Molecular networks involved in mouse cerebral corticogenesis and spatio-temporal regulation of Sox4 and Sox11 novel antisense transcripts revealed by transcriptome profiling

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Abstract

Background: Development of the cerebral cortex requires highly specific spatio-temporal regulation of gene expression. It is proposed that transcriptome profiling of the cerebral cortex at various developmental time points or regions will reveal candidate genes and associated molecular pathways involved in cerebral corticogenesis.

Results: Serial analysis of gene expression (SAGE) libraries were constructed from C57BL/6 mouse cerebral cortices of age embryonic day (E) 15.5, E17.5, postnatal day (P) 1.5 and 4 to 6 months. Hierarchical clustering analysis of 561 differentially expressed transcripts showed regionalized, stage-specific and co-regulated expression profiles. SAGE expression profiles of 70 differentially expressed transcripts were validated using quantitative RT-PCR assays. Ingenuity pathway analyses of validated differentially expressed transcripts demonstrated that these transcripts possess distinctive functional properties related to various stages of cerebral corticogenesis and human neurological disorders. Genomic clustering analysis of the differentially expressed transcripts identified two highly transcribed genomic loci, Sox4 and Sox11, during embryonic cerebral corticogenesis. These loci feature unusual overlapping sense and antisense transcripts with alternative polyadenylation sites and differential expression. The Sox4 and Sox11 antisense transcripts were highly expressed in the brain compared to other mouse organs and are
Complex behavioral tasks, from perception of sensory input and the control of motor output to cognitive functions such as learning and memory, are dependent on the precise development of innumerable interconnections of neuronal networks in the cerebral cortex. The development of the cerebral cortex (also known as cerebral corticogenesis) involves the specific influence of intrinsic and extrinsic mechanisms, which are triggered spatio-temporally [1-4]. Between embryonic day 11 (E11) and 18 (E18), the mouse cerebral cortex develops from a relatively homogenous band of mitotic multipotent progenitor cells into a complex laminated structure containing various classes of neuronal cells [2,4-6]. Cerebral corticogenesis involves: proliferation of multipotent progenitors (E11 to E16.5); migration of postmitotic cells (E11 to E17); cell morphogenesis (E13 to E18); gliogenesis and synaptogenesis (E16 until early postnatal period); and reorganization, elimination and stabilization of neuronal networks (up to adulthood).

The mouse cerebral cortex develops in the latero-medial and rostro-caudal axes [7,8]. At E11, the primordial plexiform layer begins to form in the most lateral part of the neural wall. Its growth continues in the latero-medial axis to the medial part of the telencephalon by E13. The primordial plexiform layer is also expanded in the rostro-caudal axis. The growth in this axis is always less than the growth in the latero-medial axis. The first wave of migratory neuronal cells form the cortical plate 2 days later after the development of the primordial plexiform layer. These events are followed by the development of the cortical plate into an organization of six distinct layers that forms the adult cerebral cortex. Generally, the rostral-most regions of the adult cerebral cortex consist of areas involved in executive functions and motor coordination, whilst the caudal-most regions consist of areas involved in sensory perception such as visual function. Although distinct functional arealization of the cerebral cortex do not fully apply to rodents, mounting evidence suggesting that regulated arealization exists has been shown in mice involving transcription factors such as empty spiracles homolog 2 (Emx2; Drosophila) [9,10], paired box gene 6 (Pax6) [9], COUP transcription factor 1 (Coup-tf1) [11], Sp8 transcription factor (Sp8) [12,13], distal-less homeobox 1/2 (Dlx1/2) and gastrulation brain homeobox 2 (Gbx2) [14]. The extensive cyto-architectural and anatomical changes occurring in a spatio-temporal manner during the peak (E15) and at the end (E17) of embryonic cerebral neurogenesis as well as during early postnatal (P1) corticogenesis through to adulthood involves complex underlying molecular regulatory networks.

**Conclusions:** We report validated gene expression profiles that have implications for understanding the associations between differentially expressed transcripts, novel targets and related disorders pertaining to cerebral corticogenesis. The study reports, for the first time, spatio-temporally regulated 5ox4 and Sox1 antisense transcripts in the brain, neural stem/progenitor cells and P19 cells, suggesting they have an important role in cerebral corticogenesis and neuronal/glial cell differentiation.

**Background**

Complex behavioral tasks, from perception of sensory input and the control of motor output to cognitive functions such as learning and memory, are dependent on the precise development of innumerable interconnections of neuronal networks in the cerebral cortex. The development of the cerebral cortex (also known as cerebral corticogenesis) involves the specific influence of intrinsic and extrinsic mechanisms, which are triggered spatio-temporally [1-4]. Between embryonic day 11 (E11) and 18 (E18), the mouse cerebral cortex develops from a relatively homogenous band of mitotic multipotent progenitor cells into a complex laminated structure containing various classes of neuronal cells [2,4-6]. Cerebral corticogenesis involves: proliferation of multipotent progenitors (E11 to E16.5); migration of postmitotic cells (E11 to E17); cell morphogenesis (E13 to E18); gliogenesis and synaptogenesis (E16 until early postnatal period); and reorganization, elimination and stabilization of neuronal networks (up to adulthood).

The mouse cerebral cortex develops in the latero-medial and rostro-caudal axes [7,8]. At E11, the primordial plexiform layer begins to form in the most lateral part of the neural wall. Its growth continues in the latero-medial axis to the medial part of the telencephalon by E13. The primordial plexiform layer is also expanded in the rostro-caudal axis. The growth in this axis is always less than the growth in the latero-medial axis. The first wave of migratory neuronal cells form the cortical plate 2 days later after the development of the primordial plexiform layer. These events are followed by the development of the cortical plate into an organization of six distinct layers that forms the adult cerebral cortex. Generally, the rostral-most regions of the adult cerebral cortex consist of areas involved in executive functions and motor coordination, whilst the caudal-most regions consist of areas involved in sensory perception such as visual function. Although distinct functional arealization of the cerebral cortex do not fully apply to rodents, mounting evidence suggesting that regulated arealization exists has been shown in mice involving transcription factors such as empty spiracles homolog 2 (Emx2; Drosophila) [9,10], paired box gene 6 (Pax6) [9], COUP transcription factor 1 (Coup-tf1) [11], Sp8 transcription factor (Sp8) [12,13], distal-less homeobox 1/2 (Dlx1/2) and gastrulation brain homeobox 2 (Gbx2) [14]. The extensive cyto-architectural and anatomical changes occurring in a spatio-temporal manner during the peak (E15) and at the end (E17) of embryonic cerebral neurogenesis as well as during early postnatal (P1) corticogenesis through to adulthood involves complex underlying molecular regulatory networks.

Complex molecular regulatory elements are important determinants in both spatial and temporal cerebral corticogenesis. These elements regulate gene expression at the chromatin, DNA or RNA, and protein levels through chromatin packaging or remodeling, histone acetylation and deacetylation, chromatin insulation, DNA methylation, post-transcriptional regulation and post-translational protein modification or degradation signaling pathways [15-17]. Other processes involved in such regulation include pre-mRNA processing and nuclear mRNA retention by nuclear-specific paraspeckle complexes [18,19], microRNAs (miRNAs) that interfere with mRNA translation [20-22], and a new class of under-characterized non-coding RNA transcripts known as natural antisense transcripts (NATs) [23,24]. These regulatory networks play a pivotal role in establishing when, where and how a multipotent progenitor cell should proliferate, migrate and settle at a designated position in the cortex. The information regarding regulatory networks during cerebral corticogenesis, however, remains incomplete and does not provide a comprehensive view of the underlying regulatory elements throughout this complex event.

In this study, we employed both short and long 3’ serial analysis of gene expression (SAGE) technologies [25,26] to identify differentially expressed regulatory elements by comparing transcriptomes of cerebral cortices generated from four selected developmental stages: E15.5, E17.5, P1.5 and 4 to 6 months old. We also compared rostral to caudal regions of E15.5 and left to right regions of adult cerebral cortices. We report temporally co-regulated gene clusters, novel molecular networks and associated pathways, novel candidates in regionalized development and genomic clustering of SRY-box containing gene 4 (Sox4) and SRY-box containing gene 11 (Sox11) sense and antisense transcripts. The genomic clustering analysis led us to the discovery of spatio-temporal regulation of novel Sox4 and Sox1 antisense transcripts as well as differential regulation of these transcripts in proliferating and differentiating neural stem/progenitor cells (NSPCs) and P19 (embryonal carcinoma) cells.
Results

Generation and analysis of SAGE tags

We constructed 10 SAGE libraries from the cerebral cortex of E15.5, E17.5 and 4- to 6-month-old adult C57BL/6 mice (N = 10; Table 1). The data from two additional SAGE libraries generated from E15.5 and P1.5 cerebral cortices from Gundersen et al. [27] were also incorporated into our analysis. These SAGE libraries represent key stages of cerebral cortico genesis and are accessible from the Gene Expression Omnibus (GEO) website [GEO: GSE15031] [28]. The libraries contain a total of 531,266 SAGE tags (approximately 44,000 tags per SAGE library), 223,471 of which are unique (non-redundant) after screening for artifacts and mapping of short SAGE tags to long SAGE tags (Table 1). The number of unique tags in each library ranges from approximately 7,200 to 32,000 due to the variation in library size (approximately 13,500 to 70,000). The distribution of tag abundance, however, is similar in all libraries (Figures S1 and S2 in Additional data file 1), in which the majority of tags were detected only once (58 to 76% or approximately 5,500 to approximately 24,000 tags), representing a trend comparable with previously reported SAGE analyses of mouse neocortices [27,29]. Of all unique tags, only 5,199 (approximately 2.4%) are common to all developmental stages. The low number of common unique tags is most probably due to the high abundance of tags that occur only once.

Table 1

| SAGE librarya | Sex | Age   | Tissue           | Generated sequences | Generated SAGE tag (library) | Unique tags | Unique tags (after scaling to 100,000 tags/library) | GEO accession number |
|---------------|-----|-------|------------------|---------------------|-----------------------------|-------------|---------------------------------------------------|---------------------|
| E15_1† U      | E15.5 | Rostral cerebral cortex | 2,044              | 43,327             | 15,664                      | 36,153      | GSM375449                                         |
| E15_2† U      | E15.5 | Caudal cerebral cortex | 2,925              | 39,314             | 19,929                      | 50,692      | GSM375450                                         |
| E15_3‡ U      | E15.5 | Cerebral cortex       | 1,920              | 44,332             | 15,787                      | 35,611      | GSM375451                                         |
| E15_4‡ U      | E17.5 | Cerebral cortex       | 384                | 13,573             | 7,214                       | 53,150      | GSM375638                                         |
| P1.5 U        | P1.5  | Cerebral cortex       | 1,053              | 47,733             | 19,508                      | 40,869      | GSM375639                                         |
| P1.5‡ U       | P1.5  | Cerebral cortex       | 4,194              | 42,869             | 20,465                      | 47,738      | GSM375640                                         |
| Ad_1 M        | 4 months | Left cerebral cortex | 2,016              | 50,760             | 19,032                      | 37,494      | GSM375458                                         |
| Ad_2 M        | 4 months | Left cerebral cortex | 1,536              | 52,476             | 19,157                      | 36,506      | GSM375459                                         |
| Ad_3 F        | 5-6 months | Left cerebral cortex | 2,688              | 30,914             | 15,998                      | 51,750      | GSM375460                                         |
| Ad_4 F        | 5 months | Left cerebral cortex | 1,728              | 43,592             | 17,262                      | 39,599      | GSM375461                                         |
| Ad_5 F        | 5-6 months | Right cerebral cortex | 2,684              | 53,292             | 21,693                      | 40,706      | GSM375462                                         |
| Ad_6 F        | 6 months | Right cerebral cortex | 3,264              | 69,084             | 31,762                      | 45,976      | GSM375463                                         |

aEach library was constructed by using an independent mouse cerebral cortex. †Short tags were generated from these libraries. ‡These libraries were obtained from [27]. F: female; M: male; U: undetermined sex.

Analysis of differentially expressed transcripts/tags

To identify differentially expressed tags/transcripts (DETs), we considered only those 25,165 unique tags with a count >2 across all libraries. Under stringent analyses (Table S1 in Additional data file 1), we identified a total of 561 DETs in various comparisons between developmental stages (Table 2; Figure S3 in Additional data file 1). A full list of DETs with assigned IDs is provided in Additional data file 2. Greater numbers of DETs are observed when the interval of two comparative developmental stages becomes wider. We find the largest number of DETs (approximately 59% or 328 DETs) in the embryonic versus adult stages (E versus Ad) followed by P1.5 versus Ad (approximately 34% or 192 DETs), E15.5 versus P1.5 (approximately 6% or 36 DETs) and E15.5 versus E17.5 (approximately 7% or 38 DETs) comparisons. These indicate distinctive transcript signatures during cerebral cortex development. Comparisons between rostral and caudal E15.5 (R versus C), and left and right adult cerebral cortices (L versus Ri) are described in a different section below.

Approximately 69% of DETs have been assigned to known genes and 6% to expressed sequence tags (ESTs), while 10% of DETs have multiple matches (tag matching multiple gene identifiers) and 8% having ambiguous matches (tag matching the same gene identifier at multiple chromosome loci). Approximately 8% of DETs have no matches and it is most
Hierarchical clustering of DETs

To identify co-regulated genes, all 561 DETs were hierarchically grouped into 12 clusters based on the calculation of the Euclidean distance of logged normalized counts (Figure 1). Clusters 1, 5 and 6 consist of embryonic-specific DETs that exhibit the highest expression during embryonic development of the cerebral cortex. DETs in cluster 1 are expressed throughout all stages of development but exhibit the lowest expression in the adult cortex. Expression of DETs in cluster 5 ceases prior to birth, whereas DETs in cluster 6 are expressed up to early postnatal stage. On the other hand, clusters 4, 8 and 10 consist of adult specific DETs, showing very similar temporal expression profiles, but with different magnitudes (for example, highest expression in cluster 10). Clusters 2 and 7 are termed ‘gene-switching’ clusters as they show interesting expression-switching profiles. Cluster 2 shows an expression switch between P1.5 and adult stages whilst cluster 7 shows an expression switch between embryonic and adult stages. Clusters 3 and 9 consist of DETs showing region- (caudal region of E15.5 cerebral cortex) and stage-specific (P1.5 only) expression. Clusters 11 and 12 were excluded from subsequent analyses because they contain very few annotated tags. DETs within the same cluster may be co-regulated and/or involved in similar biological functions during cerebral corticogenesis.

We performed a systematic gene ontology functional clustering using the standardized Gene Ontology term analysis tool DAVID (Database for Annotation, Visualization and Integrated Discovery) [30] (Additional data file 3). Functional analysis of these gene clusters reveals that they have distinctive roles during cerebral corticogenesis. Embryonic-specific gene clusters (1, 5 and 6) are dominated by genes associated with cellular protein and macromolecule metabolic processes or biosynthesis, and nervous system and neuron development. These results match with the expected functional ontologies during embryonic cerebral cortex development in which neuronal migration, differentiation and axonogenesis events are at their peak. In contrast, adult-specific gene clusters (4, 8 and 10) consist of genes related to biological processes in the mature cerebral cortex, such as ion homeostasis, synaptic transmission and regulation of neurotransmitter level. In addition, these gene clusters are also enriched for ribonucleotide/nucleotide binding activity and components of cytoplasmic membrane-bound vesicles. These functional ontologies are in accordance with adult cerebral cortex function, which features synaptogenesis and nerve impulse transmission at synapses. Gene-switching clusters 2 and 7 are enriched with gene ontologies that are similar to both the embryonic- and adult-specific gene clusters. In addition, these gene clusters are also enriched for microtubule cytoskeleton organization and biogenesis, nucleotide biosynthesis and regulation of mRNA translation processes.

Quantitative RT-PCR validation of DETs and gene clusters

To ascertain the robustness of the SAGE datasets, we selected 136 candidate DETs and two additional genes of interests (ATPase, Cu++ transporting, alpha polypeptide, Atp7a, and cullin-associated and neddylation-dissociated 2, Cand2) for validation after considering both stage-to-stage and hierarchical based analyses (Table S2 in Additional data file 1). The selected DETs are ESTs, which have been identified in brain-related mouse cDNA libraries or transcriptomes. Independent quantitative RT-PCR (RT-qPCR) validation was carried out using three biological repli-
cates of unpooled cerebral cortex total RNA for each developmental stage. We validated 70 DETs (including 10 ESTs) from SAGE profiles of comparisons between two developmental stages after considering various stringent criteria and cutoffs. The RT-qPCR results for all the 70 validated DETs, Atp7a and Cand2, are presented in Tables 3, 4, 5 and 6 (Additional data file 4). To validate the expression profiles of gene clusters from the hierarchical clustering analysis, we performed additional RT-qPCR analyses on 65 validated DETs (based on the analysis of two developmental stages) Atp7a and Cand2 by including other developmental stages. The analysis validated 62 out of 65 DETs (Figure 2). Of these, 22 are from embryonic gene clusters, 26 from adult gene clusters, and 14 from geneswitching clusters. We assigned Atp7a and Cand2 to clusters 2 and 5, respectively, based on their RT-qPCR expression profiles across all the developmental stages. See Tables 3, 4, 5 and 6 for complete list of DETs and their full gene names.

According to the comparison between two developmental stages, the most abundantly expressed and validated DETs in the E versus Ad analysis are Camk2a, Egr1 and Plp1 (Table 3). In the adult cerebral cortex, the expression of these DETs is more than 100-fold greater than that at E15.5. Other validated adult-specific DETs with expression levels of approximately tenfold or greater than those in the embryonic brain are Camk2n1, Cryab, Nrgn, BQ176089, CD802535, Sncb and Nptxr. Conversely, Sox11, an embryonic specific DET, is expressed in the E15.5 and E17.5 cerebral cortices with an expression level of at least 100-fold greater than that in the adult. Other validated embryonic specific DETs are Dcx, Zfp57, Ezh2, Sfrp1 and Cdk4, which have expression levels approximately tenfold or greater than those in the adult.

In the P1.5 versus Ad analysis, Dcx is expressed at a level approximately 80-fold greater in P1.5 compared to the adult cerebral cortex (Table 4). Other P1.5 enriched and validated
Figure 2
High-throughput RT-qPCR validation of gene clusters. All validations were based on DETs for canonical mRNA. Failed validation of DETs according to hierarchical clustering expression profiles is indicated by arrows. N = 3 and data are presented as mean ± standard error of the mean.
### Table 3

**RT-qPCR validation of SAGE profile for E versus adult comparison**

| SAGE tag          | RefSeq accession | Gene ID | Ad/E15.5 (SAGE) | Ad/E17.5 (SAGE) | Ad/E15.5 (RT-qPCR) | Ad/E17.5 (RT-qPCR) |
|-------------------|------------------|---------|-----------------|-----------------|-------------------|-------------------|
| gcttccccccaccccttt | NM_177407        | calcium/calmodulin-dependent protein kinase II alpha, Camk2a | As              | As              | 11.159            | 54.74             |
| gpatatggtggtctac  | NM_007913        | early growth response 1, Egr1 | As              | As              | 108.47            | 38.93             |
| aaattatggpaaatcc  | NM_011123        | proteolipid protein (myelin) 1, Plp1 | 28.77           | As              | 103.15            | 89.13             |
| gatattgcacaaaaaa  | NM_025451        | calcium/calmodulin-dependent protein kinase II inhibitor 1, Camk2n1 | 69.59           | 59.76           | 40.08             | 21.46             |
| gcttcatctccagggag | NM_009964        | crystallin, alpha B, Cryab | As              | 11.59           | 30.47             | 20.58             |
| ttcatactcagggtagg  | NM_022029       | neurogranin, Ngn | 24.67           | 8.47            | 43.67             | 17.00             |
| ccttctttccctgttc  | BQ176089        | EST from adult C57BL/6 subfornical organ and postrema tissues | As              | As              | 23.01             | 8.91              |
| accggcctagtagga   | NM_011129        | septin 4, Sept4 | 18.97           | 16.29           | 22.38             | 12.96             |
| cctattataatacaaga | CD802535        | EST from 27-32 days C57BL/6 hippocampus tissue | As              | 9.05            | 22.03             | 13.25             |
| aataagcacaattcgc  | NM_033610        | synuclein, beta, Snch | 20.89           | 7.66            | 18.20             | 15.83             |
| gctttgatactccttc  | NM_030689        | neuronal pentraxin receptor, Nptpr | 17.88           | 12.24           | 15.02             | 10.93             |
| tccctttcttagatcct | NM_144828        | protein phosphatase 1, regulatory (inhibitor) subunit 1B, Ppp1r1b | As              | As              | 10.47             | 6.88              |
| gcccctcttcctattgsc | NM_010358       | glutathione S-transferase, mu 1, Gstm1 | As              | As              | 9.87              | 5.58              |
| tgactagctgacgcgag  | NM_007694       | chromogranin B, Chgb | 6.13            | 6.30            | 9.43              | 7.22              |
| attttctttctggaggg  | NM_010585        | inositol 1,4,5-triphosphate receptor 1, Itpr1 | 14.34           | 6.16            | 9.29              | 6.12              |
| acrttgagattgtcctt | NM_009062        | regulator of G-protein signaling 4, Rgs4 | 12.23           | 21.01           | 8.84              | 5.32              |
| aataattgacctattgtg | AK139402        | Mus musculus 10 days neonate cortex cDNA | As              | As              | 8.28              | 7.55              |
| ctagacagagcattat  | NM_019634        | tetraspanin 7, Tspan7 | 13.08           | 5.61            | 7.99              | 5.38              |
| tggtagcacaatcgggt  | NM_007547        | signal-regulatory protein alpha, Sirpa | As              | As              | 7.18              | 4.63              |
| tgacagacatcctcgg  | AU258168        | EST from mouse brain | As              | As              | 6.49              | 4.58              |
| cttaccccgagcgagg  | NM_008913        | protein phosphatase 3, catalytic subunit, alpha isoform, Ppp3ca | As              | 5.43            | 5.48              | 3.45              |
| atagccttccaccaact  | NM_007471        | amyloid beta (A4) precursor protein, App | 3.98            | 2.74            | 4.56              | 3.49              |
| ttcagcagttggtcct  | NM_013556        | hypoxanthine guanine phosphoribosyltransferase 1, Hprt1 | 6.90            | 8.87            | 3.45              | 2.85              |
| aggtatgcacaaagttt  | NM_016886        | glutamate receptor, ionotropic, AMPA3 (alpha 3), Gria3 | 4.97            | 8.51            | 3.40              | 1.81*             |
| tccacctgctacatcta  | NM_009790        | calmodulin 1, Calm1 | 4.23            | 3.17            | 3.24              | 2.27              |
Table 3 (Continued)

| RT-qPCR validation of SAGE profile for E versus adult comparison |
|---------------------------------------------------------------|
| Cctcagctggggtaga   NM_009983         cathepsin D, Ctsd      | 3.46  | 2.04  | 3.22  | 3.84 |
| Gtctgctcacaacagc   NM_010777         myelin basic protein, Mbp  | 202.46 | 173.85 | 3.19  | 2.05 |
| Tttaaartgtcttttt   NM_080555         phosphatidic acid phosphatase type 2B, Pop2b | 5.71  | 2.44  | 3.02  | 2.76 |
| Cttatccctacccgc    NC_005089         NADH dehydrogenase 6, mitochondrial, mt-ND6 | As   | As   | 2.65  | 2.13 |
| Caaacctccaaaaacc   AK140219         Mus musculus adult male corpora quadrigemina cDNA | 29.22 | 18.62 | 2.63  | 2.03 |
| Agttgcataaggctgt   NM_009900         chloride channel 2, Clcn2 | 14.55 | 4.16  | 2.23  | 2.18 |
| Acaaatgaaaaaaga    AK154943         Mus musculus NOD-derived CD11c +ve dendritic cells cDNA | 109.40 | 51.13 | 1.76* | 1.35 (NS) |
| Ccactacgtgaaaaa    NM_008453         Kruppel-like factor 3 (basic), Klf3 | As   | -52.97 | -1.47‡ | -1.27 (NS) |
| Aagaaaaacattaata   NM_012010         eukaryotic translation initiation factor 2, subunit 3, structural gene X-linked, Elf2s3x | -7.70 | -10.38 | -1.72* | -1.74* |
| Cacctcggggggctcct  AK172656         amyotrophic lateral sclerosis 2 (juvenile) chromosome region, candidate 2 (human), Als2cr2 | -11.03 | -12.88 | -1.75* | -2.48 |
| Cttccctctttatact   NM_009536         tyrosine 3-monoxygenase/tryptophan 5-monoxygenase activation protein, epsilon polypeptide, Ywhae | -3.19  | -2.52  | -1.82* | -2.66 |
| Tgtgtttccttgctta   NM_008683         neural precursor cell expressed, developmentally down-regulated gene 8, Nedd8 | -4.73  | -8.41  | -1.83  | -1.86 |
| Cttctgaagccatgtt   NM_009454         ubiquitin-conjugating enzyme E2E 3, UBC4/5 homolog (yeast), Ube2e3 | -4.14  | -5.52  | -2.51  | -3.22 |
| Gtgaactaaaaaaa     NM_009094         ribosomal protein S4, X-linked, Rps4x | -10.46 | -19.19 | -2.76  | -3.35 |
| Aatgctctctcttaca   NM_011045         proliferating cell nuclear antigen, PcnA | -14.21 | -8.30  | -3.09  | -2.62 |
| Cgaggactctctgctg   NM_008976         cyclin-dependent kinase inhibitor 1C (P57), Cdkn1c | -13.68 | -7.16  | -3.39* | -3.30* |
| Cctttgagctctggc    NM_025635         ZW10 interactor, Zwint | 7.29  | 8.84  | -3.47‡ | -4.80‡ |
| Gaagccagtgggctcct  NM_001032273      RIKEN cDNA S031439G07 gene, S031439G07Rik | -9.10  | -10.00 | -4.13  | -3.65 |
| Gctgggggtccttgctg  NM_010561         interleukin enhancer binding factor 3, Irf3 | As   | As   | -4.15‡ | -2.97‡ |
| Accccggacccctgttt  NM_016707         B-cell CLL/lymphoma 11A (zinc finger protein), Bcl11a | -7.88  | -5.52  | -4.42  | -4.78 |
| RT-qPCR validation of SAGE profile for E versus adult comparison |
|---------------------------------------------------------------|
| cggtgtcccccaacctcc NM_012015 | H2A histone family, member Y, H2afy | -21.48 | -27.88 | -4.62 | -3.70 |
| cagttgcaataaaaata NM_010894 | neurogenic differentiation 1, Neurod1 | -7.54 | -4.96 | -5.62 | -5.10 |
| aagtttgctcttctcca NM_008538 | myristoylated alanine rich protein kinase C substrate, Marcks | -24.87 | -11.76 | -5.72 | -5.56 |
| ttgcctggcttttataa NM_053104 | RNA binding motif protein 9, Rbm9 | -6.10 | -7.82 | -6.50 | -5.97 |
| gttttcggttgggac NM_019653 | WD repeat and SOCS box-containing 1, Wsb1 | -7.88 | -11.96 | -6.54 | -7.00 |
| tatattgtgggac NM_007569 | B-cell translocation gene 1, anti-proliferative, Btg1 | -15.28 | -16.48 | -6.93 | -5.58 |
| taaggaacct NM_019413 | roundabout homolog 1 (Drosophila), Robo1 | -28.64 | -16.73 | -8.23 | -11.35 |
| gctgtgcgctgccctctct | AA122503 | EST from M2 cells of skin melanoma | -17.17 | -18.68 | -9.88 | -10.15 |
| ttggagctgttgctrat NM_009870 | cyclin-dependent kinase 4, Cdk4 | -114.55 | -80.88 | -14.77 | -10.00 |
| ctttcccgtgcaaatgta NM_013834 | secreted frizzled-related protein 1, Sfrp1 | Es | Es | -16.48 | -16.37 |
| tgtcgtttctgttcNM_007971 | enhancer of zeste homolog 2 (Drosophila), Ezh2 | -10.63 | -5.52 | -21.36 | -17.59 |
| cacagacccccccccacc NM_009559 | zinc finger protein 57, Zfp57 | -30.72 | -42.33 | -29.13 | -44.46 |
| cgagctgaggtgagta NM_010025 | doublecortin, Dcx | -44.41 | -58.81 | -73.76 | -91.10 |
| cagaggtgaggtgagta NM_009234 | SRY-box containing gene 11, Sox11 | Es | Es | -140.05 | -120.35 |

All RT-qPCR data are statistically significant at \( P < 0.001 \) unless specified: * \( P < 0.01 \); † \( P < 0.05 \). ‡ A disagreement between RT-qPCR and SAGE data. As, adult-specific expression; Es, embryonic-specific expression; NS, no statistically significant difference between two developmental stages. Fold change values of <1.0 are presented in a negative fold change format.
## Table 4

### RT-qPCR validation of SAGE profile for P1.5 versus adult stages

| SAGE tag      | RefSeq accession | Gene ID | Fold change | Adult/P1.5 (SAGE) | Adult/P1.5 (RT-qPCR) |
|---------------|------------------|---------|-------------|-------------------|----------------------|
| aaattatggaaaatcc | NM_011123       | proteolipid protein (myelin) 1, Plp1 | 38.9 | 43.33          |
| ttcacaggtggtgct | NM_013556       | hypoxanthine guanine phosphoribosyl transferase 1, Hprt1 | 6.99 | 2.26           |
| acctggagccacagac | NM_009790       | calmodulin 1, Calm1 | 4.57 | 1.83           |
| gctcgctccacacagcg | NM_010777      | myelin basic protein, Mbp | As | 1.79           |
| tccccctcctat | NM_026106       | down-regulator of transcription 1, Dr1 | Ps | -1.46          |
| ggagaaactaggagagagag | NM_172503      | zinc finger, SWIM domain containing 4, Zswim4 | -42.42 | -1.96*         |
| gacgccagctcgcgcc | NM_031404       | actin-like 6B, Act6b | -5.51 | -2.26          |
| gccaatccaaaataaaa | NM_009094      | ribosomal protein S4, X-linked, Rps4x | -4.72 | -2.27          |
| Nil | NM_009726       | ATPase, Cu++ transporting, alpha polypeptide, Atp7a | Nil | -2.91          |
| agaatgggtaggtt | NM_008253       | high mobility group box 3, Hmgb3 | -20.99 | -3.53          |
| cctggctcagtcgcctc | NM_025635      | ZW10 interactor, Zwint | 4.65 | -4.04†         |
| acacgtcttgctggagag | BQ17786/NM_010487 | C57BL/6 whole brain E15.5 (or known as embryonic lethal, abnormal vision, Drosophila-like 3 (Hu antigen C), Elav3) | -41.99 | -4.06          |
| gataactgggtagacta | NM_007393       | actin, beta, cytoplasmic, Actb | -18.49 | -4.35          |
| ctggctcctctt | NM_008538       | myristoylated alanine rich protein kinase C substrate, Marcks | Ps | -4.76          |
| Nil | NM_025958       | cullin-associated and neddylation-dissociated 2 (putative), Cand2 | Nil | -5.62          |
| ggctgcgctctctcttc | NM_009870      | cyclin-dependent kinase 4, Cdk4 | -5.65 | -5.77          |
| ccctagtaag | AA122503        | EST from M2 cells of skin melanoma | -12.28 | -11.12          |
| tggaagctggctgtctat | NM_009238      | SRY-box containing gene 4, Sox4 | -31.51 | -11.74          |
| cagcagcaccccccccacc | NM_007792     | cysteine and glycine-rich protein 2, Csrp2 | Ps | -26.10          |
| tgtggagggaggtgta | NM_009559      | zinc finger protein 57, Zfp57 | -16.33 | -32.74          |
| gggcccttcggagagg | NM_010025      | doublecortin, Dcx | -24.56 | -82.11          |

All RT-qPCR data are statistically significant at $P < 0.001$ unless specified; * $P < 0.01$. †A disagreement between RT-qPCR and SAGE data. As: adult-specific expression; Nil: SAGE data not available; Ps: P1.5-specific expression. Fold change values of <1.0 are presented in a negative fold change format.

## Table 5

### RT-qPCR validation of SAGE profile for E15.5 versus P1.5 stages

| SAGE tag      | RefSeq accession | Gene ID | Fold change | P1.5/E15.5 (SAGE) | P1.5/E15.5 (RT-qPCR) |
|---------------|------------------|---------|-------------|-------------------|----------------------|
| Attcctcttggtatttt | NM_010838       | microtubule-associated protein tau, Mapt | 5.51 | 1.53*          |
| Gcagtggaacagagt | NM_009234       | SRY-box containing gene 11, Sox11 | -2.17 | -3.32          |

All RT-qPCR data are statistically significant at $P < 0.001$ unless specified; * $P < 0.05$. Fold change values of <1.0 are presented in a negative fold change format.
DETs are (in descending order of enrichment) Zfp57, Csrp2, Aa122503, Cdk4, Sox4, Markes, Actb, BQ177889, Hmgkb3, Rps4x, Actl6b, Zswim4 and Drt1, whose expression ranges from 33- to 1.4-fold greater than in the adult. On the other hand, the Plpi1 is expressed at a level 40 times greater in the adult cerebral cortex compared to P1.5. Other validated genes that are enriched in the adult cerebral cortex include (in descending order) Hprt1, Calm1 and Mbp, with a 2.3- to 1.8-fold greater expression than the P1.5 cerebral cortex. Comparison between E15.5 and P1.5 shows that Mapt has a 1.5-fold greater expression level in the P1.5 cerebral cortex while Sox11 expression is 3.3-fold lower (Table 5).

We were unable to validate all 17 DETs from L versus R1 regions, suggesting the left and right hemispheres of the adult mouse cerebral cortex are highly similar and indistinguishable at the molecular level. SAGE and RT-qPCR analyses for R versus C regions of E15.5 are discussed in a separate section below.

Functional analysis of validated gene clusters using Ingenuity Pathway Analysis

The validated DETs of embryonic, adult and gene-switching clusters were functionally characterized using proprietary software, Ingenuity Pathway Analysis (IPA) from Ingenuity Systems®, to identify enriched molecular networks and canonical pathways. Given a list of input genes (known as focus genes), IPA mapped these genes to a global molecular network developed from information contained in the Ingenuity knowledge base (a manually curated database of experimentally proven molecular interactions from published literature). Networks of these focus genes were then algorithmically generated based on their connectivity. IPA determined the most significantly enriched biological functions and/or related diseases by calculating the P-value using Fisher’s exact test. Using similar methods, significantly represented canonical pathways in a set of focus genes were also determined using IPA (Section C in Additional data file 1).

From the embryonic-specific gene clusters, we identified two statistically significant molecular networks (made up of 19 focus genes and 47 associated nodes; networks 1 and 2 in Figure 3: Figures S4 and S5 in Additional data file 1). The networks are interconnected through two genes, Markes and Neurod1. In general, these networks are associated with cell cycle and DNA replication, recombination and repair processes. Three statistically significant (using P < 0.05 as a cutoff) canonical pathways are enriched in these networks (Figure 3); Wnt/β-catenin signaling (Sox4, Sox11 and Sfrp1), F53 signaling (Cdk4 and Pena) and tight junction signaling (Cdk4 and Actb) pathways. Validated DETs such as Btg1, Cdk4, Cdkn1c, Csrp2, Ezh2, Neurod1, Pena, and Rps4x are associated with cell cycle control whereas Actb, Ezh2, Als2cr2, Markes, Robo1 and Dcx are associated with cellular assembly and organization. These processes are important in the formation of filopodia, membrane blebs and growth cones during neuronal growth, migration and axonogenesis. Known human neurological disorders associated with the networks, particularly network 2, include X-linked lissencephaly (Online Mendelian Inheritance in Man [OMIM:300067]; DCX), juvenile onset dystonia ([OMIM:607371]; ACTB) and Beckwith-Wiedemann syndrome ([OMIM:130600]; CDKN1C). All the DETs implicated in these networks are expressed in the cortical plate with the exception of Pena (Table S5 in Additional data file 1) [31-42].

In adult-specific gene clusters, two molecular networks (18 focus genes and 50 associated nodes; networks 3 and 4 in Figure 3) were identified and interconnected via a single gene, Mbp (Figures S6 and S7 in Additional data file 1). These molecular networks enrich for nine statistically significant canonical pathways (P < 0.05) such as synaptic long-term potentiation and depression, calcium signaling, B cell receptor signaling, cAMP-mediated signaling, GM-CSF signaling, amyotrophic lateral sclerosis signaling, G-protein-coupled receptor signaling and xenobiotic metabolism signaling pathways. Validated DETs such as Camk2a, Gria3, Itpr1, Egri,

### Table 6

| SAGE tag       | RefSeq accession | Gene ID                                      | Caudal/rostral (SAGE) | Caudal/rostral (RT-qPCR) |
|----------------|------------------|----------------------------------------------|-----------------------|--------------------------|
| gtgttcctccagctgg | NM_016916        | bladder cancer associated protein homolog    | 2.68                  | 1.43                     |
| gccatagcctgtcctgg | BC025816         | EST sequence BC025816                        | Cs                    | 1.31 (NS)                |
| aagctgacatgtagaa | NM_026187        | ankyrin repeat and zinc finger domain         | Rs                    | 1.17 (NS)                |
| gatactggagactgacta | NM_007393       | actin, beta, cytoplasmic, Actb                | -2.63                 | -1.32                    |
| tggggaagaaaaaac | NM_021278        | thymosin, beta 4, X chromosome, Tmsb4x        | -2.33                 | -2.13 (NS)               |

All RT-qPCR data are statistically significant at P < 0.05 unless specified. †A disagreement between RT-qPCR and SAGE data.

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**Genome Biology 2009, Volume 10, Issue 10, Article R104, Ling et al. R104.11**
Rgs4, Gstm1 and Ppp3ca are associated with cell-to-cell communication in the adult cerebral cortex. Biological functions such as cell proliferation, movement and death processes are also linked to these networks through the genes Camk2a, Cryab, Egr1, Gstm1, Itp1, Mbp, Nrgn, Ppp1r1b, Ppp3ca, Rgs4, Sept4, Sirpa and Snch. Known human neurological disorders associated with Network 3 include Pelizaeus-Merzbacher disease ([OMIM:312080]; PLP1) and dementia with Lewy bodies ([OMIM:127750]; SNCA), whereas X-linked mental retardation ([OMIM:300253]; TSPAN7 or TM4SF2) is associated with network 4. MBP, found in both network 3 and 4, is associated with genetic susceptibility to multiple sclerosis [OMIM:126200]. All the DETs implicated in these networks are expressed in layers I to VI of the cerebral cortex, with the exception of Gstm1 (layers II to V), Sirpa and Ppp3ca (layers II to VI), and Nptxr (no expression data available) (Table S5 in Additional data file 1) [32-34,43-52].

Both networks 5 and 6 (Figure 3) are linked to gene-switching clusters. These two molecular networks (14 focus genes and 52 associated nodes) are associated with cellular morphogenesis, amino acid metabolism, cell death processes and developmental disorders (Figures S8 and S9 in Additional data file 1). These networks are connected by Ctsd and Ywhae and are implicated in amyloid processing (App and Mapt), aryl hydrocarbon receptor signaling (Ctsd and Nedd8), and cell cycle G2/M DNA damage checkpoint regulation pathways (Ywhae, App, Atg7, Hprt1, Mapt, Ctsd and Cln2) are involved in cell morphogenesis, assembly and organization. Among all the networks in the study, network 5 has the greatest number of DETs associated with known human neurological disorders with six out of ten focus genes being associated with known neurological disorders such as neuronal ceroid lipofuscinosis ([OMIM:610127]; CTSD), Alzheimer’s disease ([OMIM:104300]; APP), Menkes disease ([OMIM:309400]; ATP7A), X-linked mental retardation ([OMIM:300253]; TSPAN7 or TM4SF2), Supranuclear palsy progressive ([OMIM:601104]; 6) and Pick disease ([OMIM:172700]).
lesy ([OMIM:606904, 607628 and 607631]; CLCN2), Lesch-Nyhan syndrome ([OMIM:300322]; HPRT1), frontotemporal dementia ([OMIM:600274]; MAPT), progressive supranuclear palsy ([OMIM:601104]; MAPT) and Pick disease of the brain ([OMIM:172700]; MAPT). All the DETs implicated in these networks are expressed in the cortical plate during embryonic development with the exception of Ube2e3. In the adult, all DETs are placed into layers I to VI of the cerebral cortex except for Bel11a, Cln2, Hprt1 (layers II to VI), Atp7a and Ube2e3 (no expression was detected in the adult cerebral cortex) (Table S5 in Additional data file 1) [32-35,46,53-61].

To refine the functional analysis to a cellular level, we grouped all 40 RT-qPCR validated DETs from networks 3, 4, 5 and 6 (adult specific and gene-switching gene clusters) into three groups according to where they are expressed: only in cortical neurons (N group); only in cortical glia (G group); and in both cortical neurons and glia (B group). None of the DETs from networks 1 and 2 (embryonic-specific gene clusters) were analyzed because most of the cells within the cerebral cortex are committed to the neuronal lineage at E15.5. The methods used to tabulate and group validated DETs are detailed in Section D in Additional data file 1. DETs classified as part of the N group are Bel11a, Calm1, Camk2a, Camk2n1, Hprt1, Htr1a, Mapt, Nedd8, Nrgn, Ppp1r1b, Ppp3ca, Rbm9, Rs45, Sncb and Yuhae, whereas those in the G group include Crub1, Mbp, Nptx2, Plp1, Ppap2b, Sept4 and Tsap7. The B group consists of App, Atp7a, Chgb, Ctsd, Egr1, Gria3, Gstm1 and Sirpa. DETs without any cellular expression information, such as Actl6b, Cln2, Hmgb3, Ube2e3, AK138272, AK139402, AK140219, AK154943, AU258168 and BO175689, were placed into group B to facilitate downstream analysis. Of the 40 DETs, only 35 were mapped to the IPA knowledgebase and subjected to further analysis. Functional analysis of these 35 DETs show that the neuron-based DETs (N and B groups) are enriched for various human neurological disorders, such as schizophrenia (9 DETs; \( P = 4.36 \times 10^{-8} \)), Huntington’s disease (8 DETs; \( P = 1.03 \times 10^{-5} \)), X-linked mental retardation (4 DETs; \( P = 3.78 \times 10^{-7} \)), Parkinson’s disease (3 DETs; \( P = 6.99 \times 10^{-3} \)) and Alzheimer’s disease (3 DETs; \( P = 1.52 \times 10^{-2} \)) (Figure S10 in Additional data file 1). Thirteen DETs associated with these neurological disorders are also implicated in processes related to cell death. Of these, eight DETs are expressed in neurons only, two in glia only and three in both neurons and glia.

From the above IPA analysis, CAND2 (embryonic gene clusters), CAMK2N1 (adult gene clusters), HMG1 (gene-switching clusters) and 10 ESTs (BO175689, CD802535, AK139402, AU258168, AK138272, AK140219, AK154943, AA125253, NM_001033273 and BO177886) are not currently connected to any networks.

Regionalized expression of DETs in the E15.5 cerebral cortex

Early regionalized development is an important event that could lay the foundations for adult arealization of cerebral functions. To identify genes with regionalized expression profiles, we compared SAGE libraries generated from rostral and caudal regions (equivalent to anterior and posterior regions of the human cerebral cortex) of the E15.5 cerebral cortex. We identified 44 DETs and selected 25 DETs (22 known genes, 1 EST and 2 ambiguous genes; Additional data file 2) and 2 genes of interest (bladder cancer associated protein, Bcap, and ankyrin repeat and zinc finger domain containing 1, Ankzf1) for RT-qPCR validation and further detailed region-based analysis. This was done using independent biological triplicates of clearly defined regions/quadrants, such as rostro-lateral (RL), rostro-medial (RM), caudo-lateral (CL) and caudo-medial (CM) of the E15.5 cerebral cortex (see Materials and methods). Two positive controls with known regionalised expression were included in the RT-qPCR: RAR-related orphan receptor beta (Rorb) and nuclear receptor subfamily 2, group F, member 1 (Nr2f1). Rorb is highly expressed in the rostral region whereas Nr2f1 is highly expressed in the caudal region of the cerebral cortex [1,62].

An initial RT-qPCR analysis of combined RL and RM (rostral) as well as CL and CM (caudal) regions shows upregulation of Rorb and Nr2f1 in rostral and caudal regions, respectively (based on fold change direction and magnitude of 1.3 times). The same analysis also confirmed the SAGE data for 3 out of 25 DETs (Actb, Tmsb4x and BC025816) and Bcap (Table 6; Additional data file 6). Both Actb and Tmsb4x have greater expression in the rostral region whereas Bcap and BC025816 are greater in the caudal region. To identify expression profiles in a more refined area and prevent regional compensation due to combined quadrant analysis, we performed a quadrant versus quadrant multiple regions comparison. The largest number of DETs were found in the RL versus CM comparison, as they are the two developmentally most distinct regions within the cerebral cortex at E15.5 compared to others; RL versus CL > RM versus CL > RL versus RM > CM versus CL versus CM. The region-specific expression profiles were plotted for each of the DETs (Figure 4) and grouped into two categories: RL-specific DETs, such as Actb, Tmsb4x and cytochrome b-245, beta polypeptide (Cybb); and CM-specific DETs, including Bcap, EST BC025816, Ankzf1 and cytochrome c oxidase I, mitochondrial (Cox1) (Additional data file 6).

To visualize the regionalized expression profiles, we performed in situ RNA hybridization (ISH) on all DETs validated by quadrant versus quadrant RT-qPCR analysis, Bcap, Ankzf1 as well as the positive controls Rorb and Nr2f1. We performed ISH on sagittal and coronal sections (from rostral to caudal regions) of the E15.5 mouse brain (Figure 5). Under dark-field microscopic examination, we confirmed the regionalized expression of Rorb (at the rostral cortical plate;
Figure 5a) and *Nrxz1* (at the caudal ventricular zone; Figure 5f). From the analysis, *Actb* is highly expressed at the cortical plate and the subplate (Figure 5b). *Tmsb4x* is highly expressed at the cortical plate and the intermediate zone (Figure 5c-e).

ISH analysis showed that both *Blcap* and *Ankzf1* are caudal specific. Serial coronal sections from rostral to caudal regions of the brain (Figure 5g-i) show *Blcap* is weakly expressed in the rostral cerebral cortex, particularly at the intermediate zone, subplate and the cortical plate, but is highly expressed in the hippocampus and thalamus (Figure 5i). *Ankzf1* is expressed specifically in the ventricular zone as well as the cortical plate towards the caudal region of the cerebral cortex (Figure 5j-m). The distinctive expression of *Ankzf1* in both the ventricular zone and cerebral cortex prompted us to extend our ISH analysis to various developmental stages starting from E11.5 to adulthood (Figure 5n-t). In the adult, *Ankzf1* expression is obvious in the piriform cortex, hippocampus and cerebellum (Figure 5r-t).

Genomic clustering of sense-antisense SAGE tags at the *Sox4* and *Sox11* loci
We performed genomic clustering analysis of the SAGE tags to determine any actively transcribed chromosomal loci throughout cerebral corticogenesis. Probabilities for chance occurrences of two, three, four, and five DETs being clustered within a window of ten adjacent tags present within each chromosomal location, irrespective of genetic distance, were calculated. This analysis was based on the DET lists described above (Additional data file 2). The analysis showed two over-represented chromosome loci at *Sox4* and *Sox11*, which derive from embryonic-specific gene clusters.

At both loci, we observed multiple SAGE tags with both sense and antisense orientations, which signify alternative polyadenylation sites, differential splicing and overlapping antisense transcription. As an initial validation of the antisense messages, we performed strand-specific RT-PCR (Figure 6) using cDNA synthesized from equally pooled total RNAs (three
Figure 5
In situ RNA hybridization of selected R versus C regions of E15.5 DETs. ISH was performed on (a-m) E15.5 and (n-t) E11.5 to P150 brains. (g-m) Coronal sections that are generated from the rostral to caudal axis; (a-f, s-t) sagittal sections. Micrographs of higher magnification are presented directly after any micrographs with an inset box (d-e, m, t). All micrographs are in dark-field except for (e, m), which are bright-field micrographs. C: caudal; CB: cerebellum; CP: cortical plate; DG: dentate gyrus; HPf: hippocampal formation; IZ: intermediate zone; OB: olfactory bulb; R: rostral; VZ: ventricular zone. Arrows show the region with expression or silver grains.
mice from each of E15.5, E17.5, P1.5 and adult stages). Three primer sets were used for each locus (designed at the middle, 5'- and 3'-ends of the canonical transcripts). For both Sox4 and Sox11 loci, the analysis showed positive signals from all primer sets used that were complementary to the antisense strand, therefore confirming the presence of one or more antisense transcript(s) that span the canonical transcript. Hydroxymethylbilane synthase gene (Hmbs) served as a negative control and there were no positive bands in the antisense strand RT-PCR, confirming the absence of antisense transcripts.

**Genomic cluster at the Sox4 gene locus**

We mapped SAGE tags from the genomic cluster to the Sox4 gene locus using the University of California Santa Cruz (UCSC) genome browser (Figure 7a; Additional data file 7) [63]. Only tags within and around the Sox4 canonical transcript are shown. Evidence of mapped mouse mRNAs within this locus further justifies the existence of multiple SAGE tags in addition to the canonical Sox4 transcript. Subsequent validation of the genomic clusters was solely based on the SAGE tag information, 6 out of 12 tags are DETs. These include four DETs within the canonical transcript (Figure 7b), with sox4_tag10 and sox4_tag15 having greater expression in P1.5 compared to P150 (adult stage), whereas sox4_tag12 and sox4_tag16 are both abundantly expressed in the caudal region of the E15.5 cerebral cortex. Based on the RT-qPCR analysis (Figure 7c), sox4_tag10 has greater expression in E15.5 compared to E17.5 (1.76-fold change), P1.5 (3.72-fold change) and Ad (43.67-fold change). For sox4_tag12, sox4_tag15 and sox4_tag16, differences are seen only in P1.5 (-2.00-fold, -1.57-fold and -1.84-fold changes, respectively) and Ad (-82.08-fold, -68.92-fold and -69.41-fold changes, respectively) when compared to E15.5. RT-qPCR analysis on the same tags did not find any differences between rostral and caudal cerebral cortices of E15.5. The differences in fold change between DETs suggest irregular overlapping of various transcript variants at different Sox4 gene loci.

To further validate the expression profiles of the multiple Sox4 DETs, we performed 3' rapid amplification of cDNA ends (RACE)-Southern analysis using pooled adaptor-oligo-d[T]15 synthesized cDNAs from three mice at each developmental stage. Based on this method, we were able to semi-quantitatively and accurately measure the expression levels of individual SAGE tags at the locus. To show that the amplification was cDNA specific, we performed PCR by using the same primer sets on mouse genomic DNA under the same conditions. In all cases, no amplification was observed (data not shown). This analysis confirmed the presence of four of seven alternative transcripts for Sox4 (Figure 7d). Corresponding tags were determined by estimating the amplion sizes between the strand-specific primers used, the next downstream AUAAA/AUAAA polyadenylation signal (if any) and succeeding CATG sequence or SAGE tags. Figure 7d(1)-d(3) confirms the existence of sox4_tag10, sox4_tag12 and sox4_tag15. Of these tags, SAGE expression profiles of sox4_tag10 and sox4_tag15 were validated (embryonic-specific and reduced expression after P1.5) but not sox4_tag12 (E15.5 caudal region-specific). 3' RACE-Southern analysis using a sense probe detected bands in the rostral and caudal regions of E15.5, E17.5 and E17.5 cerebral cortices and, therefore, confirmed the existence of the Sox4 antisense transcripts (Figure 7d(4)). Even though none of these tags were differentially expressed in between these regions based on the SAGE analysis, our findings show distinctive regionalization for sox4_tag14 expression at the E15.5 rostral cerebral cortex. Proteasome (prosome, macropain) subunit, beta type 2 gene (Psmb2) and Hmbs were used as controls and no antisense or alternative transcripts were identified at these gene loci (Figure 7d(5)-d(8)).

Since 3' RACE-Southern analysis was dependent on oligo-[dT]15 priming, we could not rule out the possibility of amplions that were generated by false priming on homopolymer-A stretches. Therefore, Northern analyses were performed on equally pooled total RNA extracted from the cerebral cortices of seven mice at E15.5, E17.5 and P150 (negative control). By using a double-stranded DNA probe at the 3' untranslated region (UTR) of Sox4 (Additional data file 8), we identified six bands ranging from approximately 2 kb to approximately 4.7 kb (Figure 7e). Sox4 sense transcripts are weakly expressed in
Figure 7 (see legend on next page)
The number of bands observed is similar and corresponds to overlapping mouse mRNAs as well as publicly available paired-end diTag (PET) sequences downloaded from Ensembl (Figure 7a; Table S6 in Additional data file 1) [64]. Taken together, the analysis confirmed the existence of multiple overlapping variants of Sox4 sense transcripts at this gene locus.

To confirm the rostro-caudal expression of Sox4 sense transcripts, we performed ISH on sagittal sections of mouse brains using a Sox4 antisense riboprobe that spanned across the sox4_tag10, sox4_tag12, sox4_tag15 and sox4_tag16 SAGE tags. Sox4 showed regionalization expression at E15.5 and E17.5 (Figure 7f). At E15.5, Sox4 sense transcripts are expressed more in both the rostral- and caudal-end regions of the cortical plate compared to the intermediate region between them (Figure 7f(1)). By E17.5, expression of Sox4 sense transcripts is obvious in the rostral cortical plate (Figure 7f(2)). At both stages of development, Sox4 sense transcripts are uniformly expressed in the intermediate zone of the cerebral cortex. These findings correspond to the SAGE tag counts for E15.5 rostro-caudal regions of the cerebral cortex (Figure 7t). These observations explain the averaged total tag count per 100,000 tags for different Sox4 sense transcripts, which are predominantly expressed in both rostral and caudal regions of the cerebral cortex. The regionalized expression of Sox4 in the cortical plate is obvious only at E15.5 and E17.5, but not at other stages of development (Figure S11 in Additional data file 1).

In situ RNA hybridization of Sox4 sense and antisense transcripts
To further ascertain the antisense expression of Sox4 in a spatio-temporal manner, we performed ISH on coronal sections obtained from E11.5, E13.5, E15.5, E17.5, P1.5 and P150 mouse brains. Sense and antisense RNA probes were generated from the same clone used in the Northern analysis. At E11.5, Sox4 sense transcripts are confined to the primordial plexiform layer (Figure 8a). From E13.5 to P1.5, the sense transcripts are expressed throughout the cortical plate (Figure 8b-e). Expression of sense transcripts in the subventricular zones is observed at E17.5 and P1.5 only (Figure 8d, e). There is no observable sense expression in the adult stage (Figure 8f). Sox4 antisense transcripts are expressed throughout the telencephalon at E11.5 (Figure 8g). From E13.5 to P1.5, Sox4 antisense expression is confined to the cortical plate only (Figure 8h-k). There is no obvious antisense expression in the cerebral cortex in the adult stage (Figure 8l). A microscopic examination at high magnification showed that Sox4 antisense transcripts are predominantly localized in the nucleus whereas Sox4 sense transcripts are found in both the nucleus and cytoplasm (Figures S12, S13 and S14 in Additional data file 1). We used hemoglobin alpha, adult chain 1 (Hba-a1) of the corresponding brain region and time-point as a control in the analysis (Figure 8m-r).

Furthermore, Sox4 antisense expression occurs in the piriform cortex layer II (Figure 9a-c) and dentate gyrus (Figure 9g-i) in the adult brain; however, no sense expression is observed in these regions. At P1.5, we identified complementary expression between Sox4 sense and antisense transcripts in the olfactory bulb (Figure 9d-f). Sox4 sense expression was confined to the granular and glomerular layers of the olfactory bulb whereas antisense expression was found only in the outer plexiform layer. We used either Sox11 or Hba-a1 of the corresponding brain region and time-point as a control in the analysis (Figure 9c, f, i).

Analysis of the Sox11 genomic cluster
SAGE tags, which represent multiple overlapping sense and antisense transcript variants at the Sox11 genomic cluster, were validated using 3’ RACE-Southern analysis as described above. See Section F in Additional data file 1 for a full description of the Sox11 results. ISH analysis did not confirm the expression of antisense transcripts of Sox11, but the presence of PETs spanning three out of five antisense tags confirmed the existence of Sox11 antisense transcripts (Table S8 and Figures S16, S17 and S18 in Additional data file 1). The discrepancy between ISH and RT-qPCR or 3’ RACE-Southern analysis suggests that Sox11 antisense transcripts might be expressed at low levels or at specific locations of the cerebral cortex, and hence can be detectable only by using serial sections or whole mount ISH.
Screening of Sox4 and Sox11 antisense transcripts in the adult mouse brain, organs, P19 cell line and neurospheres

We screened various adult brain regions (olfactory bulb, cerebellum, medulla, hippocampus, thalamus and cerebral cortex) and selected mouse organs (E15.5 whole brain, heart, kidney, liver, skeletal muscle, skin, spleen, stomach, testis and thymus) for the expression of Sox4 and Sox11 antisense transcripts by strand-specific RT-qPCR. Within the adult brain, Sox4 sense and antisense transcripts are expressed in all regions, with the highest level found in the olfactory bulb, which is approximately four- to nine-fold greater than those in other brain regions (Figure 10a). Expression of Sox4 antisense transcripts occurs in all mouse organs, with the highest level in the thymus followed by E15.5 whole brain, testis and skin (Figure 10b). Sox4 sense and antisense expression profiles are similar throughout the entire series of samples screened, with the sense transcripts being consistently expressed at a greater level than the antisense transcripts (approximately 1.7-fold in various brain regions and approximately 2- to 14-fold in various organ comparisons).

Sox11 sense transcripts are expressed at the highest level in the olfactory bulb, approximately two- to seven-fold greater than those in other brain regions (Figure 10a). Sox11 antisense transcripts, on the other hand, are expressed in all brain regions screened and at a comparable level in the olfactory bulb, hippocampus, thalamus and cerebral cortex. In comparison to other adult mouse organs, Sox11 sense and antisense transcripts are highly expressed in the E15.5 whole brain, with Sox11 sense transcript levels at least 100-fold greater than those in other mouse organs (Figure 10b). On the other hand, Sox11 antisense expression is observed only in the E15.5 whole brain, skin and stomach. Notably, Sox11 sense

![ISH analysis of Sox4 transcripts in E11.5 to P150 mouse brains](http://genomebiology.com/2009/10/10/R104/figure8.png)

**Figure 8**

ISH analysis of Sox4 transcripts in E11.5 to P150 mouse brains. (a-f) Expression of the sense transcript for Sox4. (g-l) The expression of the antisense transcript for Sox4. (m-r) Hba-a1 antisense expression (negative control). All micrographs were taken from coronal sections. CP: cortical plate; IZ: intermediate zone; MZ: marginal zone; PP: primordial plexiform layer; SVZ: subventricular zone; VZ: ventricular zone.
transcripts are expressed more highly than antisense transcripts in the E15.5 whole brain and skin (23- and 4-fold, respectively).

Since both Sox4 and Sox11 are implicated in neuronal differentiation and glial maturation processes [65,66], we examined both Sox4 and Sox11 sense and antisense transcript expression in proliferating and differentiating P19 (embryonal carcinoma cells) and in embryonic NSPCs grown as neurospheres. Both Sox4 sense and antisense transcripts are upregulated during P19 cell differentiation (approximately 5.7- and 1.6-fold upregulation, respectively; Figure 11a) and neurosphere differentiation (approximately 1.9- and 1.8-fold upregulation, respectively; Figure 11b). For Sox11, both sense and antisense transcripts are upregulated in the differentiating compared to the proliferating P19 cells by approximately 2.3- and 4.2-fold, respectively (Figure 11c). Both the Sox11 sense and antisense transcripts are, however, downregulated in the differentiating neurospheres (approximately 2.6- and 1.5-fold, respectively; Figure 11d).

**Discussion**

In this study, SAGE was used to analyze global gene expression in the normal mouse cerebral cortex at various developmental stages. We report validated spatio-temporal regulation of genes involved in mouse cerebral cortex development from embryo to adulthood. The study highlights four main findings: association of DETs from different gene clusters with known functional processes or signaling pathways and disease-causative genes that are involved in cerebral corticogenesis; Ank2fl and Sox4 sense transcripts are regionally expressed in the E15.5 cerebral cortex; multiple overlapping Sox4 and Sox11 sense and antisense transcripts are spatio-

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**Figure 9**

*Sox4 antisense expression in other brain regions.* (a-i) Sox4 antisense expression is also observed in other regions such as the piriform cortex (a-c; arrows), olfactory bulb (d-f) and dentate gyrus (g-i). (a, d, g) Sox4 sense expression; (b, e, h) Sox4 antisense expression. (c, f, i) Hba-a1 or Sox11 antisense expression (negative controls). All micrographs were taken from sagittal sections except (a-c), which were taken from coronal sections. gl: glomerular layer; gr: granule layer; Opl: outer plexiform layer; PIR2: piriform cortex layer II.
Expression of Sox4 and Sox11 transcripts in various mouse organs. (a) Strand-specific RT-qPCR screening of Sox4 and Sox11 sense and antisense transcript expression in various adult mouse brain regions. N = 2 and data are presented as mean ± standard error of the mean (SEM). (b) Strand-specific RT-qPCR screening of Sox4 and Sox11 sense and antisense transcripts in various adult mouse organs. N = 3 and data are presented as mean ± SEM.
temporally regulated during cerebral corticogenesis; and Sox4 and Sox11 antisense transcripts are differentially regulated in both proliferating and differentiating embryonic-derived neurospheres and P19 cells.

We have shown that most tags generated in all libraries were singletons. The number of singletons could be reduced by increasing the number of tags sequenced. In mammalian cells, the number of additional unique transcripts identified approached zero when the number of SAGE tags sequenced reached approximately 600,000 [67]. Increasing the number of tags sequenced could improve the sensitivity of the technique to identify weakly expressed or novel transcripts, and the application of massively parallel signature sequencing [68] using a next-generation sequencer would be an ideal solution to accomplish this. In addition, one of the benefits of SAGE is that datasets generated from different groups or in public repositories such as SAGE Genie [69] and GEO [28] are readily comparable and, hence, can increase the tag count and sensitivity of the technique in discovering DETs between SAGE libraries. However, any meta-analyses involving various SAGE datasets are affected by experimental and biological variation; thus, a careful selection of matching libraries is crucial to limit systematic error or biases.

Our SAGE analysis robustly detected DETs with a low false positive rate (for example, <0.001% for comparison between left and right hemispheres of the adult cerebral cortex). Of all the identified DETs, approximately 8% were not mapped to either a single locus in the mouse genome or any unique annotation. This problem could be overcome by generating additional information from the 5’ end of the transcript through alternative techniques such as PET sequencing [70], cap analysis gene expression (CAGE) [71] and 5’ LongSAGE [72].

We have identified functional ontologies, molecular interactions and enriched canonical pathways that are distinct to the

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**Figure 1**

**Expression of Sox4 and Sox11 transcripts in neurospheres and P19 cells.** (a-d) The figure shows strand-specific RT-qPCR screening of Sox4 (a, b) and Sox11 (c, d) sense and antisense transcripts expression in proliferating and differentiating P19 cells (a, c) and neurospheres (b, d). N = 3 for P19 cells and N = 2 for neurospheres. All data are presented as mean ± standard error of the mean. Fold change values (normalization to proliferating cells) are presented above the comparative bar and any values <1 are presented in the negative fold change format. *P < 0.05; ***P < 0.01; ****P < 0.001.
stage-specific gene clusters of validated DETs. The IPA network analysis generated connections between validated DETs across various developmental stages in relation to well-established proteins or molecules and neurological disorders. In the study, members of the Wnt/β-catenin signaling pathway were enriched in networks 1 and 2 (embryonic-specific gene clusters). In neural development, Wnt/β-catenin signaling plays an important role in regulating regional specification of the cortex along the rostro-caudal and dorso-ventral axes, and proliferation of cortical progenitors [73]. IPA highlighted three genes (Sox4, Sox11 and Sfrp1) associated with this pathway. In humans, the SFRP1 protein (secreted frizzled-related protein 1) stabilizes β-catenin and increases transcription from β-catenin-responsive promoters [74]. In β-catenin-deficient mouse mutants, expression of both Sox4 and Sox11 is downregulated [75,76]. Sox4 and Sox11 proteins play an important role in establishing neuronal properties, pan-neuronal gene expression and proper myelination of the central nervous system [65,66]. This suggests that the role of the Wnt/β-catenin signaling pathway in regulating neuronal development could be mediated, at least in part, by the Sox4 and Sox11 proteins.

The role of Wnt/β-catenin signaling in regulating DETs (Btg1, Cdk4, Cdkn1c, Csrp2, Ezh2, Neurod1, Pena, and Rps4x) involved in cell cycle and proliferation remains unclear. Gain-and-loss-of-function studies have established that Wnt/β-catenin signaling is essential to maintain the pool of precursors for proper development of the cerebral cortex [77,78]. To date, there is no direct evidence to show that Ezh2, Pena, Rps4x and Btg1 are involved in cell cycle regulation during early embryonic neurogenesis. But their expression in the ventricular/subventricular zone of the E15.5 developing mouse cerebral cortex [33,35,36] suggests that they may be involved in regulating cell proliferation during neurogenesis. The role of these DETs and their association with the Wnt/β-catenin signaling pathway remains unclear and requires further experimentation.

Networks 5 and 6 are enriched for genes in the amyloid processing signaling pathway. App and Mapt are associated with this pathway. Under normal circumstances, the App protein is required for proper migration of neuronal precursors into the cortical plate in early embryonic corticogenesis [82]. The Mapt protein, on the other hand, plays an important role in maintaining the architecture of the neuronal cytoskeleton and intracellular trafficking. Overexpression of App protein and hyperphosphorylation of the Mapt protein have been implicated in the pathologies of Alzheimer's disease [83,84]. Interestingly, Ctsd, Atp7a, Clcn2 and Hprt1, the genes responsible for other human neurological disorders such as neuronal ceroid lipofuscinosis (Ctsd), Menkes disease (Atp7a), epilepsy (Clcn2), and Lesch-Nyhan syndrome (Hprt1), are associated with App and Mapt. These candidate genes are also involved in cell morphogenesis, assembly and organization and could be linked to deterioration of neurons during the pathologic progression of these disorders.

Pathway analysis of DETs classified into the N, G and B groups showed DETs in the neuron (N and B groups) are associated with Huntington's disease and schizophrenia, which were not previously identified in networks 3 to 6. Our analysis showed that both disorders share three common DETs, namely Rgs4, Ppp1r1b and Chgb, whose expression is downregulated in humans with Huntington's disease or schizophrenia [85-88]. A proportion of patients with Huntington's disease also develop schizophrenia [89,90]. Taken together, downregulation of Rgs4, Ppp1r1b and Chgb expression in neurons may contribute to the common symptoms in these disorders. Our findings imply that many DETs (including App, Hprt1 and Sncb) associated with both Huntington's disease and schizophrenia are also involved in neuronal/cell death processes [91-93]. Other DETs in the N group, not previously implicated in neuronal cell death, may serve as novel potential candidates during pathologic development in these disorders.

Regionalized development of the cerebral cortex involves the differential regulation of cell cycle exit, early migration and attainment of positional identity in neuronal fated cells. To date, only few genes have been associated with regionalized development of the cerebral cortex [3,94]. In the regionalization analysis, we identified the highest number of DETs in the comparison of the RL and CM libraries, which signifies that these two regions of the cerebral cortex are the most different. This finding supports the notion that the cerebral cortex is developed in a latero-medial axis followed by a rostro-caudal axis [7,8]. At E15.5, both Actb and Tmsb4x were expressed greater in the rostral cerebral cortex than in the caudal region. Both Actb and Tmsb4x proteins are involved in the actin cytoskeleton-signaling pathway [95]. In particular, the Tmsb4x protein has been shown to promote cardiomyocyte migration [96] and axonal tract growth in zebrafish [97]. Therefore, co-expression of Actb and Tmsb4x in the E15.5 mouse cortical plate suggests that they may have a synergistic
role in early cortical cell development. Conversely, Bcap and Ank2f1 were expressed more highly in the caudal than in the rostral region of the E15.5 cerebral cortex. To date, the function of both Bcap and Ank2f1 in the cerebral cortex remains uncharacterized. This study provides the first comprehensive expression profile of Ank2f1 and suggests it could be an important transcription factor in cerebral corticogenesis.

At E15.5, Sox4 sense transcripts were expressed in a high-rostral and high-caudal manner with lesser expression within the intermediate region. By E17.5, Sox4 expression becomes obvious at the rostral cortical plate, which is similar to Rorb. But, we did not find that the regionalized expression of Sox4 sense transcripts resembles that of the restricted Rorb expression at E15.5 or in the postnatal brain [1,62]. This finding could be caused by the combined expression profiles of different Sox4 sense transcripts that are present across the rostro-caudal axis of the cortical plate. The regionalized expression of Sox4 sense transcripts occurs only between E15.5 and E17.5. Because the thalamic axon innervates the cortical plate after E17.5 [98], the regionalization of Sox4 sense transcripts in early cortical development could be an outcome of an intrinsic instead of an extrinsic mechanism that regulates early patterning of the cerebral cortex.

Genomic clustering of DETs identified the differentially regulated Sox4 and Sox11 gene loci. These genomic clusters imply that there are multiple overlapping sense and antisense transcripts surrounding the same gene locus that are co-transcribed simultaneously during cerebral cortex development. Both Sox4 and Sox11 are single exon genes and these transcript variants are therefore likely to be generated due to alternative polyadenylation. The 3' UTRs of both Sox4 and Sox11 have tandem terminal polyadenylation signals on both sense and antisense strands (data not shown), which supports the occurrence of multiple transcript forms or SAGE tags. Multiple mRNA forms with different 3' UTRs can lead to cell-specific regulation, different nuclear or cytoplasmic mRNA stability and translation rates [99,100]. The 3' UTR of Sox4 and Sox11 may contain AU-rich elements that play an important role in determining mRNA stability through deadenylation, decapping or 3' → 5' decay [101]. Besides, different 3' UTR lengths may be targeted by different miRNAs, thus interfering with the transcription process. Both Sox4 and Sox11 transcripts may be targeted by various miRNAs at different predicted positions across the 3' UTR (Tables S7 and S9 and Figures S15 and S18 in Additional data file 1). Therefore, 3' UTR lengths of Sox4 and Sox11 may be an important feature in the regulation of their protein expression during cerebral corticogenesis.

In the study, NATs were found at both the Sox4 and Sox11 gene loci overlapping the sense transcripts. Overlapping NATs may function as templates for the generation of pre-miRNA and mature miRNA with exceptional high sequence conservation that complement the overlapping sense protein-coding transcripts [102]. To date, no mature or pre-miRNAs have been predicted on the Sox4 and Sox11 sense and antisense strands (data not shown) or have been reported in miRBase [103]. In addition, NATs can self-complement to form double stranded RNA or pair with sense transcripts and function as templates for the generation of endogenous small interfering RNAs, which could subsequently interfere with translation or transcription of multiple protein-coding transcripts [104]. Because both Sox4 and Sox11 proteins are highly expressed in the cerebral cortex, the overlapping NATs do not seem to be involved in the regulation of Sox4 and Sox11 through miRNA- or small interfering RNA-mediated translation repression mechanisms, but rather through antisense-regulated sense transcription within the nucleus.

Our ISH analysis showed complementary cellular expression profiles of Sox4 sense and antisense transcripts at the piri-form cortex, olfactory bulb and dentate gyrus. This finding implies that Sox4 antisense transcripts may be essential in intracellular and interlocus negative feedback loop regulation of the Sox4 sense transcripts. Similar expression profiles of Sox4 sense and antisense transcripts in multiple mouse organs and brain regions, however, suggest that these transcripts may be co-expressed. This observation is also supported by the temporal co-expression of Sox4 sense and antisense transcripts in the cortical plate or layers I to III of the cerebral cortex. Taken together, the sense and antisense transcripts of Sox4 are co-expressed in some cells and expressed complementarily in other cells, suggesting crucial cell-type-specific regulation.

Sox4 and Sox11 have been shown to have redundant roles during mouse development [105], and Sox11 may play a compensatory role in the absence of Sox4 during brain development [106]. We demonstrated that Sox4 and Sox11 sense and antisense transcripts have a similar expression in the brain, but not in other organs, suggesting a compensatory role for Sox11 only in the brain. Sox11 antisense transcripts were expressed in the brain, skin and stomach only, suggesting organ-specific regulation.

Our data show upregulation of Sox4 and Sox11 sense transcripts in differentiating P19 cells, consistent with the findings of others [107,108], and demonstrate upregulation of antisense transcripts as well. We also find both Sox4 and Sox11 sense transcripts expressed in the NSPCs cultured as neurospheres, which is in agreement with Dy et al. [109]. Furthermore, we identify upregulation of both Sox4 sense and antisense transcripts but downregulation of Sox11 sense transcripts in differentiating neurospheres. Taken together, our findings show that there are potentially common and distinct roles for Sox4 and Sox11 sense and antisense transcripts during neuronal and non-neuronal cell proliferation and differentiation. The underlying regulatory mechanism of these transcripts, particularly the antisense ones, remains unknown and requires further investigation.
Conclusions
This study provides avenues for future research focus in understanding the fundamental processes and development of neurological disorders related to the cerebral cortex. We confirm the regionalized expression of new candidate genes in the E15.5 cerebral cortex as well as differential regulation of multiple overlapping sense and novel antisense transcripts within Sox4 and Sox11 gene loci during cerebral corticogenesis. We also report for the first time the spatio-temporal regulation of Sox4 antisense transcripts in the brain as well as differential regulation of novel Sox4 and Sox11 antisense transcripts in various mouse organs and in proliferating and differentiating NSPCs and P19 cells. The finding provides an insight for future investigations into the role of antisense transcripts during cerebral corticogenesis and neuronal differentiation.

Materials and methods
Handling of animals and dissection of the cerebral cortex
All experiments that involved animal breeding and handling were performed according to protocols approved by the Melbourne Health Animal Ethics Committee (Project numbers 2001.045 and 2004.041). All animals involved in the study were C57BL/6 mice unless specified otherwise. All mice were kept under conditions of a 12-h light/12-h dark cycle with unlimited access to food and water. All mice were culled by cervical dislocation prior to dissection. Cortical tissue was procured in the following fashion. For adult samples, after removal of the meninges, coronal cuts were used to excise the olfactory bulb from the rostral region, and the superior colliculus from the caudal region. A sagittal cut to separate the two cortical hemispheres was performed. The cortical pallium was dissected from the subpallial striatum and the septum. The neocortex was then dissected away from the cingulate cortex and the entorhinal cortex. For embryonic samples, the cortical tissue was dissected free from the underlying ganglionic eminences at the pallial-subpallial border. An orthogonal cut was made to remove the presumptive striatum and the overlying pifiform cortex. On the medial aspect, the medial limbic cortex was included for analysis, but the adjacent hippocampal primordium, including the cortical hem, was excluded. For the E15.5 cerebral cortex, the resulting hemispheres containing cortical tissue only were placed on the bottom of the Petri dish and, using a fine scalpel, divided into four equal quadrants per hemisphere, namely RL, RM, CL and CM. Rostral and caudal quadrants from both hemispheres were pooled for SAGE library construction but separately tracked for RT-qPCR analysis. Procurement of other adult brain tissues and related mouse organs for Sox4 and Sox11 antisense transcript screening was carried out according to the standard mouse necropsy protocol accessible at the National Institute of Allergy and Infectious Diseases (NIAID) website [110].

Identification of differentially expressed tags
Library comparisons were performed using two methods. Fisher’s exact test was used to compare two individual SAGE libraries. In the analysis, multiple testing correction [114] was carried out to control for false-discovery rate and adjusted P-value cutoffs (Q-values) were used to select DETs. In cases where several libraries were combined to focus on a specific biological comparison (for example, different stages of development), a Bayesian model, as described previously [115], was used to integrate multiple libraries in pairwise comparisons involving biological replicates of libraries. The model accounts for within-class variability by means of mixture distributions. The resulting E-values were used to select DETs. A table of all relevant comparisons, the comparison method and Q- or E-value cutoffs is provided in Table S1 in Additional data file 1.

Hierarchical clustering of SAGE tags
To identify co-regulated genes, the clustering of DETs was performed based on the log2 of normalized counts. Each library was normalized to 100,000 tags per library to account for size differences. A pseudocount of 0.5 was added before taking the log2 of the normalized tag counts. The tag-wise mean was subtracted from the log2 tag intensities before computing the Euclidean distance of the individual tag profiles. Hierarchical clustering was performed on the tags using

SAGE libraries and analysis of tags
Ten SAGE libraries were constructed from the cerebral cortex of E15.5, E17.5 and 4- to 6-month-old (Ad) mice according to either one of the two methods described previously [25,26], using I-SAGE™ or I-SAGE™ Long Kits (Invitrogen, Mulgrave, Victoria, Australia). Additional libraries from E15.5 and P1.5 of the cerebral cortex described previously [27] were also included in the analysis. These libraries contain a total of 26,436 traces. SAGE tags were preprocessed - that is, TAGs were extracted and corrected for sequencing errors, and artifacts like SAGE linkers, ribosomal RNA and duplicated ditags were removed using the ‘sagenhaft’ package, which is available from the Bioconductor website [111,112]. To compare libraries that contain long tags with those that contain short tags, all short tags were mapped to the existing long tags from the other libraries. A table for all libraries containing the unique long or short tags was generated and redundant tags were removed. Only tags with a total count >2 (across all libraries) were considered for subsequent comparisons. Each unique tag was mapped to the mouse genome using ESTgraph, which employs ESTs and their genomic position information. ESTgraph was created by Tim Beissbarth (unpublished) [113]. Identity was assigned to these tags and they were further grouped into the following categories: matching to a gene, a genomic sequence, or an EST, or ambiguous matches or no alignment at all. All annotations were based on the latest mouse assembly (mm9 released in July 2007) accessible from the UCSC Genome Bioinformatics website [63].
the ‘hclust’ function and complete linkage, which was implemented using the statistical computing environment of R [116].

Genomic clustering of SAGE tags
To assess whether there was any genomic clustering of tags, a method previously described [117] was adopted. In brief, first gene lists (based on all DETs in both pairwise and multiple library comparisons as well as gene lists from the hierarchical clustering analysis) were selected. The genomic clustering of either of these selections was compared to the total unique tag list (all 25,165 unique tags). The tags were mapped to the mouse genome. The number of selected tags in ten consecutive tag positions for each window of the chromosome was calculated. One thousand permutations were used to compute the null distribution of maximum tag counts per window. The method was implemented using the statistical computing environment of R [116].

Functional classification and characterization of DETs
Gene ontology enrichment analysis
The DET lists generated from various comparisons were subjected to systematic functional annotation using the standardized Gene Ontology term analysis tools at the DAVID [30]. Functional clustering was performed using high stringency with a kappa similarity threshold of 0.85 and a minimum term overlap of 3. Classification was carried out using a multiple linkage threshold of 0.5 with both numbers of initial and final group members set to 3. A term was considered statistically significant when the computed P-value was < 0.05. All queries were performed in September 2009.

Molecular interactions and pathway analysis
Identification of molecular network interactions and pathway analysis of validated DETs or co-regulated genes was completed using the IPA [118] tools from Ingenuity Systems® (Redwood City, California, USA). Accession numbers for all genes with their corresponding fold changes or normalized counts were imported into the IPA software. No focus genes were set at the beginning of the analysis. To start building network graphs, the application queries the list of input genes and all other gene objects stored in the Ingenuity knowledge base. Networks with a maximum of 30 genes or proteins were constructed, and scores were computed based on the likelihood of the genes being connected together due to random chance. A score of 2 indicates that there is a 1/100 chance that these genes are connected in a network due to random chance. Therefore, any networks with a score of 2 or above are considered statistically significant (with >99% confidence). The most significant novel networks and their interactions with existing canonical pathways were investigated further.

Quantitative RT-PCR
Total RNA isolation and first strand cDNA synthesis
RT-qPCR was carried out to validate all selected candidate or co-regulated DETs. Biological triplicates from E15.5, E17.5, P1.5 and adult (5 to 6 months old) cerebral cortices were used. Genomic-free total RNA from independent mice was extracted using the RNeasy® Lipid Tissue Midi Kit (Qiagen, Doncaster, Victoria, Australia) according to the manufacturer’s protocol (Section G in Additional data file 1). First strand cDNA was synthesized from high quality total RNA [119] using random hexamers (unless specified otherwise) and the SuperScript™ III Reverse Transcriptase Kit (Invitrogen, Mulgrave, Victoria, Australia), according to the manufacturer’s protocol, under an RNase-free environment.

Primer design and RT-qPCR
All primers were designed using ProbeFinder 2.35 at the Assay Design Center for Universal ProbeLibrary Assay provided by Roche Applied Science [120]. All RT-qPCR reactions were prepared in 10 μl volumes in a 384-well plate format consisting of LC480 Master Probe Mix (Roche Diagnostics, Castle Hill, New South Wales, Australia), Universal ProbeLibrary (Roche Diagnostics, Castle Hill, New South Wales, Australia) and forward and reverse primers (GeneWorks, Hindmarsh, South Australia, Australia or Bioneer Corporation, Daejeon-gu, Daejeon, Korea) according to the manufacturers’ protocols. RT-qPCRs were performed using the LightCycler® 480 System (Roche Diagnostics, Castle Hill, New South Wales, Australia) and forward and reverse primers (GeneWorks, Hindmarsh, South Australia, Australia or Bioneer Corporation, Daejeon-gu, Daejeon, Korea) according to the manufacturer’s protocol (Section G in Additional data file 1). A full list of primers and probes used in this study is included in Additional data file 8.

Relative quantification using a standard curve method
The crossing point (Cp) from each signal was calculated based on the Second Derivative Maximum method [121]. A set of serially diluted cDNAs was used to construct a four-data point standard curve for every PCR system in each run. A total of three reference genes (from Hprt1, Psmb2, phosphoglycerate kinase 1 gene (Pglyk) or Hmbs) were used as endogenous controls. An estimated starting amount of each target gene was calculated and intra-samples multiple reference genes normalization was performed (Section G in Additional data file 1). A linear model was fitted to the time course of expression values for each gene. Genes differentially expressed between the various stages of development or regions were selected using empirical Bayesian moderated t-statistics, which borrow information between genes [122]. Standard errors for the mean expression at various developmental stages were obtained from the linear model. For each comparison, P-values were adjusted using the Benjamini and Hochberg [114] method to control the false discovery rate. See Section H in Additional data file 1 for the R code used to execute the analysis.

Validation of sense-antisense and multiple overlapping transcripts in genomic clusters

Strand specific RT-PCR
All RNA was prepared as described above. Total RNA from all developmental stages (N = 3 per developmental stage) was
equally pooled prior to cDNA synthesis. Four first strand cDNA synthesis reactions were prepared for each cluster as follows: with a primer complementary to the sense strand only; with a primer complementary to the antisense strand only; with oligo-d(T)$_{18}$ as a positive control; and without any primers as a negative control. In all four reactions, both primers were added in subsequent PCRs (Section G in Additional data file 1). PCR amplifications were carried out using FastStart PCR High Fidelity System (Roche Diagnostics, Castle Hill, New South Wales, Australia) according to the manufacturer’s protocol. More than one primer set was used in the sense-antisense strand specific RT-PCR (Additional data file 8).

**RACE**

First strand cDNA synthesis was carried out using pooled total RNA extracted from three biological replicates of rostral and caudal E15.5 and whole E17.5, P1.5 and adult (5 to 6 months old) cerebral cortices. Oligo-d(T)$_{18}$ with an adaptor sequence (5’-TACGACGTCTGCTAGGACTG-3’) was used to prime the first strand cDNA synthesis. Second strand synthesis or PCR was then carried out using a strand-specific primer and the adaptor primers (Additional data file 8). All specific primers were designed to be complementary to the SAGE tags or their upstream sequences. PCR amplifications (Section G in Additional data file 1) were carried out using FastStart PCR High Fidelity System (Roche Diagnostics, Castle Hill, New South Wales, Australia) according to the manufacturer’s protocol.

**Southern blotting analysis**

Amplified 3’ RACE products were transferred to Hybond N+™ (GE Healthcare, Rydalmere, New South Wales, Australia) nylon membrane using the neutral transfer method. Prehybridization and hybridization steps were performed in Rapid-Hyb buffer (GE Healthcare, Rydalmere, New South Wales, Australia) according to the manufacturer’s protocol. All oligonucleotides were designed to be complementary to sequence between the specific primer-priming site and the tag of interest. Synthetic oligonucleotides were 5’ end-labeled using T4 polynucleotide kinase (Promega, Alexandria, New South Wales, Australia) and [γ-32P]ATP (GE Healthcare, Rydalmere, New South Wales, Australia) with 0.5 M phosphate). After hybridization, blots were washed using 1% w/v SDS at 65°C for 5 to 6 times until the background signal was low.

**In situ RNA hybridization**

ISH was carried out using paraffin sections (5 μm) of embryonic, postnatal and adult brains (E11.5, E13.5, E15.5, E17.5, P1.5 and P150) and a related [35S]UTP-labeled complementary RNA probe (Additional data file 8) as described previously (Section G in Additional data file 1) [123].

**Screening of Sox4 and Sox11 sense and antisense transcript expression in the adult mouse brain, organs and both the proliferating and differentiating P19 cells and neurospheres**

**Strand-specific RT-qPCR**

Total RNA was extracted from harvested organs using the TRIzol® reagent (Invitrogen, Mulgrave, Victoria, Australia) according to the manufacturer’s protocol. To avoid genomic DNA contamination, all isolated total RNA was treated with the recombinant DNase I enzyme provided by the DNAfree™ kit (Applied Biosystems, Scoresby, Victoria, Australia) according to the manufacturer’s protocol. First strand cDNA synthesis was carried out using strand-specific primers followed by qPCR analysis as described above.

**Embryonic neural stem cells grown as neurospheres**

Mouse used for generation of neurospheres had a mixed genetic background including MF1, 129SvEv, C57BL/6 and CBA. Cerebral cortices from E14 embryos were dissected out into calcium-magnesium-free phosphate-buffered saline. The tissue was mechanically dissociated and centrifuged. The cells were plated in complete neuroculture medium (Section G in Additional data file 1) for 4 days followed by induction of neuronal differentiation. These cells were then plated on poly-D-lysine (catalogue number P6407, Sigma Aldrich, Castle Hill, New South Wales, Australia) and laminin (catalogue number 23017-015, Invitrogen, Mulgrave, Victoria, Australia) coated culture dishes in neuroculture medium with the presence of 2% (v/v) fetal bovine serum but not epidermal growth factor and basic fibroblast growth factor. The differentiation was allowed to proceed for 5 days. Total RNA was extracted from both proliferating and differentiating cells using TRIzol® reagent as described above.

**P19 embryonal carcinoma cells**

P19 mouse embryonal carcinoma cells were cultured and differentiated into neurons as described previously [124].
Briefly, P19 cell cultures were maintained in P19GM complete medium (Section G in Additional data file 1). For induction of neuronal differentiation, 1 × 10^6 P19 cells were cultured in suspension form using bacteriological Petri dishes. The P19GM medium with additional supplementation of 5 × 10^-7 M all-trans retinoic acid (catalogue number R-2625; Sigma Aldrich, Castle Hill, New South Wales, Australia) was used for the induction. After 4 days, P19 cells formed embryoid body stages. Embryoid bodies were collected from suspension cultures and re-plated in adherent culture flasks in the P19GM medium with only 5% (v/v) fetal bovine serum and without retinoic acid supplementation. The cells were allowed to differentiate for 5 days. Total RNA was extracted from both proliferating and differentiating cells using TRIzol® reagent as described in above.

Abbreviations
CL: caudo-lateral; CM: caudo-medial; DAVID: Database for Annotation, Visualization and Integrated Discovery; DET: differentially expressed transcript/tag; E: embryonic day; EST: expressed sequence tag; GEO: Gene Expression Omnibus; IPA: Ingenuity Pathway Analysis; ISH: in situ hybridization; LTP: long term potentiation; miRNA: microRNA; NAT: natural antisense transcript; NSPc: neural stem/progenitor cell; OMM: Online Mendelian Inheritance in Man; P: postnatal day; PET: paired-end dTag; RACE: rapid amplification of cDNA ends; RL: rostro-lateral; RM: rostro-medial; RT-qPCR: quantitative RT-PCR; SAGE: serial analysis of gene expression; SDS: sodium dodecyl sulphate; UCSC: University of California Santa Cruz; UTR: untranslated region.

Authors’ contributions
KHL performed all the SAGE validation experiments. CAH, PZC and SST procured the mouse cerebral cortex and constructed the SAGE libraries. KHL, TB, LH and GKS designed, performed and supervised the SAGE, RT-qPCR and IPA analyses. KHL and TT performed all the ISH studies. KHL, KB, PSC, CNH and PQT carried out the expression studies on Sox4 and Sox11 transcripts. KHL, CAH and CNH drafted the manuscript. CAH, GKS, TT and HSS conceived of the study, and participated in its design and coordination. All authors read and approved the final manuscript.

Additional data files
The following additional data are available with the online version of this paper: analysis of SAGE, DETs, IPA, Sox4 and Sox11 genomic cluster analysis, and R script for implementing empirical Bayesian moderated t-test on multiple groups (Additional data file 1); SAGE tag information for 561 DETs (Additional data file 2); functional annotations clustering analysis using DAVID (Additional data file 3); RT-qPCR validation of DETs based on multiple comparisons between two developmental stages (E versus Ad, PN1.5 versus Ad and E15.5 versus PN1.5) (Additional data file 4); RT-qPCR validation of gene clusters based on hierarchical clustering analysis (Additional data file 5); RT-qPCR validation of DETs based on the rostral versus caudal E15.5 cerebral cortex comparison (Additional data file 6); statistically significant over-represented genomic loci based on genomic clustering of tags (Additional data file 7); list of primers, probes, clones and assays designed for RT-qPCR, RACE, Southern, Northern and ISH analysis (Additional data file 8).

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