Cux2 (Cutl2) integrates neural progenitor development with cell-cycle progression during spinal cord neurogenesis

Angelo Iulianella1, Madhulika Sharma2, Michael Durnin1, Greg B. Vanden Heuvel2 and Paul A. Trainor1,2,*

Neurogenesis requires the complex integration of neural progenitor proliferation, cell-cycle exit, migration and differentiation. In the developing nervous system, neural stem cells are present in embryonic day (E) 8.5-9.5 mouse embryos within the neuroepithelium, where they proliferate extensively through symmetric cell divisions. Shortly thereafter, these neural stem cells are referred to as radial glia, reflecting their generation of a neuron followed by a radial glial cell through asymmetric cell division (McConnell, 1995; Temple, 2001). The coupling of cell-cycle exit to differentiation programs ensures the correct numbers of neuronal subtypes are consistently generated to form functional neural populations by the G1 cyclin inhibitor p27Kip1 (Fero et al., 1996; Gui et al., 2007; Kiyokawa et al., 1996; Nakayama et al., 1996). Although it is not clear how the generation of cell type diversity is coupled to cell-cycle withdrawal during neurogenesis, it has been suggested that the length of the cell cycle may impact directly on cell-fate determination (Shen et al., 2006; Wilcock et al., 2007).

The first neurons to be born in the spinal cord are interneurons, followed by ventral motoneurons (Sechrist and Bronner-Fraser, 1991), and central to the formation of both interneurons and motoneurons is the control of cell-cycle exit in neural progenitor populations by the G1 cyclin inhibitor p27Kip1 (Fero et al., 1996; Quaggin et al., 1996; Zimmer et al., 2004). In vertebrates, two Cut homologs Cux1 (Cutl1) and Cux2 (Cutl2) exist (Neufeld et al., 1992; Quaggin et al., 1996; Tavares et al., 2000; Valarche et al., 1993). Cux1 has been hypothesized to function in cell-cycle control, in part by regulating the G1 cyclin inhibitors p21Cip1 and p27Kip1 (Coqueret et al., 1998; Ledford et al., 2002), and consistent with this idea Cux1 mutants displayed reduced growth and organ hypoplasia (Ellis et al., 2001; Luong et al., 2002; Sinclair et al., 2001), whereas Cux1 overexpressing transgenics exhibit multiorgan hyperplasia (Ledford et al., 2002).

In contrast to Cux1, the role of Cux2 remains poorly characterized, particularly in the nervous system, where it is expressed at high levels during neurogenesis (Iulianella et al., 2003; Nieto et al., 2004; Quaggin et al., 1996; Zimmer et al., 2004). Cux2 also be important for directing both ventral and dorsal interneuron fate (Mizuguchi et al., 2006; Yang et al., 2006); however, its primary role is to promote neural progenitor maintenance (Androussellis-Theotokis et al., 2006).

The spinal cord has been used extensively as a model of homeodomain transcription factor family member, Cux2 (Cutl2), regulates cell-cycle progression and development of neural progenitors. Cux2 loss-of-function mouse mutants exhibit smaller spinal cords with deficits in neural progenitor development as well as in neuroblast and interneuron differentiation. These defects correlate with reduced cell-cycle progression of neural progenitors coupled with diminished Neurod and p27Kip1 activity. Conversely, in Cux2 gain-of-function transgenic mice, the spinal cord is enlarged in association with enhanced neuroblast formation and neuronal differentiation, particularly with respect to interneurons. Furthermore, Cux2 overexpression induces high levels of Neurod and p27Kip1. Mechanistically, we discovered through chromatin immunoprecipitation assays that Cux2 binds both the Neurod and p27Kip1 promoters in vivo, indicating that these interactions are direct. Our results therefore show that Cux2 functions at multiple levels during spinal cord neurogenesis. Cux2 initially influences cell-cycle progression in neural progenitors but subsequently makes additional inputs through Neurod and p27Kip1 to regulate neuroblast formation, cell-cycle exit and cell-fate determination. Thus our work defines novel roles for Cux2 as a transcription factor that integrates cell-cycle progression with neural progenitor development during spinal cord neurogenesis.

KEY WORDS: Cut-like, Cux, Spinal cord, Neurogenesis, Interneurons, Motoneurons, Neurod1, p27Kip1, Cell cycle, Mouse

INTRODUCTION

Neurogenesis requires the complex integration of neural progenitor proliferation, cell-cycle exit, migration and differentiation. In the developing nervous system, neural stem cells are present in embryonic day (E) 8.5-9.5 mouse embryos within the neuroepithelium, where they proliferate extensively through symmetric cell divisions. Shortly thereafter, these neural stem cells are referred to as radial glia, reflecting their generation of a neuron followed by a radial glial cell through asymmetric cell division (McConnell, 1995; Temple, 2001). The coupling of cell-cycle exit to differentiation programs ensures the correct numbers of neuronal subtypes are consistently generated to form functional neural circuits (Kintner, 2002). Of central importance to this process is the decrease in expression of progenitor determinants, the increase in cell-cycle inhibitors and the implementation of cell-fate specification programs (Durand and Raff, 2000; Jessell, 2000). How these processes are coordinated remains the focus of active investigation.

The spinal cord has been used extensively as a model of neuronal differentiation and much is known about the regulatory mechanisms coordinating these distinct cellular activities are poorly understood. Here we demonstrate for the first time that a Cut-like homeodomain transcription factor family member, Cux2 (Cutl2), regulates cell-cycle progression and development of neural progenitors. Cux2 loss-of-function mouse mutants exhibit smaller spinal cords with deficits in neural progenitor development as well as in neuroblast and interneuron differentiation. These defects correlate with reduced cell-cycle progression of neural progenitors coupled with diminished Neurod and p27Kip1 activity. Conversely, in Cux2 gain-of-function transgenic mice, the spinal cord is enlarged in association with enhanced neuroblast formation and neuronal differentiation, particularly with respect to interneurons. Furthermore, Cux2 overexpression induces high levels of Neurod and p27Kip1. Mechanistically, we discovered through chromatin immunoprecipitation assays that Cux2 binds both the Neurod and p27Kip1 promoters in vivo, indicating that these interactions are direct. Our results therefore show that Cux2 functions at multiple levels during spinal cord neurogenesis. Cux2 initially influences cell-cycle progression in neural progenitors but subsequently makes additional inputs through Neurod and p27Kip1 to regulate neuroblast formation, cell-cycle exit and cell-fate determination. Thus our work defines novel roles for Cux2 as a transcription factor that integrates cell-cycle progression with neural progenitor development during spinal cord neurogenesis.

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1Stowers Institute for Medical Research, 1000 E. 50th Street, Kansas City, MO 64110, USA. 2Department of Anatomy and Cell Biology, University of Kansas Medical Center, Kansas City, KS 66160, USA.

*Author for correspondence (e-mail: pat@stowers-institute.org)
marks interneurons in the marginal zone (mz) of the developing spinal cord (Iulianella et al., 2003) as well as progenitor cells in the subventricular zone (svz) and their descendants in the outer layers of the mouse cortex (Nieto et al., 2004; Zimmer et al., 2004). Here we reveal, through gain- and loss-of-function approaches in mouse models, novel roles for Cux2 in regulating neurogenesis. Cux2 directs neuroblast development and neuronal differentiation and cell-fate determination in the spinal cord by coupling cell-cycle progression in neural progenitors with differentiation through the direct activation of Neurod and p27kip1.

MATERIALS AND METHODS

Construction of full-length Cux2 expression vectors

Full-length mouse Cux2 cDNA was reverse transcribed from E13.5 embryo head total RNA using Superscript II Reverse Transcriptase (Invitrogen, Carlsbad, CA), oligo dT and random primers (Gene Racer Kit, Invitrogen), supplemented with mouse Cux2-specific antisense primers against the 5’ and 3’ ends of Cux2 cDNA (GTCCCTGGGACCTGGCAGGTGTTA and GGGACGTGGGCCCCATCATATGGTTT, respectively), using Quaggin et al. (Quaggin et al., 1996) as the reference sequence. Full-length Cux2 cDNA was amplified using AmpliTag Supermix (Invitrogen) and a Cux2 5’ sense primer (GAGCGATGTAATGCTCCGGTCTGAA) and 3’ antisense primer (GGACGTGGGCCCCATCATATGGTTT), allowing for sufficient time during elongation to amplify the 4.6 kb mCux2 cDNA. A 4.6 kb full-length Cux2 cDNA was TA cloned into the TOPO PCR II vector (Invitrogen) and the entire cDNA was sequenced using a series of primers covering the full-length coding sequence. Errors in the coding sequence were corrected using the Quickchange XL kit (Stratagene, La Jolla, CA), and subcloned into pBluescript II. A Kozak consensus (GCGTAATATGGCCACAACC) was present in the gene trap vector using the following primers: AACA-GCAATCGGCGTCTGCTG and TCCACCATGTAATCCGGCA. Cux2neo/neo were maintained on a Sv129 background and mated to generate homozygous mutant pups. The frequency of Cux2neo/neo pups was 20.5% less than expected from Mendelian segregation, indicating some prenatal lethality.

Generation of a Cux2 mutant mouse line

A Cux2 gene trap mouse embryonic stem (ES) cell line was identified by searching for Cux2 sequences in the Lexicon Genetics Omnimbank library (http://www.lexicon-genetics.com/discovery/omnimbank.htm). Gene trap ES clone OSTM40231 was identified as having a VICTR48 MTII gene trap insertion in the third intron of mouse Cux2 gene on chromosome 5 (Fig. 2B) (Zambrowicz et al., 1998). This clone was purchased from Lexicon Genetics, thawed, expanded on feeder layers using standard ES culture procedures (Nagy et al., 2003) and injected into C57BL/6 recipient blastocysts. Two strong chimeric mice were subsequently used to generate agouti progeny carrying the gene trap insertion in the Cux2 locus.

Based on the sequence surrounding the insertion site, we developed a genotyping strategy using a primer specific to the LTR2 region of the gene trap (AAATGGCGTTACTAAGCTAGCTTG) and two locus-specific primers upstream and downstream of the insertion site (GTCGCCATATGCCCTCC and GTCCCTTGAGCCTGGCC, respectively). A 392 bp wild-type allele was amplified using the latter two primers, while a mutant allele of approximately 180 bp was generated using TGTTGCAATATCCCTG and AAATGGCGTTACTAAGCTAGCTTG primers. Heterozygotes were confirmed by amplifying for the neomycin cassette present in the gene trap vector using the following primers: AACAGCAATCGGCGTCTGCTG and TCCACCATGTAATCCGGCA. Cux2neo/neo were maintained on a Sv129 background and mated to generate homzygous mutant pups. The frequency of Cux2neo/neo pups was 20.5% less than expected from Mendelian segregation, indicating some prenatal lethality.

Antibody production

For Cux2 polyclonal antibody production, a synthetic peptide (Q-NEKGTGEQVHSEPLS-C) derived from the C-terminal region of murine Cux-2 (amino acids 1305-1322) (Quaggin et al., 1996) was synthesized (Synpep, Dublin, CA), conjugated to KLH, and used to immunize New Zealand White rabbits (Covance, Denver, PA). The resulting antisera were purified against the immunizing peptide by affinity chromatography. The antibody was used at 1/3000 dilution on frozen sections and revealed with either goat anti-rabbit Alexa Fluor 594 or goat anti-rabbit Alexa Fluor 488 (Molecular Probes/Invitrogen, Carlsbad, CA). The specificity of the anti-Cux2 antibody was examined by immunohistochemistry following Cux2 overexpression in both embryonic chick and mouse neural tubes, and in Cux2neo/neo hypomorphic neural tubes (see Fig. S3 in the supplementary material). In addition, the specific Cux2 signal in sections was abolished by incubation with a Cux2 C-terminal peptide (see above; data not shown).

Also, western blots of E12.5 embryonic brains using the anti-mCux2 antibody identified an endogenously expressed doublet migrating at 110 kDa on SDS-PAGE (Fig. 2B), in agreement with the expected size for the full-length Cux2 protein.

Western blot analysis

Western blot analysis on SDS-PAGE was performed on protein extracts from E12.5 embryonic heads from two separate wild-type (+/+), Cux2neo/neo (+/-) and Cux2neo/neo (-/-) mutant littersmates using the Cux2 polyclonal antibody we generated. Briefly, two samples from each genotype were loaded on a 7.5% polyacrylamide gel along with loading controls (BioRad, Hercules, CA), resolved using SDS-PAGE and transferred onto nitrocellulose membranes (Biorad). Blots were blocked with Odyssey blocking buffer (Licor Biosciences, Lincoln, NE) and incubated in 1/2000 dilution of the rabbit anti-Cux2 antibodies overnight at 4°C. The blot was subsequently washed with TBST, and incubated with goat anti-rabbit IRDye 800 secondary antibodies (Licor Biosciences) diluted at 1/7500 in blocking buffer at room temperature for 1-2 hours. After washes in TBST, bands were visualized using the Licor Odyssey Visualization System (Licor Biosciences). To control for sample loading, following anti-mCux2 immunoblotting, the membrane was washed, re-blocked and then incubated in a 1/10000 dilution of anti-alpha Tubulin antibody (Sigma, St Louis, MO) at room temperature for 1 hour. Anti-alpha Tubulin signal was revealed using the SuperSignal West Pico kit (Pierce, Rockford, IL), with a brief exposure to detection film. In addition, Coomasie Blue staining of replicate polycrylamide gels indicated approximately equal protein loading across the different samples (data not shown).

In ovo electroporations of chick neural tubes

Cux2-NeoCIG and pCIG (empty control vector) expression plasmids were prepared at 3-5 μg/μl concentration in water, and 0.1% Fast Green solution was added to visualize injection into HH10-12 stage chick neural tubes. Eggs were windowed and prepared for electroporation as described (Krnail, 2004). Five millisecond pulses of 20 volts each were applied using a CUY-21 electroporator and platinum electrodes. Eggs were re-sealed with cellophane tape and allowed to incubate for another 24-48 hours at 37°C under humidified conditions.
ChIP assays
Chromatin immunoprecipitation (ChIP) assays were performed using the EZ ChIP Kit (Upstate, Lake Placid, NY) according to the manufacturer’s directions, with the following modifications. Brains dissected from E12.5 CD1 mice were used for ChIP analysis. The native protein-DNA complexes were cross-linked by treatment with 1% formaldehyde for 15 minutes. Briefly, equal aliquots of isolated chromatin were subjected to immunoprecipitation with an anti-Cux2, anti-RNA polymerase II or IgG antibodies. DNA associated with immunoprecipitates was used as a template for PCR analysis with primers producing a 248 bp fragment encompassing the Neurod promoter spanning +48 to –201, relative to the transcription start site, or with primers producing a 227 bp fragment of the p27Kip1 promoter spanning –707 to –481, relative to the transcription start site. Primers used were: Neurod 5′-TACTGTTGGGTTAGGGGAATGTGGT-3′ and 5′-TGAGAGAGGCTACTTCCTCCAGC-3′; and p27Kip1 5′-CAGACAGCTTTGTGTCACGTC-3′ and 5′-GCTGACAGAAGAAGATGATTG-5′. To control for non-specific immunoprecipitation of chromatin by the Cux-2 antibody, the same DNA was used as a template for PCR analysis with primers producing a 250 bp fragment –5557 to –5308 relative to the Neurod transcription start site, or with primers producing a 148 bp fragment –5241 to –5094 relative to the p27Kip1 transcription start site was performed. Primers used were: Neurod 5′-AGCGAGCTCTACATTCTCCAG-3′ and 5′-TAGCAGAATCTCTCCAGC-3′, and p27Kip1 5′-TCCAGTGTGATGCTGTCCTG-3′ and 5′-CAAGTCTGTGAGAAGAGAGTGTGGC-3′. Results obtained from unrelated antibody controls combined with enrichment when the Cux2 antibody is used confirmed they were in the linear range of product amplification and not a consequence of specifically immunoprecipitating chromatin.

Determination of cell-cycle parameters
Proliferation differences between Cux2neo/neo mutants and control wild-type littermates were assessed by counting the mitotic nuclei at E10.5 and 11.5 stained using a rabbit anti-phosphohistone H3 (ph3) antibody (Upstate) and goat anti-rabbit IgG Alexa Fluor 488 secondary antibodies (Molecular Probes/Invitrogen; Fig. 6D-E). Sections were counterstained with DAPI. Counts were made unilaterally in the medial neural tube, with pixel area being constant between control and mutant samples. The average values for protein is normally abundant (see Fig. S1 in the supplementary material), stained sections in the ventral half of the E10.5 neural tube, where Cux2 regulates neural differentiation.

Immunohistochemistry
Specimens were processed for cryostat and cut at 10 µm. Sections were blocked in 10% goat serum (Zymed/Invitrogen, Carlsbad, CA) for 30 minutes to 1 hour at room temperature. Primary antibodies were diluted in 10% goat serum and incubated on sections overnight at 4°C. Sections were subsequently rinsed with PBS + 0.05% Triton X-100 and incubated with the following secondary antibodies diluted at 1/250 with 10% goat serum for 1 hour: goat anti-mouse IgG Alexa Fluor 594, goat anti-mouse IgM Alexa Fluor 594, chicken anti-rabbit IgG Alexa Fluor 488, goat anti-mouse IgG Marina Blue or goat anti-guinea pig Alexa Fluor 594 (Molecular Probes/Invitrogen).

For the following primary antibodies were used: Rabbit anti-Cux2 at 1/3000, mouse anti-TuJ1 at 1/500-1/1000 (Covance, Berkeley, CA), mouse anti-p27Kip1 at 1/200-1/300 (BD Transduction Laboratories, BD Biosciences/Pharmingen), mouse anti-NeuN at 1/500 (Chemicon, Temecula, CA), neuronal rabbit anti-Ki67 at 1/40 (Neomarkers, LabVision, Fremont, CA), guinea pig anti-mouse Olig2 at 1/10000 (kind gift from Dr Ben Novitch, Northwestern University, Chicago, USA), guinea pig anti-Chx10 at 1/5000 (kind gift from Dr Sam Pfaff, Salk Institute, California, USA) and rabbit-anti-NeuroD1 at 1/1000 (kind gift of Dr Jacques Drouin, IRCM, Montreal, Canada). The following mouse monoclonal antibodies were obtained from the Developmental Studies Hybridoma Bank developed under the auspices of the NICHD and maintained by the University of Iowa, Department of Biological Sciences (Iowa City, IA 52242): Lim1/Lhx1 (4F2), Neurod neo/neo, and Cux2 transgenics. Statistical relevance was tested using a one-tailed Student’s t-test with the level of significance set at <0.05. Differences between the Cux2 loss-of-function and gain-of-function experiments were expressed as percentage differences between the average value from the experimental and control samples (see Table S1 in the supplementary material).

The effect of Cux2 overexpression on TuJ1 expression in sectioned chick neural tubes was assessed by counting the number of GFP- and TuJ1-double-positive cells and expressed as a percentage of total GFP-positive cells. The effect of Cux2 overexpression was measured using seven different specimens and compared to 14 different specimens for control electroporations. Standard error was computed using regression analysis of GFP/TuJ1-double-positive versus GFP-total-positive cell numbers. Statistical significance was measured using a one-tailed Student’s t-test with significance taken at <0.05.

The effect of Cux2 loss and overexpression on motoneuron and interneuron formation in embryonic mouse spinal cords was assessed by counting Isl1-positive, Lhx1-positive and Chx10-positive cells in the ventral region of thoracic and lumbar-level sections of E10.5 neural tubes. The pixel area used in the cell counts was kept constant between the Cux2neo/neo mutant and wild-type or heterozygous control littermates, and encompassed the entire ventral neural tube from the dorsal limit of the ventral horn to the floor plate (fp) (Fig. 7), which corresponds to the domain of high Cux2 protein expression (Fig. 1A and see Fig. S1A in the supplementary material). All counts were
done unilaterally. For Isl1 counts, a total of 19 sections from eight different E10.5 wild-type and Cux2neo/neo heterozygote neural tubes, 34 sections from 11 different Cux2neo/neo mutants and nine sections from four different Nestin-enhancer-driven Cux2-IREs-EGFP transgenics were used. For Lhx1, eight sections from four different E10.5 wild type, 15 sections from eight different Cux2neo/neo mutants and nine sections from four different Cux2 transgenic embryos were used. Significant differences from controls were determined using an one-tailed Student’s t-test with the level of significance taken at <0.05 (see Table S2 in the supplementary material). For Chx10 number, sections from forelimb regions of five different E10.5 wild type and eight different Cux2neo/neo littermates were used (Fig. 8).

**RESULTS**

**Distribution of Cux2 in the developing spinal cord**

We initially characterized the activity of Cux2 during murine spinal cord neurogenesis by profiling its distribution between E9.5 and 11.5 with a Cux2 polyclonal antibody (see Materials and methods). At E9.5, Cux2 protein was observed only in the roof plate (rp) of the neural tube and dorsal epidermis (data not shown). Interestingly, by E10.5, high levels of Cux2 protein were detected in neuronal precursors in the vz and in nascent neurons exiting the cell cycle in the intermediate zone (iz) (arrowhead in Fig. 1A) at the forelimb bud level. Importantly, Cux2 was expressed in many proliferating progenitor cells of the vz, demarcated by S-phase nuclei that incorporated BrdU over a period of 30 minutes (Fig. 1B). Cux2 was also detected in the developing ventral interneurons of the mz, but only in the occasional cell in the ventral horn of the E10.5 spinal cord, where motoneurons are typically generated. By E11.5, Cux2 protein was abundantly detected in both the rp and fp in progenitor cells in the vz, in nascent neurons of the iz and in post-mitotic interneurons in the mz (Fig. 1C,F,I; see Fig. S1A in the supplementary material). Interestingly, Cux2 protein was notably absent from most post-mitotic motoneuron populations in forelimb bud regions of the spinal cord (see Fig. S1A in the supplementary material). Thus Cux2 is expressed in proliferating neural progenitors and in nascent neurons (especially interneurons) during the peak phase of spinal cord neurogenesis.

The high level of Cux2 activity observed in the vz and iz was intriguing because this is the location where proliferative neuroblasts undergo cell-cycle exit and respond to signals that trigger terminal differentiation (Gui et al., 2007; Rao and Sockanathan, 2005). This implied that Cux2 might play a role in cell-cycle regulation and/or differentiation during neurogenesis. Previous work has established the importance of the Cip/Kip family of proteins in promoting cell-cycle withdrawal (Gui et al., 2007) and in particular p27Kip1, a G1-cyclin-dependent kinase inhibitor, in regulating cell cycle exit and post-mitotic differentiation during spinal cord neurogenesis (Fero et al., 1996; Kiyokawa et al., 1996; Nakayama et al., 1996). P27Kip1 protein is abundantly detected in the vz, rp and fp of E11.5 wild-type embryos (Fig. 1D,G,J; see Fig. S1B in the supplementary material). In the ventral region of the neural tube we frequently observed nuclei exiting the vz that were co-stained with p27Kip1 and Cux2 (arrowhead, Fig. 1K). Particularly striking was the co-localization of p27Kip1 with Cux2 (Fig. 1E,H,K; see Fig. S1C in the supplementary material) within a region of the vz 2-4 cell layers thick (Fig. 1F-H; see Fig. S1A-C in the supplementary material) that correlates with neural progenitor cells undergoing cell-cycle exit and terminal differentiation. These results suggested that Cux2 might play important roles during spinal cord neurogenesis.

Cux2 is required for neuronal development

To functionally test the role of Cux2 during neurogenesis, we used gene trapped ES cells (Zambrowicz et al., 1998) to generate a Cux2 mutant mouse line (Fig. 2A). Cux2neo/neo adults were produced at less than expected Mendelian frequency (see Materials and methods), which suggested the presence of an embryonic lethal phenotype. Western blots of E12.5 head lysates from two individual wild-type (+/+), Cux2neo/neo (+/–) and Cux2–/– (–/–) mutants (Fig. 2B, lanes 5 and 6, respectively). The severe reduction of Cux2 protein levels in Cux2neo/neo embryos was confirmed by immunoprecipitation (data not shown) and immunohistochemistry (see Fig. S3G,H in the supplementary material), and indicates that the Cux2neo/neo gene trap mutant is a severe hypomorph.

E11.5 Cux2neo/neo mutant embryos exhibited hypoplasic neural tube compared with their wild-type or heterozygous littermates at E11.5 (Fig. 2C,D). TUNEL staining revealed no significant differences in the amount of cell death between Cux2neo/neo mutants and control littermate neural tubes at E10.5 and 11.5 (data not shown), suggesting that loss of Cux2 resulted in proliferation and/or differentiation deficiencies in the developing spinal cord. Histological analysis revealed that Cux2 loss greatly affected axonal mass in the mz of the neural tube and in the ventral commissure, which preferentially stain with eosin (black arrowhead, Fig. 2D). These defects were exemplified with the definitive pan neural marker TuJ1...
was much narrower throughout the entire dorsoventral extent of TuJ1 immunostaining correlating with the mz of the neural tube demarcates the boundaries of these structures (Fig. 2E,J). The domain neurons within the mz and dorsal root ganglia (drg) and clearly differentiated mz layer in and TuJ1 (red) further illustrated the preferential reduction of the mutant embryos (Fig. 2J). Double immunostaining with Ki67 (green) by 13%, the mz was 49% smaller, reflecting a preferential loss of post-mitotic populations (see Table S1 in the supplementary material). Additional defects seen in E11.5 loading control westerns for the embryo lysates showed anti-alpha Tubulin levels migrate near 50 kDa. (neuronal-specific β-tubulin), which labels axons of post-mitotic neurons within the mz and dorsal root ganglia (drg) and clearly demarcates the boundaries of these structures (Fig. 2E,J). The domain of TuJ1 immunostaining correlating with the mz of the neural tube was much narrower throughout the entire dorsoventral extent of Cux2 mutant embryos (Fig. 2J). Double immunostaining with Ki67 (green) and TuJ1 (red) further illustrated the preferential reduction of the differentiated mz layer in Cux2neo/neo mutants (Fig. 2J, compare with 2E). Cux2 mutants exhibited a 27.8% reduction in overall neural tube area compared with controls; however, whereas the vz was diminished by 13%, the mz was 49% smaller, reflecting a preferential loss of post-mitotic populations (see Table S1 in the supplementary material). Additional defects seen in E11.5 Cux2neo/neo embryos included drg, which were evident by TuJ1, p27^Kip1, NeuN (Neuna60 – Mouse Genome Informatics) and NeuN immunostaining (Fig. 2J,K,M; see Fig. S2 in the supplementary material). All the post-mitotic markers examined revealed a requirement for Cux2 function in influencing the size of the drg, but not necessarily in the establishment of early patterning, which remained grossly intact.

**Cux2 influences progenitor pool size through promoting cell-cycle length**

To determine whether the neural differentiation defects observed in Cux2 mutant embryos were primarily due to alterations in neural progenitor populations, we used Pax6 to investigate the integrity of ventral spinal cord progenitors (Fig. 3). Cux2 was co-expressed with Pax6 in ventral neural progenitors at E10.5 and 11.5 (data not shown). In comparison to wild type, Cux2 mutant embryos exhibited a mediolateral reduction in Pax6-positive nuclei in the neural tube at E11.5 (Fig. 3A,B), demonstrating a requirement for Cux2 in the formation and/or maintenance of spinal cord progenitors.

Given the requirement for Cux2 function in spinal cord progenitors, we next evaluated the progression of the cell cycle in the vz cells of E10.5 embryos via sequential pulsing of neural progenitors, we next evaluated the progression of the cell cycle in the vz cells of E10.5 embryos via sequential pulsing of neural progenitors with IDU and BrdU as previously described (Quinn et al., 2007). Briefly, the G2/M phase of the cell cycle was labeled through a 2 hour pulse with IDU (green/yellow in Fig. 4A,C), while the proliferating nuclei localized to the lateral half of the vz were labeled by a 30 minute pulse with BrdU (red/yellow in Fig. 4A). By counting the number of nuclei in S phase (called Scells; i.e. IDU label alone, Fig. 4A), the S-phase length (called Ts) can be determined (Fig. 4C). Cux2neo/neo mutants (n=8) displayed a significantly greater number of nuclei in S phase, and a reduced number of nuclei in G2/M phase (i.e. nuclei that have left the S phase) relative to controls (n=14; Fig. 4B; Table 1). This resulted in a significant 62% increase in neural progenitor S-phase length in Cux2neo/neo mutant embryos relative to controls (P=0.0001, Table 1), and reflected a slowing of the cell cycle in Cux2 mutants.
To determine if the alteration of cell-cycle dynamics in the Cux2 mutants affected neural progenitor proliferation, we examined the number of neuroepithelial mitoses in Cux2neo/neo and control littermates. Anti-pH3 antibodies labeled mitotic nuclei at the luminal edge of the vz (green, Fig. 4D,E). At E10.5, proliferation levels were comparable between Cux2neo/neo mutants (n=18) and control (n=9) littermates (Fig. 4F). However, by E11.5 the Cux2 hypomorphs (n=5) displayed significantly reduced pH3 staining (P=0.009) relative to littermate controls (n=6; Fig. 4F). Thus the reduced G2/M progression observed in the Cux2 mutants at E10.5 resulted in reduced proliferation by E11.5. This correlates with the onset and peak temporal period of Cux2 activity (Fig. 1) and can account for the mediolateral reduction in Pax6-positive progenitors observed at the same developmental stage. A consequence of slowing down the cell-cycle progression in the vz progenitors is decreased post-mitotic neurons in the mz, and indeed TuJ1 immunostaining illustrated the greatly reduced mz in Cux2neo/neo mutant embryos (Fig. 2E,J).

Cux2 is required for cell-cycle exit and neuronal differentiation

The effect of Cux2 on Pax6-positive neural progenitors raised the possibility that Cux2 influences the formation of neuroblasts. Here we define neuroblasts as migratory immature neuronal descendants of progenitor cells that express basic helix-loop-helix (bHLH) proteins such as Neurod1 (Lee et al., 2000). Interestingly, Neurod promotes neuronal differentiation in part through the activation of p27Kip1 (Farah et al., 2000; Lee et al., 1995), and p27kip1 is upregulated and co-expressed with Cux2 in the iz, which corresponds with cell-cycle withdrawal and terminal differentiation of neural progenitors (Fig. 1C-K, Fig. 2F,G; see Fig. S1A-C in the supplementary material). We therefore assessed whether the reduction in the size of the differentiated mz layer in Cux2 mutants embryos was due to specific effects on Neurod and p27kip1.

We initially examined the relationship between Neurod1 and p27kip1 in the developing spinal cord (see Fig. S4 in the supplementary material). At E9.5, Neurod1-positive nuclei were detected at the lateral edges of the vz in a pattern that was mostly non-overlapping with p27kip1-labeled cells (see Fig. S4A in the supplementary material). The occasional co-labeled nuclei were

Fig. 3. Cux2 regulates neural progenitors and neuroblasts. (A-D) Pax6 expression in spinal cords of E11.5 wild-type (A), Cux2neo/neo (B) and Nestin–Cux2-ires-EGFP (C,D) embryos. (D) GFP (green) overlay on anti-Pax6 immunohistochemistry (red) revealed a mediolateral expansion of Pax6-labeled vz cells following Cux2 overexpression (arrows). (E-H) Neurod immunostaining of neuroblasts exiting the vz and also cells in the drg in E11.5 control (E), Cux2neo/neo (F) and Nestin–Cux2-ires-EGFP (G,H) spinal cords. (IJ) Anti-Neurod1 (green) labeling of neuroblasts in the iz (arrow) adjacent to anti-p27kip1 (red) immunohistochemistry in E10.5 ventral spinal cords of control and Cux2neo/neo embryos. (K,L) Enhanced Neurod1 (red) activity (arrows) in an E10.5 Cux2 transgenic embryo overexpressing Cux2-ires-EGFP (green). Scale bars: 250 μm in A,E; 500 μm in I.

Fig. 4. Reduced cell cycle progression in Cux2 mutants. (A,B) Detection of M phase by IDU (green), S phase by BrdU (red) and G2/S phase by IDU/BrdU (yellow/orange) labeling in E10.5 forelimb bud level neural tubes of control Cux2+/− (A) and Cux2neo/neo mutant (B) littermates. (C) Schematic of IDU and BrdU pulsing regime of E10.5 pregnant dams from Cux2+/− intercrosses. (D,E) G2/S- and M-phase labeling by BrdU (fuchsia) and pH3 (green) immunohistochemistry, respectively, in neural tubes of E10.5 control (D) and Cux2neo/neo mutants. (F) Bar chart summarizing pH3 counts in forelimb level neural tubes at E10.5 and 11.5. Cux2neo/neo mutants displayed normal levels of pH3 staining at E10.5, but showed significant decreases in pH3 counts at E11.5 (P=0.009).
detected in ventral pseudostratified neuroepithelium (arrow in Fig. S4A in the supplementary material). By E10.5, the bulk of Neurod-positive neuroblasts resided in the vz immediately adjacent to p27Kip1-positive post-mitotic neurons in the iz and mz (Fig. 3J; see Fig. S4C in the supplementary material), indicating that the activity of Neurod1 and p27Kip are largely independent of one another. However, small numbers of Neurod1/p27Kip1-double-positive cells were observed in the iz (arrow, Fig. 3I; see Fig. S4C in the supplementary material). These double-labeled cells were neuroblasts withdrawing from the cell cycle and undergoing terminal differentiation. Neurod1 levels were only modestly attenuated in E9.5 Cux2neo/neo mutants (see Fig. S4 in the supplementary material). By contrast, E10.5 Cux2neo/neo mutant embryos exhibited a striking reduction of Neurod-labeled cells (Fig. 3J; see Fig. 5D in the supplementary material). E11.5 wild-type embryos expressed Neurod1 specifically in neuroblasts located within the ventral half of the neural tube and at the lateral edge of the vz (Fig. 3E), and the severe reduction of Neurod1 expression at this stage in Cux2 mutants highlighted their deficient ability to generate neuroblasts (Fig. 3F).

In comparison with Neurod1, a greater reduction of p27Kip1 was observed upon Cux2 loss at E9.5 (see Fig. S4B in the supplementary material). Furthermore, fewer p27Kip1-expressing cells were also observed in the vz of Cux2neo/neo mutants at E10.5 relative to controls (arrow, Fig. 3J; see Fig. S4D in the supplementary material). By E11.5, the effect of Cux2 loss on p27Kip1-expressing cells in the vz was even more pronounced (Fig. 2F,K,G,L). Thus, Cux2 loss attenuated the formation of neuroblasts and also affected their exit from the cell cycle. Collectively, these results indicate that the reduction in spinal cord size in Cux2neo/neo embryos was not simply the result of alterations in the progenitor pool size, but also reflected a requirement for Cux2 in neuroblast formation and differentiation.

Cux2 is present in activator complexes bound to the native promoters of Neurod1 and p27Kip1

Our Cux2 loss-of-function analyses highlighted both Neurod1 and p27Kip1 as potential direct targets of Cux2 during neurogenesis. To directly assess if Cux2 interacts with the native Neurod1 and p27Kip1 promoters, we performed ChIP analysis using chromatin isolated from brains of E12.5 embryos, a tissue that normally expresses high levels of Cux2. The ChIP assays were carried out using IgG (negative control), anti-Cux2 and anti-polymerase II (positive control) antibodies. We focused on the proximal regions of the Neurod1 and p27Kip1 promoters, which have several putative AT-rich Cux-binding sites. A clear PCR product for Neurod1 was observed in the high concentration anti-Cux2 ChIP (Fig. 5A, lane 10). Similarly, a PCR product for p27Kip1 was detected in both the high and low concentration of anti-Cux2 ChIP (Fig. 5C, lanes 9 and 10). In control experiments, amplification of sequences 5 kb upstream from the Neurod1 (Fig. 5B) or p27Kip1 (Fig. 5D) transcription start sites produced no Cux2-bound products. These results clearly show that Cux2 is part of a complex bound to the Neurod1 and p27Kip1 promoters in their native chromatin configuration. Therefore, Neurod1 and p27Kip1 are direct in vivo transcriptional targets of Cux2, which mechanistically accounts for the functional properties of Cux2 in regulating neuroblast formation and cell-cycle exit during mammalian spinal cord neurogenesis.

Cux2 overexpression results in enlarge neural tubes and enhanced neurogenesis

To further validate a requirement for Cux2 during spinal cord neurogenesis, we investigated the effect of overexpressing Cux2 in neural progenitors via mouse transgenesis using the Nestin intron II enhancer (Zimmerman et al., 1994). We were unable to obtain postnatal Nestin-Cux2-ires-EGFP-expressing transgenics due to late gestation embryonic lethality and therefore restricted our analyses to transient transgenic embryos at E10.5-11.5. Seven independent integrants appropriately expressing high levels of GFP and thus Cux2 protein in neural progenitors were selected for analysis (see Fig. S3 in the supplementary material). Cux2 transgenic embryos consistently exhibited modest increases in total neural tube size (11% larger than controls), with the mz being preferentially affected relative to the vz (14 versus 8% respectively; see Table S1 in the supplementary material). These observations complemented the Cux2 mutant phenotype and indicated that Cux2 regulates spinal cord neurogenesis.

We next used Pax6 to investigate neural progenitor development within the ventral domain of E11.5 spinal cords (Fig. 3). In comparison with wild-type littermates, Cux2 transgenic embryos displayed a mediolateral expansion of Pax6-positive progenitor cells (arrows, Fig. 3C,D), but no dorsoventral expansion, despite strong GFP expression throughout the entire extent of the neural tube. These results contrast well with the reduction in Pax6 progenitors observed in stage-matched Cux2 mutants. Thus Cux2 dosage is an important regulator of progenitor pool size.

Given that Cux2 binds to the proximal promoters of Neurod1 and p27Kip1, we examined the effect of manipulating Cux2 levels on the formation of neuroblasts and p27Kip1-mediated cell-cycle exit. Cux2 mutants displayed a severe reduction in Neurod1-positive neuroblasts relative to wild-type controls (Fig. 3E,F). By contrast, Cux2 overexpression dramatically enhanced neuroblast formation in a cell-autonomous manner in the spinal cord of E10.5 and 11.5 embryos (Fig. 3G-L). Neurod-positive cells are normally found only in the most apical portion, where progenitor cells reside (Fig. 3E). Although Cux2 overexpression enhanced Neurod1-positive neuroblast formation, it did so only in the lateral domain of the vz and did not force the apical

### Table 1. Cux2neo/neo mutants display reduced progression through mitosis in the developing spinal cords at E10.5

<table>
<thead>
<tr>
<th>Control E10.5 (n=14) *</th>
<th>Cux2neo/neo E10.5 (n=8)</th>
<th>P value (t-test) †</th>
</tr>
</thead>
<tbody>
<tr>
<td>Scells (IDU+BrdU)</td>
<td>Lcells (IDU only)</td>
<td>Ts (S-phase length)</td>
</tr>
<tr>
<td>33.3±7.2†</td>
<td>20.6±5.0</td>
<td>2.4±0.5</td>
</tr>
<tr>
<td>43.1±7.7</td>
<td>16.4±2.7</td>
<td>3.9±0.7</td>
</tr>
<tr>
<td>0.005</td>
<td>0.01</td>
<td>0.0001</td>
</tr>
</tbody>
</table>

Embryos were pulsed with IDU for a total of 2 hours and BrdU for 0.5 hours prior to harvesting. The fraction of cells within S phase of the cell cycle (Scells) is represented by the total of IDU and BrdU double-labeled nuclei (yellow signal in Fig. 5), whereas the fraction of cells in G2/M (Lcells) was obtained from counting the nuclei labeled with IDU alone (green in Fig. 5). The S-phase length was determined using the following formula: Scells/Lcells×1.5 hours.

*For control littermates, a total of 14 sections from four different embryos were used. For Cux2neo/neo embryos, a total of eight sections from three different mutants were used. IDU and BrdU positive nuclei were counted from the unilateral halves of ventral neural tubes at E10.5.

†Figures are represented as average values±s.d.

‡P values were determined using a one-tailed Student’s t-test with two samples, unequal variance.
progenitor cells to ectopically adopt a neuroblast fate. This may explain why Cux2 overexpression did not automatically lead to a premature depletion of the stem cell pool.

Our Cux2 mutant analyses and ChIP assays uncovered p27Kip1 and Neurod1 as important downstream targets of Cux2 that are crucial for cell-cycle exit. We therefore analyzed E11.5 Cux2-overexpressing transients for markers of post-mitotic neurons and definitive neurogenesis. In control embryos, p27Kip1 activity is prominently expressed in the iz, corresponding with neural progenitor cell-cycle exit and terminal differentiation (Fig. 6A). Cux2 overexpression in neural progenitors induced high levels of p27Kip1 activity in cells in the iz and mz, and to a lesser extent in the vz (Fig. 6B,C). Taken together with the co-localization of Cux2 and p27Kip1, this supports the argument in favor of a role for Cux2 in regulating cell-cycle exit of neuronal precursors.

Cux2 transgenic embryos displayed ectopic NeuN (Fig. 6E,F, versus 6D), neurofilament (Fig. 6H,I, compare with G) and TuJ1 staining (data not shown) throughout the mz of the spinal cord, consistent with an enhancement of neurogenesis. Surprisingly, many of the axon filaments appeared to be disorganized, projecting laterally instead of ventrally (Fig. 6H,I versus 6G). This suggested that Cux2 not only enhanced neural differentiation but also influenced neuronal migration, maturation and/or patterning. Importantly, the markers of post-mitotic neurons (e.g. NeuN and neurofilament) were not ectopically activated in the vz of Cux2 transgenics, despite strong Cux2-GFP expression there (Fig. 6D-I). Thus even though Cux2 promotes cell-cycle exit and interneuron formation, it is not at the expense of transforming all of the neural stem or progenitor cells prematurely into post-mitotic neurons. This is consistent with the exit of quiescent cell populations from the proliferative vz occurring before neuronal maturation (Fig. 6) (Gui et al., 2007).

In an effort to quantify the effect of Cux2 on neurogenesis, we constitutively overexpressed Cux2 in the neural tube of HH7-8-stage chick embryos via in ovo electroporation and analyzed the embryos with TuJ1 immunostaining 24 hours later. In Cux2-ires-EGFP electroporated neural tubes, 14.2% of Cux2/GFP labeled cells were also TuJ1 positive (n=7; Fig. 6J-L). By contrast, in control electroporated neural tubes, only 5.8% of GFP-labeled cells were TuJ1 positive (n=14; Fig. 6L). Our results therefore demonstrate that Cux2 acts as an important regulator of neural differentiation throughout the developing spinal cord.

### Cux2 regulates ventral interneuron and motoneuron formation

Cux2 mutant spinal cords displayed a strikingly specific reduction of p27Kip1 in the iz and mz (arrowhead, Fig. 2L versus 2G) but surprisingly not in the ventrolateral motoneuron domain (Fig. 2K, compare with 2F). These results were suggestive of a selective loss of interneurons in Cux2neo/neo mutant embryos and indicated that Cux2 may upregulate p27Kip1 in neural progenitors to force their exit from the cell cycle and initiate differentiation. Further evidence supporting selective interneuron loss came from analyses of the levels of the phosphoprotein NeuN (Lind et al., 2005). NeuN demarcates mature post-mitotic neurons and is initially observed in the ventral
motoneuron population before expanding to dorsal neuron populations in conjunction with ventral to dorsal maturation of the spinal cord (Mullen et al., 1992). We observed that NeuN immunostaining was more intense in the lateral motoneuron domain of Cux2 mutant embryo spinal cords compared with wild-type littermates (Fig. 2M-N versus 2H-I). This implied that the loss of Cux2 led to an increase in the formation of motoneurons and suggested that Cux2 normally acts as a cell-fate determinant by promoting interneuron formation and limiting motoneuron generation.

To better substantiate a functional role for Cux2 in fate determination, we characterized the pattern of interneuron and motoneuron development in our gain- and loss-of-function models (Fig. 7). Lhx1 labels ventral interneuron populations (v1, v2) at E10.5 (Fig. 7A) (Tsuchida et al., 1994) and Cux2neo/neo mutants (n=15) displayed a significant decrease (16%, P=0.007) in Lhx1-positive neurons in the ventral neural tube (Fig. 7B,N; see Table S2 in the supplementary material). We next examined the expression of Chx10 protein, a homeodomain transcription factor, in V2 interneurons (Fig. 8). Loss of Cux2 led to a significant reduction of Chx10-positive cells in the ventral spinal cord at E10.5 (Fig. 8B). Quantification of Chx10-positive cells in Cux2neo/neo mutants (n=8) revealed a 45% reduction (P=0.002) relative to control littermates (n=5). Collectively this supports the argument for a role for Cux2 in promoting interneuron formation.

Fate-mapping studies have demonstrated that Olig2 progenitors give rise to motoneurons (Masahira et al., 2006; Mukouyama et al., 2006), whereas Nkx2.2 labels the ventral most progenitor domain in the neural tube that contributes to the formation of v3 interneurons (Briscoe et al., 1999). We used Olig2 in combination with Isl1/2 to characterize progenitor and post-mitotic motoneuron patterning (Fig. 7E,F) and Nkx2.2 to identify v3 progenitors (Fig. 7I,J) (Mizuguchi et al., 2001; Novitch et al., 2001). E10.5 Cux2neo/neo mutant embryos displayed an expansion of Olig2-positive motoneuron progenitors (Fig. 7F) and a concomitant reduction in the dorsal extent of the Nkx2.2 progenitor domain (Fig. 7J). This correlated with an increase in Isl1/2-positive motoneurons at the ventrolateral margin of the spinal cord (n=11; Fig. 7F; see Table S2 in the supplementary material). These effects were independent of any alterations of Shh levels in the ventral neural tube, which were comparable between E10.5 Cux2neo/neo mutant embryos and control littermates (data not shown). Quantification of the effect of Cux2 loss revealed a highly significant (P<0.001) 32% increase in Isl1/2-positive motoneurons (n=11, Fig. 7M; see Table S2 in the supplementary material), demonstrating that Cux2 normally acts to limit motoneuron formation. Taken together, these results indicate that Cux2 acts as a cell-fate determinant, promoting the generation of interneurons but limiting motoneuron development.

Cux2 overexpression promotes ventral interneuron but not motoneuron formation

Given the sensitivity of neurogenesis to Cux2 gene dosage, we next examined whether Cux2 gain of function impacted on cell-fate determination in a complementary fashion to Cux2 mutants (Fig. 7). Cux2 overexpression resulted in an increase in Lhx1-positive ventral...
interneurons (n=4, Fig. 7C,D; see Table S2 in the supplementary material), indicating that Cux2 can indeed promote interneuron formation. We quantified this effect and observed that Cux2 overexpression in neural progenitors led to a 43% increase (P=0.0058) in the formation of ventral interneurons (n=4; Fig. 7N; see Table S2 in the supplementary material). This result was complementary to that obtained in isochronic Cux2neo/neo mutants, which displayed a significant loss of Lhx1-positive neurons (Fig. 7N; see Table S2 in the supplementary material). Furthermore, a comparison of Cux2 mutants to transgenic embryos revealed 70% fewer (P=0.007) Lhx1-positive cells (Fig. 7N; see Table S2 in the supplementary material), supporting a role for Cux2 in promoting interneuron development.

Lastly, we evaluated the effect of Cux2 gain of function on the formation of motoneurons. Cux2 overexpression in ventral progenitors led to a 20% decrease (P=0.0053) in Isl1/2-positive motoneurons in the ventrolateral margin of the spinal cord relative to littermate controls (n=4, Fig. 7G,H; see Table S2 in the supplementary material). By contrast, as described above, Cux2 loss resulted in a 32% increase in Isl1/2-positive motoneurons (n=11, Fig. 7M; see Table S2 in the supplementary material). The effects on motoneuron formation were even more pronounced when gain- and loss-of-function embryos were compared. A highly significant increase (61%) in motoneuron production was observed in the Cux2neo/neo mutants relative to Cux2 transgenics (P<0.001; Fig. 7M; see Table S2 in the supplementary material). These results further illustrate a cell-fate determinant function for Cux2 during spinal cord neurogenesis.

**DISCUSSION**

The acquisition of cell-type-specific neuronal fate is coupled to the differentiation of neural precursors in vertebrates (Cremisi et al., 2003; Ohnuma et al., 2002); however, the nature of the intrinsic signals that integrate cell-cycle control with these processes during neurogenesis remain rudimentary. Our results demonstrate that Cux2 is an important mediator of spinal cord neurogenesis, acting through the direct regulation of the proneural gene NeuroD and cell-cycle inhibitor p27Kip1 in spinal cord progenitors. We show for the first time that a single factor, Cux2, can promote both progression of cell cycle in neural progenitors and stimulate neuronal differentiation in the developing mammalian spinal cord. Cux2 mouse mutants exhibited fewer Pax6-, Chx10- and Nkx2.2-positive progenitors, diminished neuroblast formation and p27Kip1-driven cell-cycle exit correlating with a loss of ventral interneurons. However, not all post-mitotic neurons were decreased, as we noted a surprising enhancement of motoneuron formation preceded by an expansion of olig2-positive motoneuron progenitor domain. Interestingly, Cux2 is expressed in post-mitotic interneurons but is conspicuously...
weak or absent from the lateral motoneuron domain. Given that Shh protein levels remained robust in the Cux2 hypomorphs, this supports the argument that Cux2 directly influences the acquisition of cell fate in ventral progenitors of the developing spinal cord and may normally act to promote interneuron formation at the expense of motoneurons.

It is possible that the enhanced formation of motoneurons in the Cux2 mutants may be due to changes in neural progenitor cycle length. The generation of different types of neurons in the central nervous system has been purported to be influenced in part by the length of the cell cycle, which progressively increases during embryogenesis (Durand et al., 1998; Gao et al., 1997; Raff et al., 1998; Shen et al., 2006; Wilcock et al., 2007), and furthermore, interneuron generation commences before motoneuron formation in the spinal cord (Sechrist and Bronner-Fraser, 1991). We documented a significant lengthening of the S phase in the spinal cord progenitors of Cux2 mutants, suggesting that Cux2 influences the balance of interneurons and motoneurons at least in part by regulating the length of the progenitor cell cycle. In agreement with these observations, overexpressing Cux2 in the vz of the developing neural tube enhanced progenitor development and promoted Neurod1-positive neuroblast formation and p27Kip1-driven cell-cycle exit as well. Indeed, neurogenesis was enhanced, particularly with respect to the formation of interneurons. Importantly, using ChIP assays from embryonic brain extracts we determined that the regulation of Neurod1 and p27Kip1 by Cux2 was direct, as Cux2 bound the transcriptionally active proximal promoters of these genes. Although neurogenesis defects have been reported for Neurod1 null mutants (Goebels et al., 2005; Liu et al., 2000; Miyata et al., 1999), no spinal cord defects have been reported, perhaps reflecting redundancy with other widely expressed bHLH factors (Helms et al., 2005; McCormick et al., 1996). Similarly, whereas p27Kip1 appears to be dispensable for spinal cord neurogenesis, p27Kip1/p57Kip2 double mutants show defects in cell-cycle exit of neuronal progenitors, which are associated with reduced formation of ventral interneurons (Gui et al., 2007). This phenotype is reminiscent of the Cux2neo/neo mutant described here. For both Neurod1 and p27Kip1, altering Cux2 levels genetically in spinal cord progenitors led to dramatic alterations in the expression of these key neurogenic factors, particularly in nascent neurons undergoing cell-cycle exit. Whether Neurod1 regulates p27Kip1 also remains a possibility, as there is limited co-expression in nascent neurons from E9.5 onwards. However, recent work has demonstrated that p27Kip1 engages neurogenesis by promoting cell-cycle exit and stabilization of proneural proteins (Nguyen et al., 2006). Thus, Cux2-mediated upregulation of p27Kip1 in the iz may also promote neurogenesis by maintaining robust bHLH gene expression in neuroblasts exiting the cell cycle, thereby accounting for the relative specificity of Cux2 mutant phenotype. Our findings thus establish for the first time the importance a Cut homeodomain transcription factor in a genetic hierarchy acting upstream of a bHLH neurogenic factor during vertebrate neurogenesis.

In the current study, we show that Cux2 is crucial in establishing and/or maintaining the spinal cord progenitor pool. Importantly, individual cells within the undifferentiated neuroepithelium are not necessarily equivalent, and only some cells have neuron-generating potential (Wilcock et al., 2007). These so-called neurogenic cells divide to generate a progenitor and a neuron and as such influence patterning and development of both the vz and iz/mz. In Cux2 gain- and loss-of-function embryos, we observed complementary alterations to both progenitor and differentiated neuronal populations, implying that Cux2 may impart neurogenic potential to undifferentiated neuroepithelial cells. It is perhaps in these populations that the loss of Cux2 is most acutely felt. Cux2 therefore appears to be required for both neural progenitor maintenance and neural differentiation. Given that these two processes are often


Cux2 (Cut2) regulates neural differentiation


Table S1. Difference in neural tube, mz and vz size in E11.5 neural tubes from multiple sections from Cux2 transgenic and Cux2neo/neo mutants relative to control littermates

<table>
<thead>
<tr>
<th></th>
<th>VZ size</th>
<th>MZ size</th>
<th>Total NT size</th>
<th>NT height</th>
<th>NT width</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control versus Cux2 transgenic (% difference; n=7)</td>
<td>108%</td>
<td>114%</td>
<td>111%</td>
<td>107%</td>
<td>110%</td>
</tr>
<tr>
<td>Control versus Cux2neo/neo (% difference; n=7)</td>
<td>87%*</td>
<td>51%*</td>
<td>72.2%*</td>
<td>98%</td>
<td>79.3%*</td>
</tr>
</tbody>
</table>

VZ, ventricular zone; MZ, marginal zone; NT, neural tube.
*Statistical significance (P<0.05) by Student's t-test.

Table S2. Effect of Cux2 loss- and gain-of-function on neuronal cell fate in the spinal cord

<table>
<thead>
<tr>
<th></th>
<th>Control E10.5</th>
<th>Cux2neo/neo mutants E10.5</th>
<th>Cux2 Tg E10.5</th>
</tr>
</thead>
<tbody>
<tr>
<td>Isl1</td>
<td>67.12±14.6*</td>
<td>88.7±14.5</td>
<td>53.7±16.1</td>
</tr>
<tr>
<td>P value (t-test)†</td>
<td>0.000003</td>
<td>0.053</td>
<td></td>
</tr>
<tr>
<td>Lhx1</td>
<td>101.1±21.2</td>
<td>85.2±30.7</td>
<td>144.6±38.1</td>
</tr>
<tr>
<td>P value (t-test)</td>
<td>0.0007</td>
<td>0.0058</td>
<td></td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th></th>
<th>Cux2neo/neo/Ctrl (%)</th>
<th>Cux2 Tg/Ctrl (%)</th>
<th>Cux2neo/neo/Cux2 Tg (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Isl1</td>
<td>32</td>
<td>-20†</td>
<td>61</td>
</tr>
<tr>
<td>P value (t-test)</td>
<td>0.000002</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Lhx1</td>
<td>-16</td>
<td>43</td>
<td>-70</td>
</tr>
<tr>
<td>P value (t-test)</td>
<td>0.0007</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Cux2neo/neo mutants display increased numbers of Isl1-positive cells and decreased Lhx1 numbers in the ventral neural tube at E10.5, relative to control littermates. Cux2 transgenics (Tg), however, display decreased numbers of Isl1-positive cells and increased numbers of Lhx1-positive cells relative to control embryos in E10.5 ventral neural tubes.
*Figures are represented as average values±s.d.
†P values were determined using a one-tailed Student’s t-test with two samples, unequal variance.
‡Negative sign denotes decrease.