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Zic1 represses *Math1* expression via interactions with the *Math1* enhancer and modulation of *Math1* autoregulation

Philip J. Ebert[1], John R. Timmer[2], Yuji Nakada[1], Amy W. Helms[1], Preeti B. Parab[1], Ying Liu[1], Thomas L. Hunsaker[1] and Jane E. Johnson[1,*]

[1]Center for Basic Neuroscience, University of Texas Southwestern Medical Center, Dallas, TX 75390-9111, USA
[2]Developmental Biology Program, Memorial Sloan-Kettering Cancer Center, New York, NY 10021, USA
*Author for correspondence (e-mail: jane.johnson@utsouthwestern.edu)

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SUMMARY

Math1 is a basic helix-loop-helix transcription factor expressed in progenitor cells that give rise to dorsal commissural interneurons in the spinal cord, granule cells of the cerebellum, and sensory cells in the inner ear and skin. Transcriptional regulation of this gene is tightly controlled both temporally and spatially during nervous system development. The signals that mediate this regulation are likely integrated at the *Math1* enhancer, which is highly conserved among vertebrate species. We have identified the zinc-finger transcription factor Zic1 as a regulator of *Math1* expression. Zic1 binds a novel conserved site within the *Math1* enhancer, and represses both the expression of endogenous Cath1 (chicken homolog of Math1) and the activity of a *Math1* enhancer driven *lacZ* reporter when expressed in chick neural tubes. Repression by Zic1 blocks the autoregulatory activity of Math1 itself. Although previous reports have shown that Zic1 and Math1 are both induced by BMP signaling, these genes appear to have opposing functions, as Math1 acts to promote neuronal differentiation in the chick neural tube and excess Zic1 appears to block differentiation. Zic1-mediated repression of *Cath1* transcription may modulate the temporal switch between the progenitor state and differentiating dorsal cell types during neural tube development.

Key words: bHLH transcription factors, Transcriptional regulation, Transcription repressors, Chick electroporation, Dorsal spinal cord development, Neuronal differentiation, Math1/Cath1, Mash1/Cash1, Ngn1, Ngn2

INTRODUCTION

The differentiation of a proliferating neural progenitor into a postmitotic neuronal cell is a critical transition during mammalian neural development. During this time period, extensive cell lineage commitments are formed based on signals from dorsoventral and anteroposterior signaling centers (Altmann and Brivanlou, 2001; Briscoe et al., 2000; Chiang et al., 1996; Lee et al., 2000). These signals are integrated with cues controlling the timing of differentiation, such as those modulated by the effects of lateral inhibition (Cai et al., 2000; Gaiano et al., 2000; Morrison et al., 2000). The neural bHLH family of transcription factors, which is expressed in specific populations of neural precursors, figure prominently in this process. The regulation of these transcription factors, including Mash1 (Ascl1 – Mouse Genome Informatics), Math1 (Atoh1 – Mouse Genome Informatics) and Ngn1 (Neurog1 – Mouse Genome Informatics) may serve to translate dorsoventral positional information into specific neural cell-type identity (Fode et al., 2000; Gowan et al., 2001; Lee et al., 2000; Lee et al., 1998). Accordingly, the expression of bHLH genes in a restricted pattern appears to be required for the generation of a diverse population of adult neurons. Loss-of-function mutations in these factors result in the loss of specific subsets of neurons and neonatal lethality (Ben-Arie et al., 1997; Bermingham et al., 1999; Bermingham et al., 2001; Fode et al., 1998; Guillemot et al., 1993; Ma et al., 1998). Overexpression of these neural bHLH factors appears to induce neuronal differentiation (Cai et al., 2000; Farah et al., 2000; Kim et al., 1997; Lee et al., 1995; Ma et al., 1996; Sun et al., 2001). In addition, substantial defects in neural development arise when the expression of *Math1* is deregulated spatially, temporally or quantitatively (Helms et al., 2001; Isaka et al., 1999). Thus, the proper initiation, maintenance and termination of neural bHLH transcription factor expression are essential for normal neural development.

The bHLH factor Math1 is expressed transiently in proliferating neural precursors in multiple domains of the developing murine nervous system, including the dorsal hindbrain and neural tube, hair cells of the vestibular and auditory systems, mechanoreceptor (Merkel) cells of the hairy skin, and the cells of the external granule layer of the developing cerebellum (Akazawa et al., 1995; Ben-Arie et al., 2000; Ben-Arie et al., 1996; Helms and Johnson, 1998). Analysis of the *Math1* gene has identified two discrete ~500 bp sequences 3′ of the *Math1*-coding sequence that exhibit significant homology to sequences flanking the human ortholog *HATH1* (*ATOH1* – Human Gene Nomenclature

Database) (Helms et al., 2000). These sequence domains are sufficient to direct heterologous *lacZ* reporter activity to domains of native *Math1* expression in transgenic mice. The activity of the *Math1* enhancer in transgenic mice requires both a conserved E-box (binding site for bHLH factors) and *Math1* expression (Helms et al., 2000). Similar autoregulation has been reported for the *Drosophila* homolog *atonal* (Sun et al., 1998) and a related chick gene *CATH5* (Matter-Sadzinski et al., 2001). Thus, sustained expression of *Math1*, as with its homologs, appears dependent on previous *Math1* expression. Little is known, however, about other factors controlling *Math1* expression. Initiation of *Math1* expression appears to be dependent on BMP signaling from the roof plate. Ablation of the roof plate in mouse embryos resulted in the absence of multiple dorsal markers, including *Math1* (Lee et al., 2000). In naive neural tube explants, application of BMP factors induced *Math1* expression (Alder et al., 1999). In chick neural tube, expression of constitutively active BMP receptor results in increased *Math1* expression (Timmer et al., 2002). The direct effectors of regulation of *Math1* expression by BMP signaling are unknown.

In a screen to isolate factors involved in the regulation of *Math1* expression, we have identified Zic1, a zinc-finger transcription factor that is also induced by BMPs in the neural tube (Alder et al., 1999; Aruga et al., 2002b; Aruga et al., 1994). It belongs to a family of related proteins including Zic2, Zic3, Zic4 and Zic5, all of which share homology to the *Drosophila* gene *odd paired* (Aruga et al., 1996a; Aruga et al., 1996b; Nakata et al., 2000), in addition to weaker homology to the Gli family of vertebrate genes and their *Drosophila* homolog *cubitus interruptus* (Aruga et al., 1994). Loss-of-function and gain-of-function studies with Zic factors in the neural tissue of *Xenopus*, mouse, and chicken have suggested that Zic1 inhibits neuronal differentiation and maintains cells as progenitors (Aruga et al., 2002b; Brewster et al., 1998; Kuo et al., 1998; Mizuseki et al., 1998). Although Zic1 and Math1 are both expressed in the dorsal neural tube, and both are induced in response to BMP signaling, we have found that high expression of Zic1 and Math1 occurs only in distinct cells. We demonstrate that in the chick neural tube Zic1 represses Math1 expression. This repression blocks Math1 auto-activation and inhibits bHLH induced neuronal differentiation. Thus, BMP signaling induces both Zic1 (Aruga et al., 2002b) and Math1 (Alder et al., 1999; Timmer et al., 2002), setting up antagonistic pathways that must be in balance to form a spinal cord with the correct cell number and cell type.

MATERIALS AND METHODS

Yeast one-hybrid screen

The yeast one-hybrid screen was carried out using the Matchmaker one-hybrid kit (Clontech, Palo Alto, CA). The yeast strain YM4271 was used to generate a dual His/*lacZ* reporter strain as follows. *Math1* enhancer region 898-1271 (AF218258) (Helms et al., 2000) was cloned in either one or four copies into the pHISi and pLACi vectors (details available upon request). The vectors were linearized and sequentially integrated into YM4271. Ectopic activity of either vector following integration was minimal (pHISi-Math required 15 mM 3-aminotriazole to suppress nonspecific growth). An E10.5 mouse embryo library (a gift from E. Olson) was screened. Putative *Math1* enhancer-binding factors were isolated from His+, *lacZ*+ clones, transformed into DH5α *E. coli* and sequenced.

Plasmid construction

Math1/lacZ transgenes were constructed by PCR from the *Math1* enhancer (Helms et al., 2000) (Accession Number, AF218258) and cloned into the BGZA reporter construct (Yee and Rigby, 1993), which places the enhancer region 5′ to the β-globin basal promoter, the *lacZ*-coding region and SV40 polyadenylation sequences. Specific enhancer fragments used and mutations within these fragments are shown in Fig. 5 and Fig. 1B. Tg22 also contains a mutation in a possible Zic1-binding site within the non-conserved regions between enhancers A and B. Templates for in vitro transcription coupled translation (TNT) were generated as follows. The truncated *Zic1* construct was derived by PCR using the *Zic1*-containing yeast one-hybrid clone and primers 5′ ACCATGGGCCCACACGGCCATAC 3′ (sense) and 5′ GGGTCGGCATGTTTTGTTTC 3′ (antisense), and then subcloned into pSP64-TEN. Full-length *Zic1* was generated by hybrid PCR from a genomically derived *Zic1* fragment corresponding to the 5′ region of the coding sequence, as well as the yeast one-hybrid clone used to generate the truncated *Zic1* construct (details available upon request). All chick expression plasmids are in pMIWIII (Muramatsu et al., 1997). pMIWIII-^{myc}Zic1 was constructed by cloning the full-length coding sequences in frame into pMIWIII-^{myc}Ngn1, after the removal of the Ngn1-coding sequence (Gowan et al., 2001). The co-electroporation vector EGFP-N1 is from Clontech. pMIWIII-^{myc}control contains a Myc tag fused to a missense Ngn1 (Gowan et al., 2001). The Math1 expression construct is from Gowan et al. (Gowan et al., 2001). All constructs were sequenced to confirm the lack of PCR incorporation errors.

Electrophoretic mobility shift assay

Electrophoretic mobility shift assays (EMSA) were performed using protein generated via in vitro transcription coupled translation starting with the templates described above using the TNT SP6 kit (Promega, Madison, WI). EMSA was performed in 1×Zic1 buffer (Aruga et al., 1994), which contained 20 mM HEPES (pH 7.9), 10 mM $MgCl_2$, 50 mM KCl, 0.5 mM DTT, 0.1 mM EDTA, 10 μM $ZnCl_2$, 10% glycerol, 50 μg/ml poly dI/dC and 10^5 cpm probe. Oligonucleotide probes were generated by annealing complementary oligonucleotides (see Fig. 3 for probe sequence), followed by radiolabeling in 1×PNK buffer containing 50 ng annealed oligonucleotides, 50 mM Tris-HCl, 10 mM $MgCl_2$, 0.1 mM EDTA, 5 mM DTT, 0.1 mM spermidine, 50 μCi γ^{32}P ATP (Amersham, Piscataway, NJ), 10 U polynucleotide kinase (Roche, Indianapolis, IN). Labeled oligonucleotides were purified over a G-50 sephadex column and quantified by scintillation analysis. The enhancer B (374 bp) probes were generated by adding 50 μCi α^{32}P dCTP to a standard PCR reaction with primers 5′ AAGCCG-AGTGTACGTTTAGT 3′ and 5′ TAAATGTGGCCGCTCAGCTC 3′. The reaction was purified using High Pure PCR Product Purification kit (Roche) to reduce primer-dimer contamination, and quantified by scintillation analysis. Pre-binding reactions of unlabeled competitor oligonucleotides at ~100-fold molar excess occurred for 15 minutes at room temperature. Binding reactions were performed for 30 minutes at room temperature, followed by electrophoresis on a pre-run 5%-0.08% acrylamide-bisacrylamide non-denaturing 0.5×TBE gel for 3-5 hours at 20 mA constant current, followed by drying and exposure to X-ray film for several hours.

Chick electroporation

White leghorn eggs were obtained from SPAFAS (Norwich, CT) or Texas A&M (College Station, TX). DNA solutions (concentrations ranged from 1-3 μg/λ) were injected into the lumen of the neural tube using a General Valve Corporation Picospritzer II (Fairfield, NJ) and pulled glass pipettes. Electroporations were performed using a BTX ECM 830 power supply and BTX Genetrodes electrodes (San Diego, CA) with 3-50 ms pulses at 25 V. Embryos used for *lacZ* reporter

construct analysis were electroporated at stages 14-17, and harvested for β-gal activity assays 24-36 hours later as previously described (Timmer et al., 2001). Embryos used for analysis of ectopic expression of Zic1 were electroporated at stages 13-14 and harvested at 24 hours for either β-gal activity or immunocytochemistry. Embryos used to assess contribution to neural crest were electroporated at stages 11-13 and harvested 48 hours later. All electroporations included the EGFP-N1 plasmid (Clontech) to mark cells that took up the DNA.

Immunocytochemistry, β-gal activity and in situ hybridization

Mouse and chick embryos were dissected in 1×PBS on ice, rinsed, fixed in 2-4% formaldehyde for 2-4 hours at 4°C, rinsed with 1×PBS and sunk in 30% sucrose at 4°C overnight. Embryos were embedded in OCT compound (Tissue Tek) and cryosectioned at 30 μm. Slides were incubated in the appropriate dilution of primary antibody in PBS/1% goat serum/0.1% triton X-100, followed by either goat anti-rabbit or goat anti-mouse IgG, conjugated to Alexa Fluor 488 and 594 (Molecular Probes). Primary antibodies used for this study include: rabbit anti-Math1 (Helms and Johnson, 1998), mouse anti-Math1 (Gowan et al., 2001), rabbit anti-Zic (gift of R. Segal), rabbit anti-β-galactosidase (5prime-3prime), mouse anti-c-myc (9E10 Santa Cruz Biotechnology), mouse anti-Mash1 (Lo et al., 1991) and mouse anti-islet1 (Developmental Studies Hybridoma Bank). Fluoresence imaging was carried out on a BioRad MRC 1024 confocal microscope. EGFP signal was imaged using a standard FITC filter. For detection of β-gal activity, chick embryos were dissected in room temperature 1×PBS, fixed for 30 minutes at room temperature in 2% formaldehyde, rinsed and stained in X-gal solution as described (Timmer et al., 2001).

In situ hybridization was performed as described (Gowan et al., 2001) using in situ probes for Cath1 (PCR generated), Cash1 (obtained from T. Reh), and chick Ngn1 and Ngn2 (obtained from D. Anderson).

RESULTS

Math1 and *Cath1* enhancers have conserved sequence and function

In previous studies, a *Math1* enhancer that resides ~3.4 kb 3′ of the *Math1*-coding sequence was sufficient to direct restricted expression of a *lacZ* reporter to most domains of *Math1* expression in transgenic mice (Helms et al., 2000). This enhancer contains two discrete blocks of sequence (denoted enhancer A and B) that are highly conserved with sequence near the human Math1 ortholog, HATH1. To delineate important sequence elements further, we analyzed the Math1 gene of a more evolutionarily distant organism. We cloned the chick homolog of *Math1*, *Cath1*, and identified genomic sequence 3′ of the coding region conserved with the *Math1* enhancer (AF467292). Like human *HATH1*, *Cath1* contains two extensive blocks of homology with the *Math1* enhancer, and this homology is located ~1.7 kb 3′ of the *Cath1* coding sequence. The *Cath1* enhancer sequence is less conserved with *Math1* (75% and 62% for the two regions) relative to the conservation between *Math1* and *HATH1* (91% and 87% for the two regions) (Fig. 1A). Notably, an E-box site, previously shown to be essential for *Math1* enhancer function in transgenic mice (Helms et al., 2000), and the Zic1 binding site described below, are conserved between all three species (Fig. 1B).

We then tested for functional conservation by assaying the activity of the chick *Cath1* enhancer in mouse transgenic assays, and conversely, testing the activity of the mouse *Math1* enhancer in the chick neural tube by in ovo electroporation. The sequences with highest homology between the chick and mouse enhancers were tested for activity. A 1.2 kb *Math1* enhancer sequence (which corresponds to the region conserved with the chick enhancer) was tested using the BGZA reporter (Yee and Rigby, 1993) both in transgenic mice, and in the chick neural tube after electroporation. Expression of a 1.2 kb *Math1/lacZ* transgene in mouse E10.5 embryos resulted in restricted expression of *lacZ* in the dorsal neural tube (Fig. 1C,E) as had been seen previously with a longer sequence (Helms et al., 2000). Electroporation of this same construct in the chick neural tube (stage HH14-17) resulted in *lacZ* expression on the electroporated side of the neural tube in a

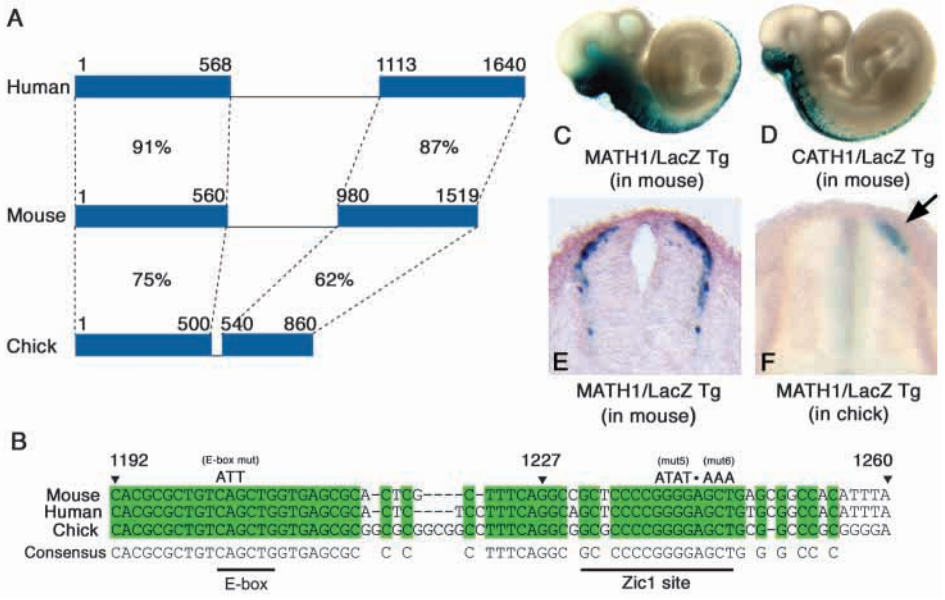

Fig. 1. Sequence and functional conservation between *Math1* and *Cath* enhancers. (A) The percent sequence conservation between the *Math1* enhancer (Accession Number, AF218258) and sequences in human (*HATH1*, Accession Number, AF18259) and chicken (*Cath1*, Accession Number, AF467292). These sequences are found 3′ to the coding sequences, ~3 kb for *Math1*, ~3.4 kb for *HATH1* and ~1.7 kb for *Cath1*. (B) Sequence from 1192 to 1260 comparing mouse, human and chick sequences across the region containing the essential E-box and the Zic1-binding site in the *Math1* enhancer. Details of the mutations in the constructs shown in Fig. 5 are shown. (C) The *Math1* enhancer (*Math1/lacZ* Tg) and (D) the *Cath1* enhancer (*Cath1/lacZ* Tg) have the same activity in E10.5 transgenic embryos as shown with whole-mount X-gal stained embryos. (E) Cross-section of the neural tube of a *Math1/lacZ* transgenic mouse embryo showing the dorsal pattern of expression for comparison with F, which is the expression of the same transgene after electroporation into one side of a chick neural tube (arrow). Note the DNA enters cells along the DV axis but expression of the transgene is restricted dorsally.

similar dorsal pattern when assayed 24-36 hours after electroporation (Fig. 1F). The activity of this 1.2 kb *Math1* enhancer in chick is identical to previously published results using a 1.7 kb enhancer sequence (Timmer et al., 2001). Conversely, we tested the activity of a 950 bp *Cath1* sequence with the BGZA reporter in transgenic mice. This *Cath1/lacZ* construct resulted in *lacZ* expression in a dorsally restricted pattern in the neural tube of E10.5 embryos from the midbrain/hindbrain boundary caudally to the tail (Fig. 1D), identical to that seen with the comparable *Math1/lacZ* transgene (Fig. 1C). Thus, conserved sequences between *Math1* and *Cath1* reflect a functional conservation that suggests conserved mechanisms control *Cath1/Math1* transcription in chick and mouse.

Zic1 is identified as a candidate upstream regulator of *Math1* expression

To identify factors that interact with the *Math1* enhancer, we initiated a yeast one-hybrid screen using a 374 bp region of the enhancer (nucleotides 898-1271) to screen an E10.5 mouse embryo library (a gift from Dr Eric Olson). Upon screening ~1×10^6 independent clones for binding to the 374 bp sequence, the zinc-finger transcription factor Zic1 was identified six times. This study addresses the possible role of this factor in the regulation of *Math1* transcription.

Zic1 is a zinc-finger transcription factor originally identified based on enriched expression in the cerebellum (Aruga et al., 1994). Published reports on the expression of *Zic1* are consistent with a role for this molecule in the regulation of *Math1* expression. In mouse neural tissue, *Zic1* mRNA is detected in the neural plate at E7.5 (Nagai et al., 1997), in advance of the initiation of *Math1* expression at E9.0 (Akazawa et al., 1995; Ben-Arie et al., 1996). By E10.5, *Zic1* expression is restricted to the dorsal third of the neural tube, including spinal and hindbrain regions that overlap the domain of *Math1* expression (Aruga et al., 1994; Nagai et al., 1997). Like *Math1*, *Zic1* is also expressed in cerebellar granule cell progenitors from rhombic lip stages through postnatal stages (Akazawa et al., 1995; Aruga et al., 1994; Ben-Arie et al., 2000; Helms and Johnson, 1998; Nagai et al., 1997). The multiple overlapping domains of *Zic1* and *Math1* expression are consistent with Zic1 playing a role in the regulation of *Math1*.

To compare the distribution of Math1 to the Zic factors at the single cell level, we used double immunofluorescence with a rabbit polyclonal antisera that recognizes multiple members of the Zic factor family (gift of R. Segal) and mouse monoclonal antibodies specific to Math1 (Gowan et al., 2001). At E10.5 in mouse, Math1 is restricted to the dorsal neural tube in cells adjacent to the roof plate (Fig. 2B) (Akazawa et al., 1995; Ben-Arie et al., 1996; Helms and Johnson, 1998). Expression of Zic factors is broader, extending from the roof plate through the dorsal third of the neural tube with the highest expression within the roof plate region (Fig. 2A) (Aruga et al., 1994; Nagai et al., 1997). Unexpectedly, Zic expression appears to be diminished specifically in the dorsal domain where Math1 is expressed (Fig. 2C). Analysis at higher magnification reveals cells co-expressing low levels of both Zic and Math1, but cells co-expressing Zic and Math1 at high levels are not detected (Fig. 2D-F). This inverse expression pattern suggests a negative role for Zic1 in *Math1* transcription. Conversely, as seen in the *Math1* mutant, the Zic expression pattern is uniform in the dorsal neural tube (Fig. 2G), suggesting Math1 may inhibit Zic expression as well.

Identification of a novel binding site for Zic1 in the *Math1* enhancer 30 bp from the autoregulatory E-box site

To confirm and characterize the specific Zic1-binding site within the *Math1* enhancer, we used electrophoretic mobility shift assays (EMSA). The *Zic1* clones identified in the yeast one-hybrid screen did not contain a sequence encoding the full-length *Zic1*; they lacked the domain N-terminal to the Zn-finger domain. In all previous published DNA-binding studies with Zic factors, similarly truncated forms of the proteins were used (Aruga et al., 1996a; Mizugishi et al., 2001; Salero et al., 2001). We in vitro transcribed and translated this partial *Zic1* clone and tested it for protein/DNA interactions in EMSA. The enhancer sequence used to identify Zic1 in the yeast-one-hybrid screen does not contain any site resembling published Zic1-binding sites (Aruga et al., 1994; Mizugishi et al., 2001). We identified an oligonucleotide (Z-site) within the *Math1* enhancer that could be bound by Zic1 at an apparent

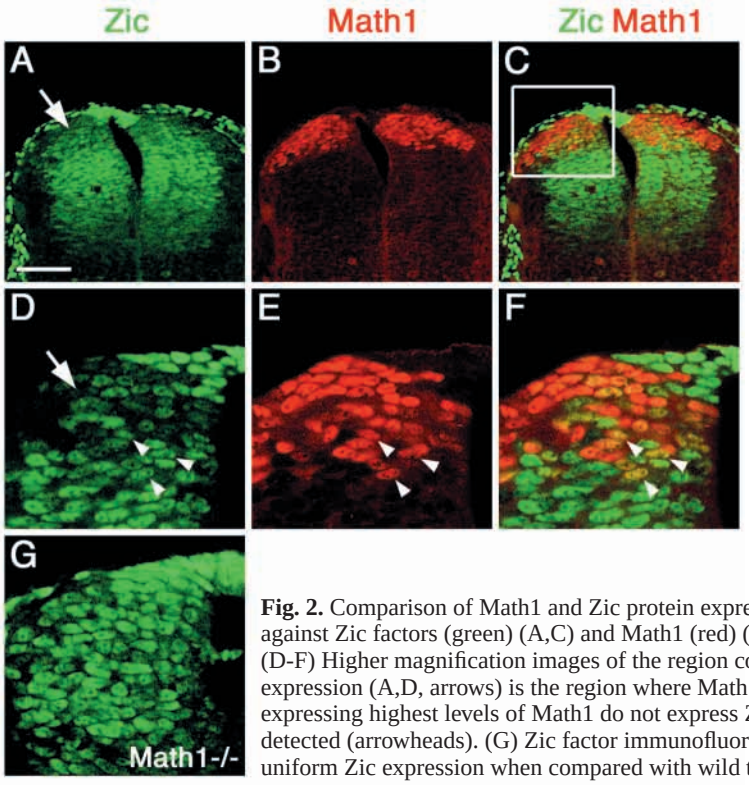

Fig. 2. Comparison of Math1 and Zic protein expression. Double immunofluorescence labelling with antisera against Zic factors (green) (A,C) and Math1 (red) (B,C) in cross-sections of mouse E10.5 dorsal neural tube. (D-F) Higher magnification images of the region comparable with the boxed region in C. The region of lowest Zic expression (A,D, arrows) is the region where Math1 is expressed in the dorsal neural tube (C,F). Note that the cells expressing highest levels of Math1 do not express Zic factors. Cells expressing low levels of both factors are detected (arrowheads). (G) Zic factor immunofluorescence in E10.5 Math1 mutant dorsal neural tube. Note the uniform Zic expression when compared with wild type (D). Scale bar: 100 μm in A-C; 35 μm in D-G.

higher affinity than binding to the previously defined consensus (Aruga et al., 1994) (Fig. 3A, compare lanes 2 and 3). Addition of unlabeled wild-type oligonucleotides competed for this binding, while addition of unlabeled mutated oligonucleotides failed to compete (Fig. 3A, lanes 4, 5, and 6). This novel Zic1 binding site is located ~25 bp from the previously identified E-box site required for *Math1* enhancer activity (see Fig. 1B). No additional Zic1 binding sites within this enhancer fragment were identified as shown using EMSA with the 374 bp *Math1* enhancer probe and Zic1 extract. A single complex, in addition to that seen with control extract, was detected (Fig. 3B, lane 2). This complex was lost when the identified Zic1 site was mutated (Fig. 3B, lanes 2, 4, and 6). Thus, this enhancer sequence contains only one Zic1-binding site.

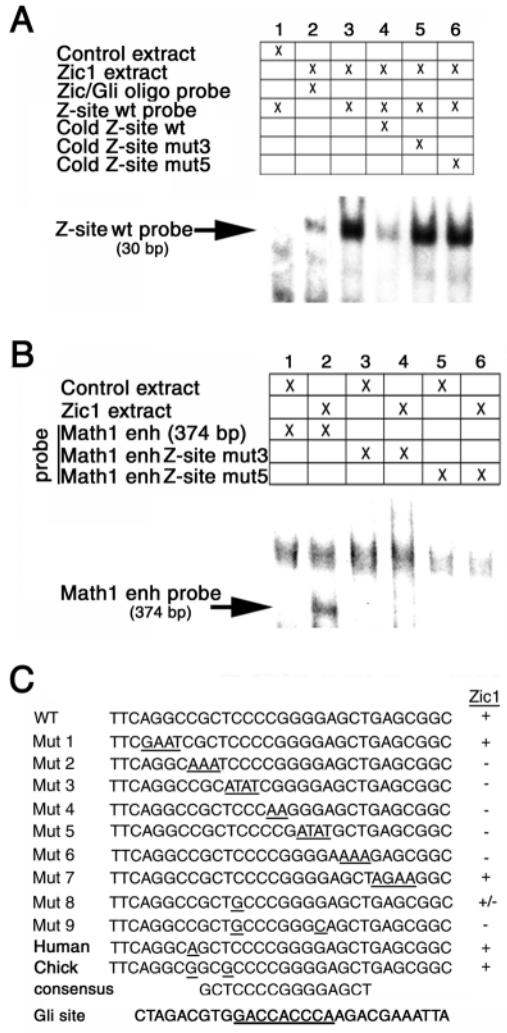

Fig. 3. A novel Zic1 site identified within the *Math1* enhancer. Gel shift assays with in vitro transcribed and translated Zic1 (lacking the N-terminal 110 amino acids) and probes generated from (A) a 30 bp sequence from the *Math1* enhancer containing the newly identified Zic1-binding site (Z-site wt), or (B) the wild-type 374 bp enhancer B (Math1 enh probe) and Math1 enh probes mutated in the Zic1 site (mutations shown in C). Sequence of the oligonucleotide probe and the cold competitor DNAs are shown in C. Control extract is the reticulocyte extract with no added template. Zic/Gli probe is the previously published Zic consensus binding site. (C) Nucleotide requirements for Zic1 binding defined by EMSA with mutant oligonucleotides are shown. The novel Zic1 site defined here (consensus) has little similarity to the published Zic binding site (Gli site).

To characterize the novel Zic1-binding site further in the *Math1* enhancer, we performed EMSA with mutant oligonucleotide probes spanning the Z-site oligonucleotide. This analysis defined a 15 bp region required for Zic1 binding (Fig. 3C, consensus). EMSA using probes from the homologous human (HATH1) and chick (Cath1) regions revealed that the ability to interact with Zic1 is conserved among all three species (Fig. 3C). This consensus has some similarity (8 out of 12 nucleotides) to a recently published Zic1 binding site in the apolipoprotein E enhancer (Salero et al., 2001) and no significant similarity to the published Gli site. In conclusion, a novel Zic1 binding site conserved between multiple species is present in the *Math1* enhancer.

Ectopic expression of Zic1 inhibits expression of *Cath1* and a *Math1/lacZ* transgene *in vivo*

To test whether Zic1 functions in vivo in regulating *Math1/Cath1* expression, we examined *Cath1* expression when Zic1 was overexpressed in the chick neural tube. A construct containing the full-length *Zic1*-coding sequence fused to five N-terminal Myc tags in the pMiWIII vector was electroporated into stage HH13-14 chick neural tubes and assayed for *Cath1* expression by immunofluorescence at 24 hours post-electroporation (HH20-21). Neural tubes ectopically expressing ^{myc}Zic1 had a dramatic decrease in the number of cells expressing Cath1 (Fig. 4B). This loss of Cath1-positive cells was not seen with neural tubes electroporated with a control expression vector (Fig. 4A). These data support a negative role for Zic1 in the regulation of Cath1 expression. Electroporation of ^{myc}Zic1 after normal initiation of Cath1 expression (HH18) had no effect on Cath1 expression (data not shown). Thus, Zic1 appears to repress expression of Cath1 but this repression is stage dependent and occurs during early stages of Cath1 expression.

As suggested by the binding activity described above, Zic1 also represses a *lacZ* reporter transgene driven by the *Math1* enhancer (nucleotides 1-1365). The *Math1* enhancer directs *lacZ* expression to the dorsal neural tube when co-electroporated into the chick neural tube at HH14-17 with a control vector, pMiWIII expressing five Myc tags and assayed at 24 hours (Fig. 4C, n=6). By contrast, co-electroporation of the *Math1/lacZ* transgene with the ^{myc}Zic1 expression construct dramatically reduces the activity of the enhancer (Fig. 4D, n=6). These data support the conclusion that Zic1 represses *Math1/Cath1* expression and this activity may be through the 1365 bp *Math1* enhancer.

The Zic1-binding site in the *Math1* enhancer is required for enhancer activity in chick neural tube

Zic1 represses Cath1 and *Math1/lacZ* transgene expression in the chick neural tube. This suggests that mutation of the Zic1 binding site in the *Math1* enhancer might result in increased enhancer activity due to loss of Zic1 binding. To test this, we mutated or deleted specific nucleotides to disrupt Zic1 binding, and tested enhancer activity in the chick electroporation assay. Electroporation of multiple *Math1 enhancer/lacZ* constructs that include the Zic1 site into chick embryos resulted in consistent restricted expression of *lacZ* in the dorsal neural

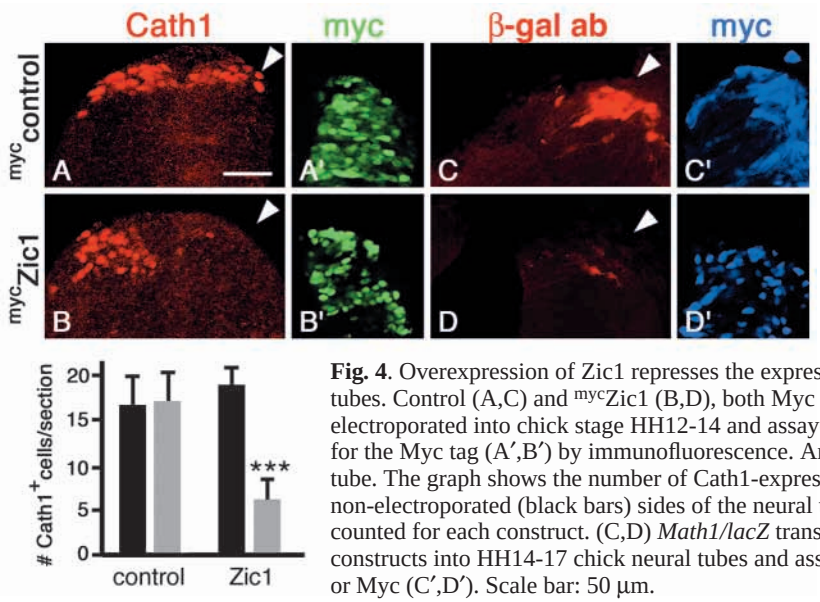

Fig. 4. Overexpression of Zic1 represses the expression of Cath1 and the *Math1* enhancer in chick neural tubes. Control (A,C) and ^{myc}Zic1 (B,D), both Myc tagged and in expression vector pMiWIII were electroporated into chick stage HH12-14 and assayed at 24 hours for endogenous levels of Cath1 (A,B) and for the Myc tag (A′,B′) by immunofluorescence. Arrowheads indicate the electroporated side of the neural tube. The graph shows the number of Cath1-expressing cells/section on the electroporated (gray bars) and non-electroporated (black bars) sides of the neural tube. At least three sections each from three embryos were counted for each construct. (C,D) *Math1/lacZ* transgene was co-electroporated with the different expression constructs into HH14-17 chick neural tubes and assayed at 24 hours by immunofluorescence for β-gal (C,D) or Myc (C′,D′). Scale bar: 50 μm.

tube (Fig. 1F, Fig. 4C and Fig. 5; Tg18 and 20) (Timmer et al., 2001). Contrary to our prediction, mutation (Fig. 5, Tg19 and 22) or deletion (Fig. 5, Tg21) of the Zic1 site within the context of the *Math1* enhancer completely eliminated expression of the construct. Thus, the Zic1 site is necessary for enhancer activity in the chick neural tube, suggesting a positive role for this site in enhancer function. Given our findings that Zic1 is acting to repress *Cath1* expression, the loss of enhancer activity when Zic1-binding site is lost suggests that Zic1 may either compete for this site with an activator, or that Zic1 also has a role as an activator, but only in the presence of a co-factor.

Zic1 represses the auto-activation of *Math1* expression

We have previously demonstrated that the *Math1* enhancer requires Math1 for activity and contains an essential E-box element (the binding site for Math1) supporting the involvement of autoregulation in *Math1* expression (Helms et al., 2000). We have found a similar requirement for the E-box for activity of the enhancer in the chick neural tube (Fig. 5, Tg23). Further data supporting positive autoregulation is seen by the increase in expression of endogenous *Cath1* upon electroporation of *Math1* in the chick neural tube (Fig. 6A, the endogenous increase in *Cath1* was distinguished from the exogenous *Math1* using a *Cath1*-specific probe for mRNA in situ analysis). The increase in *Cath1* was mostly seen in the dorsal neural tube, consistent with previous results showing context dependent activity of Math1 in this assay (Gowan et al., 2001). An even more dramatic increase was seen in the expression of the *Math1/lacZ* transgene upon co-electroporation with *Math1* (Fig. 6B). Note that the expression is throughout the DV axis, in contrast to the normal dorsal-

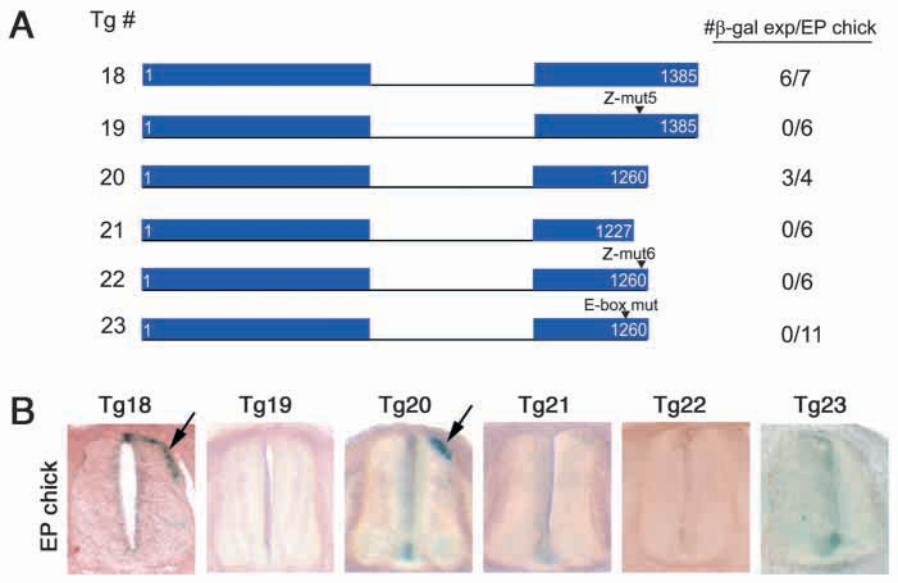

Fig. 5. Characterization of the role of the Zic1-binding site for enhancer activity using electroporation of chick neural tube. (A) The transgenic constructs used in this study (Tg#) and a summary of their activity (β-gal exp) when electroporated into chick neural tube (EP chick). Each *Math1* enhancer fragment was cloned 5′ to the promoter region in the BGZA reporter. The numbers in the diagram relate to the published nucleotide sequence of the *Math1* enhancer (Accession Number, AF218258). The specific mutations are shown in Fig. 1B. Representative data for each construct are shown in B as cross-sections of chick neural tubes after electroporation and X-gal staining. Electroporated side is always on the right. Arrows indicate expression of the transgene on the electroporated side.

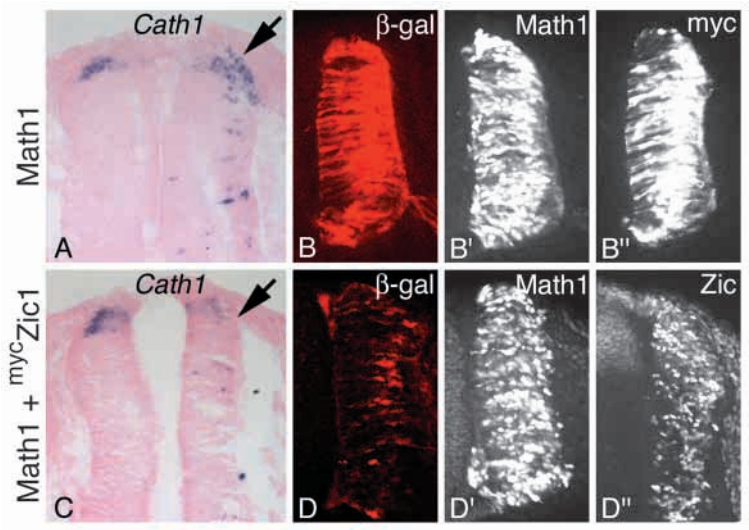

Fig. 6. Zic1 represses Math1/Cath1 autoregulation. Chick neural tubes electroporated with Math1 (A) or Math1 plus ^{myc}Zic1 (C) at HH14 and assayed at 24 hours for Cath1 expression by mRNA in situ hybridization. Arrows indicate the electroporated side. Math1 activates *Cath1* expression (A) but this activity is inhibited by ^{myc}Zic1 (C). Chick neural tubes co-electroporated with a *Math1/lacZ* transgene plus Math1 and Myc-tagged control vector (B), or Math1 and ^{myc}Zic1 (D). Immunofluorescence of adjacent sections to detect β-gal (B,D), Math1 (B′,D′), Myc (B″), or Zic (D″) are shown. Note the dramatic activation of the *Math1/lacZ* transgene when co-electroporated with Math1 (compare Fig. 3E or 5C with B) and the block of this activation in the presence of ^{myc}Zic1 (D).

restricted expression of the *Math1/lacZ* transgene (Fig. 1F, Fig. 4C, Fig. 5B). These data further support our previous result that demonstrated the involvement of positive autoregulation in controlling *Math1* expression.

To address the interplay of autoregulation with the repression caused by Zic1, we electroporated *Math1* and *Zic1* together, and assayed for expression of both *Cath1* and the *Math1/lacZ* transgene. The autoactivation of *Cath1* by Math1 is clearly blocked by ^{myc}Zic1 (Fig. 6C). Furthermore, the dramatic increase in *Math1/lacZ* transgene expression seen with electroporated Math1 was completely blocked by co-electroporating ^{myc}Zic1 (Fig. 6D). Similar results were seen with embryos electroporated at HH14 (Fig. 6) or at the later HH18 stage (data not shown). Thus, one mechanism for Zic1 repression of *Math1/Cath1* expression is by blocking positive autoregulation.

Contrasting roles for Math1 and Zic1 in maintaining progenitors versus inducing neuronal differentiation

Roles for Math1 and Zic1 in the developing dorsal neural tube have been suggested previously from loss-of-function studies in mouse (Aruga et al., 2002b; Bermingham et al., 2001; Gowan et al., 2001) and overexpression studies in *Xenopus* (Brewster et al., 1998; Kim et al., 1997; Kuo et al., 1998; Mizuseki et al., 1998). Studies of the *Math1* mutant mouse demonstrated that Math1 is required for the formation of a specific population of interneurons (Bermingham et al., 2001; Gowan et al., 2001), and in *Xenopus*, Xath1, drives ectopic neuronal differentiation (Kim et al., 1997). By contrast, overexpression of Zic factors in *Xenopus* have suggested that Zic factors mediate neural competence, but also induce dorsal cell fates such as neural crest. The consequences for dorsal neural tube neuronal differentiation are not clear, as the previous referenced reports suggest that dorsal cell types may be induced, repressed or not affected (Brewster et al., 1998; Kuo et al., 1998; Mizuseki et al., 1998). Studies of the *Zic1* mutant mouse suggest that Zic1 normally represses neuronal differentiation and maintains cells as neural progenitors (Aruga et al., 2002b). Electroporation of Math1 and Zic1 into the chick neural tube provides another paradigm to address the function of these factors in dorsal neural tube development.

Electroporation of a GFP expression construct alone at HH11-12 and analysis 48 hours later illustrates the distribution of cells that have taken up the DNA. Many GFP-expressing cells are found in the dorsal root ganglia (DRG), a neural crest derivative, on the electroporated side (Fig. 7A,B). A few cells are found in the DRG opposite to the electroporated side, suggesting that neural crest cells can cross the midline. Within the neural tube, the GFP is distributed throughout the ventricular zone, presumably in neural progenitor cells, as well as cells that are found laterally where the differentiated neurons reside (Fig. 7A,B; asterisk marks the midline). By contrast, when a Math1 expression construct is co-electroporated with the GFP expression construct, cells expressing GFP are rarely found in either DRG (Fig. 7C,D). Furthermore, within the neural tube, the GFP is no longer evenly distributed within ventricular zone but rather is mostly found lateral to this progenitor domain (Fig. 7C,D, asterisk indicates the midline). This effect is seen within 24 hours of the electroporation (data not shown). The distribution of the GFP-expressing cells in the presence of Math1 suggests that Math1 promotes neuronal differentiation within the neural tube in a manner that prevents these cells from adopting the neural crest fate.

Based on previous overexpression studies with Zic factors in *Xenopus* (Brewster et al., 1998; Kuo et al., 1998; Mizuseki et al., 1998), electroporation of Zic1 in the chick neural tube might be expected to increase the number of cells joining the neural crest lineage and inhibit neuronal differentiation in the neural tube. We found that indeed, neuronal differentiation is blocked as illustrated by the lack of GFP expressing cells lateral to the ventricular zone in the neural tube (Fig. 7E,F). This is consistent with Zic1 mediating the repression of the neuronal differentiation factor Math1, and similar to recent reports expressing chick Zic1 in the chick neural tube (Aruga et al., 2002b). However, the cells did not preferentially choose the neural crest lineage, but seem to actively avoid it, as seen by the absence of GFP expressing cells in the DRG. Rather, all GFP expressing cells appear to be restricted to the ventricular zone along the midline. This suggests that Zic1 may function to maintain the cells in a progenitor state. Furthermore, the midline location and change in morphology of the electroporated cells suggests these progenitors have additional

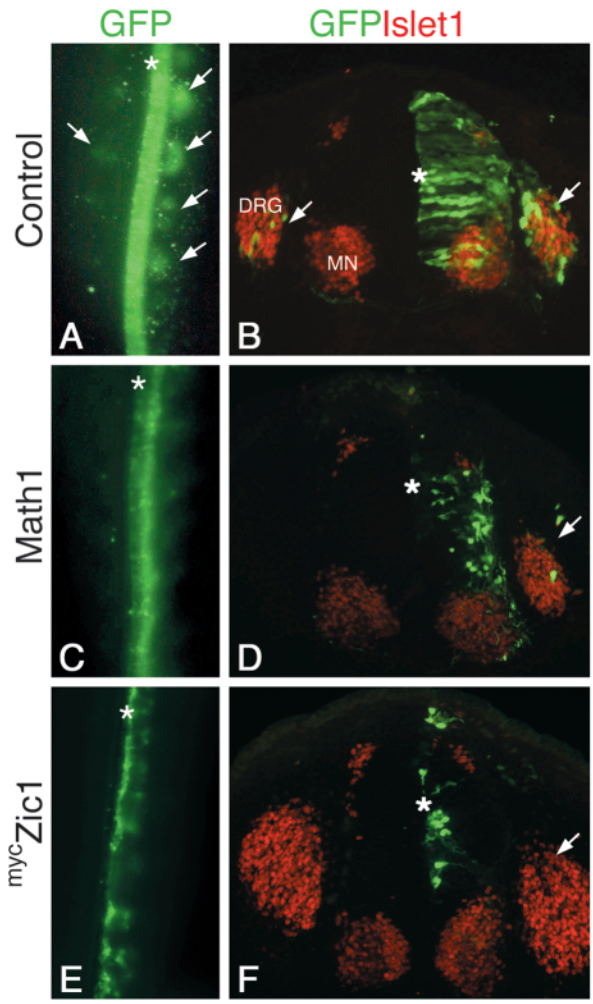

Fig. 7. Math1 promotes cell movement out of the ventricular zone while Zic1 maintains cells within this zone, both at the expense of neural crest cells. Neural tubes of HH12 chick embryos were electroporated (right side as shown) with CMVpN1-EGFP alone (A,B), with Math1 (C,D) or with ^{myc}Zic1 (E,F) expression constructs and harvested at 48 hours. Whole-mount GFP images (A,C,E) or transverse sections imaged for GFP (green) and islet1 (red) immunofluorescence (B,D,F) are shown. The islet1 staining allows visualization of the motor pools (MN) and the neural crest-derived dorsal root ganglia (DRG). Arrows in A,B indicate clear GFP in DRG on the electroporated side with lower levels in DRG on the non-electroporated side. Math1 and ^{myc}Zic1 biases cells away from DRG as seen in the near absence of GFP in this structure (C-F). Within the neural tube, GFP in controls spans the width of the neural tube (A,B), whereas Math1 overexpressing cells are biased towards the lateral tube where differentiated cells reside (C,D) and ^{myc}Zic1 overexpression biases cells to the ventricular side where progenitors reside (E,F). The asterisks indicate the location of the apical surface of the neural tube. Arrows in D,F indicate DRG.

perturbations and may be blocked in a particular phase of the cell cycle.

Differential effects by Zic1 on bHLH factor expression

The data presented here, and in previous studies, suggest that Zic1 plays a role in maintaining cells as neural progenitors, and thus may play a role in the timing of differentiation. We have shown that Zic1 represses expression of the neuronal differentiation factor Math1. However, Zic1 is expressed not only in the region encompassing the Math1 domain, but also extends ventrally to the dorsal regions of expression of three other bHLH transcription factors: Ngn1, Ngn2 and Mash1 (Gowan et al., 2001). To examine whether the Zic1 repression is specific to Cath1, or whether repression of bHLH transcription factors is a general mechanism for inhibiting differentiation, we analyzed expression of chick *Ngn1*, chick *Ngn2* and *Cash1* in the ^{myc}Zic1 electroporated chick neural tubes. As seen previously with Cath1 immunostaining (Fig. 4), *Cath1* mRNA is repressed relative to the non-electroporated side when ^{myc}Zic1 is electroporated (Fig. 8A). Similarly, *Cash1* expression is repressed with ^{myc}Zic1 (Fig. 8B). By contrast, *Ngn1* and *Ngn2* appear to be unaffected (or even slightly induced) by ^{myc}Zic1 (Fig. 8C,D). Thus, Zic1 differentially regulates these bHLH factors at this stage in the chick neural tube.

We used double label immunofluorescence to examine if Zic factors and Mash1 are co-expressed, or whether they are mutually exclusive like Zic factors and Math1. The Mash1 and Zic domains of expression overlap in the dorsal half of the Mash1 expression domain (Fig. 8E). Within the overlapping domain, individual cells co-express Mash1 and Zic factors (Fig. 8F-H). Thus, in contrast to the direct repression of Math1/Cath1 by Zic1, the repression of Cash1/Mash1 is likely to be through a distinct indirect mechanism.

DISCUSSION

During development of the mammalian nervous system, regulation of the spatial and temporal transcription pattern of neural bHLH transcription factor genes is required for the correct differentiation of neural progenitors. We have begun to probe the mechanism of this transcriptional regulation by identifying enhancers in the *Math1* gene required for tissue- and temporal-specific expression, and using these enhancer sequences to identify transcription factors that play a role in this process. Using this strategy, we identified Zic1 as a repressor of *Math1* expression. Zic1, like Math1, is induced by BMPs. Thus, BMP signaling activates pathways that both promote and inhibit neuronal differentiation. Math1 promotes differentiation, and Zic1 suppresses it, in part, through inhibition of bHLH genes such as *Math1*. The balance between these two BMP-induced pathways is likely to be central in obtaining the correct composition of dorsal cell-types in the spinal cord.

Zic1: a repressor of *Math1/Cath1* and *Cash1* expression

The inverse relationship in expression of Zic factors and Math1 in the dorsal neural tube is consistent with an inhibitory role for Zic1 in *Math1* expression. In ovo experiments analyzing Zic1 function demonstrated that overexpression of Zic1 can repress both the endogenous *Cath1* and *Cash1* genes, as well as a *Math1* enhancer/*lacZ* reporter construct. Expression of related genes encoding the bHLH factors Ngn1 and Ngn2 were not repressed. These data suggest that Zic1 does not mediate global repression of neural differentiation, but instead is directed at

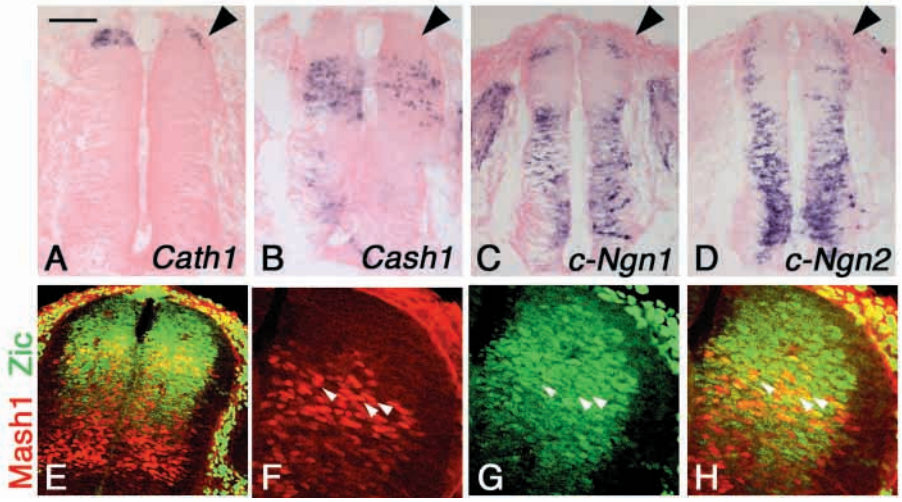

Fig. 8. Zic1 represses *Cath1* and *Cash1* but not chick *Ngn1* and *Ngn2*. (A-D) Neural tubes of HH14 chick embryos were electroporated on the right side (arrowheads) with ^{myc}Zic1 and harvested at 24 hours. mRNA in situ hybridization with *Cath1* (A), *Cash1* (B), *Ngn1* (C) and *Ngn2* (D) are shown. Relative to the control side (left) in each case, *Cath1* and *Cash1*, but not *Ngn1/2*, are repressed by overexpression of ^{myc}Zic1. (E-H) Double label immunofluorescence using anti-Mash1 (red) and anti-Zic (green) antisera on E11.5 mouse spinal neural tube. Yellow cells indicate overlap in expression in the dorsal Mash1 domain (F-H, arrowheads). Scale bar: 100 μm in A-E; 50 μm in F-H.

distinct neuronal subtypes, in particular those descendant from Math1/Cath1- and Mash1/Cash1-expressing cells.

The data presented here strongly support a model in which Zic1 directly represses Math1 expression. This repression could function to block initiation of Math1 expression, to modulate the autoregulation phase of expression, or both. It is intriguing that the Zic1 binding site and E-box site required for autoregulation are separated by only 25 bp in the *Math1* enhancer. It is possible that the two sites cannot be occupied at the same time, thus providing an explanation for why Zic1 was unable to repress endogenous *Cath1* expression when Zic1 was added after *Cath1* was already present. Localized repressive effects on the chromatin catalyzed by Zic1 could undermine the ability of factors binding the E-box to activate transcription. Consistent with this possibility, factors related to Zic proteins, the Gli factors, recruit components of the mouse Sin3/histone deacetylase complexes by interaction with the Fused kinase (Cheng and Bishop, 2002). Gli and Zic factors interact in specific cell contexts, and this interaction can be direct, as shown in immunoprecipitation and yeast two-hybrid experiments (Koyabu et al., 2001). These data suggest that a combination of Zic/Gli factors (or possibly Zic alone) could act to recruit complexes to chromatin that repress transcription activity, suppressing activation of the autoregulatory E-box in the *Math1* enhancer, and thus block or limit the establishment of a Math1 expressing domain in the dorsal neural tube.

Although it is clear that Cath1 is directly repressed by Zic1, the repression of *Cash1* by overexpression of Zic1 may be indirect, based on the relatively abundant co-expression of these two molecules in the dorsal neural tube. An increase in expression of the neurogenic repressor Notch1 and its effector Hes1 was seen in Zic1 overexpression paradigms (Aruga et al., 2002b), providing a possible link to the indirect repression of *Cash1* expression. The observation that *Cash1* but not *Ngn1/2* was repressed by Zic1 could reflect a difference in how these genes are regulated by Notch signaling. Such differences in sensitivity of bHLH genes to inhibition by Notch signaling have been seen previously (Cau et al., 2002; Chitnis and Kintner, 1996; Lo et al., 2002).

Zic1: a bifunctional protein?

The conclusion that Zic1 acts to repress *Math1* expression was confounded by our findings that the *Math1* enhancer lost activity in the chick neural tube when the Zic1 site was mutated. Loss of activity of the mutated enhancer suggests the Zic1-binding site is also required for activating *Math1* expression. We propose alternate mechanisms by which Zic1 may temporally modulate *Math1* transcription through binding to the *Math1* enhancer. First, Zic1 may compete with a positive regulator for binding to the *Math1* enhancer. Mutation of nucleotides critical for Zic1 binding to the *Math1* enhancer compromises the activity of the enhancer in assays in vivo, suggesting that nucleotides bound by the putative activator of *Math1* transcription and Zic1 are similar. Alternatively, a positive factor interacting with Zic1, or post-translational modifications of Zic1, may convert it to an activator, explaining the requirement for the Zic1-binding site for enhancer activity. Zic genes share homology to the Gli family of transcription factors, which can alternately act as repressors or activators of transcription depending on whether the repressor domain of the protein has been proteolyticly removed (Ruiz i Altaba, 1999; Sasaki et al., 1999). Zic factors may have a similar function, as they have been shown to either repress or activate expression of reporter constructs containing Zic/Gli-binding sites in a cell-type-specific manner (Brewster et al., 1998; Mizugishi et al., 2001; Salero et al., 2001; Yang et al., 2000; Kuo et al., 1998). In the Zic1 mutant, the number of Math1-expressing cells in the dorsal neural tube was decreased at E11.5 (Aruga et al., 2002b). This is consistent with a role for Zic1 in activating *Math1* expression. However, this interpretation is complicated by the fact that in the absence of Zic1 premature neuronal differentiation occurs. Thus, the progenitor pool will be diminished and result in fewer cells that are competent to express *Math1*, particularly when looking at the later stages of development of this progenitor population. Further research into regulation of Zic1 protein function will be required to reveal if Zic1 can act as a bifunctional protein.

Zic1 represses neuronal differentiation in the dorsal neural tube via repression of Math1

What is the importance of repression of *Math1* expression by Zic1? One possibility is that a delay in *Math1* expression would allow expansion of the progenitor population needed to generate the correct number of cells prior to cells exiting the

cell cycle and differentiating. Because Math1 is a regulator of neuronal differentiation of dorsal neuronal cell types (Bermingham et al., 2001; Gowan et al., 2001), a Zic1-mediated delay in the onset of *Math1* expression would allow dorsal progenitors to proliferate to the needed population size. When the repression of *Math1* by Zic1 is subsequently overcome, possibly by the expression of a positive factor that shares the binding site with Zic1, or a modification of Zic1 itself, and *Math1* expression is initiated, the differentiation of dorsal neuronal cell types results. Such a model is supported by multiple data generated in *Xenopus*, where ectopic expression of Xzic factors result in the inhibition of neuronal differentiation (Brewster et al., 1998; Kuo et al., 1998; Mizuseki et al., 1998). A role for Zic1 and Zic2 in inhibition of neuronal differentiation has also been suggested in mice, as in *Zic1* and *Zic2* mutants, premature neuronal differentiation was detected (Aruga et al., 2002a; Aruga et al., 1998; Aruga et al., 2002b). The role for Zic1 in controlling the timing of differentiation is similar to functions attributed to Notch. The increase in expression of Notch and its effector Hes1 when Zic1 was overexpressed suggests this function of Zic1 in inhibiting differentiation may be mediated by the Notch pathway. Furthermore, the negative regulation of Zic factors by Math1, suggested by the increased expression of Zic factors in the Math1 mutant, may contribute to the progression of neural progenitors towards the differentiated state.

An additional consequence for Zic1-based repression of *Math1* expression may be to allow early-born cells of the dorsal neural tube to differentiate into a neural crest cell fate. Cells of the neural crest begin to migrate from the dorsal regions of the neural tube shortly after fusion of the neural tube, continuing up to ~E9.0-E9.5 in the mouse. This latter time period coincides with the induction of *Math1* expression in the dorsal neural tube. Electroporation of Math1 into the chick neural tube (prior to native Cath1 induction) prevents transfected cells from accumulating in the DRG, consistent with Math1 acting to bias cells toward neuronal cell fates and blocking the adoption of neural crest fates. Zic repression of *Math1* expression may allow the generation of neural crest cells early in embryonic development. Overcoming this repression allows the induction of *Math1* expression and subsequent generation of dorsal interneurons. In conclusion, the role of Zic1 may be to control the timing of the initiation of *Math1* expression, to modulate the number of cells that take on a neural crest fate versus those remaining in the neural tube that will adopt a dorsal interneuron fate.

Concluding remarks

The regulation of *Math1* expression is complex in that it both integrates signals that pattern the neural tube and responds to signals that initiate neuronal differentiation. Thus, the spatial and temporal components of the *Math1* expression pattern must be tightly controlled. The size of the conserved enhancer suggests that the integration of spatial and temporal signals may occur at the level of the enhancer. Identification of autoregulation (Helms et al., 2000), cross-inhibition between neural bHLH classes (Gowan et al., 2001) and the role of Zic1 in regulating different aspects of the *Math1* expression pattern provide inroads into our understanding of these processes. Further experiments should be able to address how BMP signaling functions in *Math1* regulation, and how it relates to Zic1 function. In addition, the studies to date have been focused on *Math1* expression in the dorsal neural tube at E10.5. Will the same regulatory relationships function in the other domains of *Math1* expression including the EGL of the cerebellum, and sensory cell progenitors in the inner ear and skin? Many transcription factors and signaling pathways functioning in the dorsal neural tube are present in these tissues as well, suggesting that common themes in regulation are likely.

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