The Folate Metabolic Enzyme ALDH1L1 Is Restricted to the Midline of the Early CNS, Suggesting a Role in Human Neural Tube Defects

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ABSTRACT

Folate supplementation prevents up to 70% of human neural tube defects (NTDs), although the precise cellular and metabolic sites of action remain undefined. One possibility is that folate modulates the function of metabolic enzymes expressed in cellular populations involved in neural tube closure. Here we show that the folate metabolic enzyme ALDH1L1 is cell-specifically expressed in PAX3-negative radial glia at the midline of the neural tube during early murine embryogenesis. Midline restriction is not a general property of this branch of folate metabolism, as MTHFD1 displays broad and apparently ubiquitous expression throughout the neural tube. Consistent with previous work showing antiproliferative effects in vitro, ALDH1L1 up-regulation during central nervous system (CNS) development correlates with reduced proliferation and most midline ALDH1L1+ cells are quiescent. These data provide the first evidence for localized differences in folate metabolism within the early neural tube and suggest that folate might modulate proliferation via effects on midline Aldh1l1+ cells. To begin addressing its role in neurulation, we analyzed a microdeletion mouse strain lacking Aldh1l1 and observed neither increased failure of neural tube closure nor detectable proliferation defects. Although these results indicate that loss-of-function Aldh1l1 mutations do not impair these processes in mice, the specific midline expression of ALDH1L1 and its ability to dominantly suppress proliferation in a folate responsive manner may suggest that mutations contributing to disease are gain-of-function, rather than loss-of-function. Moreover, a role for loss-of-function mutations in human NTDs remains possible, as Mthfr null mice do not develop NTDs even though MTHFR mutations increase human NTD risk. J. Comp. Neurol. 500:368–383, 2007. © 2006 Wiley-Liss, Inc.

Indexing terms: radial glial cell; folic acid; glia; BAC transgenic; Chromosome 6; CNS injury; reactive astrocyte; NEUT2; Pax3

Neural tube defects (NTDs) are among the most common human birth defects, occurring in ~1 in 1,000 pregnancies in the United States (Cragan et al., 1995) and an estimated 300,000 or more newborns worldwide each year (Shibuya and Murray, 1998). NTDs are polygenic, with no single dominant or recessive causative gene yet identified (Juriloff and Harris, 2000); accordingly, the risk of recurrence in sibships is ~2–5% (McBride, 1979; Campbell et al., 1986). Although folate taken during the periconceptual period has been shown to prevent the recurrence of up to 70% of human NTDs (Smithells et al., 1981; Wald et al., 1991; Czeizel and Dudas, 1992), the mechanisms underlying these protective effects have remained unclear.

As folate deficiency alone is insufficient to cause NTDs in mice (Heid et al., 1992), one possible mode of action is that folate supplementation acts to correct inherited defects of folate metabolism. This idea is supported by studies using the mouse mutant splotch (SpH1), which has a
deletion within the paired homeodomain of the transcription factor Pax3 (Epstein et al., 1991). Homozygous Sp²H embryos develop exencephaly and/or spina bifida (Epstein et al., 1991; Fleming and Copp, 1998), and ~40% of these can be prevented when folate is delivered in utero (Fleming and Copp, 1998). Moreover, analysis of cultured embryos demonstrated that folate cycling is compromised in Sp²H/Sp²H mice but can be restored to levels indistinguishable from wildtype mice when exogenous folic acid is added (Fleming and Copp, 1998). The normalization of both neural tube closure and folate cycling suggests that folate might exert its protective effects in splotch mice by supplementing impaired metabolic pathways. Although this mechanism may explain NTD prevention in some cases, there is evidence that folate does more than just correct biochemical deficiencies. In particular, mice lacking the Cited2 gene develop exencephaly and die at late gestation due to failed neural tube closure in the midbrain and hindbrain (Bamforth et al., 2001; Barbera et al., 2002); despite normal folate cycling in these mutants, the NTDs can be prevented by exogenous folate (Barbera et al., 2002). These data suggest that, in addition to rescuing impaired metabolism, folate supplementation could also act by modulating folate-dependent reactions in populations of cells that are involved in neural tube closure (Barbera et al., 2002; Copp et al., 2003). Currently, it is not known upon which cellular population(s) folate acts; which reactions might be modulated, or how such effects can prevent NTDs.

One approach toward gaining insight into these issues is to study the expression and function of folate metabolic enzymes present in the developing nervous system. We previously found that the expression of aldehyde dehydrogenase 1 family, member L1 (Aldh1l1) is developmentally regulated in the cerebellum, with high mRNA levels postnatally and decreased levels in the adult (Kuhan et al., 1993); this observation suggested that Aldh1l1 might fulfill a unique developmental role. Aldh1l1 (EC 1.5.1.16; a.k.a. 10-formyltetrahydrofolate dehydrogenase, FTHFD) is a cytosolic folate binding protein that irreversibly converts 10-formyltetrahydrofolate (10-FTHF) to tetrahydrofolate (THF) and CO₂ (Kutzbach and Stokstad, 1971; Cook, 2001a), and which may also be responsible for regulating cellular THF levels (Kim et al., 1996). As the reaction catalyzed by Aldh1l1 results in permanent removal of one-carbon units from the cellular pool, it has been suggested that Aldh1l1 may function primarily as a regulatory molecule (Krebs et al., 1976; Scrutton and Beis, 1979). Moreover, as 10-FTHF is required during two steps of purine synthesis, it has been proposed that Aldh1l1 might restrict proliferation. This idea is supported by the findings that overexpression of Aldh1l1 suppresses proliferation of certain cell lines, and that Aldh1l1 is significantly downregulated in several different tumor tissues (Krupenko and Oleinik, 2002; Oleinik and Krupenko, 2003). However, the precise physiological role played by Aldh1l1 in vivo is unknown.

In the course of analyzing the embryonic expression pattern of Aldh1l1, we observed its specific localization in radial glia at the ventral and dorsal midlines of the E12 spinal cord. As no other enzyme directly involved in folate metabolism has previously been shown to have such restricted expression within the developing neural tube, and as a role for localized differences of folate metabolism during neural tube closure has not been explored, we investigated Aldh1l1 expression and function further.

**MATERIALS AND METHODS**

**Mice and BAC transgenesis**

Mice were maintained and utilized according to protocols approved by the Institutional Animal Care and Use Committee at Rockefeller University. Wildtype C57B6/J mice were used for expression analysis. NEUT2 mice were previously generated as described (Champion et al., 1994) and were obtained from Charles River Laboratories (Wilmington, MA) with the permission of Dr. Carol Giometti. NEUT2 mice were bred to C57B6/J for three generations and then the F₃s intercrossed for maintenance. To restore Aldh1l1 expression onto a NEUT2 background, the BAC clone RP23-7M9 (Krupenko and Oleinik, 2003) was introduced into NEUT2 oocytes generated from a NEUT2/~− × B6CBA F1 cross using standard transgenic techniques (Hogan et al., 1994). Positive founders were then bred to NEUT2 homozygous mice to generate transgene positive NEUT2 homozygotes; these are denoted ~−/−;TgN(RP23-7M9). All quantitative analysis was done using mice from these crosses, and therefore have a mixed genetic background of C57B6, CBA, and BALB/C.

**Immunostaining**

Tissues were either immersion-fixed (embryos) or perfused (postnatal/adult) with 4% paraformaldehyde (PFA) and postfixed overnight at 4°C. Tissues were then washed in phosphate-buffered saline (PBS, pH 7.4) and cut either on a cryostat (20 μm) or vibratome (75 μm); tissues to be cryosectioned were first cryoprotected in 15% sucrose overnight at 4°C. Details on source and specificity of the antibodies used are as follows: Rabbit α-Aldh1l1 was used at 1:1,000 and was a gift of Dr. Robert Cook. This antiserum was generated against full-length recombinant rat Aldh1l1; specificity was confirmed by a complete lack of staining in NEUT2 homozygotes (Aldh1l1 null mice, Fig. 5). Rat α-Brdu (1:100, Oxford Biotechnology, Kidlington, UK) was generated against bromodeoxyuridine (Brdu) coupled to keyhole limpet hemocyanin (KLH), and yielded no staining in mice not administered Brdu. Rabbit α-GFAP (1:500, Dako, Carpenteria, CA) was generated against glial fibrillary acidic protein (GFAP) isolated from bovine spinal cord; mouse α-GFAP (1:200, Sternberger Monoclonals, Baltimore, MD) was generated against human GFAP. Both of these antibodies labeled only cells with the morphology of fibrillary astrocytes. Rabbit α-MTHFD1 (1:500, Newcastle upon Tyne, UK) was generated against a 1,086-bp KI67 motif-containing cDNA fragment, gave the expected nuclear staining pattern and was restricted to areas of active proliferation. Rabbit α-MTHFD1 (1:2,000, gift of Dr. Dean Appling) was generated against full-length enzyme purified from rat liver; Western analysis of crude liver extracts demonstrated that the antiserum labeled a single band of ~116 kDa that comigrated with purified MTHFD1 (Cheek and Appling, 1989). Mouse α-Nestin/rat-401 (1:4, Developmental Studies Hybridoma Bank, Iowa City, IA) was generated against homogenized Sprague-Dawley rat spinal cord (Hcockfield and McKay, 1985) and gave characteristic filamentous staining of cells in germinal zones. Monoclonal mouse α-PAX3 (1:2,000 and TSA-amplified,
Developmental Studies Hybridoma Bank) was generated against amino acids 298–481 of quail PAX3. This antibody has been previously used to immunolabel PAX3 (Baker et al., 1999; Venters et al., 2004), and in our hands yielded staining in the murine spinal cord that was nuclear and in a pattern essentially identical to that reported for PAX3 mRNA and protein (Li et al., 1999; Mansouri et al., 2001; Milewski et al., 2004). Rabbit α-phosphorylated histone H3 (Ser10) (PH3, 1:250, Upstate Biotechnology, Lake Placid, NY) was generated against a peptide (ARKpSTG-GKAPRKLQ) corresponding to amino acids 7–20 of human histone H3, and gave completely nuclear staining present only in actively proliferating regions. All secondary antibodies (Cy2 and Cy3) were from Jackson Immunoresearch (West Grove, PA) and used at 1:500. Sections were preblocked in 5% donkey serum, 0.1% Triton X-100 in PBS, and incubated with primary antibodies overnight at 4°C. After washing, sections were incubated with secondary antibodies generated in donkeys for 2 hours in the same solution at room temperature. BrdU immunostaining was done as follows: sections were incubated in 0.5% H2O2 in methanol for 15 minutes, washed in PBS, treated in 1 mg/ml sodium borohydride in PBS for 20 minutes, washed again, denatured in 2 N HCl for 40 minutes, then washed extensively in PBS before being used for immunostaining. Sections to be used for PAX3 immunostaining were microwaved at full power for 10 minutes in 10 mM sodium citrate, pH 6.0, prior to application of primary antibody. Confocal imaging was done on an LSM 510 Axioplan (Zeiss, Thornwood, NY) in the Rockefeller University Bioimaging Facility, and full resolution images were exported directly into Adobe Photoshop (San Jose, CA) for figure construction.

Mapping, complementation, and genotyping the NEUT2 microdeletion

Since the full-length Aldh111 cDNA hybridizes to NEUT2 homozygous genomic DNA (Champion et al., 1994), one end of the deletion must be within the Aldh111 gene. PCR against the first and last exons demonstrated that the deletion was in the 5′ part of the gene. By designing PCRs against the most 3′ end of the gene and walking progressively 5′, the 3′ breakpoint could be mapped to within 200 bp, in the intron between exons 10 and 11. Next, NEUT2 −/− DNA was digested separately with three different restriction enzymes and nested ligation mediated PCR was performed using the following primers located in the 3′ breakpoint region: 5′-AAGCCTGTAG-GGAGACAGTGG-3′ and 5′-CCCAAGCCATGTCAGAG-GTC-3′ as the 3′ primers and oligos provided in the Vectorette II Kit (Sigma Genosys, St. Louis, MO) as the 5′ primers. Products were sequenced from all three reactions and each yielded the same result of an 841,250 bp deletion. The 5′ breakpoint is: 5′-TAACCCAAAATATGCT-GCTTATAACT-3′ deletion. The 3′ breakpoint is: deletion 5′-GGATTTCTAGACAACAGTCAGAG-3′. This deletion removes all but the last two exons of the Aldh111 gene, the entire 5′ flanking genomic sequence (putative promoter region), as well as 27 other upstream genes (see text and Table 1). Neither Aldh111 mRNA (Champion et al., 1994) nor protein (Fig. 5) is detectable in NEUT2 −/− mice.

Once the deletion breakpoints were determined, mice were typed by PCR with four sets of primers. The first two primer sets are against portions of the deleted chromosome; generation of product indicates that the sample is either wildtype or heterozygous: 1_forward 5′-CCACCTATAAGGTTGCAG-3′ and 1_reverse 5′-GGATGGACCA-TCTAGAGAC-3′, 241 bp product; forward 2 5′-TGT-GTGTGCCACAGTGCTC-3′, 252 bp product. Both of these primer sets amplify a deleted region of NEUT2 mice that is not present in BAC RP23-7M9; thus, they can be also be used for typing −/−:Tg(NRP23-7M9) mice. The second two primer sets amplify across the deletion breakpoint; generation of product indicates that the sample is either heterozygous or homozygous: 3_forward 5′-CTGCACAT-AAGCACAACCACGAGCTC-3′ and 3_reverse 5′-TGTA-GTTCCACCGAGCTC-3′, 295 bp product. BAC transgenic mice were identified by PCR against the backbone of the RP23 library vector, pBAc3.6 using the following two primers: c3.6 for 5′-CAAGGTGTCTGATGCCGTCGGGACCCAGGG-ATTCAGG-3′ and c3.6 rev 5′-AGAGCAATATATGTCCTACAATGTCAGAGCTC-3′.

Cortical injury model

Injury experiments were done using the method previously described (Koguchi et al., 2002). Briefly, adult mice were deeply anesthetized with avertin and a small hole was drilled in the skull over the cortex using a 21G needle. Cortical wounds were inflicted by inserting a 27G needle into the cortex to a depth of 2 mm, and animals then allowed to recover on a heating pad. Proliferating astrocytes were marked 3 days after injury by administration of a single pulse of BrdU at 0.5 mg/10 grams body weight to label cells in S-phase. Two and a half hours after receiving BrdU, mice were deeply anesthetized with nembutal at 60 mg/kg, followed by transcardial perfusion with 4% PFA. The percentage of GFAP-positive astrocytes that were also-BrdU positive (labeling index for astrocytes) was then determined by immunostaining as described above.
RESULTS

ALDH1L1 is restricted to midline radial glia in the early neural tube and is developmentally regulated in the central nervous system (CNS)

To determine the timing and location of ALDH1L1 onset in the neural tube, we immunostained cryosections from embryonic day (E)8.5 to E12.5. Using TSA-amplified immunofluorescence, ALDH1L1 was not detected at E8.5 (prior to neural tube closure) but was present by E9.5 in cells flanking the ventral midline of the neural tube in the hindbrain and cervical spinal cord (Fig. 1A,B); the ventral midline in this region of the neural tube is also known as the median hinge point (MHP), which is one of two major sites where the neural plate bends during neurulation (Copp et al., 2003). Expression was further upregulated in these ventral midline cells by E10.5 and could be detected using standard immunofluorescent staining (Fig. 1C,D). Progressive dorsal expansion of staining was observed over the next few days in adjacent ventral regions, and by E12.5 cells flanking the dorsal midline (the site of neural fold fusion; Copp et al., 2003) were also labeled (arrow in Fig. 1E). Interestingly, all ALDH1L1+ cells have the dis-
Distinctive morphology of radial glia, cells that play both structural and precursor roles in the developing nervous system (Fishell and Kriegstein, 2003; Rakic, 2003; Ever and Gaiano, 2005). To confirm their identity, we double-labeled with nestin, a gene expressed in all radial glia but absent from neurons; as expected, all ALDH1L1+ cells contain high levels of nestin, demonstrating cell-specific expression (Fig. 1F–H). ALDH1L1 expression continues to be upregulated in more dorsally situated cells, and by E14.5 essentially all spinal cord radial glia at all rostro-caudal levels of the CNS are ALDH1L1+ (Fig. 1I).

Similar spatiotemporal gradients of staining were observed in other brain regions. For example, high levels of ALDH1L1 protein are detected in midline radial glia of the E14.5 tectum and hypothalamus (Fig. 1J,K). As in the spinal cord, ALDH1L1 becomes induced in lateral regions over the next couple of days, and extensive expression in these areas is seen by E16.5 (Fig. 1L and data not shown). Given that broad and high levels of ALDH1L1 are not seen until relatively late stages of embryogenesis, these data suggest that ALDH1L1+ radial glia might be developmentally more mature than ALDH1L1- cells, and that radial glia at the ventral and dorsal midlines may differentiate earlier than intervening populations.

Given its role in folate metabolism, the above-described restricted expression near sites involved in neurulation suggests that ALDH1L1 might play a role in neural tube closure. Regarding the timing of ALDH1L1 onset, it should be noted that two different mouse mutants have increased frequencies of NTDs after initial neural tube closure. In T
−/− compound mutants, neural folds in the caudal CNS fuse but then subsequently rupture and reopen (Park et al., 1989). Furthermore, a substantially larger percentage of Cited2 null embryos display obvious exencephaly at E10.5 (~80% of embryos) than at E9.5 (~50%) (Barbera et al., 2002). These examples demonstrate that successful neural tube closure requires processes that are active both before and after neural fold fusion. Accordingly, the onset of ALDH1L1 expression after neural fold fusion does not rule out a potential role in neurulation.

MTHFD1 is broadly expressed throughout the developing CNS

ALDH1L1 is the first folate metabolic enzyme shown to display restricted expression at sites known to be involved in neurulation. Although correlative, potential involvement of ALDH1L1 in neural tube closure is also suggested by the fact that a single nucleotide polymorphism (R653Q) in the methylenetetrahydrofolate dehydrogenase 1 gene (MTHFD1) has been shown to associate with increased risk of NTDs and decreased embryo survival in humans (Brody et al., 2002). MTHFD1 is a trifunctional enzyme that possesses two activities that generate 10-THF: 1) 10-formyl-THF synthetase (EC 6.3.4.3), which converts formate and THF to 10-THF; and 2) 5,10-methylenetetrahydrofolate cyclohydrolase (EC 3.5.4.9), which converts 5,10-methylenetetrahydrofolate to 10-THF (Cheek and Appling, 1989; Cook, 2001a). ALDH1L1 and MTHFD1 therefore have opposing effects on cellular levels of the same folate derivative and likely affect a similar set of metabolic reactions. Although the functional consequences of the R653Q polymorphism are unknown, one could predict that reduced MTHFD1 activity (loss-of-function) might have similar phenotypic effects as increased ALDH1L1 activity (gain-of-function), and vice versa.

Given the restricted expression of ALDH1L1 at the midline and association of an MTHFD1 allele with human NTDs, we compared their expression patterns to determine how they might interact to regulate folate metabolism and neural tube closure. In contrast to ALDH1L1, MTHFD1 does not show regionally restricted expression in the embryonic CNS (Fig. 2). Broad Mthfd1 staining is observed in neuroepithelial cells at all rostro-caudal levels of the CNS, both prior to (E8.5, Fig. 2A) and after (E9.5, Fig. 2B) neural tube closure. Moreover, MTHFD1 is not cell-specifically expressed; examination after the start of neurogenesis demonstrates that MTHFD1 is present in both neurons (Fig. 2D–G,L–N) and radial glia (Fig. 2D,H–K). One additional difference between ALDH1L1 and MTHFD1 that was particularly obvious in neocortex at later stages of embryogenesis is their subcellular distribution. ALDH1L1 protein is detectable in cell bodies and throughout radial glial processes (Fig. 2Q,R), whereas MTHFD1 is largely restricted to cell bodies in both neurons and radial glia (Fig. 2P,R). This raises the interesting possibility that 10-THF concentrations may vary within different subcellular compartments of radial glia expressing both enzymes.

ALDH1L1 upregulation correlates with decreased proliferation

The above expression data indicate that MTHFD1 activity fulfills general metabolic requirements for 10-THF throughout the embryonic CNS, while ALDH1L1 modulates 10-THF-dependent processes in a spatiotemporally dynamic manner. Two lines of evidence suggest that proliferation may be one of the processes affected by ALDH1L1 activity. First, ALDH1L1 overexpression has been shown to repress proliferation in vitro (Krupenko and Oleinik, 2002; Oleinik and Krupenko, 2003). Second, radial glial maturation is accompanied by an increase in cell cycle length and a decrease in the percentage of mitotic cells (Takahashi et al., 1995; Noctor et al., 2002), and ALDH1L1 becomes broadly expressed at relatively late stages of radial glial development (Fig. 1). Therefore, we next examined the proliferative status of regions that have upregulated ALDH1L1. To do this, adjacent sections of different CNS regions were immunostained for ALDH1L1 and either Ki67 (expressed by proliferating cells during all stages of the cell cycle) or PH3 (a marker for cells in mitosis).

In the E12.5 spinal cord, ALDH1L1 is restricted to radial glia flanking the ventral and dorsal midlines (Fig. 3A). Strikingly, these regions are largely devoid of proliferating cells: a gradient of Ki67+ staining in the spinal cord is observed, with virtually no cycling cells at the most ventral portion of the spinal cord, increasing numbers in more dorsally situated cells, but a sharp decrease again at the most dorsal point (Fig. 3A'). This relative lack of dividing cells at the dorsal midline is also clearly observed in the midbrain at the tectal midline; whereas ALDH1L1 is expressed specifically in midline radial glia (Fig. 3B), essentially all proliferating cells lie outside this region (Fig. 3B'). To determine how proliferation changes as ALDH1L1 is upregulated, we compared the number of mitotic cells in the caudal ganglionic eminence (CGE) at E12.5 (prior to ALDH1L1 expression) and E14.5 (after ALDH1L1 upregulation). As shown in Figure 3C,D, upregulation of ALDH1L1 correlates with a sharp decrease in the number of mitotic cells in the ventricular zone, the
Fig. 2. MTHFD1 is broadly expressed in both radial glia and neurons throughout the embryonic CNS. Immunofluorescent staining for MTHFD1 (A–E,G,H,J–L,N,P,R), β-III Tubulin (F,G,M,N), Nestin (I,J), and ALDH1L1 (O,Q). High-power double-labeling is shown for regions in D with star (E–G) and box (H–J). Broad and apparently ubiquitous expression of MTHFD1 is observed prior to neural tube closure at E8.5 (A) and is similar at E9.5 (B), a timepoint when ALDH1L1 (in contrast) displays highly localized expression in ventral midline radial glia (Fig. 1A,B). Arrowhead points to region shown at higher power in box at lower right; note the characteristic cytoplasmic staining. MTHFD1 is present in both neurons and radial glia, as is evident from its distribution in E12.5 spinal cord (C,D) and neocortex (K,P); double-labeling with β-III Tubulin (E–G,L–N) and nestin (H–J) confirms this. This contrasts sharply with ALDH1L1 expression, which is confined to radial glial cells (O,Q and Fig. 1). The subcellular distributions of ALDH1L1 and MTHFD1 are also distinct, and were particularly noticeable during late stages of cortical development (E17.5 is shown in O–R). Whereas ALDH1L1 is present throughout radial glial cell bodies and processes (O,Q), MTHFD1 is contained only in neuronal and radial glial cell bodies (P,R). SVZ, subventricular zone (location of radial glial cell bodies); CP, cortical plate (location of neuronal cell bodies). Scale bar = 100 μm for C,O,P; 90 μm for B; 50 μm for A; 40 μm for K,Q,R; 25 μm for B inset, D,L–N; 15 μm for E–J.
location of ALDH1L1\(^+\) radial glial cell bodies. It should be noted that subpallial expression is not unique to the CGE; ALDH1L1 is highly expressed in all ventral telencephalic radial glia at E14.5 (e.g., the lateral and medial ganglionic eminences, Fig. 5E,G). Moreover, as observed in the spinal cord, expression is progressively upregulated in more dorsal structures and is detectable in pallial (neocortical) radial glia by E17.5 (Fig. 2O).

These data demonstrate that cell division is minimal or absent in ALDH1L1\(^+\) midline radial glia, and that upregulation of ALDH1L1 correlates temporally with a decrease in the percentage of mitotic radial glia. Furthermore, the inverse relationship between ALDH1L1 expression and proliferation suggests that a shift in folate metabolism could be involved in regulating proliferation in ALDH1L1\(^+\) midline radial glia.

**ALDH1L1 and PAX3 expression are inversely correlated in the developing spinal cord**

ALDH1L1 expression is restricted to radial glia in the ventral portion of the spinal cord from E9.5–12.5 (Fig. 1), and its inverse correlation with proliferation (Fig. 3) is consistent with its known antiproliferative activity (Krupenko and Oleinik, 2002; Oleinik and Krupenko, 2003). Interestingly, these characteristics are opposite those of the transcription factor PAX3. In the spinal cord, PAX3 expression is restricted to mitotic dorsal progenitors (Goulding et al., 1991). Moreover, significantly fewer cells are born in the dorsal spinal cord at E10 in Pax3 mutant mice (Keller-Peck and Mullen, 1997), suggesting that Pax3 might positively regulate proliferation in the early neural tube. The relationship between ALDH1L1 and Pax3 is of potential interest, as Spr\(^{\text{H}}\) embryos (Pax3 mutants) develop NTDs that can be rescued by exogenous folic acid, and this folate-responsiveness is due at least in part to disrupted folate metabolism (Fleming and Copp, 1998). However, the link between PAX3 function and folic acid metabolism remains unknown. We therefore directly examined the relationship between the expression of PAX3 and ALDH1L1 using double immunofluorescence. At E11.5, ALDH1L1 was detected only in a small population of radial glia flanking the floor plate (Fig. 4A), whereas Pax3 was highly expressed in progenitors throughout the dorsal spinal cord (Fig. 4B). At E12.5, ALDH1L1 expression increased considerably in the ventral midline radial glia and had begun to spread to more dorsally located radial glia (Fig. 4C). However, a sharp boundary was observed between the regions containing ALDH1L1 immunopositive and negative cells that appeared to correlate tightly with Pax3 expression (Fig. 4C,D). Examination of this boundary at higher magnification showed that ALDH1L1\(^+\) cells were essentially Pax3\(^-\) and vice versa (Fig. 4E–G). Moreover, ALDH1L1 expression was upregulated in radial glia throughout the dorsal–ventral extent of the spinal cord by E14.5 (Fig. 4H); by this age, Pax3 can no longer be detected (Fig. 4I). These data demonstrate a strong inverse relationship between Pax3 and ALDH1L1 expression, and raise the possibility that Pax3 might negatively regulate Aldh111 transcription.
Another radial glial gene previously reported to have restricted ventral expression in the developing spinal cord is the glutamate transporter, GLAST (Shibata et al., 1997). To determine if PAX3 might also be absent from GLAST<sup>+</sup> cells, we double-immunolabeled for these proteins. At E12.5, low-magnification images showed that GLAST<sup>+</sup> regions are largely devoid of PAX3<sup>+</sup> cells and vice versa (Fig. 4J,K). However, examination of the boundary region at higher magnification clearly showed that strong PAX3 immunoreactivity was present in some GLAST<sup>+</sup> cells (Fig. 4L,M). These results could suggest that while PAX3 negatively regulates both ALDH1L1 and GLAST in dorsal radial glia, it exerts tighter suppression over the Aldh1l1 locus.

Characterization and complementation of an Aldh1l1 null mouse strain

Either loss- or gain-of-function mutations in Aldh1l1 could potentially affect neural tube closure. Modeling gain-of-function in the absence of known human or mouse mutations is complicated, as it is difficult to predict what relevant alleles might look like. Therefore, we chose to assess the effects of Aldh1l1 loss-of-function by analyzing NEUT2 mice; this strain contains a microdeletion in the region containing the Aldh1l1 gene. These mice were generated by exposing mice to fission spectrum neutrons and then breeding the mutations to homozygosity. Two-dimensional electrophoresis of liver proteins in one of the homozygous lines (called NEUT2) demonstrated the absence of a protein that sequencing established was ALDH1L1; Northern blotting later confirmed the lack of Aldh1l1 mRNA (Champion et al., 1994; Giometti et al., 1994). Viable NEUT2 homozygotes could be generated and biochemical studies showed that hepatic folate pools are distorted, with a threefold decrease of THF and similar increase of 10-FOXH levels (Champion et al., 1994). In addition, hepatic histidine catabolism via...
Fig. 5. Characterization and complementation of NEUT2 homozygous mice. A: A diagram (not to scale) depicting the NEUT2 microdeletion on mouse chromosome 6D1. The breakpoints are denoted by arrows, and each deleted gene is shown as a shaded circle. Of the 28 genes deleted, 21 code for vomeronasal receptors (gray circles); all genes contained within the deletion are listed in Table 1. BAC clone RP23-7M9 is aligned below; note that this clone contains the entire Aldh111 gene but none of the other genes deleted in NEUT2 mice. B–J: Immunostaining for ALDH111 in wildtype (B,E,H), NEUT2 +/- (C,F,I), and NEUT2 +/-;TgN(RP23-7M9) (D,G,J) mice. Clone RP23-7M9 was sufficient to restore ALDH111 expression in the wildtype pattern to NEUT2 +/- mice throughout the CNS; shown here are radial glia in the E14.5 tectum (B–D) and forebrain (E–G), and adult cerebellar Bergmann glia and internal granular layer (IGL) astroglia (H–J). Note the restricted expression in both wildtype and BAC transgenic mice at the tectal midline (arrows in B,D). Scale bar = 100 μm for E–G; 40 μm for B–D; 20 μm for H–J.
Folate deficiency induced malformations resembling NTDs at low frequency on the NEUT2 background. Whole mount normal (A) and malformed (B) E12.5 embryos derived from dams maintained on an amino acid diet with 0.4 mg/kg folate (mutant embryo shown in B is representative of the types of defects observed). The embryo in B developed a malformation resembling rachischisis (failure of neural tube closure over a large portion of the spinal cord), but appeared otherwise normal. Scale bar = 750 μm.

Folate-dependent deamination is abnormal in NEUT2 homozygotes (Cook, 2001b). Thus, NEUT2 mice have disruptions of folate metabolism, the consequences of which have not yet been determined in the developing CNS.

As the extent of the chromosomal deletion (and hence the total number of deleted genes) had not been previously determined, we mapped the deletion breakpoints using ligation-mediated PCR as described in Materials and Methods. This analysis demonstrated an 841,250 bp deletion present on mouse chromosome 6D1 in NEUT2 mice; examination of the deleted region using public databases demonstrated the loss of 28 genes (Fig. 5, Table 1). Of these, 21 code for vomeronasal receptors, genes that are expressed in the vomeronasal organ and that appear to be primarily involved in detecting pheromones (Dulac, 2000; Del Punta et al., 2002). Of the remaining seven genes, the only deleted gene other than Aldh111 that has any known effect on folate metabolism is urocanase domain containing 1 (Uroc1), which is required for folate-dependent histidine catabolism. However, the absence of Uroc1 in NEUT2 homozygotes has been proposed to actually lessen the disturbance to folate metabolism by reducing the load on the cellular THF pool (Cook, 2001b). Therefore, the previously described distortions in folate pools are most likely due to the absence of ALDH1L1, making NEUT2 mice a potentially useful model for studying the physiological effects of altered folate metabolism in tissues expressing ALDH1L1.

In order to identify which phenotypes present in NEUT2 mice are due to loss of Aldh111, it was necessary to restore its expression onto a NEUT2 homozygous background. To accomplish this, BAC transgenic mice were generated using the clone RP23-7M9. Importantly, this clone spans only the most telomeric extent of the NEUT2 deletion, containing the entire Aldh111 gene and ~40 kb of 5’ flanking genomic sequence, but none of the other genes present in the deletion (Fig. 5A). This results only in the complementation of the Aldh111 mutation and rescue of any phenotype in NEUT2 mice carrying the BAC transgene can be attributed to restoration of Aldh111. As shown in Figure 5B–J, RP23-7M9 is sufficient to drive ALDH1L1 expression in NEUT2 homozygotes in the same pattern as in wildtype mice throughout the embryonic and postnatal CNS. Thus, NEUT2 homozygotes carrying the RP23-7M9 BAC transgene (−/−;TgN(RP23-7M9)) serve as an appropriate control to assess the specific effects of ALDH1L1 deficiency in NEUT2 mice.
**Aldh111 null mice do not have increased incidence of NTDs**

Mendelian ratios of mice generated from heterozygous crosses did not statistically differ from the expected 1:2:1 wildtype:heterozygous:homozygous proportions (n = 58:91:48) when typed at 10 days of age ($\chi^2 > 0.5$). As NTD penetrance is variable in both humans and mice (Juriloff and Harris, 2000), we screened E12 embryos to see if low penetrant NTDs might occur in NEUT2 homozygotes. No NTDs were found for any genotype (n = 58:98:119 wt:het: homo), indicating that Aldh111 is not critical for neural tube closure in mice under normal circumstances.

Although low folate status alone is not sufficient to induce NTDs (Heid et al., 1992), the finding that some mothers of affected pregnancies have low serum folate suggests that on some genetic backgrounds maternal folate levels could be an important determinant of disease (Daly et al., 1995). To determine if NEUT2 homozygotes might be susceptible to NTDs in the presence of low maternal serum folates, dams were placed on a defined amino acid diet containing folic acid at 0.4 mg/kg (906 nmol/kg); this is ~7.5-fold less than normal mouse chow, and has previously been shown to significantly reduce maternal folate profiles without effects on litter size, resorption rate, or embryonic weight (Heid et al., 1992). E12.5 embryos examined from dams fed this diet developed malformations at low frequency that superficially appeared to resemble NTDs (Fig. 6), but there was no significant difference in occurrence among the three genotypes (#malformations/#embryos: wildtype, 1/54 ~ 1.8%; heterozygous, 6/184 ~ 3.3%; homozygous, 4/116 ~ 3.5%). Although the trend appears to be toward Aldh111 deficiency increasing risk of malformations on a low folate diet, the chi-square test statistic cannot be applied to such a low pen-

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**Fig. 7.** No morphological or proliferation defects are observed in NEUT2 $+/-$ embryos. Immunostaining for nestin (A–C), Ki67 (D,E), and PH3 (F,G). Regions shown are the E14.5 lateral ganglionic eminence VZ (A–C,F,G) and tectal dorsal midline (D,E); genotypes are stated in each panel. Staining for nestin revealed no obvious defects in the radial glial scaffolding at any CNS site, and density of radial glial fibers appeared unaffected by loss of ALDH1L1 function (A–C). The relative absence of proliferation at midline structures is preserved (arrows in D,E), and no apparent increase in the number of mitotic cells was observed in regions that would normally have upregulated ALDH1L1 (F,G). Scale bar = 50 μm.

**Fig. 8.** ALDH1L1 is induced in reactive astrocytes but is not essential for regulating their proliferation. Immunostaining for ALDH1L1 (A–D,F,G,I), BrdU (E,F,K,L), and GFAP (H–J,L) in the injured adult neocortex 3 days post lesion. Following neuronal injury, ALDH1L1 is upregulated in astrocytes specifically in the ipsilateral hemisphere (A,B; the asterisk in B marks the wound site). The morphology of ALDH1L1$^+$ reactive astrocytes is shown in C. Note that one of these cells (arrow) has nuclear immunoreactivity; this was observed in many cells surrounding the wound site, but rarely in normal brains and not at all in Aldh111 null mice or when preimmune antisera was used (data not shown). Immunostaining for BrdU administered 2 and ½ hours prior to perfusion demonstrates the presence of ALDH1L1$^+$BrdU$^+$ cells (D,F; arrows in F). As expected, some BrdU$^+$ cells are not positive for ALDH1L1 (arrowhead in F); the small size of this nucleus suggests it is a microglial cell. Double labeling for ALDH1L1 and GFAP confirms that all ALDH1L1$^+$ cells are GFAP$^+$ astrocytes and vice versa (G–I); this allowed GFAP/BrdU double staining (J–L) to be used in NEUT2 $+/-$ mice to quantitate proliferation in Aldh111 null reactive astrocytes. Scale bar = 100 μm for A,B; 25 μm for J–L; 20 μm for D–I; 10 μm for C.
No morphological defects due to absence of *Aldh1l1* are detectable

Grossly, NEUT2 homozygous embryos derived from dams on both normal and folate-deficient diets appeared normal. Immunostaining for nestin revealed no apparent defects in the radial glial scaffold in any CNS region (Fig. 7A–C). Postnatally, hydrocephalus of varying severity was observed specifically in homozygotes (#hydrocephalic/#animals: wildtype, 0/10; heterozygous, 0/14; homozygous, 21/21), which caused death by 1 month of age in ~18.5% of homozygous mice (27/146). However, hydrocephaly was not rescued in −/−;TgN(RP23-7M9) mice (16/16 displayed hydrocephalus), demonstrating that it is not due to loss of *Aldh1l1*. Detailed analysis (including hematology, clinical chemistry, urinalysis, and histological examination of all tissues) of 3-month-old mice maintained for 6 weeks on a folate-deficient diet (0.4 mg/kg amino acid diet) revealed no significant differences between −/− and +/+. TgN(RP23-7M9) mice (two mice each genotype analyzed).

No proliferation defects are detectable in *Aldh1l1* null mice

Initial analysis of radial glial proliferation in embryos taken from dams fed a normal diet did not show any obvious difference in the absence of *Aldh1l1*. However, it remained a possibility that ALDH1L1 function might only be rate-limiting under certain conditions, i.e., reduced availability of folic acid. To test this idea, dams were placed on low folate diets as described above and embryos were analyzed for proliferation. As was the case for embryos on normal diets, immunostaining revealed no obvious expansion of proliferation into the midlines of either the tectum (Fig. 7DE) or spinal cord (data not shown) in low-folate embryos. To obtain quantitative data on proliferation in low-folate embryos, we looked at the lateral ganglionic eminences (LGE). Similar to the CGE (Fig. 3CD), ALDH1L1 is absent from the LGE at E12.5 but strongly upregulated by E14.5, correlating with a decrease in the number of mitotic cells in this region. To determine if ALDH1L1 is required for this decrease, we counted the number of PH3+ cells (mitotic cells) present in the ventricular zone at E14.5 and E15.5 (Fig. 7FG). Importantly, we compared −/− and +/−;TgN(RP23-7M9) littermates, sectioned coronally and counted only in the most rostral portion of the LGE (up the MGE) to avoid ambiguities due to possible regional proliferative differences; three embryos per genotype for each timepoint were stained. No differences were observed at either timepoint (PH3+ cells/section ± standard deviation, SD: E14.5 = 7.9 ± 6.15; E15.5 = 7.7 ± 6.15; TgN(RP23-7M9) 64.5 ± 7.9 (n = 7 sections), E15.5 = 46.3 ± 4.8 (n = 9 sections), E15.5 = 7.0 ± 4.8 (n = 9 sections)). These data indicate that *Aldh1l1* is not essential for the normal reduction in proliferation that occurs as radial glia mature.

An alternative possibility that existed was that ALDH1L1 might be required to prevent excessive proliferation in response to mitogenic signals after most developmental processes were finished. One situation where such signaling takes place is following neuronal injury, where damaged tissues induce astroglial proliferation as part of the repair process (Amat et al., 1996; Stoll et al., 1998; Raiach et al., 1999). The expression of ALDH1L1 in mature astrocytes (Neymeyer et al., 1997) suggested that it might be part of a pathway that limits proliferation of reactive astroglia following neuronal injury. To test this idea, we used an established injury model in which mice receive a cortical stab wound (Amat et al., 1996; Koguchi et al., 2002). Staining of injured adult wildtype mice demonstrated that ALDH1L1 protein is upregulated in astrocytes surrounding the wound site, but not on the contralateral side of the cortex (Fig. 8A–C). Upregulation could be detected as early as 1 day postlesion, and strong ALDH1L1 expression was seen by 3 days postlesion (data not shown). To determine the percentage of reactive astrocytes in 5-phase, BrdU was administered to injured mice at 3 days postlesion, and animals were anesthetized and perfused 2 and ½ hours later; this time point was chosen since astroglial proliferation has previously been shown to be maximal 3 days after injury (Amat et al., 1996). Double immunofluorescence for ALDH1L1 and BrdU identified numerous ALDH1L1+ BrdU+ cells, demonstrating that upregulation of ALDH1L1 is not incompatible with proliferation. To determine if ALDH1L1 might be involved in regulating the rate of reactive astroglial division, we compared the percentage of BrdU+ astrocytes in −/− and +/−;TgN(RP23-7M9) adult mice at 3 days postlesion (three mice per genotype). Since all ALDH1L1+ astrocytes surrounding the wound site were found to be GFAP+ and vice versa (Fig. 8G–I), double labeling for GFAP and BrdU was used for quantitation (Fig. 8J–L). No significant differences in the percentage of BrdU+ astrocytes were observed between the two genotypes [BrdU+GFAP+ cells/GFAP+ cells ± SD: −/− 16.5% ± 1 (n = 317 GFAP+ cells), +/−;TgN(RP23-7M9) 15.1% ± 1.8 (n = 425 GFAP+ cells)]. These results demonstrate that loss of ALDH1L1 activity is not required to regulate reactive astroglial proliferation.

Ventral midline radial glia are labeled in *Aldh1l1*::eGFP mice

*Aldh1l1*+ midline radial glia are a potential cellular target upon which supplemental folate may act. Because RP23-7M9 (the BAC used to restore *Aldh1l1* expression onto NEUT2 homozygotes) drove correct transgenic expression, ALDH1L1+ midline radial glia can now be genetically manipulated to test their role in neural tube closure. In addition, these cells can be isolated for transcriptional profiling in order to determine what other components of folate metabolism might be specifically expressed at the midline in normal or mutant mice. To facilitate this, we generated BAC transgenic mice that express eGFP from the *Aldh1l1* genomic locus. Although the full characterization of these mice will be presented elsewhere, we report here that eGFP expression in *Aldh1l1*::eGFP mice is detected specifically in midline radial glia in the early neural tube (Fig. 9). Use of fluorescence-activated cell sorting to purify *Aldh1l1*+ midline cells will enable comparisons with *Aldh1l1*+ cells within the neural tube both in normal mice as well as in folate-responsive NTD mouse models. These mice have been transferred to the publicly available repository maintained by the Mutant Mouse Regional Centers (MMRRC), and should provide a valuable tool for the dissection of metabolic pathways involved in neuralization as well as those that prevent disease.
DISCUSSION

In this study we provide the first evidence that an enzyme involved in folate metabolism has restricted expression in the developing neural tube. We have shown that ALDH1L1 is expressed in radial glia flanking the ventral midline from as early as E9.5, and becomes up-regulated at the dorsal midline soon after. Comparison with markers of dividing cells showed that regions expressing ALDH1L1 were largely nonproliferative and vice versa, demonstrating that proliferation is repressed in specific midline populations and raising the possibility that restricted expression of folate metabolic enzymes could be a mechanism controlling proliferation in subdomains of the neural tube. Finally, we demonstrated that Aldh1l1 null mice are not prone to NTDs and do not have detectable proliferation defects, indicating that ALDH1L1 activity is not essential for these processes.

It is important to note that although NEUT2 homozygous mice do not develop NTDs, Aldh1l1 remains a candidate for involvement in human NTDs. One obvious reason for this is that Aldh1l1 mutations that contribute to disease could be gain-of-function. Support for this idea comes from the metabolic effects of the teratogen valproic acid (VPA), which is an anticonvulsant that increases risk of human NTDs (Lammer et al., 1987). VPA is also teratogenic in mice and causes NTDs that can be prevented with folate supplementation (Padmanabhan and Shafiullah, 2003). When VPA-treated embryos were biochemically analyzed, they were found to contain reduced levels of 10-FTHF and increased levels of THF (Greene and Copp, 2005). These changes to cellular folate pools are the opposite of those observed in NEUT2 homozygous livers (Champion et al., 1994), and the same as what would be predicted if ALDH1L1 were overexpressed or overactive. Considering that we did not detect proliferation defects in Aldh1l1 null mice, whereas overexpressing ALDH1L1 suppresses proliferation (Krupenko and Oleinik, 2002; Oleinik and Krupenko, 2003), the available data suggest that if Aldh1l1 does affect neurulation it is gain-of-function mutations that would be most likely to cause disease.

If loss-of-function mutations in Aldh1l1 are relevant, there are several reasons why significant phenotypes were not observed in NEUT2 mice. First, the NEUT2 deletion eliminates both Aldh1l1 and Uroc1 (described above). Loss of Uroc1 is believed to mitigate the disruption of folate metabolism in the absence of Aldh1l1 by reducing the load on the cellular THF pool (Cook, 2001b). Therefore, it remains possible that a specific targeted mutation in the Aldh1l1 gene could reveal a role in neural tube closure that cannot be readily observed in NEUT2 mice. With regard to this latter point, it should be noted that our analysis of NTD incidence in NEUT2 mice maintained on low folate included only 354 embryos; as the NTD penetrance was very low, this number was insufficient to provide a definitive conclusion on the potential role of Aldh1l1. As the mitigating effects of the Uroc1 deletion may have contributed to the observed low

Fig. 9. Aldh1l1::eGFP BAC transgenic mice drive expression in midline spinal cord radial glia. Immunostaining for the radial glial marker BLBP (A,C) and eGFP (B,C) in the E12.5 spinal cord of Aldh1l1::eGFP BAC transgenic embryos. Note that, whereas BLBP is expressed in all radial glia, eGFP is restricted to cells in the most ventral and dorsal areas; this is identical to endogenous ALDH1L1 expression (compare with Fig. 1). Scale bar = 50 μm.
penetration, either targeted mutation of Aldh1l1 or analysis of a significantly larger number of NEUT2 embryos will be required to resolve this issue. It should also be noted that the etiology of human NTDs is complex and depends on both genetic and environmental factors (Botto et al., 1999; Juriloff and Harris, 2000). Accordingly, it is possible that the mixed NEUT2 genetic background studied here is not susceptible to Aldh1l1 deficiency under the conditions tested. This issue is clearly relevant to modeling folate-preventable NTDs in mice, as genetic background has been observed to alter the frequency of cranial NTDs in Sp^{2H}/Sp^{2H} embryos (Fleming and Copp, 2000). Another factor to consider is the difference in the amount of time neurulation takes in mice compared with humans. Whereas neurulation in the mouse is complete in less than 2 days (Harris and Juriloff, 1999), humans require nearly 2 weeks to complete the process (Nolte, 1993). Efforts to model human neurodegenerative diseases in mice have often required drastic increases in the toxicity of disease-causing molecules (above that already present in the forms that are pathogenic in humans) in order to see a phenotype in the short lifespan of a mouse (Watase and Zoghbi, 2003). Similarly, multiple folate-dependent pathways may need to be disrupted in mice in order to mimic the effects of prolonged deficiency of a single mutant gene in humans. Additionally, and as in other cases of mouse mutants without detectable phenotypes, compensation by or alteration of the activity in other components of folate metabolism may have obscured a role for Aldh1l1 in neurulation. Finally, we note that other folate metabolizing enzymes implicated in human NTDs have been knocked out in mice with no apparent detrimental effect on neural tube closure. For example, numerous studies have shown that a thermolabile allele of the 5,10-methylenetetrahydrofolate reductase gene (Mthfr) is associated with increased risk of NTDs in multiple human populations (reviewed in Boyles et al., 2005); however, Mthfr null mice do not develop NTDs (Chen et al., 2001). This example in particular makes it clear that negative results in mouse models must be interpreted with caution.

Although we did not detect increases in radial or astroglial proliferation in NEUT2 homozygotes, overexpression of Aldh1l1 has previously been shown to suppress proliferation in vitro (Krupenko and Oleink, 2002; Oleink and Krupenko, 2003). Moreover, multiple tumor tissues were shown to downregulate Aldh1l1 expression (Krupenko and Oleink, 2002). These data indicate that while Aldh1l1 is not an essential regulator of proliferation, its presence may be restrictive. If correct, this would suggest a testable mechanism by which folic acid might prevent NTDs in the absence of defective folate metabolism. By restricting proliferation in and around the midline, ALDH1L1 may limit critical compensatory proliferative adjustments necessary in the presence of NTD-inducing mutations. However, the presence of high levels of exogenous folate could overcome the repressive effects of ALDH1L1, allowing compensatory reactions to proceed. Experimental support for such a model comes from several sources. First, excessive apoptotic cell death was observed in Cited2 null mice specifically at the midline of the tectum (Barbera et al., 2002), a region in which we have shown both restricted expression of ALDH1L1 and minimal proliferation. Although folate does not prevent this excessive cell death (Barbera et al., 2002), apoptosis is likely causative, as crossing splotch mice onto a p53 null background completely prevents NTDs (Pani et al., 2002).

Thus, these data argue that folate rescues NTDs in Cited2 null mice by driving compensatory processes in or near ALDH1L1-"midline" cells. Moreover, exogenous folic acid has been shown to be capable of overcoming ALDH1L1-mediated inhibition in vitro: overexpression of ALDH1L1 represses growth of several cell types, but addition of folinic acid (5-FTHF) abolishes this repression (Krupenko and Oleink, 2002). A direct genetic test for an inhibitory role of ALDH1L1 would be to determine if the NEUT2 microdeletion can rescue NTDs in folate-responsive mouse models, particularly the Cited2 knockout, which has no inherent defects in folate cycling. Similarly, it would be interesting to know whether ALDH1L1 overexpression can increase incidence of disease in mouse strains that develop folate-responsive NTDs (e.g., by placing the RP23-7M9 BAC transgene onto a Cited2 null background).

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LITERATURE CITED


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