BM88/CEND1 coordinates cell cycle exit and differentiation of neuronal precursors

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During development, coordinate regulation of cell cycle exit and differentiation of neuronal precursors is essential for generation of appropriate number of neurons and proper wiring of neuronal circuits. BM88 is a neuronal protein associated *in vivo* with terminal neuron-generating divisions, marking the exit of proliferative cells from the cell cycle. Here, we provide functional evidence that BM88 is sufficient to initiate the differentiation of spinal cord neural precursors toward acquisition of generic neuronal and subtype-specific traits. Gain-of-function approaches show that BM88 negatively regulates proliferation of neuronal precursors, driving them to prematurely exit the cell cycle, down-regulate Notch1, and commit to a neuronal differentiation pathway. The combined effect on proliferation and differentiation results in precocious induction of neurogenesis and generation of postmitotic neurons within the ventricular zone. The dual action of BM88 is not recapitulated by the cell cycle inhibitor p27^{Kip1}, suggesting that cell cycle exit does not induce differentiation by default. Mechanistically, induction of endogenous BM88 by forced expression of the proneural gene *Mash1* indicates that BM88 is part of the differentiation program activated by proneural genes. Furthermore, BM88 gene silencing conferred by small interfering RNA in spinal cord neural progenitor cells enhances cell cycle progression and impairs neuronal differentiation. Our results implicate BM88 in the synchronization of cell cycle exit and differentiation of neuronal precursors in the developing nervous system.

neural progenitor cells | neurogenesis | p27^{Kip1} | proneural genes | spinal cord development

In the vertebrate spinal cord, neurogenesis occurs in the ventricular zone (VZ), where neuronal precursors initially undergo rapid proliferation and subsequently give rise to postmitotic cells that differentiate into distinct neuronal phenotypes. The early neural tube is patterned by extrinsic signals that activate hierarchies of transcription factors expressed in a region- and cell-specific manner (1, 2). These transcription factors act to subdivide the VZ into defined progenitor domains with restricted developmental potentials and subsequently establish distinct differentiation programs in the neurons that emerge from each domain. Current evidence suggests that progression of progenitor cells toward neuronal differentiation is tightly linked with cell cycle control and that the two events may be coordinately regulated (3). However, the molecular machinery that couples cell cycle control with neuronal differentiation remains largely unknown.

Here, we report the involvement of BM88 [cell cycle exit and neuronal differentiation 1 (Cend1); National Center for Biotechnology Information nomenclature at www.ncbi.nih.gov] in such cross talk. BM88 has recently emerged as part of a protein interaction network associated with inherited human ataxias, a group of diseases characterized by degeneration of cerebellar Purkinje cells (4). BM88 cloned from mammalian and chick brain (5, 6) is an integral membrane protein composed of two 23-kDa polypeptide chains linked together by disulfide bridges. It is anchored to the membrane of intracellular organelles, including the outer membrane of mitochondria and endoplasmic reticulum, via a transmembrane domain so that the bulk of the protein faces toward the cytoplasm (6, 7). It was first described as a neuronal protein widely expressed in the adult nervous system (7). However, subsequent studies revealed that BM88 marks neuronal cells all along the neuronal lineage both in rodents and the chick (8, 9). In particular, BM88 is expressed in neuroepithelial progenitors and radial glia in the developing forebrain and spinal cord at a time window when these cells are destined to generate neurons, whereas it ceases to be expressed when they give rise to glial cells. Most important, BM88 is associated with neurogenic cell divisions, marking the exit of proliferative cells from the cell cycle (9). In addition, we and others (10) have shown that BM88 expression is distinctly elevated on precursor to neuron switch and can be induced by retinoic acid. This intimate correlation between BM88 expression and the progression of progenitor cells toward neuronal differentiation suggested that BM88 may be functionally involved in this process. In support, we have recently shown (11) that BM88 is sufficient and necessary for accomplishment of cell cycle exit and differentiation of mouse neuroblastoma cells toward a neuronal phenotype via (*i*) activation of the p53–pRb signaling pathway that controls the balance between cell cycle progression and exit and (*ii*) cyclin D1 down-regulation and cytoplasmic sequestration, which is a key event for progenitor cell survival and differentiation into postmitotic neurons (12).

Together, these data raised the possibility that BM88 may participate in the proliferation/differentiation transition of neural stem/progenitor cells during embryonic development. Here, we demonstrate that forced expression of BM88 in the neural tube of the chicken embryo has a strong antiproliferative effect, causing neural precursors to prematurely exit the cell cycle and commit to specific differentiation pathways. Conversely, loss-of-function conferred by siRNA targeting BM88 in neural progenitor cells (NPCs), enhanced proliferation and impaired neuronal differentiation, suggesting that BM88 participates in the molecular machinery that coordinates cell cycle exit and differentiation of neuronal precursors.

Results

BM88 Knockdown Enhances Proliferation and Impairs Differentiation of NPC. To evaluate whether the observed correlation between BM88 expression and neuronal lineage progression has func-

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Abbreviations: VZ, ventricular zone; MZ, mantle zone; NPC, neural progenitor cell; E*n*, embryonic day *n*; bFGF, basic FGF-2; a.e., after electroporation; pMN, motor neuron progenitor domain; MN, motor neuron; ChAT, choline acetyltransferase.

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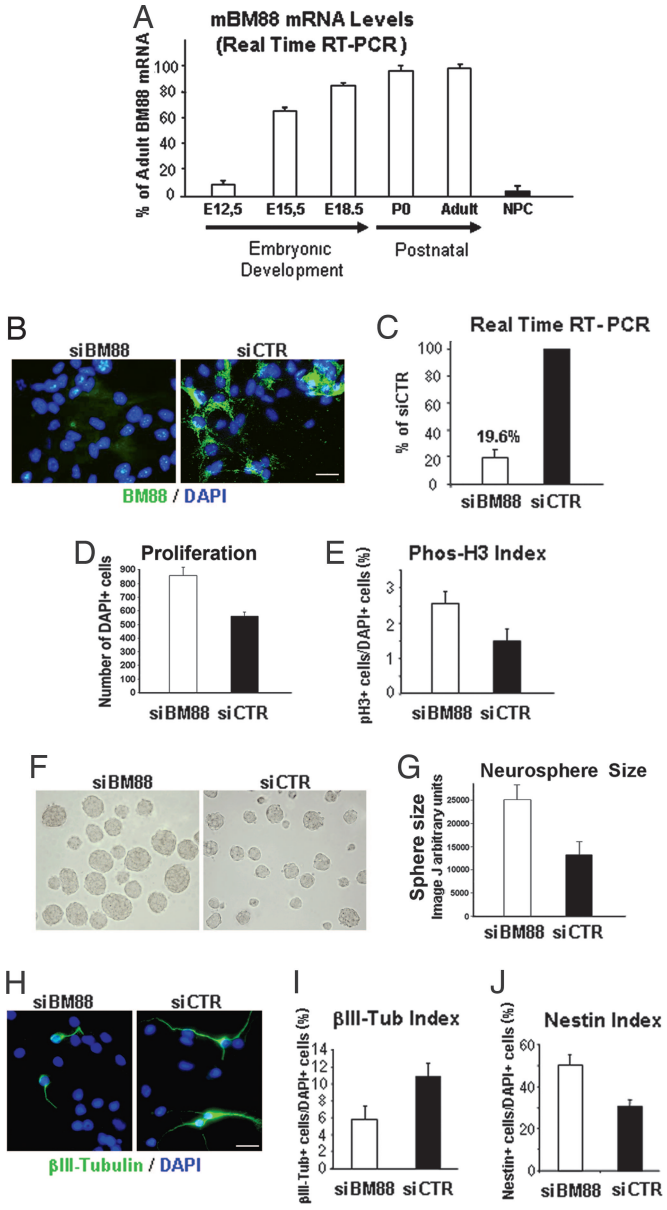

Fig. 1. BM88 knockdown in NPCs induces proliferation and impairs neuronal differentiation. (*A*) Relative expression levels of BM88 mRNA in the developing and postnatal mouse spinal cord and isolated NPCs, measured with quantitative real-time RT-PCR. (*B*) BM88 immunostaining (green) of dissociated NPCs 72 h a.e. with BM88-specific siRNA (siBM88) or control siRNA (siCTR); DAPI, blue. (Scale bar: 40 μm.) (*C*) Quantification of BM88 mRNA by real-time RT-PCR (siBM88, 19.6 ± 4.7% of the siCTR mBM88 mRNA levels). (*D–G*) BM88 knockdown in NPCs enhances proliferation. Proliferation was measured by counting the numbers of DAPI+ cells after equal plating (siBM88, 855 ± 63.2; siCTR, 560 ± 30.2; $P < 0.01$) (*D*), the index of Phos-H3+ cells (siBM88, 2.53 ± 0.35; siCTR, 1.51 ± 0.33; $P < 0.01$) (*E*), and neurosphere size 96 h a.e. (siBM88, 100 ± 11.1%; siCTR, 52.2 ± 9.1%; $P < 0.05$) (*F* and *G*). (*H* and *I*) BM88 knockdown reduced the potential of NPCs for neuronal differentiation. Immunostaining for βIII-tubulin (*H*; green) and index of βIII-tubulin+ cells (siBM88, 5.85 ± 1.62; siCTR, 10.95 ± 1.56; $P < 0.01$) (*I*). (Scale bar: 40 μm.) (*J*) siBM88 transfected NPCs show increased numbers of Nestin+ cells compared with siCTR-treated cells (siBM88, 49.3 ± 7.1; siCTR, 30.7 ± 6.8; $P < 0.01$).

tional significance (refs. 8 and 9; Fig. 1*A*), we used neural progenitors derived from embryonic day 12.5 (E12.5) mouse spinal cord as a model system to analyze cell proliferation and differentiation. These cells fulfill the definition of multipotent neural progenitors because they self-renew when cultured with basic FGF-2 (bFGF) either as neurospheres or after plating as single cells and can differentiate into neurons, astrocytes, and oligodendrocytes on bFGF withdrawal [supporting information (SI) Fig. 6]. To assess the contribution of BM88 in neuronal lineage progression, we manipulated BM88 expression by siRNA gene silencing, which resulted in down-regulation of the endogenous BM88 protein to nondetectable levels and an 80.4 ± 4.7% reduction of BM88 mRNA (Fig. 1 *B* and *C*). We first examined the effect of BM88 knockdown on NPC proliferation. NPCs cultured as neurospheres were electroporated with BM88 or control siRNAs, maintained for a further 24 h as floating spheres, and then plated in equal numbers as single cells in the presence of bFGF. Population analysis after 48 h revealed a 53% increase in cell numbers on BM88 knockdown (Fig. 1*D*), indicative of increased proliferative capacity. In addition, staining for phosphorylated histone H3 showed an increased proportion of NPCs undergoing mitosis (Fig. 1*E*). Finally, when NPCs were allowed to reform floating spheres for 96 h after electroporation (a.e.), a 1.9-fold increase in sphere size was noted in response to siBM88 (Fig. 1 *F* and *G*). We next asked whether BM88 silencing also influences neuronal differentiation. To induce NPC differentiation, neurosphere cultures electroporated with BM88 or control siRNAs were plated as single cells in the absence of bFGF for 72 h. Under these conditions, induction of neuronal differentiation was significantly impaired (by ≈40%) in the BM88-siRNA electroporated NPCs, as evidenced by measuring the index of βIII-tubulin+ cells (Fig. 1 *H* and *I*), with no indication of increased cell death as estimated by staining for activated caspase 3 (data not shown). Conversely, a significant increase in the proportion of nestin+ cells was observed in the BM88 knockdown cells (Fig. 1*J*), indicating that a larger fraction remained in a nondifferentiated progenitor state. The index of GFAP+ cells was not significantly affected by BM88 ablation, suggesting that BM88 is not involved in astrocyte lineage progression. A mirror image phenotype, involving reduction in cell proliferation and enhancement in neuron generation, was obtained by overexpression of BM88 in NPCs (SI Fig. 7). Taken together, our results demonstrate that BM88 is sufficient and necessary for proper regulation of cell cycle withdrawal and differentiation of NPCs toward a neuronal phenotype.

BM88 Is Sufficient to Arrest Proliferation of Neuronal Progenitors *in Vivo*. To explore the role of BM88 in withdrawal of neuronal progenitors from the cell cycle, we misexpressed BM88 unilaterally in the neural tube by *in ovo* electroporation of Hamburger–Hamilton stage 12–13 (E2) chick embryos (13). A plasmid driving expression of GFP was coelectroporated. Virtually all GFP-expressing cells coexpressed BM88 (SI Fig. 8), which allowed to use either the BM88 transgene or GFP reporter gene as markers for the transfected cells. A striking reduction in the number of BrdU-incorporating cycling progenitors was observed in the neural tube of BM88-transfected embryos 24 h a.e. compared with control GFP-transfected embryos (Fig. 2 *A–E*). The percentage of BrdU+/GFP+ cells in BM88-transfected embryos was reduced ≈9-fold in response to BM88 compared with GFP. This effect was similar in the dorsal and ventral halves of the neural tube. Immunostaining for phosphorylated histone H3 (Phos-H3), an M-phase marker of the cell cycle, revealed practically no overlap with cells misexpressing BM88 (Fig. 2 *F* and *G*). To exclude the possibility that cells were depleted because of an apoptotic effect of BM88, we performed TUNEL assays. No evidence of induction of apoptosis was observed either at 24 h or earlier, at 8 h a.e, which corresponds to Hamburger–Hamilton stage 15–16 when apoptosis peaks under normal conditions *in vivo* (14) (SI Fig. 9 *A–E*). These results are consistent with the hypothesis that BM88 participates in the switch to the postmitotic state of neural progenitors *in vivo*.

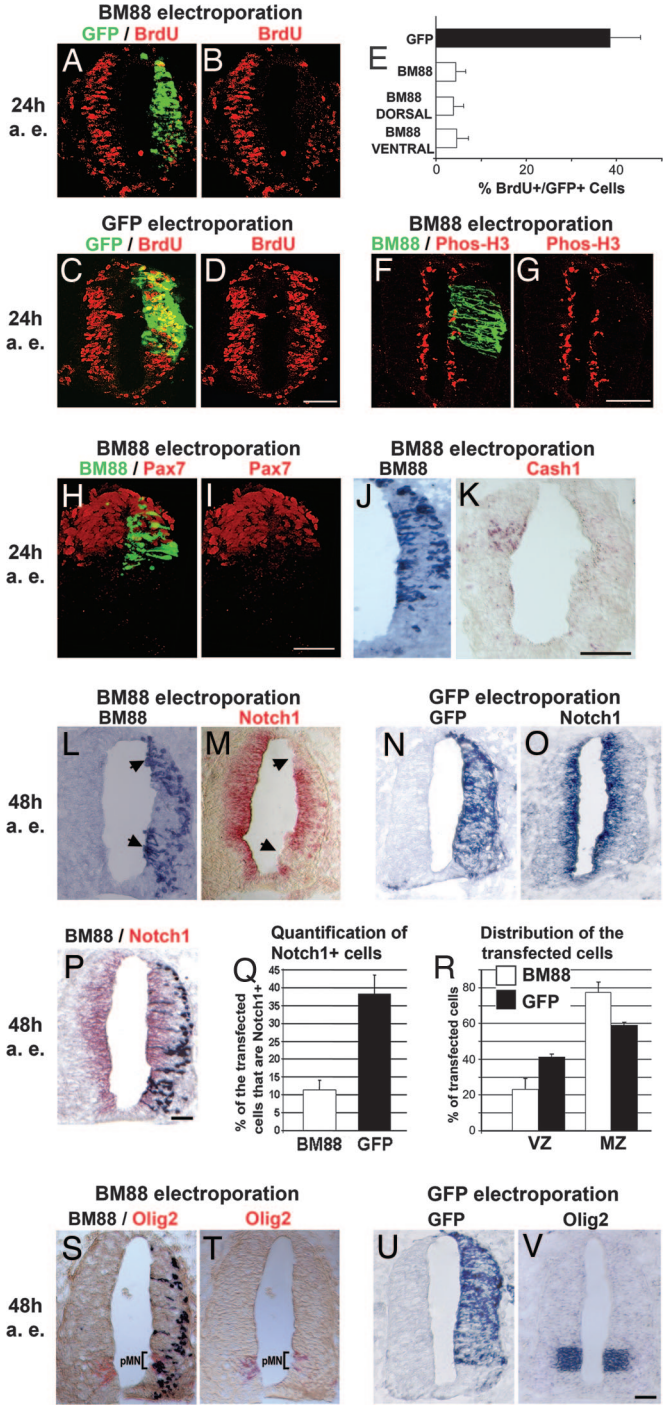

Fig. 2. Forced expression of BM88 reduces the number of cycling progenitors. (*A–D*) Double GFP fluorescence/BrdU immunostaining 24 h a.e. with BM88/GFP or GFP alone followed by 2-h BrdU pulse. Note the marked reduction in double-labeled cells (yellow) between *A* and *C*. (*E*) Quantitative analysis of BrdU incorporation. The number of BrdU+ transfected cells (GFP+/BrdU+) 24 h a.e. with BM88/GFP or GFP alone is expressed as percentage of the total number of transfected (GFP+) cells [$n = 5$ embryos; $P < 0.001$ for GFP vs. BM88; $P = 0.4$ for BM88 (dorsal) vs. BM88 (ventral)]. (*F* and *G*) BM88-electroporated side shows reduced numbers of phos-H3+ cells. Double BM88/phos-H3 immunostaining 24 h a.e. (*H–K*) BM88 electroporation reduced expression of markers for neuronal precursors such as Pax7 and Cash1 24 h a.e. Note the depletion of dorsal Pax7+ (*H* and *I*; double immunofluorescence) and Cash1+ precursors (*J* and *K*; *in situ* hybridization). (*L–Q*) BM88 overcomes lateral inhibition by down-regulating Notch1. (*L–O*) *In situ* hybridization for Notch1 (*M* and *O*) and transgene BM88 (*L*) or GFP immunolabeling (*N*) in consecutive spinal cord sections 48 h a.e. Arrows in *L* and *M* point to Notch1-depleted/BM88+ areas. (*P*) Double *in situ* hybridization for BM88 (blue) and Notch1 (red) 48 h a.e. (*Q*) Quantification of the transfected cells that are Notch1+ by double *in situ* hybridization. (*R*) The majority of BM88-transfected cells tend to migrate outside the Notch1+ VZ. Quantification of this effect in comparison with GFP control electroporations ($BM88_{VZ}$, 23 ± 6.3, vs. GFP_{VZ}, 41.1 ± 1.7; $BM88_{MZ}$, 77.2 ± 6.1, vs. GFP_{MZ}, 58.9 ± 1.7; $n = 5$; for both cases, $P < 0.01$). (*S–V*) BM88 expression reduced the numbers of Olig2-positive cells 48 h a.e. (*S* and *T*) Double *in situ* hybridization for BM88 (blue) and Olig2 (red). (*U* and *V*) GFP and Olig2 *in situ* hybridization in consecutive sections. [Scale bars: *D*, *G*, *I*, *P*, and *V* (for *A–U*), 40 μm.]

BM88 Counteracts Notch Signaling and Down-Regulates Markers for Progenitor Cells. We next asked whether the capacity of BM88 to drive cells out of the cell cycle might be achieved by interfering with the Notch signaling pathway, thus providing a link to neurogenesis. Forced expression of BM88 in VZ cells was able to overcome lateral inhibition by driving large groups of neighboring progenitor cells to down-regulate Notch1 (Fig. 2 *L–O*). Quantification of the effect (see *SI Materials and Methods*) showed that only 11.4 ± 2.7% of BM88-transfected cells expressed Notch1 compared with 38.2 ± 5.3% of GFP-transfected cells (Fig. 2 *P* and *Q*). A concomitant reduction in expression of the Notch1 target gene Hes5 was also observed (SI Fig. 10). Moreover, BM88 misexpression caused down-regulation of basic helix–loop–helix proneural and homeodomain genes associated with progenitor domain specification, an observation consistent with cell cycle exit and initiation of differentiation. In particular, we examined expression of the homeodomain gene Pax7 and the basic helix–loop–helix genes Cash1 and Olig2, 24 or 48 h a.e. In each domain, whether dorsal or ventral, a reduction in the expression levels and/or number of cells that express these genes was observed in areas where BM88 was misexpressed, whereas precursor cells negative for the BM88 transgene expressed the appropriate precursor markers (Fig. 2 *H–K*, *S*, and *T*, and data not shown). Thus, BM88 appears not only to interfere with proliferation of neural progenitors but also to initiate their differentiation program, which is reflected by down-regulation of Notch1, Hes5, Olig2, Cash1, and Pax7.

Newly born neurons migrate out of the VZ and differentiate. Thus, whereas 24 h a.e. BM88-transfected cells were distributed similarly between the VZ and mantle zone (MZ), at 48 h a.e. most BM88-transfected cells had migrated to the MZ (Fig. 2*P*). As an index to assess the differentiation potential of BM88-transfected cells (15), we estimated their distribution in the Notch1+ VZ and in the Notch1- MZ 48 h a.e. Interestingly, a higher percentage of BM88-transfected cells was found in the MZ, compared with GFP cells in control embryos (Fig. 2*R*), indicating that BM88 positively regulates the cell decision to down-regulate Notch1, become postmitotic, and initiate neuronal differentiation. Yet a minority of BM88-positive cells stalled within the VZ, most frequently in the motor neuron progenitor domain (pMN; Fig. 2 *P* and *S*). These cells did not express Olig2, the characteristic progenitor marker of this domain (Fig. 2 *S* and *T*), indicative of a possible advanced differentiation state.

BM88 Drives VZ Precursors Toward Neuronal Differentiation. To evaluate whether BM88 induces neurogenesis *in vivo*, we examined expression of βIII-tubulin, an early marker of postmitotic neurons. Misexpression of BM88 caused precocious neuronal differentiation, as evidenced by overt induction of βIII-tubulin 24 h a.e., whereas in control embryos βIII-tubulin expression was hardly detectable at this time (Fig. 3 *A–D*). By 48 h a.e., βIII-tubulin+ cells were mostly found in the MZ of BM88-electroporated embryos. To our surprise, however, a minority of βIII-tubulin+ cells remained in the VZ. Thus, BM88 misexpression generated ectopic βIII-tubulin+ cells within the VZ (Fig. 3 *E* and *F*), where only actively dividing progenitors exist under

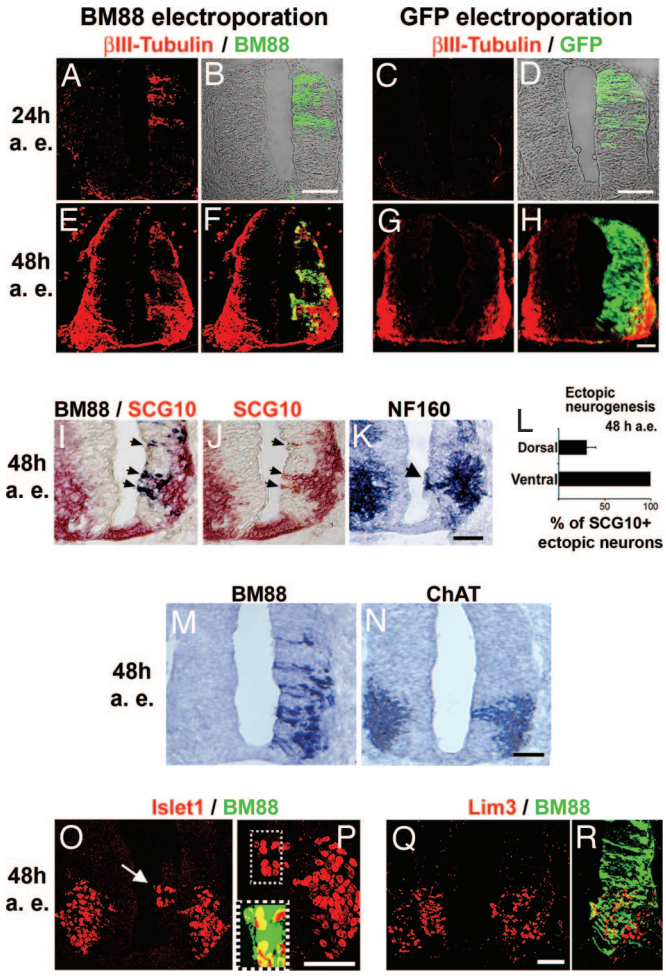

Fig. 3. Induction of precocious and ectopic neurogenesis by BM88. Double labeling of spinal cord sections for βIII-tubulin and transgene BM88 from BM88-electroporated (*A* and *B*; *E* and *F*) and GFP control embryos (*C* and *D*; *G* and *H*), 24 and 48 h a.e. (*I* and *J*) Double *in situ* hybridization of spinal cord section for BM88 (blue) and SCG10 (red) 48 h after BM88 electroporation. Arrows point to ectopic terminally differentiated SCG10+ neurons in the VZ. (*K*) A section consecutive to *I* and *J* was stained for NF160 by *in situ* hybridization. (*L*) Percentage of ventral over dorsal ectopic SCG10+ neurons per embryo (15 sections per embryo; $n = 4$ embryos). (*M–R*) BM88 is sufficient to induce MN markers in the VZ. Consecutive sections from BM88-electroporated embryos were stained 48 h a.e. for BM88 transgene (*M*) and the MN markers ChAT (*N*), Islet1 (*O* and *P*), and Lim3 (*Q* and *R*). *P* is a larger magnification of the area pointed by the arrow in *O*, and the inset shows at larger magnification an overlay of Islet1/BM88 immunolabeling corresponding to the area marked by the dotted white rectangle in *P*. (*R*) Double immunofluorescence for Lim3 and BM88. [Scale bars: *B*, *D*, *H*, and *N* (for *A–N*), 40 μm; *O* (for *O*, *Q*, and *R*), 40 μm; and *P*, 40 μm.]

normal conditions. This effect was particularly evident in the pMN domain, in agreement with the prominent ablation of Notch1 and Olig2 in this domain. By contrast, in control embryos all βIII-tubulin+ descendants of the GFP-expressing cells migrated to the MZ (Fig. 3 *G* and *H*).

We next examined two later markers, SCG10 and neurofilament 160 (NF160), that identify terminally differentiated neurons. As with βIII-tubulin, BM88 misexpression caused ectopic appearance of SCG10+ and NF160+ cells in the VZ (Fig. 3 *I–K*), whereas GFP had no similar effect (SI Fig. 11). In general, the incidence of SCG10+ cells in the VZ was more pronounced in the ventral neural tube 48 h a.e. (Fig. 3*L*). This suggests that the ectopic neuronal differentiation induced by BM88 is more

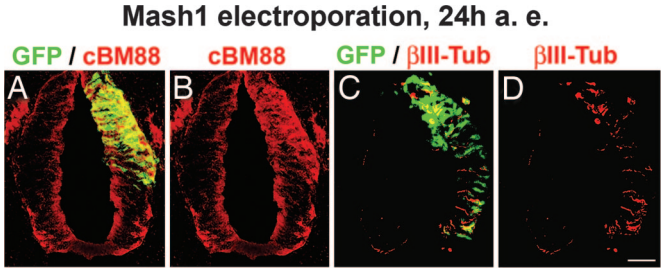

Fig. 4. Mash1 misexpression in the chick neural tube enhances expression of endogenous cBM88. (*A* and *B*) Double immunostaining for GFP and cBM88 of a spinal cord section 24 h after Mash1 electroporation. (*C* and *D*) Double immunostaining for GFP and βIII-tubulin in a neighboring section to *A* and *B*. (Scale bar: 40 μm.)

successfully terminated ventrally, correlating inversely with the dorsoventral proliferation gradient (16). Besides, an increase in the number of apoptotic cells was observed in the dorsal part of the spinal cord 48 h a.e., in contrast to 24 h a.e., which might also contribute to reduced numbers of dorsalmost ectopic neurons (SI Fig. 12). The combined BM88 effect on cell cycle exit, differentiation, and possibly the contribution of dorsal apoptosis eventually resulted in reduction of the spinal cord size by ≈30% (SI Fig. 13).

BM88 Directs Ectopic Generation of Motor Neurons (MNs) in the VZ. The finding that ectopic neurogenesis was most pronounced in the ventral spinal cord raised the question whether BM88 induces MN generation in the VZ. To test this, a number of general and subtype-specific MN markers were used. We observed ectopic expression of the LIM homeodomain transcription factors Islet1 and Lim3, and the choline acetyltransferase enzyme (ChAT) in the VZ of the ventral spinal cord, but not in more dorsal regions (Fig. 3 *M–R*). These data suggest that BM88 is able to induce generation of ectopic MNs within, but not outside, the pMN area. Despite ectopic generation of MNs in the VZ, the total number of Islet1+ MNs was reduced in response to BM88 (SI Fig. 14) because of premature progenitor cell depletion, which also resulted in reduction of the spinal cord size (SI Fig. 13).

To determine whether BM88 is able to ectopically activate initial steps of MN specification in dorsal regions, we tested the expression boundaries of Nkx6.1, Olig2, and MNR2 after BM88 electroporation. None of these factors was detected in a more dorsal position than their wild-type expression pattern (SI Fig. 15). Neither does BM88 expand the interneuron progenitor domains defined by the transcription factors Nkx2.2 and Pax7 (1, 2) (Fig. 2 *H* and *I* and SI Fig. 15), demonstrating that it does not act instructively in favor of either MN or interneuron phenotypes.

BM88 Is Induced by Proneural Genes. We next asked whether BM88 is induced by proneural genes, as part of signaling cascades activated to sustain neuronal differentiation. To this end, we tested the effect of Mash1 misexpression on cBM88. We found that Mash1 was sufficient to induce endogenous cBM88 as early as 24 h a.e. with simultaneous induction of precocious neurogenesis (Fig. 4). However, the extent of βIII-tubulin induction was not as prominent as BM88 induction, suggesting that the effect on BM88 is an early step in the Mash1-dependent differentiation events. In agreement, we have recently shown that neurogenin-1, another member of the proneural family, has direct action on the human BM88 promoter (17), further supporting regulation of BM88 by proneural genes.

Forced Cell Cycle Exit of Neuronal Progenitors Is Not Sufficient to Induce Neurogenesis. Because cell cycle exit is tightly coupled to neuronal differentiation, we asked whether the antiproliferative

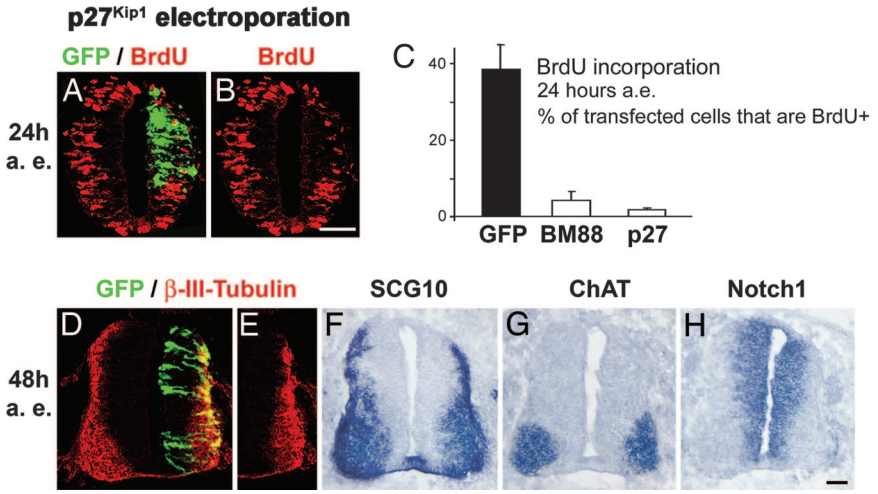

Fig. 5. The BM88 effect on neurogenesis is not recapitulated by forced cell cycle exit of neuronal precursors. (*A* and *B*) Misexpression of $p27^{Kip1}$ results in markedly reduced BrdU incorporation as revealed by immunohistochemical detection of BrdU 24 h a.e. (*C*) Quantification of BrdU incorporation due to GFP control, BM88, and $p27^{Kip1}$ electroporation. The numbers of GFP+/BrdU+ cells are expressed as percentage of the total number of GFP+ transfected cells (GFP, 38.6 ± 6.64%; BM88, 4.34 ± 2.27%; $p27^{Kip1}$, 1.82 ± 0.45%; $n = 5$ embryos; $P < 0.001$ for GFP vs. BM88 or $p27^{Kip1}$). (*D* and *E*) Forced expression of $p27^{Kip1}$ in the chick neural tube is not sufficient to reproduce the ectopic neurogenesis effect of BM88 48 h a.e. (*D* and *E*) βIII-tubulin immunolabeling/GFP fluorescence. (*F–H*) *In situ* hybridization for SCG10 (*F*), ChAT (*G*), and Notch1 (*H*). [Scale bars: *B* (for *A* and *B* and *D–H*), 40 μm.]

effect of BM88 is sufficient to drive ectopic neurogenesis. To address this, the cell cycle inhibitor $p27^{Kip1}$ (18) was ectopically expressed in the chick neural tube. Forced expression of $p27^{Kip1}$ caused neuronal progenitors to exit the cell cycle in a manner similar to BM88 (Fig. 5 *A–C*). However, in contrast to BM88, $p27^{Kip1}$-transfected cells were not prematurely differentiated to generate ectopic neurons. Even though a significant number of $p27^{Kip1}$-expressing cells stayed in the VZ, similarly to BM88, they did not express any markers indicative of either generic or subtype-specific neuronal differentiation, and $p27^{Kip1}$, unlike BM88, did not interfere with the Notch pathway (Fig. 5). Thus, $p27^{Kip1}$ misexpression is able to reproduce only the antiproliferative effect of BM88, but not the effect on neuron differentiation. This suggests that BM88 acts not only to cause exit of neuronal progenitors from the cell cycle, but also to coordinately induce neuronal differentiation.

Discussion

This study provides functional evidence that BM88 exerts dual action on neuron generation: first, BM88 strongly increases the probability that neuronal precursors exit the cell cycle and start differentiating and, second, drives postmitotic cells to become terminally differentiated neurons. The first indication that BM88 may be linked *in vivo* to mechanisms regulating neurogenic processes, came from previous studies showing that (*i*) BM88 is broadly expressed in the developing neuroepithelium of vertebrates around the time window that neurogenesis occurs and (*ii*) its expression in the chick and mouse CNS is associated with the neuronal, but not the astroglial or oligodendroglial, lineage (8, 9). Moreover, BM88 is associated with the dynamics of differentiative neuron-generating divisions (9). During development, the progression of progenitor cells from a symmetric to an asymmetric pattern of divisions marks the onset of neurogenesis (19). Interestingly, recent findings suggest that lengthening of the G_1 phase of the cell cycle is responsible for the onset of neuron-generating divisions (20). These observations support the involvement of cell cycle mediators in coupling the mode of progenitor cell division with differentiation. Here, we demonstrated that BM88 is necessary for proper regulation of proliferation and neuronal differentiation of NPCs derived from embryonic mouse spinal cord. In agreement, we have recently reported that BM88 knockdown in a neuroblastoma cell line enhanced proliferation and impaired retinoic acid-induced neuronal differentiation (11). Collectively, these data implicate BM88 in synchronization of cell cycle exit and neuronal lineage progression.

Forced ectopic expression of BM88 in the neuroepithelium of the chick neural tube just a few hours before the initiation of its endogenous expression (8), led to a large reduction in the numbers of cycling progenitors without any significant enhancement of apoptosis. This suggests that the expression level of endogenous BM88, which is low in neuroepithelial precursors compared with differentiated neurons (8), is intimately associated with the antiproliferative function of this molecule. This raised the question of how this function is achieved. Recently reported data suggest that BM88 promotes cell cycle exit through the p53/cyclin D1/pRb signaling pathway (11). In neuroblastoma cells, BM88 is sufficient to induce up-regulation and nuclear accumulation of p53, as well as activation of its downstream mediator $p21^{WAF/Cip1}$ and dephosphorylation of pRb, known to be responsible for growth arrest and G_1 accumulation of neuronal and nonneuronal cells in response to growth arrest signals (21). Consistent with these observations, we (11) and others (10) have shown that BM88 is sufficient to reduce cyclin D1 expression and induce its cytoplasmic sequestration, which appears to be a key event for cell cycle withdrawal and neuronal differentiation (12). Thus, BM88 appears to be a neuronal lineage-specific regulator of the cell cycle machinery that controls cell cycle withdrawal of neuronal progenitors.

Gain- and loss-of-function data presented in this article suggest that BM88 couples cell cycle exit to generic neuronal differentiation. BM88 is sufficient to down-regulate Notch1 signaling, cause precocious and ectopic neurogenesis, and additionally induce ectopic generation of MNs in the VZ. Interestingly, glycerophosphodiester phosphodiestarase 2, which like BM88 is a retinoid-inducible neuronal membrane protein, generates a similar to BM88 phenotype by inducing MN generation in the chick spinal cord (22). The ability of BM88 to induce ectopic neurons within the VZ appears to be context dependent. Thus, although its effect on cell cycle exit is similar along the dorsoventral axis of the neural tube, the effect on neuron generation is more successfully terminated in the ventral spinal cord within the MN domain. A similar context-dependent

requirement for the neurogenic potential of BM88 was noted *in vitro* (11). Thus, BM88 induces neurogenesis in neural cells possessing the appropriate machinery for neuronal differentiation, whereas it seems to direct nonneural cells toward cell cycle exit and, eventually, to a proapoptotic pathway. It is also possible that the ability of BM88 to induce MNs in the VZ is related to its endogenous expression pattern, which is higher in ventral compared with dorsal spinal cord (8).

A key question arising from our observations is whether inhibition of cell cycle progression by BM88, is in itself sufficient to cause premature differentiation. To address this, we used $p27^{Kip1}$ as a means to interfere with cell proliferation. In line with recently reported data (23), forced cell cycle exit of neuronal precursors by means of misexpression of $p27^{Kip1}$ alone, failed to reproduce the effect of BM88 on neuronal differentiation. However, failure of $p27^{Kip1}$ to induce neuronal differentiation in the chick neural tube does not preclude its involvement in differentiation/migration events within a different cellular context, such as that of the mammalian cerebral cortex (24). Nevertheless, our data imply that in the chick spinal cord ectopic neurogenesis is not caused by the action of BM88 on cell cycle exit alone, consistently with reports suggesting the existence of a molecular machinery dedicated in coordinating cell cycle exit and differentiation (1–3, 15, 23, 25–27).

Considering the negative correlation between the functions of Notch and BM88, it seems a reasonable assumption that BM88 might be the target of basic helix–loop–helix proneural genes, thus defining a late molecular switch for generic neurogenesis. Because proneural genes are expressed transiently in neural progenitors and are usually down-regulated before progenitor cells exit the VZ and begin to differentiate (28–30), their ability to potentiate full neuronal differentiation relies on the induction of downstream differentiation genes that can further implement neuronal differentiation programs (26). BM88 is a strong candidate for such a function, whereas induction of endogenous BM88 after misexpression of Mash1 in the chick neural tube supports this hypothesis. In agreement, neurogenin-1 and Olf-1 binding sites are present on the minimal BM88 promoter, whereas neurogenin-1 directly transactivates the BM88 promoter (17). Neurogenins and Olf-1 act upstream of NeuroD to promote neurogenesis (31–33). Although the exact mechanism of action of BM88 is not known, an interesting speculation is the participation of BM88 in proneural gene networks for controlling neurogenesis. An additional attractive possibility is destabilization/stabilization by BM88 of cellular components involved in cell cycle/neuronal lineage progression via the ubiquitin/proteasome pathway, a speculation that stems from its recent identification as potential partner of ataxin-1 interacting ubiquitin-like protein A1UP, which plays a crucial role in proteasome-mediated activities (4, 34). These nonexclusive alternatives are being investigated.

Methods

RNA Extraction and Real-Time RT-PCR Analysis. Total RNA was isolated by using the RNAeasy kit (Qiagen, Valencia, CA) followed by treatment with RQ1 DNase. Quantitative real-time RT-PCR analysis was performed as described in ref. 11.

BM88 siRNA in NPCs. NPCs were prepared from E12.5 mouse embryo spinal cords and passaged at least three times prior to electroporation with siRNA molecules. Detailed methods for NPC preparation, electroporation, and measurements of proliferative and differentiative capacity of NPCs are listed in *SI Materials and Methods*.

***In Ovo* Electroporation.** Detailed methods are listed in *SI Materials and Methods*.

***In Situ* Hybridization on Cryosections.** Nonradioactive *in situ* hybridization on cryosections and preparations for digoxigenin- or fluorescein-labeled probes were carried out as described in ref. 8.

Immunohistochemistry. Detailed methods are listed in *SI Materials and Methods*.

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1. Marquardt T, Pfaff SL (2001) *Cell* 106:651–654.
2. Jessell TM (2000) *Nat Rev Genet* 1:20–29.
3. Ohnuma S, Harris WA (2003) *Neuron* 40:199–208.
4. Lim J, Hao T, Shaw C, Patel AJ, Szabo G, Rual JF, Fisk CJ, Li N, Smolyar A, Hill DE, *et al.* (2006) *Cell* 125:801–814.
5. Gaitanou M, Buanne P, Pappa C, Georgopoulou N, Mamalaki A, Tirone F, Matsas R (2001) *Biochem J* 355:715–724.
6. Mamalaki A, Boutou E, Hurel C, Patsavoudi E, Tzartos S, Matsas R (1995) *J Biol Chem* 270:14201–14208.
7. Patsavoudi E, Merkouri E, Thomaidou D, Sandillon F, Alonso G, Matsas R (1995) *J Neurosci Res* 40:506–518.
8. Politis PK, Rohrer H, Matsas R (2007) *Gene Expr Patterns* 7:165–177.
9. Koutmani Y, Hurel C, Patsavoudi E, Hack M, Gotz M, Thomaidou D, Matsas R (2004) *Eur J Neurosci* 20:2509–2523.
10. Cesi V, Tanno B, Vitali R, Mancini C, Giuffrida ML, Calabretta B, Raschella G (2002) *Cell Death Differ* 9:1232–1239.
11. Georgopoulou N, Hurel C, Politis PK, Gaitanou M, Matsas R, Thomaidou D (2006) *J Biol Chem* 281:33606–33620.
12. Sumrejkanchanakij P, Tamamori-Adachi M, Matsunaga Y, Eto K, Ikeda MA (2003) *Oncogene* 22:8723–8730.
13. Hamburger V, Hamilton HL (1951) *J Morphol* 88:49–92.
14. Hirata M, Hall BK (2000) *Int J Dev Biol* 44:757–768.
15. Dubreuil V, Hirsch MR, Pattyn A, Brunet JF, Goridis C (2000) *Development (Cambridge, UK)* 127:5191–5201.
16. Megason SG, McMahon AP (2002) *Development (Cambridge, UK)* 129:2087–2098.
17. Papadodima O, Sergaki M, Hurel C, Mamalaki A, Matsas R (2005) *J Neurochem* 95:146–159.
18. Mitsuhashi T, Aoki Y, Eksioglu YZ, Takahashi T, Bhide PG, Reeves SA, Caviness VS, Jr (2001) *Proc Natl Acad Sci USA* 98:6435–6440.
19. Chenn A, McConnell SK (1995) *Cell* 82:631–641.
20. Calegari F, Huttner WB (2003) *J Cell Sci* 116:4947–4955.
21. Gill RM, Slack R, Kiess M, Hamel PA (1998) *Exp Cell Res* 244:157–170.
22. Rao M, Sockanathan S (2005) *Science* 309:2212–2215.
23. Paris M, Wang WH, Shin MH, Franklin DS, Andrisani OM (2006) *Mol Cell Biol* 26:8826–8839.
24. Nguyen L, Besson A, Heng JI, Schuurmans C, Teboul L, Parras C, Philpott A, Roberts JM, Guillemot F (2006) *Genes Dev* 20:1511–1524.
25. Dyer MA, Livesey FJ, Cepko CL, Oliver G (2003) *Nat Genet* 34:53–58.
26. Bertrand N, Castro DS, Guillemot F (2002) *Nat Rev Neurosci* 3:517–530.
27. Mizuguchi R, Sugimori M, Takebayashi H, Kosako H, Nagao M, Yoshida S, Nabeshima Y, Shimamura K, Nakafuku M (2001) *Neuron* 31:757–771.
28. Ben-Arie N, McCall AE, Berkman S, Eichele G, Bellen HJ, Zoghbi HY (1996) *Hum Mol Genet* 5:1207–1216.
29. Gradwohl G, Fode C, Guillemot F (1996) *Dev Biol* 180:227–241.
30. Ma Q, Kintner C, Anderson DJ (1996) *Cell* 87:43–52.
31. Fode C, Gradwohl G, Morin X, Dierich A, LeMeur M, Goridis C, Guillemot F (1998) *Neuron* 20:483–494.
32. Dubois L, Bally-Cuif L, Crozatier M, Moreau J, Paquereau L, Vincent A (1998) *Curr Biol* 8:199–209.
33. Dubois L, Vincent A (2001) *Mech Dev* 108:3–12.
34. Riley BE, Xu Y, Zoghbi HY, Orr HT (2004) *J Biol Chem* 279:42290–42301.