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The GATA2 transcription factor negatively regulates the proliferation of neuronal progenitors

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Postmitotic neurons are produced from a pool of cycling progenitors in an orderly fashion that requires proper spatial and temporal coordination of proliferation, fate determination, differentiation and morphogenesis. This probably relies on complex interplay between mechanisms that control cell cycle, specification and differentiation. In this respect, we have studied the possible implication of GATA2, a transcription factor that is involved in several neuronal specification pathways, in the control of the proliferation of neural progenitors in the embryonic spinal cord. Using gain- and loss-of-function manipulations, we have shown that *Gata2* can drive neural progenitors out of the cycle and, to some extent, into differentiation. This correlates with the control of cyclin D1 transcription and of the expression of the p27/Kip1 protein. Interestingly, this functional aspect is not only associated with silencing of the Notch pathway but also appears to be independent of proneural function. Consistently, GATA2 also controls the proliferation capacity of mouse embryonic neuroepithelial cells in culture. Indeed, *Gata2* inactivation enhances the proliferation rate in these cells. By contrast, GATA2 overexpression is sufficient to force such cells and neuroblastoma cells to stop dividing but not to drive either type of cell into differentiation. Furthermore, a non-cell autonomous effect of *Gata2* expression was observed in vivo as well as in vitro. Hence, our data have provided evidence for the ability of *Gata2* to inhibit the proliferation of neural progenitors, and they further suggest that, in this regard, *Gata2* can operate independently of neuronal differentiation.

KEY WORDS: GATA2, Cell cycle, Neuronal progenitors, Posterior neural tube, Mouse, Chick

INTRODUCTION

The nervous system is a complex tissue including multiple types of neurons organised in elaborate circuits. All neurons are derived from the neuroectoderm and are composed of multipotent proliferating neural progenitors that can individually give rise to both neuronal and glial cell types in a characteristic order of cell differentiation events. This implies that progenitor cell proliferation versus cell cycle exit and differentiation must be precisely coordinated during nervous system development in order to avoid either the depletion of progenitors before the end of histogenesis (Ohnuma et al., 2002) or, conversely, an excess of later-born cell types (Cremisi et al., 2003; Galderisi et al., 2003; Ohnuma and Harris, 2003). Correct coordination between proliferation and differentiation is achieved through different signalling pathways. The most widely implicated pathway in sustaining progenitor proliferation is mediated downstream of Notch receptor activation. This pathway underlies two major mechanisms involved in the choice between maintenance and arrest of proliferation: lateral inhibition and asymmetric division (Artavanis-Tsakonas et al., 1999; Roegiers and Jan, 2004). Upregulation of proneuronal genes, such as *Mash1* (*Ascl1* – Mouse Genome Informatics) or neurogenins, promotes commitment of progenitors to a neuronal fate and cell cycle exit (Bertrand et al., 2002; Blader et al., 1997). Newborn neurons transiently express high levels of either of two types of Notch ligand, delta or jagged (Dll1 and Jag1), which will activate Notch in neighbouring cells and prevent them from differentiating; a phenomenon known as lateral inhibition of neurogenesis (Artavanis-Tsakonas et al., 1999). In the case of asymmetric division, only one daughter cell inherits components inhibiting Notch signalling and as a result differentiates, whereas the other daughter cell keeps dividing and remains a precursor (Petersen et al., 2002; Roegiers and Jan, 2004). Other extrinsic and intrinsic cues are believed to govern the progression of a multipotent neural progenitor towards a post-mitotic neuronal precursor state (Edlund and Jessell, 1999). Indeed, crosstalk between Notch signalling and other signalling pathways, acting in specific regions, have been reported (Franco et al., 1999; Hofmann et al., 2004; Tsuda et al., 2002).

Regulatory proteins involved in neuron specification have also been shown to influence the cell cycle (Dubreuil et al., 2000; Edlund and Jessell, 1999; Tanabe et al., 1998). However, data concerning the genetic events, which most probably link specification, modulation of Notch signalling, pro-neural gene trans-activation and the cell cycle machinery, are still limited. Pax6 has been shown to regulate the expression of neurogenin 2 (*Ngn2*; *Neurog2* – Mouse Genome Informatics) in several populations of neuronal precursors in both the brain and the spinal cord (Scardigli et al., 2003), whereas *Mash1* has been placed upstream of genetic cascades leading to the differentiation of serotoninergic (Pattyn et al., 2004) and sympathetic (Tsarovina et al., 2004) neurons. In the retina, cyclin D1 and cell cycle inhibitors, such as the p57/Kip2 (Cdkn1c – Mouse Genome Informatics) and p27/Kip1 (Cdkn1b – Mouse Genome Informatics) proteins, have been shown to be differentially involved in distinct differentiation pathways, suggesting that the expression of cell cycle regulators may be dependent on specification genes (Dyer and Cepko, 2001; Livesey and Cepko, 2001). Deciphering crosstalk between specification genes, proneural genes and the machinery of the cell cycle appears therefore to be a key issue.

Here, we report several lines of data supporting the involvement of GATA2, a transcription factor that contains two C4 zinc fingers, in such crosstalk. GATA2 was first described as a regulatory protein

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that plays a major role in the proliferation of progenitors and in lineage specification during early hematopoiesis. Indeed *Gata2* loss of function in the mouse leads to the death of embryos between E9.5 and E11, owing to severe defects in primary hematopoiesis (Tsai et al., 1994). *Gata2* also appears to be indispensable for the differentiation of several other tissues during embryogenesis, including the uro-genital (Zhou et al., 1998) and the nervous systems. In the latter case, *Gata2* is known to participate in the differentiation pathway of different types of neurons in the ventral hindbrain and spinal cord, namely cranial nerves (Nardelli et al., 1999; Pata et al., 1999), serotoninergic neurons (Craven et al., 2004; Pattyn et al., 2004) and V2 interneurons (Karunaratne et al., 2002; Zhou et al., 2000). The fact that, in each of these lineages, *Gata2* activation is turned on in early precursors raised the possibility that *Gata2* may influence the cell cycle and probably, like other specification genes, participate in its withdrawal. In order to gain further insight into this possible new aspect of *Gata2* function, we carried out analyses aimed at a better understanding of how *Gata2* is linked to the cell cycle. Our data have established that *Gata2* expression is sufficient to force cycling neural progenitors to switch to a quiescent and eventually differentiating state in vivo. In addition, we show that *Gata2* acts as a potent inhibitor of proliferation in embryonic undifferentiated neuroepithelial cells in culture. Such negative control on the proliferation of neuronal progenitors appears to be exerted by *Gata2* by interfering with the regulation of cell cycle components and by shutting off the Notch pathway.

MATERIALS AND METHODS

Mouse embryos

Mice heterozygous for the *Gata2*-null mutation (Tsai et al., 1994) were maintained in a mixed C57Bl6/DBA2 background, which was found to slightly improve the survival of the *Gata2*$^{-/-}$ embryos in comparison with the pure C57Bl6 background. The day of the vaginal plug was considered to be E0.5 and embryos were collected at E10.5, a stage at which the survival of the homozygous embryos starts to decrease significantly. Only surviving embryos, identified by their beating heart, were included in our studies. Animals and embryos were genotyped as described (Tsai et al., 1994). For BrdU incorporation, pregnant females were injected intraperitonally (2 g/kg) 1 hour before sacrifice.

Electroporation of chick embryos in ovo

In order to obtain the PAdRSV-GATA2HA expression plasmid, the entire human sequence encoding *Gata2* was amplified by PCR so as to introduce a *Nco*I site at the 5′ end, one copy of the HA epitope and an *Eco*RV site at the 3′ end of the cDNA. The PCR product was cloned into PGEM3, entirely checked by sequencing and transferred into pAdRSV-SP (Giudicelli et al., 2001) by *Nco*I and *Eco*RV digestions. The pCAGGS-cId2 (Dubreuil et al., 2000), pAdRSV-bGal (Giudicelli et al., 2001) and pAdRSVGFP plasmids were kindly provided by Christo Goridis, Pascale Gilardi and Philippe Ravassard, respectively. Fertilised eggs from *Gallus gallus domesticus* hens were incubated at 38°C in a humidified oven. The DNA solution (2-3 mg/ml) was injected in the hindbrain and the spinal cord with an Eppendorf Femtojet injector at stage HH12 or HH15. Six pulses of 25 V for 50 mseconds each were then applied with a BTX ECM 830 electroporator. Embryos were further incubated for either 24 or 48 hours (occasionally for 6-12 hours) before being fixed as described below. For BrdU incorporation, a single injection of a 20 mg/ml BrdU solution was injected into the heart or the umbilical vein 30 minutes prior to collecting the embryos.

Immunohistology and in situ hybridisation

Embryos, fixed in paraformaldehyde (PFA) 4% for 2-3 hours for immunostaining and overnight for in situ hybridisation, were equilibrated in PBS 30% sucrose, embedded in PBS containing 15% sucrose and 7% gelatin and frozen at –50°C. Serial sections (12 μm) were then cut with a LEICA CM 3000 cryostat.

For immunostaining, sections were saturated with PBS/0.1% Triton/10% foetal calf serum (FCS) (saturation solution) for 30 minutes and incubated with the primary antibody diluted in the same solution. After several washes in PBS/0.1% Triton, the secondary antibody, diluted in the saturation solution, was added for 30 minutes at room temperature. Slides were then mounted in Fluorescent mounting medium (DAKO). Black and white pictures were obtained with a TCS Leica confocal microscope and false-coloured in Photoshop (Adobe).

BrdU staining was performed as described (Ravassard et al., 1997), except that the secondary antibody was coupled to Alexa Fluor 488 or 546. The nuclei were stained with DAPI before mounting. To assess the percentage of *Gata2*-expressing cells being in S phase in E10.5 mouse embryos, BrdU-positive cells were counted among 850 *Gata2*-positive cells, distributed into 10 spinal cord sections from two different E10.5 embryos. In chick embryos misexpressing GATA2-HA, all the nuclei and the BrdU-positive nuclei were counted on the electroporated and the control sides on at least four sections in three different embryos. Counting was carried out using Metamorph software. The same approach was applied to calculate the percentage of phospho-Histone3-positive cells.

TUNEL stainings were performed with the Apodetect^{plus} kit (Qbiogen) following the supplier's instructions.

In situ hybridisation on sections were performed according to Ravassard et al. (Ravassard et al., 1997). For further immunostaining, sections were processed as described earlier. Staining was analysed with a BX60 Olympus epifluorescence microscope, and pictures were taken with a black and white Cool Snap camera (Ropper Scientific) and false-coloured in Photoshop (Adobe).

Primary and secondary antibodies

The following primary antibodies were used: mouse monoclonal anti-p27/Kip1 (BD Bioscience, 1/2000), anti-βIII-Tubulin (Babco, 1/10,000), anti-BrdU (DAKO, 1/1000), anti-Isl1 (DSHB; 1/20), 3A10 anti-neurofilament-associated protein (1/500; DSHB), anti-NeuN (Chemicon, 1/1000), rat monoclonal anti-HA epitope (Roche, 1/2000), rabbit polyclonal anti-phosho-Histone3 (Upstate Biotechnology, 1/1000), anti-p57/Kip2 (BD Bioscience, 1/2000), anti-GATA2 (Santa-Cruz, 1/400), anti-GFP (Abcam, 1/1000) and anti-Sox2 (Abcam, 1/1000). The fluorescent secondary antibodies, goat anti-mouse, -rabbit and -rat IgG, all highly absorbed against other species and coupled either to Alexa Fluor 488 or 546 (Molecular Probes) were diluted 1/2000.

In situ probes

The mouse probes were: *Gata2* (Nardelli et al., 1999); cyclin D1, cyclin D2 and cyclin D3 (Wianny et al., 1998); full-length *p27* cDNA (Toyoshima and Hunter, 1994); 700 bp *Acc*I/*Xba*I fragment for *Sox2*; rat *Notch1* (Lindsell et al., 1996); *Dll1* (Campos et al., 2001); and *Jag1*, full-length *Hes5* cDNA, *Mash1* and *Ngn2* (Gradwohl et al., 1996). The chick probes were: full-length cDNA for *Gata2*; *Sox2*, *Dll1* and *Jag1* (obtained from Nicole Le Douarin's laboratory); *Ngn2* (Novitch et al., 2001); *Cash1* (Groves et al., 1995); and cyclin D1 (Lobjois et al., 2004). The full chick *Hes5*-coding sequence was cloned by RT-PCR from E6.5 chick retina total RNA, using the following primers: 5′-GCGGCTCGAGAGCCAGCTTCGTGC-3′ (forward) and 5′-CCTCTGGAATGTGCACCAGTGTCC-3′ (reverse). The cDNA was checked by sequencing. To monitor the activation of cyclin D1 and *Cash1*, antisense probes devoid of bacterial sequence were synthesised from cDNA fragments amplified by PCR. Primers for cyclin D1 amplification were as described (Lobjois et al., 2004); *Cash1* primers were 5′-TGATGCG-CTGCAAGAGGCGG-3′ (forward) and 5′-GTAATACGACTCACTAT-AGCACCGGGAAACGTGGTCCAGA-3′ (reverse). Likewise, the chick *Id2* probe was synthesised by PCR amplification of the cDNA included in pCAGGS-cId2, using the following primers: 5′-AAGCTTTCAGCCCCGT-GCGGT-3′ (F) and 5′-GTTAATACGACTCACTATAGAGCGTGGATT-CCTCCCCTCC-3′ (R).

Cell culture

Mouse embryonic neuroepithelial cell cultures (ENC) were initiated from neural tube explants of E9.5 wild-type and *Gata2*$^{-/-}$ embryos, generated in the same genetic background and maintained as described (Nardelli et

al., 2003). All comparative experiments between wild-type and *Gata2*$^{-/-}$ cells were performed at matching numbers of passages, which never exceeded 12. To compare the proliferation rate by BrdU incorporation, cells were seeded on glass cover-slips in 24-well plates. At 50% confluency, the cells were placed in medium containing 0.5% FCS. Twenty-four hours later, BrdU (10 μg/ml of culture medium) was added with 10% FCS. The cells were then fixed at different time intervals and treated as previously described for tissue sections. The nuclei were stained with DAPI before the slides were mounted with Dako Fluorescence Mounting Medium. Experiments for each time point were carried out in duplicate, and all experiments were repeated three times. Nuclei were then counted using Metamorph software. Counting was carried out on at least five fields, including more than 200 cells selected on different coverslips. Transfection experiments and immunostaining were performed as described (Nardelli et al., 2003). During GATA2-HA transient expression experiments, BrdU incorporation in transfected and control cells was started 30 hours after transfection and allowed for 15 hours. In situ hybridisation on transfected cells was performed as described (Nardelli et al., 2003). As this protocol does not permit further immunostaining, pAdRSV-βGal plasmid was added to pAdRSV-GATA2HA to detect transfected cells by X-Gal coloration before in situ hybridisation (Nardelli et al., 2003). Control cells were transfected with pAdRSV-βGal alone. The culture medium was changed 6 hours after transfection, replaced by fresh medium and collected 24 hours later. This conditioned medium was diluted twice with fresh medium and applied for 24 hours on *Gata2*$^{-/-}$ cells, plated on glass cover-slips in 24-well plates. NB2a neuroblastoma cells were cultured in DMEM supplemented with 10% serum and processed as ENC for immunostaining, transfection and BrdU incorporation.

Semi-quantitative and quantitative PCR reactions

Total RNA from wild type or *Gata2*$^{-/-}$ embryonic neuroepithelial cells was prepared by adding TRIZOL (Invitrogen) directly onto the cells in the tissue-dish and following the manufacturer's instructions for extraction. RNA was further purified on RNeasy columns (QIAGEN), treated with DNAse, then checked and quantified on Agilent chips. Experiments were repeated twice on three different RNA preparations. Quantitative RT-PCR was performed using a SIGMA SYBR Green Quantitative RT-PCR kit.

Reverse-transcription was performed from 5 μg RNA, with Superscript III (Invitrogen). Semi-quantitative PCR was performed with 0.5 μl of reverse-transcription reaction, using FastStart Taq polymerase (Roche). The PCR program included an initial denaturation step of 95°C for 4 minutes, then 45 seconds at 95°C, 45 seconds at 65°C and 30 seconds at 72°C for actin and cyclin D2 (20 cycles), cyclin D1 and Notch1 (23 cycles), cyclin D3 and *p27* (27 cycles), and for cyclin E1 (30 cycles). The PCR products, all sized between 300 and 350 bp, were separated in BEt-agarose gels and then analysed with Gel-Doc camera and soft-ware (BioRad) to calculate the relative amount of each product.

The quantitative PCR reactions (20 μl) were performed in a MX 4000 apparatus (Stratagene). The program was as follows: an initial step at 95°C for 10 minutes; 30 seconds at 95°C, 1 minute at 65°C and 30 seconds at 72°C for 36 cycles; then 95°C for 1 minute and a 1°C increment/minute from 55°C up to 95°C to generate denaturation curves.

The primer sequences were as follows: cyclin D1, 5′-TGTGGCC-CTCTGTGCCACAG-3′ (forward) and 5′-TCTCGCACGTCGGTGGG-CGT3 (reverse); cyclin E1, 5′-TCAGGAGCAGCAGGGGTCTGC-3′ (forward) and 5′-CTGTGGGCTCTGCATCCCACA-3′ (reverse); *p27*, 5′-TGGAGAGGGGCAGCTTGCCC-3′ (forward) and 5′-AGGCCGGG-CTTCTTGGGCGT-3′ (reverse); cyclin D2, 5′-TGGCCGCAGTCAGGG-CTCACG-3′ (forward) and 5′-TCAGGGCATCACACGTGAGTGTGT-3′ (reverse); cyclin D3, 5′-CCTGGCCTTGATTCTGCACCG-3′ (forward) and 5′-GCTTCCCTGAGGCTCTCCCTG-3′ (reverse); actin, 5′-GTGG-GCCGCTCTAGGCACCAA-3′ (forward) and 5′-CTCTTTGATGTC-ACGCACGATTTC-3′ (reverse); Notch1, 5′-CAGTCGTCCGACTG-GGGACA-3′ (forward) and 5′-TCGTCCGTGTGAGCACCCAC-3′ (reverse).

RESULTS

Gata2 expression is predominantly associated with newborn neuronal precursors

In the hindbrain and the spinal cord, *Gata2* is ventrally expressed in the presumptive domain of V2 interneuron precursors that abut the dorsal limit of the motoneuron domain (Fig. 1A,B) (Briscoe et al., 2000; Karunaratne et al., 2002; Zhou et al., 2000). In the hindbrain, further expression is observed in a thin domain adjacent to the floor-plate corresponding to the p3 domain described in the spinal cord (Fig. 1A) (Briscoe et al., 2000) and generating serotoninergic neurons (Craven et al., 2004; Nardelli et al., 1999; Pattyn et al., 2004). For the sake of homogeneity, we principally present data relative to the spinal cord, although our analyses were carried out in both the caudal hindbrain and the spinal cord. As shown in Fig. 1A,B,D, the onset of *Gata2* activation occurs in cells localised in medial and intermediate positions, corresponding to cells either still proliferating or newly withdrawn from division and engaged in the differentiation pathway. Using transgenic mice in which *lacZ* expression was driven by *Gata2* cis-regulating elements, Zhou et al. (Zhou et al., 2000) reported a very restricted overlap between proliferating cells, as evidenced by BrdU incorporation, and β-galactosidase expression in the spinal cord. Using in situ hybridisation we confirmed that, at E10.5, only 9% (75 among 850) of the GATA2-positive cells in the spinal cord appeared to be cycling (Fig. 1C,F). Furthermore, we observed a similarly restricted overlap in all the other sites of *Gata2* expression (data not shown). Such observations, and the fact that *Gata2* has been implicated upstream of several differentiation pathways, raised the hypothesis that *Gata2* could participate in coordinating commitment and cell cycle exit.

Gata2 is sufficient to arrest the proliferation of neuronal progenitors in vivo

To assess the possible role of *Gata2* in the withdrawal of neuronal progenitors from the cell cycle, we first induced *Gata2* misexpression in the neural tube of chick embryos by electroporation of pAdRSV-GATA2HA, a plasmid that drives ubiquitous expression of the human GATA2 protein tagged with the HA epitope. Compared with the non-electroporated side of the neural tube, GATA2-HA expression caused a marked decrease (5% versus 11%) in cells containing phospho-Histone3, a pan-marker of the M phase (Fig. 1G-I,M). Likewise, short pulses of BrdU incorporation, performed for half an hour before collecting the embryos, demonstrated a significant decrease in BrdU-positive cells on the electroporated side (28% compared to 47%) and a very limited coincidence with GATA2-overexpressing cells (Fig. 1J-L,N). In addition, we observed that GATA2-HA caused a marked downregulation of *Sox2*, a pan-marker of the neural progenitor stage (Fig. 1O,P). These results were consistent with the hypothesis that GATA2 can behave as an inhibitor of the cell cycle and may thus actively participate in the switch to the post-mitotic stage in neural progenitors.

Interaction between *Gata2* and the cell cycle machinery

To confirm such a role, we investigated possible interactions between *Gata2* and the cell cycle. In most cases, cells leave the cell cycle in G1, when the concentration of cell cycle inhibitors has increased sufficiently to prevent them from reaching the restriction point, thus forcing them to take the G0 branch. These inhibitors principally impair the activity of cyclin-dependent kinases, such as CDK4 and CDK6, which is dependent on interaction with D-type cyclins, namely cyclin D1, D2 and D3 (Cunningham and Roussel,

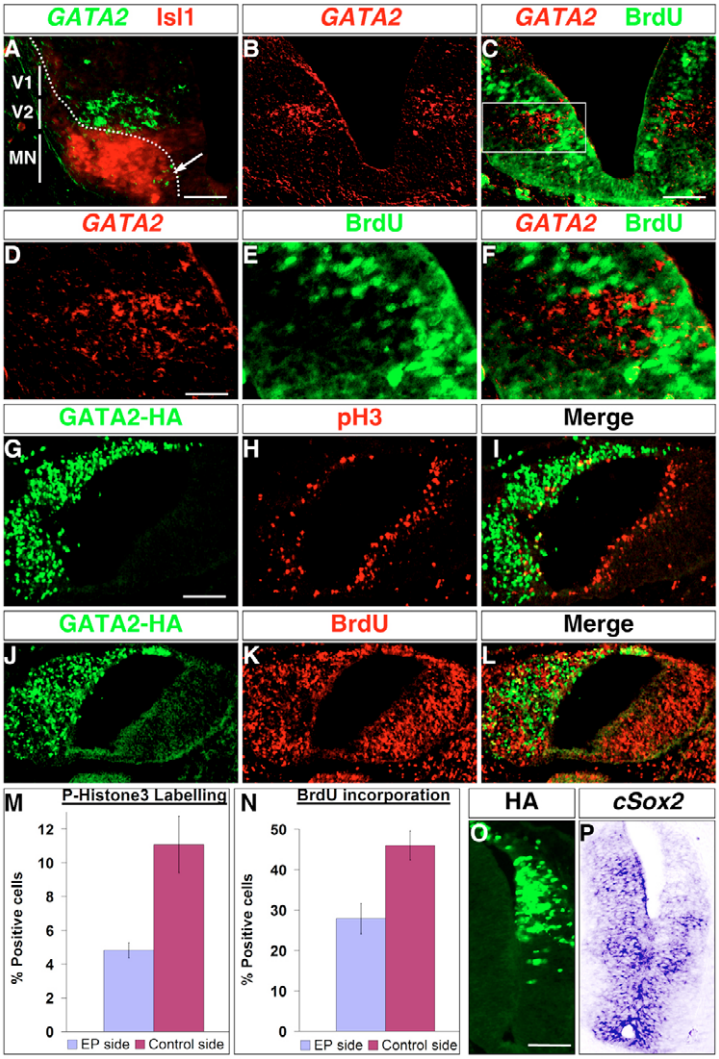

Fig. 1. GATA2 expression is turned on in newborn progenitors and is sufficient to arrest proliferation. (**A**) Transverse section of the caudal hindbrain of a E10.5 mouse embryo, first hybridised with *Gata2* anti-sense RNA (green), then stained with antibodies against Isl1 (red), a pan-marker of motoneurons. The presumptive domain of V2 precursors, which express *Gata2*, is located between the respective domains of the motoneuron and V1 interneuron precursors; in the hindbrain, *Gata2* is also activated to a lesser extent in the p3 domain, indicated by the white arrow. (**B**,**C**) Transverse section of the spinal cord of a E10.5 mouse embryo injected with BrdU. Hybridisation with *Gata2* antisense RNA (B) was followed by BrdU immunostaining (C). (**D-F**) Higher magnification of the area included in the white rectangle in C. (**G-L**) Transverse sections of the spinal cord of chick embryos 24 hours after electroporation with the pAdRSV-GATA2HA plasmid, double-stained with anti-HA antibodies (G,J) and either with anti-phospho-Histone3 (pH3) (H) or anti-BrdU antibodies (K), and analysed by confocal microscopy. (I,L) Superimpositions of G,H (I) and J,K (L). (**M**,**N**) The percentages of phospho-Histone3- and BrdU-positive cells in the control (red, M) and the transfected sides (blue, N) are compared. (**O**,**P**) Adjacent sections of chick embryos misexpressing GATA2-HA, stained with anti-HA-antibodies and hybridised with chick *Sox2* anti-sense RNA. Scale bar: in A, 70 μm in A-C; in D, 20 μm in D-F; in G, 20 μm in G-L; in O, 50 μm in O,P.

2001; Murray, 2004). However, in addition to their active role during the progression towards the G1/S transition, D-cyclins have also been reported, at high expression levels, to exert the opposite effect and to sustain the maintenance of quiescence (Meyyappan et al., 1998; Pagano et al., 1994). Speculating that *Gata2* could probably affect D-cyclin expression, we first closely examined the distribution of their respective transcripts in the ventral neural tube. At E10.5, surprising distinguishing features were observed in the expression patterns of cyclin D1 and D2. As shown in Fig. 2A,B, cyclin D1 transcripts were detected at low levels (cyclin $D1^{low}$) in the ventricular zone and not at all in the marginal layer, which contains maturing neuron precursors. Strikingly, within the ventricular zone, a high level of expression (cyclin $D1^{high}$) was observed in a domain which abuts the dorsal limit of motoneurons (Fig. 2A,E). This domain most probably includes the presumptive domain of *Gata2*-expressing V2 interneurons and more dorsal interneurons, such as V1. Furthermore, the semi-lateral position of the majority of these cells suggested that this high level of expression was associated with newborn precursors. At the same stage, cyclin D2, like cyclin D1, showed two levels of transcription. In contrast to the general low activation observed in the progenitors, transcripts were conspicuously detected in the marginal layer along the entire dorsoventral axis, although to a lesser extent at the level of motoneurons and more ventral precursors (Fig. 2C). Cyclin D3 expression was restricted to the ventricular zone (Fig. 2D). To summarise, the transcription levels of the three cyclin D genes at E10.5 appeared to vary individually according not only to the phase of the cell cycle, but also to cell specificity. In E10.5 $Gata2^{-/-}$ embryos, the transcription pattern of neither cyclin D2 nor cyclin D3 seemed to be significantly affected (data not shown). By contrast, we observed a decrease in the number of newborn precursors distinguished by cyclin $D1^{high}$ transcription (Fig. 2B). This suggested that *Gata2* might participate in the upregulation of cyclin D1 in newly committed precursors. This hypothesis was further assessed in *Gata2* gain-of-function experiments. Indeed, GATA2-HA misexpression in the spinal cord of chick embryos drove ectopic activation of cyclin D1 (Fig. 2F-H). Surprisingly, such a regulation occurred not only in GATA2-HA-expressing cells but also in neighbouring non-expressing cells (Fig. 2H). Wondering whether this effect was related to the activation of the endogenous *Gata2* gene, we checked its expression in electroporated embryos. Indeed, as in the case of cyclin D1, we observed a clear activation of the endogenous *Gata2* gene not only in GATA2-HA-positive cells, but also in the neighbouring negative cells (Fig. 2J-L). To exclude the possibility that the lack of GATA2-HA detection was due to its degradation, we provided evidence that endogenous chick *Gata2* activation was induced in cells that were not stained by the anti-HA antibody 6 and 12 hours after electroporation (data not shown). In

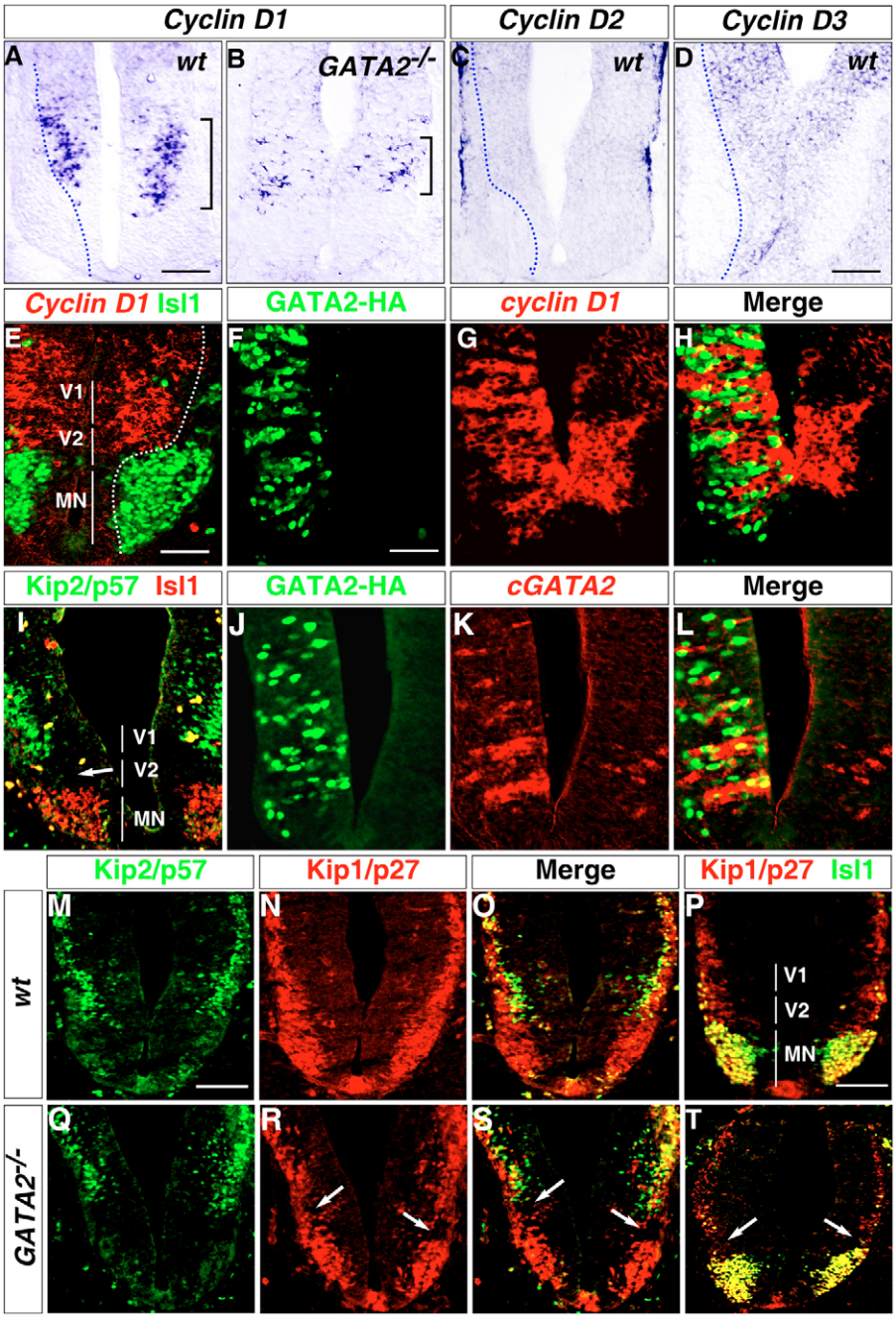

Fig. 2. Analysis of the expression of cell cycle regulators in the context of GATA2 loss- and gain-of-function.
(**A-E**) transverse sections of the spinal cord of E10.5 mouse embryos, wild type (A,C,D,E) or *Gata2*$^{-/-}$ (B); in situ hybridisation was performed with RNA anti-sense probes for cyclin D1 (A,B,E), cyclin D2 (C) and cyclin D3 (D) genes. In E, further staining, performed with anti-islet 1 antibodies (green), shows that the domain of higher concentration of cyclin D1 transcripts abuts the dorsal limit of motoneurons and overlaps V2 and V1 precursors. Broken lines in A,C-E delineate the presumptive limit between the ventricular and the marginal zones. Brackets in A,B indicate the domain where cyclin D1 transcription is upregulated. (**F-H**,**J-L**) Transverse sections of the spinal cord of electroporated chick embryos, hybridised with a chick cyclin D1 (G) or *Gata2* (K) antisense RNA probe, then immunostained with anti-HA antibodies (F,J). (H,L) superimposition of E-G (H) and J-K (L). (**I**,**M-T**) Confocal analysis of double immunofluorescent staining performed on transverse spinal cord sections of wild-type (I,M-P) or *Gata2*$^{-/-}$ (Q-T) E10.5 mouse embryos. Anti-Kip2/p57 antibodies (I,M,Q, green) were coupled with either anti-Isl1 (I, red) or anti-Kip1/p27 (N,R). (O,S) Superimpositions of M,N (O) and Q,R (S). (P,T) Double immunostaining with anti-p27 and anti-Isl1 antibodies, respectively visualised with anti-mouse IgG1 coupled to Alexa Fluor 546 and anti mouse IgG2b coupled to Alexa Fluor 488. The respective presumptive domains of V1, V2 and motor (MN) neurons are indicated in I and P. White arrows in I indicate that the V2 presumptive domain does not express p57/Kip2, and in R,S,T, that the same domain lacks p27 expression in *Gata2*$^{-/-}$ embryos. Scale bar: 70 μm in A-D; 60 μm in E-L; 80 μm in M-T.

conclusion, GATA2 misexpression led to activation of the endogenous *Gata2* and cyclin D1 genes, and some aspects of this activation appears to be generated by a non-cell autonomous process.

To further understand the impact of GATA2 expression on the control of the cell cycle, we next examined the expression of Kip proteins, which are inhibitors of CDK-cyclin D complexes (Cunningham and Roussel, 2001) and markers of the post-mitotic stage in several neuronal differentiation processes (Dyer and Cepko, 2001; Livesey and Cepko, 2001). Although p27/Kip1 protein was continuously expressed in the marginal zone (Fig. 2N,P), thus appearing to be a general marker of post-mitotic precursors in the spinal cord, *p27* transcripts were equally distributed in the neural tube (data not shown). By contrast, p57/Kip2 was barely detected in motor and V2 neuron precursors (Fig. 2I,M; note the expression gap observed between Isl1 and p57 in 2I). Higher expression was observed in more dorsal regions and appeared to precede that of p27 (Fig. 2O). In *Gata2* mutant embryos, p27/Kip1 protein was missing or markedly reduced in a domain dorsally abutting the motoneuron precursors, where *Gata2* is normally expressed (compare Fig. 2O with 2S and Fig. 2P with 2T), whereas the distribution of *p27* transcripts did not seem to be altered (data not shown). Interestingly, expression of p57/Kip2 did not appear to compensate for the lack of p27/Kip1 protein (compare Fig. 2O with 2S) and did not seem to be affected (compare Fig. 2M with 2Q). The ectopic induction of the Kip1/p27 protein we then obtained by *GATA2*-HA misexpression (Fig. 3A-C) further strengthened the hypothesis that GATA2 can participate in the positive control of p27 protein expression and, accordingly, in the engagement towards the post-mitotic stage.

Gata2 control on cell cycle can be uncoupled from neuronal differentiation

We next wanted to know whether such a role was coupled to neuronal differentiation. Twenty-four hours after electroporation, GATA2-HA misexpression was found to induce the expression of early markers of neuronal differentiation, such as βIII-Tubulin/Tuj1 (Fig. 3D-F) and neurofilament (Fig. 3G-I), whereas NeuN expression was not detected (Fig. 3J-L). βIII-Tubulin/Tuj1 induction appeared to overlap partially with cyclin D1 activation (see Fig. S1 in the supplementary material). Further analyses carried out 48 hours after electroporation showed that some of the GATA2 misexpressing cells had migrated into the marginal zone. These cells continued to express p27 (Fig. 4A-C), βIII-Tubulin/Tuj1 (Fig. 4D-F) and neurofilament (Fig. 4G-I), and had activated NeuN expression (Fig. 4J-L). At this stage, the expression of these markers appeared to be exclusive of that of cyclin D1 (see Fig. S1 in the supplementary material). By contrast, the rest of the GATA2 misexpressing cells had not migrated to the marginal zone, were not dividing (data not shown) and were not expressing p27/Kip1 (Fig. 4A-C) or neuronal differentiation markers such as βIII-Tubulin (Fig. 4D-F), neurofilament (Fig. 4G-I) or NeuN (Fig. 4J-L). GATA2-HA misexpressing precursors thus appeared either to differentiate normally or to become blocked in the ventricular zone and probably unable to progress along the differentiation pathway.

In conclusion, gain-of-function studies established that *Gata2* is sufficient to stop proliferation but not to systematically induce neuronal differentiation. Notably this effect was not regionally restricted along the dorsoventral axis of the neural tube and was therefore independent of the cellular context.

Gata2 counteracts Notch signalling

GATA2 capacity to drive cells out of the cell cycle might be achieved through inhibition of the Notch pathway. We therefore analysed the impact of *Gata2* loss- and gain-of-function on the expression of effectors of the Notch pathway such as *Notch1*, *Dll1*, *Hes5*, *Mash1* and *Ngn2*. In this regard, loss-of-function studies did not turn out to be informative as only slight differences could be observed (data not

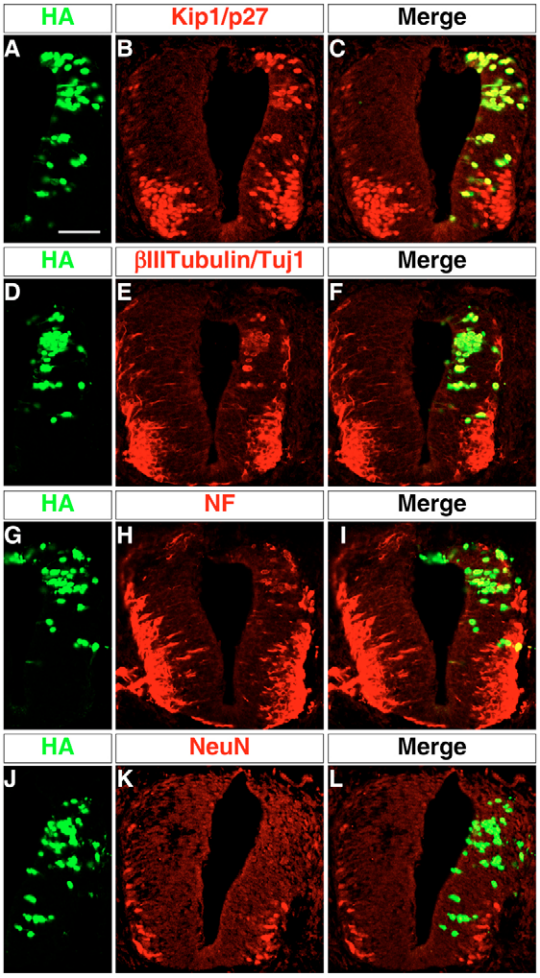

Fig. 3. GATA2-HA misexpression can induce the expression of postmitotic differentiation markers 24 hours after electroporation. (**A-L**) Confocal analysis of adjacent spinal cord sections of a chick embryo misexpressing GATA2-HA 24 hours after electroporation. HA immunostaining (A,D,G,J, green) was coupled with p27/Kip1 (B), Tuj1/βIIItubulin (E), neurofilament (NF) (H) or NeuN (K) immunostaining. (C,F,I,L) Superimpositions of A,B (C), D,E (F), G,H (I) and J,K (L). Scale bar: 80 μm.

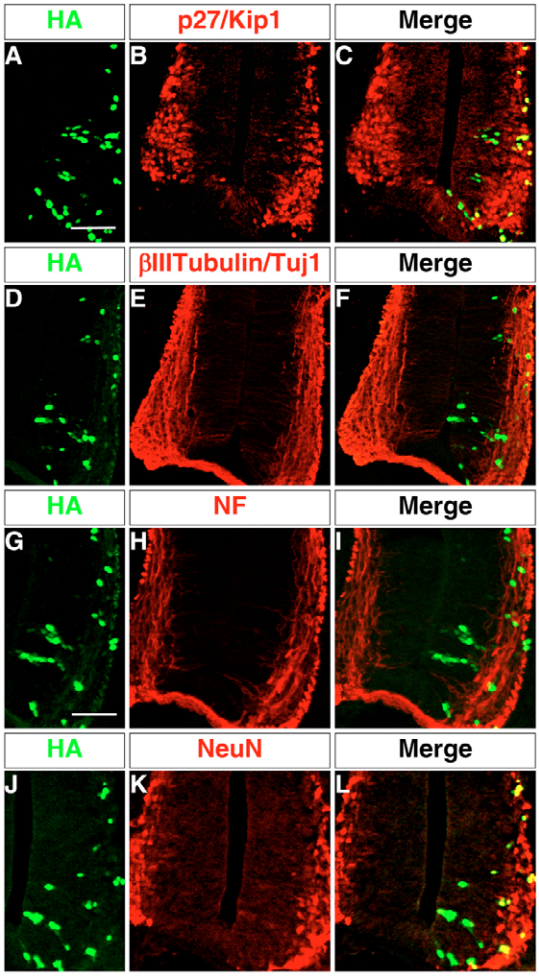

Fig. 4. Cells misexpressing GATA2-HA can fail to further progress into the differentiation pathway. (**A-L**) Confocal analysis of double immunofluorescent stainings performed on adjacent sections of spinal cord of chick embryos 48 hours after electroporation. HA immunostaining (A,D,G,J, green) was coupled with p27/Kip1 (B), Tuj1/βIIItubulin (E), neurofilament (NF) (H) or NeuN (K) immunostaining. (C,F,I,L) Superimpositions of A,B (C), D,E (F), G,H (I) and J,K (L). Scale bar: 80 μm in A-F; 90 μm in G-L.

shown), probably owing to the low distribution of *Gata2*-expressing cells at E10.5. By contrast, gain-of-function studies showed that *Gata2* misexpression caused a clear repression of *Dll1* (Fig. 5A,B), *Ngn2* (Fig. 5D,F), *Notch1* (Fig. 5G,H), *Hes5* (Fig. 5G,I) and even of *Serrate1*/*Jag1* (Fig. 5A,C), the expression of which does not overlap that of *Gata2*. Only *Cash1*, the chick *Mash1* homologue, appeared to be activated in response to GATA2 misexpression (Fig. 5D,E). Thus, GATA2 appeared to be a strong inhibitor of Notch signalling, which raised the possibility that it could function in concert with proneural activity. To address this question specifically, we decided to analyse the consequences of inhibiting proneural function in the *Gata2* gain-of-function context. To this end GATA2-HA was misexpressed with Id2, a bHLH regulatory protein that has been shown to inhibit the function of proneural proteins. We then observed that GATA2-HA was still able to induce *Cash1* (Fig. 6A,B) activation and chick *Hes5* repression (Fig. 6C,D). Similarly, GATA2-induced neuronal differentiation was not abolished by Id2, as assessed by βIII-tubulin expression (data not shown)**.** The sole contrasting effect of Id2 co-expression was the activation of *Ngn2* (Fig. 6E,F). This can be assigned to Id2 function as it was also observed in the presence of Id2 alone (Fig. 6G,J). By contrast, *Cash1* and *Hes5* did not appear to be affected by Id2 misexpression (Fig. 6G-I). These results established that: (1) proneural activity is not required for GATA2 to extinguish Notch signalling and to induce *Mash1* and neuronal differentiation; and (2) Id2 appears to alleviate the repression of *Ngn2* transcription observed in the presence of GATA2 alone.

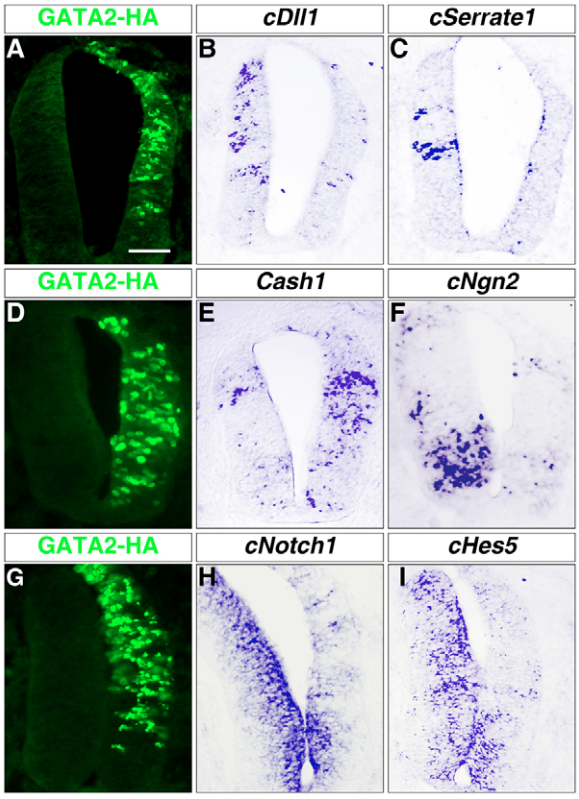

Fig. 5. The Notch pathway is shut off in the context of *Gata2* misexpression. (**A-I**) Spinal cord cross-sections of chick embryos 24 hours after electroporation, hybridised with chick *Dll1* (B), *Serrate1*/*Jag1* (C), *Cash1* (E), *Ngn2* (F), *Notch1* (H) and *Hes5* (I) antisense RNA probes, or stained with anti-HA antibodies (A,D,G). (B,C,E,F,H,I) Adjacent sections to A,D,G. Scale bar: 70 μm.

Gata2 impedes the proliferation of neural cells in culture

Another consequence that could be expected from defective cell cycle exit in vivo is increased proliferation. However, despite different attempts, it was not possible to provide evidence for such an increase in *Gata2* knockout (KO) embryos. First, the expression pattern of phospho-Histone3 and *Sox2* was found to be unaltered (data not shown). Second, as the *Gata2* RNA probe we had been using allows detection of transcripts generated from both the wild-type and the mutated *Gata2* allele (Tsai et al., 1994), we could assess the distribution of transcripts in both wild-type and *Gata2* KO embryos. We found that the distribution of the transcripts in *Gata2* mutant embryos was in fact more restricted than in wild-type embryos (data not shown). TUNEL staining excluded the possibility that cell death could account for such a difference (data not shown). To further investigate this aspect, we switched our investigations to cultured cells, specifically to mouse embryonic neuroepithelial cells (ENC). Indeed, ENC isolated from either the hindbrain or the spinal cord do express *Gata2* in culture, albeit at low levels [Fig. 7A and Nardelli et al. (Nardelli et al., 2003)]. In addition, these cells exhibit a poor proliferation rate if they do not express an immortalising agent such as a mutated version of SV40T (Nardelli et al., 2003). Speculating that *Gata2* expression could be involved in the restriction of proliferation, we decided to assess the impact of *Gata2* inactivation on the proliferation rate of ENC in culture. To this end, ENC cultures were generated from posterior neural tube explants dissected either from wild-type or *Gata2*$^{-/-}$ embryos at E9.5. In accordance with our previous experiments, only 11 out of the 25 wild-type explants survived and could be expanded in culture. The cells derived from these cultures divided very slowly, allowing a passage, at 1:4 dilution, every two to three weeks. By contrast, five out of six cultures originating from *Gata2*$^{-/-}$ explants survived, and in all five cases the mutated cells manifested a significantly higher proliferation rate. Indeed, the mutant cells reached confluency in less than a week when seeded at a 1:5 dilution. Moreover, they did not flatten and spread as much as wild-type cells, remaining smaller and more rounded. Widespread nestin (Fig. 7B,C) and Sox2 expression (Fig. 7D and data not shown), and lack of βIII-Tubulin expression (Fig. 7F and data not shown) confirmed the neuroepithelial character of the wild-type and mutant cells, and their common low propensity to differentiate.

The marked difference in the proliferation rate between *Gata2* wild-type and mutant ENC was probably due to a shortening of the division time in the absence of GATA2. In order to confirm this, BrdU incorporation was performed in two cultures representative of the wild-type and of the mutant ENC. Both cultures were driven into quiescence by serum deprivation for 24 hours. ENC were allowed to re-enter the cell cycle by addition of foetal calf serum and BrdU in the culture medium, and the rate of BrdU incorporation was compared at time intervals. As shown in Fig. 7G, the percentage of BrdU-positive ENC increased steadily in the mutant cells upon re-entry into the cell cycle. By contrast, such an increase was clearly delayed in the wild-type ENC. Indeed, 60% of the mutant cells, but only 10% of the wild-type cells, were BrdU positive 8 hours after cell cycle reactivation. These results established that the time necessary to reach the G1/S phase was shorter in mutant ENC, thus implying a shortening of the G1 phase in the absence of GATA2. This prompted us to compare the status of cyclin D transcription in wild-type and mutant cells. By performing semi-quantitative RT-PCR, we found that the levels of *cyclin D1* and *cyclin D2* were higher in the wild-type than in the *Gata2*$^{-/-}$ ENC, three- and fourfold respectively, whereas no significant differences were observed in the

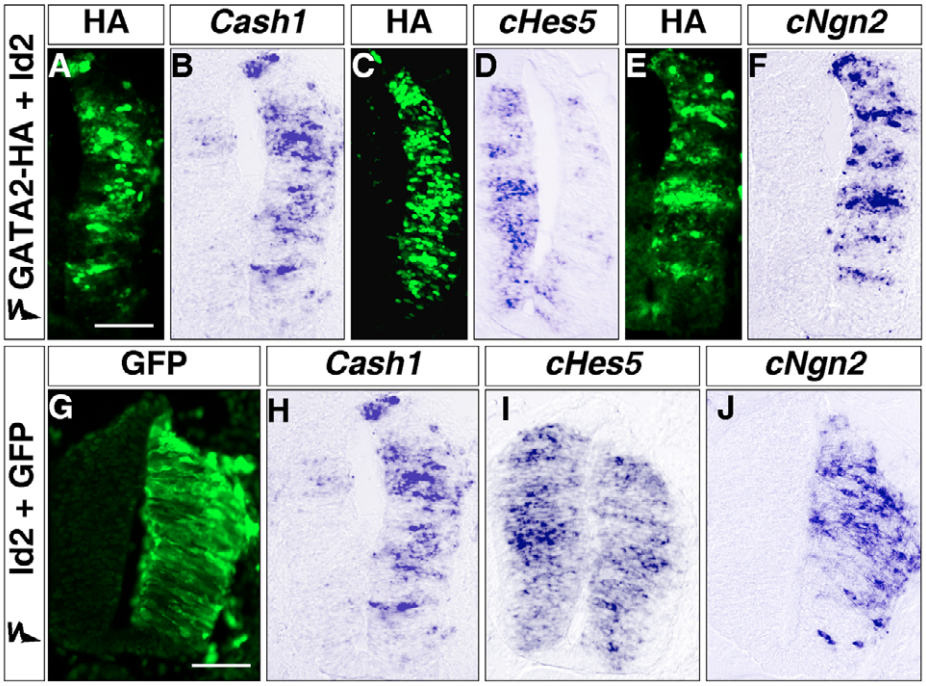

Fig. 6. *Gata2* function does not require proneural activity. (**A-J**) Spinal cord sections of chick embryos 24 hours after electroporation either with pAdRSV-GATA2HA and pACGGScId2 (A-F) or with pACGGScId2 and pAdRSV-GFP (G-J). (A,C,E) Immunostaining with anti-HA antibodies and (G) with anti-GFP antibodies. In situ hybridisation was performed with chick RNA antisense probes for *Cash1* (B,H), *Hes5* (D,I) and *Ngn2* (F,J). (A,B; C,D; E,F; G-J) Adjacent sections. Scale bar: 70 μm in A-F; 60 μm in G-J.

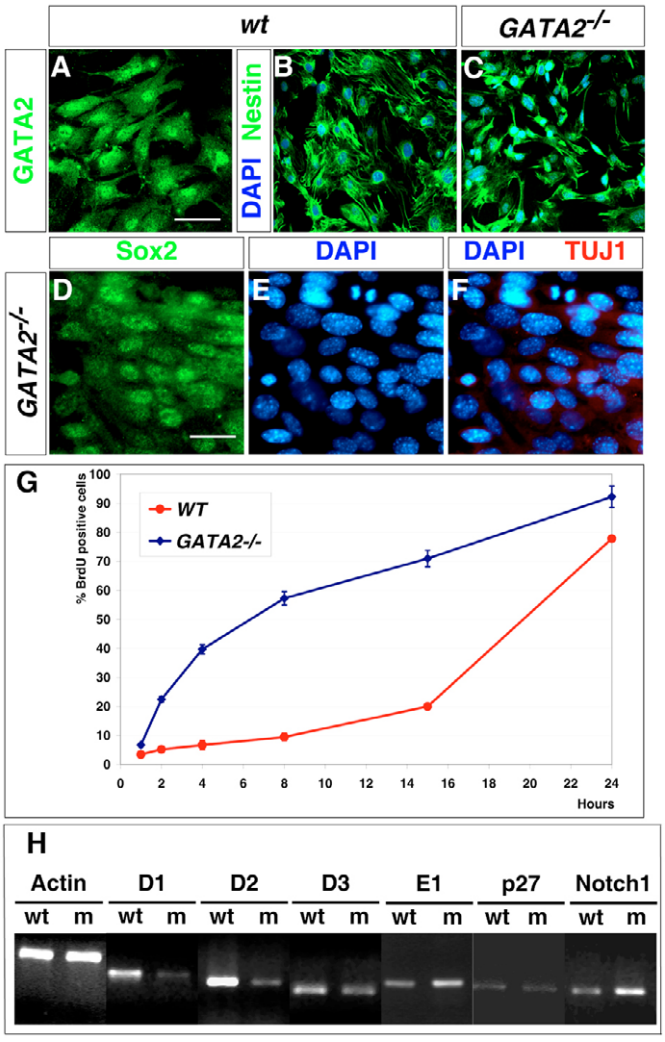

Fig. 7. GATA2 inhibits the proliferation of mouse embryonic neuroepithelial cells in culture. (**A-F**) Wild-type (A,B) and *Gata2*$^{-/-}$ (C-F) embryonic mouse neuroepithelial cells (ENC); immunostaining with anti-GATA2 (A), anti-nestin (B,C, green), anti Sox2 (D, green) and anti-Tuj1/βIII-tubulin (F, red) antibodies. Nuclei were stained with DAPI (blue) in B,C,E,F. Wild-type (B) or *Gata2*$^{-/-}$ (C) ENCs express Nestin. Sox2 (D), but no Tuj1/βIII-Tubulin (F), can be detected in *Gata2*$^{-/-}$ cells, as in wild-type cells (not shown); D-F correspond to the same field. (**G**) Comparison of the percentage of serum-starved cells reaching the S phase at different time intervals after cell cycle release in the presence of serum and BrdU. (**H**) Comparison of the transcription level of cyclin D1, cyclin D2, cyclin D3 and cyclin E1, *p27* and *Notch1* genes by semi-quantitative RT-PCR. PCR products were separated on BEt agarose gels, then analysed and quantified with GelDoc camera and software (BioRad). Scale bar: 40 μm in A-C; 20 μm in D-F.

case of cyclin D3 and of *p27* (Fig. 7H). In addition, the same approach demonstrated that the level of cyclin E1 transcripts was increased by threefold in *Gata2* mutant ENC (Fig. 7H). The results concerning cyclin D1 and cyclin D2 were further confirmed by performing quantitative RT-PCR experiments that showed that the level of transcripts in mutant cells was decreased three- and fivefold, respectively, in comparison with wild-type cells. Consistent with the observations we made in vivo, the concentration of *Notch1* transcripts in wild type ENC cells was found to be 4.5-fold lower than in *Gata2*-deficient ENC.

Consistently, *Gata2* complementation in mutant ENC, achieved by transient transfection with the pAdRSV-GATA2HA construct, appeared to markedly and widely upregulate cyclin D1 and D2 transcription (Fig. 8A,C), which was detected at very low levels in control cells (Fig. 8B,D). Similar upregulation was obtained in non transfected cells cultured in medium conditioned by transfected cells (see Fig. S2 in the supplementary material). These experiments

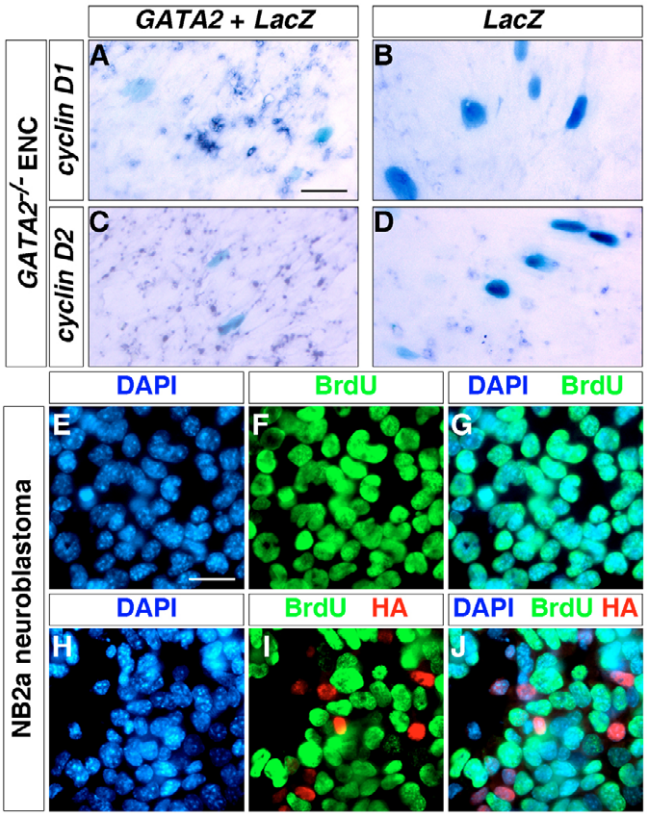

Fig. 8. *Gata2*-deficient ENC complemented with *Gata2* exhibit much more active cyclin D1 and cyclin D2 transcription, while *Gata2* transient overexpression blocks the proliferation of mouse neuroblastoma cells. (**A-D**) *Gata2*$^{-/-}$ ENC were transfected either with pAdRSV-GATA2HA and pAdRSV-βGal plasmids (A,C) or with pAdRSV-βGal alone, then treated for X-Gal colouration and in situ hybridisation with cyclin D1 (A,B) or cyclin D2 (C,D) antisense RNA probes. (**E-J**) NB2a mouse neuroblastoma cells, either control (E-G) or transiently transfected with pAdRSV-GATA2HA (H-J). BrdU was added in the culture medium 30 hours after transfection, cells were recovered 15 hours later, processed for BrdU staining (F,I) and further HA-immunostained (I, red); nuclei were stained with DAPI (E,H) before mounting. (G,J) Superimpositions of E,F (G) and H,I (J). In the control culture (E-G), cells have not stopped growing and are all BrdU positive; whereas in transfected cultures (H-J), cells have not reached the same density and, especially GATA2-HA expressing cells, have not incorporated BrdU (J). Scale bar: 45 μm in A-D; 15 μm in E-J.

provided evidence that GATA2 induces cyclin D1 and D2 transcription in ENC cells in a non-cell-autonomous manner, as observed for cyclin D1 in vivo. Furthermore, they strongly support that this non-cell-autonomous effect is mediated by a secreted factor. GATA2 transient expression also caused the arrest of the proliferation of *Gata2*$^{-/-}$ ENC (see Fig. S3 in the supplementary material) and notably of highly proliferating NB2a mouse neuroblastoma cells. Indeed, in both cases, whereas the control cells continued to grow normally and most of them were BrdU positive (Fig. 8E-G; see Fig. S3 in the supplementary material), transfected cultures did not grow as well, and GATA2-HA expressing cells did not incorporate BrdU or express phospho-Histone 3 (Fig. 8H-J and data not shown). However, this clear inhibition of cell proliferation was not associated with the induction of p27/Kip1 expression or with neuronal differentiation, as deduced by the lack of βIII-Tubulin expression in these cells (data not shown).

DISCUSSION

Gata2 has been demonstrated to be a determining factor in molecular cascades that take place during the early steps of several neuronal differentiation pathways (Craven et al., 2004; Karunaratne et al., 2002; Pattyn et al., 2004; Zhou et al., 2000). The fact that only a minority of cells expressing *Gata2* were found to be mitotic raised the possibility that *Gata2* is activated during final cell division prior to differentiation, and this instructs cells to exit the cell cycle in order to engage in terminal differentiation. This has previously been described for other ventral specification genes, such as *MNR2/HB9* during motoneuron specification (Tanabe et al., 1998) or *Phox2b* (Dubreuil et al., 2000). Using both loss- and gain-of-function studies, we have established that *Gata2* exerts negative control on the division of neuronal progenitors, which can lead to cell cycle exit in vivo and restricts the proliferation rate in vitro. Interestingly, this effect was observed both in concert with and independently of neuronal differentiation.

Gata2 is sufficient to inhibit cell proliferation but may require further instruction to induce neuronal differentiation

As estimated by BrdU incorporation, GATA2 expression can be sufficient to impede the proliferation of neural progenitors, in vivo as in culture, in ENC or NB2a cells. As visualised by p27, βIII-tubulin, neurofilament and NeuN expression, this effect can be further associated with cell cycle exit and neuronal differentiation in vivo. However, if outsets of early neuronal differentiation are primarily observed in GATA2-misexpressing cells in vivo, it appears that part of these cells are not able to keep differentiating properly and become negative for such markers 48 hours after electroporation. Furthermore, no differentiation was observed in culture. Consistently, we suggest that GATA2 may not be always sufficient to consolidate the post-mitotic stage and enable cells to irremediably progress along the differentiation pathway, which may require further molecular events obviously absent in our culture conditions.

Gata2 interferes with the control of expression of components of the cell cycle machinery

To gain further insight into how GATA2 can eventually participate in cell cycle withdrawal, we focused our studies on D-cyclins and Kip proteins, which are known to play a crucial role during the G1 phase in committing the cells towards either the G0 phase or, conversely, the G1/S transition. According to our expression studies, a low level of activation of the D-cyclins may be sufficient to enable them to participate in the maintenance of cell division, whereas distinct upregulation features appear to be involved in other functions. Indeed, previous reports have shown that high levels of D-cyclins, which can result from either more active gene transcription or further stabilisation of the transcripts, impede cell cycle progression and sustain the maintenance of quiescence (Meyyappan et al., 1998; Pagano et al., 1994). Cyclin D2 transcription pattern is consistent with a general function during the maturation phase of neurons, which could thus be related to consolidation of the postmitotic stage. In addition, it can eventually denote a more specific role, which remains to be understood, during neuronal differentiation. By contrast, the region-specific aspect of cyclin D1 upregulation indicates that environmental cues are implicated in the regulation of this cyclin gene, which is consistent with the fact that growth factors and signalling pathways (Lobjois et al., 2004) have been shown to largely account for distinct expression features of D-cyclins in the early neural plate (Lobjois et al., 2004;

Wianny et al., 1998). Downstream of signalling pathways, specification genes such as *Gata2* may be implicated in the regulation of transcription activation. Further analysis will be necessary to gain more insight into the eventual role of this cyclin during the specification of neural progenitors. With regard to this, we have shown that GATA2 is able to cause transient upregulation of cyclin D1 in vivo and may thus participate in this upregulation in V2 precursors. The same influence of GATA2 on cyclin D1 and D2 transcription has been established in ENC cells. In the case of cyclin D2, this was in discrepancy with the fact that the expression pattern of this cyclin did not seem to be altered in vivo in *Gata2* mutant embryos. This could possibly be explained in a number of ways: (1) the distribution of cells expressing GATA2 at E10.5 and of ventral cells exhibiting the higher level of *cyclin D2* transcription which might be too low to provide clear evidence of altered expression in absence of GATA2; (2) compensatory mechanisms taking place in vivo, as described in KO mice for D-cyclins (Kozar et al., 2004); and (3) the existence of a distinct regulatory mechanism involving *Gata2* and taking place only in culture. The downregulation of cyclin E observed in ENC could be a consequence of the higher expression of cyclin D1 and D2. Indeed, at high concentrations, D-cyclins can inactivate CDK2 by disrupting the complex it forms with cyclin E. This can result in the inhibition of events underlying the progression towards the S phase, in particular the upregulation of cyclin E (Meyyappan et al., 1998; Pagano et al., 1994).

Once cells have been committed to leave the cell cycle, further events may be required to consolidate the post-mitotic stage, such as the expression of p27/Kip1 and/or p57/Kip2 proteins. In fact, p27 continuously delineates the marginal zone, whereas p57/Kip2 is barely detected in the motoneuron and V2 domains. Although the functional consequences of such distinct regional features have not yet been elucidated, they denote the potential existence of different mechanisms underlying cell cycle withdrawal among neural progenitors with respect to their position along the dorsoventral axis. Furthermore, the fact that *p27* transcripts were found to be widely distributed in the neural tube supports the idea that the protein synthesis in the marginal layer relies on post-transcriptional controls. Indeed, translational (Kullmann et al., 2002) and post-translational (Tsvetkov et al., 1999; Zhang et al., 2005) events largely account for the control of p27/Kip1 protein expression. Our gain- and loss-of-function analyses strongly support that *Gata2* can contribute to this control in vivo. Furthermore, the lack of p27 protein occurring in GATA2-deficient precursors does not appear to be compensated for by p57/Kip2, and this raises the issue of the alteration of cell cycle exit in these precursors. The expected consequence of such a situation could be increased proliferation. This was overtly manifested by ENC in culture but was not observed in vivo. This failure might be due to the low distribution of cells activating *Gata2* in the ventral spinal cord at E10.5, which was found to be even more restricted in *Gata2* mutant embryos. Considering that cell death could not explain it, this pronounced restriction could be accounted for by two non-mutually exclusive hypotheses: (1) a possible switch in cell fate and the subsequent downregulation of the *Gata2* locus; and (2) the lack of auto-activation potentially induced by GATA2. The latter hypothesis is strongly supported by the fact that exogenous GATA2 expression induced activation of the endogenous *Gata2* and cyclin D1 genes in both a cell-autonomous and non-cell-autonomous manner. Complementation experiments in $Gata2^{-/-}$ ENC, which confirmed the existence of this non-cell autonomous effect, suggest that endogenous functional *Gata2* is not necessary to mediate this effect, which appears to function in absence of GATA2 and to be mediated by a secreted factor not yet identified. This is also consistent with the hypothesis that the transcription of cyclin D1 and D2 may not be directly regulated by GATA2. It will be interesting to gain further insight into this transcriptional control and into how this non-cell-autonomous mechanism is controlled in the spinal cord so that *Gata2* activation is restricted to V2 precursors.

Gata2 interferes with the Notch pathway

The Notch silencing caused by *Gata2* gain-of-function manipulations is consistent with the arrest of proliferation and the induction of neuronal differentiation. This effect on the Notch pathway may occur in different ways. First, Notch signalling involves an auto-regulatory loop, which means that alteration of the expression of any component of the loop will alter the expression of other components (Huppert et al., 1997) (for a review, see Artavanis-Tsakonas et al., 1999). Second, Notch silencing may simply be a consequence of negative regulation of the cell cycle by *Gata2* as interference between Notch signalling and cell cycle components has been reported (Wai et al., 1999). Finally, we cannot rule out that it may rely on degradation processes that control Notch turnover (Oberg et al., 2001; Schweisguth, 1999). Although our data do not allow us to determine the way in which *Gata2* impedes Notch signalling, despite *Cash1* activation, they support the idea that this functional aspect of *Gata2* does not require proneural activity. Indeed, in addition to its proneural activity, *Mash1/Cash1* is involved in neuron-type specification. The two functional aspects have been described to be mechanistically distinct (Parras et al., 2002; Pattyn et al., 2004), and we think that *Cash1* activation by GATA2 is associated with a function in neuronal specification (Craven et al., 2004; Pattyn et al., 2004) and not with proneural activity.

In conclusion, our studies have revealed new aspects of the function of *Gata2* during neurogenesis related to the control of the proliferation of neural progenitors. They provide further evidence of a possible crosstalk between proneural and specification genes that intervene during neuronal fate determination.

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Supplementary material

Supplementary material for this article is available at
http://dev.biologists.org/cgi/content/full/133/11/2155/DC1

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