Genomic characterisation of a Fgf-regulated gradient-based neocortical protomap

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Summary

Recent findings support a model for neocortical area formation in which neocortical progenitor cells become patterned by extracellular signals to generate a protomap of progenitor cell areas that in turn generate area-specific neurons. The protomap is thought to be underpinned by spatial differences in progenitor cell identity that are reflected at the transcriptional level. We systematically investigated the nature and composition of the protomap by genomic analyses of spatial and temporal neocortical progenitor cell gene expression. We did not find gene expression evidence for progenitor cell organisation into domains or compartments, instead finding rostrocaudal gradients of gene expression across the entire neocortex. Given the role of Fgf signalling in rostrocaudal neocortical patterning, we carried out an in vivo global analysis of cortical gene expression in *Fgfr1* mutant mice, identifying consistent alterations in the expression of candidate protomap elements. One such gene, *Mest*, was predicted by those studies to be a direct target of Fgf8 signalling and to be involved in setting up, rather than implementing, the progenitor cell protomap. In support of this, we confirmed *Mest* as a direct transcriptional target of Fgf8-regulated signalling in vitro. Functional studies demonstrated that this gene has a role in establishing patterned gene expression in the developing neocortex, potentially by acting as a negative regulator of the Fgf8-controlled patterning system.

Key words: Neocortex, Protomap, Microarrays, Patterning, Mouse

Introduction

Composed of six cellular layers, the neocortex is a modular structure, with anatomically distinct areas devoted to different functions (Mountcastle, 1998). Two distinct models have been proposed for the formation of neocortical areas. The protomap hypothesis proposes that cortical progenitor cells are intrinsically specified early in development to contribute to a given region, and their radially arranged neural progeny inherit this spatial information (Rakic, 1988). An alternative protocortex model proposes that areal identity is conferred by signals extrinsic to the neocortex, including incoming thalamocortical axons, with the developing cortex effectively being a naïve structure (O'Leary, 1989). Data from several studies suggest that the protomap hypothesis may describe early neocortical patterning, and that innervation is an important regulator of neocortical maturation and the maintenance of areal identity (for a review, see Grove and Fukuchi-Shimogori, 2003).

The early formation of cortical areas, as assessed by gene expression, occurs normally in the absence of thalamocortical innervation, suggesting that arealisation is a process intrinsic to the neocortex (Miyashita-Lin et al., 1999; Nakagawa et al., 1999). Two homeobox transcription factors, Pax6 and Emx2, are expressed in opposing rostrocaudal gradients in the developing neocortex (Bishop et al., 2000; Mallamaci et al., 2000). Mice carrying mutations in either *Pax6* or *Emx2* have reductions in the relative sizes of the cortical areas in which those genes are normally highly expressed, and expansions of parts of the neocortex in which they are expressed at low levels (Bishop et al., 2000; Mallamaci et al., 2000; Muzio et al., 2002). A similar finding has been made in the case of another trancription factor, COUP-TFI (Nr2f1 – Mouse Genome Informatics), which is expressed in a high caudal to low rostral gradient (Liu et al., 2000; Zhou et al., 2001). Recent data indicate that altering the absolute levels of Emx2 in the developing neocortex is sufficient to alter rostrocaudal patterning, such that caudal areas are expanded at the expense of rostral areas (Hamasaki et al., 2004). These opposing gradients of transcription factor expression are reminiscent of transcription factor expression in progenitor cells in the spinal cord and the retina (Jessell, 2000; Koshiba-Takeuchi et al., 2000). In the developing spinal cord, combinatorial expression of a set of transcription factors divides progenitor cells into several discrete domains along the dorsoventral axis of the

neural tube, with the progenitor cells in each domain giving rise to a particular class of neuron (Briscoe et al., 2000).

Areal identity is conferred, at least in part, by extracellular signals. Heterotopic transplantation of presumptive cortical areas early in development results in the transplanted cortices assuming the identity of their destination (Cohen-Tannoudji et al., 1994). However, mouse neocortical tissue becomes intrinsically specified with respect to spatial identity by embryonic day 13, two days after the initiation of neurogenesis, at which point it becomes refractory to transplantation (Gitton et al., 1999). Similar findings have been reported in the developing rat cortex (Gaillard et al., 2003). It has been proposed that there are three potential signalling centres around the margins of the neocortex: the anterior neural ridge, the cortical hem and the cortical-subcortical boundary region (Grove and Fukuchi-Shimogori, 2003; O'Leary and Nakagawa, 2002; Ragsdale and Grove, 2001). Several BMP family members are produced at the cortical hem, dorsal midline and caudal cortex (Furuta et al., 1997), and there is evidence for a role for these proteins in mediating mediolateral patterning of the neocortex (Monuki et al., 2001), although studies of Bmp receptor mutants suggest that the primary role of Bmp signalling is local patterning around the dorsal midline (Hebert et al., 2003a; Hebert et al., 2002).

Fgf8 appears to be a primary regulator of rostral areal identity in the neocortex. Expressed initially in the anterior neural ridge and then by progenitor cells in the rostral pole of each cerebral hemisphere (Crossley and Martin, 1995), Fgf8 is necessary for patterning the rostral neocortex and is capable of repressing caudal and inducing rostral neocortical identities (Fukuchi-Shimogori and Grove, 2001). These findings have been confirmed in an analysis of hypomorphic *Fgf8* mutants, in which there is both a relative reduction in the domains of progenitor cells expressing rostral markers and a proportional increase in the domains of progenitor cells expressing caudal markers, as well as a loss of the most rostral cortical structure, the olfactory bulbs (Garel et al., 2003). Fgf8 has been proposed to regulate area formation by repressing progenitor cell expression of the transcription factor Emx2, which is expressed in a high caudal to low rostral gradient (Fukuchi-Shimogori and Grove, 2003).

Therefore, a current model for cortical area formation proposes that signalling centres co-ordinately determine the spatial or areal identity of a neocortical progenitor cell, which is read out as spatially specific gene expression (O'Leary and Nakagawa, 2002). By analogy with spinal cord development (Jessell, 2000), neocortical progenitor cells then produce area-specific neurons based on their positional identity. However, little is known of the components or the nature of the proposed progenitor cell protomap. We report the results of a genomics-based strategy for characterising the nature and composition of the protomap, in which we identified known and novel rostrocaudal gradients of neocortical progenitor cell gene expression.

Given the role of Fgf8 signalling in rostrocaudal patterning in the neocortex, we proposed that protomap components would alter their expression in response to changes in Fgf signalling. Therefore, we studied the transcriptional consequences of forebrain-specific loss of Fgf receptor 1 (Fgfr1) function during the period of Fgf8-regulated patterning in the neocortex, as the abnormalities in cortical development in these animals are very similar to those occurring in Fgf8 hypomorphic mutants (Garel et al., 2003; Hebert et al., 2003b). In *Fgfr1* mutant mice, a subset of the proposed protomap genes change in expression in a manner consistent with their positive and negative regulation by Fgf8-mediated signalling. One gene, *Mest* (also known as *Peg1*), was identified by those analyses as a strong neocortical patterning candidate gene, as in addition to its rostral expression and Fgf regulation in vivo, we found that *Mest* expression is directly induced by Fgf8 signalling in neocortical explant cultures. Anatomical and genomic analysis of mice mutant for *Mest*/*Peg1* identified significant alterations in patterned gene expression in the developing neocortex. Therefore, we propose that the early neocortical protomap is composed, at least in part, of gradients, rather than domains, of gene expression along the rostrocaudal axis that are regulated by an Fgf signalling system.

Materials and methods

Expression profiling

Neocortices were dissected from the forebrains of embryos at embryonic day (E) 11.5 or E13.5. All animal handling was carried out within UK Home Office guidelines. Tissues were pooled from groups of embryos and RNA extracted by the guanidinium-acid phenol method [Trizol, Invitrogen (Chomczynski and Sacchi, 1987)]. This RNA was used to make double-stranded cDNA and was amplified with the SMART method (Matz et al., 1999) (Clontech). The resulting cDNA was used to synthesise fluorescently labelled microarray probes and to probe microarrays as described (Livesey et al., 2000). Microarrays were constructed using a library of 22,000 65-mer 5′ amino-linked oligonucleotides (Sigma-Genosys/Compugen Mouse Oligolibrary), printed on Codelink slides (Amersham) using a QArray microarraying system (Genetix, UK).

Microarray data analysis

Hybridised microarrays were scanned on a GenePix 4000B microarray scanner (Axon Instruments) and the resulting images were analysed with GenePix 5 array analysis software. Image data were archived and analysed in the Acuity system (Axon Instruments). Individual arrays were lowess normalised by individual printing block to correct for both intensity and position-dependent variations in expression measurements. Data from each hybridisation were filtered to remove array features that were not detectable significantly above local background. To identify reproducible rostrocaudal differences in gene expression, all hybridisations were carried out as dye-swapped technical replicates. For statistical analysis, data from these technical replicates were inverted and the average value of each pair of hybridisations was used in subsequent analyses. The significance analysis of microarrays (SAM) algorithm was used to identify significant rostracaudal differences in expression, using a false discovery rate (FDR) cutoff of 5% (Tusher et al., 2001). Statistical analysis of expression data from the rostral/middle/caudal screen and the *Fgfr1* and *Mest* mutant cortices was carried out using the *t*-test algorithm implemented in Acuity. Data from each analysis were stored in Acuity and subsequently analysed by hierarchical and k-means cluster analysis. Gene ontology annotation was assigned to gene sets using the online FatiGO tool (http://www.fatigo.org/) (Al-Shahrour et al., 2004). The complete microarray datasets were deposited in the NCBI Gene Expression Omnibus (GEO) data repository (http://www.ncbi.nlm.nih.gov/projects/geo/index.cgi), with accession numbers: GSE2854-GSE2858.

Analysis of gene expression in mutant mice

The dorsal telencephalon was dissected from subcortical structures from single embryos of a litter of E12.5 *Fgfr1* conditional mutant

mice (Hebert et al., 2003b), RNA was extracted, and cDNA synthesised and amplified using the SMART system. To control for differences in gene expression due to the presence of the modified *Fgfr1* allele and *Foxg1* heterozygosity, gene expression was compared among single embryos of two key genotypes, $Foxg1^{+/Cre};Fgfr1^{+/fl}$ and $Foxg1^{+/Cre};Fgfr1^{fl/fl}$; that is, heterozygous and homozygous *Fgfr1* mutants. A total of nine different pair-wise hybridisations was carried out, four of which were dye-reversed relative to the other five. For the Peg1/Mest analysis, cortices were dissected from null and wild-type littermate embryos at E12.5, and cDNA synthesised and amplified from tissues from single embryos using the SMART system. As *Mest* is a paternally imprinted gene, gene expression was compared between functional null and wild-type tissues.

In situ hybridisation

Non-isotopic in situ hybridisation to whole-mount embryos and parasagittal embryo sections was carried out as described (Brent et al., 2003). Probes were prepared from cDNA clones for the genes studied, selected from a Brain Molecular Anatomy Project clone set (kind gift of Dr Bento Soares, University of Iowa). All clones were sequenced and compared to GenBank sequences using BLAST to confirm clone identity. For genes not represented in that set, PCR primers were designed to amplify approximately 1 kb in the 3′ region of the corresponding RefSeq cDNA, the amplicons were cloned into TA cloning vectors (Invitrogen) and the inserts sequenced. Whole-mount in situ hybridisation was carried out as described using the same probes as were used for sections (Bao and Cepko, 1997). Fluorescent in situ hybridisation was carried out using Tyramide Signal Amplification (TSA; Perkin Elmer) according to the manufacturer's instructions with minor modifications. Cy3- and Cy5-labelled hybridisation probes were visualised using the Axon 4000B microarray scanner.

Neocortical explant culture and real-time PCR

Explants of the middle third of the E11.5 neocortex were dissected free of surrounding tissues and placed on polycarbonate filters floating on serum-free medium consisting of DMEM/F-12 and the supplements B-27 and N2 (Invitrogen), to which poly-L-glutamine and heparin were added. At least two explants were cultured on each filter. Recombinant mouse Fgf8b (R&D Systems) was added to a concentration of 10 ng/ml. Explants were harvested 4 hours after Fgf8 addition, RNA extracted (Trizol, Invitrogen) and cDNA synthesised by oligo-dT priming. Real-time PCR was carried out on a Roche LightCycler according to the manufacturer's instructions. Expression of each gene was calculated relative to that of the mRNA for the abundant ribosomal protein rpS17 in the same sample and in three independent experiments.

Bioinformatics

Mest homologues were identified by BLAST comparison of the mouse and human Mest protein sequences to the predicted proteins encoded by the other genomes shown (see text and figures), using Ensembl and the public *Caenorhabditis elegans* and *Drosophila melanogaster* genome databases (FlyBase and WormBase). The best-scoring hits were compared by BLAST back against the mouse genome to confirm the true homologue of Mest in each organism. Multiple sequence cluster analysis was carried out by ClustalW on the EBI server.

Results

A genomics strategy for identifying components of the neocortical protomap

Little is known about spatial differences in gene expression across the field of neocortical progenitor cells. The majority of the few known spatial differences have been described along the rostrocaudal (anteroposterior, or long) axis of the neocortex, and include genes encoding the transcription factors Pax6, Emx2, Lhx2 and COUP-TFI (Bishop et al., 2000; Donoghue and Rakic, 1999b; Liu et al., 2000; Mallamaci et al., 2000; Monuki et al., 2001; Zhou et al., 2001). Similarly, the majority of described spatial gene expression patterns in the adult neocortex are organised along the rostrocaudal axis, such as those for Cadherin8 and EphrinA5 (Mackarehtschian et al., 1999; Nakagawa et al., 1999), reflecting the anatomical organisation of the primary motor, sensory and visual areas in a rostrocaudal series. Therefore, we designed a screening strategy to identify genes with differential expression along the rostrocaudal axis of the neocortex (Fig. 1). As many of the genes involved in patterning progenitor cell populations in the neocortex and other parts of the nervous system are expressed in domains that include one boundary or edge of the tissue, we designed the initial screen to compare gene expression at the rostral and caudal poles of the neocortex.

To identify genes with spatial differences in expression in the developing neocortex, we used oligonucleotide microarrays representing over 22,000 of the genes expressed from the mouse genome. We analysed differences in rostrocaudal neocortical gene expression prior to the arrival of the thalamocortical afferents to the cortex, and at the onset and in the early phases of neurogenesis (Molnar et al., 1998; Takahashi et al., 1999). In order to systematically characterise rostrocaudal differences in gene expression, we performed a detailed comparison of rostral and caudal pools of E11 and E13 neocortex, applying the strategy illustrated in Fig. 1. Two different strains of mice, one inbred (C57Bl/6), the other outbred (MF1), were used to control for strain-dependent variations in patterns of gene expression (Pavlidis and Noble, 2001; Sandberg et al., 2000). For each strain at each timepoint, three sets of pooled rostral and caudal tissue were collected from single litters. Gene expression screens were carried out within single litters to match embryos by developmental age as closely as possible. Individual pools contained material from the left and right cortices of at least two embryos, to correct for variation among dissections. Given the limited amount of RNA available when using such a screening strategy, the corresponding cDNA was amplified using the SMART system to generate enough material for synthesising array probes (Matz et al., 1999), as has been used successfully for our previous studies (Livesey et al., 2000; Livesey et al., 2004).

To assess the efficacy of the screening strategy, we investigated the expression of a set of known differentially expressed genes in our dataset: Pax6, Emx2, Lhx2, COUP-TFI and Sfrp1. All five genes are represented on the array, and four were reliably detected by the arrays, with all four (Pax6, Lhx2, Sfrp1 and COUP-TFI) demonstrating the predicted expression patterns (Fig. 1B). Emx2 was detected in a subset of four arrays, and was detected as caudally expressed in all four (data not shown). Thus, the array strategy accurately identified the majority of known protomap components. Statistical analysis of the array data, both within and across timepoints, identified marked rostrocaudal differences in gene expression at each age, including temporally stable differences in gene expression between the rostral and caudal neocortex (Fig. 2).

Given the three rostrocaudal domains of gene expression observed in the adult neocortex within neurons (Bishop et al., 2000; Garel et al., 2003; Nakagawa et al., 1999), one

possibility is that the progenitor cells that generate those neurons are themselves organised into the three rostrocaudal domains reflected in their gene expression. In order to look for genes differentially expressed between the rostral, middle and caudal neocortex, we carried out a further expression screen at E13.5, dissecting the neocortex into thirds along the rostrocaudal axis (Fig. 2), and collecting three pools of rostral, middle and caudal tissue. Each pool contained tissue from four different embryos. Gene expression was compared between each pair of groups (rostral versus middle; middle versus caudal; rostral versus caudal) in a set of 18 independent hybridisations. Although we found many genes to be differentially expressed in rank order between the rostral, middle and caudal thirds, consistent with graded expression along the rostrocaudal axis, we did not find any genes with significant peaks or troughs of expression in the middle of the neocortex.

Differential expression between the rostral and caudal thirds of the neocortex could occur for a number of reasons, including as a result of genes with spatial differences in the expression in progenitor cells or neurons, the developmental gradient across the neocortex and the differing ratio of neurons to progenitor cells along the rostrocaudal axis (related to the developmental gradient). An analysis of temporal changes in gene expression in the neocortex over the period studied here demonstrated some overlap between spatially and temporally expressed transcripts, as had been expected (data not shown).

Genes expressed differentially between the rostral and caudal neocortex are expressed in gradients across the neocortex

A secondary in situ hybridisation screen was carried out to confirm differential expression along the rostrocaudal axis, to assign expression to cell types (progenitor cells, newly born neurons, differentiated neurons) and to assess the nature of the rostrocaudal expression pattern (graded expression or discrete domain of expression). Thirty-eight genes were selected for this secondary expression screen according to two criteria: the rank q-value of differential expression in the combined E11.5/E13.5 dataset, as calculated by SAM (q-values

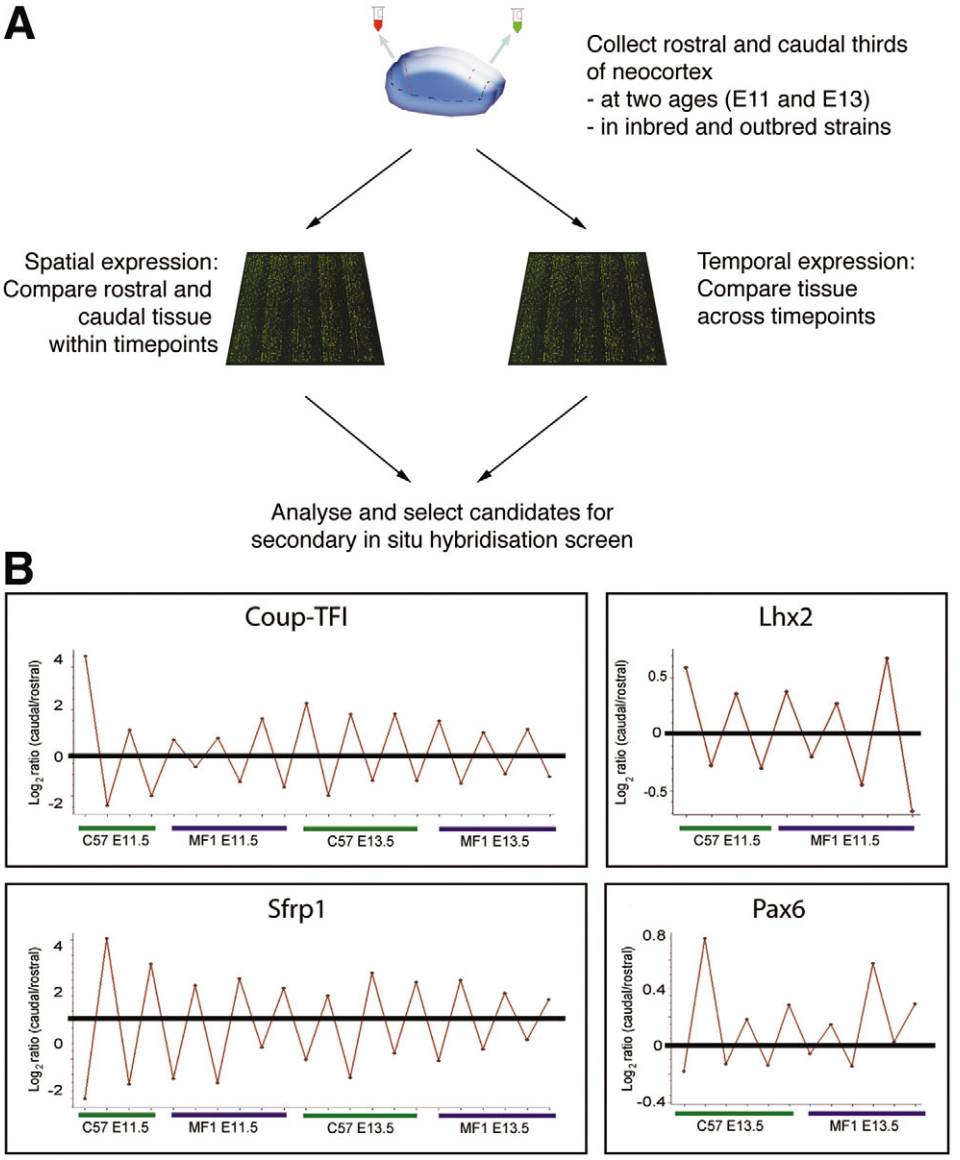

Fig. 1. A genomics-based investigation into the nature and composition of a neocortical protomap successfully identified genes with described spatial differences in expression. (A) Strategy for a genomics-based identification of protomap components. Rostral and caudal thirds of the neocortex were dissected from mouse embryos at two ages (E11.5 and E13.5) in both inbred and outbred strains. Comparison of gene expression between rostral and caudal tissue was carried out within single litters of embryos to reduce variability in the developmental stages of embryos. In addition to spatial gene expression, the same samples were also used to analyse temporal changes in gene expression. (B) Screen efficacy was demonstrated by the successful identification of almost all of the genes that have been previously described as having differential rostrocaudal expression, including *COUP-TFI*, *Lhx2*, *Sfrp1* and *Pax6*. Plots of gene expression ratios (expressed in base 2) between rostral and caudal neocortex are shown. The graphs show changes in gene expression for single genes for multiple dye-swapped samples. The stage and strain of origin of the samples is as indicated, with each datapoint representing a measurement from a single microarray. Measurements are presented as dye-swapped pairs, producing the alternating positive and negative expression patterns. The two caudally expressed genes *COUP-TFI* and *Lhx2* show an opposite expression pattern to the two rostral genes *Sfrp1* and *Pax6*.

approximate to false discovery rates), and functional annotation. In the case of functional annotation, a thorough bioinformatics annotation of all of the differentially expressed genes was carried out (data not shown). For putative protomap components, by analogy with other systems, transcription factors and signalling pathway components were prioritised for the secondary screen.

Of the set of 38 genes selected by these criteria, including those encoding the transcription factors Klf3, COUP-TFII (Nr2f2 – Mouse Genome Informatics), Hey1, Tcf4 and Fez-

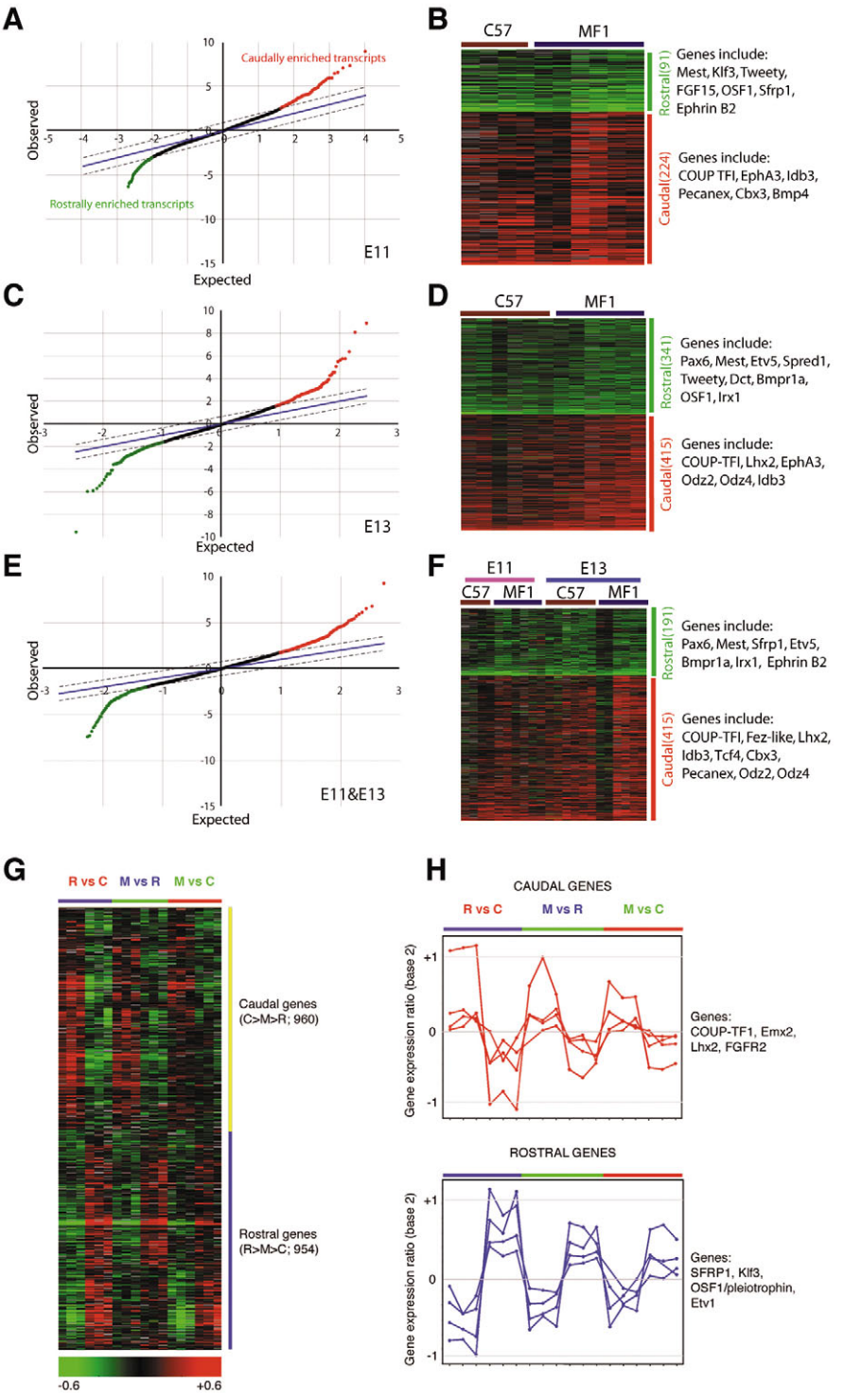

Fig. 2. Comprehensive identification of genes demonstrating differences in expression along the rostrocaudal axis of the developing neocortex. (A) Rostrocaudal gene expression differences in the neocortex at E11.5. Scatter plot of observed gene expression ratios against expected ratios, as calculated using the significance analysis of microarrays algorithm (SAM, see text for details). (B) Clustering of genes from A with rostrocaudal expression differences at E11.5 passing a 5% false discovery rate cutoff in SAM. Each column represents a single microarray and each row expression data for a single gene. Alternate microarrays are dye-swaps (technical replicate) of the preceeding microarray, the ratios of which have been reversed. The 10 microarrays shown here represent five dye-swapped pairs, and the analysis of five separate samples of rostral and caudal neocortical tissue. The numbers of rostral and caudal genes identified are indicated in brackets. By convention, positive differences in expression (upregulation) are represented in red, and negative differences (downregulation) in green, with the colour intensity reflecting the magnitude of the underlying expression ratio. (C) Rostrocaudal gene expression differences at E13.5. Scatter plot of observed gene expression ratios against expected ratios, as calculated using the significance analysis of microarrays algorithm (SAM, see text for details). (D) Clustering of genes from C with rostrocaudal expression differences at E13.5 passing a 5% false discovery rate cutoff in SAM. (E) Combined analysis of E11.5 and E13.5 data. Scatter plot of observed gene expression ratios against expected ratios, as calculated using the significance analysis of microarrays algorithm (SAM, see text for details). (F) Clustering of genes passing 5% false discovery rate cutoff in SAM from E. (G) An array screen for genes demonstrating periodic gene expression along the rostrocaudal axis of the developing neocortex. E13 neocortices were divided into thirds along the rostrocaudal axis, and tissue pooled from single cortical hemispheres from four different embryos to generate three separate pools of tissue for each third (three pools each of rostral, middle and caudal tissue). Global gene expression was compared between every possible pair of types of pool in a set of 18 independent array hybridisations. The resulting cluster of genes identified as reproducibly rostrally or caudally enriched, compared with middle and rostral or caudal tissue, is shown. (H) Genes identified as more highly expressed in the caudal neocortex and expressed in gradients, or as more highly expressed in the rostral neocortex and expressed in gradients, show the appropriate rank order of expression by microarray analysis. For example, the magnitude of the expression ratio is highest comparing caudal and rostral samples, and these genes are expressed at higher levels in caudal samples than middle, and in middle samples than rostral.

like, spatial differences in expression were confirmed for 23 genes, of which 10 were expressed rostrally and 13 caudally (Fig. 3; Table 1). All of these genes were expressed in clear rostrocaudal gradients across the field of progenitor cells (Fig. 3). The transcription factor Klf3 is expressed in a rostrocaudal gradient extending throughout the neocortex before the onset of neurogenesis, in a pattern consistent with that of a gene encoding positional information in neocortical progenitor cells. By contrast, Mest/Peg1 is expressed in a rostrocaudal gradient at E11 that has retracted by E13, which is suggestive of a gene encoding a protein in a pathway for setting up the initial domains of gene expression in the rostral neocortex. Conversely, the transcription factors Hey1, Fez-like and Tcf4 are all expressed in high caudal to low rostral gradients.

We also studied the expression of three genes whose expression we predicted to reflect the neurogenetic gradient, based on their temporal and spatial expression: Fabp7, GLAST (Slc1a3 – Mouse Genome Informatics) and pleiotrophin. All three of these genes are expressed in rostrocaudal gradients at both E11.5 and E13.5, with the expression gradient expanded at E13.5 relative to E11.5 to extend along the entire rostrocaudal length of the neocortex (Fig. 3). This pattern of expression is consistent with the neurogenetic gradient and contrasts with that of *Klf3*, which is expressed in a temporally stable high rostral to low caudal gradient that extends throughout the neocortex at E11.

We did not find compelling evidence for discrete spatial groups of progenitor cells (based on their shared combinatorial expression of genes with restricted domains of expression), as seen in the developing spinal cord (Briscoe et al., 2000). To investigate this directly, we carried out two-colour, fluorescent in situ hybridisation for pairs of caudally expressed genes at both E11.5 and E13.5 (data not shown). As suggested from the initial in situ hybridisation screen, there was little evidence for

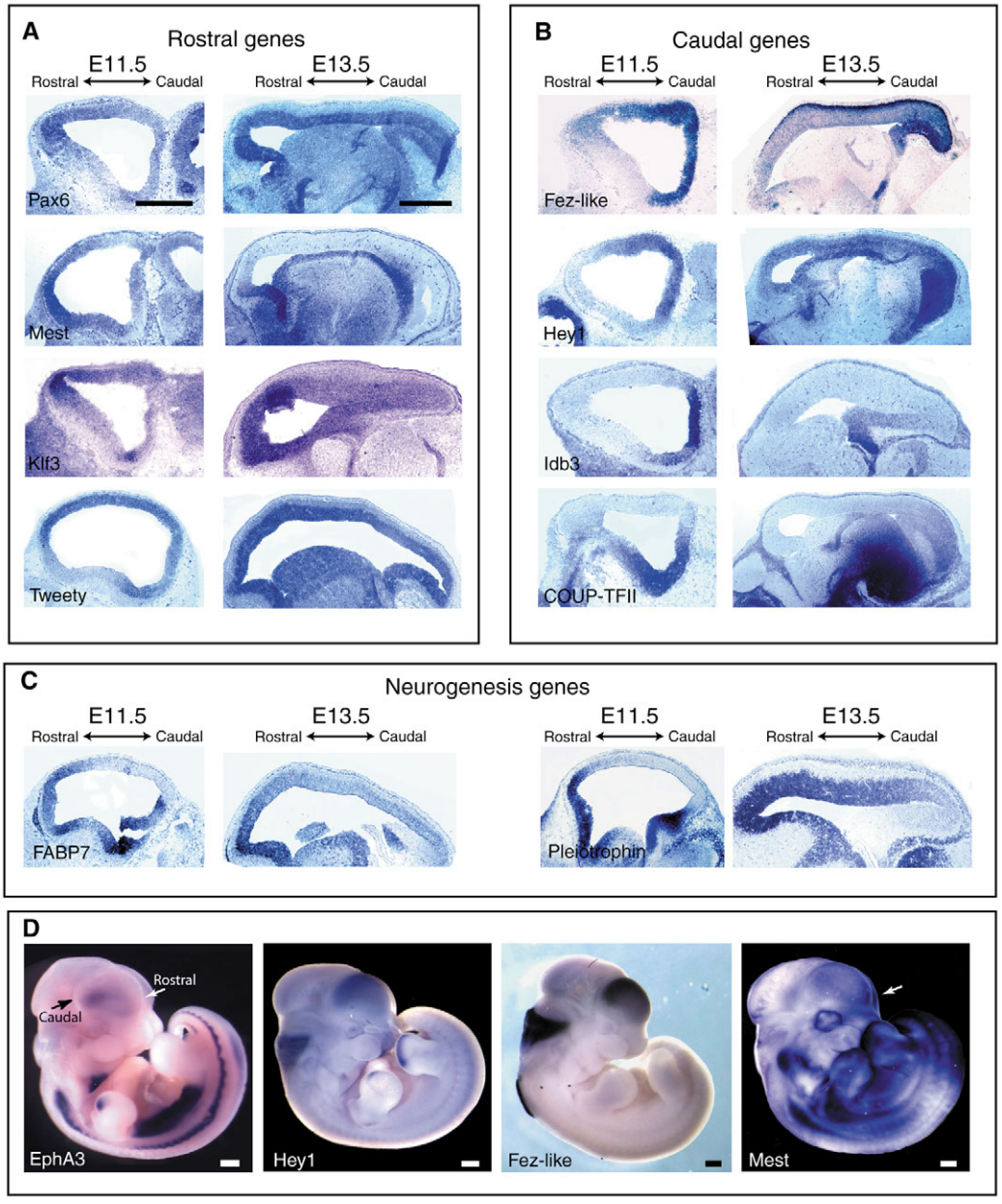

Fig. 3. Spatial gene expression across the field of neocortical progenitor cells is in gradients, rather than discrete domains. (A) Expression of rostrally enriched transcripts in neocortical progenitor cells at E11.5 and E13.5. In situ hybridisation on 14-μm parasagittal sections at each age. Rostrocaudal orientation and gene identifiers are as indicated. (B) Expression of caudally enriched transcripts at E11.5 and E13.5. (C) Expression of genes predicted from array analyses as temporally regulated and correlating with the neurogenetic gradient. Both genes are expressed rostrally initially (E11.5) and subsequently (E13.5) expression spreads caudally. (D) Whole-mount in situ hybridisation for caudally enriched transcripts. With the exception of the EphA3 whole-mount, which was carried out at E12, the embryos are all E11.5. Genes are as labelled in each panel. Arrow indicates rostral forebrain expression. Scale bars: 0.5 mm.

domains or compartments of neocortical progenitor cells, as defined by gene expression.

Many transcription factors and signalling proteins are differentially expressed along the rostrocaudal axis during early neocortical development

The set of differentially expressed genes appears to be functionally diverse, as it includes genes encoding transcription factors, cell surface proteins and signalling molecules, as well as many unannotated genes. At each timepoint, over half of the differentially expressed transcripts encode unannotated genes that encode mRNAs for which there are considerable expressed sequence tag (EST) or full-length cDNA data, underlining the importance of previously unstudied genes in cortical development. To analyse the functional breakdown of the known genes in more detail, we assigned Gene Ontology (GO) annotation to all genes for which it is currently available (data not shown). Notably, genes involved in transcriptional regulation and neurogenesis are highly represented.

Particularly noteworthy is the considerable number of differentially expressed transcription factors. A large set of transcription factors are expressed in high caudal to low rostral gradients, with Lhx2, Emx2, COUP-TFI, COUP-TFII, Hey1, Tcf4 and Fez-like all showing this expression pattern. Conversely, there are several transcription factors expressed in a high rostral to low caudal gradient, including Klf3, Pax6, Irx1, Etv5 (ERM) and Etv1 (ERF/ER81). All of the rostral and caudal transcription factors studied are expressed in neocortical progenitor cells in gradients that extend the entire length of the neocortex.

In addition to transcription factors, there is a marked enrichment of extracellular signalling molecules and key components of their intracellular signalling pathways in both rostral and caudal neocortex. Caudal neocortex shows high expression of two bone morphogenetic proteins, Bmp2 and Bmp4, as well as Tgfβ3 and a key transcriptional regulator in the Bmp pathway, Smad5. Furthermore, there is high caudal expression of both Lef1 and Tcf4, key factors in the Wnt pathway that also integrate signals from different pathways. In

Table 1. Genes identified by the genomics screens and confirmed as being differentially expressed

Accession	Unigene	Name	Expression ratio	q-value (%)	Stage	Reference
Rostral genes						
NM_007666	Mm.57048	Cadherin 6	1.17	0.30	E11 and E13	Bishop et al., 2000; Bishop et al., 2002
NM_010024	Mm.19987	Dopachrome tautomerase	1.62	0.30	E11 and E13	Guyonneau et al., 2002; Steel et al., 1992
AY036118	–	*Etv1* (*Erf/ER81*)	1.22	1.62	E13	Fukuchi-Shimogori and Grove, 2003
AY004174	Mm.155708	*Etv5* (*ERM*)	1.39	0.30	E11 and E13	Fukuchi-Shimogori and Grove, 2003
NM_008003	Mm.3904	*Fgf15*	1.66	0.63	E11	McWhirter et al., 1997
NM_010329	Mm.2976	Glycoprotein 38	1.45	0.63	E11	+
NM_008453	Mm.221757	Kruppel-like factor 3 (*Klf3*)	1.29	2.45	E11	+
NM_008590	Mm.335639	*Mest*	1.73	0.30	E11 and E13	+
NM_009717	Mm.5106	*Math2*	1.15	5.04	E13	Schwab et al., 2000
NM_013627	Mm.3608	*Pax6*	1.18	1.76	E11 and E13	+; Bishop et al., 2000
NM_008901	Mm.56946	*Pou3f4/Brn4*	1.25	0.63	E11	Heydemann et al., 2001
NM_009052	Mm.336245	Reduced expression 3	1.23	1.76	E11 and E13	+
NM_020599	Mm.41653	Retinaldehyde binding 1	1.78	0.28	E13	+
NM_013834	Mm.281691	*Sfrp1*	2.10	0.30	E11 and E13	Leimeister et al., 1998
NM_011897	Mm.89982	Sprouty homolog 2	1.35	0.28	E13	+; Minowada et al., 1999
NM_021324	Mm.29729	Tweety homolog 1	1.32	0.30	E11 and E13	+
Caudal genes						
NM_007585	Mm.238343	Annexin A2	1.38	0.90	E11 and E13	+
NM_007553	Mm.235230	*Bmp2*	1.11	0.74	E11 and E13	Furuta et al., 1997
NM_007554	Mm.6813	*Bmp4*	1.09	2.80	E11 and E13	Furuta et al., 1997
NM_009866	Mm.1571	Cadherin 11	1.18	5.04	E13	Nakagawa et al., 1999
NM_007624	Mm.280968	Chromobox homolog 3	1.26	0.63	E11	+
M68513	Mm.1977	Eph receptor A3	1.57	0.30	E11 and E13	+
NM_007936	Mm.3249	Eph receptor A4	1.21	0.30	E11 and E13	+
AB042399	Mm.34644	Fez-like	1.18	0.54	E11 and E13	+; Matsuo-Takasaki et al., 2000
M23362	Mm.16340	*Fgfr2*	1.41	3.40	E11 and E13	Hebert et al., 2003b
NM_010423	Mm.29581	*Hey1*	1.32	–	E11 and E13	+
NM_010496	Mm.34871	*Idb2*	1.15	0.30	E11 and E13	Jen et al., 1997
NM_008321	Mm.110	*Idb3*	1.48	0.30	E11 and E13	+; Jen et al., 1997
NM_010710	Mm.142856	*Lhx2*	1.33	0.30	E11 and E13	Nakagawa et al., 1999
AJ296304	–	Lmo1/Rhombotin 1	1.63	0.28	E13	Rabbitts and Boehm, 1990; Bulchand et al., 2003
U28068	Mm.4636	*Neurod1*	1.34	0.30	E11 and E13	+
NM_010151	Mm.14297	*Nr2f1* (*COUPTFI*)	1.81	0.28	E13	Garel et al., 2003
NM_009697	Mm.270109	*Nr2f2* (*COUPTFII*)	2.81	0.30	E11 and E13	+
NM_011856	Mm.40599	Odd Oz/ten-m homolog 2	1.68	0.30	E11 and E13	Zhou et al., 2003
D87034	Mm.254610	Odd Oz/ten-m homolog 4	1.33	0.30	E11 and E13	Zhou et al., 2003
NM_026211	Mm.45233	RIKEN 2400003B06 gene	1.27	0.63	E11	+
NM_018804	Mm.259316	Synaptotagmin 11	1.24	0.74	E11 and E13	+
NM_013685	Mm.4269	*Tcf4*	1.25	0.30	E11 and E13	+
NM_011594	Mm.206505	*Timp2*	1.26	0.30	E11 and E13	+

+, denotes in situ hybridisation confirmation carried out for this report.

rostral neocortex, Fgf15 and the Wnt antagonist Sfrp1 are highly expressed. Notably, sprouty 2 and Spred1, negative-feedback regulators of Fgf signalling, are also highly expressed at the rostral pole of the neocortex. The presence of Fgf15, Sprouty2 and Spred1, along with the Ets transcription factors Etv5 and Etv1, is indicative of the active Fgf signalling taking place around the rostral midline of the neocortex at this time in development (Fukuchi-Shimogori and Grove, 2001; Fukuchi-Shimogori and Grove, 2003; Storm et al., 2003).

The expression of putative protomap components is regulated by Fgf signalling in the developing neocortex in vivo

Fgf8 signalling has a central role in controlling neocortical pattern formation, as it is capable of both inducing rostral and repressing caudal neocortical identities (Fukuchi-Shimogori and Grove, 2001; Fukuchi-Shimogori and Grove, 2003; Grove and Fukuchi-Shimogori, 2003; Ragsdale and Grove, 2001). In agreement with this, the expression gradients of caudally expressed genes are shifted rostrally in the neocortex of *Fgf8* hypomorphic mutants (Garel et al., 2003). Genes encoding protomap components that are regulated by Fgf signalling should change their expression pattern in predictable ways upon altering cortical Fgf signalling, and this would serve to confirm their involvement in neocortical patterning. To investigate this, we took an unbiased approach to identify gene expression changes in the neocortex in response to altered Fgf signalling. *Fgfr1* mutant mice have a very similar phenotype to that of the *Fgf8* hypomorphic mutant, most notably the loss of the olfactory bulbs (Garel et al., 2003; Hebert et al., 2003b). Therefore, we carried out a global analysis of changes in gene expression in the dorsal telencephalon of forebrain-specific *Fgfr1* mutant mice. Gene expression was compared between the dorsal telencephalons of single E12.5 embryos of two key genotypes: *Fgfr1* heterozygous and homozygous mutants that were both heterozygous for the Foxg1 transcription factor, as Cre recombinase expression is driven off the endogenous *Foxg1* locus in these animals (Hebert et al., 2003b; Hebert and McConnell, 2000). Comparing *Foxg1* heterozygous null tissues controlled for possible changes in cortical gene expression due to *Foxg1* heterozygosity. Comparisons were carried out at E12.5, as this developmental stage is early in the neurogenetic period, precedes the arrival of incoming thalamocortical axons and falls between the two stages at which we carried out the spatial expression screen.

Statistically significant differences in expression between the two genotypes were identified, including both up- and downregulated transcripts (Fig. 4). Notably, among the genes whose expressions were altered in the homozygous null mutants were three Ets-domain transcription factors, Etv1, Etv5 and ELF2A2, found rostrally expressed in the array screen (Fig. 4). Two of these, Etv1 and Etv5, have been shown to be regulated by Fgf8 in vivo (Fukuchi-Shimogori and Grove, 2003). In addition, several other rostrally expressed candidate protomap genes, including Mest/Peg1 and Tweety, were downregulated in the *Fgfr1* mutant cortex. Conversely, caudally expressed proposed patterning genes, most notably the transcription factors COUP-TF1 and Tcf4, were upregulated in the *Fgfr1* mutant cortex (Fig. 4).

A striking additional finding in the *Fgfr1* E12.5 mutant cortex was the significant upregulation of expression of a set of neurogenic genes, including many normally expressed in

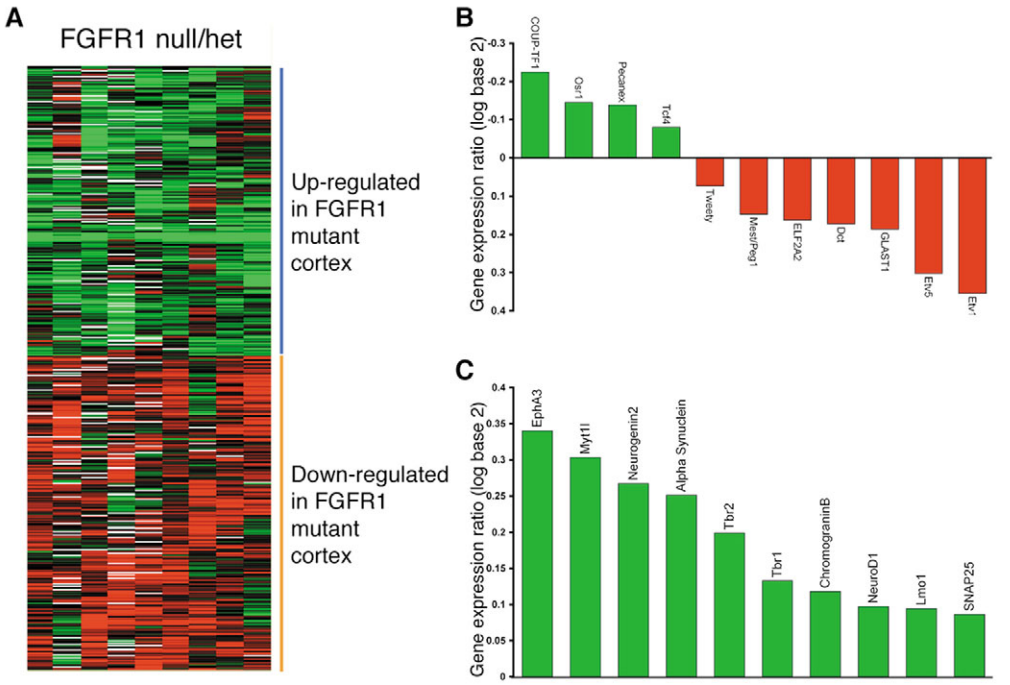

Fig. 4. Candidate protomap genes are up- and downregulated in *Fgfr1* mutant cortices in a manner consistent with their predicted regulation. (A) Cluster of genes up- and downregulated in the conditional *Fgfr1* mutant dorsal telencephalon at E12.5. Nine independent comparisons of gene expression between single homozygous and heterozygous null mutant embryos were carried out and the data analysed by *t*-test to identify reproducible differences in expression between those two genotypes. (B) Examples of the expression in the *Fgfr1* mutant cortex of genes normally expressed caudally (green) and rostrally (red) in the E13 neocortex. Caudal genes are upregulated, whereas rostral genes are downregulated in the *Fgfr1* E12.5 mutant cortex. Average log base 2 (mean from nine independent hybridisations) gene expression values are shown. Gene names are as indicated: *Osr1*, odd-skipped related 1; *Dct*, dopachrome tautomerase. (C) Neurogenic genes and genes expressed in differentiating neurons are upregulated in the *Fgfr1* mutant E12.5 neocortex. Genes shown include a neurogenic gene expressed in progenitor cells (neurogenin 2), transcription factor genes expressed in differentiating neurons (*Neurod*, *Myt1l*, *Tbr1*, *Tbr2*, *Lmo1*) and genes expressed in differentiated neurons (*Epha3*, alpha-synuclein, chromogranin B, *Snap25*).

differentiating neurons (Fig. 4). These genes include several transcription factor genes, such as Neurod1 (Schwab et al., 1998), Myt1-like (S. Rahman and F.J.L., unpublished) and Tbr2 (Bulfone et al., 1999), that are all expressed in newly born, differentiating neurons. The coherent upregulation of this set of genes suggests that, in addition to the changes in spatial gene expression, there is an increase in neurogenesis and neural differentiation in the E12.5 *Fgfr1* mutant cortex.

Expression of *Mest*, a candidate protomap component gene, is directly regulated by Fgf8 signalling

Given the effects of loss of Fgfr1 function in the developing cortex (see above) (see also Hebert et al., 2003b), Fgf signalling may indirectly regulate neocortical pattern formation by regulating progenitor cell proliferation or neural differentiation. Alternatively, Fgf8 regulation of progenitor cell gene expression may involve an intermediary signal or cell type, such as the cortical hem (Shimogori et al., 2004). To address these questions, we used an in vitro explant culture system to investigate whether neocortical progenitor cell expression of a candidate gene for formation of the protomap, *Mest*/*Peg1*, as well as that of several other previously described protomap genes, is directly regulated by Fgf8 (Fig. 5).

Expression of a set of six genes, including known (*COUP-TF1*, *Emx2*, *Pax6*) and novel potential protomap components (*Mest*, *Klf3*, *Hey1*), was studied following brief (4 hour) Fgf8 treatment of neocortical explants in defined media in vitro. Real-time PCR analysis of the expression levels of the three rostrally expressed genes, Etv5/ERM, Mest and Pax6, found an Fgf8-induced upregulation of expression of all of these genes within 4 hours. Conversely, of the three caudally expressed genes examined, *COUP-TF1*, *Hey1* and *Emx2*, only *COUP-TF1* expression was significantly repressed in neocortical progenitor cells within four hours of Fgf8 exposure. At this developmental stage, the overwhelming majority of neocortical cells are progenitor cells with a cell cycle length of approximately 10 hours (Takahashi et al., 1996). Together with the short time period over which these changes occur, we conclude that Fgf8 directly regulates the expression of *Mest* within neocortical progenitor cells.

In vivo loss of function of Mest results in abnormal neocortical development, including changes in cortical patterning

To validate the approach taken to identify protomap components, we analysed neocortical development in mice mutant for one of the strongest candidate neocortical patterning genes, *Mest*/*Peg1*. *Mest*/*Peg1* mRNA is expressed in a rostrocaudal gradient in neocortical progenitor cells at E11, a gradient that has substantially retracted rostrally by E13 to leave a relatively small region of the extreme rostral neocortex in which progenitor cells express *Mest*. The rostral expression of this gene is dependent on Fgf8 signalling, as it is significantly downregulated in *Fgfr1* mutant cortices in vivo, and is upregulated in neocortical progenitor cells by Fgf8 treatment in vitro. Notably, adult *Mest* mutant mice have a behavioural phenotype in which *Mest* mutant females fail to nurture newborns (Lefebvre et al., 1998). Furthermore, there is an unexplained increase in perinatal mortality in *Mest*/*Peg1* mutant newborns (Lefebvre et al., 1998).

A cellular function for Mest is not suggested by homology searches. *Mest* encodes a protein composed almost completely of a hydrolase-like domain, with an amino-terminal hydrophobic region that is predicted to act as either a signal peptide or a transmembrane domain (Fig. 6). Such a composition indicates that Mest is an enzyme, but it does not indicate a subcellular localisation or any possible substrates, as this hydrolase fold is very common in prokaryotic and eukaryotic proteins. However, although the cellular function of *Mest*/*Peg1* is unknown, this protein is highly conserved throughout its length in vertebrates (Fig. 6). Notably, an

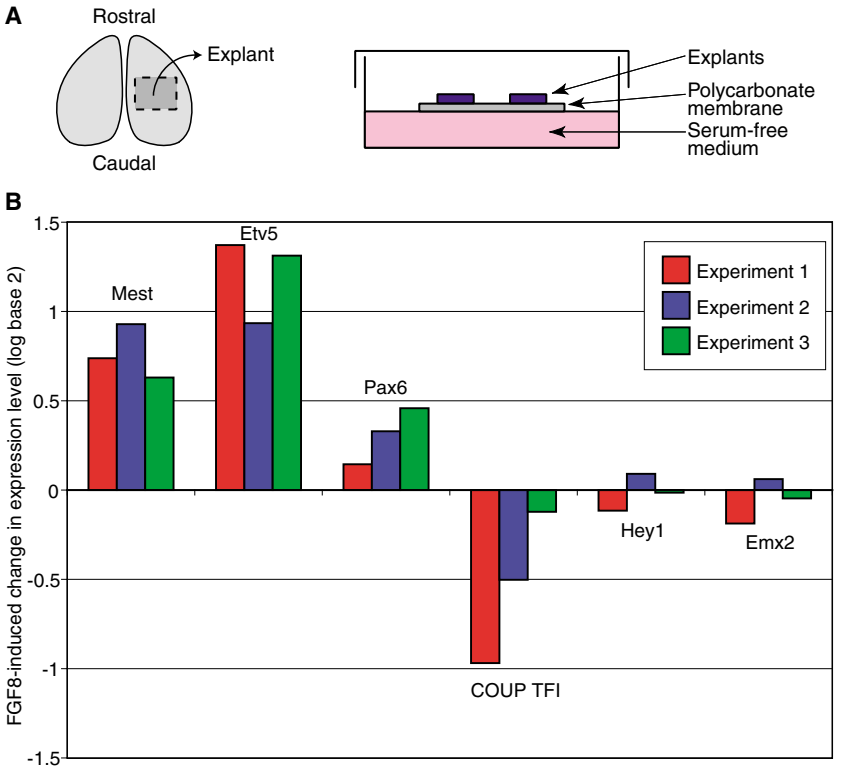

Fig. 5. Neocortical progenitor cell expression of *Mest* is directly regulated by Fgf8 signalling. (A) Explants of the middle third of the E11.5 neocortex were cultured on polycarbonate membranes in defined, serum-free medium, as shown. Two explants were used for each single treatment. (B) Four hours of Fgf8 exposure strongly induced the expression of *Mest*, *Etv5* and *Pax6* and strongly repressed expression of *COUP-TF1*. No significant changes in the expression of *Hey1* and *Emx2* were seen. Histograms of the expression levels of all six genes relative to the average expression level of each gene from four control, non-Fgf8-treated experiments are shown. Results from three independent experiments are shown, with each experiment containing two neocortical explants. Expression levels within each sample were normalised to that of the abundant transcript encoding the ribosomal protein rpS17, whose expression does not change in this system.

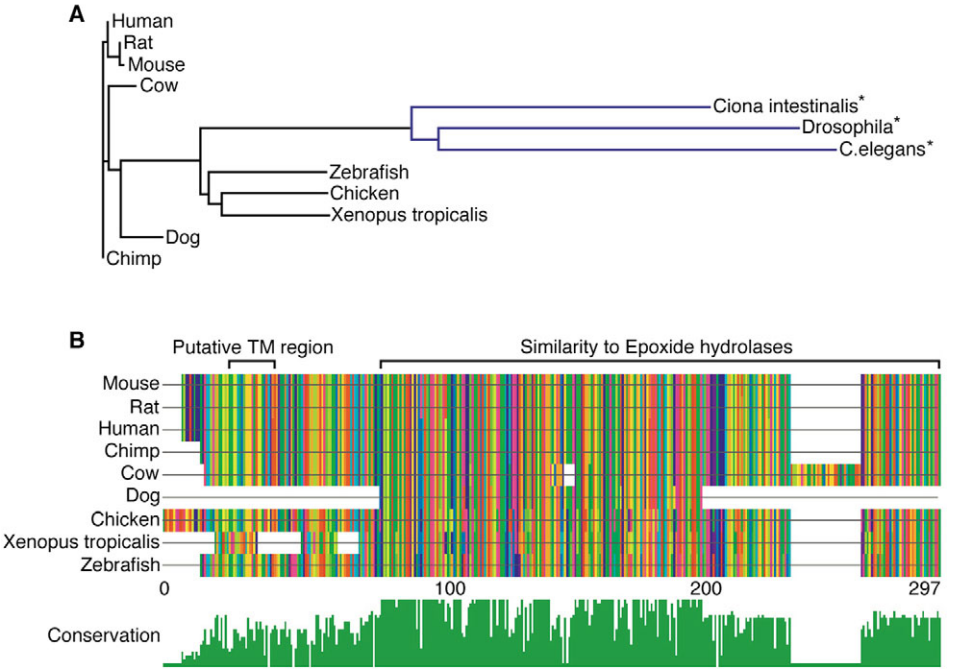

Fig. 6. *Mest* encodes a highly conserved, vertebrate-specific hydrolytic enzyme. (A) Phylogram of the multiple sequence alignment of the predicted protein sequences for Mest from several vertebrate and invertebrate genomes. Organisms are as labelled. Note that the most similar proteins from *C. elegans*, *D. melanogaster* and *Ciona intestinalis* group together and are relatively dissimilar from the other proteins. In all three organisms, the most similar protein in the mouse or human genome is not Mest but is instead a hydrolase lacking the hydrophobic N terminus. (B) Multiple sequence alignment of the vertebrate Mest protein sequences demonstrate a very high level of conservation among the different organisms.

orthologous protein cannot be found in the *Drosophila* or *C. elegans* genomes (Fig. 6), suggesting that this particular protein is vertebrate specific.

To analyse the effects of loss of Mest function in vivo in an unbiased manner, we carried out a microarray analysis of gene expression in *Mest* mutant cortices from single embryos compared with single wild-type littermates at E12.5 (Fig. 7). As *Mest* is an imprinted gene that is only expressed from the paternal allele, mutants were generated by crossing males carrying a *lacZ*-targeted allele in which exons 3-8 were removed to wild-type females, as described (Lefebvre et al., 1998). Statistically significant changes in gene expression in the *Mest* mutant cortex were detected at this age (E12.5), including the expected reduction in *Mest* transcript levels (Fig. 7).

To characterise the nature of the changes in expression in the *Mest* mutant, we carried out an analysis of the intersection between genes identified as showing spatial expression at E13 and those genes showing changes in expression in the *Mest* mutant cortex (Fig. 7). Of the 118 rostrally expressed genes that show altered expression in the *Mest* mutant cortex, 97 are downregulated and 21 are upregulated (Fig. 7). However, the 21 upregulated genes contain many candidate rostral patterning genes, including the Ets genes *Etv1* and *Etv5*, *Klf3* and *Fgf15*, as well as other rostrally expressed genes such ephrin B2 and ephrin A5. Section in situ hybridisation for the rostrally expressed gene *Klf3* confirmed its elevated expression in the rostral region of the *Mest* mutant cortex relative to that of a wild-type littermate (Fig. 7).

Conversely, of the 84 caudally expressed genes that show altered expression in the *Mest* mutant cortex, 16 are downregulated and 68 upregulated. However, as for the rostral genes, the smaller set of downregulated genes includes the candidate patterning genes *Hey1*, *Tcf4* and *Emx2* (Fig. 7). Two conclusions can be drawn from this analysis: that there appear to be two distinct neocortical phenotypes in *Mest* mutant embryos, one of which is an alteration in progenitor cell patterning; and that the patterning phenotype consists of an upregulation of rostrally expressed genes accompanied by a downregulation of caudally expressed genes.

Array analysis indicates that Mest may act as a negative regulator of Fgf-regulated neocortical patterning

The genomics screen for protomap components and the subsequent functional experiments on the Fgf regulation of neocortical gene expression indicate that the neocortex is patterned by Fgf-regulated gradients of gene expression. Furthermore, analysis of neocortical gene expression in *Mest*/*Peg1* mutant E12.5 embryos suggested that Mest/Peg1 is a potential negative regulator of the Fgf8-controlled cortical patterning pathway. To test this formally, we carried out a combined analysis of the gene expression data from both the *Fgfr1* and *Mest* mutant cortices (Fig. 8).

Statistical testing (*t*-test) for significant changes in gene expression common to both genotypes found no such changes above those expected by chance (data not shown). However, testing for significant ($P<0.05$) differences in gene expression changes between the genotypes identified 703 such genes (Fig. 8). A hierarchical cluster of those genes shows that there are over 300 genes upregulated in the *Mest* mutant that are downregulated in the *Fgfr1* mutant, and conversely over 300 genes that show the opposite behaviour (Fig. 8).

The genes showing opposite behaviours in the two mutant genotypes include several candidate rostral and caudal patterning genes (Fig. 8). Notably, the rostral patterning genes show a marked reversal in their expression change in the two mutants, such that genes in this category were upregulated in the *Mest* mutant and downregulated in the *Fgfr1* mutant cortex. These genes include several readouts of Fgf signalling, including Ets genes and sprouty 2, suggesting that there is increased Fgf signalling in the *Mest* mutant cortex.

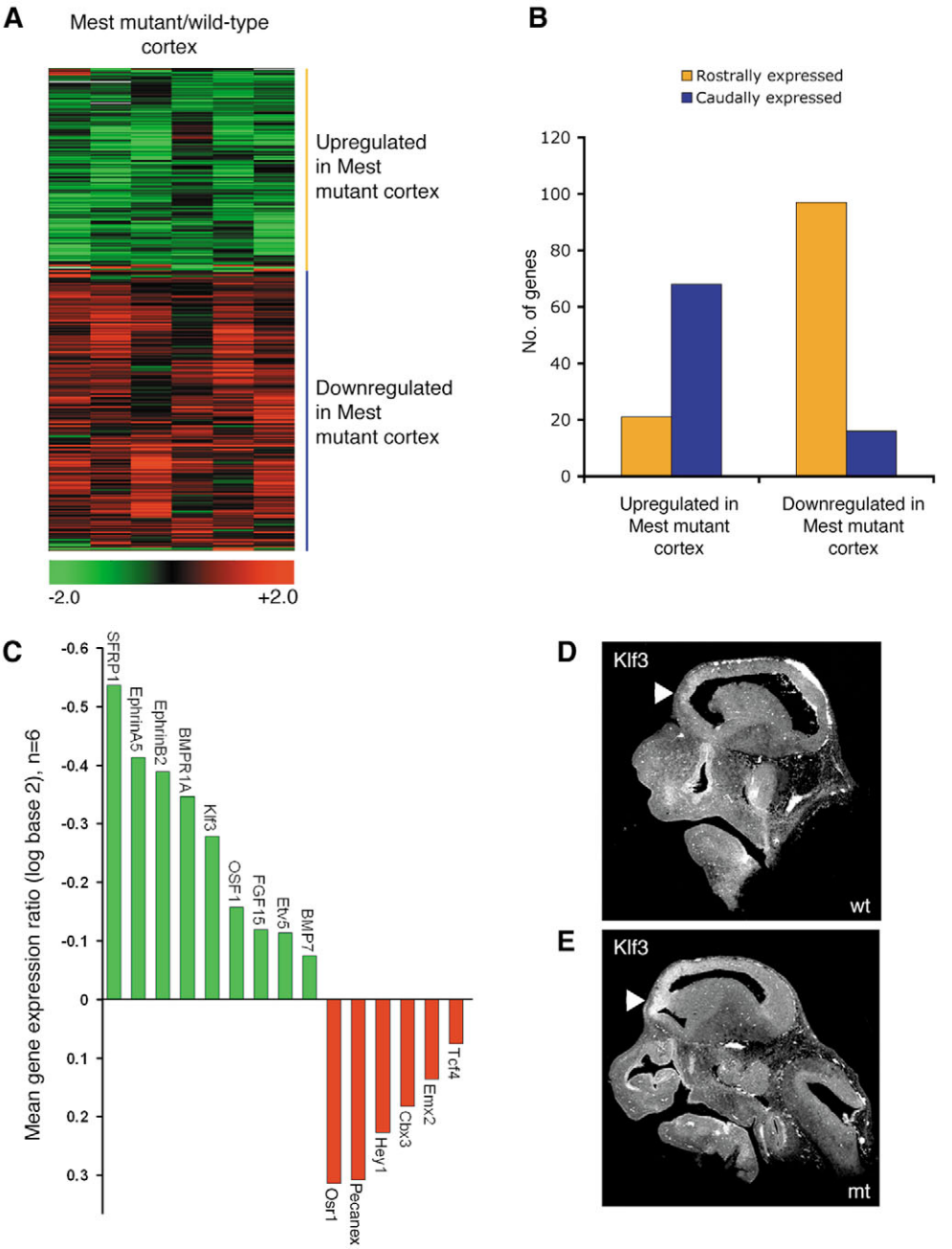

Fig. 7. Functional analysis of a candidate protomap component, Mest/Peg1, reveals a role in Fgf8-mediated patterning of rostral cortex. (A) Gene expression changes in the E12.5 *Mest* mutant neocortex. A hierarchical cluster analysis of the set of genes showing statistically significant differences in gene expression between E12.5 *Mest* mutant and wild-type cortices. (B) Changes in the expression of genes showing higher rostral and caudal expression in the E13 microarray screen in the *Mest* mutant cortex. Note that approximately 20% of such rostral genes are upregulated in the *Mest* mutant and a similar percentage of caudal genes are downregulated. (C) Caudal patterning genes (red) are downregulated and rostral patterning genes are upregulated (green) in the *Mest* mutant E12.5 cortex. The average gene expression change (n=6 hybridisations) for each gene is shown. (D,E) A comparison of Klf3 expression in E12.5 wild-type and *Mest* mutant neocortex shows marked changes in both the level of expression and the extent of the rostrocaudal gradient, with a caudal shift of the gradient in the mutant cortex. Fluorescent in situ hybridisation on parasagittal sections from mutant (E) and wild-type (D) E12.5 embryos is shown, with rostral to the left. Arrowheads indicate the rostral region of Klf3 expression in each section.

Discussion

Genomics screens enable the characterisation of the nature and composition of a neocortical progenitor cell protomap

The existence of a protomap of neocortical progenitor cells was first proposed over a decade ago (Rakic, 1988). Gradients of gene expression across the field of neocortical progenitor cells have been reported (for reviews, see Grove and Fukuchi-Shimogori, 2003; O'Leary and Nakagawa, 2002), but a comprehensive analysis of the spatial organisation of neocortical progenitor cells, as reflected in gene expression, has been lacking. To address this, we systematically investigated the nature and composition of the cortical protomap at the transcriptional level. By analogy with patterning in other parts of the nervous system, we proposed that the identification of genes that show spatial expression in neocortical progenitor cells would enable the definition of the nature and topography of the cortical protomap, in addition to identifying protomap components. We took a genomics approach to identify comprehensively the genes demonstrating rostrocaudal differences in gene expression in the early developing neocortex, before the arrival of ingrowing thalamocortical axons.

The efficacy of this screen was underlined by the identification of almost every known rostrocaudal gene expression difference in the neocortex, including the transcription factors Emx2, Pax6, COUP-TFI and Lhx2 (Bishop et al., 2000; Liu et al., 2000; Mallamaci et al., 2000; Monuki et al., 2001; Nakagawa et al., 1999; Zhou et al., 2001). Our criteria for protomap components were spatial expression in neocortical progenitor cells that was not related to the

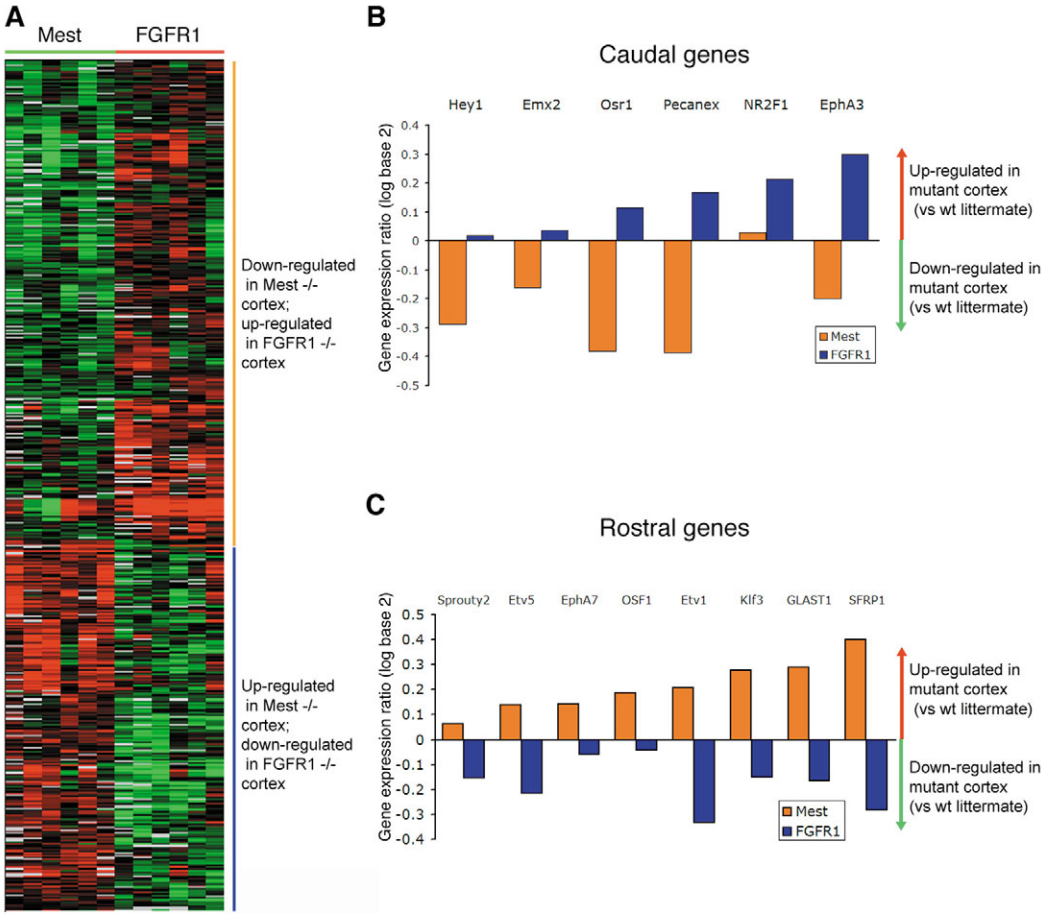

Fig. 8. Mest is a potential negative regulator of an Fgf-regulated neocortical patterning pathway. (A) Genes upregulated in the *Fgfr1* mutant E12.5 cortex are downregulated in the E12.5 *Mest* mutant cortex. Hierarchical cluster of the set of genes showing significantly different changes in gene expression between the *Fgfr1* and *Mest* mutant cortices ($P<0.05$). (B) Candidate caudal neocortical patterning genes (as labelled) are downregulated in the *Mest* mutant cortex but upregulated in the *Fgfr1* mutant cortex. Average fold changes (log base 2) from multiple independent hybridisations are shown (*Mest*, $n=6$; *Fgfr1*, $n=9$). (C) Candidate rostral neocortical patterning genes (as labelled) are upregulated in the *Mest* mutant cortex but downregulated in the *Fgfr1* mutant cortex.

developmental gradient across this structure, and this identified many candidate transcripts. These included a set of transcription factor genes with graded rostrocaudal expression across the field of neocortical progenitor cells, such as *Klf3*, *Hey1* and Fez-like, which are novel candidates for genes encoding progenitor cell positional identity.

Consistent with a subset of those genes being elements of a neocortical protomap, we found that many of these genes, including the transcription factor genes, change their expressions in a predictable manner in the neocortex following changes in Fgf signalling. Functional analysis of one such gene, *Mest*/*Peg1*, that is a candidate for a gene involved in setting up the protomap, demonstrated that this rostrally expressed gene is required for the establishment of normal patterned gene expression in the neocortex. Overall, we find evidence for a cortical protomap that is composed of gradients of gene expression across the field of neocortical progenitor cells, rather than spatially discrete populations of progenitor cells defined by combinatorial gene expression.

Gene expression gradients and compartments in the developing neocortex

At least two alternative systems for conferring spatial identities on neural progenitor cells have been described in other systems. Dorsoventral gradients of expression of transcription factors have been found in retinal progenitor cells (Barbieri et al., 1999; Koshiba-Takeuchi et al., 2000; Ohsaki et al., 1999; Schulte et al., 1999), which are required for the graded expression of the ephrins and eph receptors in ganglion cell neurons. This in turn is the basis for the retinotopic mapping of spatial information from retinal ganglion cells onto the tectum (McLaughlin et al., 2003). Within the developing spinal cord, progenitor populations are defined by the combinatorial expression of several transcription factors along the dorsoventral axis of the neural tube, with each population giving rise to defined classes of neurons (Briscoe et al., 2000; Jessell, 2000).

It has been proposed that a similar system of transcriptionally encoded positional information is likely to operate in neocortical progenitor cells (Grove and Fukuchi-Shimogori, 2003). Opposing gradients of expression of Pax6 and Emx2 have been found in the neocortex, and loss of function of either gene results in shifts in the relative sizes of neocortical areas (Bishop et al., 2000; Mallamaci et al., 2000). Alterations in the absolute levels of Emx2 result in shifts in neocortical areas, leading to the recently proposed cooperative-concentration model for neocortical patterning, in which it is proposed the spatial identity of a neocortical progenitor cell is encoded by the absolute levels of expression of the patterning transcription factors (Hamasaki et al., 2004).

Consistent with this, we found little evidence for discrete domains of gene expression across the field of neocortical progenitor cells, as described in the developing spinal cord (Briscoe et al., 2000). The initial genomics screens and the in situ hybridisation studies together demonstrate that the characteristic patterns of gene expression across the field of

neocortical progenitor cells are gradient based. We did not find any clear boundaries of gene expression within the neocortex at these stages. This is in contrast with the sharp gene expression boundaries between the developing hippocampus, a cortical structure, and subcortical structures and the neocortex. This is illustrated by the expression of the transcription factors Fez-like and COUP-TFII: at embryonic day E13.5, both of these genes have sharp caudal boundaries of expression, whereas they are expressed in gradients across the neocortex.

Given the comprehensive, unbiased array design used for the screens reported here, the accuracy of which were confirmed by in situ hybridisation data for the majority of the top protomap candidate genes, we conclude that few genes are expressed in discrete domains in the field of neocortical progenitor cells in the first half of the neuronogenetic interval. It is possible that a small number of genes that encode spatial identity are expressed in domains and were not detected by this screen. However, the screens reported here identified almost all of the known genes that show differential expression along the rostrocaudal axis, and the arrays represent over 22,000 genes expressed from the mouse genome, suggesting that gradients are the dominant pattern of gene expression along the rostrocaudal axis of the neocortex at the stages examined.

Fgf8 signalling and the formation of the neocortical protomap

A central tenet of our strategy for identifying protomap components is that regionally expressed genes have roles in setting up or encoding the protomap, and that a subset of these genes will be regulated directly or indirectly by Fgf8 signalling. Several of the genes identified in our screen have been shown to have roles in neocortical area formation, most notably *Pax6*, *Emx2* and *COUP-TFI* (Bishop et al., 2000; Mallamaci et al., 2000; Zhou et al., 2001). By expression profiling of the neocortices of *Fgfr1* mutant mice, we found that many genes showing patterned gene expression in the neocortex (some of which encode proposed protomap components) are regulated by Fgf signalling in vivo. However, we also found a marked change in the expression of neurogenic and neural differentiation genes, suggesting that there are alterations in progenitor cell proliferation or neurogenesis, or both, in this mutant. Such a phenotype is similar to that observed in the neocortex of *Fgf2* mutant mice, in which neocortical progenitors generate too many neurons at the expense of the progenitor cell pool early in neocortical neurogenesis (Korada et al., 2002), resulting in a reduction in total numbers of glutamatergic neurons in the mature frontal cortex. Similarly, overexpression of a dominant-negative Fgfr1 in the early developing cortex results in an overall reduction of pyramidal neurons in the adult frontal cortex (Shin et al., 2004).

Those findings raised the possibility that Fgf signalling may indirectly regulate cortical patterning by controlling the relative growth of the frontal cortex. We therefore tested whether Fgf8 could directly regulate neocortical progenitor cell expression of known and proposed neocortical patterning genes, finding that Fgf8 could induce or repress expression of a subset of such genes by as much as twofold within 4 hours. Together with the in vivo analysis of Fgf-regulated gene expression, we conclude that Fgf8 can directly regulate the graded expression of protomap components.

Mest/Peg1, an Fgf8-regulated gene, is a part of the rostral neocortex patterning system

To validate the strategy taken to identify protomap components, we carried out a functional in vivo analysis of a leading candidate for a gene involved in protomap formation, *Mest/Peg1*. This gene is rostrally expressed normally, is induced almost twofold by Fgf8 treatment for 4 hours in vitro and is downregulated in the *Fgfr1* mutant. Mest loss of function results in striking changes in neocortical patterning during development: a set of rostrally expressed genes, downregulated in the *Fgfr1* mutant, is upregulated, whereas caudally expressed genes are downregulated.

Of the rostrally expressed genes upregulated in the Mest mutant, several are well-characterised targets genes for Fgf signalling, including the Ets transcription factor genes *Etv1* and *Etv5* and the Fgf-induced negative regulator sprouty 2. By contrast, all of these genes are downregulated in the *Fgfr1* mutant neocortex. These opposite phenotypes are consistent with Mest being part of a pathway that negatively regulates Fgf-mediated rostral patterning in the neocortex. However, the cellular function of Mest is not clear from the nature of the Mest protein. The predicted signal peptide/transmembrane domain indicates that Mest may act in an intracellular compartment or may act extracellularly. Future experiments will clarify the cellular localisation and possible function of Mest as a negative regulator of Fgf signalling.

Rostrocaudal gradients and the nature of the neocortical protomap

The gradient-based system identified here is consistent with previous gene expression analyses in primates (Donoghue and Rakic, 1999a; Donoghue and Rakic, 1999b). In situ hybridisation studies of a set of genes identified the characteristic spatial expression pattern across the field of neocortical progenitor cells as being gradient based, although there was strong evidence for domains of gene expression in developing neurons (Donoghue and Rakic, 1999a; Sestan et al., 2001), as also seen in rodents (Miyashita-Lin et al., 1999; Nakagawa et al., 1999), corresponding to anatomical and functional neocortical areas.

Further support for a gradient-based system for positional information comes from in vivo manipulation of Fgf8 levels in the developing neocortex, which alters the position of neocortical areas along the rostrocaudal axis (Fukuchi-Shimogori and Grove, 2001). Detailed analyses of the morphology of one major component of the somatosensory cortex, the whisker barrels, revealed that this manipulation does not simply shift areas, but also alters their relative proportions. In the case of the whisker barrels, the individual whisker barrels are expanded or compressed with the reduction or augmentation of the endogenous Fgf8 source (Fukuchi-Shimogori and Grove, 2001), leading to the suggestion that progenitor cell spatial identity may be gradient based (Grove and Fukuchi-Shimogori, 2003).

Patterning of developing structures by gradients of extracellular factors and morphogens is a common theme in many developmental systems, including the neural tube and developing limb (Echelard et al., 1993; Pearse and Tabin, 1998; Placzek et al., 1991; Riddle et al., 1993; Roelink et al., 1994). However, a classical question in developmental biology is how such graded signals generate cells with discrete positional

identities, as reflected in the spatial expression of Hox genes in the developing limb (Johnson et al., 1994). This is distinct from the findings on neocortical progenitor cell spatial identity, which suggest that these cells are not organised into discrete spatial populations based on graded extracellular signals. Rather, it appears that each progenitor has a unique spatial identity, based on the gradient of gene expression across the field of neocortical progenitor cells. Recent data have clearly demonstrated that altering the nature of the gradient of expression of the protomap gene *Emx2*, rather than removing its expression completely, leads to shifts in both the position and morphology of neocortical areas (Hamasaki et al., 2004). In this case, it appears that the absolute level of *Emx2* expression at any point along the gradient is a key regulator of neocortical pattern formation (Hamasaki et al., 2004).

In conclusion, from the combined genomic and genetic analysis presented here, we propose that the protomap is composed of transcription factors with gradients of expression across the field of neocortical progenitor cells. Furthermore, we conclude that a key element in the nature of the protomap is gradient-based positional information within neocortical progenitor cells, the encoding of which is controlled via an Fgf8-regulated pathway of which Mest is a potential negative regulator.

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