesting given that the coordinated expression of an entire set of mature microRNAs might theoretically achieve the simultaneous modulation of the fate of hundreds of mRNA targets.

A typical example of a microRNA gene is the early embryonic microRNA cluster (EEmiRC) identified in ESCs of placental mammals, and showing a remarkable cross-eutherian species conservation at the levels of both pre-miRNA hairpins and the core-promoter region (6,7). EEmiRC encodes seven microRNAs (miR-290, -291a, -292, -291b, -293, -294 and -295), which have been labeled as ESC-specific/pluripotency-associated microRNAs controlling cell-cycle progression, proliferation and DNA methylation (8–10) in undifferentiated/pluripotent cells. Therefore, understanding the biology of ESCs requires the detailed knowledge of the mechanisms regulating EEmiRC expression.

Surprisingly little is known about this subject. High through-put genome wide ChIP analysis showed that the sequences upstream to the EEmiRC promoter contains active binding sites for Nanog, Oct3/4, Sox2, Tcf3, c-Myc and 4n-Myc, and is H3K4 trimethylated in ESCs and H3K27 trimethylated in differentiated cells (2,11,12). However, attempts to activate EEmiRC expression by ectopic expression of these individual TFs in fibroblasts

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were unsuccessful and it has been suggested that EEmiRC expression is under epigenetic control (12). In this study, we have identified within the EEmiRC a previously uncovered region able to regulate the expression of miR290s, which involves pluripotency factors and epigenetic mechanisms in pluripotent and differentiated cells.

## MATERIALS AND METHODS

### Cell culture and differentiation

Mouse ESCs, P19CL6 carcinoma cells, mouse embryonic fibroblasts (MEF) and HEK293T cells were cultured following standard procedures. ZHBTc4 ES cells were described previously (13). Differentiation of ES cells into neural progenitor cells was performed essentially as described in ref. (14) with some modifications.

### miRNA and RNA isolation and quantitative RT-PCR

miRNA and total RNA were isolated using *miRVana* micro RNA isolation kit (Ambion). cDNA synthesis was carried out using standard procedures. miRNA quantification was done using Taqman assays (Applied biosystems).

### Plasmids, transfection and reporter assays

All the reporter constructs were cloned into either pGL-4.23 or pGL-4.23dMP plasmids. pEEmiRC plasmid was described previously (7). Transfection of all reporter plasmids, pEEmiRC-EGFP, pEEmiRC-dEn-II.1a-GFP and pEEmiRC-dTATA-GFP constructs was performed using Lipofectamine LTX reagent. Renilla luciferase served as transfection control and luciferase activities were represented as relative light units.

### Bisulphite sequencing and *in vitro* methylation

Bisulfite sequencing was performed using the EZ methylation kit (Zymo research). *In vitro* methylation of pGL-4.23 empty vector and EnII.1a fragment containing vector was done using *M.SssI* (New England Biolabs) and the efficiency of *in vitro* methylation was checked using methyl sensitive restriction enzyme *HpaII*.

### Chromatin immunoprecipitation

Chromatin immunoprecipitations (ChIPs) were performed as described previously (11). Briefly, formaldehyde cross-linked cells were lysed and sonicated to shear the DNA. The sonicated DNA was pre cleared using agarose beads and then immuno complexes were formed using respective antibodies. The immuno complexes were collected using protein A/G agarose beads and finally DNA was eluted. The eluted DNA and 1% of respective input DNA was reverse cross-linked and used for the qPCR using SYBR Green qPCR mix with ROX (Fermentas).

### Statistical analysis

All experiments were performed at least in triplicates. Statistical significance was tested using the *t*-test (paired, two-tailed).

A detailed description of all materials and methods used can be found in the Supplementary Text.

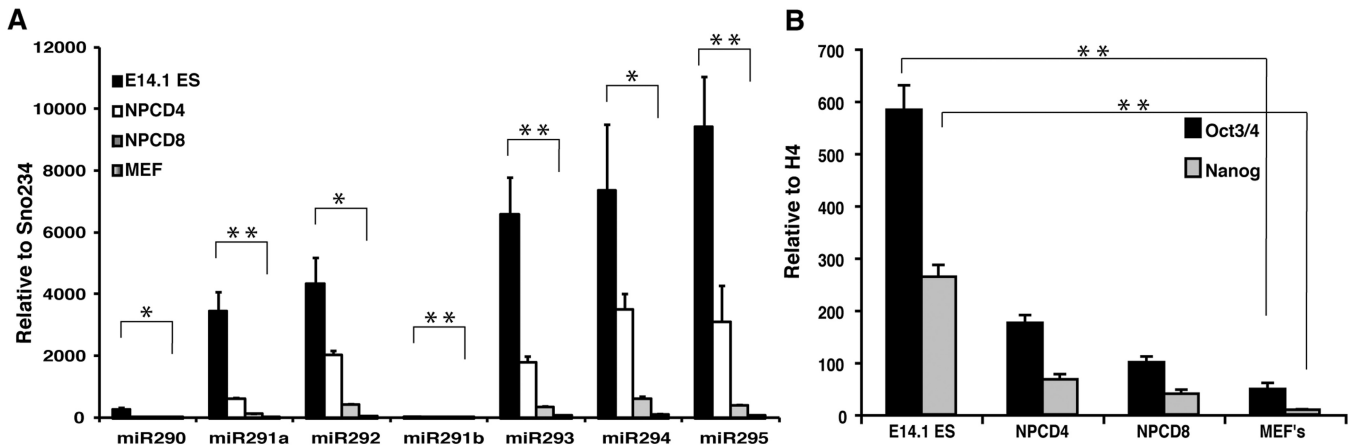
## RESULTS

### Transcriptional attenuation of EEmiRC during differentiation

The EEmiRC genomic locus is spread over 3.2kb on chromosome 7 and is organized into two hemiclusters (hC) separated by a stretch of 782nt: 5'-hC (containing pre-miR-290, -291a, -292 and -291b) and 3'-hC (containing pre-miR-293, -294 and -295). Quantitative RT-PCR analysis of EEmiRC mature microRNAs levels in ESCs showed strong discrepancies between the expression levels of the miR290s, with a minimum for miR-291b (less than 1/10 of the Sno243 level) and a maximum for miR-295. For the moment we can only speculate on these discrepancies as reflecting the differences in the turnover of the miR290s, since microRNA stability has been shown to be an important component of microRNA homeostasis (15,16). However, the expression of all miRs is gradually attenuated along ESCs differentiation (e.g. towards neural progenitor cells, NPC), becoming negligible in MEF (Figure 1A and B).

### An intragenic enhancer within the EEmiR cluster

In an attempt to find novel regulatory sequences governing the transcription of EEmiRC locus, we examined the genomic region separating the two hemiclusters. We found that two partially overlapping inter-hC DNA fragments, namely En-II (from +1419 to +2205, overlapping with the pre-miR291b locus) and En-II-4 (from +1828 to +2205) are able to drive *Luciferase* transcription when cloned in a pGL-4.23 vector, with En-II activity being significantly higher than En-II-4. In strong contrast, an En-I fragment (from -3423 to -240) covering a region previously shown to harbor Oct-4/Sox2 active binding sites (2) failed to activate luciferase transcription when cloned into the same vector (Figure 2A and B). Furthermore, the En-II fragment was not able to activate *Luciferase* transcription when cloned into a promoter-less vector pGL-4.23dMP, indicating enhancer activity. Deletion analysis of En-II ultimately led to the identification of a 332 bp fragment En-II.1a (from +1419 to +1751) able to drive *Luciferase* transcription with the same efficiency as the entire En-II fragment when expressed from a pGL-4.23 construct driven by a minimal promoter in either HEK293T, mouse ESCs or P19CL6 undifferentiated embryonal carcinoma cells (Figure 2B). Experiments performed in ESCs showed that when cloned downstream of the *Luciferase* in a pGL-4.23dMP-miR290-Pro vector, the En-II-1.a fragment is able to interact with and enhance the transcription from the upstream endogenous EEmiRC promoter (Figure 2C). Further more, in order to mimic the *in vivo* context of the EEmiR Cluster, we have deleted En-II.1.a from a pEEmiRC (7) construct holding a EGFP-tag fused downstream of miR295 and found a significant decrease in the level of *EGFP* transcripts, further suggesting that the 332 bp fragment is required for



**Figure 1.** Expression of members of the miR-290s and pluripotency factors gradually decreases during E14.1 ES cells differentiation. (A) Quantitative RT-PCR analysis of EEmiRC miRs in ES (black bar), neural progenitor cell at Day 4 of differentiation (NPCD4) (white), neural progenitor cell at Day 8 of differentiation NPCD8 (gray) and MEFs (white/gray); expression levels were normalized relative to Sno234. (B) Expression levels of Oct-3/4 and Nanog (relative to Histone4, H4) in ES, NPCD4, NPCD8 and MEF's. Data are represented as means  $\pm$  SEM of at least three independent experiments. \* $P < 0.05$ ; \*\* $P < 0.01$ .

enhancing the transcription from the miR290 promoter (Figure 2D).

We noticed that the luciferase activity readings were consistently higher in ESCs and P19CL6 experiments compared to the HEK293T ones (Figure 2B), suggesting that En-II.1a transcriptional activity benefits from the presence of pluripotency factors in ESCs and P19CL6 cells. This hypothesis would be in concordance with data showing that the decrease in the expression of pluripotency factors Oct3/4 and Nanog during ESC differentiation correlates with the decrease in miR290s expression (Figure 1A and B) (7). Of note, Marson *et al.* (2) described Oct3/4, Nanog and Sox2 ChIP-enrichment 5'-upstream of the transcription initiation site of the EEmiRC, but not within the cluster. Since the construct lacking the En-II.1a (pEEmiRC-dEnII.1a-EGFP) expresses the EGFP at significantly lower levels when transfected into mESC (Figure 2D), we were interested in understanding the mechanisms by which En-II.1a exerts its effects upon EEmiRC transcription.

#### Binding of Oct-3/4, Sox2 and CTCF is enriched at the IE

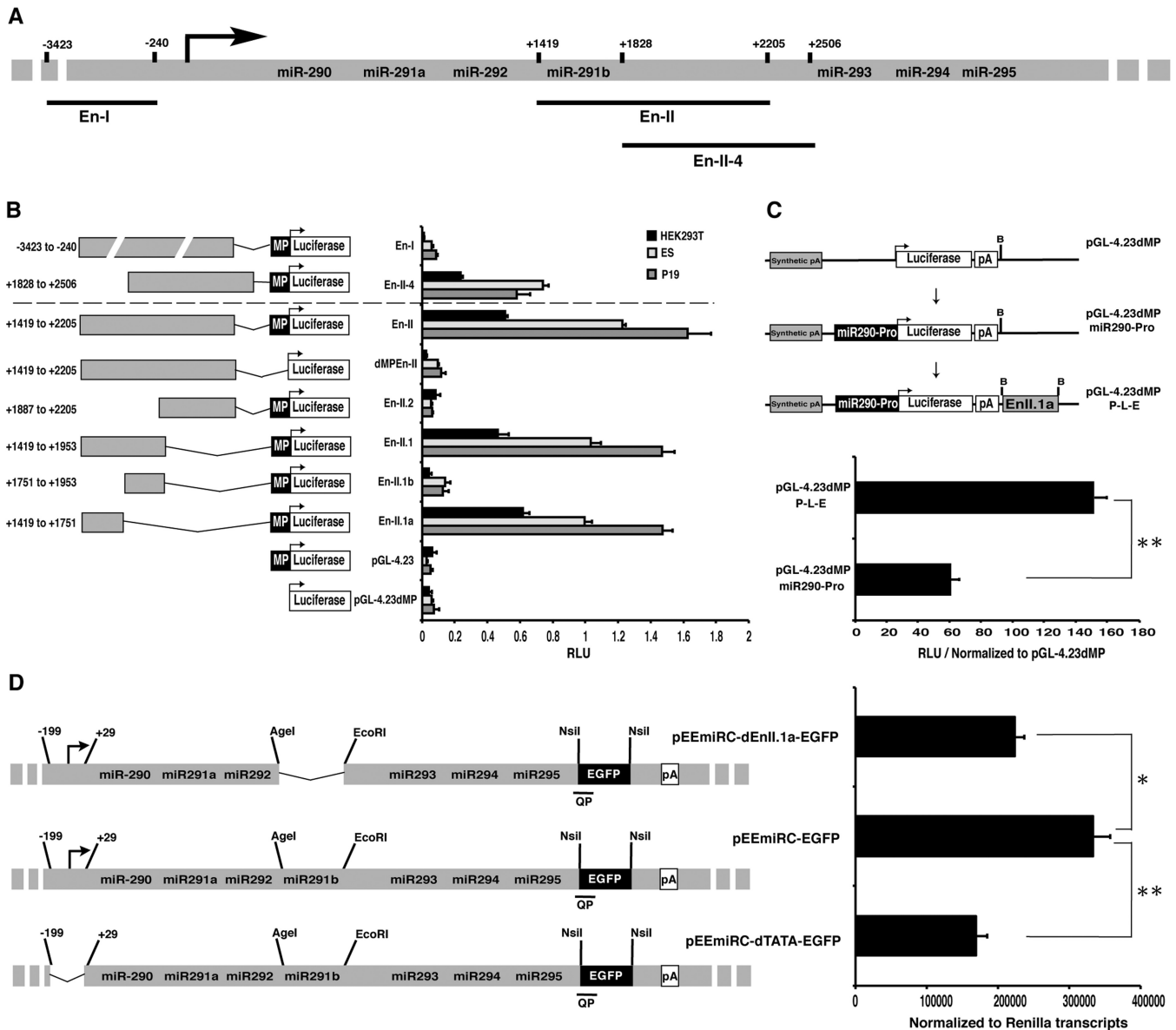
We asked whether the En-II.1a or its vicinity is occupied by factors currently known to modulate pluripotency. ChIP analysis of the genomic region separating the two hCs showed a significant enrichment in binding events for Oct3/4, Sox2 and CCCTC-binding factor (CTCF) in two specific domains: within the En-II.1a (designated P3) and 3' of En-II.1a (designated P4) (Figure 3). Surprisingly, the DNA regions upstream (P1) and downstream (P2) of the transcription initiation site show a much lower level of occupancy as it might have been anticipated from previously published Oct3/4 and Sox2 genome-wide ChIP experiments (3). Consistent with previous data showing that Oct3/4 cooperates with Sox2 and CTCF to drive transcription of target genes (11,17,18), the level of occupancy of both P3 and P4 domains gradually and dramatically decreases during ESC differentiation, suggesting a functional role for these transcription factors in regulating

EEmiRC expression (Figure 3). Within the inter-hC region (overlapping En-II and En-II-4), the binding sites for Oct4, Sox2 and CTCF are situated in close proximity, suggesting a cooperative mode of action (Supplementary Figure S1). Furthermore, Oct3/4 appears to coordinate the recruitment of both Sox2 and CTCF to P3 and P4 regions, as the occupancy for these factors is strongly reduced in doxycycline-inducible Oct3/4-knockout ESCs (ZHBTc4; 13) upon addition of doxycycline (Figure 3F-H), while the total protein levels of Sox2 and CTCF are minimally changed (Supplementary Figure S2).

#### Epigenetic marks at the EEmiRC IE

A characteristic of the genes responsible for early development of the embryos is their ambivalent epigenetic signature, i.e. associate both repressive marks (like H3K27me3) and active marks (like H3K4me3), suggestive for a ready-to-transcribe status of these genes in the highly dynamic chromatin context of pluripotent cells (19). Many of the ambivalently marked chromatin regions also associate binding-enrichment of Oct4/Sox2 transcription factors (20) while Sox2 has been shown to directly activate histone demethylases involved in ESC self-renewal (21,22). CTCF has an ambivalent role. CTCF binding sites have been shown to be associated to H3K27me3-marked chromatin domains (23,24); CTCF is also supposed to protect unmethylated CpG-containing DNA regions from silencing through DNA methylation by blocking PARP1-Dnmt1 crosstalk (25). We have therefore asked whether En-II.1a also associates epigenetic marks like DNA methylation and histone-3 methylation.

Bioinformatics analysis of the EEmiRC locus revealed the presence of 12 CG repeats in a 222 bp CpG island within the En-II.1a (Supplementary Figure S1). We have analyzed the methylation status of this CpG island by bisulphate sequencing and found that the decrease in miR290's expression during ESCs differentiation associates a progressive methylation of the 12 CG-repeats. To confirm the functional role of the CpG island, we

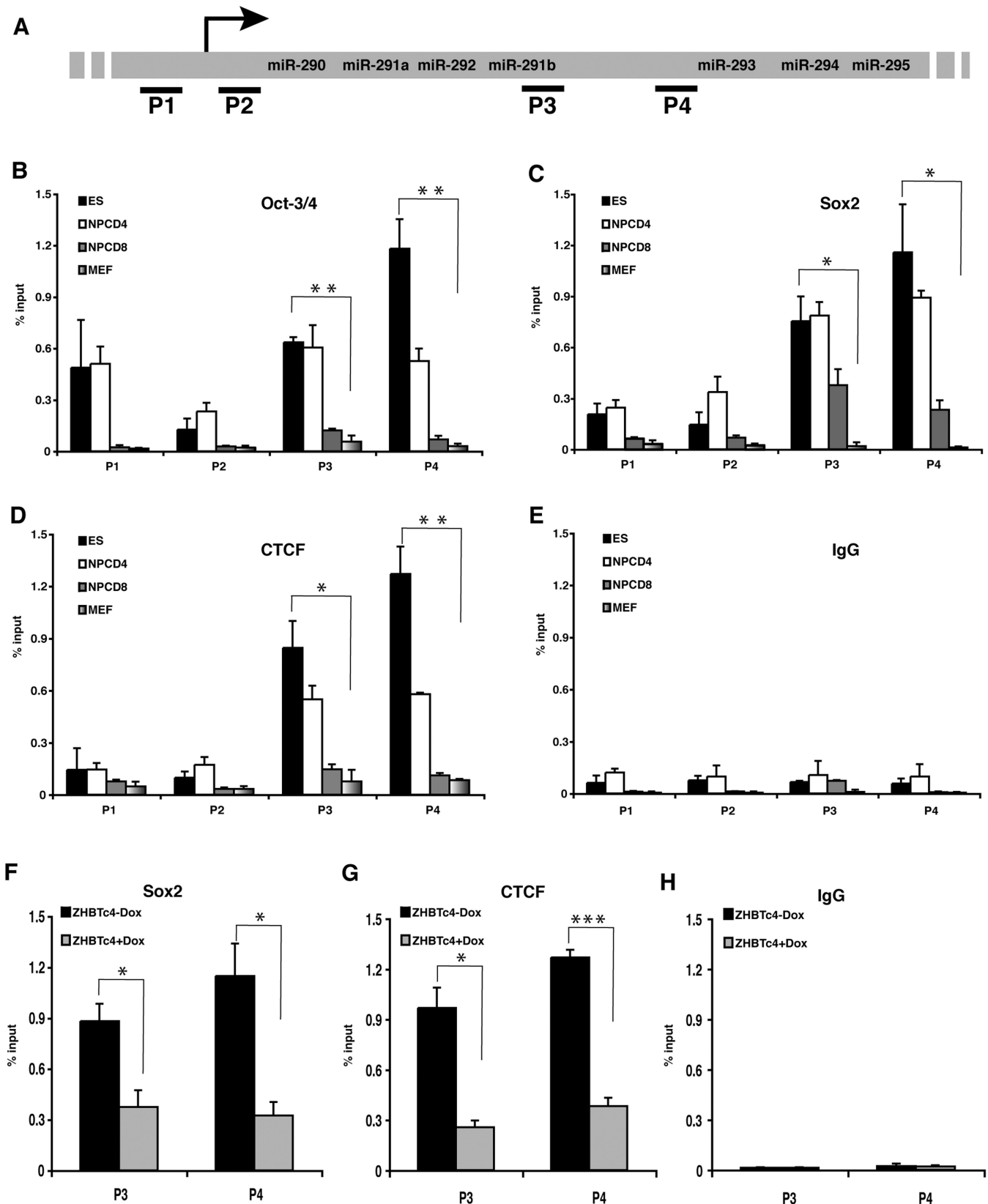


**Figure 2.** Identification of En-II.1a enhancer within the EEmiR cluster. (A) Schematic representation of genomic regions used in the luciferase reporter assays; nucleotide positions are relative to the EEmiRC transcriptional start site. The different genomic regions of the inter-hC were cloned in to pGL-4.23 and pGL-4.23dMP (without the minimal promoter) vector and tested for the ability to promote luciferase transcription after transfection in HEK293T, mESCs and P19CL6 cells. (B) Delineation of the inter-hC enhancer using deletion constructs. The 332bp fragment (En-II.1a) shows significant enhancer activity in HEK293T, mESCs and P19CL6 cells. (C) The En-II.1a fragment cloned downstream of *Luciferase* enhances transcription from the endogenous miR290 promoter in a pGL-4.23dMP vector transfected in mESCs. Data are normalized to pGL-4.23dMP readings and represented as mean  $\pm$  SD from three different experiments. (D) Deletion of En-II.1a significantly reduces the level of expression of *EGFP* from a pEEmiRC-EGFP construct transfected into mESCs. Data are normalized to *Renilla* transcripts and represented as mean  $\pm$  SD from three different experiments. \* $P < 0.05$ ; \*\* $P < 0.01$ .

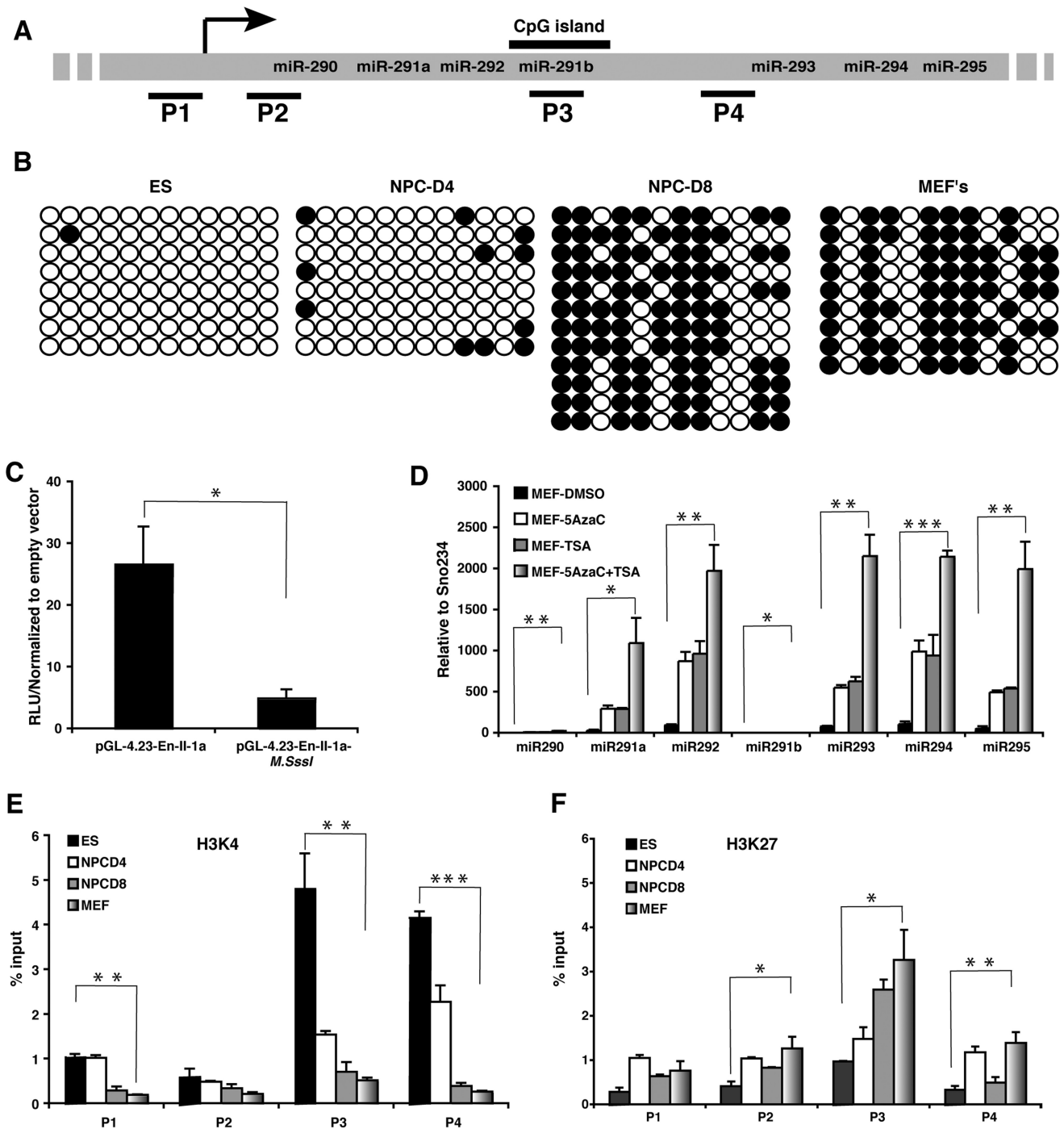
performed *in vitro* methylation of the pGL-4.23-En-II.1a construct using the CpG methyltransferase *M.SssI* (26), and found a strong decrease of *Luciferase* activity after methylation of the pGL-4.23-En-II.1a vector (Figure 4C and Supplementary Figure S3). Conversely, culturing MEFs in the presence of the demethylating agent 5-azacytidine (27) partially restored the expression of EEmiRC; this effect that was further enhanced by the addition of histone deacetylase inhibitor Trychostatin A (TSA) (Figure 4D), a synergistic effect previously

shown to resuscitate the transcription of epigenetically silenced genes (28,29). Of note, in our assay, miR-291b expression cannot be restored either by 5-Aza, TSA nor by the combination of the two compounds. ChIP analysis of the P3 and P4 domains of the inter-hCs region shows that the H3K4me3 (30) enrichment found in ESCs is gradually lost during differentiation in favor of silencing marks like H3K27me3 (31), further proving that the region is subjected to heavy epigenetic regulation (Figure 4E and F).





**Figure 3.** En-IL1a and its vicinity are highly enriched in Oct3/4, Sox2 and CTCF. (A) Schematic representation of the EEmiRC genomic regions (P1–P4) subjected to ChIP-qPCR analysis. P3 and P4 are highly enriched in Oct3/4 (B), Sox2 (C) and CTCF (D) when compared to IgG control (E). Doxycyclin-induced depletion of Oct3/4 in ZHBTc4 ES cells strongly reduces the recruitment of Sox2 (F) and CTCF (G) to P3 and P4 regions when compared to IgG controls (H). Data are represented as mean  $\pm$  SD from three different experiments. \* $P < 0.05$ ; \*\* $P < 0.01$ ; \*\*\* $P < 0.001$ .



**Figure 4.** En-II.1a enhancer is subject to epigenetic regulation. (A) Schematic representation of the 222 bp CpG island within the inter-hC region. (B) DNA methylation analysis of the 12 CG dinucleotides of the CpG island in ES, NPCD4, NPCD8 and MEFs using the bisulphite-sequencing approach. Each column represents a CpG dinucleotide, each row represents the number of clones analysed. Open circles represents the unmethylated CpGs, closed circles represents the methylated CpGs. (C) *In vitro* methylation of pGL-4.23-En-II.1a construct using *M.SssI* leads to a loss of the enhancer activity as assessed by luciferase assay. The enhancer activities were normalized to *M.SssI*-treated and -untreated empty vectors and represented as relative luciferase units (RLU). (D) Treatment of MEFs with the DNA methyltransferase inhibitor 5'-azacytidine (5'-azaC), HDAC inhibitor trichostatin A (TSA) or a combination of both reactivates the expression of the members of the EEmiRC. (E and F) ChIP-qPCR analysis of P1-P4 enrichment in H3K4 (E) and H3K27 (F) trimethylation in ESCs (black bar), NPCD4 (white bar), NPCD8 (gray bar) and MEFs (white/gray bar). Data in (C-F) are represented as mean  $\pm$  SD from three different experiments. \* $P < 0.05$ ; \*\* $P < 0.01$ ; \*\*\* $P < 0.001$ .

## DISCUSSION

EEmiRC represent not only the majority (over 70%) of the microRNAs in ESCs (6), but also one of the best-characterized (so far) ES-specific miRNAs: it promotes proliferation of ESCs by regulating G1-S transition (8), controls *de novo* DNA methylation (including Oct3/4 promoter) (9,10) and enhances the ability of Oct3/4, Sox2 and Klf4 to re-programme MEFs (12). Genome-wide ChIP experiments suggested that EEmiRC expression is controlled by Oct3/4 (3) through binding (together with other pluripotency factors like Sox2 and Nanog) to the promoter region (2,11), which also bears histone methylation marks: H3K4-me3 (activation mark) in ESCs and H3K27me3 (repressive mark) in differentiated cells (17).

Our study shows that an intragenic enhancer (IE) residing in the inter-hC region and not only the EEmiRC promoter is required for the integration of miR290s in the ESCs specific auto-regulatory circuit. The binding events to IE of all the transcription factors used in our work (Oct3/4, Sox2, CTCF) are much more abundant when compared to the region comprising the putative EEmiRC promoter as defined by previous studies (2,7,12). Our *in silico* analysis suggests that these TF binding sites are densely clustered in two regions at the 5' and 3' of the inter-hC region, implying an extensive local inter-cooperativity. Therefore, the EEmiRC inter-hC region harbors a multiple transcription factor-binding locus with ESC enhancer activity (12), in which the recruitment of TFs (and most probably their subsequent transcriptional synergy) is governed by Oct3/4. We have also shown that in ESCs, Oct4/Sox2/CTCF clustering to inter-hC associates H3K4me3 mark characteristic for active genomic regions, thus portraying the IE as a *bona fide* ESC-specific enhanceosome as described by Chen and colleagues (12). From this point of view, it would be interesting to analyze the functional significance and hierarchy of occupancy for all the TFs predicted to bind to the IE, including the functionally versatile CCCTC-binding factor, CTCF (32).

The cross-talk between microRNAs and the epigenetic effectors has lately received considerable attention, since its perturbation has been shown to be associated with malignant characteristics of tumors in humans (33). miR290s have been shown to be modulators of epigenetic machinery (9,10), but the epigenetic control of their expression has been only marginally investigated (12). Our data show that IE confers epigenetic sensitivity to EEmiRC through both histone modifications and the methylation of a novel CpG island within the En-II.1a. As a rule of thumb, the promoters and enhancers of pluripotency associated factors are hypomethylated in ESCs (but hypermethylated in their differentiated derivatives) and associate H3K3 trimethylation (progressively replaced by H3K27 trimethylation during differentiation) (34,35). In this respect, the epigenetic signature of IE in EEmiRC makes no exception from the rule, being similar to that of Oct3/4 (36) or Nanog (37): is CpG hypomethylated and associates H3K4me3 in pluripotent ESCs and CpG hypermethylated and associates H3K27me3 in differentiated NPCs.

Therefore, the EEmiRC-IE allows the Dnmts to negatively feed back on EEmiRC and functionally close a feedback loop with effects on the level of miR290s expression. However, since the effect of miR290s on Dnmts has been shown to be specific to ESC behavior (10), we suggest that this negative feedback loop is governed by pluripotency factors such as Oct3/4 and Sox2.

By integrating inputs from Oct3/4, Sox2 and CTCF with epigenetic cues (through methylation of the CpG island and association with histone3 methylation marks), the EEmiRC-IE represents the module required for the insertion of the miR290s into the complex stem cell-specific regulatory circuit of pluripotency factors, epigenetic mechanisms and microRNAs (38).

## SUPPLEMENTARY DATA

Supplementary Data are available at NAR Online.

## ACKNOWLEDGEMENTS

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## REFERENCES

- Smith,A.G. (2001) Embryo-derived stem cells: of mice and men. *Annu. Rev. Cell Dev. Biol.*, **17**, 435–462.
- Marson,A., Levine,S.S., Cole,M.F., Frampton,G.M., Brambrink,T., Johnstone,S., Guenther,M.G., Johnston,W.K., Wernig,M., Newman,J. *et al.* (2008) Connecting microRNA genes to the core transcriptional regulatory circuitry of embryonic stem cells. *Cell*, **134**, 521–533.
- Tay,Y., Zhang,J., Thomson,A.M., Lim,B. and Rigoutsos,I. (2008) MicroRNAs to Nanog, Oct4 and Sox2 coding regions modulate embryonic stem cell differentiation. *Nature*, **455**, 1124–1128.
- Kim,V.N., Han,J. and Siomi,M.C. (2009) Biogenesis of small RNAs in animals. *Nat. Rev. Mol. Cell. Biol.*, **10**, 126–139.
- Liu,N., Okamura,K., Tyler,D.M., Phillips,M.D., Chung,W.J. and Lai,E.C. (2008) The evolution and functional diversification of animal microRNA genes. *Cell Res.*, **18**, 985–996.
- Houbaviy,H.B., Murray,M.F. and Sharp,P.A. (2003) Embryonic stem cell-specific MicroRNAs. *Dev. Cell*, **5**, 351–358.
- Houbaviy,H.B., Dennis,L., Jaenisch,R. and Sharp,P.A. (2005) Characterization of a highly variable eutherian microRNA gene. *RNA*, **11**, 1245–1257.
- Wang,Y., Baskerville,S., Shenoy,A., Babiarz,J.E., Baehner,L. and Blelloch,R. (2008) Embryonic stem cell-specific microRNAs regulate the G1-S transition and promote rapid proliferation. *Nat. Genet.*, **40**, 1478–1483.

9. Benetti,R., Gonzalo,S., Jaco,I., Muñoz,P., Gonzalez,S., Schoeftner,S., Murchison,E., Andl,T., Chen,T., Klatt,P. *et al.* (2008) A mammalian microRNA cluster controls DNA methylation and telomere recombination via Rbl2-dependent regulation of DNA methyltransferases. *Nat. Struct. Mol. Biol.*, **15**, 268–279.
10. Sinkkonen,L., Hugenschmidt,T., Berninger,P., Gaidatzis,D., Mohn,F., Artus-Revel,C.G., Zavolan,M., Svoboda,P. and Filipowicz,W. (2008) MicroRNAs control de novo DNA methylation through regulation of transcriptional repressors in mouse embryonic stem cells. *Nat. Struct. Mol. Biol.*, **15**, 259–267.
11. Chen,X., Xu,H., Yuan,P., Fang,F., Huss,M., Vega,V.B., Wong,E., Orlov,Y.L., Zhang,W., Jiang,J. *et al.* (2008) Integration of external signaling pathways with the core transcriptional network in embryonic stem cells. *Cell*, **13**, 1106–1117.
12. Judson,R.L., Babiarz,J.E., Venero,M. and Belloch,R. (2009) Embryonic stem cell-specific microRNAs promote induced pluripotency. *Nat. Biotechnol.*, **27**, 459–461.
13. Niwa,H., Miyazaki,J. and Smith,A.G. (2000) Quantitative expression of Oct-3/4 defines differentiation, dedifferentiation or self-renewal of ES cells. *Nat. Genet.*, **24**, 372–376.
14. Bibel,M., Richter,J., Lacroix,E. and Barde,Y.A. (2007) Generation of a defined and uniform population of CNS progenitors and neurons from mouse embryonic stem cells. *Nat. Protocols*, **2**, 1034–1043.
15. Chatterjee,S. and Grosshans,H. (2009) Active turnover modulates mature microRNA activity in *Caenorhabditis elegans*. *Nature*, **461**, 546–549.
16. Bail,S., Swerdel,M., Liu,H., Jiao,X., Goff,L.A., Hart,R.P. and Kiledjian,M. (2010) Differential regulation of microRNA stability. *RNA*, **16**, 1032–1039.
17. Rizzino,A. (2009) Sox2 and Oct-3/4: a versatile pair of master regulators that orchestrate the self-renewal and pluripotency of embryonic stem cells by functioning as molecular rheostats. *Wiley Interdiscip. Rev. Syst. Biol. Med.*, **1**, 228–236.
18. Donohoe,M.E., Silva,S.S., Pinter,S.F., Xu,N. and Lee,J.T. (2009) The pluripotency factor Oct4 interacts with Ctf and also controls X-chromosome pairing and counting. *Nature*, **460**, 128–132.
19. Bernstein,B.E., Mikkelsen,T.S., Xie,X., Kamal,M., Huebert,D.J., Cuff,J., Fry,B., Meissner,A., Wernig,M., Plath,K. *et al.* (2006) A bivalent chromatin structure marks key developmental genes in embryonic stem cells. *Cell*, **125**, 315–326.
20. Abu-Remaileh,M. and Bergman,Y. (2009) Epigenetic signature of embryonic stem cells: a DNA methylation perspective. In Rajasekhar,V.K. and Vemuri,M.C. (eds), *Regulatory Networks in Stem Cells*. Humana Press, New York, pp. 247–256.
21. Loh,Y.H., Wu,Q., Chew,J.L., Vega,V.B., Zhang,W., Chen,X., Bourque,G., George,J., Leong,B., Liu,J. *et al.* (2006) The Oct4 and Nanog transcription network regulates pluripotency in mouse embryonic stem cells. *Nat. Genet.*, **38**, 431–440.
22. Loh,Y.H., Zhang,W., Chen,X., George,J. and Ng,H.H. (2007) Jmjd1a and Jmjd1c histone H3 Lys 9 demethylases regulate self-renewal in embryonic stem cells. *Genes Dev.*, **21**, 2545–2557.
23. Li,T., Hu,J.F., Qiu,X., Ling,J., Chen,H., Wang,S., Hou,A., Vu,T.H. and Hoffman,A.R. (2008) CTCF regulates allelic expression of Igf2 by orchestrating a promoter-polycomb repressive complex 2 intrachromosomal loop. *Mol. Cell. Biol.*, **28**, 6473–6482.
24. Cuddapah,S., Jothi,R., Schones,D.E., Roh,T.Y., Cui,K. and Zhao,K. (2009) Global analysis of the insulator binding protein CTCF in chromatin barrier regions reveals demarcation of active and repressive domains. *Genome Res.*, **19**, 24–32.
25. Guastafierro,T., Cecchinelli,B., Zampieri,M., Reale,A., Riggio,G., Sthandier,O., Zupi,G., Calabrese,L. and Caiafa,P. (2008) CCCTC-binding factor activates PARP-1 affecting DNA methylation machinery. *J. Biol. Chem.*, **283**, 21873–21880.
26. Renbaum,P., Abrahamov,D., Fainsod,A., Wilson,G.G., Rottem,S. and Razin,A. (1990) Cloning, characterization, and expression in *Escherichia coli* of the gene coding for the CpG DNA methylase from *Spiroplasma* sp. strain MQ1(M.SssI). *Nucleic Acids Res.*, **18**, 1145–1152.
27. Christman,J.K. (2002) 5-Azacytidine and 5-aza-2'-deoxycytidine as inhibitors of DNA methylation: mechanistic studies and their implications for cancer therapy. *Oncogene*, **21**, 5483–5495.
28. Jones,P.L., Veenstra,G.J., Wade,P.A., Vermaak,D., Kass,S.U., Landsberger,N., Strouboulis,J. and Wolffe,A.P. (1998) Methylated DNA and MeCP2 recruit histone deacetylase to repress transcription. *Nature Genet.*, **19**, 187–191.
29. Cameron,E.E., Bachman,K.E., Myöhänen,S., Herman,J.G. and Baylin,S.B. (1999) Synergy of demethylation and histone deacetylase inhibition in the re-expression of genes silenced in cancer. *Nat. Genet.*, **21**, 103–107.
30. Santos-Rosa,H., Schneider,R., Bannister,A.J., Sherriff,J., Bernstein,B.E., Emre,N.C., Schreiber,S.L., Mellor,J. and Kouzarides,T. (2002) Active genes are tri-methylated at K4 of histone H3. *Nature*, **419**, 407–411.
31. Cao,R., Wang,L., Wang,H., Xia,L., Erdjument-Bromage,H., Tempst,P., Jones,R.S. and Zhang,Y. (2002) Role of histone H3 lysine 27 methylation in Polycomb-group silencing. *Science*, **298**, 1039–1043.
32. Phillips,J.E. and Corces,V.G. (2009) CTCF: master weaver of the genome. *Cell*, **137**, 1194–1211.
33. Valeri,N., Vannini,I., Fanini,F., Calore,F., Adair,B. and Fabbri,M. (2009) Epigenetics, miRNAs, and human cancer: a new chapter in human gene regulation. *Mamm. Genome*, **20**, 573–580.
34. Ram,E.V.S.R. and Meshorer,E. (2009) Transcriptional competence in pluripotency. *Genes Dev.*, **23**, 2793–2798.
35. Efroni,S., Duttgupta,R., Cheng,J., Dehghani,H., Hoepfner,D.J., Dash,C., Bazett-Jones,D.P., Le Grice,S., McKay,R.D., Buetow,K.H. *et al.* (2008) Global transcription in pluripotent embryonic stem cells. *Cell Stem Cell*, **2**, 437–447.
36. Hattori,N., Nishino,K., Ko,Y.G., Ohgane,J., Tanaka,S. and Shiota,K. (2004) Epigenetic control of mouse Oct-4 gene expression in embryonic stem cells and trophoblast stem cells. *J. Biol. Chem.*, **279**, 17063–17069.
37. Hattori,N., Imao,Y., Nishino,K., Ohgane,J., Yagi,S., Tanaka,S. and Shiota,K. (2007) Epigenetic regulation of Nanog gene in embryonic stem and trophoblast stem cells. *Genes Cells*, **12**, 387–396.
38. Kashyap,V., Rezende,N.C., Scotland,K.B., Shaffer,S.M., Persson,J.L., Gudas,L.J. and Mongan,N.P. (2009) Regulation of stem cell pluripotency and differentiation involves mutual regulatory circuit of the Nanog, OCT4 and SOX2 pluripotency transcription factors with polycomb repressive complexes and stem cell microRNAs. *Stem Cells Dev.*, **18**, 1093–1108.