Cux2 functions downstream of Notch signaling to regulate dorsal interneuron formation in the spinal cord

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Obtaining the diversity of interneuron subtypes in their appropriate numbers requires the orchestrated integration of progenitor proliferation with the regulation of differentiation. Here we demonstrate through loss-of-function studies in mice that the Cut homeodomain transcription factor Cux2 (Cutl2) plays an important role in regulating the formation of dorsal spinal cord interneurons. Furthermore, we show that Notch regulates Cux2 expression. Although Notch signaling can be inhibitory to the expression of proneural genes, it is also required for interneuron formation during spinal cord development. Our findings suggest that Cux2 might mediate some of the effects of Notch signaling on interneuron formation. Together with the requirement for Cux2 in cell cycle progression, our work highlights the mechanistic complexity in balancing neural progenitor maintenance and differentiation during spinal cord neurogenesis.

KEY WORDS: Cux, Spinal cord, Neurogenesis, Interneurons, Notch signaling, Mouse

INTRODUCTION

The embryonic dorsal spinal cord is functionally organized as six discrete parallel layers of interneurons (d11-d16), which are distinguishable by their location, morphology and projections. Establishing the specific architecture of the spinal cord requires the precise integration of cell proliferation, cell cycle progression and neuronal differentiation through the complex interplay of multiple signaling systems. Dorsal interneurons arise from a common progenitor pool that requires the function of basic helix-loop-helix proneural transcription factors such as Mash1 (Ascl1), Olig3 and Ngn2 (Neurog2) (Helms et al., 2005; Muller et al., 2005; Nakada et al., 2004; Wildner et al., 2006). Dorsal interneuron subtypes subsequently become distinct from one another through the activity of unique combinatorial codes of homeodomain transcription factors such as Lhx1, Pax2, Brn3a (Pou4f1) and Tlx3 (Helms and Johnson, 2003).

Notch signaling is thought to be required for the maintenance of the neural progenitor state (Androussellis-Theotokis et al., 2006; Bolos et al., 2007; Handler et al., 2000; Henrique et al., 1997; Hitoshi et al., 2002; Yoon and Gaiano, 2005). However, Notch also controls the differentiation of progenitors into neuronal subtypes in the developing spinal cord and cortex (Chambers et al., 2001; Del Barrio et al., 2007; Machold et al., 2007; Peng et al., 2007; Yang et al., 2006) through regulating the activities of proneural genes (Fischer and Gessler, 2007; Gaiano and Fishell, 2005). It therefore appears that Notch activity can selectively control both the maintenance and differentiation of neural progenitors, but how this balance is achieved is currently poorly understood.

The Cut gene family comprises homeodomain transcription factors that contain one or more Cut repeat DNA-binding domains. Previously, we determined that Cux2 (cut-like homeobox 2) regulates cell cycle progression and cell fate acquisition during ventral spinal cord neurogenesis (Iulianella et al., 2008). Here we characterize a role for Cux2 as a putative Notch effector gene that regulates dorsal interneuron development. Collectively, our work demonstrates that by balancing cell cycle progression with neuronal differentiation, Cux2 plays a crucial role in integrating Notch signaling during spinal cord neurogenesis.

MATERIALS AND METHODS

Mouse strains

The generation, genotyping and phenotyping of Cux2, Rbpjk; flox-stop Notch1 intracellular domain (NICD)-ires-EGFP, Wnt1-Cre and Nestin-Cre lines of mice were performed as previously described (Iulianella et al., 2008; Oka et al., 1995; Murtaugh et al., 2003; Chai et al., 2000; Dubois et al., 2006).

Mouse embryo DAPT treatment

To abrogate Notch signaling, E11.5 CD1 mouse embryos were cultured in the presence of the γ-secretase inhibitor DAPT (10 μM; Sigma, St Louis, MO) or DMSO (control carrier) for 16 hours. Embryos were then rinsed in PBS, fixed and processed for cryosectioning. Cux2 immunohistochemistry was performed using Cux2 antibodies (Iulianella et al., 2008). Mouse embryos were cultured in a whole-embryo roller culture incubator (BTC Engineering) in 100% rat serum supplemented with 2 mg/ml glucose and penicillin-streptomycin (R-100 Medium) maintained in a 60% O2/5% CO2/35% N2 atmosphere at 37°C. Embryos were then rinsed in PBS, fixed with paraformaldehyde and processed for cryosectioning.

Immunohistochemistry

Immunohistochemistry and statistics were performed on 10-12 μm sections of E11.5 forelimb bud-level spinal cords as described (Iulianella et al., 2008). Primary antibodies used included anti-Cux2 (Iulianella et al., 2008), anti-Mash1 (BD Biosciences, San Jose, CA, USA), anti-Lhx1 (Lim1) [4F2, Development Studies Hybridoma Bank (DSHB), University of Iowa], anti-neurofilament (2H3, DSHB), anti-Tag1 (4D7, DSHB), anti-Lbx1 and anti-Tlx3 (kind gifts of Drs C. Birchmeier and T. Muller, Max-Delbrueck-Center for Molecular Medicine, Berlin, Germany), anti-Sox1 (kind gift of Dr R. Lovell-Badge, National Institute for Medical Research, Mill Hill, UK), anti-Pax2 (Zymed, South San Francisco, CA, USA), anti-Notch1 (ICD) mN1A (BD Biosciences), anti-cyclin D1 (Upstate, Lake Placid, NY, USA), mouse anti-p57Kip2 (LabVision/Neomarkers, Fremont, CA, USA), anti-p27Kip1 (BD Biosciences), anti-GFP-FITC (Abcam, Cambridge, MA, USA), anti-Brn3a (Millipore, Temecula, CA), anti-Lhx2 (Santa Cruz Biotechnology, Santa Cruz, CA, USA), anti-Hes1 (Neuromics, Edina, MN, USA) and anti-Math1 (kind gift of Dr J. Johnson, University of Texas Southwestern Medical Center, Dallas, TX, USA). Secondary antibodies included species-specific Alexa Fluor 594 or 488-conjugated antibodies (Invitrogen, Carlsbad, CA, USA).
USA). Sections were counterstained with DAPI (Sigma) prior to mounting with fluorescent mounting medium (DakoCytoMation, Carpinteria, CA, USA) and were visualized using a Zeiss upright fluorescence microscope with 10× or 20× objectives.

**Statistics**
Counts of cells positive for cyclin D1, p57Kip2, Mash1, Lbx1, Pax2 or Tlx3 were performed on two or three sections from three or more distinct E11.5 Cux2neo/neo mutants or control Cux2+/- littermates. For Bmi1a, Lhx2 and Math1 cell counts, two or three serial sections from a total of 12 different E11.5 Cux2neo/neo mutants and nine Cux2+/- littermate controls were used to quantify an effect on commissural interneurons. Pixel area was kept constant, and cell counts were performed unilaterally and included both differentiated neurons in the marginal zone and adjacent ventricular domains for each marker. Differences were plotted as the mean ± variance, and significance was examined using a one-tailed Student’s t-test with significance set at P<0.01.

**Chromatin immunoprecipitation**
Chromatin immunoprecipitation (ChIP) assays were performed on E12.5 heads from CD1 mice using the EZ-Chip Assay Kit (Upstate) as previously described (Iulianella et al., 2008). DNA-associated immunoprecipitates were used as templates for PCR analysis with primers producing a 251 bp fragment that encompasses the Mash1 promoter, spanning -13 to -263 relative to the transcription start site. Primers: 5′-TTATTCGCCG-GGAGTCCCGGTTCG-3′ and 5′-GGTAACTTTTCCGCCGGCGT-3′. To control for non-specific immunoprecipitation of chromatin by the Cux2 antibody, the same DNA was used as a template for PCR analysis with primers producing a 471 bp fragment spanning +838 to +1308 relative to the Mash1 transcription start site. Primers: 5′-GGAACGTGCGGCTGCAAAAC-3′ and 5′-CCTGTTTCAAGTCCATTCCC-3′.

**RESULTS AND DISCUSSION**
**Cux2 regulates Mash1-positive dorsal spinal cord progenitors**
Cux2 is dynamically expressed during vertebrate neurogenesis. In embryonic day (E) 10.5 mouse embryos, proliferating progenitor cells in the ventricular zone of the spinal cord and in nascent neurons exiting the cell cycle exhibit high levels of Cux2 activity (Iulianella et al., 2008; Iulianella et al., 2003; Nieto et al., 2004; Zimmer et al., 2004). To date, however, it is not known whether Cux2 plays a functional role in interneuron formation and patterning during dorsal spinal cord neurogenesis. To address this question, we took advantage of a hypomorphic Cux2 loss-of-function mouse model (Iulianella et al., 2008) and examined its impact on interneuron patterning in the developing dorsal spinal cord.

E11.5 Cux2neo/neo mutant embryos exhibit hypoplasic neural tubes in which both the ventricular (vz) and marginal (mz) zones are reduced, the cause of which is not differential cell death (Iulianella et al., 2008) (Fig. 1A,B). Sox1 immunostaining revealed that the vz progenitor pool is diminished in the spinal cord of E11.5 Cux2 mutant embryos (Fig. 1A,B). Cux2 therefore regulates the size of the progenitor pool, consistent with its requirement in cell cycle progression and proliferation (Iulianella et al., 2008).

We previously determined that the mz of Cux2neo/neo spinal cords was more severely affected than the vz, principally owing to the dependency of two key neurogenic factors, NeuroD1 and p27Kip1 (Cdkn1b), on Cux2 function (Iulianella et al., 2008). Similarly, both cyclin D1-expressing and p57Kip2 (Cdkn1c)-expressing cells were significantly decreased in the dorsal spinal cord of Cux2 mutants (see Fig. S1 in the supplementary material), p27Kip1 and p57Kip2 regulate the timing of cell cycle withdrawal in spinal cord progenitors (Gui et al., 2007), confirming a role for Cux2 in cell cycle progression and exit of nascent neurons in the dorsal spinal cord.

The development of the six distinct layers of dorsal interneurons (d11-d16) along the dorsal-to-ventral axis of the spinal cord requires the activity of proneural genes between E9.5 and E12.5. Mash1 functions in both neuronal differentiation and specification (Helms et al., 2005; Wildner et al., 2006) and is expressed in the precursors that will become d13-d16 (Gowan et al., 2001; Lo et al., 1991). Mash1 loss-of-function results in a diminished capacity to undergo neuronal differentiation (Battiste et al., 2007). In dorsal neural progenitors, Cux2 co-localizes with 16.4% of Mash1-positive cells in the lateral-most edge of the vz in the dorsal spinal cord, where cells undergo cell cycle exit and begin terminal differentiation (Fig. 1C-E, bracket; see Fig. S2A-D in the supplementary material). In comparison to Cux2neo/+ littermates, Cux2neo/neo mutants displayed a 48% reduction in the number of Mash1-positive progenitors at E11.5 (Fig. 1F,G; P<0.01), consistent with a role for Cux2 in dorsal spinal cord neurogenesis.

To determine whether the reduction of Mash1 in Cux2 mutants was due to direct regulation of the Mash1 gene by Cux2, we performed chromatin immunoprecipitation (ChIP) analysis using chromatin isolated from E12.5 mouse heads, which express high levels of Cux2 protein. The proximal region of the Mash1 promoter contains putative Cux binding sites, which were weakly amplified as a 221 bp PCR product via anti-Cux2 ChIP (see Fig. S2E, lane 8, in the supplementary material). The extent of Mash1 promoter amplification was similar to that of the IgG control, arguing against a direct interaction. This suggests that the reduction of Mash1-positive cells in Cux2 hypomorphs is not due to a direct regulation by Cux2, but instead arises as a consequence of a hypoplastic vz and reduced cell cycle exit (Iulianella et al., 2008).
Cux2 is required for dorsal interneuron development

Mash1-positive progenitors, which give rise to the dI3-dI6 groups of interneurons, are further refined by the activity of homeodomain transcription factors (Helms et al., 2005; Helms and Johnson, 2003). *Lhx1*, for example, is expressed in dI4-dI6 (Muller et al., 2002), *Tlx3* is expressed in dI3 and dI5 (Muller et al., 2005; Wildner et al., 2006), and *Pax2* is expressed in dI4 and dI6 (Burrill et al., 1997).

Immunostaining of E11.5 embryos revealed that Cux2 is extensively co-expressed with *Lbx1* (Fig. 2A-C) and *Lhx1* (data not shown) in dI4-dI6. Cux2 was also expressed in the dorsal-most *Lbx1*-positive interneurons, which are mostly dI4 nascent interneurons and possibly a sub-population of dI3s (Fig. 2A-C). Cux2 co-localized with *Tlx3* in dI3 (Fig. 2F-H). *Tlx3* also marks dI5, where we observed little or no co-staining with Cux2 (Fig. 2F-H).

In comparison to Cux2 heterozygous littermates, E11.5 *Cux2neo/+* control (A-C,F-H,K) and *Cux2neo/neo* mutant (D,I,L) mouse spinal cords immunostained for *Lbx1* (red; A,C,D), *Tlx3* (red; F,H,I), Cux2 (green; B,C,G,H) and *Pax2* (red; K,L). The dotted lines indicate boundaries of one half of the spinal cord. (E,J,M) Bar charts quantifying the reduction of cells positive for *Lbx1* (E), *Tlx3* (J) or *Pax2* (M) in *Cux2neo/neo* mutants. *, P<0.01. dI, dorsal interneuron; d, dorsal progenitor.

In contrast to the role of *Mash1* in dI3 and dI5 interneurons, Cux2 appears to be more broadly required for the generation of dI3-dI6 populations and their progenitors (Fig. 2). The reduction in dorsal interneuron populations in *Cux2neo/neo* hypomorphs might reflect dual roles of Cux2 in the cell cycle progression of progenitors and their differentiation into neurons. Consistent with this, Cux2 directly regulates *p27kip1* expression in spinal cord progenitors undergoing cell cycle withdrawal (Iulianella et al., 2008). Furthermore, both the nascent dorsal interneurons and their immediate progenitors were greatly attenuated in Cux2 mutants (Fig. 2D,L; see Fig. S1 in the supplementary material). We therefore favor a role for Cux2 in mediating both the induction and cell cycle exit of newborn neurons in the developing spinal cord.

Cux2 promotes the formation of commissural interneurons

We hypothesized that the effects on dorsal progenitor and interneuron development observed in Cux2 mutants should lead to deficits in mature dorsal interneurons. We focused on the generation of commissural interneurons, the origins and morphology of which are well characterized. Commissural interneurons are derived from dI1-dI3 (Helms and Johnson, 1998; Muller et al., 2002) and dI5 and dI6 (Silos-Santiago and Snider, 1992) interneuron subgroups, which were greatly reduced in *Cux2neo/neo* mutant embryos.

*Brn3a*, *Lhx2* and *Math1* (Atoh1) demarcate dI1 and dI2 commissural interneuron populations (Fedtsova and Turner, 1997; Helms and Johnson, 1998), and *Cux2neo/neo* mutants consistently showed reductions in all commissural markers examined relative to their *Cux2* heterozygous littermates (Fig. 3A-I). Specifically, *Cux2* loss resulted in 29.2%, 41.0% and 26.5% reductions in cells positive for *Brn3a* (Fig. 3B,C; *P<0.01*), *Lhx2* (Fig. 3E,F; *P<0.01*), and *Math1* (Fig. 3H,I; *P<0.01*), respectively. These findings, in addition to those from the analysis of dI3-dI6 populations in *Cux2* mutants (Fig. 2), argue that Cux2 is broadly required for the generation of most dorsal spinal cord interneurons, including commissural interneurons.
Notch signaling regulates Cux2 levels in spinal cord progenitors

In *Drosophila*, *cut* was initially identified as a Notch effector in wing development (de Celis et al., 1996; Doherty et al., 1996; Michell et al., 1997). Consistent with the role of Notch in controlling both the maintenance and differentiation of neural progenitor populations, we investigated whether Cux2 was regulated by Notch signaling in the developing spinal cord. Using an antibody that detects the cleaved intracellular domain of Notch1, we observed strong nuclear and cytoplasmic staining, with processes extending to the apical surface of the vz, consistent with a neural progenitor phenotype (Fig. 4A). Co-immunostaining with Cux2 revealed an association between activated Notch1 and the induction of high levels of Cux2 in the vz of the dorsal spinal cord at E11.5 (Fig. 4B).

To validate the regulation of Cux2 by Notch signaling we conditionally overexpressed the Notch intracellular domain [NICD-ires-EGFP (Murtaugh et al., 2003)] in neural progenitors throughout the developing spinal cord via Nestin-Cre (Dubois et al., 2006), or only in the dorsal neural tube with Wnt1-Cre (Chai et al., 2000). Cux2 is normally expressed mosaically in the vz (n=6; Fig. 4C) and Nestin-Cre-mediated NICD-ires-EGFP overexpression induced high levels of Cux2 expression throughout the vz at E11.5 (n=4) (Fig. 4D) (Dubois et al., 2006). We also examined the levels of Cux2 following Wnt1-Cre-mediated NICD induction at E12, when Cux2 levels are robust in the roof plate but have begun to recede in the dorsal progenitors and nascent neurons (n=8; Fig. 4E). Upon NICD overexpression, a dramatic induction of Cux2 levels was observed throughout the dorsal spinal cord (n=4; Fig. 4F).

We then examined the effect of blocking Notch signaling in vivo. We cultured mouse embryos at E11 for 16 hours in the presence of either DMSO (carrier control) or 10 μM DAPT, which prevents γ-secretase-mediated cleavage of Notch and blocks Notch signaling (Geling et al., 2002). In E11.5 control cultured embryos, Cux2 was expressed in the nascent neurons at the lateral edge of the vz (4/5 embryos; Fig. 4G). By contrast, DAPT inhibition of Notch signaling effectively abrogated Cux2 levels (7/9 embryos; Fig. 4H). Consistent with these findings, we also observed a dramatic downregulation of Cux2 mRNA expression in embryos with a null mutation in the Notch effector transcription factor Rbpj (see Fig. S3 in the supplementary material). Collectively, these findings demonstrate that Cux2 is regulated by Notch signaling in neural progenitors during spinal cord development.

Interestingly, the GFP reporter used in the conditional transgenesis assays revealed that constitutive activation of NICD inhibits neuronal differentiation and maintains vz cells in a progenitor state (Fig. 4D,F, inset). We confirmed this by examining the levels of the Notch target Hes1 and of the proneural transcription factor Mash1 following Wnt1-Cre-mediated NICD-ires-EGFP overexpression in the dorsal spinal cord at E11.5 (n=8). Hes1 is required for the maintenance of neural progenitors during development (Hatakeyama et al., 2004). Its expression can therefore be used as a measure of the neural progenitor state in the developing spinal cord. As expected, NICD overexpression in the dorsal spinal cord induced increased Hes1 levels and expanded the vz, demonstrating that NICD maintains progenitors in an undifferentiated state (see Fig. S4A-D in the supplementary material). Consistent with this, Mash1-positive cells were greatly attenuated following NICD overexpression (see Fig. S4E-H in the supplementary material). Thus, NICD overexpression promotes the maintenance of neural progenitors at the expense of the activation of a Mash1-dependent neurogenic program.

The axonal projections from commissural interneurons can be labeled with the cell adhesion molecule Tag1 (Ctnn2) (Fig. 3J). In comparison to Cux2 heterozygous controls at E11.5 (Fig. 3J,L), Cux2<sup>neo/neo</sup> mutants displayed diminished numbers of Tag1-positive axons projecting ventrally to the floor plate and commissure (Fig. 3K,M). This defect was particularly obvious at high magnification of Tag1-positive axons crossing the ventral commissure underneath the floorplate at E11.5 (Fig. 3L,M). Further examination of the axon network using anti-neurofilament antibodies confirmed reductions in the axonal plexus in the developing mz, and highlighted aberrant ventral root formation in Cux2<sup>neo/neo</sup> mutants (Fig. 3N,O). Given that Cux2 is required for the development of spinal cord progenitors, the reduction in commissural interneurons in Cux2 mutants is primarily a consequence of a diminished progenitor population. Furthermore, as Cux2 regulates cell cycle progression, this phenotype also reflects the reduced ability of progenitors to exit the cell cycle and mature into post-mitotic neurons.
To confirm that sustained Notch1 activation was inhibitory to neuronal differentiation, we examined the activity of p27^Kip1 (see Fig. SSA-C in the supplementary material) and p57^Kip2 (see Fig. SSD-F in the supplementary material). These two key regulators of cell cycle exit were both reduced in the dorsal spinal cord following Wnt1-Cre-driven NICD overexpression (see Fig. S5 in the supplementary material). Finally, we evaluated the formation of Brn3a-, Lhx2- and Math1-positive dorsal interneurons in NICD transgenic embryos at E11.5 (see Fig. S6 in the supplementary material). NICD overexpression greatly reduced the levels of these markers of dI1 and dI2 interneurons. Taken together, our findings demonstrate that constitutive Notch activity inhibits neuronal differentiation by maintaining vz cells in a progenitor state.

Although NICD signaling positively regulates Cux2 in spinal cord progenitors, the constitutive activation of NICD overrides Cux2 promotion of neuronal differentiation through the aberrant activation of Hes1 (see Fig. S4 in the supplementary material). However, Notch signaling is normally only transiently activated in dividing progenitor populations, resulting in stochastic cell fate determination (Kageyama et al., 2008). This process is subsequently stabilized by lateral inhibition among neighboring cells and results in the acquisition of asymmetric cell fate, such as the formation of a Hes1-positive progenitor cell alongside a proneural daughter. Although the Notch pathway is involved in the initial regulation of proneural gene expression, other mechanisms are required to increase or maintain the levels of proneural gene expression in selected progenitors so as to stabilize the neuronal differentiation program (Bertrand et al., 2002; Kageyama et al., 2008). Interestingly, Cux2 is expressed in a salt-and-pepper manner in the developing nervous system, as is the case for several Notch1 target genes, including Dll1 and Hes1 (Kageyama et al., 2008; Shimojo et al., 2008). Our data imply that Notch activity, which is normally transient, results in the induction of a Cux2-positive interneuron progenitor in the vz, which then goes on to promote neuronal maturation. Continued Cux2 activity then acts to force cell cycle withdrawal of these nascent neurons through p27^Kip1 and p57^Kip2 activation, resulting in interneuron maturation.

Cux2 has been shown to regulate both cell cycle progression and the balance between interneuron and motoneuron differentiation in the ventral spinal cord (Julianella et al., 2008). Notch signaling also regulates the formation of interneurons in the developing spinal cord (Mizuguchi et al., 2006; Yang et al., 2006), and might do so at least in part via the regulation of Cux2. Our findings suggest that Cux2 acts downstream of the Notch pathway to stabilize the neurogenic program and promote cell cycle exit in dorsal interneuron progenitors.

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Supplementary material
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