Math1 controls cerebellar granule cell differentiation by regulating multiple components of the Notch signaling pathway

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Summary

Cerebellar granule cells (CGC) are the most abundant neurons in the mammalian brain, and an important tool for unraveling molecular mechanisms underlying neurogenesis. *Math1* is a bHLH transcription activator that is essential for the genesis of CGC. To delineate the effects of *Math1* on CGC differentiation, we generated and studied primary cultures of CGC progenitors from *Math1*/*lacZ* knockout mice. Rhombic lip precursors appeared properly positioned, expressed CGC-specific markers, and maintained *Math1* promoter activity in vivo and in vitro, suggesting that *Math1* is not essential for the initial stages of specification or survival of CGC. Moreover, the continuous activity of *Math1* promoter in the absence of MATH1, indicated that MATH1 was not necessary for the activation of its own expression. After 6, but not 3, days in culture, *Math1* promoter activity was downregulated in control cultures, but not in cells from *Math1* null mice, thus implying that *Math1* participates in a negative regulatory feedback loop that is dependent on increased levels of MATH1 generated through the positive autoregulatory feedback loop. In addition, *Math1* null CGC did not differentiate properly in culture, and were unable to extend processes. All Notch signaling pathway receptors and ligands tested were expressed in the rhombic lip at embryonic date 14, with highest levels of *Notch2* and *Jag1*. However, *Math1*-null rhombic lip cells presented conspicuous downregulation of *Notch4* and *Dll1*. Moreover, of the two transcriptional repressors known to antagonize *Math1*, *Hes5* (but not *Hes1*) was downregulated in *Math1*-null rhombic lip tissue and primary cultures, and was shown to bind MATH1, thus revealing a negative regulatory feedback loop. Taken together, our data demonstrate that CGC differentiation, but not specification, depends on *Math1*, which acts by regulating the level of multiple components of the Notch signaling pathway.

Key words: Rhombic lip, Cerebellum, Cerebellar granule cells, Neurite, Notch, Delta, Jagged, Hes, Knockout, Mouse

Introduction

The highly ordered cytoarchitecture and the relative simplicity of cerebellar development make it one of the best studied systems for neurogenesis. Most of the cerebellar neurons (e.g. Purkinje cells, deep cerebellar nuclei and interneurons) arise at a ventricular zone located at the edge of the fourth ventricle (Hatten and Heintz, 1995). Precursors of the cerebellar granule cells (CGC) are born in a second proliferative zone, the rhombic lip, where they proliferate and later migrate via a rostral movement over the surface of the embryonic cerebellum (Altman and Bayer, 1997; Gilthorpe et al., 2002; Wingate, 2001). Consequently, these CGC precursors yield the external granule/germinate layer (EGL) of the cerebellum, a displaced germinal zone, where proliferation continues and peaks at postnatal day 7 (P7) in mouse (Altman and Bayer, 1997; Hatten et al., 1997; Hatten and Heintz, 1995). Postmitotic cells congregate in the inner EGL, and then migrate into the cerebellar cortex along Bergman radial glia towards their final destination: the cerebellar internal granule layer (IGL) (Edmondson et al., 1988; Fishman and Hatten, 1993; Hatten and Heintz, 1995). The later stages of CGC development – EGL formation and migration towards the IGL – have been extensively studied (reviewed by Goldowitz and Hamre, 1998; Hatten and Heintz, 1995; Millen et al., 1999; Wang and Zoghbi, 2001), in contrast to the earlier stages of precursor specification and differentiation, which are less characterized.

Math1 (*Atoh1* – Mouse Genome Informatics) encodes a murine basic helix-loop-helix (bHLH) transcription activator (Akazawa et al., 1995; Ben-Arie et al., 1996), orthologous to the *Drosophila atonal*. In the developing cerebellum, *Math1* is expressed in mitotic CGC at the rhombic lip and in the outer EGL (Akazawa et al., 1995; Ben-Arie et al., 1997; Ben-Arie et al., 2000; Ben-Arie et al., 1996; Helms et al., 2000). Genomic disruption has proven that *Math1* is essential for proper development of CGC, as *Math1* null mice lack the EGL (Ben-Arie et al., 1997; Ben-Arie et al., 2000). However, overexpression of *Math1* resulted in cerebellar abnormalities without extra neurogenesis (Helms et al., 2001; Isaka et al.,

1999), arguing against a proneural role for *Math1* in the developing nervous system of the mouse.

The Notch signaling pathway is a crucial mechanism for controlling cell specification and differentiation in both invertebrates and vertebrates (Artavanis-Tsakonas et al., 1999; Beatus and Lendahl, 1998; de la Pompa et al., 1997; Frisen and Lendahl, 2001; Gaiano and Fishell, 2002; Justice and Jan, 2002). Notch signaling components, such as the receptors Notch1 and Notch2, the ligands Delta1 (Dll1 – Mouse Genome Informatics), Dll3, Jag1 and Jag2, the DNA-binding protein interactor *Cbf1* (*Rbpsuh* – Mouse Genome Informatics), and the effectors *Hes1* and *Hes5* were found to be expressed in the EGL of neonatal mice (Irvin et al., 2001; Kusumi et al., 2001; Solecki et al., 2001; Tanaka et al., 1999). Moreover, activation of Notch and overexpression of its effector *Hes1*, maintained the proliferation of CGC EGL precursors (Solecki et al., 2001). Loss of *Notch1* was shown to result in a premature onset of neurogenesis, which resulted in a reduced number of neurons in the adult cerebellum (Lutolf et al., 2002). Similarly, the importance of *Hes1* and *Hes3* in cerebellar development was identified in knockout mice (Hirata et al., 2001).

Links between Notch signaling pathway and *Math1* were identified in various tissues. *Math1* was shown to be essential for the generation of inner ear hair cells (Bermingham et al., 1999; Chen et al., 2002; Kawamoto et al., 2003; Shou et al., 2003; Zheng and Gao, 2000). Moreover, activation of Notch via *Jag2* was shown to inhibit expression of *Math1* in cochlear progenitor cells, possibly through the activity of *Hes5* (Lanford et al., 2000). Indeed, upregulation of *Math1* in *Hes1* and *Hes5* mutant cochleae suggested that Hes genes regulate hair cell differentiation by antagonizing *Math1* expression (Zine and de Ribaupierre, 2002). Notch pathway components were similarly found to be variably expressed in the mouse small intestine (Schroder and Gossler, 2002). Notably, in the small intestine of *Math1*-null mice, which lack secretory cells, the expression of *Dll3* was halved, while *Dll1*, *Hes1*, *Notch1*, *Notch2*, *Notch3* and *Notch4* expression was unaffected (Yang et al., 2001).

In this study we aimed to deepen our insight into CGC neurogenesis, by taking advantage of *Math1*-null mice, in which this process is arrested. The development of CGC precursors in *Math1*-null mice was followed by examination of *Math1* promoter activity. Rhombic lip cells were then cultured and analyzed for their survival, specification and differentiation in vitro. Our data show that lack of *Math1* did not affect the viability of CGC or their specification. Rather, CGC progenitors were abnormal in their differentiation, as evident molecularly (by the continuous activation of *Math1* promoter) and morphologically (by their inability to extend processes in culture). Among all Notch receptors and ligands expressed in the rhombic lip, *Notch4* and *Dll1* showed the most pronounced downregulation in *Math1*-null mice. Moreover, by testing two Notch effectors we have discovered that the expression of *Hes5*, but not *Hes1*, is *Math1* dependent, and that MATH1 can bind directly *Hes5*, thus demonstrating a novel negative autoregulatory loop of *Math1* expression. The feedback mechanism requires an accumulation of MATH1, and therefore provides an explanation for the delayed downregulation of *Math1* in cultured cells. Taken together, our data reveal that *Math1* controls cerebellar granule cell differentiation as well as its own expression, at least in part, through the Notch signaling pathway.

Materials and methods

Math1 null mice

The generation of *Math1*-null allele mice has been previously described (Ben-Arie et al., 2000). In this line, the entire coding region of *Math1* has been removed, and replaced by a pSAβgal/PGK-neo cassette, such that *lacZ* expression is driven by the endogenous control elements of *Math1*. As $Math1^{\beta\text{-}gal/\beta\text{-}gal}$ mice are not viable, heterozygous mice were mated to obtain all *Math1* genotypes. The morning of vaginal plug appearance was considered as embryonic day (E) 0.5. Experiments were conducted according to an ethical approval from the Hebrew University of Jerusalem, according to the Israeli laws.

X-Gal staining

Whole embryos or tissue staining was previously described (Ben-Arie et al., 2000). To stain cultured cells the wells were washed twice in PBS, fixed by 0.05% gluteraldehyde in PBS for 5 minutes at room temperature, and washed three times in PBS. Staining was performed at 37°C for about 10 hours, in solution of 1 mg/ml X-Gal, 5 mM potassium ferricyanide, 5 mM potassium ferrocyanide and 1 mM $MgCl_2$ in PBS. After postfixation in 4% paraformaldehyde in PBS, cells were counterstained by Nuclear Fast Red (Aldrich) and clarified in 75% Glycerol in PBS.

Rhombic lip primary cultures

Culturing of cerebellar granule cells is based on a previously described procedure (Alder et al., 1996; Hatten et al., 1998). Briefly, embryos were collected in ice-cold CMF-PBS (Hatten et al., 1998), and the cerebellum isolated under a dissecting microscope by two incisions across the mesencephalon/metencephalon border and across the fourth ventricle. The rhombic lip tissue was pealed off with fine forceps, placed in CMF-PBS and stored on ice. Dissociation was performed by incubation of the tissue in 0.08% Trypsin (Biological Industries, Beit-Haemek, Israel), 0.02% EGTA, 0.05 mg/ml *DNase*I (Sigma) in CMF-PBS, for 15 minutes at 37°C; which was then changed to 0.05 mg/ml *DNase*I, 0.45% Glucose in ice cold Eagle's basal medium (BME). The tissue was triturated by passing through a pipettor tip, centrifuged at 700 ***g*** at 4°C for 5 minutes, and pellets resuspended in 50 μl granule cell medium (Hatten et al., 1998) supplemented by 5% fetal calf serum and 10% horse serum (Biological Industries, Beit-Haemek, Israel). Cells were diluted to 1200-1300 cell/μl before plating into Terasaki Micro Plate (#1006-01-3, Robbins, Sunnyvale, CA). Normally, four or five wells were plated from each embryo (22×10^3 cells/well). Cultures were grown in 95% air/5% CO_2 humidified incubator, at 37°C. Half the medium was changed on the next day after plating and every other day thereafter.

Quantification of β-galactosidase activity

Liquid assay for the *lacZ* reporter activity was performed using the All-in-One Mammalian β-Galactosidase Assay Kit (Pierce, Rockford, IL). Cultured rhombic lip cells grown in Terasaki plates were washed with PBS, lysed by the addition of 29 μl M-PER (Pierce, Rockford, IL) per well and incubated for 5 minutes. An aliquot of 20 μl was transferred into a 96-well plate, and 180 μl All-in-One reagent added. Reaction was carried out at 37°C for 6 hours and color development was measured every hour at 405 nm. A second aliquot of 8 μl was used for protein quantification; using Protein-Assay Reagent (BioRad, Hercules, CA).

Immunohistochemical analysis of primary cultures

Cultured cells were fixed by 4% paraformaldehyde in PBS for 15 minutes at room temperature, washed three times with PBS, and blocked by 5% normal goat serum, 2% BSA, 0.1% Triton X-100 in PBS for 1 hour at room temperature. Primary antibodies were diluted in blocking buffer and incubated overnight at 4°C, then for 1 hour at

room temperature. The antibodies used were: mouse anti-β-tubulin (1:10, DSHB, E7), rabbit anti-NF160 (1:100, Sigma, N4142), mouse anti-phosphorylated neurofilaments (1:5, DSHB, RT97) and mouse anti-NCAM (1:5, DSHB, 5B8). Cells were washed four times with 0.1% Triton X-100 in PBS; before the addition of secondary antibodies conjugated to FITC or Biotin (Sigma), and incubated for 2 hours at room temperature, after which they were washed three times with PBS. For Biotin-conjugated antibodies StreptAvidin-TexasRed (Vector Laboratories, Burlingame, CA) was used for visualization. Counterstaining by DAPI was performed before mounting with 1% n-propyl-galate (Sigma) in 90% glycerol. Pictures were taken under an Axioskop2 microscope (Zeiss, Germany), using a DP10 digital camera (Olympus, Germany). Images were assembled using NIH ImageJ software (http://rsb.info.nih.gov/nih-image/index.html).

For quantification of processes the cultures were grown for 6 days, fixed, blocked and stained with mouse anti-β-tubulin as above. Then, cells were washed, incubated for 2 hours at room temperature with a secondary antibody conjugated to peroxidase (Jackson ImmunoResearch, West Grove, PA) and washed. The cells were then lysed by CytoBuster (Novagene, Milwaukee, WI) and the content of each two wells combined. A colorimetric reactions was initiated by the addition of 1mg/ml ABTS (2,2′-Azino-bis(3-ethylbenzothiazoline-6-sulfonic acid, diammonium salt)), 0.003% H_2O_2 (Sigma), 28 mM citric acid and 44 mM Na_2HPO_4. The O.D (405 nm) was measured every 15 minutes to ensure that the values are within the linear range.

RT-PCR analysis

RNA was extracted as described (Chirgwin et al., 1979). Cultured cells were lysed with 25 μl/well of lysis buffer (4 M guanidine thiocyanate, 25 mM sodium citrate, 17 mM N-laurylsarcosine) for 5 minutes at room temperature and kept at –70°C. After genotyping lysates were thawed and mixed with 1 μl β-mercaptoethanol, 12.5 μl 2M sodium acetate pH 4.0, 125 μl acidic phenol, 25 μl chloroform-isoamyl alcohol (49:1). The aqueous phase was extracted twice using chloroform-isoamyl alcohol, precipitated by isopropanol with glycogen as a carrier, washed by 70% ethanol, dried, dissolved in 25 μl water, and *DNase*I treated using the DNA-free kit (Ambion, Austin, Texas). Reverse transcription was carried out by RevertAid H Minus First Strand cDNA Synthesis Kit (Fermentas MBI, Vilnius, Lithuania).

PCR amplifications were performed using FastStart Taq DNA polymerase (Roche, Germany), 0.2 mM dNTPs, 1.5 mM $MgCl_2$ and 1 μM each primer. The thermocycling parameters for *Zic1*, *Zipro1* and β-actin (set A) were: 94°C/4 minutes, 40 cycles of 94°C for 30 seconds, 55°C for 30 seconds, 72°C for 30 seconds, and 72°C for 3 minutes; and for *Hes1*, *Hes5* and β-actin (set B): 94°C for 4 minutes; 34 cycles of 94°C for 30 seconds, 68°C for 120 seconds and 68°C for 5 minutes.

Real-time amplifications were performed on Rotor-Gene machine (Corbett Research, Australia) using 2 mM $MgCl_2$ and ×0.3 SYBR I Green. Thermocycling conditions were 94°C for 4 minutes, then 45 cycles of 96°C for 25 seconds, 60°C for 20 seconds, 72°C for 30 seconds; 72°C for 1 minute. Amplification of a single product was verified by melting curves, and the correct product size by gel separation. For quantification, calibration curves were run simultaneously with experimental samples and C_t calculations were performed by the Rotor-Gene software.

The primers used were as follows: *Zic1*, (F) GGCCAACCCCAAAAAGTC, (R) CGTTAAAATTCGAAGAGAGCG; *Zipro1*, (F) CCAGACTCCAAAGCGGTTCTGAG, (R) AGTGTCATGGTACCCAAATTG; β-actin (A), (F) TGTTACCAACTGGGACGACA, (R) TGTTACCAACTGGGACGACA; β-actin (B), (F) GTGGGCCGCTCTAGGCACCAA, (R) CTCTTTGATGTCACGCACGATTTC; *Hes1*, (F) AGCTGGAGAGGCTGCCAAGGTTT, (R) ACATGGAGTCCGAAGTGAGCGAG; *Hes5*, (F) TTAAGCAAGTGACTTCTGCGAAGTTC, (R) GGCCATGTGGACCTTGAGGTGAG; *Notch1*, (F) AGAGATGTGGGATGCAGGAC, (R) CACACAGGGAACTTCACCCT; *Notch*2, (F) TGTACCAGATCCCAGAGATGC, (R) GTCAGATGCAGAGTGTGGTGA; *Notch3*, (F) AATCCTGTAGCTGTTCCCCTC, (R) CTGGGCTAGGTGTTGAGTCAG; *Notch4*, (F) ATCACAGGATGACTGGCCTC, (R) ACTCGTACGTGTCGCTTCCT; *Dll*1, (F) CTGAGGTGTAAGATGGAAGCG, (R) CAACTGTCCATAGTGCAATGG; *Dll*3, (F) CACCAGTAGCTGCCTGAACTC, (R) GTTAGAGCCTTGGAAACCAAG; *Dll4*, (F) CCTCTAGGCAAGAGTTGGTCC, (R) TAGAAAGGCCAGTGCTTCTGA; *Jag1*, (F) TGACATGGATAAACACCAGCA, (R) GCAGCCCACTGTCTGCTATAC; *Jag2*, (F) ATTGTAGCAAGGTATGGTGCG, (R) GCACAGTTGTTGTCCAAATGA.

Electrophoretic mobility shift assay (EMSA)

Full length Math1 and E47 cDNAs were cloned into pGEX-3X and pET28(a) expression vectors, respectively. MATH1/GST and E47/6xHIS fusion proteins were purified from IPTG-induced BL21 bacteria by agarose-Glutathione (Sigma, USA) or Co Talon Affinity Resin (Clontech, USA), respectively.

For EMSA, two oligonucleotides CAGGAGCCCTGCCAGGCAGCTGGTGGCATTCTCCA and GTGGAGAATGCCACCAGCTGCCTGGCAGGGCTCCTG were annealed and labeled by Klenow enzyme in the presence of [α-^{32}P]dCTP. A positive control probe was E1 according to (Akazawa et al., 1995). EMSA was carried out as previously described (Ben-Porath et al., 1999).

Results

CGC precursors are present in the rhombic lip in *Math1*$^{β\text{-}gal/β\text{-}gal}$ mice, but do not proceed to generate the EGL after E14.5

Targeted deletion of *Math1* (*Math1*$^{-/-}$) or a total replacement of the coding region by a reporter gene (*Math1*$^{β\text{-}gal/β\text{-}gal}$) was shown to cause lack of the EGL at the time of birth (Ben-Arie et al., 1997; Ben-Arie et al., 2000). Here, we further examined *Math1*$^{β\text{-}gal/+}$ (which displayed a normal phenotype and could serve as controls) and *Math1*$^{β\text{-}gal/β\text{-}gal}$ mice by whole-mount X-Gal staining of the brain.

As seen in Fig. 1A-D, by E14.5 CGC precursors occupy the cerebellar rhombic lip, as revealed by *Math1/lacZ* activity (*lacZ* expression under *Math1* endogenous control elements). Similar staining pattern in *Math1*$^{β\text{-}gal/+}$ (Fig. 1A,C) and *Math1*$^{β\text{-}gal/β\text{-}gal}$ (Fig. 1B,D) indicated that in *Math1*-null mice CGC precursors were born and reached a state of differentiation that required *Math1* expression. At E16.5, *Math1*$^{β\text{-}gal/+}$ displayed staining all over the surface of the developing cerebellum (Fig. 1E), consistent with the formation of EGL by a rostromedial migration of CGC progenitors from the rhombic lip (Altman and Bayer, 1997; Gilthorpe et al., 2002; Hatten and Heintz, 1995). By contrast, in *Math1*$^{β\text{-}gal/β\text{-}gal}$ there were less *Math1/lacZ*-positive cells at the cerebellar surface, although the rhombic lip continued to include surviving progenitors (Fig. 1F). At both stages, the rhombic lip was smaller in *Math1*-null embryos when compared with the heterozygous littermate. This was in agreement with the previous histological analysis of sectioned cerebella and proliferation rate measured by BrdU incorporation (Ben-Arie et al., 1997; Ben-Arie et al., 2000) and suggested that CGC progenitors were viable even without *Math1* expression. Moreover, examination of the entire brain revealed no ectopic migration in *Math1*-null mice, excluding such an explanation for the lack of EGL.

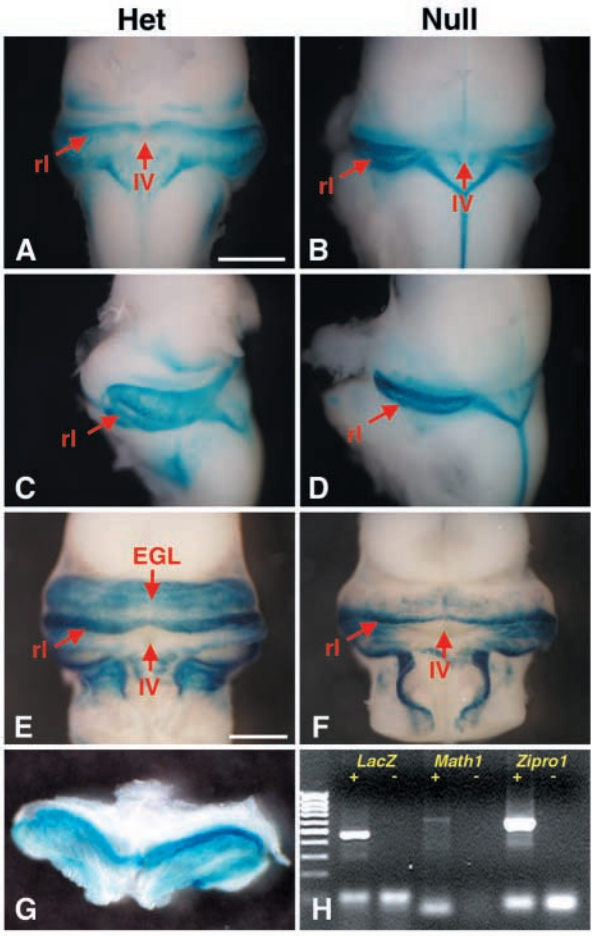

Fig. 1. Existing rhombic lip precursors fail to form an EGL in *Math1*$^{\beta\text{-gal}/\beta\text{-gal}}$ cerebellum. Whole-mount X-Gal staining of brains from E14.5 (A-D) and E16.5 (E,F) mice. Expression of a *lacZ* reporter under the endogenous control of *Math1* promoter is seen in the rhombic lip of E14.5 *Math1*$^{\beta\text{-gal}/+}$ (Het, A,C) and *Math1*$^{\beta\text{-gal}/\beta\text{-gal}}$ (Null, B,D). Stained progenitors are seen in both genotypes, although the rhombic lip seems smaller in *Math1* null cerebellum. At E16.5, *lacZ* expression is detected in CGC progenitors migrating over the cerebellar surface to generate the EGL in *Math1*$^{\beta\text{-gal}/+}$ (E) but not in a *Math1*$^{\beta\text{-gal}/\beta\text{-gal}}$ littermate (F). The rhombic lip was dissected out from E14.5 *Math1*$^{\beta\text{-gal}/+}$ brain and subjected to X-Gal staining (G). The large proportion of stained cells indicates that the isolated tissue is enriched with CGC progenitors. RT-PCR on the isolated rhombic lip verifies the expression of *lacZ*, *Math1* and *Zipro1* (H). + and – indicate the presence and absence of reverse transcriptase, respectively. (A,B,E,F) Dorsal views; (C,D) Lateral views. rl, rhombic lip; IV, fourth ventricle of the brain, EGL, external granule layer. Scale bars: 1 mm.

Math1 null CGC survive normally in primary cultures

To investigate the origin of the EGL agenesis in *Math1*-null mice, we attempted to separate the complex processes they normally undergo in vivo, by examining the CGC progenitors in vitro. In addition, culturing allowed us to follow cells isolated from the rhombic lip, which is a transient structure that disappears during normal embryogenesis.

Based on the spatiotemporal expression pattern of *Math1/lacZ* (Fig. 1), we chose to examine CGC precursors at E14.5, as an advanced stage in which the rhombic lip progenitors are present in both *Math1*$^{\beta\text{-gal}/\beta\text{-gal}}$ and *Math1*$^{\beta\text{-gal}/+}$, and the abnormal phenotype is only emerging. A typical example of a dissected rhombic lip from *Math1*$^{\beta\text{-gal}/+}$ cerebellum, which was subsequently stained by X-Gal, showed that an enriched source of *Math1/lacZ*-expressing cells could be obtained (Fig. 1G). Isolation of a totally pure CGC population from individual embryos was impractical, but not essential, as similar proportions of *Math1/lacZ*-negative cells were present in the different cultures compared, regardless of *Math1* genotype. As the isolated tissues may contain CGC precursors as well as other cell types, we use the term ‘rhombic lip cells’. Further confirmation for the enrichment of the isolated rhombic lip tissue by CGC was obtained by RT-PCR. Isolated rhombic lips from *Math1*$^{\beta\text{-gal}/+}$ expressed *lacZ* and *Math1*, as expected (Fig. 1H). An independent verification was provided by the expression of *Zipro1* (RU49/Zfp38), a zinc-finger transcription factor specifically expressed in CGC from early stages (Yang et al., 1996) (Fig. 1H).

We followed the expression of *Math1/lacZ* over time in cultures obtained from individual embryos of the three *Math1* genotypes. No notable differences, such as density of cells or increased number of dead cells, were observed in cultures from controls and *Math1*$^{\beta\text{-gal}/\beta\text{-gal}}$ (data not shown). Staining for *lacZ* after 3 days in culture (Fig. 2A-F) revealed no background in cultures from *Math1*$^{+/+}$, although most cells from *Math1*$^{\beta\text{-gal}/+}$ (Fig. 2B,E) and *Math1*$^{\beta\text{-gal}/\beta\text{-gal}}$ (Fig. 2C,F) appeared blue. Comparison of cell density, proportion of stained cells and staining intensity did not imply any major difference between *Math1* null and control cells at this stage. Moreover, *Math1/lacZ* expression indicated that CGC precursors lacking *Math1* survived after 3 days in vitro and continuously maintained *Math1* promoter activity. Hence, it was concluded that *Math1* was not essential for the survival of CGC precursors.

Math1 is required for downregulation of its expression

As no differences were visible between *Math1*-null and control cells after 3 days in vitro, we challenged the cells with a longer culturing period (Fig. 2G-L). After 6 days in vitro, *Math1/lacZ* expression was dramatically decreased in cultures from *Math1*$^{\beta\text{-gal}/+}$ (Fig. 2H,K). This observation was consistent with the expression of *Math1* in the outer EGL, and its downregulation in differentiating cells at the inner EGL (Helms and Johnson, 1998). Surprisingly, *Math1/lacZ* activity in *Math1*$^{\beta\text{-gal}/\beta\text{-gal}}$ was still strong after 6 days in culture (Fig. 2I,L). Thus, *Math1* promoter activity remained high in cells derived from *Math1*$^{\beta\text{-gal}/\beta\text{-gal}}$, while downregulated in cells from *Math1*$^{\beta\text{-gal}/+}$ littermates.

To refine this observation, we used a quantitative colorimetric assay for β-galactosidase activity in the cultured cells. After 3 days in culture, *Math1/lacZ* activity was very similar in *Math1*$^{\beta\text{-gal}/+}$ and *Math1*$^{\beta\text{-gal}/\beta\text{-gal}}$ cultures, much above the background measured in *Math1*$^{+/+}$ (Fig. 2M). However, after 6 days in culture a significantly higher level of β-galactosidase activity remained in *Math1*$^{\beta\text{-gal}/\beta\text{-gal}}$ cells, in contrast to the significant reduction of activity in *Math1*$^{\beta\text{-gal}/+}$ cultures ($P<0.001$, *t*-test). *Math1* was shown before to act as a positive autoregulator (Helms et al., 2000), and our data demonstrated for the first time a role for *Math1* also in a negative autoregulation of its own expression.

Specification of CGC is independent of *Math1*

The absence of an essential transcription factor may change the fate of neural precursor cells (Guillemot, 1999; Hassan and Bellen, 2000). Moreover, culturing of normal neural precursors may lead to alteration of the cellular identity that may result in a fate switch, by accelerating differentiation or causing de-differentiation (Anderson, 2001). Therefore, we studied cell fate specification of the rhombic lip cells in *Math1*-null mice.

As *Math1* is expressed in a limited time window during CGC development, both accelerated differentiation and de-differentiation of the progenitors may silence *Math1* promoter, resulting in a decreased *Math1/lacZ* expression. Therefore, we examined the expression of two CGC-specific transcription factors *Zic1* and *Zipro1*, which are expressed in rhombic lip precursors, as well as in mature CGC in the IGL (Aruga et al., 1994; Nagai et al., 1997; Yang et al., 1996). RT-PCR analysis was performed on cells cultured for 3 and 6 days from all *Math1* genotypes (Fig. 3). Similar levels of *Zic1* and *Zipro1* transcripts were detected in *Math1*$^{\beta\text{-gal}/\beta\text{-gal}}$, when compared with *Math1*$^{+/+}$ and *Math1*$^{\beta\text{-gal}/+}$ littermates at both time points. These data revealed that the initiation and maintenance of the correct fate of rhombic lip cells destined to become CGC, was independent of *Math1*, and was not lost upon prolonged growth in vitro.

CGC from *Math1*-null mice fail to differentiate

As specification was not altered in *Math1*-null CGC, we examined the in vitro differentiation capability of the cells. Embryonic CGC precursors have been shown before to be able to differentiate in culture (Alder et al., 1996). We chose to examine process extension as a pronounced phenotype of neuronal maturation.

Immunofluorescent detection of β-tubulin, which is known to be expressed in CGC processes (Alder et al., 1999; Helms et al., 2001), showed that a large number of processes have developed from *Math1*$^{+/+}$ and *Math1*$^{\beta\text{-gal}/+}$ rhombic lip cells, when cultured for 6 days, but not in cultures from *Math1*$^{\beta\text{-gal}/\beta\text{-gal}}$ (Fig. 4A-C). Quantification of the processes evaluation was achieved by β-tubulin staining of similarly grown cultures followed by a colorimetric assay. The absorbance of control cultures from *Math1*$^{+/+}$ and *Math1*$^{\beta\text{-gal}/+}$

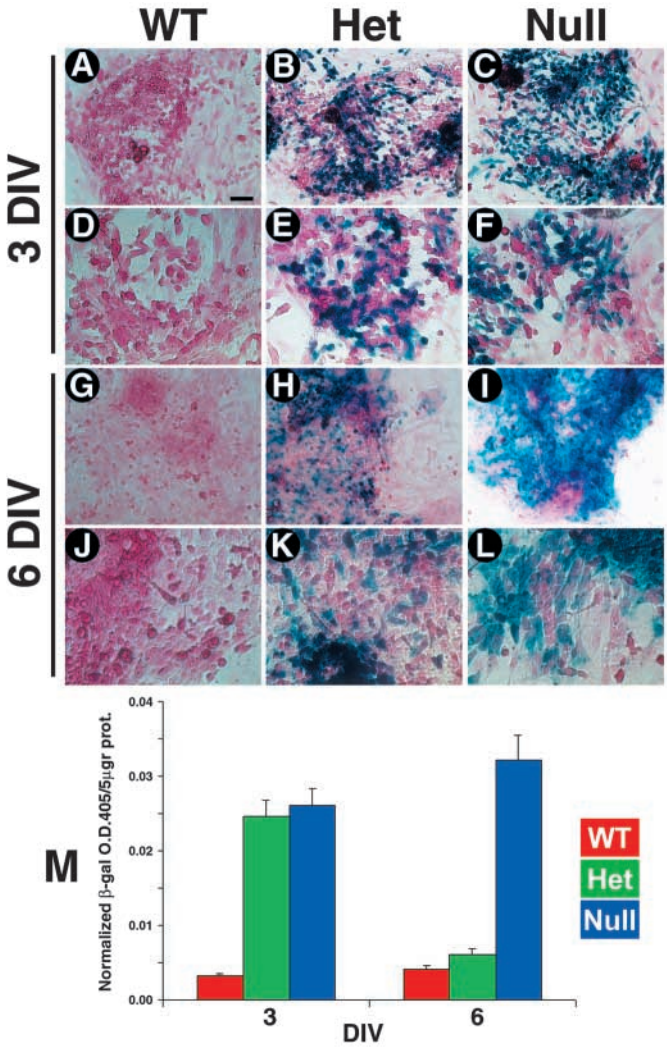

Fig. 2. *Math1* promoter activity is maintained in rhombic lip cultures, and is downregulated only in *Math1*-expressing cells. (A-L) CGC were cultured and grown for 3 days (A-F) or 6 days (G-L), and *Math1* promoter activity detected by X-Gal staining. No background is seen in cells from *Math1*$^{+/+}$ (WT, A,D). Rhombic lip cells from both *Math1*$^{\beta\text{-gal}/+}$ (Het, B,E) and *Math1*$^{\beta\text{-gal}/\beta\text{-gal}}$ (Null, C,F) continue to express similar levels of *lacZ* after 3 days in vitro. By contrast, after 6 days, the rhombic lip cells from *Math1*$^{\beta\text{-gal}/\beta\text{-gal}}$ display numerous positive cells (I,L), while in the *Math1*$^{\beta\text{-gal}/+}$ a notable decrease in stained cells is observed (H,K). (M) Quantification of *Math1* promoter activity presented as normalized activities +s.e.m. from cultures after 3 and 6 days in vitro. *Math1*$^{\beta\text{-gal}/+}$ and *Math1*$^{\beta\text{-gal}/\beta\text{-gal}}$ have very similar *Math1* promoter activity after 3 days in culture, in contrast to a significant decrease in *Math1*$^{\beta\text{-gal}/+}$ cells, and a significantly high level in *Math1*$^{\beta\text{-gal}/\beta\text{-gal}}$ after 6 days in culture (P<0.001, t-test). Scale bar in A: 50 μm for A-C,G-I; 25 μm for D-F,J-L.

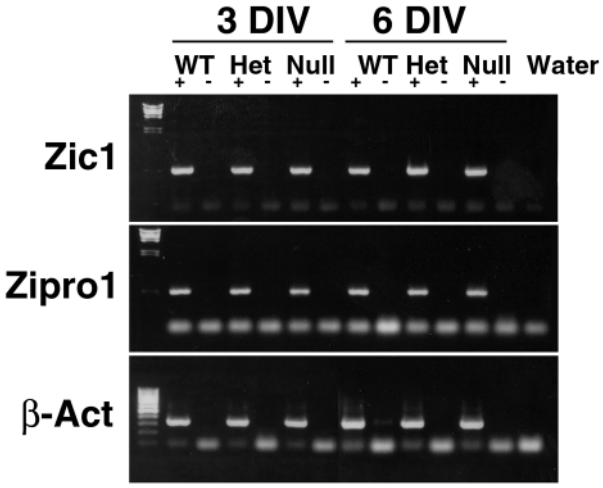

Fig. 3. Specification of CGC is maintained in RL cultures independently of *Math1* expression. Rhombic lip cells from *Math1*$^{+/+}$ (WT), *Math1*$^{\beta\text{-gal}/+}$ (Het) and *Math1*$^{\beta\text{-gal}/\beta\text{-gal}}$ (Null) were cultured and analyzed by RT-PCR with *Zic1*, *Zipro1* and β-actin-specific primers after 3 and 6 days in vitro. The expression of *Zic1* and *Zipro1* is constant in cultures from all genotypes and along the culturing periods. + and – indicate the presence and absence of reverse transcriptase, respectively.

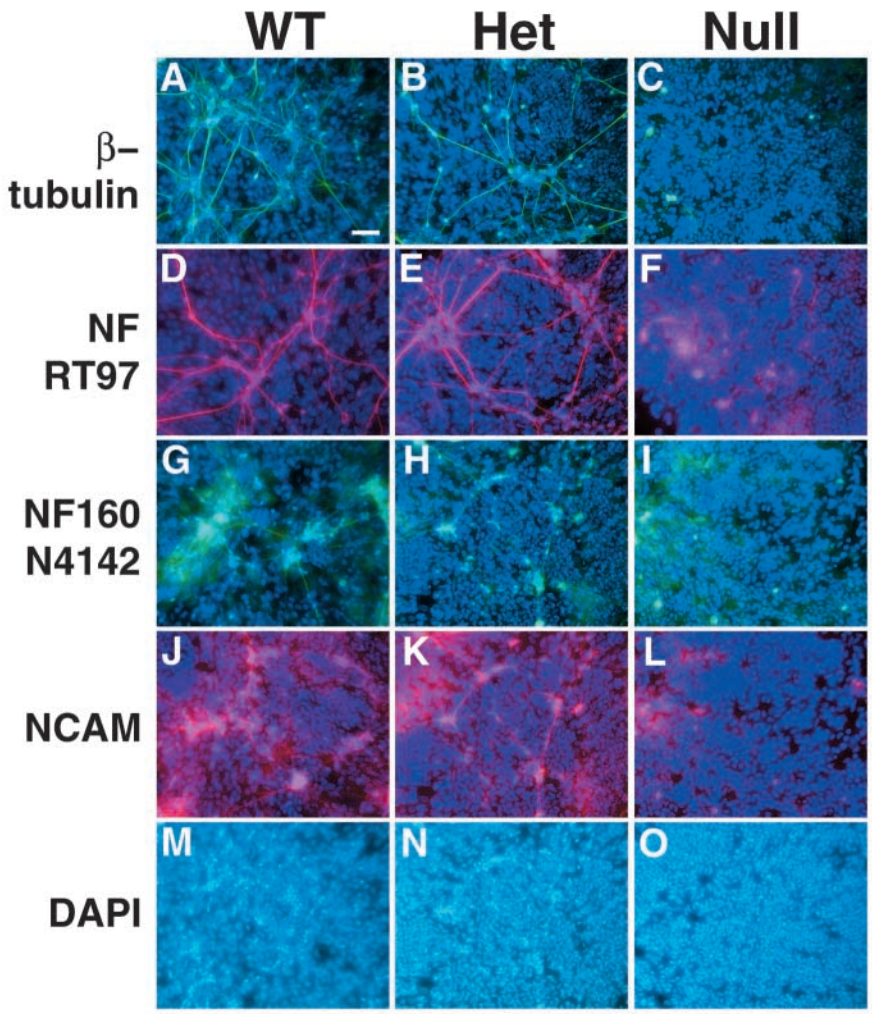

Fig. 4. *Math1* is necessary for process outgrowth in rhombic lip cultured cells. Immunodetection of β-tubulin (A-C), phosphorylated neurofilaments (D-F), 160 kDa neurofilament (G-I) and NCAM (J-L) in rhombic lip cells after 6 days in culture. The antibodies decorate process extensions from cells from *Math1*$^{+/+}$ (WT, A,D,G,J) and *Math1*$^{β\text{-}gal/+}$ (Het, B,E,H,K), but not *Math1*$^{β\text{-}gal/β\text{-}gal}$ (Null, C,F,I,L). Counterstaining by DAPI (M-O) displays similar cell densities in all cultures. Scale bar: 50 μm.

(n=15) was 0.30 (±0.04), and was reduced to 0.13 (±0.02) in *Math1*$^{β\text{-}gal/β\text{-}gal}$ (n=11), which is significantly lower (P<0.001, t-test). The difference is smaller than visualized by immunostaining, as staining of both the soma and processes were measured. Staining against phosphorylated neurofilaments (Fig. 4D-F), NF160 (Fig. 4G-I) and NCAM (Fig. 4J-L) illustrated long processes in *Math1*$^{+/+}$ and *Math1*$^{β\text{-}gal/+}$, but not in *Math1*$^{β\text{-}gal/β\text{-}gal}$. Control nuclear staining by DAPI showed a uniform cell density in all genotypes (Fig. 4M-O, and Fig. 4A-L as counterstaining), indicating a similar survival of cells after 6 days in culture. The neural phenotype displayed by only a fraction of the cultured cells was consistent with previous reports that only some of rhombic lip precursors are competent to differentiate in vitro (Alder et al., 1996).

The molecular and phenotypic manifestation of neural differentiation was detected in cultured rhombic lip cells from wild-type and heterozygous, but not *Math1*-null cultures, although the specification and survival of cells appeared similar. These finding are compatible with the hypothesis that *Math1* is essential for neural differentiation of CGC progenitors. The molecular mechanisms underlying this ability should be further pursued.

Math1 regulates the expression of Notch receptors, ligands and the *Hes5* effector

Accumulating data support the involvement of the Notch signaling pathway in cerebellar development, and connect *Math1* to this pathway in various organs during embryogenesis. Therefore, we first analyzed the expression of various receptors (*Notch1* to *Notch4*) and ligands (*Dll1*, *Dll3*, *Dll4*, *Jag1* and *Jag2*) in the rhombic lip at E14 by quantitative real-time RT-PCR. We assumed that analyzing the absolute level of each transcript combined with a comparison of its amount in *Math1*$^{+/+}$ and *Math1*$^{β\text{-}gal/β\text{-}gal}$ is indicative of its importance for CGC development.

Among all Notch receptors tested, the level of *Notch2* was the highest, being 145-fold higher than *Notch1* and more than 20-fold higher than *Notch3-4* (Fig. 5A). A striking difference was detected also for Notch ligands, where the level of *Jag1* and to a lower extent *Dll1* was the highest among the five ligands tested (Fig. 5A).

When expression of the receptors was tested in *Math1*$^{+/+}$ and *Math1*$^{β\text{-}gal/β\text{-}gal}$ littermates the largest reduction of 2.8-fold was detected for *Notch4* (Fig. 5B). Among the Notch ligands, the level of *Dll1* was reduced by 2.5 fold, while *Dll3*, *Dll4* and *Jag2* transcript levels were also significantly decreased by 1.7-fold (Fig. 5C). Overall, all Notch receptors and ligands tested were expressed in the developing cerebellum. However, the differences in the level of downregulation in *Math1*$^{β\text{-}gal/β\text{-}gal}$ implied that only some of the Notch receptors and ligand were related to *Math1* function.

Seeing that the Notch signaling pathway was related to CGC development, we next examined two Notch effectors *Hes1* and *Hes5* in E14.5 rhombic lips and primary cultures from *Math1*$^{+/+}$ and *Math1*$^{β\text{-}gal/β\text{-}gal}$ littermates by RT-PCR (Fig. 6). Both *Hes1* and *Hes5* were found to be expressed in wild-type rhombic lip, with a higher level of the latter. Although the expression of *Hes1* and β-actin was similar in the two genotypes, *Hes5* expression was reduced in *Math1*$^{β\text{-}gal/β\text{-}gal}$ rhombic lip, when compared with *Math1*$^{+/+}$ (Fig. 6). Moreover, the decrease in *Hes5* expression level in *Math1*$^{β\text{-}gal/+}$ was even more pronounced in rhombic lip cells cultured for 3 and 6 days (Fig. 6). The reduction of *Hes5* in CGC progenitors from *Math1* null suggested a positive control of *Math1* over *Hes5*, but not *Hes1*, expression, which was not identified previously.

To establish a more causal relationship between Math1 and

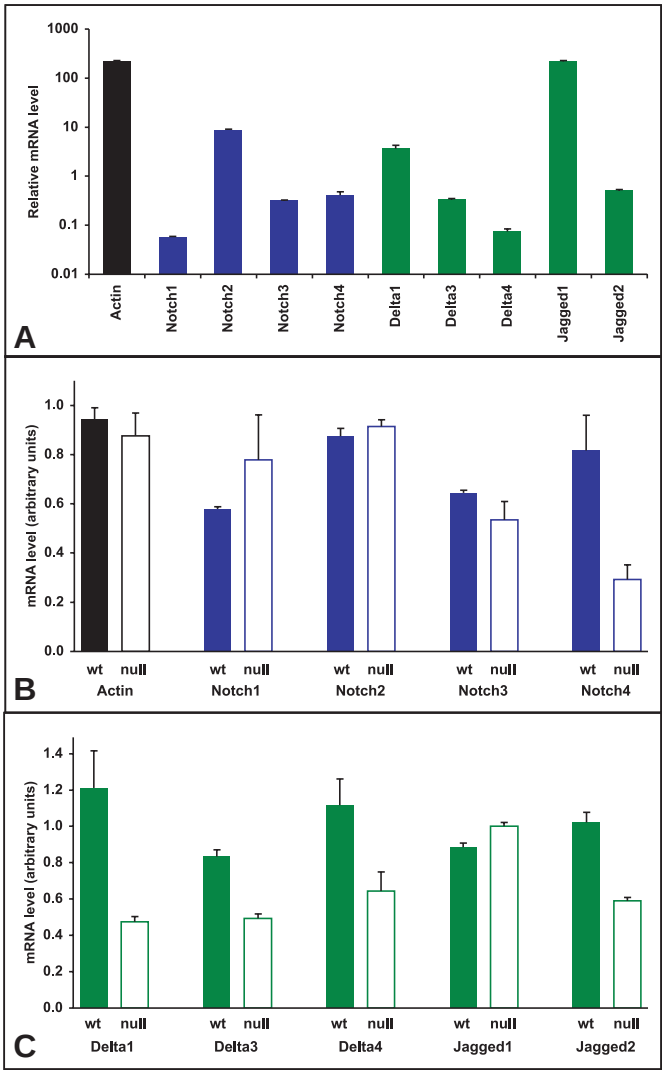

Fig. 5. Notch receptors and ligands are differentially expressed in *Math1*$^{\beta\text{-}gal/\beta\text{-}gal}$ and *Math1*$^{+/+}$ rhombic lip cells. (A) Expression level of Notch signaling components was tested by real-time quantitative RT-PCR on E14.5 rhombic lips. All Notch receptors and ligands tested were expressed in the rhombic lip, although at various levels (note logarithmic scale). Expression level of Notch receptors (B) and ligands (C) was compared between rhombic lip from *Math1*-null (open bars) and wild-type (closed bars) littermates at E14.5. β-actin was used as a control. Values are the mean of at least three measurements +s.e.m.

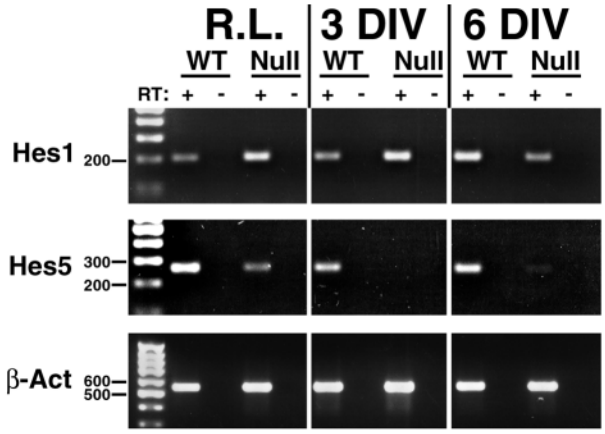

Fig. 6. *Hes5* expression is reduced in *Math1*$^{\beta\text{-}gal/\beta\text{-}gal}$ CGC. Semi-quantitative RT-PCR analysis of *Hes1*, *Hes5* and β-actin expression in E14.5 rhombic lip (R.L.) tissue, and after 3 and 6 days (3 DIV and 6 DIV, respectively) in culture. *Hes5* expression is greatly reduced in *Math1*$^{\beta\text{-}gal/\beta\text{-}gal}$ (Null) compared with *Math1*$^{+/+}$ (WT). In contrast, *Hes1* expression is not significantly altered between *Math1*$^{+/+}$ and *Math1*$^{\beta\text{-}gal/\beta\text{-}gal}$ cells. The β-Actin control indicates similar level of starting material. + and – indicate the presence and absence of reverse transcriptase, respectively.

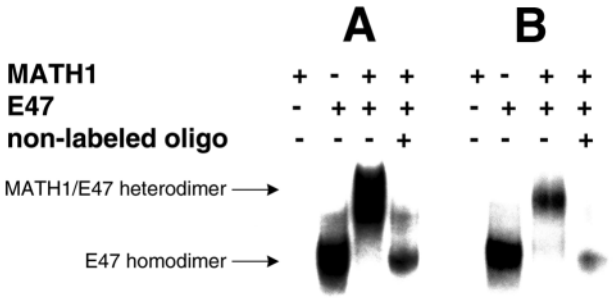

Fig. 7. MATH1/E47 heterodimers bind an E-box-containing sequence flanking *Hes5*. The DNA-binding activity of MATH1 was examined by electrophoretic mobility shift assay with or without E47. ^{32}P-labeled E-box-containing targets were from *asense*, shown before to bind MATH1 (Akazawa et al., 1995) (A) and Hes5 (B). MATH1 binds both targets in its heterodimer, but not monomeric, form.

Hes5, we have tested the ability of *Math1* gene product to recognize and bind *Hes5* (Fig. 7). Recombinant and purified MATH1 and E47 were allowed to heterodimerize and then subjected to an electrophoretic mobility shift assay. As targets we have used a proven MATH1 target (Fig. 7A) (Akazawa et al., 1995) and an E-box located downstream to *Hes5*, similar to the position of the E-box-containing enhancer involved in the positive autoregulation of *Math1* (Helms et al., 2000). Although MATH1/E47 could bind directly *Hes5*, MATH1 by itself did not, in contrast to the known capability of the homodimeric E47 (Akazawa et al., 1995; Helms et al., 2000). A cold oligonucleotide containing the E-box target blocked the binding, which indicated the specificity of the protein/DNA interaction. The ability of *Hes5* and *Math1* gene products to affect the transcription of each other by a luciferase reporter assay is currently not feasible as *Math1* promoter has not been identified yet.

Discussion

In the fly *atonal* has a proneural role in the PNS, but not in the CNS, where it controls arborization. The question therefore arose as to the function of *Math1* in the developing cerebellum: does it have a proneural role in the specification of rhombic lip stem cells or progenitors, or does it work later in development, during differentiation? Moreover, as *Math1* was found to participate in the Notch signaling in inner ear hair cells and in the intestine, we were interested in learning whether this is a general theme that takes place also during CGC development. By studying the effect of *Math1* knockout on rhombic lip

development in vivo and in vitro, we found that *Math1* was not essential for the specification of rhombic lip cells, but for their proper differentiation. The lack of *Math1* interrupted the normal downregulation of *Math1* promoter activity, and inhibited the ability of rhombic lip cells to develop processes in culture. Moreover, *Math1*-null mice displayed a selective downregulation of Notch receptors and ligands in the rhombic lip, and revealed a novel negative autoregulatory loop controlling *Math1* expression through the *Hes5* effector.

Generation and specification of cerebellar granule cell progenitors is *Math1*-independent

Whole-mount X-Gal staining demonstrated clearly that rhombic lip CGC precursors were born in *Math1*$^{\beta\text{-gal}/\beta\text{-gal}}$ mice, but failed to migrate out to form the EGL, consistently with previous studies (Ben-Arie et al., 1997; Ben-Arie et al., 2000). The thinner rhombic lip identified by whole-mount staining of *Math1*-null mice was in full agreement with previous analyses and the fact that decreased proliferation was detected by a BrdU incorporation assay (Ben-Arie et al., 1997). As we examined the entire cerebellar region, the absence of ectopic staining in *Math1*$^{\beta\text{-gal}/\beta\text{-gal}}$ mice excluded the likelihood of abnormal migration of *Math1/lacZ*-expressing cells. The possibility that ectopic cells were not stained, as they did not maintain *Math1* promoter activity, is less probable, as the rhombic lip kept staining until E18.5 in vivo, and the precursors maintained *Math1/lacZ* expression for an extended period in vitro.

The fact that rhombic lip CGC precursors activated *Math1*/*lacZ* expression did not provide a definite answer to the question of the specification status of the progenitors. To address whether *Math1* is needed for proper fate determination we examined the expression of two more transcription factors, *Zipro1* and *Zic1* known to be expressed in CGC and their progeny (Alder et al., 1999; Aruga et al., 1998; Aruga et al., 1994; Nagai et al., 1997; Yang et al., 1999). The continuous expression of both genes in the rhombic lip and in cultured progenitors was shown to be *Math1* independent, which lead us to the conclusion that *Math1* was not required for the initial specification of granule cell progenitors and for the maintenance of granule identity, both in vivo and in vitro.

The relationship between *Math1*, *Zic1* and *Zipro1* is noteworthy. We show that in *Math1*-null mice both *Zic1* and *Zipro1* were normally expressed in CGC in vivo and in vitro, which may indicate that they act upstream to *Math1*. However, this notion is contradicted by other data. First, *Zic1* expression in the developing neural tube is broad and becomes confined to the rhombic lip only by E12 (Aruga et al., 1994), whereas *Math1* expression at the neural tube begins at E9 (Akazawa et al., 1995; Ben-Arie et al., 1997; Ben-Arie et al., 2000). Similarly, *Zipro1* is expressed also in granule cells of the olfactory bulb and dentate gyrus, where no *Math1* expression was reported (Yang et al., 1996). Second, *Zic1* and *Zipro1* knockout and overexpression in mice demonstrated that these genes regulate cerebellar patterning and EGL proliferation at stages later then those affected by *Math1* deletion (Aruga et al., 1998; Yang et al., 1999). Third, *Zic1* was recently shown to bind an enhancer of *Math1* and to downregulate *Math1* expression. However, *Zic1* acts through repression of the positive autoregulation of *Math1* itself (Ebert et al., 2003), which is not the major regulatory element of *Math1* expression, as the autoregulation depends on initial activation of *Math1* by independent upstream genes. Taken together, *Math1*, *Zic1* and *Zipro1* seem to affect cerebellar development through parallel, yet crosstalking, signaling pathways.

Differentiation of CGC precursors is *Math1*-dependent

Rhombic lip cells from both *Math1*$^{+/+}$ and *Math1*$^{\beta\text{-gal}/+}$ E14 embryos reaggregated in culture, as expected (Alder et al., 1996). However, only after a longer incubation period in vitro (between 3 and 6 days) did a complex network of processes form, without the addition of supplements like BMPs of NGF. Immunoreactivity with β-tubulin, phosphorylated neurofilaments, NF160, NCAM and the distinct process morphology, confirmed a progress of the rhombic lip cells towards a neural phenotype. By contrast, *Math1*$^{\beta\text{-gal}/\beta\text{-gal}}$ cultures developed few processes and growth cones, and lacked well developed neural extensions. During normal development in vivo, CGC do not grow extensions until they are situated in the inner EGL and become competent to start the inward radial migration to form the IGL (Hatten and Heintz, 1995). Therefore, culturing and analysis of the process outgrowth were not supposed to mimic the in vivo situation, but rather allow examination of the developmental potential of the progenitors, separating it from the need to migrate to the EGL, the place at which this morphological change normally takes place.

Normally, at the rhombic lip stage, CGC undergo proliferation and consequently migrate out of the rhombic lip: two abilities that are affected in *Math1*-null mice. As both functions mark the progress in the developmental program, which require *Math1* for the regulation of its target genes, they can be regarded as *Math1*-dependent differentiation events. We propose that improper differentiation is the cause for developmental arrest in the rhombic lip. A simplistic view of the lack of EGL may suggest that *Math1* was essential for activation of genes, which convey a migratory ability, or that their products are part of the migratory machinery per se. However, as the transcription of those genes is under the control of *Math1*, directly or indirectly, the lack of migration from the rhombic lip may be regarded as the outcome of improper differentiation of the progenitors. Hence, we suggest that only after *Math1* is activated do rhombic lip cells acquire the ability to further differentiate.

Math1 is not essential for the initial activation of its promoter activity, but is necessary for its downregulation

Helms et al. (Helms et al., 2000) reported a positive autoregulation of *Math1* over its own expression, through an E-box-containing downstream enhancer, which was shown to bind *Math1*. Transgenic mice expressing a *Math1/lacZ* reporter, under various control elements flanking *Math1* ORF, recapitulated most of the endogenous *Math1* expression. However, the same transgene was not expressed when the mice were crossed with *Math1*-null mice, as no MATH1 was available to activate its enhancer (Helms et al., 2000). The fact that a *Math1/lacZ* reporter is expressed in *Math1*$^{\beta\text{-gal}/\beta\text{-gal}}$ mice, which are a completely null for *Math1*, established the

existence of additional *Math1*-independent control elements that activates *Math1* expression. Moreover, as we found a continuous expression of *Math1/lacZ* in rhombic lip cultured cells, it seemed that the major control over *Math1* expression is MATH1 independent, and that the positive autoregulation contributes mainly to the refinement of *Math1* levels.

During normal cerebellar development *Math1* is expressed in granule cell precursors and in the rhombic lip and outer EGL, and is turned off in postmitotic cells in the inner EGL (Akazawa et al., 1995; Ben-Arie et al., 2000; Helms and Johnson, 1998). However, upstream genes and control mechanisms regulating the expression of *Math1* are not yet fully identified. In the spinal cord of *Gdf7* mutant mice, *Math1* expression does not continue after E10.5, but the addition of GDF7 or BMP7 markedly increased *Math1* expression (Lee et al., 1998). Similarly, the dorsal midline cells adjacent to the rhombic lip express GDF7, BMP6 and BMP7, which were demonstrated to induce *Math1*, *En1/2*, *Zic1* and *Wnt3a* in the ventral mesencephalon/metencephalon neural tube. The induction of those genes normally confined to dorsal cells that develop into CGC precursors indicates the ability of BMP factors to determine the neural subtype fate, and suggests that BMPs regulate *Math1* expression (Alder et al., 1999).

Math1 acts via Notch signaling by activating *Hes5* transcription during CGC development

The evolutionarily conserved Notch signaling pathway mediates cell-to-cell communication to regulate cell fate decisions and patterning in both invertebrates and vertebrates. In the developing nervous system Notch signaling was classically regarded as a mechanism that keeps cells in an undifferentiated state. However, recently Notch signaling was found to be important for differentiation of glial cells and the organization of neuronal processes (Frisen and Lendahl, 2001; Justice and Jan, 2002). To shed light on the role of Notch signaling in CGC development, and based on the observations that various components of the pathway are expressed in various stages of cerebellar development, we analyzed their expression in the rhombic lip. We have found that the *Notch2* receptor and *Jag1* ligand are the most abundant species, although all known receptors and ligands tested were expressed. Our findings are in agreement with previous studies that were mostly concerned with later stages of cerebellar development (Irvin et al., 2001; Kusumi et al., 2001; Solecki et al., 2001; Tanaka et al., 1999). Moreover, we have identified a selective downregulation of *Notch4*, *Dll1*, *Dll3*, *Dll4* and *Jag2* in the rhombic lip of *Math1*-null mice.

Because in *Math1*-null mutants the level of Notch receptors and ligands was affected, we examined whether *Math1* had a transcriptional control over the Notch effectors *Hes1* or *Hes5* in the rhombic lip. RT-PCR analysis of *Hes1* and *Hes5* expression in rhombic lip tissue and in cultured cells after 3 and 6 days demonstrated a continuous downregulation of *Hes5*, but not of *Hes1*, in *Math1*-null mice. EMSA analysis has indicated that MATH1 can bind an E-box-containing sequence flanking *Hes5*, which suggests a novel control mechanism of *Math1* over the transcription of *Hes5*, which is known to act as *Math1* suppressor. Taking the new and established data together, we suggest a possible model linking some of the genes and interactions involved in CGC development (Fig. 8). According to our hypothesis, *Hes5* normally downregulates

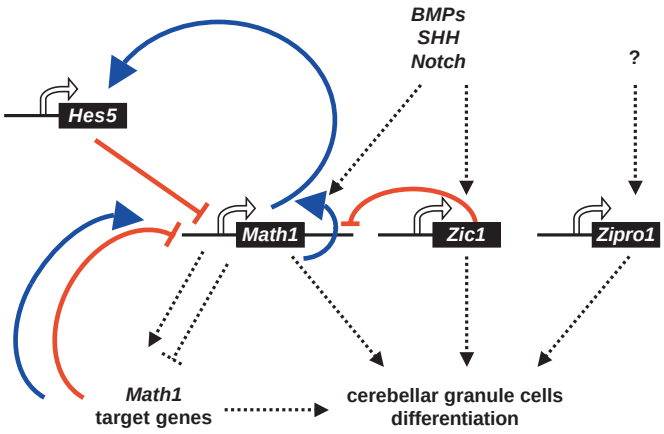

Fig. 8. A schematic representation of a possible model of genes and interactions involved in CGC development. Early cerebellar dorsoventral patterning genes and pathways involved in determination of hindbrain boundaries and fate specification, like BMPs, sonic hedgehog (SHH) and Notch are presented (reviewed by Wang and Zoghbi, 2001). Specifically, *Bmp7* was shown to activate *Math1* and *Zic1* expression (Alder et al., 1999). *Math1* is subjected to further positive autoactivation through binding to an E-box motif in a downstream enhancer (Helms et al., 2000). However, *Math1* transcription and binding activities are known to be downregulated by the *Hes* gene products (Akazawa et al., 1995). As shown here, *Math1* may also have a negative autoregulatory loop, through a direct or indirect transcriptional activation of *Hes5*, which further elevates the level of *Hes5*, leading to downregulation of its transcription. Moreover, *Zic1* can bind directly to *Math1* enhancer and repress *Math1* positive autoregulation (Ebert et al., 2003). However, we assume that unidentified *Math1* target genes are also involved in a complete attenuation of *Math1* expression, cell cycle exit and further differentiation. White arrows indicate transcription, and blue and red arrows indicate activation and suppression, respectively. Broken arrows indicate pathways that act up- and downstream of CGC transcription factors.

Math1, which in turn further activates *Hes5* transcription (directly or indirectly), and thus an increasing suppression of *Math1* develops. However, in *Math1*$^{\beta\text{-gal}/\beta\text{-gal}}$ cells, this feedback loop is interrupted, as there is no *Math1* gene product to further activate *Hes5*. Therefore, the level of *Hes5* gene product cannot increase, and *Math1* promoter remains active, which is in full agreement with our observations. The model also provides an explanation for the delay in the downregulation of *Math1* promoter activity, seen in cultured rhombic lip cells from *Math1*$^{\beta\text{-gal}/+}$ mice after 6, but not 3, days in vitro. Accordingly, at E14.5 there is a balance between MATH1 and HES5 levels, in which both the positive and negative regulatory loops take place. However, with time, the level of MATH1 increases due to the positive autoregulation, which finally leads to an increase in the level of HES5 until it reaches the threshold needed to attenuate *Math1* transcription. Further experiments are needed in order to establish and verify the interplay between all the genes and proteins presented in the suggested model.

An inhibitory effect of *Hes1* over *Math1* activity was previously demonstrated, as transfection of a mouse pluripotent cell line with *Math1* induced transcriptional activation of a luciferase reporter, which was inhibited by

cotransfection with *Hes1* (Akazawa et al., 1995). *Hes1* and *Hes5* knockout mice have supernumerary hair cells, which express *Math1* (Zine et al., 2001). Based on the characterization of both lines, it was suggested that in inner ear hair cells *Math1* controls *Jag2* expression, which is repressed by *Hes1* and *Hes5* through the inhibition of *Math1* activity (Zine et al., 2001). Moreover, *Hes1* was also demonstrated to highly repress *Math1*-induced hair cell generation in cochlear explants (Zheng et al., 2000). However, the expression level and cellular localization of *Hes1* were unaffected in the intestine of *Math1*-null mice (Yang et al., 2001), suggesting that the interrelations between the genes are also context dependent and vary in different tissues.

Mutual effects between bHLH factors and *Hes* genes are not limited to *Math1* (reviewed by Guillemot, 1999; Kageyama et al., 1997). Cau et al. (Cau et al., 2000) have demonstrated a complex interplay between Hes genes and *Mash1* in the olfactory epithelium. *Mash1* was expressed ectopically in *Hes1* mutants, but normally in *Hes5* mutants. By contrast, in *Mash1* knockout the expression of *Hes1* was unaffected, while *Hes5* level was severely reduced (Cau et al., 2000). However, retroviral overexpression of *Hes5* repressed *Mash1* expression in oligodendrocytes precursors (Kondo and Raff, 2000). It was therefore proposed that *Hes1* represses *Mash1*, while *Mash1* activates *Hes5*, which in turn represses *Mash1*. This mode of action is very similar to the model we propose for *Math1* action.

Interestingly, during recent years Notch signaling has been linked not only to neural and glial cell fate determination, but also to the control of process outgrowth (Frisen and Lendahl, 2001). Upregulation of Notch was shown to inhibit process extension or even cause their retraction, while repression of Notch signaling enhanced process outgrowth (Berezovska et al., 1999; Franklin et al., 1999; Sestan et al., 1999). However, in cultured CGC we have noticed that downregulation of Notch receptors and ligands in *Math1*-null mice was accompanied by a reduction in process outgrowth. However, the exact molecular mechanism underlying the relationship between this downregulation and process extension should be further examined.

The correlation between the expression of Notch effectors, such as *Hes1* and *Hes5*, in controlling process outgrowth has been demonstrated in various experimental systems. Expression of *Hes1* in PC12-E2 cells inhibits NCAM-dependent process outgrowth (Jessen et al., 2003), and its expression inhibits both the intrinsic and NGF-induced process outgrowth of embryonic day-17 rat hippocampal neurons in culture (Castella et al., 1999). Similarly, constitutive expression of the intracellular domain of *Notch1*, which activates *Hes1* promoter in SH-SY5Y neuroblastoma cells, inhibits their spontaneous and induced process outgrowth (Grynfeld et al., 2000). Interestingly, axonal injury of corticospinal and dorsal root ganglion neurons suppresses Hes gene expression, possibly as part of the initiation of a regenerative response (Kabos et al., 2002). Hence, our data support the hypothesis that *Math1* influences process outgrowth, via the Notch pathway, by regulation of *Hes5* expression.

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