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dILA neurons in the dorsal spinal cord are the product of terminal and non-terminal asymmetric progenitor cell divisions, and require Mash1 for their development

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dILA and dILB neurons comprise the major neuronal subtypes generated in the dorsal spinal cord, and arise in a salt-and-pepper pattern from a broad progenitor domain that expresses the bHLH factor Mash1. In this domain, Mash1-positive and Mash1-negative cells intermingle. Using a Mash1^{GFP} allele in mice, we show here that Mash1+ progenitors give rise to dILA and dILB neurons. Using retroviral tracing in the chick, we demonstrate that a single progenitor can give rise to a dILA and a dILB neuron, and that dILA neurons are the product of asymmetric progenitor cell divisions. In *Mash1*-null mutant mice, the development of dILA, but not of dILB neurons is impaired. We provide evidence that a dual function of Mash1 in neuronal differentiation and specification accounts for the observed changes in the mutant mice. Our data allow us to assign to Mash1 a function in asymmetric cell divisions, and indicate that the factor coordinates cell cycle exit and specification in the one daughter that gives rise to a dILA neuron.

KEY WORDS: Asymmetric cell division, Neuronal specification, Spinal cord, Mash1 (Ascl1), Mouse, Chick

INTRODUCTION

The dorsal horn of the spinal cord is the first relay station for somatosensory perception. Neurons located in the dorsal horn receive and process information from the periphery, integrate this sensory information and relay it to higher brain centers. Morphologically and physiologically, dorsal horn neurons are diverse, and they remain ill-defined on a molecular level (Gillespie and Walker, 2001; Hunt and Mantyh, 2001; Julius and Basbaum, 2001). The developmental mechanisms that generate this neuronal diversity are incompletely understood.

Neuroepithelial cells in the developing nervous system produce a remarkable variety of neural cell types in a spatially and temporally controlled manner. Many spinal cord neuronal subtypes are generated from distinct stripes of progenitors that have been patterned by dorsal and ventral signals (Caspary and Anderson, 2003; Helms and Johnson, 2003; Jessell, 2000; Lee and Jessell, 1999). In the dorsal spinal cord, six dorsal neuronal cell types (dI1-dI6) arise from stripes of progenitors during the early developmental phase (E10-E11.5). As development of the dorsal spinal cord proceeds, pronounced temporal and spatial changes in specification of neural cells occur. At E12-E14.5, the majority of the dorsal progenitor domain produces two neuronal subtypes, dILA and dILB, which arise in a salt-and-pepper pattern (Gross et al., 2002; Müller et al., 2002). dILA and dILB neurons are defined by the expression of the homeodomain factors Pax2/Lhx1/5/Lbx1 and Tlx3/Lmx1b/Lbx1, respectively. Homeodomain factors that first appear in postmitotic dILA and dILB neurons determine their further differentiation program. Lbx1 acts as an upstream regulator, and dILA and dILB neurons are not correctly specified in *Lbx1* mutant mice (Gross et al., 2002; Müller et al., 2002). Recently, the essential role of *Tlx3* for the generation of excitatory glutamatergic neurons in the dorsal spinal cord and of *Lbx1* and *Pax2* in the specification of inhibitory GABAergic neurons has been demonstrated (Cheng et al., 2004; Cheng et al., 2005). *Lmx1b* and *Drg11* are essential for terminal differentiation of neurons that settle in the uppermost layers of the spinal cord, which process information from nociceptive sensory neurons (Chen et al., 2001; Ding et al., 2004). dILA and dILB neurons arise from a progenitor domain that expresses *Mash1* (*Ascl1* – Mouse Genome Informatics) and *Gsh1/2*. The role of *Mash1* in the development of dILA and dILB neurons has not been assessed.

Transcription factors of the basic helix-loop-helix family have important roles in the development of the nervous system. Such genes can control the acquisition of a pan-neuronal character, i.e. exit from the cell cycle and neuronal differentiation, as well as the specification of particular neuronal cell-types in vertebrates and invertebrates (Bertrand et al., 2002). *Mash1* encodes a bHLH factor that controls important steps in development of the nervous system. Mutation of *Mash1* reduces neurogenesis in the telencephalon (Casarosa et al., 1999) and interferes with the differentiation of sympathetic neurons (Guillemot et al., 1993; Hirsch et al., 1998; Sommer et al., 1995). Conversely, mis-expression of *Mash1* in the chick spinal cord during the early developmental phase induces premature neuronal differentiation of progenitor cells and the production of supernumerary Isl1/2+ and Tlx3+ neurons (Müller et al., 2005; Nakada et al., 2004). *Mash1* is expressed in progenitors of the dorsal spinal cord and is essential for the development of the dorsal Tlx3+ neuronal subtypes (dI3 and dI5) produced during the early developmental phase.

Asymmetric cell divisions occur during neuronal development in invertebrates and vertebrates (Wodarz and Huttner, 2003). Non-terminal asymmetric progenitor cell divisions generate one progenitor and one differentiating neural cell. They allow

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differentiation concomitant with the maintenance of the progenitor pool. Asymmetric terminal divisions generate two different neural cells and have not been assessed in the development of the vertebrate nervous system. Such divisions have been extensively characterized in sensory organ development of *Drosophila* (Lai and Orgogozo, 2004). The fact that dILA and dILB neurons arise in a salt-and-pepper pattern raises the possibility that they are produced by asymmetric terminal cell divisions. Using a *Mash1*^{GFP} allele in mice, we show that Mash1+ progenitors give rise to dILA and dILB neurons. Furthermore, we demonstrate by retroviral tracing in the chick that a single progenitor can give rise to dILA and dILB neurons, and that dILA neurons are always the product of asymmetric cell divisions. Our analysis of the *Mash1*$^{-/-}$ mice shows that in the absence of *Mash1*, the generation of dILA neurons is severely impaired, which is accompanied by the presence of supernumerary neural progenitors. By contrast, development of dILB neurons is not affected in *Mash1* mutant mice. Thus, despite the fact that *Mash1* is expressed in progenitors of dILA and dILB neurons, it exerts its essential function only in the dILA lineage. Mash1 appears thus to act in an asymmetric manner to coordinate cell cycle exit and specification of the dILA daughter.

MATERIALS AND METHODS

Mouse strains and chick in ovo electroporation

The generation and genotyping of *Mash1* and *Mash1*^{Ngn2} alleles has been previously described (Guillemot et al., 1993; Parras et al., 2002). To generate the *Mash1*^{GFP} allele, Mash1-coding sequences were replaced by Gap43-GFP cDNA (kindly provided by U. Mueller, The Scripps Research Institute, La Jolla, CA) and a floxed neomycin (neoLoxP) cassette. For this, a 3 kb fragment containing the Gap43-GFP sequence and neoLoxP was amplified by PCR, and primers were used that also introduced in addition a sequence of 45 nucleotides homologous to Mash1. We used homologous recombination in bacteria to introduce into a 14 kb genomic subclone of Mash1 this Gap43-GFP neoLoxP fragment, which replaced the Mash1-coding sequence and resulted in the Mash1^{GFPneoLoxP} targeting vector (Lee et al., 2001; Yu et al., 2000). The sequence of the GAP43-GFP fragment in the targeting vector was verified. E14.1 cells were used to introduce the targeting vector by electroporation. ES cell colonies that had inserted the targeting vector into their genome were selected by G418 and analyzed for homologous recombination events by Southern hybridization (Torres and Kühn, 1997). For removal of the neoLoxP cassette, the Cre-deleter strain was used (Schwenk et al., 1995); animals without a neo cassette (Mash1^{GFP} allele) were identified and used for further experiments. Routine genotyping was performed by PCR; occasionally, genotypes were verified by Southern hybridization. PCR-based genotyping for the Mash1^{GFP} allele was performed with the following primers: 5′-AAACCTCCCACACCTCCCCCTGAA-3′ and 5′-ATGCCT-CACCTCGACCTTCT GCTC-3′.

The full-length mouse *Mash1*-coding sequence was cloned into the pCIG vector (pCAGGS-IRES-nucEGFP) (cf. Megason and McMahon, 2002). The *Ptf1a* expression vector was a gift from Jane Johnson. These vectors were electroporated unilaterally into the spinal cord of chick embryos at a concentration of 1 μg/μl using a T820 electro-squareporator (BTX). To determine the role of *Mash1* and *Ptf1a* on neuronal specification during the second developmental phase, the *Mash1* and *Ptf1a* expression constructs were electroporated at Hamburger Hamilton stage (HH26) and the spinal cords were analyzed at HH29-30. Embryos that did not express EGFP dorsally were excluded from the analysis. The effects of the electroporated factors on neuronal specification were quantified as follows: the numbers of Pax2+/GFP+, Tlx3+/GFP+ or Ptf1a+/GFP+ cells were determined on the electroporated side of embryos. Furthermore, the absolute numbers of Pax2+ neurons were determined. A minimum of 10 sections from at least three independently electroporated embryos were counted.

Production of replication-incompetent retrovirus, in ovo injection and lineage analysis

The avian replication-incompetent retroviral vector used, pRAVE GFPnLacZ (a kind gift from M. Samson and C. Cepko), corresponds to a modified version of pRAVE nLacZ (Peters and Cepko, 2002) and contains IRES-GFP. Viruses were pseudotyped with the VSV-G envelope protein and produced in DF1 cells as previously described (Chen et al., 1999). Fertilized White Leghorn eggs (SPAFAS, CT) were used either at HH23-24 or at HH stages 25-26+. The lumen of the spinal cord was injected with RAVE GFPnLacZ virus, and the embryos were analyzed at HH30-31. The rare clones derived from infected cells were identified by anti-β-galactosidase immunohistology, and the cells were also analyzed for the expression of Lbx1 and Lhx1/5. Embryos injected at HH 25-26+ were used for the analysis of the two-cell clones. Progenitor cells were identified by the following criteria, location in the ventricular zone of the dL domain and a lack of Lbx1 and Lhx1/5 expression. dILA and dILB neurons were identified by the expression of Lbx1+/Lhx1/5+ and Lbx1+/Lhx1/5–, respectively, and by a location in the mantle zone lateral of the dL domain. Clones containing cells whose identity was ambiguous were discarded. Non-ambiguous clones were examined in 35 μm frozen sections using a confocal microscope (LSM510, Zeiss). For this, stacks of 0.84 μm optical sections were analyzed.

In situ hybridization, immunofluorescence, BrdU labeling and histology

For in situ hybridization, embryonic tissues were embedded into OCT compound (Sakura) and cryosectioned. Hybridization was performed with DIG-labeled riboprobes, many of which were generated from plasmids derived from other laboratories (see acknowledgements).

Immunofluorescence staining was performed on 12 μm cryosections of mouse and chick embryos fixed with 4% paraformaldehyde in 0.1 M sodium phosphate buffer (pH 7.4). The following antibodies were used on mouse tissue: rabbit and guinea-pig anti-Lbx1 (Müller et al., 2002); rabbit and guinea-pig anti-Tlx3 (Müller et al., 2005); rabbit anti-Olig3 (Müller et al., 2005); mouse anti-Mash1 and anti-Ngn2 (David Anderson); guinea-pig anti-Isl1 and guinea-pig anti-Lmx1b (Tom Jessell, Columbia University, New York, USA); rabbit anti-Ngn1 (Jane Johnson); rabbit anti Gsh1/2 (Martin Goulding); rabbit anti-Ptf1a (Helena Edlund); rabbit anti-GFP (Abcam); rabbit anti-peripherin and mouse anti-NeuN (Chemicon); rabbit anti-Pax2 (Zymed); rabbit anti β-galactosidase (CAPPEL); mouse anti-Tuj1 (Babco); mouse and rabbit anti-phospho-Histon3 (Upstate-Cell Signaling Solutions); monoclonal mouse anti-Lhx1/5, anti-Pax6 and anti-Pax7 (Developmental Studies Hybridoma Bank, University of Iowa). On chick tissue, the following antibodies were used: rabbit anti-β-galactosidase (CAPPEL); guinea-pig anti-Lbx1 (Müller et al., 2002); monoclonal mouse anti-Lhx1/5 (Developmental Studies Hybridoma Bank, University of Iowa); rabbit anti-Tlx3 (Müller et al., 2005); rabbit anti-Pax2 (Zymed) and goat anti-GFP (Abcam). In addition, various fluorophore-conjugated secondary antibodies (Dianova) were employed. For amplification of weak signals, the TSA Cy3 System was used (Perkin Elmer).

For BrdU labeling experiments, BrdU (Sigma; 75 μg/g body weight) was injected intraperitoneally at various stages. Embryos were isolated at the indicated times after injection. Sections were first treated with antibodies that specifically detect various neuronal types and subsequently postfixed (Müller et al., 2002). Incorporated BrdU was then detected with either mouse (Sigma) or rat anti-BrdU antibodies (Oxford Biotechnology). YO-PRO1 (Invitrogen) was used for nuclear counterstaining. TUNEL assays were performed using the ApopTag fluorescein in situ apoptosis detection kit (Intergen). Cell numbers for each genotype were counted on confocal images, and at least nine sections from at least three distinct animals were used for this.

RESULTS

Lineage relationship between Mash1+ progenitors and dILA and dILB neurons

Distinct phases of neurogenesis can be observed in the dorsal spinal cord. At E10.5 and E11.5, d4 and d5 progenitors give rise to one neuronal subtype each, dI4 and dI5 neurons, which express

Lbx1, Pax2 and Lhx1/5 (dI4), and Lbx1, Lmx1b, Tlx3 (dI5) (Fig. 1A,B). By contrast, at E12.5, a broad late dorsal (dL) progenitor domain generates two distinct neuronal subtypes, dILA and dILB, that arise in a salt-and-pepper pattern. dILA neurons are characterized by the expression of Lbx1, Pax2 and Lhx1/5, whereas dILB neurons express Lbx1, Lmx1b and Tlx3 (Fig. 1C,D) (see also Gross et al., 2002; Matise, 2002; Müller et al., 2002). The ratio of dILA to dILB neurons changes as development proceeds, but is constant at a given developmental stage in different individuals (Table 1). For example, pulse chase experiments using BrdU indicate that at E12.5 the ratio of newborn dILA to dILB neurons is 0.74±0.08. In the chick spinal cord, the dorsal progenitor domain undergoes a similar change as development proceeds and generates during a late phase dILA and dILB neurons, which also arise in a salt-and-pepper pattern (data not shown). At HH30, the stage that is comparable with E12.5 in mice, the ratio of newborn dILA to dILB neurons is 0.78±0.09, as assessed by BrdU pulse-chase experiments. The dL progenitor domain in mouse and chick embryos expresses Mash1 and Gsh1/2 (Fig. 1E,F). Gsh1/2 is present in all cells of the dL progenitor domain (Fig. 1E,G,G′). By contrast, Mash1 is expressed in some, but not all cells, of this domain, and Mash1+ and Mash1– cells intermingle (Fig. 1F,G,G′). Ptf1a protein is expressed transiently, and is found in a subset of Mash1+ progenitors located in the lateral progenitor domain (Fig. 1H, see also Fig. 4L and Fig. 7E-H), as well as in a subset of dILA neurons located outside but close to the progenitor domain (Fig. 1H′; see Fig. 1I for a schematic display of the neuronal subtype produced in the spinal cord at E12.5 and of the progenitor domain that generates these neurons).

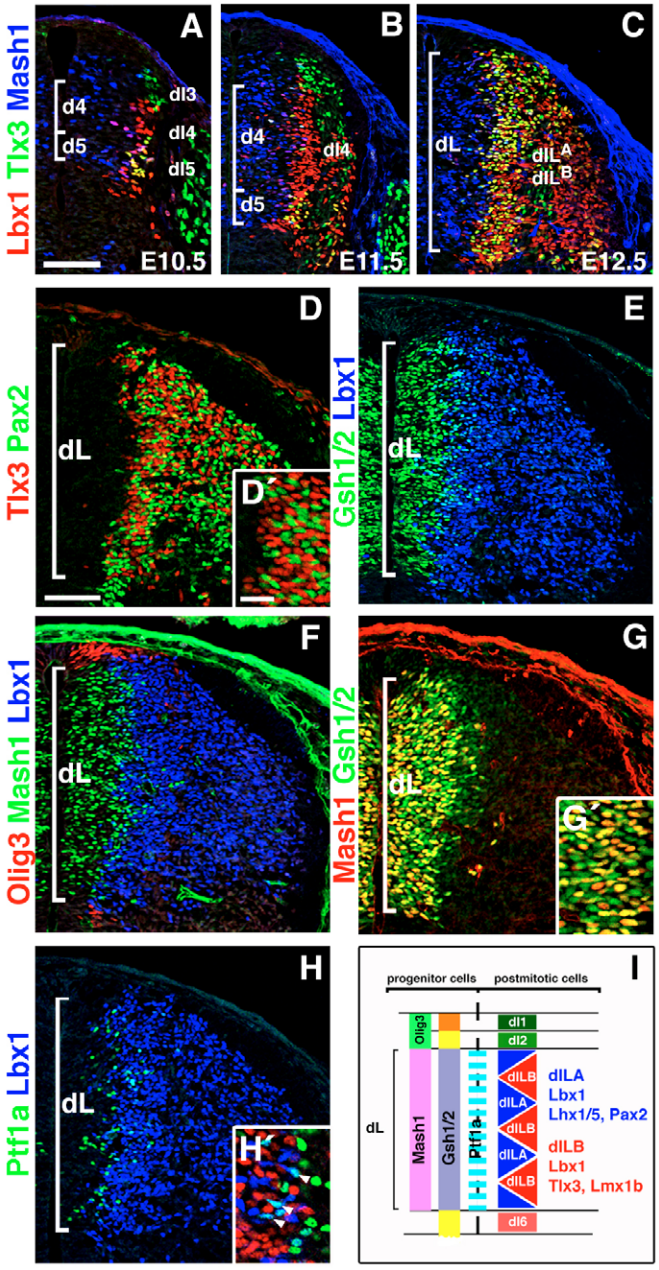

Fig. 1. Temporal and spatial changes in the specification of neurons in the dorsal spinal cord. (**A-C**) Immunohistological analysis of dorsal spinal cords of wild type mice at stage E10.5-E12.5 using antibodies directed against Mash1 (blue), Lbx1 (red) and Tlx3 (green). The d4 and d5 (early) and dL (late) progenitor domains are indicated, as well as the dI3-5, dILA and dILB neuronal subtypes generated. Immunohistological analysis of spinal cords of wild-type animals at stage E12.5 using antibodies directed against (**D**,D′) Tlx3 (red) and Pax2 (green), (**E**) Gsh1/2 (green) and Lbx1 (blue), (**F**) Mash1 (green), Olig3 (red) and Lbx1 (blue), (**G**,G′) Mash1 (red) and Gsh1/2 (green), (**H**) Ptf1a (green) and Lbx1 (blue), and (H′) Ptf1a (green), Lhx1/5 (blue) and Lmx1b (red). (**I**) Schematic display of the expression of transcription factors in progenitors of the alar plate and in dorsal neurons at E12.5. The yellow and orange bars indicate progenitor domains that express Ngn1 and Math1, respectively. Scale bars: 100 μm in A-H; 20 μm in D′,G′,H′.

To assess if Mash1+ progenitors produce predominantly either dILA or dILB neurons, we generated a Mash1^{GFP} allele. GFP is known to be stable, and its expression can be used for lineage tracing. For this, exon 1 sequences of *Mash1* were replaced by *GFP* cDNA using homologous recombination in ES cells (Fig. 2A). In situ hybridization analysis of *Mash1* and *GFP* mRNA showed indistinguishable expression patterns in the spinal cord of *Mash1*$^{GFP/+}$ mice, i.e. *Mash1* and the *GFP* transcripts were found exclusively in the progenitor zone (Fig. 2B,C). Mash1 protein was present only in progenitors. As expected, GFP protein was present in progenitors and persisted, owing to its higher stability, in postmitotic neurons (Fig. 2D-F). In *Mash1*$^{GFP/+}$ mice at E12.5, GFP was expressed in dILA and dILB neurons (Fig. 2E,F). Thus, Mash1+ progenitors generate both neuronal lineages, dILA and dILB.

dILA neurons are generated by asymmetric cell divisions

To assess whether single dorsal progenitor cells can give rise to one dILA and one dLB neuron, we used retroviral lineage tracing in chick embryos and infected progenitors with a replication incompetent RAVE-GFPnlacZ retrovirus that expresses nuclear β-galactosidase [(Peters and Cepko, 2002), for further details see Materials and methods]. Progenitor cells were infected at stage HH25-26+, and the infected cells and their progeny were analyzed

Table 1. The ratio of dILA to dILB neurons

Stage	dILA:dILB
E11.75	1.3±0.12
E12.0	1.01±0.14
E12.5	0.74±0.08
E13.5	0.88±0.06

The ratio of dILA to dILB neurons was determined at the indicated developmental stages in mice. Newborn neurons were labeled by BrdU, Pax2 and Lmx1b staining identified dILA and dILB, respectively. Their numbers were determined by counting at least 9-12 sections from three embryos. The error represents s.d.

at HH30-31 (Fig. 3). We analyzed in detail only those clones that were located in or lateral to the dL domain, and that contained two cells and at least one neuron. Thus, among 50 independently generated clones of this type, 23 contained one progenitor and one neuron, and 27 contained two neurons (Fig. 3B). These clones were analyzed by immunohistological analysis, which demonstrated that dILA neurons were generated by asymmetric cell divisions only (see Fig. 3A for the types of cell divisions observed). In particular, 60% of the dILA (Lbx1+/Lhx1/5+) neurons were generated by cell divisions that produced one dILA neuron and one progenitor, and 40% by cell divisions that produce one dILA and one dILB neuron at this developmental stage (Fig. 3B-E). dILA neurons are therefore always the product of asymmetric cell divisions, that can be terminal or non-terminal. By contrast, dILB (Lbx1+/Lhx1/5–) neurons were produced by asymmetric and by symmetric cell divisions (see Fig. 3A for a summary of all observed divisions).

Mash1 is essential but not sufficient for the generation of dILA dorsal neurons

To determine the role of Mash1 in development of the dorsal spinal cord, we used mice that carry a mutation in the *Mash1* gene (Guillemot et al., 1993). Histological and immunohistological analysis of the spinal cord indicated that the domain occupied by differentiated neurons was reduced in size at E11.5 and subsequent stages in *Mash1*$^{-/-}$ compared with control mice (Fig. 4A,B; Fig. 5A,B). By E12.5, this was morphologically apparent by the abnormal triangular shape of the dorsal horn of *Mash1*$^{-/-}$ mice (compare Fig. 4A with 4B). BrdU-labeling experiments indicated that the numbers of dILA neurons generated were severely reduced in the *Mash1*$^{-/-}$ mice, whereas the numbers of dILB neurons were not affected at E12.5 (Fig. 4A,B,D,E,G,H,I,K). Thus, the dILA neuronal subtype generated by asymmetric cell divisions is the one that is primarily affected by the *Mash1* mutation. *Ngn2* (*Neurog2* – Mouse Genome Informatics), which encodes another factor of the

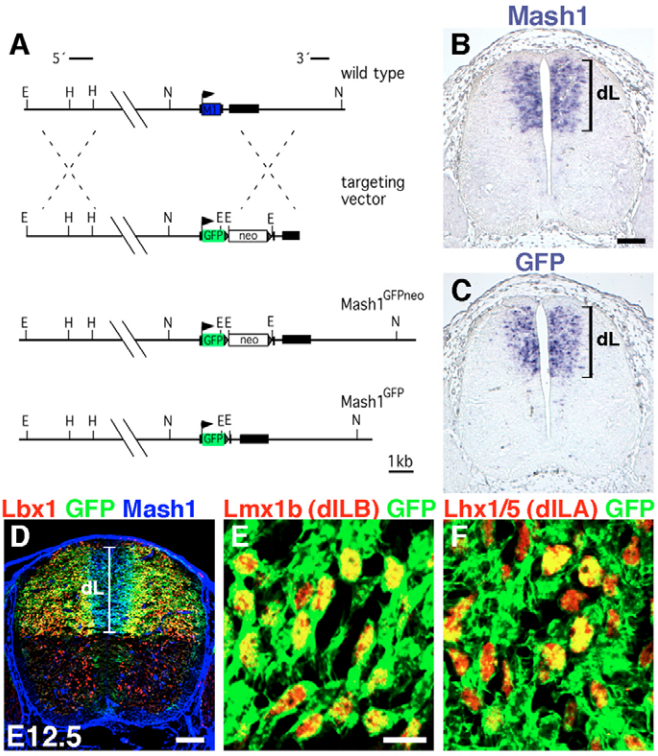

Fig. 2. *Mash1* expressing progenitors give rise to dILA and dILB neurons. (**A**) Strategy used to replace the *Mash1*-coding sequence by *GFP*. The wild type *Mash1* locus, the targeting vector, and the recombined allele before (*Mash1*^{GFPneo}) and after deletion of the neo resistance cassette (*Mash1*^{GFP}) are depicted. In the targeting vector, the *GFP* sequence (green) was fused to the initiation codon of *Mash1*, and the coding sequence of *Mash1* (blue) was deleted. No additional polyA was introduced, and the exon-intron structure of the *Mash1*^{GFP} allele is identical to the one of wild-type *Mash1*. (**B**,**C**) In situ hybridization of consecutive sections from a E12.5 *Mash1*$^{GFP/+}$ spinal cord using probes directed against *Mash1*-coding sequence (B) or *GFP* (C). The expression patterns are identical. (**D-F**) Immunohistochemical analysis of spinal cords of *Mash1*$^{GFP/+}$ mice at E12.5 with antibodies directed against (D) Lbx1 (red), GFP (green) and Mash1 (blue), (E) Lmx1b (red) and GFP (green), and (F) Lhx1/5 (red) and GFP (green). The spinal cord is shown at low (D) and high (E,F) magnification. All Lmx1b+ (dILB) neurons are also GFP+ in E; all Lhx1/5 (dILA) neurons are also GFP+ in F. Scale bars: 100 μm in B-D; 10 μm in E,F.

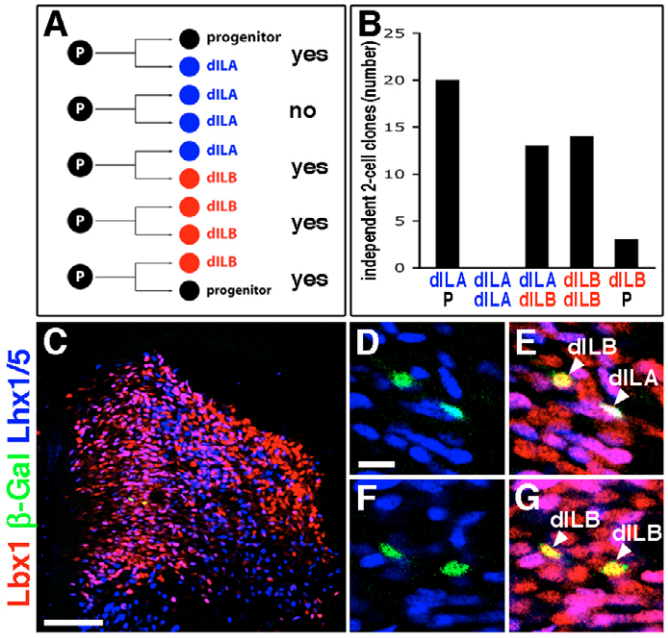

Fig. 3. dILA neurons are the product of asymmetric cell divisions. (**A**) Summary of all possible cell divisions that could generate dILA or dILB neurons. Yes/no indicates if a particular type of progenitor cell division was observed by lineage tracing experiments using retroviral infection. (**B**) Quantification of the different types of independent two-cell clones observed after retroviral infection. (**C-G**) Two-cell clones residing in the dorsal spinal cord were identified after retroviral infection by the expression of β-galactosidase, as assessed by immunohistochemisty (green). The molecular identity of cells was assessed by the use of antibodies directed against Lbx1 (red) and Lhx1/5 (blue). dILA neurons were defined as Lbx1+/Lhx1/5+ cells, dILB neurons as Lbx1+/Lhx1/5– cells, and progenitors as Lbx1-negative cells located close to the ventricle. Only those clones that contained at least one Lbx1+ neuron were assessed. Spinal cords are shown at low (C) and high (D-G) magnification. (E) High magnification of a two-cell clone containing one dILA (white) and one dILB (yellow) neuron. (G) High magnification of a two-cell clone containing two dILB neurons (yellow). Scale bar: 100 μm in A;10 μm in B-E.

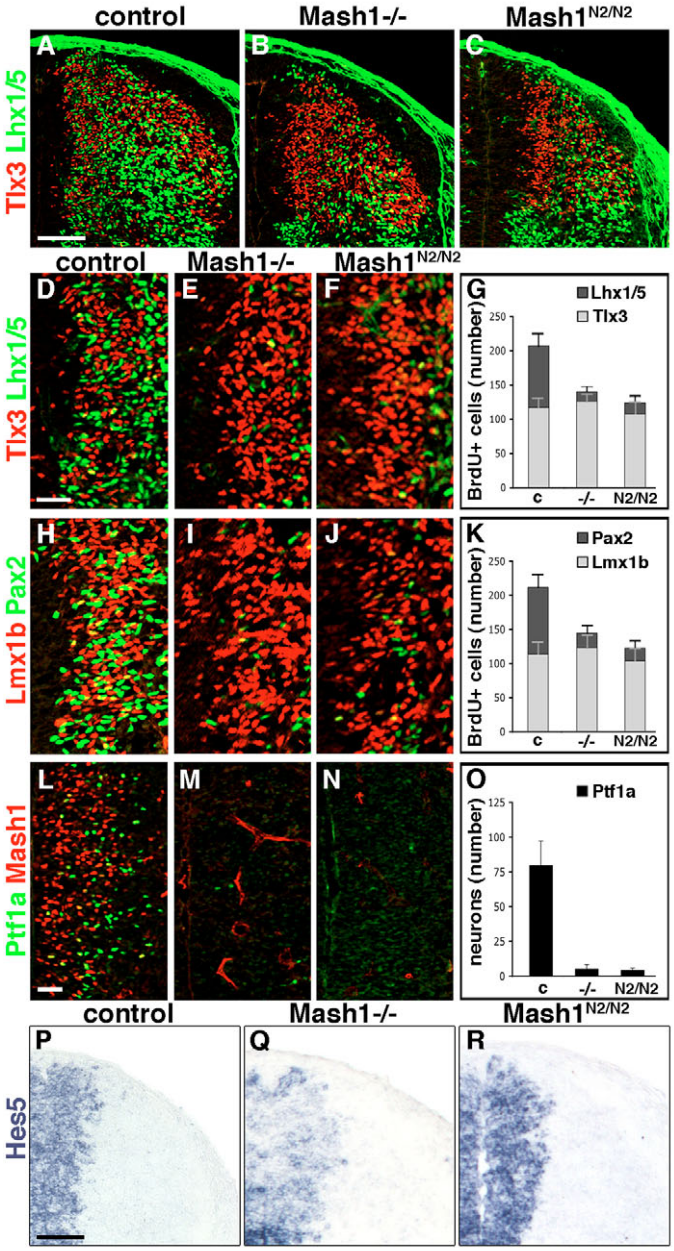

Fig. 4. Effect of the *Mash1* mutation on the generation of dILA neurons. Dorsal neurons in control, *Mash1*$^{-/-}$ and *Mash1*^{Ngn2}/*Mash1*^{Ngn2} embryos were analyzed at E12.5 using immunohistochemistry, with antibodies directed against (**A-F**) Lhx1/5 (green) and Tlx3 (red), (**H-J**) Pax2 (green) and Lmx1b (red). dILA neurons express Lhx1/5 and Pax2, and dILB neurons express Tlx3 and Lmx1b. (**G**,**K**) Numbers of BrdU+ dILA (dark-grey columns) and BrdU+ dILB (light grey columns) neurons in control (c), *Mash1*$^{-/-}$ ($^{-/-}$) and *Mash1*^{Ngn2}/*Mash1*^{Ngn2} (N2/N2) mutant mice, as assessed 24 hours after BrdU injection. The number of dILA neurons is reduced in *Mash1*$^{-/-}$ embryos. (**L-N**) Analysis of Ptf1a (green) and Mash1 (red) expression in the dorsal spinal cord of control (L), *Mash1*$^{-/-}$ (M) and *Mash1*^{Ngn2}/*Mash1*^{Ngn2} (N) embryos. (**O**) Numbers of Ptf1a+ cells (black columns) in control (c), *Mash1*$^{-/-}$ ($^{-/-}$) and *Mash1*^{Ngn2}/*Mash1*^{Ngn2} (N2/N2) mutant mice. The error bars represent the standard deviation. (**P-R**) In situ hybridization of sections from E12.5 control, Mash1$^{-/-}$ and *Mash1*^{Ngn2}/*Mash1*^{Ngn2} spinal cords using a probe directed against *Hes5* mRNA. Scale bars: 100 μm in A-C,P-R; 50 μm in D-F,H-J,L-N.

bHLH family, can replace neurogenic functions of *Mash1* in the telencephalon and hindbrain of mutant mice (Parras et al., 2002; Pattyn et al., 2004); analysis of *Mash1*^{Ngn2}/*Mash1*^{Ngn2} mice, in which coding sequences of *Mash1* are substituted by *Ngn2* cDNA, demonstrated that Ngn2 was neither sufficient to rescue the change in the ratio of dILA to dILB neurons, nor the deficit in neuron numbers (Fig. 4A,C,D-G,H,J,K). *Ptf1a* is known to be essential for the specification of dILA neurons (Glasgow et al., 2005). We also analyzed the expression of Ptf1a in the dorsal spinal cord of *Mash1*$^{-/-}$ and *Mash1*^{Ngn2}/*Mash1*^{Ngn2} mice, which was markedly reduced (Fig. 4L-O). Thus, *Mash1* is essential for Ptf1a expression, and *Ngn2* cannot rescue this. *Hes5* or *Dll1* expression was also reduced in the dorsal spinal cord of *Mash1* mutant mice, and expression of *Hes5* or *Dll1* was rescued in the *Mash1*^{Ngn2}/*Mash1*^{Ngn2} animals (Fig. 4P-R; data not shown).

The number of cells in the dorsal progenitor domain was comparable in the dorsal spinal cord of control and *Mash1*$^{-/-}$ embryos at E10.5, but was increased at E11.5 and subsequent stages (Fig. 5A,B,E). BrdU was injected at various time points, and the numbers of BrdU+ cells were determined 24 hours later to determine the differentiation capacity of dorsal progenitors (Fig. 5F). At E10.5, no change in the number of newly generated dorsal neurons was observed in *Mash1*$^{-/-}$ mice, but the number was significantly reduced at E11.5 and subsequent stages. The differentiation index (number of BrdU+ neurons/total number of BrdU+ neurons and progenitor cells) was comparable in control and *Mash1*$^{-/-}$ embryos at E10.5, but reduced at E11.5 and subsequent stages (Fig. 5H; data not shown). Thus, the probability that progenitor cells differentiate is decreased in the *Mash1*$^{-/-}$ spinal cord at E11.5 and subsequent stages. To determine if the proliferation capacity was affected, replicating progenitor cells were labeled by BrdU injection, and the numbers of BrdU+ progenitors were determined 2 hours later (Fig. 5G). The proliferation index (number of BrdU+ progenitor cells/total number of progenitor cells) was similar in control and *Mash1*$^{-/-}$ mutant mice. We conclude therefore that the increased size of the progenitor domain is caused by a reduction in the probability that the progenitors leave the cell cycle and differentiate. In the dorsal spinal cord of control mice, Pax7 and Lbx1 are expressed in progenitor cells and differentiated neurons, respectively, and cells that co-express Pax7 and Lbx1 in the progenitor domain are only rarely observed (Fig. 5C). By contrast, many cells in the progenitor domain of *Mash1*$^{-/-}$ mice co-expressed Pax7 and Lbx1 (Fig. 5D). Other proteins typically expressed by postmitotic neurons like TuJ1, NeuN, Pax2 or Lmx1b were not expressed by Pax7+ progenitors, indicating that abnormal progenitor cells in the Mash1$^{-/-}$ mice exist that have partially initiated their differentiation program but are unable to complete it. We investigated if the supernumerary cells in the progenitor zone were eliminated by cell death in *Mash1* mutant mice, and observed a significant increase in apoptosis at E11.5 and subsequent stages. For example, at E11.5 and E12.5, the number of TUNEL+ cells was increased by the factors 3.2 and 9.7, respectively (E11.5, 5.6±3.9 and 17.7±8.2 TUNEL+ cells in control and Mash1$^{-/-}$ mice, respectively; E12.5, 6.7±3.4 and 64.1±13.6 TUNEL+ cells in control and Mash1$^{-/-}$ mice, respectively). The majority of the TUNEL+ cells in *Mash1* mutant mice were located in the ventricular zone (86.6±4.8% and 13.3±4.8% of TUNEL+ cells in the ventricular and mantle zone, respectively). Thus, dILA neurons are not generated in *Mash1*$^{-/-}$ mice, and instead supernumerary cells accumulate in the progenitor zone (see Fig. 8A for a summary of the phenotype observed in the dorsal spinal cord of Mash1$^{-/-}$ mice). Analysis of *Mash1*^{Ngn2}/*Mash1*^{Ngn2} mice demonstrated that expression of *Ngn2* did not suffice to rescue the

reduction in the differentiation index (Fig. 5H). Similarly, apoptosis in the spinal cord of *Mash1*^{Ngn2}/*Mash1*^{Ngn2} animals was high (E12.5, 6.7±3.4 and 35.1±6.5 TUNEL+ cells in control and *Mash1*^{Ngn2}/*Mash1*^{Ngn2} mutant mice, respectively), and observed predominantly in the ventricular zone (89.5±5.8% and 10.5±5,8% of TUNEL+ cells in the ventricular and mantle zone, respectively).

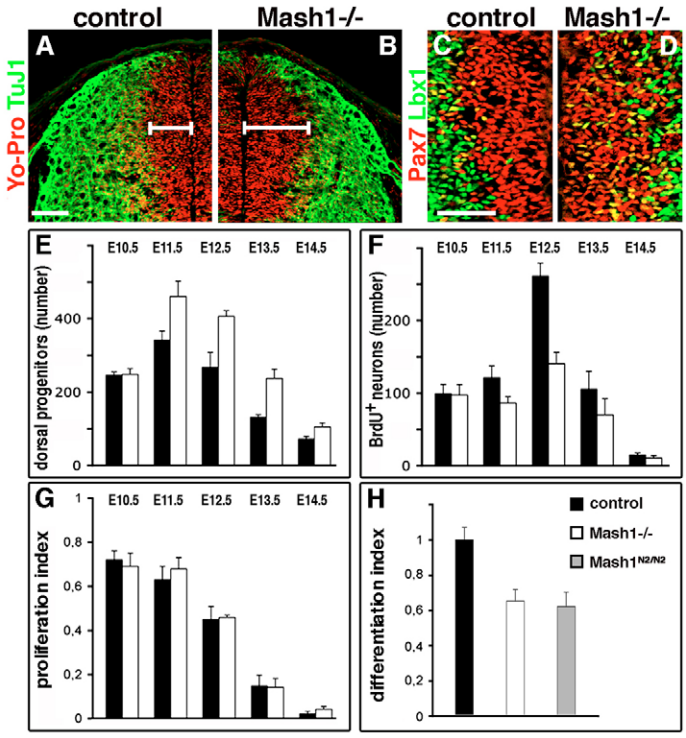

Fig. 5. Essential neurogenic function of *Mash1* at E11.5 and subsequent stages in the dorsal spinal cord. (**A-D**) Spinal cords of *Mash1*$^{-/-}$ and control embryos were analyzed by immunohistochemistry at E12.5. (A,B) Differentiated neurons were visualized using the TuJ1 antibody (green), and YO-PRO1 (red) was used to stain nuclei. The width of the progenitor domain is indicated. (C,D) In control embryos, Lbx1+ cells (green) are rarely found in the progenitor domain, marked by the expression of Pax7 (red). In *Mash1*$^{-/-}$ embryos, many Lbx1+ cells can be found in the progenitor domain co-expressing Lbx1 and Pax7 (yellow). (**E**) Numbers of Pax7+ cells in the dorsal progenitor domain were determined in *Mash1*$^{-/-}$ (white columns) and control (black columns) embryos at the indicated time points. The number of progenitor cells is increased in *Mash1*$^{-/-}$ at E11.5 and at subsequent stages. (**F**) Quantification of newborn neurons at various developmental stages; to achieve this, BrdU was injected at various time points, and the number of dorsal BrdU+/NeuN+ neurons in *Mash*$^{-/-}$ and control embryos was determined 24 hours later. There is a decrease in the number of newborn neurons at E11.5 and at subsequent stages in *Mash1*$^{-/-}$ embryos. (**G**) The proliferation index of progenitor cells (number of dorsal BrdU+ progenitor cells/number of Pax7+ progenitor cells after a 2-hour BrdU pulse) was determined in *Mash1*$^{-/-}$ and control embryos at the indicated time points. The proliferation index of progenitor cells is not altered in *Mash1*$^{-/-}$ embryos. (**H**) The differentiation index (number of BrdU+NeuN+ dorsal neurons/total number of BrdU+ dorsal cells after a 24 hours BrdU pulse) was determined in *Mash1*$^{-/-}$ (white columns), *Mash1*^{Ngn2}/*Mash1*^{Ngn2} (grey columns) and control (black columns) embryos at E12.5. The thickness of the optical section shown in A and B is 10 μm; the thickness of the optical sections shown in C and D is 1.2 μm. The error bars in E-H represent s.d. Scale bars: 100 μm in A,B; 50 μm in C,D.

We used electroporation experiments to test if *Mash1* or *Ptf1a* suffice to induce dILA neurons in the late developmental phase. For this, the dorsal spinal cord of chick embryos was electroporated at stage HH26, and the generation of dILA/dILB neurons was assessed at HH30 (Fig. 6). Electroporation of *Mash1* could neither induce the generation of dILA neurons (Fig. 6D-F), nor the expression of *Ptf1a* (data not shown). By contrast, after electroporation of *Ptf1a*, the majority of postmitotic cells that expressed Ptf1a expressed Pax2; furthermore, dILA neurons were generated in increased numbers (Fig. 6G-I; 180.4±22.6 Pax2+ cells in control embryos and 289.6±30.7 Pax2+ cells in embryos electroporated with *Ptf1a*). We conclude therefore that *Mash1* is essential, but not sufficient, to control the expression of *Ptf1a* and the specification of dILA neurons (Fig. 4).

Distinct expression of Mash1 and Ptf1a during the cell cycle

To identify if progenitor cells in a particular stage of their cell cycle express Mash1 or Ptf1a, we labeled S-phase cells by injection of BrdU and analyzed the BrdU+ cells at various time points (30 minutes, 2 hours and 8 hours) after the BrdU injections (Fig. 7). M-phase cells were identified by the immunohistological analysis of phosphorylated histone 3. These labeling experiments indicated that in the dorsal spinal cord at E12.5, the majority of the cells reach M-phase 2 hours after the injection of BrdU (Fig. 7A-D). The majority of Ptf1a+ cells were BrdU+ only 8 hours after BrdU injections, and M-phase cells never expressed Ptf1a (Fig. 7E-H). This indicates that Ptf1a is expressed mainly after the M-phase and during the G0-phase of the cell cycle (see summary in Fig. 7M). During the asymmetric cell divisions that give rise to dILA neurons, Ptf1a expression is thus induced after the division of the progenitor. This is consistent with the observation that Ptf1a expression is restricted to the dILA lineage (Glasgow et al., 2005). By contrast, Mash1+ cells were BrdU+ at all time points after the BrdU injections, and also M-phase cells were Mash1+ (Fig. 7I-L). Mash1 is therefore expressed at all stages of the cell cycle in a subset of the progenitor cells, and might mark progenitors that will undergo symmetric or asymmetric terminal divisions (see summary in Fig. 7M). We propose therefore that during the asymmetric cell divisions that generate dILA neurons, *Mash1* acts after M-phase to control Ptf1a expression and the correct specification of the dILA neuronal subtype (see also Fig. 8B and Discussion).

DISCUSSION

Neuronal specification in the dorsal spinal cord undergoes a temporal change during development. During an early phase, six neuronal subtypes are born at characteristic positions along the dorsoventral axis in a stripe-like pattern. By contrast, during a late phase, the majority of the dorsal spinal cord generates two neuronal types, dILA and dILB, which arise in a salt-and-pepper pattern. *Mash1* is expressed in the dorsal progenitor domain that generates the dILA and dILB neurons, but mRNA and protein are detected only in a subset of the progenitor cells in this domain. Using a *Mash1*^{GFP} allele, we show that the *Mash1*-expressing cells produce dILA and dILB neurons. Furthermore, we demonstrate that a single progenitor cell can generate dILA and dILB daughters. In *Mash1*$^{-/-}$ mice, neural progenitors are apparently produced at the expense of dILA neurons, whereas the mutation has little effect on the numbers of dILB neurons generated. Thus, despite the fact that endogenous *Mash1* is expressed in progenitors that will generate dILA and dILB neurons, it appears to function only during the generation of dILA neurons. We therefore propose that *Mash1* exerts asymmetric functions in the terminal cell divisions that give rise to dILA neurons, and discuss this with respect to neurogenesis and neuronal specification.

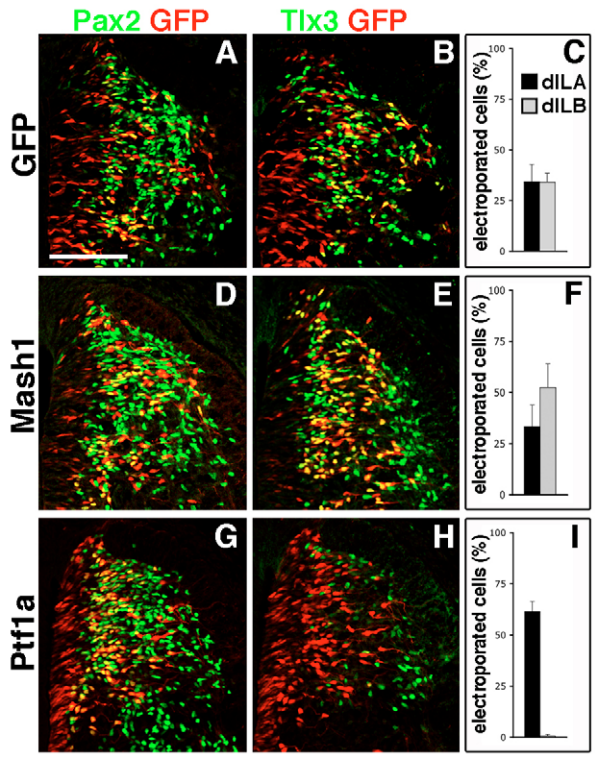

Fig. 6. Ectopic expression of *Mash1* is not sufficient to induce dILA neurons. Chick neural tubes were electroporated at HH26 with constructs expressing *GFP* (**A-C**), *Mash1* and GFP (**D-F**), or Ptf1a and GFP (**G-I**), and the effects on neuronal specification were assessed at HH29-30 using antibodies against GFP (red), Pax2 (green in A,D,G) and Tlx3 (green in B,E,H). The proportion of GFP+/Pax2+ (black columns) and GFP+/Tlx3+ cells (light-grey columns) after electroporation of the indicated constructs were determined in the alar plate. The error bars represent s.d. Scale bar: 100 μm.

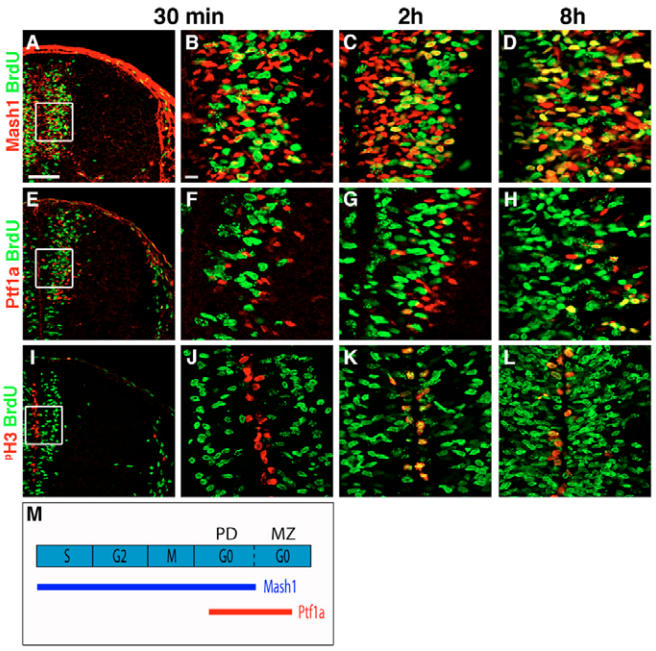

Fig. 7. Mash1 but not Ptf1a is expressed during all stages of the cell cycle. To determine at which stages of the cell cycle Mash1 or Ptf1a are expressed, BrdU was injected at the indicated time points before analysis of E12.5 embryos. (**A-D**) Analysis of dorsal spinal cords with antibodies directed against BrdU (green) and Mash1 (red). (**E-H**) Analysis of dorsal spinal cords with antibodies directed against BrdU (green) and Ptf1a (red). (**I-L**) Analysis of dorsal spinal cords with antibodies directed against BrdU (green) and phosphorylated Histone 3 (red), a marker for M-Phase cells. (**M**) Schematic display of the expression of the transcription factors Mash1 and Ptf1a during cell cycle at E12.5 (PD cell located in progenitor domain; MZ cell located in mantle zone). Scale bars: 100 μm and 10 μm.

The role of *Mash1* in neurogenesis

As development proceeds, increasing deficits in the production of dorsal neuron numbers are apparent in *Mash1* mutant mice, indicating that neurogenesis is impaired. In control mice, Notch signaling increases with progressing development, which is reflected in an increase in the expression of Notch target genes such as *Hes5* (Wu et al., 2003). Notch signals keep progenitors in an undifferentiated state and preclude neuronal differentiation (Beatus and Lendahl, 1998; de la Pompa et al., 1997; Hatakeyama et al., 2004; Hitoshi et al., 2002; Ohtsuka et al., 1999). To overcome the increased Notch signals, the pro-neural functions of factors like Mash1 might thus gain in importance. Mash1 and Ngn2 can both act as pro-neural factors (Parras et al., 2002), and both induce premature neuronal differentiation when mis-expressed in the chick (Cai et al., 2000; Lee et al., 2005; Lee and Pfaff, 2003; Mizuguchi et al., 2001; Nakada et al., 2004). In the dorsal spinal cord, aspects of the pro-neural function of *Mash1* can be rescued by the expression of *Ngn2*; for example, the expression of *Hes5* or *Dll1*. Nevertheless, *Ngn2* cannot rescue the deficits in dorsal neuron numbers that are caused by the *Mash1* mutation.

The reduction in the neuronal differentiation in *Mash1* mutant mice became apparent around E11.5 and thus around the time when the late phase of neurogenesis commences and dILA and dILB neuronal subtypes appear. Interestingly, the *Mash1* mutation only reduced the numbers of dILA neurons, whereas the numbers of dILB neurons generated were not affected at E12.5. We demonstrate that this reflects a reduced probability of differentiation, which was accompanied by an increased cell number in the dorsal progenitor zone of *Mash1*$^{-/-}$ animals. The number of cells that incorporate BrdU increased proportionally with the cell numbers, indicating that the supernumerary cells can replicate their DNA and correspond thus to progenitors. Nevertheless, some progenitor cells that inappropriately failed to differentiate in *Mash1* mutant mice might not have retained a complete progenitor character, as many Lbx1+ cells are detected in the progenitor zone of *Mash1* mutant mice. These abnormal progenitors express Lbx1, but not other markers of neuronal differentiation. The increase in cell number in the progenitor zone of the dorsal spinal cord of *Mash1* mutant mice is accompanied by an increase in apoptosis, and we suggest that many supernumerary progenitors are subsequently eliminated by cell death (see Fig. 8A for a summary).

Mash1 and the neuronal specification in the dorsal spinal cord

During the early developmental phase, six distinct neuronal types (dI1-dI6) arise in stripes in the dorsal spinal cord, and of these, dI3-dI5 are generated from a Mash1+ progenitor domain. In the *Mash1*$^{-/-}$ mice, dI3 neurons are born in reduced numbers, and dI5 neurons are not specified (Helms et al., 2005). Mis-expression experiments assign instructive functions to *Mash1* during early

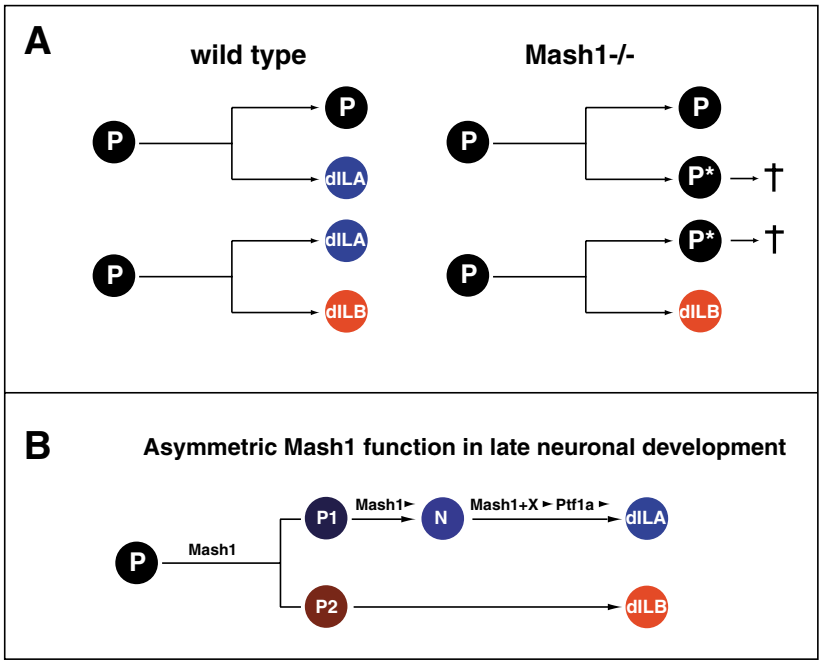

Fig. 8. Asymmetry of Mash1 function in the development of dILA neurons. Schematic diagram of progenitor cell divisions that generate a dILA daughter, and a model of the *Mash1* function in their development. (**A**) dILA neurons are generated from progenitor cells by asymmetric cell divisions in control mice (left). These divisions are either non-terminal (top) and generate one dILA neuron and one progenitor, or are terminal (bottom) and generate one dILA and one dILB neuron. In *Mash1*$^{-/-}$ mice, aberrant progenitor cells (P*) are formed at the expense of dILA neurons (right). Supernumerary dorsal progenitors of *Mash1*$^{-/-}$ mice can incorporate BrdU and do thus replicate, but many are subsequently eliminated by apoptosis. (**B**) A model of Mash1 function in the development of dILA neurons that arise by asymmetric terminal divisions. Mash1 is expressed in the progenitor cell that gives rise to a dILA and a dILB neuron. In the dILA daughter, *Mash1* exerts essential functions for neurogenesis and lineage specification. *Mash1* allows a dILA progenitor (P1) to differentiate (P1→N), and to express Ptf1a. By contrast, *Mash1* is dispensable for the development of the dILB lineage.

development in the specification of dI3 and dI5 neurons. Nevertheless, *Mash1* imposes only a partial dI3 or dI5 character, and the activity of additional factors is required for correct specification of these neurons (Helms et al., 2005; Müller et al., 2005; Nakada et al., 2004). Our previous work has identified one such factor, Olig3, that can cooperate with Mash1 to specify dI3 neurons (Müller et al., 2005), but we have to postulate others that are involved in order to explain the observed effects of Mash1 on the specification of dI5 neurons.

Analysis of the late developmental phase in *Mash1* mutant mice indicates that differentiation and specification of dILA neurons cannot occur correctly. Furthermore, *Mash1* is essential to control the expression of *Ptf1a*. *Ptf1a* is transiently expressed during neuronal differentiation and is essential for the specification of dI4 and dILA neurons (Glasgow et al., 2005). *Mash1* appears to direct the specification dILA, but not dI4 neurons, via controlling *Ptf1a* (see Fig. 8B for a summary). However, electroporation experiments indicate that Mash1 is not sufficient to induce *Ptf1a* expression, and we have to postulate an as yet unknown cooperating factor.

Two possible models can account for the function of Mash1 in development of dILA and dILB neurons. In the first model, Mash1 would be required for cell cycle exit of all neurons. In addition, in the asymmetric divisions that generate dILA neurons, Mash1 would be essential for specification of the dILA daughter. This would account for the reduction in the number of neurons in the *Mash1*$^{-/-}$ mice, and we would have to postulate that aberrantly specified dILA neurons would assume a dILB fate. In such a scenario, a mere coincidence would account for the unchanged number of dILB neurons observed in control and Mash1 mutant mice at E12.5. *Ngn2* would be expected to rescue, at least partially, cell cycle exit and the number of dorsal neurons in *Mash1* mutant mice, but not the deficit in neuronal specification (Parras et al., 2002; Pattyn et al., 2004). This should cause an increase in dILB neurons in *Mash1*^{Ngn2}/*Mash1*^{Ngn2} compared with *Mash1*$^{-/-}$ mice, which was not observed. We therefore favor a second model, in which *Mash1* exerts a function in cell cycle exit only if the progenitor produces a daughter destined to a dILA fate. Thus, in asymmetric divisions that generate dILA neurons, *Mash1* (but not *Ngn2*) would coordinate cell cycle exit and specification of the one daughter destined to generate a dILA neuron (see Fig. 8 for a summary of the second model). Notch signals control asymmetric cell divisions and the asymmetric fate specification in neural development of *Drosophila* (Lai and Orgogozo, 2004). It remains to be investigated if *Notch* is required, together with *Mash1*, in the asymmetric cell divisions that generate dILA neurons.

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