

# Reverse engineering a mouse embryonic stem cell-specific transcriptional network reveals a new modulator of neuronal differentiation

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## ABSTRACT

Gene expression profiles can be used to infer previously unknown transcriptional regulatory interaction among thousands of genes, via systems biology ‘reverse engineering’ approaches. We ‘reverse engineered’ an embryonic stem (ES)-specific transcriptional network from 171 gene expression profiles, measured in ES cells, to identify master regulators of gene expression (‘hubs’). We discovered that *E130012A19Rik* (*E13*), highly expressed in mouse ES cells as compared with differentiated cells, was a central ‘hub’ of the network. We demonstrated that *E13* is a protein-coding gene implicated in regulating the commitment towards the different neuronal subtypes and glia cells. The overexpression and knock-down of *E13* in ES cell lines, undergoing differentiation into neurons and glia cells, caused a strong up-regulation of the glutamatergic neurons marker *Vglut2* and a strong down-regulation of the GABAergic neurons marker *GAD65* and of the radial glia marker *Blbp*. We confirmed *E13* expression in the cerebral cortex of adult mice and during development. By immuno-based affinity purification, we characterized protein partners of *E13*, involved in the Polycomb complex. Our results suggest a role of *E13* in

regulating the division between glutamatergic projection neurons and GABAergic interneurons and glia cells possibly by epigenetic-mediated transcriptional regulation.

## INTRODUCTION

Embryonic stem (ES) cells derive from the inner cell mass of blastocyst-stage embryos (1,2). The ES properties to self-renew (3) and differentiate in all three germ layers both *in vitro* and *in vivo* (4,5) have made these cells a unique *in vitro* system for studying the molecular mechanisms that regulate lineage specification. High-throughput experimental techniques, combined to the use of systems biology approaches to infer gene regulatory networks (reverse engineering), have shown promise in the elucidation of stem cell renewal and differentiation (6).

In this work, starting from a collection of ~200 gene expression profiles (GEPs) generated in mouse ES cells following overexpression of single genes (7), we ‘reverse engineered’ a transcriptional network encompassing ES-specific genes to identify master regulators of gene expression in ES cells (‘hubs’). We discovered that a previously uncharacterized gene, *E130012A19Rik* (*E13*), highly expressed in mouse ES cells as compared with differentiated cells, is a central ‘hub’ of the network. We generated *E13*-overexpressing and *E13* knock-down ES clones. We performed transcriptome analysis of these

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clones and demonstrated an enrichment of differentially expressed genes involved in axon guidance and neuronal differentiation. By immune-based affinity purification, we identified protein–protein interactions of E13 with components of the Polycomb chromatin remodelling complex and proteins involved in transcriptional regulation. We found that ES cells overexpressing *E13* and differentiated into neurons and glial cells show up-regulation of the glutamatergic neurons marker *Vglut2* (8) and down-regulation of both the  $\gamma$ -aminobutyric acid (GABA)ergic neuron marker *GAD65* (9,10) and of the radial glia marker *Blbp* (11,12), as compared with wild-type ES clones. We further demonstrated that E13 is specifically expressed in the developing and adult cerebral cortex. Taken together our results show that E13 has a role in regulating the commitment towards the different neuronal subtypes and glia cells.

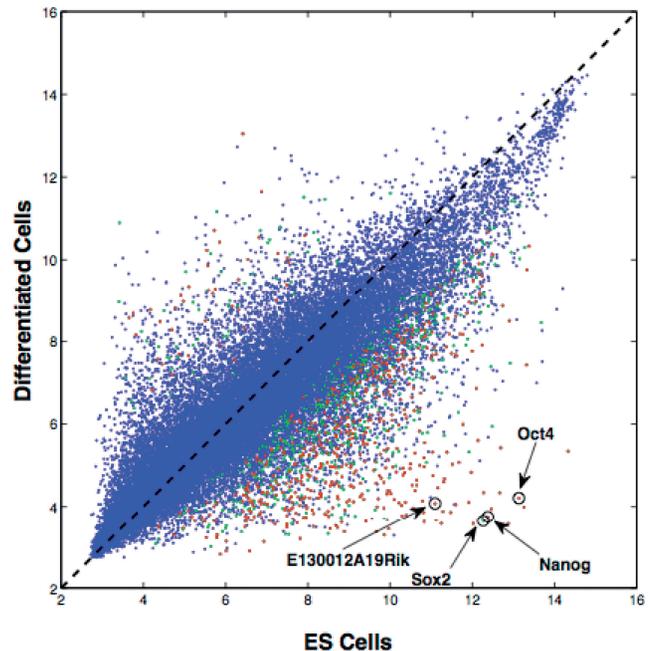
## MATERIALS AND METHODS

### Data analysis of differentially expressed genes in ES cells versus differentiated cells

We compared our collection of 171 ES-specific GEPs (GSE19836 and GSE32015) to a collection of 180 GEPs derived from normal mouse tissues and differentiated cell lines (GSE10246) (13). The two data sets were first normalized together using the RMA algorithm (14). The median was chosen as measure of the expression values for each probe set within each data set. The variability of the data was taken into account by dividing this measure by a pooled variance given by the sum median absolute deviation of the genes expression values in the two data collections. Each probe set was thus associated with two coordinates representing median expression in the ES-specific data set and in the differentiated data set, and thus represented as a dot in Figure 1. The distance from the diagonal was computed, and an empirical *P*-value and a corresponding false discovery rate (FDR) were estimated to identify ES-specific transcripts.

### Regulatory network inference

We used ARACNe (15) on 171 microarray experiments (GSE19836 and GSE32015) to reconstruct the transcriptional regulatory network (Supplementary File S1) in mouse ES cells, following the steps shown in Supplementary Figure S1. The gene network among the 45 101 transcripts (probe sets) was inferred using as a significance threshold for the mutual information (MI) a  $P < 0.001$  and setting the data processing inequality threshold to 0.01. The expression value of each probe set was averaged across biological replicates before ARACNe analysis, and a low-entropy filter was applied to remove probe sets whose changes were not significant across the data set, thus removing 4511 probe sets. The low-entropy filter removes non-informative probe sets by computing the entropy of each probe set across the data set as described in (16). Probe sets with entropy values less than the 10th percentile were removed from further analysis.



**Figure 1.** Scatter plot of the median gene expression level in mouse ES cells versus differentiated cells. The median level of expression of each gene in mouse ES cells (*x*-axis) and in differentiated cells (*y*-axis) is represented as a dot. The diagonal line corresponds to the set of genes with the same expression in ES cells versus differentiated cells. Genes corresponding to dots significantly off the diagonal (in magenta, genes with FDR < 0.025 and in green FDR < 0.05) represent either genes whose expression is lower in ES cells than in differentiated cells (above the diagonal), or genes whose expression higher in ES cells than in differentiated cells (below the diagonal). Some ES-specific markers (*Oct4*, *Nanog* and *Sox2*) and the novel ES differentially expressed gene *E130012A19Rik* are highlighted.

We validated the inferred network by computing the positive predictive value [PPV = TP/(TP + FP)] against two different 'Golden Standards' (GS): (i) the Reactome database: containing experimentally validated interactions from the literature (Supplementary File S2); and (ii) the ESCAPE (Embryonic Stem Cell Atlas from Pluripotency Evidence) database: containing putative transcription factor (TF)–messenger RNA (mRNA) regulatory interactions predicted from gene expression profiling in mouse ES cells (Supplementary File S3). The PPV represents the percentage of correctly inferred interactions, i.e. those interactions confirmed by one of the two GS. To compute the PPV, we first converted transcripts to genes and then selected only those genes present also in the 'Golden Standard' (and their inferred interactions).

The number of predicted interactions in the inferred transcript-wise network is 299 610 among 40 590 transcripts, whereas the gene-wise network has 131 587 interactions among 17 645 genes.

The ESCAPE GS and the inferred gene-wise network have in common 14 151 of 17 645 genes. Among these 14 151 genes, there are 107 663 interactions in the ESCAPE GS, and 91 925 interactions in the inferred network. Therefore, the random PPV for the ESCAPE GS is equal to  $107\,663 / [(14\,151^2 - 14\,151) / 2] = 0.0011$ .

The Reactome GS and the inferred gene-wise network have in common 3087 genes of 17 645 genes. Among those 3087 genes, there are 53 933 in the Reactome GS, and 4973 interactions in the inferred network. Therefore, the random PPV for the Reactome GS is equal to  $53\,933 / [(3087^2 - 3087) / 2] = 0.0113$ .

We also built a smaller ES-specific subnetwork by selecting only the 543 ES-specific genes (FDR < 0.025) and the genes they were connected to in the network (gene neighbours). To identify ES-specific 'hub genes', we first ranked the 5863 probe sets in the ES-specific sub-network according to their degree (i.e. the number of interactions a probe set has in the network) and retained only the top 100 probe sets with the highest degree. We then ranked the 5863 probe sets according to their ES-specific expression, i.e. with the smallest FDR (as detailed in the 'Identification of genes prevalently expressed in mouse ES cells' section) and retained only the top 100 probe sets with the most specific expression. Finally to identify ES-specific 'hub genes', we intersected the two lists of probe sets (highest degree versus the most specific expression) to obtain the 14 probe sets in Figure 2.

### Generation of ES clones

E14Tg2a.4 [E14 (17)] and EBRTcH3 [EB3 (18)] parental cell lines were used. The EB3 cell line (18) was obtained from the laboratory of Dr Hitoshi Niwa as previously described in (7). Mouse ES cells were grown as previously described (7). The two E13-inducible cell lines (not-tagged and 3xFLAG-tagged) were derived from the EB3 cell line. For the generation of two exchange vectors (pPTHC-E13 and pPTHC-E13-3xFLAG), we used the vector pPthC-Oct-3/4 obtained from the laboratory of Dr Hitoshi

Niwa (18) and modified it as in (7). The primer pair for the generation of the two E13-inducible cell lines and for the selection of positive clones, performed as previously described, (7) are reported in Supplementary Table S1.

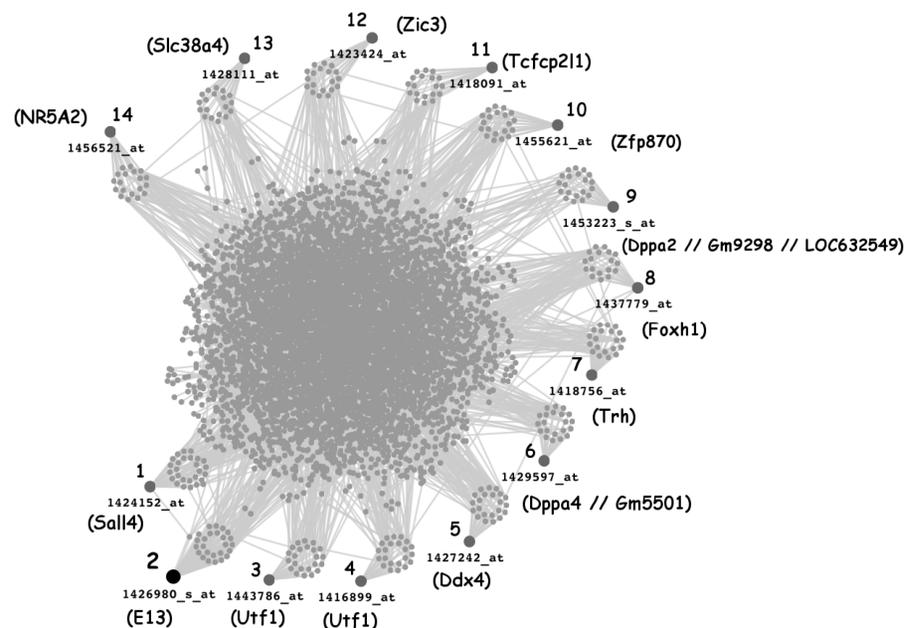
The knock-down control (shCTL) clones and the E13 knock-down (shE13) clones were derived from the E14 cell line. For the generation of the pSuper.neo-shE13 and pSuper.neo-shGFP plasmids, the pSuper.neo vector (Oligoengine, Seattle, WA, USA) was used. The knock-down of E13 mRNA was verified by quantitative real-time polymerase chain reaction (q-PCR) on total RNA extracted from three shE13 clones (shE13 A7, shE13 C1 and shE13 C4), as compared with three shCTL clones (shC B6, shC C1 and shC C3). The primer pair used for q-PCR was the E13-affy primer pair reported in Supplementary Table S1. Full details of the protocol can be found in (7).

### Induction of transgene expression in E13-inducible cell lines

Three inducible non-tagged clones (C1, C3 and C6) and two inducible 3xFLAG-tagged clones (B5 and B8) were thawed, amplified and tested for transgene induction as previously described (7). q-PCR experiments were performed using LightCycler 480 II (Roche) for signal detection. Primers are reported in Supplementary Table S1.

### Microarray hybridization and differential gene expression analysis

For the analysis of E13-inducible cell clones, microarray hybridization experiments were performed on three biological replicates of non-tagged clones (C1, C3 and C6) induced in mouse ES media deprived of tetracycline (Tc)



**Figure 2.** ES-specific subnetwork inferred from the analysis of 171 GEPs. A reverse engineering algorithm was applied to the set of 171 GEPs from ES cells comprising >45 000 transcripts. The resulting network was used to obtain an ES-specific subnetwork by selecting only the 543 genes with ES-specific expression and the genes they were connected to. We then identified 'hub' genes in the subnetwork (numbered from 1 to 14) by ranking the 543 genes according to the number of connections.

for 48 h. As control, the same clones were grown in mouse ES media containing 1 µg/ml of Tc. Similarly, for the E13 knock-down cell line, experiments were performed on three biological replicates for the shE13 clones (shE13 A7, shE13 C1 and shE13 C4) and on two biological replicates for the shCTL clones (shC C1 and shC C3). Three micrograms of total RNA from each clone were used and hybridized to the Affymetrix GeneChip Mouse Genome 430\_2 array (Mouse 430\_2) using standard protocols. Differentially expressed genes were detected by a Bayesian *t*-test method [Cyber-*t* (19)] followed by FDR correction. The thresholds used were FDR < 0.05 for the induction set and FDR < 0.10 for the knock-down set (different FDR thresholds were selected to have a comparable number of false positives in the two data sets).

For the analysis of the Sugino microarray data set [(20) GSE2882], data were normalized using the RMA method (14). The normalized microarray data relative to E13 were extracted and a one-way analysis of variance (21) was carried out. The Tukey multiple comparison procedure was then performed to identify cell types displaying a statistical difference in the expression of *E13*.

#### ES cell differentiation protocol and data analysis

Mouse ES cells were differentiated towards neurons and glial cells using the one-step differentiation method (22). The differentiation procedure was applied (i) to two E13-inducible non-tagged (C1 and C3) and to two 3xFLAG-tagged clones (B5 and B8); and (ii) to two shE13 knock-down clones (shE13 A7 and shE13 C4) and to two shCTL knock-down clones (shC C1 and shC C3). The morphology of differentiated cells was followed using a stereomicroscope (MZ16FA, Leica Microsystems, Wetzlar, Germany); images were acquired on a DFC 320 camera (Leica). For each time course expression profile of the selected markers, we used a statistical modelling approach based on Gaussian processes (GPs) to identify those markers that were significantly affected by *E13* overexpression or knock-down as described in (23). GPs enable to quantify the true signal and noise embedded in a GEP over time and moreover provide a ranking of the genes according to their differential expression. The method estimates the continuous trajectory of the gene expression by means of GP regression. In particular, given an observed GEP, two different hypothesis H1 and H2 are compared: either the gene is truly differential expressed (H1) or the observed profile is just the effect of random noise (H2). The log-ratio of the marginal likelihoods (llr) measures which of the two hypotheses is more likely, with positive values indicating that hypothesis H1 is more likely, and vice versa for negative values.

#### Western blotting and immunofluorescence analysis

Fractionated cell lysates from EB3 (CTL) cells and from two 3xFLAG-tagged clones (B5 and B8) induced for 24 and 48 h were prepared. Cytoplasmic (Cyt) and nuclear (Nuc) fractions were obtained using standard protocol (24). Forty micrograms of total protein extracts were fractionated on 8% sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) gel,

whereas 15 µg of fractionated protein extracts were separated on a 10% SDS-PAGE gel. Western blotting was then performed as previously described (7), and the following primary antibodies were used: a mouse monoclonal anti-Flag M2-peroxidase antibody (Sigma) and a mouse monoclonal anti-β-Tubulin (Sigma).

For immunofluorescence analysis, ES cell clones were plated at a density of 1000 cells/cm<sup>2</sup> on gelatine-coated 24-well plates and induced for 48 h in mouse ES media and in differentiation medium, respectively, deprived of Tc. The same clones grown in medium containing Tc were used as control. The following primary antibodies were used: an anti-Vglut2 (Abcam, ab79157), an anti-GAD65 (G1166, Sigma) and an anti-Blbp antibody (Abcam, ab32423). As secondary antibodies, we used AlexaFluor594 goat anti-mouse (1:400, Molecular Probes, Invitrogen) or AlexaFluor594 goat anti-rabbit (1:400, Molecular Probes, Invitrogen). In all immunofluorescence analysis performed, the 4',6-diamidino-2-phenylindole (10 µg/ml, Sigma) in phosphate buffered saline was used to stain the nucleus. Labelling was detected by fluorescent illumination using an inverted microscope (DMIRB, Leica Microsystems, Wetzlar, DE); images were acquired on a DC 350 FX camera (Leica).

#### Co-immunoprecipitation (co-IP), mass spectrometry and liquid chromatography

Co-immunoprecipitation (co-IP) was performed using the Sigma FLAG Immunoprecipitation Kit (Sigma Aldrich) according to the manufacturer's instructions. A total of 10<sup>7</sup> cells from two inducible 3xFLAG-tagged clones (B5 and B8) and two control clones from EB3 parental cell line (EB3/1 and EB3/2) were lysed for co-IP experiment. A cell-free negative control and a positive control with 50 ng of FLAG-tagged bacterial alkaline phosphatase (BAP) protein were applied. After the elution of immuno-precipitated proteins, the samples were fractionated on SDS-PAGE (Bio-Rad mini format) followed by coomassie staining for the visualization of the quality of the samples. Each gel strip was cut into 20 equal-sized gel slices and subjected to MS database-based protein identification after tryptic digestion. Liquid chromatography was performed on an Easy-nLC device (Proxeon, Denmark), which is directly coupled to ESI-MS analysis. Liquid chromatography (LC)/electrospray ionization mass spectrometry (ESI-MS)/MS was performed on a LCQ Deca XP ion trap instrument (Thermo Finnigan, Waltham, MA, USA). ESI-MS data acquisition was performed throughout the LC run. Three scan events, (i) full scan; (ii) zoom scan of most intense ion in (i); and (iii) MS/MS scan of most intense ion in (i) were applied sequentially. No MS/MS scan was performed on single charged ions. The raw data were extracted by TurboSEQUENT algorithm; trypsin autolytic fragments and known keratin peptides were filtered out. All DTA files of the same original sample (20 gel slices) generated by Sequest were merged and converted to mascot generic format files. The mascot generic format files were searched using our Mascot Version 2.1 in-house license.

The MS/MS ion searches against ExPasy UniProt were performed with the following parameters: taxonomy *Mus musculus*, one trypsin miss-cleavage accepted, monoisotopic mass values, peptide and fragment mass tolerance  $\pm 0.8$  Da. Oxidation of methionine and acrylamide adducts on cysteine was expected as variable modification. Protein hits corresponding to  $P < 0.05$  (Mowse score  $>28$ ) were considered as significant protein identifications. FDRs were estimated based on matches to reversed sequences in the concatenated target–decoy database. A maximum FDR of 0.01 at both peptide and protein levels was tolerated. Protein–protein interaction data were submitted directly to Database of Interacting Proteins (DIP) database.

To confirm some of the protein interactions identified by MS/MS, we proceeded as follows. Polyclonal rabbit IgGs against the following proteins were used: embryonic ectoderm development (EED) (Anti-EED, Millipore, 09-774), suppressor of zeste 12 (Anti-SUZ12 C-terminal region, Aviva Systems Biology, ARP39190\_P050), general TF IIE beta (Anti-GTF2E2, Proteintech, 11 596-1-AP). The FLAG peptide tag (rabbit IgG against FLAG peptide epitope, Sigma) was used to detect *E13* expression in the E13-inducible ES clones with the 3xFLAG before and after IP experiment. Forty micrograms of protein extracts of the following eight samples were first separated by SDS-PAGE using the Laemmli system (Bio-Rad mini electrophoresis running chamber, 12% Polyacrylamide, gel format: 7.5  $\times$  8 cm): E13-inducible clone B5 (B5) after FLAG-Tag IP, E13-inducible clone B8 (B8) after IP, control cell clone Eb3/A2 (K1) after IP, control cell clone Eb3/B2 (K2) after IP, E13-inducible clone B5 before IP, E13-inducible clone B8 before IP, control cell clone Eb3/A2 before IP and control cell clone Eb3/B2 before IP. For the western blotting against FLAG-tag, a 49-kDa FLAG-BAP fusion protein from *Escherichia coli* (Sigma-Aldrich, F7425) was used as the positive control (PK) for the immunoblotting methodology. Subsequently, proteins were transferred onto the supported nitrocellulose membrane using the standard procedure (semi-dry transfer chamber, 40 mA, 1.5 h). The One-Hour Western standard kit, including secondary rabbit antibody and TMB substrate, (GenScript L00204T, according to the manufacturer's instruction) was used for the immunodetection and visualization.

### Immunohistochemistry (IHC) and *in situ* hybridization (ISH)

Mouse tissue sections were prepared using standard protocols (25) Antisense RNA probes were labelled using a DIG-RNA labelling kit (Roche). The following probes were used: *Gad67* and *E13* (1100-nt length, containing the *E13* complete coding DNA sequence). RNA *in situ* hybridization (ISH) hybridization procedures, combined or not with standard immunohistochemistry (IHC), were performed as previously described (26). For IHC, the following primary antibodies were used: anti-Blbp, rabbit polyclonal antibody (1:100, Abcam); anti-GFAP rabbit polyclonal antibody (1:400, Dako); anti-Tbr2, rabbit polyclonal antibody (1:1000, Chemicon); anti-NeuN, mouse

monoclonal antibody, (1:200, Chemicon); anti-Vglut1, mouse monoclonal antibody, (1:100, Millipore); anti-Calbindin D-28k, rabbit polyclonal antibody (1:2500, Swant) and anti-TH, rabbit polyclonal antibody (1:200, Cell Signaling). All experiments in mice were conducted following guidelines of the Institutional Animal Care and Use Committee, Cardarelli Hospital (Naples, Italy).

## RESULTS

### Identification of genes prevalently expressed in mouse ES cells

In a previous study, we generated a collection of 120 GEPs measured using microarrays in mouse ES cells by overexpressing 20 mouse orthologous of human chromosome 21 genes [Gene Expression Omnibus (GEO) accession number: GSE19836] (7). We have now extended this collection by overexpressing eight more genes, comprising mostly TFs (Supplementary Table S2), thus generating 51 additional GEPs (GEO accession number: GSE32015).

We analysed the entire data set of 171 GEPs to identify genes whose expression is enriched in mouse ES cells, as compared with differentiated cells. To this end, we compared our collection of ES-specific GEPs (GSE19836 and GSE32015) with a collection of 180 GEPs derived from normal mouse tissues and differentiated cell lines (GSE10246) (13). Both sets were obtained with the same microarray platform (Affymetrix Mouse 430\_2), enabling a homogeneous comparison.

To identify genes that are significantly more expressed in ES cells versus differentiated cells, we computed the 'median' expression level of each probe set in the two gene expression data set (ES cells and differentiated cells), which is shown as a dot in Figure 1. We then computed the distance from the diagonal (corresponding to the set of probe sets with the same expression levels in both ES and differentiated cells). We retained for further analysis only those probe sets whose distance from the diagonal was determined to be statistically significant. This approach enabled the identification of 543 genes that were significantly more expressed in ES cells versus non-ES cells (FDR  $< 0.025$ ), independently of their absolute level of expression ('ES-specific genes'), among which many known 'stemness' genes, such as *Oct4* [*Pou5f1* (27)] and *Nanog* (28) (Supplementary Table S3). The gene ontology enrichment analysis (GOEA) (29,30) performed on the list of 543 genes confirms that this set is strongly enriched for stemness-related processes (Supplementary Table S4), such as stem cell differentiation (GO:0048863 –  $p = 9.6 \times 10^{-10}$ ), stem cell maintenance (GO:0019827 –  $p = 3.8 \times 10^{-7}$ ) and stem cell development (GO:0048864 –  $p = 5.4 \times 10^{-7}$ ), thus confirming the validity of our approach and its potential for the discovery of novel genes involved in ES cell fate regulation.

### Identification of a novel mouse gene as central 'hub' of an ES-specific gene regulatory network

'Reverse engineering' approaches allow to infer gene–gene regulatory interactions by computational analysis of

GEPs (31,32). We applied an information-theoretic approach, named ARACNe (15), which uses a generalization of pairwise correlation coefficient, called MI, to identify co-regulated genes from GEPs. ARACNe was run on the collection 171 ES-specific GEPs. The resulting gene network consists of 299 610 predicted interactions among 40 590 transcripts. To measure the reliability of the inferred network, we used two GS data sets: (i) a collection of 153 920 putative TF–mRNA regulatory interactions obtained from studies of TF loss- or gain-of-function followed by mRNA microarray profiling in mammalian ES cells (ESCAPE, <http://www.maayanlab.net/ESCAPE/index.php>); and (ii) a smaller set of high-quality experimentally verified functional interactions found in the Reactome database, an open-source, open-access, manually curated and peer-reviewed interaction database (33). We then computed the percentage of correctly inferred interactions (PPV) by ranking interactions by the value of their MI ('Materials and Methods' section). As shown in Supplementary Figure S2 for the ESCAPE database and Supplementary Figure S3 for the Reactome database, the inferred interactions are significantly enriched for functional interactions.

We then applied a hierarchical clustering algorithm based on the Jaccard distance to discover communities within the network (34–36). A community is defined as a set of genes strongly co-regulated with each other, but with few interactions with other genes in the network. We thus identified 53 communities (Supplementary Table S5). For each community, we performed GOEA to identify biological functions significantly enriched among genes within the community (Supplementary Table S6).

To better analyse this large network, we built a smaller subnetwork, as shown in Figure 2, by selecting the 543 ES-enriched genes (FDR < 0.025) described earlier and the genes they were connected to in the network (gene neighbours). This subnetwork comprises 5863 transcripts and 12 944 interactions among them. A graph containing the complete workflow, data sources and cut-offs threshold used to obtain the final ES-specific subnetwork is reported in Supplementary Figure S1.

We identified 'hub genes' in this subnetwork, by selecting genes with the highest number of inferred regulatory interactions, which were specifically expressed in ES cells ('Materials and Methods' section). As shown in Figure 2, hub genes comprise the sal-like 4 (*Sall4*) TF, a known regulator of stem cells pluripotency (37); the genes of the Dppa family, known to be involved in pluripotency and stemness (38); the zinc finger protein of the cerebellum 3 (*Zic3*), which is required for maintenance of pluripotency in ES cells (39) and the undifferentiated embryonic cell TF 1 [*UTF1*, (40–41)]. Interestingly, we found that one of the ES-specific 'hubs' was a gene with unknown function, *E130012A19Rik* (hereafter abbreviated as *E13*).

To gain initial insight into its function, we performed GOEA on the 26 genes connected to *E13* in the network (Supplementary Table S7C). Such a 'guilty-by-association' approach has already been successfully applied to predict gene function (42). The results of this analysis suggested a role of *E13* in transcriptional regulation and nucleic acid

metabolism (Supplementary Table S7C). Moreover, *E13* belongs to network community 2 (Supplementary Table S5), which is significantly enriched for biological processes such as: pattern specification process (GO:0007389 –  $p = 7e-4$ ), axon guidance (GO:0007411 –  $p = 0.02$ ), embryonic morphogenesis (GO:0048598 –  $p = 0.02$ ) and embryonic development (GO:0009790 –  $p = 0.03$ ) (Supplementary Table S6).

We also performed a bioinformatic analysis of the *E13* sequence. This gene is predicted to encode a hypothetical protein product (*LOC103551*, <http://www.ncbi.nlm.nih.gov/gene/>) that is highly conserved across vertebrates (Supplementary Figure S4A), and contains a proline-rich domain [PROSITE database (43)] (Supplementary Figure S4B). Analysis of the predicted 3D structure suggests that part of *E13* protein has similarity to the DNA-directed RNA polymerase II. However, it differs from other RNA polymerases because of the presence of additional peripheral 3D structures not present in normal RNA polymerases. Three putative phosphorylation sites (S15, S19 and S22) were annotated in PhosphoSite database (44) (Supplementary Figure S4C). In addition, *E13* may be regulated by KLF4, MYC, NANOG, OCT4, REST, SOX2, TCF3 and TRIM28 according to chromatin immunoprecipitation experiments reported in (45).

### Generation of *E13* transgenic mouse ES cell lines

We generated three stable mouse ES cell lines: two inducible cell lines overexpressing *E13*, which only differ for the presence in one of them of a 3xFLAG epitope at the C-terminus of the transgene-coding sequence, and one cell line in which *E13* was stably knocked-down (Supplementary Figure S5).

q-PCR analysis ('Materials and Methods' section) confirmed that the expression of the *E13* mRNA was induced on the removal of Tc from the medium in both the overexpressing clones (Supplementary Figure S5A and B). Moreover, we verified the correct induction of the *E13* protein product and determined its intracellular localization by western blot (Supplementary Figure S6) with a FLAG-specific monoclonal antibody in the inducible 3xFLAG-tagged cell line ('Materials and Methods' section). As shown in Supplementary Figure S6A, we detected an ~43-kDa band corresponding to the *E13*-3xFLAG expected protein product (the *E13* molecular weight is about 41 kDa plus 2.4 kDa of the 3xFLAG peptide), confirming that *E13* is a protein-coding gene. The *E13* protein appears to be present in both cytoplasmic and nuclear fractions (Supplementary Figure S6B).

The knock-down cell line (sh*E13* clones) was generated by stably expressing a specific short hairpin RNA against the *E13* sequence, thus knocking-down *E13* expression in the parental mouse ES cell line [E14 (17)]. As control, we selected a short hairpin RNA against the green fluorescent protein reporter, thus generating control knock-down clones (shCTL clones) ('Materials and Methods' section). The extent of inhibition of *E13* expression was efficient (~90%) and comparable in the three different sh*E13* clones generated (Supplementary Figure S5C).

## Transcriptome analysis of E13 transgenic mouse ES cell lines

We performed gene expression profiling experiments using Affymetrix microarrays in both inducible not-tagged clones ( $n = 3$ ) and in shE13 clones ( $n = 3$ ) (GSE31701). We found that both the overexpression and knock-down of *E13* perturbed the transcriptome in a statistically significant manner ('Materials and Methods' section). In Supplementary Table S8 and in Supplementary Table S9, we report the complete list of differentially expressed genes in the two sets of experiments.

To obtain a high-confidence set of genes responsive to *E13*, we selected only those genes ( $n = 221$ ) whose expression levels changed consistently (i.e. increased in E13 overexpressing clones and decreased in knock-down shE13 clones or vice versa—'Materials and Methods' section and Supplementary Table S10). A GOEA of the 221 high-confidence genes revealed a significant enrichment for biological processes related to axon guidance, axonogenesis and other processes involved in neurogenesis (Table 1).

To better characterize the role of *E13*, we subdivided the list of 221 genes in two sublists: the first list (UP) consists of 145 genes up-regulated by the induction of *E13* and down-regulated by its knock-down (Supplementary Table S11); the second list (DOWN) includes 76 genes down-regulated by the induction of *E13* and up-regulated by its knock-down (Supplementary Table S12). GOEA revealed that the UP list contains genes involved in anatomical structure development, axon guidance and other related processes, which suggest a possible involvement of E13 in neurogenesis (Supplementary Table S13). On the other hand, the DOWN list consists of genes involved in the regulation of biosynthetic process and regulation of gene expression, suggesting a role of E13 on global transcriptional regulation (Supplementary Table S14), and confirming the results obtained by the 'guilty-by-association' approach on the ES network.

We also explored the network of the genes surrounding *E13*, i.e. all the nodes at a Distance 2 from E13 (Figure 3), and obtained a subnetwork composed by 106 genes and 128 connections. We found that 63 of 106 genes were

indeed perturbed in their expression level by E13 (Supplementary Table S15).

## Identification of protein interaction partners of E13

We performed immuno-based affinity purification experiments followed by mass spectrometric protein identification (46) using the E13-inducible ES clones with the 3xFLAG ('Materials and Methods' section). We identified 23 potential protein partners of E13 with high-confidence (Table 2), among which there are two TFs [Gtf2e2, Btf3 (47)], several mRNA processing proteins and two components of the Polycomb complex [Eed, Suz12 (48) and the retinoblastoma binding protein Rbbp4, which may be involved in pluripotent stem cell maintenance and neuronal differentiation (49)]. Twelve of 23 transcripts corresponding to this subset of proteins were also differentially expressed following *E13* overexpression (FDR < 0.1) (Supplementary Table S16).

To further confirm these protein interactions, we selected 3 of the 23 proteins, taking into account the availability of antibodies and their biological functions: Suz12 and EED (part of the Polycomb complex) and the general TF GTF2E2. We then immune-precipitated E13 using the anti-3xFLAG antibody in E13 overexpressing clones followed by western blot analysis with the three antibodies against the selected proteins (the experiment was performed in duplicate) ('Materials and Methods' section). For all the three proteins, we were able confirm the interaction of E13 with 3 of 23 potential protein partners, as shown in Supplementary Figure S7.

The interaction of E13 with these proteins further suggests a role of E13 in mRNA processing and epigenetic regulation (Supplementary Table S17).

## E13 is a modulator of neuronal differentiation

Results of the bioinformatic analysis and of the transcriptomics and proteomics experiments suggested a role of E13 in regulating gene expression and neurogenesis. To confirm a role of E13 in this process, we specifically differentiated both E13-inducible and knock-down ES clones into neurons and glia cells using the one-step differentiation method (22).

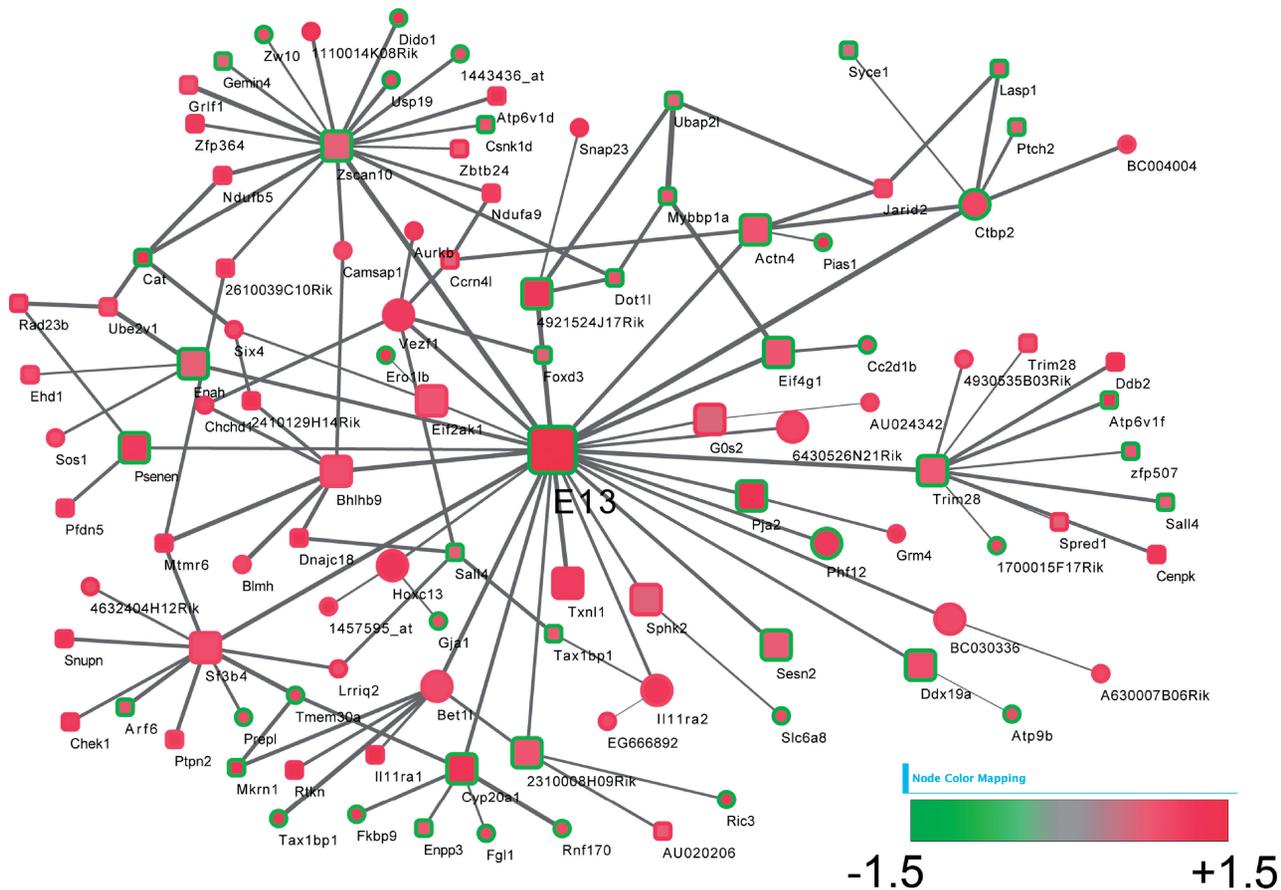
We first verified, by western blot analysis, the expression of the E13 protein, at Days 10 and 15 of the differentiation protocol (Supplementary Figure S8). We then verified by q-PCR the potential of the inducible and of the knock-down E13 clones to differentiate along the neuronal and glial cell lineages. To this purpose, we collected RNA samples at different time points and analysed the expression profiles of *E13* and of the markers of undifferentiated ES cells [*Oct4* (27)], and of neuronal precursors [*Nestin* (50) and *Neurog1* (51)].

Figure 4 shows the expression profiles of *E13* (Figure 4A), *Oct4* (Figure 4B), *Nestin* (Figure 4C) and *Neurog1* (Figure 4D) in the inducible clones (left panels) and in the knock-down clones (right panels). All of the clones displayed the expected down-regulation of the pluripotency gene *Oct4*. We observed that during differentiation the expression profiles of the neuronal precursor

**Table 1.** Significantly enriched GOEA terms for the 221 high-confidence genes

Gene ontology terms	FDR	Fold enrichment	P-value
Axon guidance	0.052	7.5	3.2E-4
Axonogenesis	0.069	4.5	4.5E-3
Anatomical structure development	0.086	1.6	5.6E-3
Neuron projection morphogenesis	0.098	4.2	6.4E-3
Cell morphogenesis involved in neuron differentiation	0.11	4.1	7.5E-3

Significant GOEA terms, the FDR, the fold enrichment and the P-values for each term. GOEA was performed with the DAVID online tool restricting the output to all biological process terms with a significance threshold of FDR  $\leq 0.1$  and fold enrichment  $\geq 1.5$ .



**Figure 3.** Subnetwork of genes surrounding E13. The network comprises all of the nodes at a Distance 2 from E13. It consists of 106 genes and 128 connections. Nodes are coloured according to their differential expression (squares if significant, circles if not) following *E13* overexpression (inner square/circle) or knock-down (outer square/circle).

markers, *Nestin* and *Neurog1* were significantly, albeit transiently, affected by E13 knock-down.

To assess whether the induction and/or the knock-down of E13 could influence the formation of radial glia cells and/or of some neuronal subtypes, we then analysed the expression of additional neuronal lineage specific markers (Figure 5). *Vglut2*, the vesicular glutamate transporter 2, which is essential for glutamate release from presynaptic vesicles in glutamatergic excitatory neurons (8), was specifically up-regulated by *E13* overexpression in the inducible clones (Figure 5A). In contrast, both *GAD65*, a glutamate decarboxylase specific for GABAergic neurons (10), and *Blbp*, the brain-specific member of the lipid-binding protein family, which is required for the establishment of the radial glial fibre system in developing brain (11,12), were significantly down-regulated (Figure 5B and C).

In addition, we also analysed the expression of *Chat* (choline acetyltransferase), a marker of mammalian cholinergic system (52), as well as that of the rate-limiting enzyme in dopamine biosynthesis, the tyrosine hydroxylase *TH* (53), and of the serotonin biosynthetic enzyme tryptophan hydroxylase *Tph2* (54). The expression of *Chat* was significantly, albeit transiently, affected by the knock-down of E13, whereas the expression of *TH* and of

*Tph2* did not change significantly following *E13* induction or knock-down (Supplementary Figure S9).

To verify whether the aforementioned variations in the expression of the glutamatergic, GABAergic and radial glial markers also resulted in a variation in the number of cells belonging to those specific subpopulations, we performed immunofluorescence experiments with anti-Vglut2, anti-GAD65 and anti-Blbp antibodies during differentiation of E13-inducible clones and knock-down clones (Supplementary Figure S10). We found that the number of Blbp-positive cells and of GAD65-positive cells in induced clones was significantly lower (negative binomial  $P = 0.02$  and  $P = 0.009$ , respectively) than in uninduced control clones (Supplementary Figure S10B). These data suggest that the down-regulation of the *GAD65* and *Blbp* transcripts (Figure 5B and C) following *E13* overexpression is because of the reduced number of GAD65-positive and Blbp-positive cells.

### Expression of E13 in brain

The aforementioned results prompted us to study the expression pattern of *E13* in the central nervous system. Towards this goal, we performed RNA ISH analysis with an *E13* probe in mouse brain starting at embryonic Day 12.0 (E12.0), (Figures 6 and 7). At this stage, the first

**Table 2.** Proteins identified from the co-IP experiment as potential partners of E13

UniProt ID	Gene symbol	Protein name	Mowse score	Molecular weight (Da)	Sample name*
BTF3_MOUSE	Btf3	Basic TF 3	47	22 017	IC
C1QBP_MOUSE	C1qbp	Complement component 1 Q subcomponent-binding protein	53	30 994	IC
CPSF6_MOUSE	Cpsf6	Cleavage and polyadenylation specificity factor subunit	31	59 116	IC
CQ096_MOUSE	<i>E130012A19Rik</i>	Uncharacterized protein C17orf96 homologue	103	38 071	IC
DDX5_MOUSE	Ddx5	Probable ATP-dependent RNA helicase DDX	57	69 277	IC
EED_MOUSE	Eed	Polycomb protein EED	53	50 166	IC
H12_MOUSE	Hist1h1c	Histone H1.2	76	21 254	IC
HS90A_MOUSE	Hsp90aa1	Heat shock protein HSP 90-alpha	35	84 735	IC
IGH1M_MOUSE	Ighg1	Ig gamma-1 chain C region, membrane-bound form	601	43 359	IC
IGKC_MOUSE	Igkc	Ig kappa chain C region	843	11 771	IC
INT3_MOUSE	Ints3	Integrator complex subunit 3	28	117 862	IC
KPRA_MOUSE	Prpsap1	Phosphoribosyl pyrophosphate synthetase-associated protein 1	142	39 407	IC
KV2A7_MOUSE	LOC636468	Ig kappa chain V-II region 26-10	796	12 265	IC
ML12B_MOUSE	My112b	Myosin regulatory light chain 12B	776	19 767	IC
MTF2_MOUSE	Mtf2	Metal-response element-binding TF 2	42	66 882	IC
MYH9_MOUSE	Myh9	Myosin-9	2487	226 232	IC
NEK9_MOUSE	Nek9	Serine/threonine-protein kinase Nek9	32	107 015	IC
NKTR_MOUSE	Nktr	NK-tumour recognition protein	32	163 341	IC
PCNA_MOUSE	Pcna	Proliferating cell nuclear antigen	72	28 766	IC
RBBP4_MOUSE	Rbbp4	Histone-binding protein RBBP4	38	47 625	IC
RL23A_MOUSE	Rpl23a	60S ribosomal protein L23a	240	17 684	IC
RL28_MOUSE	Rpl28	60S ribosomal protein L28	98	15 724	IC
RS11_MOUSE	Rps11	40S ribosomal protein S11	82	18 419	IC
RS13_MOUSE	Rps13	40S ribosomal protein S13	105	17 212	IC
SETX_MOUSE	Setx	Probable helicase senataxin	53	297 401	IC
SPTA2_MOUSE	Sptan1	Spectrin alpha chain, brain	658	284 422	IC
SPTB2_MOUSE	Sptbn1	Spectrin beta chain, brain 1	607	274 052	IC
SUZ12_MOUSE	Suz12	Polycomb protein Suz12	78	82 974	IC
T2EB_MOUSE	Gtf2e2	General TF IIE subunit 2	42	33 026	IC
THOC4_MOUSE	Thoc4	THO complex subunit 4	84	26 924	IC
UBIQ_MOUSE	Rps27a	Ubiquitin	105	8 560	IC
ACTB_MOUSE	Actb	Actin, cytoplasmic 1	247	41 710	CTL
HNRPF_MOUSE	Hnrnpf	Heterogeneous nuclear ribonucleoprotein F	170	45 701	CTL
IGH1M_MOUSE	Ighg1	Ig gamma-1 chain C region, membrane-bound form	699	43 359	CTL
IGKC_MOUSE	Igkc	Ig kappa chain C region	306	11 771	CTL
KV2A7_MOUSE	LOC636468	Ig kappa chain V-II region 26-10	796	12 265	CTL
MYH10_MOUSE	Myh10	Myosin-10	1883	228 855	CTL
PRPS1_MOUSE	Prps1	Ribose-phosphate pyrophosphokinase 1	123	34 826	CTL
RS5_MOUSE	Rps5	40S ribosomal protein S5	84	22 875	CTL
IGH1M_MOUSE	Ighg1	Ig gamma-1 chain C region, membrane-bound form	699	43 359	P-CTL
IGKC_MOUSE	Igkc	Ig kappa chain C region	310	11 771	P-CTL
KV2A7_MOUSE	LOC636468	Ig kappa chain V-II region 26-10	368	12 265	P-CTL
PPB_ECOLI	phoA	Alkaline phosphatase OS = <i>E. coli</i> (strain K12)	6665	49 408	P-CTL
IGH1M_MOUSE	Ighg1	Ig gamma-1 chain C region, membrane-bound form	625	43 359	N-CTL
IGKC_MOUSE	Igkc	Ig kappa chain C region	735	11 771	N-CTL
KV2A7_MOUSE	LOC636468	Ig kappa chain V-II region 26-10	535	12 265	N-CTL

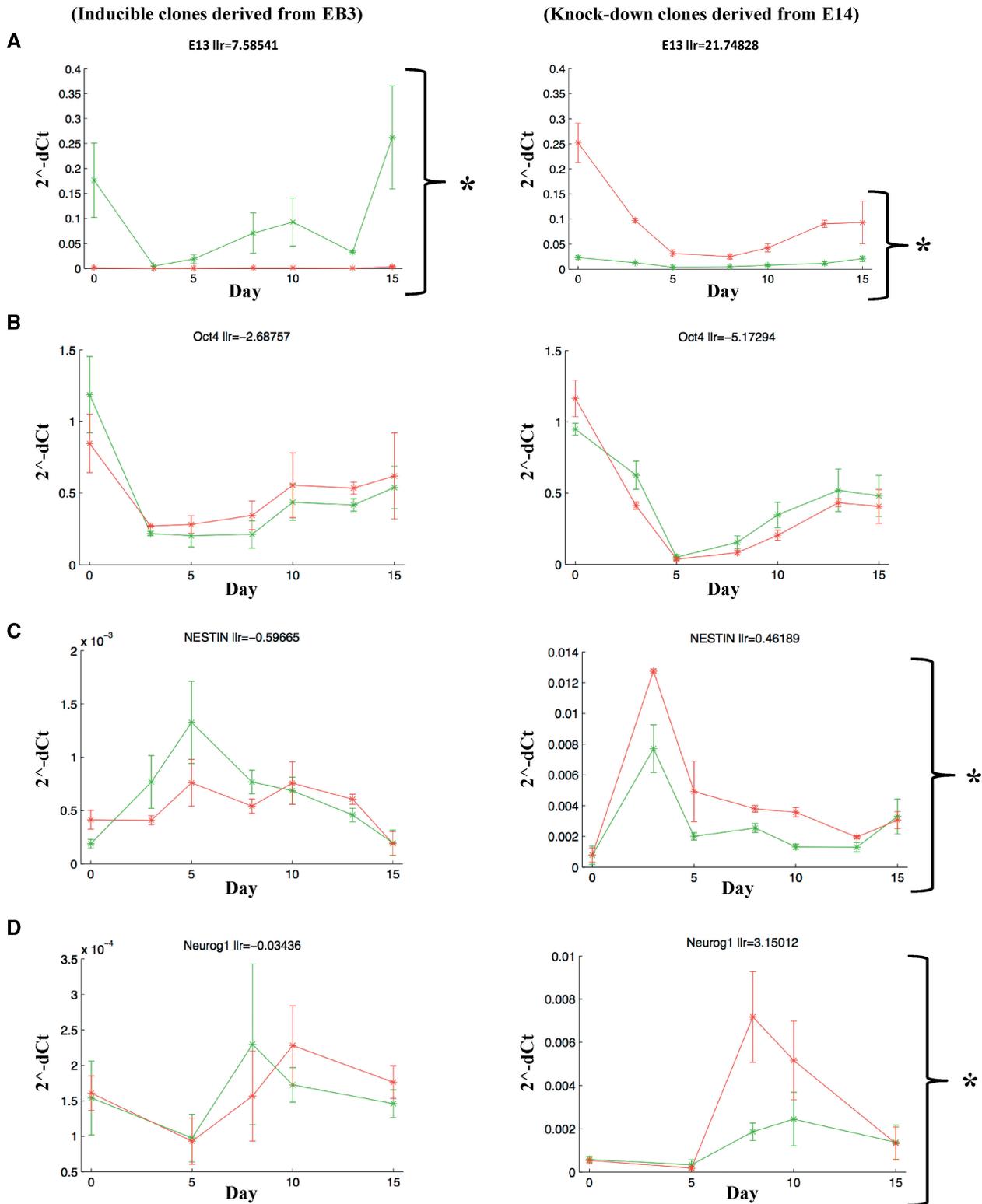
IC = inducible cell line; CTL = control cell line; P-CTL = positive control with flag-tagged BAP protein; N-CTL = negative control without protein. Two inducible cell samples, two control cell samples, one negative control and one positive control were processed simultaneously. Protein hits corresponding to  $P < 0.05$  (Mowse score  $> 28$ ) were considered as significant protein identifications.

post-mitotic excitatory neurons are clearly visible at the pial border of the dorsal pallium (55,56), while the inter-neuronal population emerging in the ganglionic eminences of the ventral pallium is approaching its tangential migration (57–59).

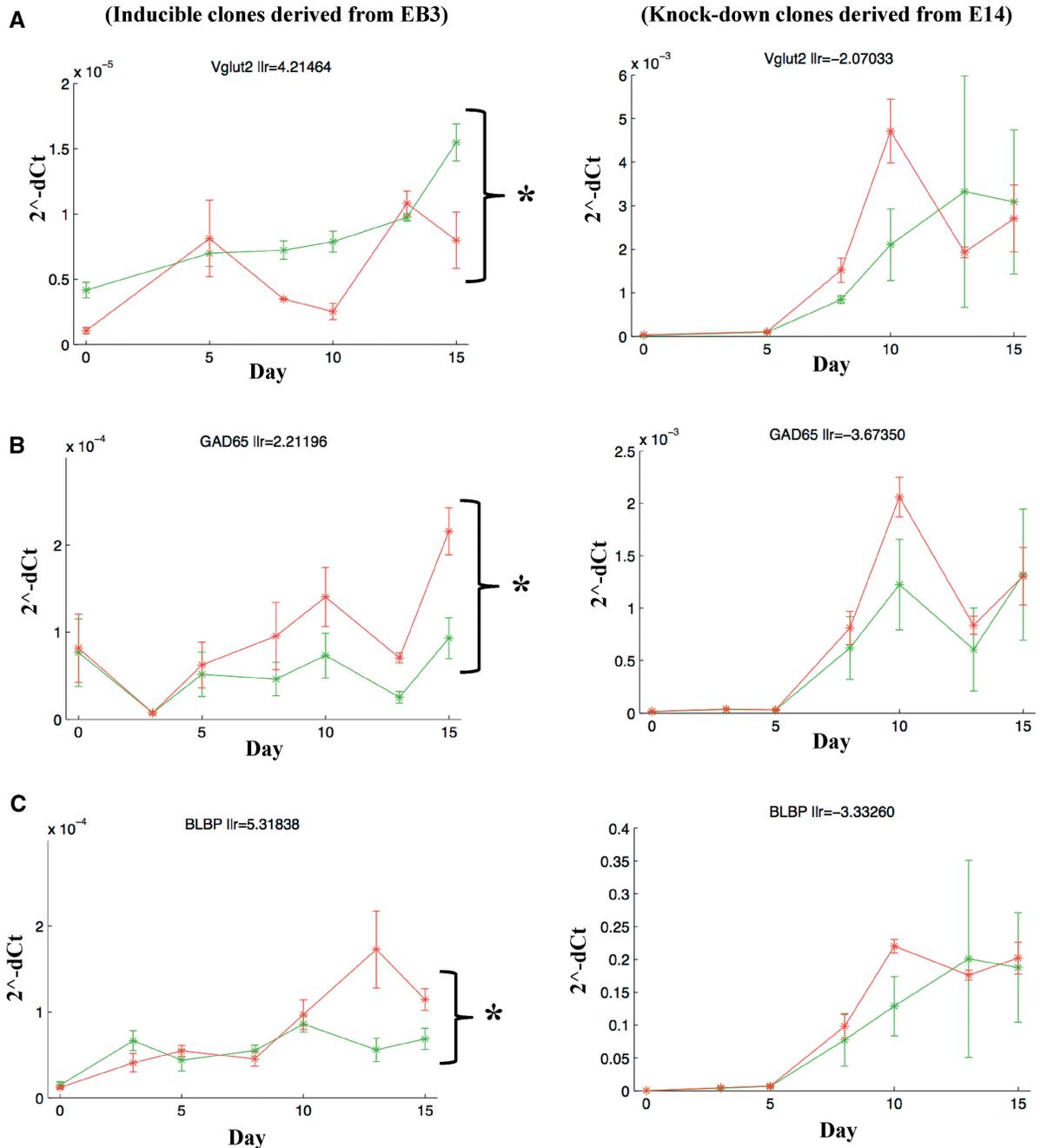
*E13* mRNA was strongly expressed in the developing cerebral cortex, from which glutamatergic neurons and glial cells derive (Figure 6A). At higher magnification, a graded expression of *E13* in the developing cortex (Figure 6B) is clearly visible, from the ventricular (low) to the pial (high) surface. By double ISH/IHC, *E13* appears to be strongly expressed in the post-mitotic neuronal population of the pre-plate, as marked by the neuronal nuclei staining [NeuN, Rbfox3 (60)]

(Figure 6B). *E13* levels gradually increase in the subventricular zone, co-localizing with the intermediate progenitor cells, which are positive for the neuronal marker *Tbr2* (Figure 6B) (61–63).

*E13* expression in adult brain was localized to cortical glutamatergic neurons by RNA ISH in P21 mouse brain, and appeared to be particularly high in the cerebral cortex, especially in cingulate (not shown), somatosensory and piriform cortex, in the hippocampal formation, and in the amygdaloid nuclei (Figure 7A). A detailed analysis of *E13* in the cortical domains indicated a large representation in all the cortical layers, showing high co-localization with the vesicular glutamate transporter 1, *Vglut1*, a marker of adult glutamatergic cells, whereas



**Figure 4.** Expression profile of *E13*, *Oct4*, *Nestin* and *Neurog1* during neuronal differentiation. The expression profiles of *E13* (A), *Oct4* (B), *Nestin* (C) and *Neurog1* (D) transcripts were evaluated by q-PCR in two inducible not-tagged clones derived from parental ES cell line EB3 (left panels) and in two knock-down clones derived from parental ES cell line E14 (right panels). For *E13* transcript, E13-3xFLAG primer pair and E13-affy primer pair were used in inducible and in knock-down clones, respectively. The red bar represents the control, the green bar represents the expression profile of each transgene in the inducible and in the knock-down clones, respectively. For each expression graph, we reported on the x-axis the days of the differentiation protocol and on the y-axis the relative expression of the transcript expressed as 2-dCt. For each expression profile, the llr was calculated, and the value was reported on the top of each graph. The llr represents the statistical significance value of the difference in the expression profiles of each transgene in the inducible and in the knock-down condition compared with each control. The statistical significance is indicated by asterisk (llr > 0).

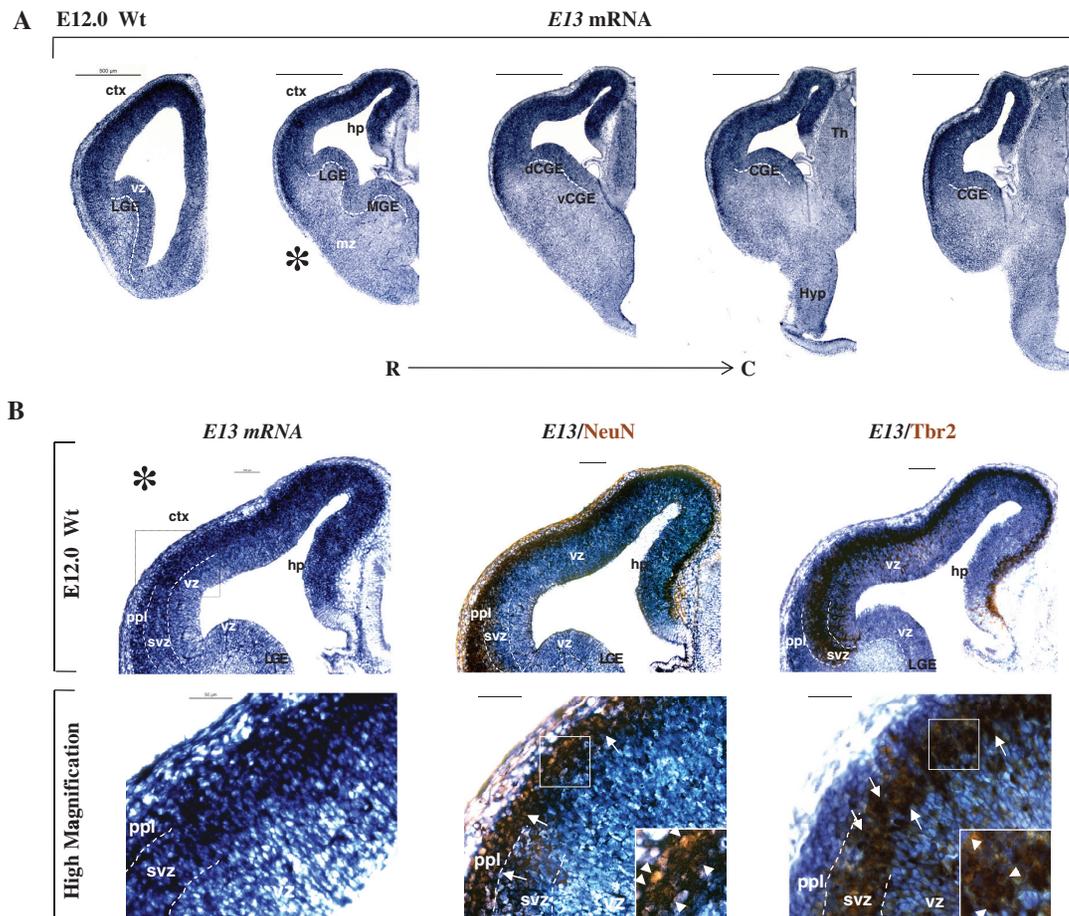


**Figure 5.** Expression profile of *Vglut2*, *GAD65* and *BLBP* during neuronal differentiation. Expression analysis in two inducible non-tagged clones derived from parental ES cell line EB3 (left panels) and in two knock-down clones derived from parental ES cell line E14 (right panels) was evaluated by q-PCR: *Vglut2* is a glutamatergic neuron marker (A), *GAD65* is a GABAergic neuron marker (B), *BLBP* is a radial glia marker (C). The red bar represents the control, the green bar represents the expression profile of each transcript in the overexpressing and in the knock-down clones. For each expression graph, we reported on the x-axis the days of the differentiation protocol and on the y-axis the relative expression of the transcript expressed as 2-dCt. For each expression profile, the *lir* was calculated, and the value was reported on the top of each graph. The *lir* represents the statistical significance value of the difference in the expression profiles of each transgene in the inducible and in the knock-down condition compared with each control. The statistical significance is indicated by asterisk ( $lir > 0$ ).

no co-localization is apparent in *Blbp*-positive (11,12), or GFAP-positive mature astroglial cells, in the cortex (64,65) (Figure 7B).

*E13* is also highly represented in the hippocampal formation, in both the glutamatergic principal neuronal populations, the pyramidal neurons of the Cornus Ammonis 1 (CA1) to the CA3 fields of the hippocampus

proper and the granule cells of the dentate gyrus (DG) (Figure 7C and D). Interestingly, in the DG, one of the two brain niches where neurogenesis occurs throughout adult life (66–70), *E13*, shows a gradient similar to that observed in the developing cortex, with high levels in the mature granule cells, as confirmed by co-localization with the calcium binding protein, Calbindin (Figure 7C) (71).



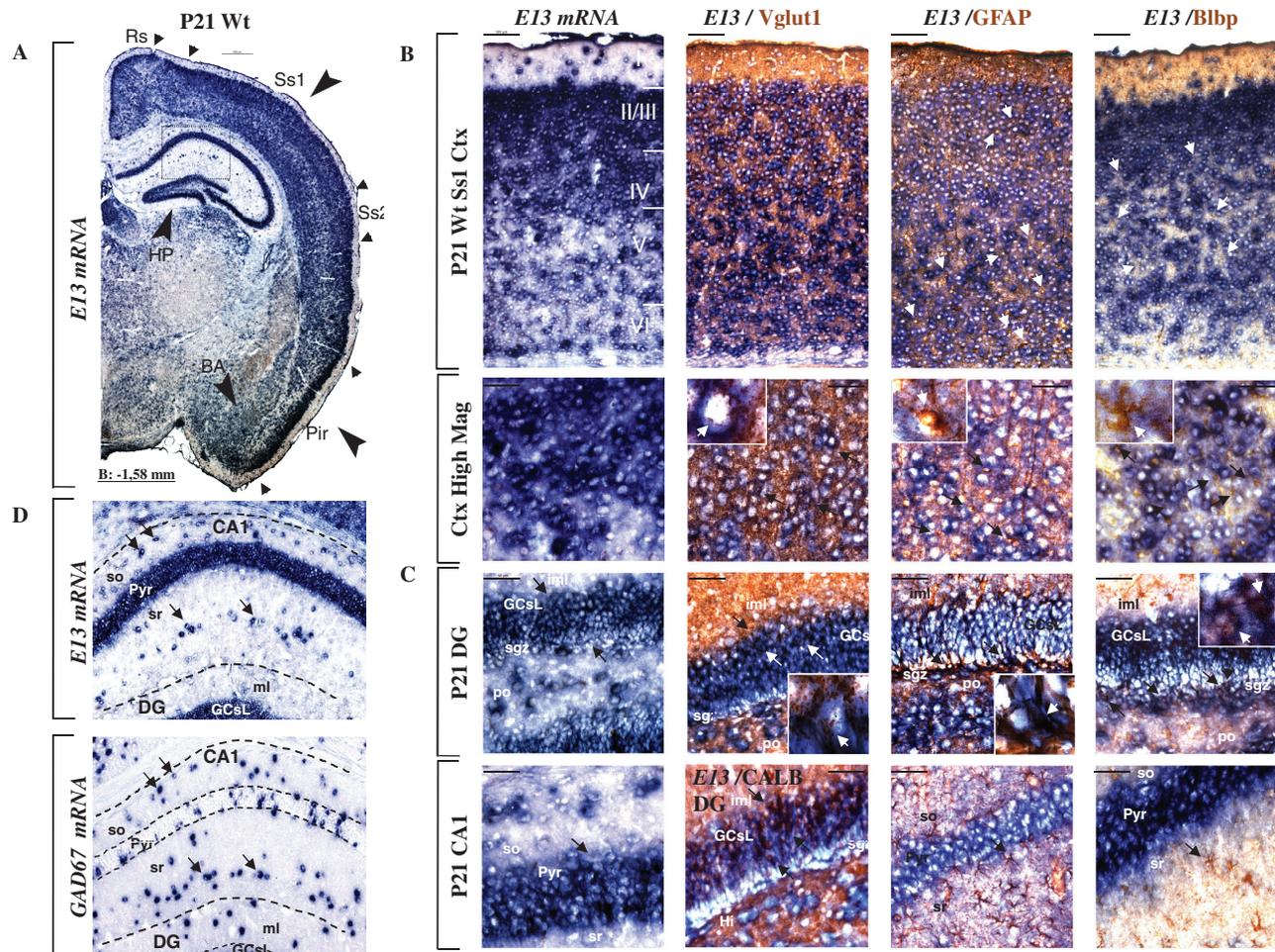
**Figure 6.** Expression of *E13* in developing mouse brain. (A) Expression of *E13* during mouse brain development evaluated by RNA ISH at embryonic Day 12.0 (E12.0). High levels of *E13* are detected in the dorsal pallium from the ventricular to the pial surface, along the entire rostro-caudal (R-C) axis. In the ventral pallium, *E13* expression is restricted to the ventricular zone of the ganglionic eminences (dashed lines). Scale bar: 500  $\mu\text{m}$ . (B) High magnification of E12.0 embryonic brain at the rostro-caudal level indicated by the asterisk in (A). A graded expression of *E13* is clearly visible in all the cortical primordium. Double ISH/IHC shows that although at low levels of expression, *E13* levels gradually increase and co-localize with the *Tbr2*<sup>+</sup> intermediate progenitor cells of the subventricular zone, which are about to undergo the final neurogenic division. The maximum rate of *E13* is evident in the post-mitotic neurons of the preplate that are positive to NeuN staining (arrows and arrow heads in the higher magnification and insets). Please note that in the ganglionic eminence, *E13* is detected only in the ventricular zone. Scale bars: 100  $\mu\text{m}$ , 50  $\mu\text{m}$  in high magnifications. ctx, cortex; hp, hippocampus; vz, ventricular zone; svz, subventricular zone; ppl, preplate; LGE, lateral ganglionic eminence; MGE, medial ganglionic eminence; CGE, caudal ganglionic eminence; mz, mantle zone; Th, thalamus; Hyp, hypothalamus.

To evaluate the expression of *E13* in neural populations whose transcriptome profiles, *in vitro*, do not appear to be influenced by *E13* manipulation, we examined the TH-positive population that correspond to dopaminergic neurons (53). We followed this population history, from the embryonic brain development to the adulthood, and we found no co-localization with *E13* neither at E12.0 (Supplementary Figure S11A), nor at P21 (Supplementary Figure S11B).

To further assess in which neuronal subtype *E13* gene was significantly expressed, we analysed 36 GEPs collected from 12 different neuronal cell types in the adult mouse forebrain (GSE2882) (72). As shown in Supplementary Figure S12, the expression of *E13* varies significantly across the 12 different neuronal cell types (analysis of variance  $P = 3 \times 10^{-6}$ ), with higher expression in the five glutamatergic pyramidal neuron populations, as compared with the six GABAergic populations of interneurons.

## DISCUSSION

Regulatory interactions among genes can be ‘reverse engineered’ by considering pairs of genes and checking whether they are co-expressed across different experimental conditions (‘co-expression’ networks). Reverse engineering is a powerful tool to generate hypotheses on gene function (73). In this work, we have produced a collection of GEPs measured in mouse ES cells from our previous study (7) together with a new collection of microarrays and used a systems biology reverse engineering approach to gain initial insight into the functional role of a previously uncharacterized gene, *E130012A19Rik*. We applied the ARACNe reverse engineering algorithm, which is based on computing a pairwise MI between two genes, as the nature of the GEPs we collected prevented use of more sophisticated strategies (such as those based on time series GEPs). We observe that there is a plethora of reverse engineering algorithms available, and new and



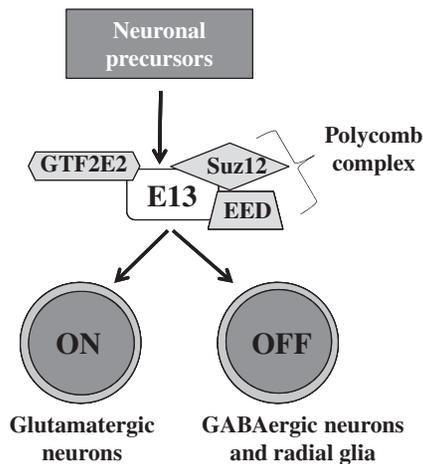
**Figure 7.** *E13* is expressed in cortical glutamatergic neurons and in the radial glia stem cells-like of the SGZ neurogenesis niche in the adult brain. (A) RNA ISH on P21 mouse brain reveals a strong signal of *E13* in the cerebral cortex, in the hippocampus and in the amygdaloid nuclei (arrowheads). Scale bar: 500  $\mu$ m. (B) A more detailed analysis of *E13* expression in primary somatosensory cortex indicates a widespread signal of *E13* in all the cortical layers. Co-localization of the vesicular glutamate transporter 1, *Vglut1*, is detected in *E13*<sup>+</sup> cortical neurons (high magnification and arrow in insets). No co-localization of *E13* with *Blbp* and *GFAP* is found (high magnification and arrow in insets). Scale bar: 100  $\mu$ m. (C) In the hippocampus, *E13* expression is present in both the glutamatergic main neurons: CA pyramidal cells and the DG granules. Interestingly in the DG, *E13* shows a gradient similar to that found in the developing cortex, namely higher in the more mature granule cells. Scale bar: 50  $\mu$ m. (D) By adjacent ISH for *E13* and *GAD67* in P21 brains, at the hippocampal level [dotted box in (A)], *E13* appears also in the in the GABAergic interneurons populating the oriens and radiatum strata of the hippocampus (arrows). so, stratum oriens; Pyr, pyramidal layer; sr, stratum radiatum; Rs, Retro splenic cortex; Ss1, primary somatosensory cortex; Ss2, secondary somatosensory cortex; Pir, Piriform cortex; BA, basal amygdaloid nuclei; Hp, hippocampal formation; CA1, Cornus Ammonis field 1 of hippocampus proper; Iml, inner molecular layer; GCsL, granule cells layer; sgz, subgranular zone; po, polymorphic layer.

improved methods are being developed (32). Therefore, different reverse engineering methods could reveal different aspects of the network, and thus either confirm or reveal additional roles of *E13*, as well as of other genes with unknown functions.

Interestingly, *E13* was the uncharacterized ES-specific 'hub' gene with the highest number of connections in the inferred network. Hub genes have been found to be master regulators of specific transcriptional programs both in normal and pathogenic conditions (74,75). Here, by transcriptomics, immuno-based affinity purification experiments and ISH, we indeed identified a role of *E13* in neuronal subpopulation specification.

The capability of neurons to adopt the correct neurotransmitter phenotype during early development is

the critical point for the proper functioning of the vertebrate adult nervous system. A multiple array of neuronal types have to arise from a field of undifferentiated progenitors; how a cell acquires a given neurotransmitter phenotype is a central issue in developmental neurobiology (76,77). This is particularly true for glutamatergic and  $\gamma$ -aminobutyric acid GABAergic neurons, which are the most abundant excitatory and inhibitory neurons, respectively, in the vertebrate's central nervous system. It is likely that only a proportion of the factors required for neuronal identity have so far been identified, and the precise way in which such factors interact to specify the timing and terminal differentiation of particular neuronal subpopulations is not yet defined (78).



**Figure 8.** Proposed mechanism of action of E13 in regulating neuronal cell differentiation. E13 may interact with proteins of PRCs to regulate neuronal specification by modulating the transcriptional program of differentiating cells.

In addition, the emerging role that epigenetic control plays in brain development implies that the interplay of TFs and epigenetic modifiers, including histone modifications, DNA methylation and microRNAs, is essential for the acquisition of specific cell fates (79). Several chromatin-modifying complexes regulate the renewal or differentiation of a range of neural stem cells, but the Polycomb repressor complexes (PRCs) are of particular interest in this context. This is particularly true for some members of PRC2, such as *EZH2*, whose altered expression is able to change the competence of cortical progenitors to generate neurons of different cortical layers, orchestrating the switch from neurogenesis to gliogenesis (80,81). Members of this Polycomb complex are also required for the subgranular zone (SGZ)-hippocampal adult neurogenesis (82,83) and impact excitatory synaptic plasticity (84,85).

Here, we show that E13 may be a component of the genetic and epigenetic networks controlling neuronal specification. Indeed, induction of the expression of E13 in stable ES cell lines results in the significant up-regulation of markers of glutamatergic excitatory neurons, and a strong down-regulation of the GABAergic neurons and radial glia markers. Our study further suggest that the effects of E13 on neuronal differentiation may be exerted via epigenetic mechanisms; the results we obtained by immune-based affinity purification show that E13 interacts with Eed (EED gene) and Suz12 (suppressor of zeste 12 homologue), which are both components of the PRC-EED-EZH2 Polycomb chromatin modelling complex (48), as well as the retinoblastoma binding protein Rbbp4, a core histone-binding protein (86).

Taken together, the interaction of E13 with these proteins suggest it as member of the epigenetic regulation machinery of the neuronal subtypes and glia commitment (Figure 8). Further studies, including mouse models and chromatin IP, are needed to clarify the specific mechanisms by which E13 exerts its function on neuron specification.

## SUPPLEMENTARY DATA

Supplementary Data are available at NAR Online: Supplementary Tables 1–17, Supplementary Figures 1–12 and Supplementary Files 1–3.

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*Conflict of interest statement.* None declared.

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