Cell

# Hmga2 Promotes Neural Stem Cell Self-Renewal in Young but Not Old Mice by Reducing $p16^{Ink4a}$ and $p19^{Arf}$ Expression

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DOI 10.1016/j.cell.2008.09.017

## SUMMARY

Stem cells persist throughout life in diverse tissues by undergoing self-renewing divisions. Self-renewal capacity declines with age, partly because of increasing expression of the tumor suppressor $p16^{Ink4a}$. We discovered that the Hmga2 transcriptional regulator is highly expressed in fetal neural stem cells but that expression declines with age. This decrease is partly caused by the increasing expression of *let-7b* microRNA, which is known to target HMGA2. *Hmga2*-deficient mice show reduced stem cell numbers and self-renewal throughout the central and peripheral nervous systems of fetal and young-adult mice but not old-adult mice. Furthermore, $p16^{Ink4a}$ and $p19^{Arf}$ expression were increased in *Hmga2*-deficient fetal and young-adult stem cells, and deletion of $p16^{Ink4a}$ and/or $p19^{Arf}$ partially restored self-renewal capacity. *let-7b* overexpression reduced Hmga2 and increased $p16^{Ink4a}$/$p19^{Arf}$ expression. Hmga2 thus promotes fetal and young-adult stem cell self-renewal by decreasing $p16^{Ink4a}$/$p19^{Arf}$ expression. Changes in *let-7* and Hmga2 expression during aging contribute to the decline in neural stem cell function.

## INTRODUCTION

Stem cells self-renew throughout life in numerous tissues, including the central (CNS) and peripheral (PNS) nervous systems (Kruger et al., 2002; Maslov et al., 2004; Molofsky et al., 2006). However, neural stem cell frequency, self-renewal capacity, and mitotic activity all decline with age along with the rate of neurogenesis (Enwere et al., 2004; Maslov et al., 2004; Molofsky et al., 2006). Declines in stem cell function within aging tissues are partially caused by increasing $p16^{Ink4a}$ expression. $p16^{Ink4a}$ generally cannot be detected in fetal and young-adult tissues but is induced in a variety of aging tissues (Zindy et al., 1997; Krishnamurthy et al., 2004; Molofsky et al., 2006). $p16^{Ink4a}$ deficiency partially rescues the decline in stem and progenitor cell function in the aging CNS and other tissues without affecting stem and progenitor cell function in young-adult tissues (Janzen et al., 2006; Krishnamurthy et al., 2006; Molofsky et al., 2006). However, it remains uncertain what regulates this change in $p16^{Ink4a}$ expression in old tissues and what mechanisms act in fetal and young-adult stem cells to increase their self-renewal relative to old-adult stem cells.

Consistent with the changes in stem cell function with age, different transcriptional programs regulate stem cell self-renewal at embryonic, fetal-neonatal, and adult stages. Fetal and neonatal hematopoietic stem cells (HSCs) depend on Sox17 for their maintenance, whereas adult HSCs do not, demonstrating the existence of a distinct fetal-neonatal self-renewal program (Kim et al., 2007). *Bmi-1*-deficient mice are born with normal numbers of stem cells but exhibit postnatal self-renewal defects that lead to the depletion of adult stem cells (Lessard and Sauvageau, 2003; Molofsky et al., 2003; Park et al., 2003). Other transcriptional regulators are also required by postnatal but not fetal stem cells (Hock et al., 2004a, 2004b; Shi et al., 2004), demonstrating the existence of a postnatal-adult self-renewal program. This raises the question of whether all stage-specific transcriptional regulators of self-renewal cluster into temporally distinct embryonic, fetal-neonatal, and postnatal-adult programs.

Bmi-1 promotes the self-renewal of postnatal stem cells largely by repressing the expression of $p16^{Ink4a}$ and $p19^{Arf}$ (Bruggeman et al., 2005; Molofsky et al., 2005). $p16^{Ink4a}$ is a cyclin-dependent kinase inhibitor that promotes Rb activation, slowing cell-cycle progression or inducing cellular senescence (Lowe and Sherr, 2003). $p19^{Arf}$ promotes p53 activity, also slowing cell-cycle progression or inducing cellular senescence. Bmi-1 represses $p16^{Ink4a}$ and $p19^{Arf}$ expression within postnatal stem cells (Molofsky et al., 2003). However, $p16^{Ink4a}$ and $p19^{Arf}$ expression increase in aging stem cells despite ongoing Bmi-1 expression (Molofsky et al., 2006). In addition to contributing to the decline in stem cell function with age, $p16^{Ink4a}$, and perhaps $p19^{Arf}$, may also contribute to diseases of aging (Sharpless and DePinho, 2007). These studies demonstrate that adult stem cell maintenance critically depends upon transcriptional mechanisms that repress $p16^{Ink4a}$ and $p19^{Arf}$; however, these studies also raise important questions. Do fetal stem cells have different mechanisms to repress $p16^{Ink4a}$/$p19^{Arf}$? Why do

old-adult stem cells express higher levels of $p16^{Ink4a}$ and $p19^{Arf}$ than young-adult stem cells?

*Hmga2* is a member of the high-mobility group A (HMGA) family that encodes a small, chromatin-associated protein that has no intrinsic transcriptional activity but can modulate transcription by altering chromatin structure (Reeves, 2001). *Hmga2* is widely expressed in undifferentiated cells during embryogenesis, but expression becomes more restricted as fetal development progresses (Zhou et al., 1995; Hirning-Folz et al., 1998). *Hmga2* is rarely detected within normal adult tissues but is expressed by a variety of benign and malignant tumors (Schoenmakers et al., 1995; Fusco and Fedele, 2007). Recent studies have demonstrated that *let-7* family microRNAs are important negative regulators of Hmga2 expression: loss of the *let-7* binding sites in *Hmga2* increases Hmga2 expression and promotes tumorigenesis (Lee and Dutta, 2007; Mayr et al., 2007; Yu et al., 2007). $Hmga2^{-/-}$ mice exhibit a dwarf phenotype (Zhou et al., 1995). In contrast, overexpression of truncated (*let-7*-insensitive) HMGA2 in mice increases body size and the incidence of certain tumors (Arlotta et al., 2000; Fedele et al., 2006). It has not yet been tested whether *Hmga2* regulates somatic stem cell function.

In a full-genome analysis, *Hmga2* was the only gene that we found to be preferentially expressed by stem cells and that showed a progressive decline in expression with age. This decline in Hmga2 expression inversely correlated with increasing *let-7b* expression during aging, and deletion of the *let-7* binding sites in the 3′ untranslated region (UTR) of *Hmga2* was required for overexpression of Hmga2 in stem cells from old mice. Hmga2 increased the self-renewal of fetal and young-adult stem cells relative to old-adult stem cells by negatively regulating $p16^{Ink4a}$ and $p19^{Arf}$ expression in fetal and young-adult, but not old-adult, stages. The requirement for Hmga2 in fetal and young-adult stem cells, along with the requirement for Bmi-1 in adult stem cells, demonstrates there are overlapping transcriptional mechanisms to prevent the expression of $p16^{Ink4a}$ and $p19^{Arf}$ in stem cells. Such pathways are temporally regulated by changes in *let-7b* and Hmga2 expression to allow $p16^{Ink4a}$ and $p19^{Arf}$ expression in aging stem cells.

## RESULTS

### *Hmga2* Expression Declines with Age in Stem Cells

We reasoned that genes that regulate changes in stem cell function with age might show progressive changes in their expression in stem cells with age. In a prior study, we identified 26 genes that were more highly expressed by fetal HSCs than by young-adult HSCs (Kiel et al., 2005). In this study, we examined these genes by quantitative (real-time) PCR (qPCR) and with additional microarray data sets (data not shown) to identify genes that fit two additional criteria. First, we identified genes that remained detectably expressed by young-adult HSCs but declined significantly further in expression in old-adult HSCs (>3-fold, $p < 0.01$). Second, we identified genes that were more highly expressed in HSCs as compared to all $CD45^+$ hematopoietic cells. Only one gene in a full-genome analysis met all of these criteria: *Hmga2* (Figures 1A and 1B).

To test whether this unusual expression pattern is observed in other tissues, we examined *Hmga2* expression in CNS stem and progenitor cells by performing qPCR on cells from embryonic day (E)14.5 telencephalon, postnatal day (P)0 lateral ventricle ventricular zone (VZ), P30 lateral ventricle subventricular zone (SVZ), P360 SVZ, and P720 SVZ (locations in which neural stem and progenitor cells reside within the forebrain). We again observed declining *Hmga2* expression with age (Figure 1C). *Hmga1* did not exhibit a similar change in expression with age by either microarray analysis in HSCs (data not shown) or by qPCR in CNS progenitors (Figure 1C). *Hmga2* was also expressed at higher levels in CNS stem and progenitor cells as compared to differentiated cells by qPCR (Figure 1D), western blot (Figure 1E), and in situ hybridization (Figure 1F). We confirmed the decline in Hmga2 expression with age in neural stem and progenitor cells by both western blot (Figure 1E) and in situ hybridization (Figure 1F): Hmga2 expression was extinguished in the SVZ of old mice by every technique. The declining *Hmga2* expression with age contrasted with the increasing expression of $p16^{Ink4a}$ and $p19^{Arf}$ with age in stem and progenitor cells (Figure 1C). These data raised the possibility that Hmga2 might contribute to the increased stem cell function in fetal and young-adult mice as compared to old-adult mice.

### Hmga2 Promotes Fetal and Adult Neural Stem Cell Self-Renewal

To assess Hmga2 function, we examined *Hmga2*-deficient mice (Zhou et al., 1995). Consistent with earlier reports (Benson and Chada, 1994; Zhou et al., 1995), *Hmga2* deficiency led to significant ($p < 0.01$) growth retardation that was evident by birth and that remained evident throughout adult life (Figures S1A–S1F available online). The brains of adult $Hmga2^{-/-}$ mice were also significantly ($p < 0.01$) smaller than the brains of littermate controls (Figures S1G and S1H). Despite this growth retardation, we detected no premature death among $Hmga2^{-/-}$ mice.

To assess whether Hmga2 regulates changes in stem cell function during aging, we elected to focus on neural stem cells from the CNS and PNS because these stem cell populations exhibit clear declines in frequency, mitotic activity, and neurogenesis in vivo during aging (Kruger et al., 2002; Molofsky et al., 2006). We cultured CNS stem cells from the forebrain of $Hmga2^{-/-}$ mice and littermate controls to test whether *Hmga2* was required for normal neural stem cell function (Figure 2A). The percentage of dissociated cells that was capable of forming multipotent neurospheres did not differ between $Hmga2^{-/-}$ and wild-type telencephalon at E11.5 or E14.5 but was significantly reduced ($p < 0.05$) in the $Hmga2^{-/-}$ P0 VZ and P49–56 SVZ as compared to littermate controls (Figure 2B). $Hmga2^{+/-}$ cells performed similarly to wild-type cells in this assay (data not shown), so cells from wild-type littermates were used as controls in subsequent experiments. To minimize fusion between neurospheres, we cultured cells at very low density in nonadherent cultures (<1 cell/μl of medium) and then transferred them to adherent secondary cultures to determine the fraction of neurospheres capable of multilineage differentiation. Fetal and adult $Hmga2^{-/-}$ neurospheres were significantly smaller ($p < 0.05$) than wild-type neurospheres and gave rise to significantly fewer multipotent secondary neurospheres ($p < 0.01$) upon subcloning

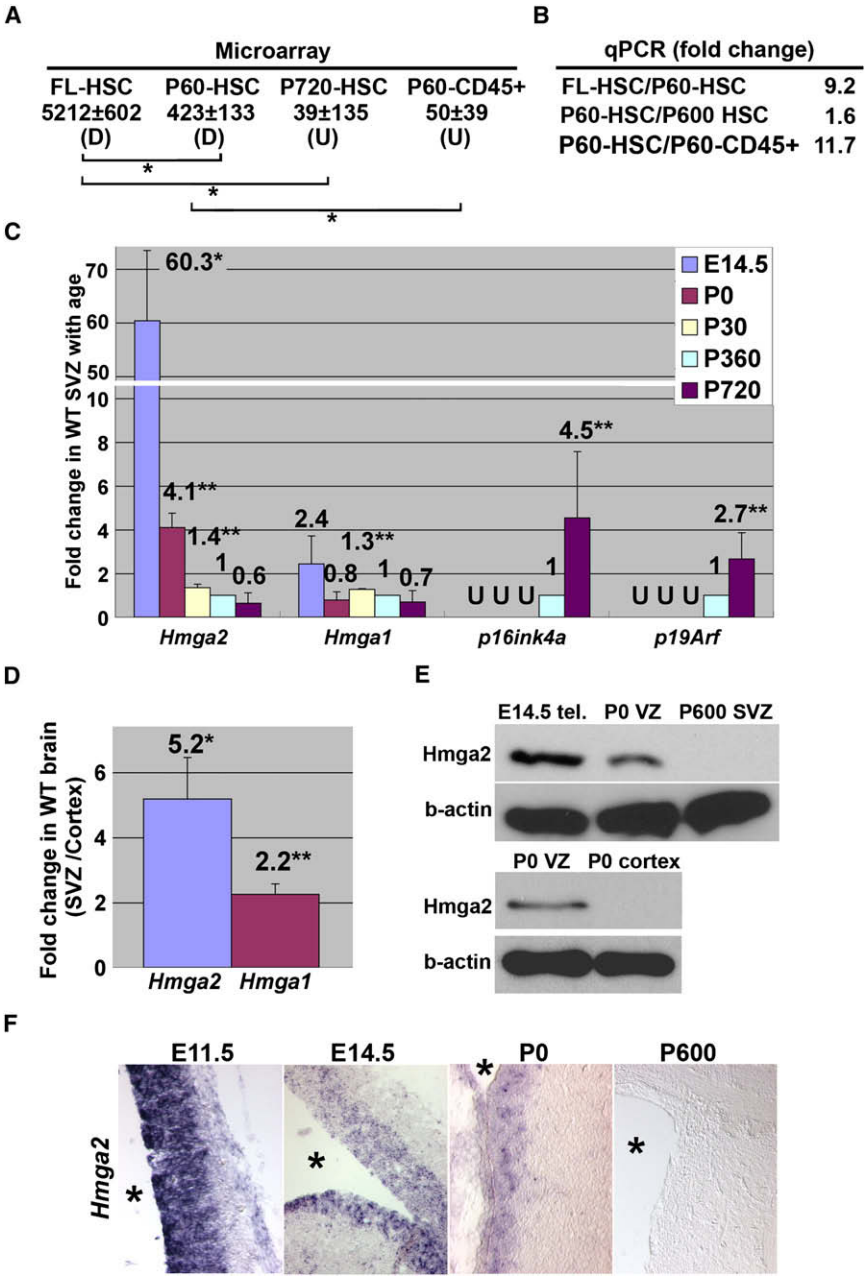


**Figure 1. *Hmga2* Expression Is Elevated in Stem Cells in the Hematopoietic and Nervous Systems but Declines with Age, in Contrast to *p16$^{Ink4a}$* and *p19$^{Arf}$***

(A and B) *Hmga2* expression by microarray (A) and qPCR (B) in E14.5 fetal liver HSCs (Thy-1$^{low}$Sca-1$^{+}$Lineage$^{-}$Mac-1$^{+}$ cells), P60 and P720 bone marrow HSCs (Thy-1$^{low}$Sca-1$^{+}$Lineage$^{-}$c-kit$^{+}$ cells), and P60 bone marrow CD45$^{+}$ hematopoietic cells. Microarray signal intensities represent mean ± SD from three independent samples of each cell type (*$p < 0.01$; D, significantly above background; U, not detectable above background). Differences were confirmed in independent samples by qPCR.

(C) qPCR in freshly dissected E14.5 telencephalon, P0 VZ, P30 SVZ, P360 SVZ, and P720 SVZ (expressed as fold change relative to P360 SVZ; mean ± SD for four to five mice per stage). *Hmga2* expression significantly declined with age (*$p < 0.01$, **$p < 0.05$). *p16$^{Ink4a}$* and *p19$^{Arf}$* expression could not be detected (U) in fetal, newborn, or young-adult samples but significantly (**$p < 0.05$) increased with age in P360 and P720 SVZ samples.

(D) *Hmga2* and *Hmga1* were elevated in SVZ versus differentiated layers of the cortex by qPCR in P30 wild-type mice (mean ± SD of three independent experiments; *$p < 0.01$,**$p < 0.05$).

(E) Western blot of E14.5 telencephalon, P0 VZ, and P600 SVZ. Consistent with results by qPCR (D) and in situ hybridization (F), Hmga2 protein levels (E) were elevated in undifferentiated cells within the VZ as compared to differentiated cells in the cortex but declined with age.

(F) In situ hybridization for *Hmga2* on sections of E11.5, E14.5, P0, and P600 forebrains (* indicates lateral ventricle). *Hmga2* transcript levels were greatly elevated in the VZ-SVZ but declined with age.

All t tests were paired, except in (C).

(Figures 2A and 2B). These data suggest that *Hmga2* is not required for the formation of stem cells during embryonic development but that *Hmga2*$^{-/-}$ deficiency reduces self-renewal potential throughout fetal development and young adulthood, reducing stem cell frequency by birth and even further by adulthood.

To test whether *Hmga2* is required for stem cell function in other tissues, we examined neural crest stem cells (NCSCs) from the guts of *Hmga2*$^{-/-}$ mice and littermate controls. NCSCs give rise to the enteric nervous system within the gut during fetal development and then persist throughout adult life in the gut wall (Kruger et al., 2002). In the PNS, the frequency of cells capable of forming multipotent neurospheres also did not differ between *Hmga2*$^{-/-}$ guts and littermate controls at E14.5 but was significantly reduced in *Hmga2*$^{-/-}$ guts at P0 and P49–56 compared to littermate controls ($p < 0.05$; Figure S2B). The *Hmga2*$^{-/-}$ neurospheres were also significantly smaller ($p < 0.01$) and gave rise to fewer secondary multipotent neurospheres ($p < 0.05$) upon subcloning at all stages examined (Figure S2B). *Hmga2* was thus required to maintain normal neural stem cell self-renewal and frequency throughout the PNS and CNS.

The reduced self-renewal of *Hmga2*-deficient CNS stem cells was associated with significantly reduced proliferation within stem cell colonies ($p < 0.05$; Figures 2C and 2F) but no detectable effect on cell death, whether this was assessed on the basis of activated caspase-3 staining (Figure 2D) or nuclear morphology (Figure 2E; cell death was rare in all colonies). In the PNS, *Hmga2* deficiency also led to the formation of smaller colonies with a lower frequency of dividing cells (Figures S2C–S2E).

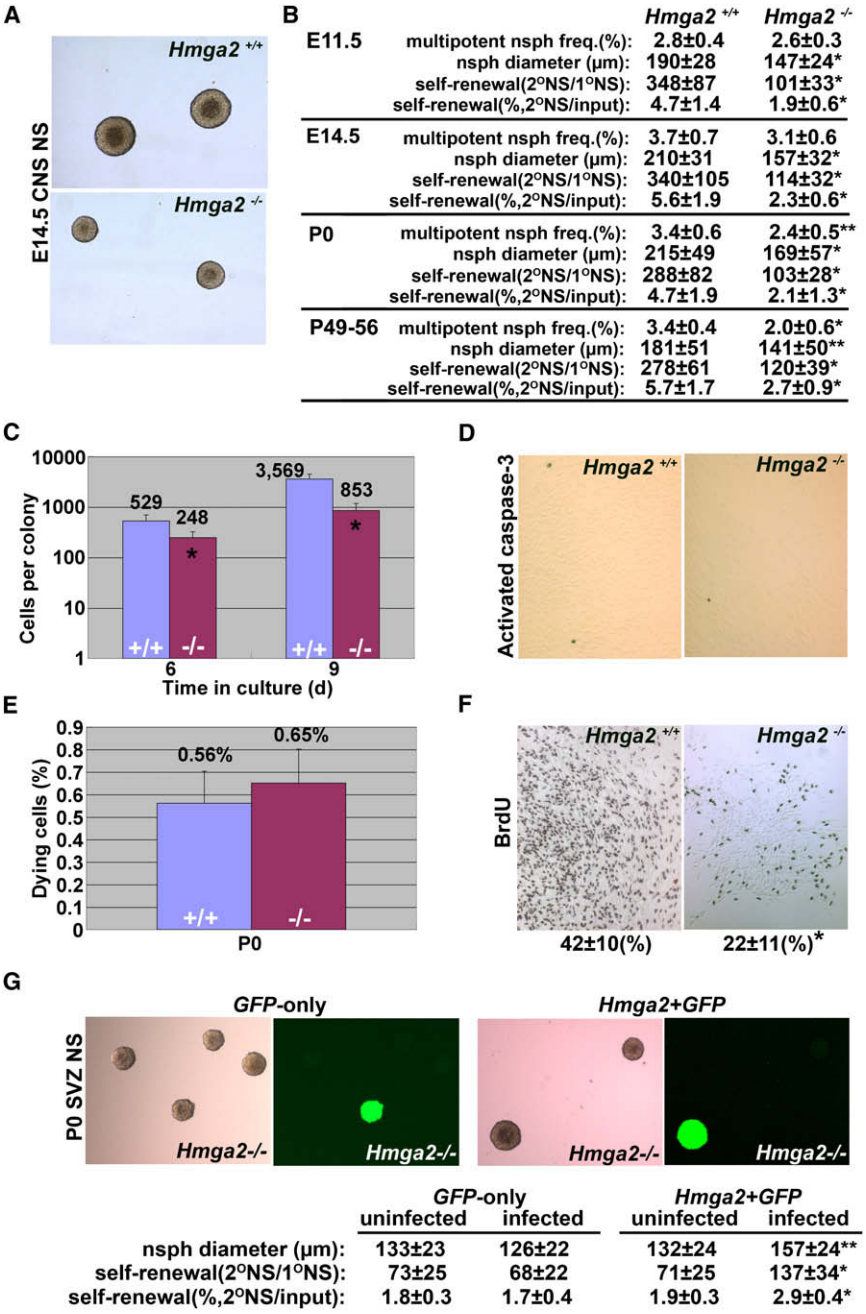


**Figure 2. Hmga2 Promotes the Self-Renewal of Fetal and Young-Adult CNS Stem Cells**

(A) Typical neurospheres that formed after 9 days in nonadherent cultures from E14.5 telencephalon cells cultured at very low cell density (~1 cell/μl).

(B) The percentage of cells from E11.5 telencephalon, E14.5 telencephalon, P0 VZ, and P49–56 SVZ of *Hmga2*$^{-/-}$ mice and littermate controls that gave rise to multipotent neurospheres in culture, the diameter of these neurospheres, and their self-renewal potential (number and percentage of cells from individual primary neurospheres that gave rise to multipotent secondary neurospheres upon subcloning). *Hmga2* deficiency significantly reduced self-renewal at all of these stages and stem cell frequency at P0 and P49–56 (mean ± SD for five to seven independent experiments per stage; *$p < 0.01$, **$p < 0.05$).

(C–E) P0 VZ cells were dissociated and plated in adherent cultures at clonal density (0.33 cells/μl) (C). Wild-type (+/+) stem cell colonies contained significantly more cells than did *Hmga2*$^{-/-}$ (−/−) colonies (three independent experiments; error bars always represent the SD). The frequency of dying cells within multilineage colonies after 10 days in culture was examined on the basis of the basis of activated caspase-3 staining (D), or the frequency of condensed, fragmented nuclei identified by 4′,6-diamino-2-phenylindole dihydrochloride (DAPI) staining (E). Only rare cells underwent cell death in *Hmga2*$^{+/+}$ and *Hmga2*$^{-/-}$ colonies (three independent experiments).

(F) A significantly lower percentage of cells within *Hmga2*$^{-/-}$ multilineage colonies (22% ± 11%, mean ± SD) incorporated a 20 min pulse of BrdU as compared to *Hmga2*$^{+/+}$ colonies (42% ± 10%; three experiments; *$p < 0.01$).

(G) P0 *Hmga2*$^{-/-}$ VZ cells were infected with either *GFP-only* control lentivirus or with *Hmga2+GFP* lentivirus. *Hmga2* overexpression significantly increased the size and self-renewal of *Hmga2*$^{-/-}$ neurospheres (three experiments; $p < 0.01$, **$p < 0.05$).

All t tests were paired.

These data suggest that Hmga2 promotes neural stem cell self-renewal by promoting division.

To assess whether the reduced self-renewal of *Hmga2*$^{-/-}$ neural stem cells could be rescued by *Hmga2* overexpression, we infected P0 VZ cells from an *Hmga2*$^{-/-}$ mouse with a dual-promoter lentivirus expressing *Hmga2* and *GFP*, or control virus lacking *Hmga2* (*GFP-only*). After infection, the cells were subcloned into secondary cultures to form neurospheres. *GFP-only* vector did not affect the diameter or self-renewal of neurospheres as compared to uninfected neurospheres within the same cultures (Figure 2G). In contrast, *Hmga2* overexpression significantly increased the diameter and self-renewal of neurospheres as compared to both uninfected neurospheres within the same cultures and *GFP-only* neurospheres ($p < 0.05$; Figure 2G). *Hmga2* overexpression can thus rescue the self-renewal defect in *Hmga2*$^{-/-}$ neural stem cells.

To assess the effect of *Hmga2* deficiency on neural stem cell differentiation, we adherently cultured cells from the P0 VZ at clonal density. We first examined multilineage colonies 7 days after plating. *Hmga2*$^{-/-}$ colonies showed no signs of premature differentiation as only rare cells expressed neuronal or astrocytic markers in either *Hmga2*$^{-/-}$ or wild-type colonies and no cells expressed the oligodendrocyte marker O4 (Figure 3A). We cultured cells for another 7 days in medium that promotes differentiation and observed similar multilineage differentiation to

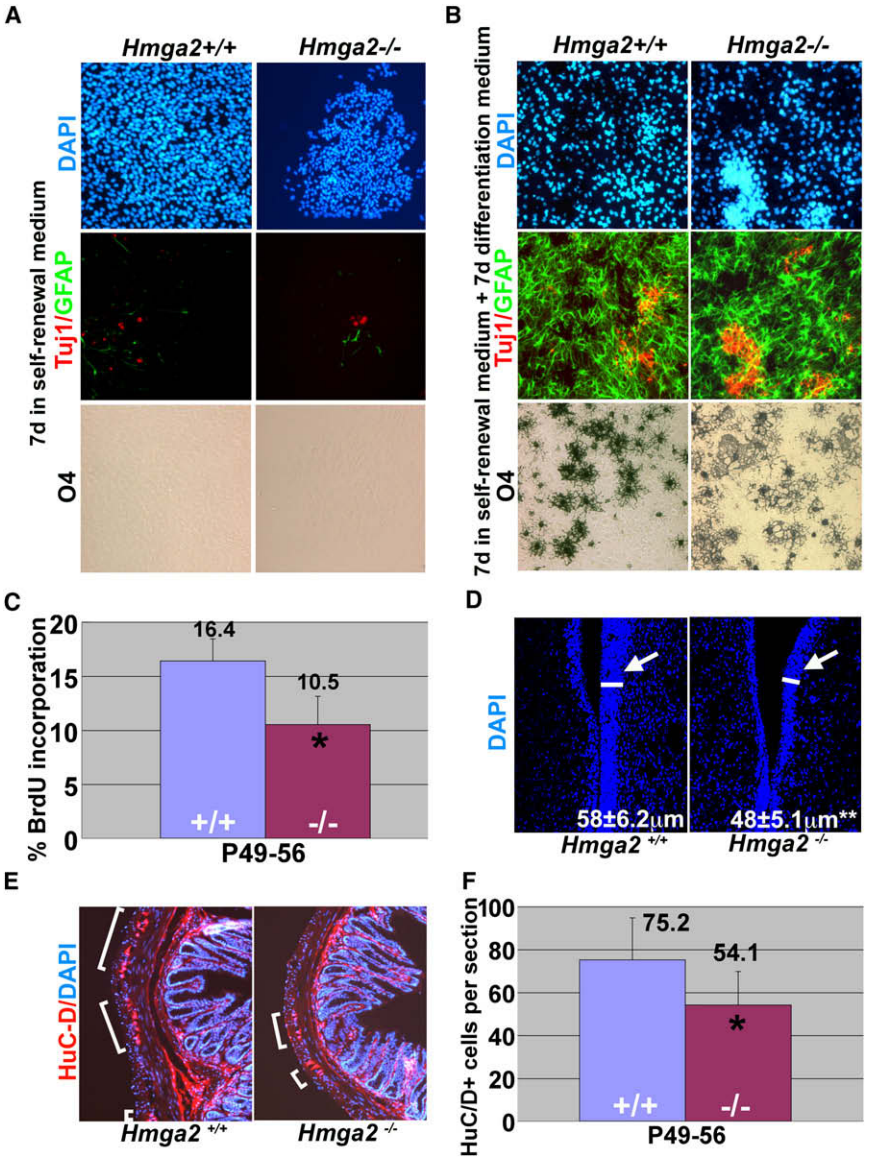


**Figure 3. *Hmga2* Deficiency Does Not Affect the Ability of CNS Stem Cells to Undergo Multilineage Differentiation in Culture but Does Reduce Proliferation In Vivo**

(A) P0 VZ cells were dissociated and cultured adherently at clonal density for 7 days in a medium that promotes self-renewal (see the Experimental Procedures). $Hmga2^{+/+}$ and $Hmga2^{-/-}$ colonies contained few cells that exhibited signs of neuronal ($TuJ1^+$) or astrocytic ($GFAP^+$) differentiation and no oligodendrocytes ($O4^+$), suggesting that *Hmga2* deficiency does not cause premature differentiation.

(B) Some colonies were cultured for another 7 days in a medium that favors differentiation (see the Experimental Procedures). $Hmga2^{+/+}$ and $Hmga2^{-/-}$ stem cell colonies contained similar proportions of neurons ($Tuj1^+$), astrocytes ($GFAP^+$), and oligodendrocytes ($O4^+$).

(C) The percentage of P49–56 SVZ cells that incorporated a 2 hr pulse of BrdU in vivo was significantly reduced in $Hmga2^{-/-}$ mice (−/−) as compared to wild-type (+/+) controls (five mice per genotype, six to ten sections per mouse; $^*p < 0.01$; error bars always represent the SD).

(D) Cross-sections through the P0 lateral ventricle. The lateral wall (white arrows and bars) was significantly thinner in the $Hmga2^{-/-}$ brain as compared to littermate controls ($^{**}p < 0.05$; three brains per genotype, six to eight sections per brain).

(E) Transverse sections through the P49 distal ileum. $HuC/D^+$ neurons in myenteric plexi are indicated by white brackets.

(F) $Hmga2^{-/-}$ mice had significantly fewer $HuC/D^+$ neurons per transverse gut section than did wild-type mice (n = 5 guts for the wild-type, n = 4 for $Hmga2^{-/-}$, eight to ten sections counted per gut; $^*p < 0.01$).

All t tests were paired.

neurons, astrocytes, and oligodendrocytes in $Hmga2^{-/-}$ and wild-type CNS stem cell colonies (Figure 3B). Similar results were observed in NCSC colonies (data not shown). Indeed, the vast majority of neurospheres cultured from the CNS and PNS at all stages of development underwent multilineage differentiation irrespective of *Hmga2* genotype (Figure S3). *Hmga2* deficiency therefore does not affect the ability of neural stem cells to undergo multilineage differentiation. However, adherent stem cell colonies, like neurospheres, were significantly smaller in the absence of Hmga2 (Figure 3A).

## *Hmga2* Deficiency Leads to Defects in the CNS and PNS In Vivo

We examined proliferation in the SVZ of young-adult mice by administering a pulse of BrdU. $Hmga2^{-/-}$ mice exhibited a significantly lower overall rate of proliferation in the SVZ in vivo ($p < 0.01$; Figure 3C). This was consistent with the reduced proliferation within $Hmga2^{-/-}$ CNS stem cell colonies in culture (Figure 2F) and the reduced brain size of $Hmga2^{-/-}$ mice (Figures S1G and S1H). The P0 VZ was also slightly but significantly thinner in $Hmga2^{-/-}$ mice than in littermate controls (Figure 3D), consistent with the reduced stem cell frequency (Figure 2B) and overall mitotic activity (Figure 3C) in these mice. In the PNS, we counted the number of myenteric plexus neurons per transverse section through the distal ileum. Defects in NCSC self-renewal, such as those observed in the absence of *Bmi-1*, lead to a reduction in the number of neurons per section (Molofsky et al., 2003, 2005). Young-adult $Hmga2^{-/-}$ mice also had significantly fewer neurons per section than did littermate controls ($p < 0.01$; Figures 3E and 3F). $Hmga2^{-/-}$ mice exhibit phenotypes in vivo consistent with reduced proliferation by CNS and PNS neural stem cells.

## *Hmga2* Is Not Required for the Proliferation of Many Restricted Neural Progenitors

We examined the effect of *Hmga2* deficiency on the proliferation of restricted neuronal progenitors (that make neuron-only

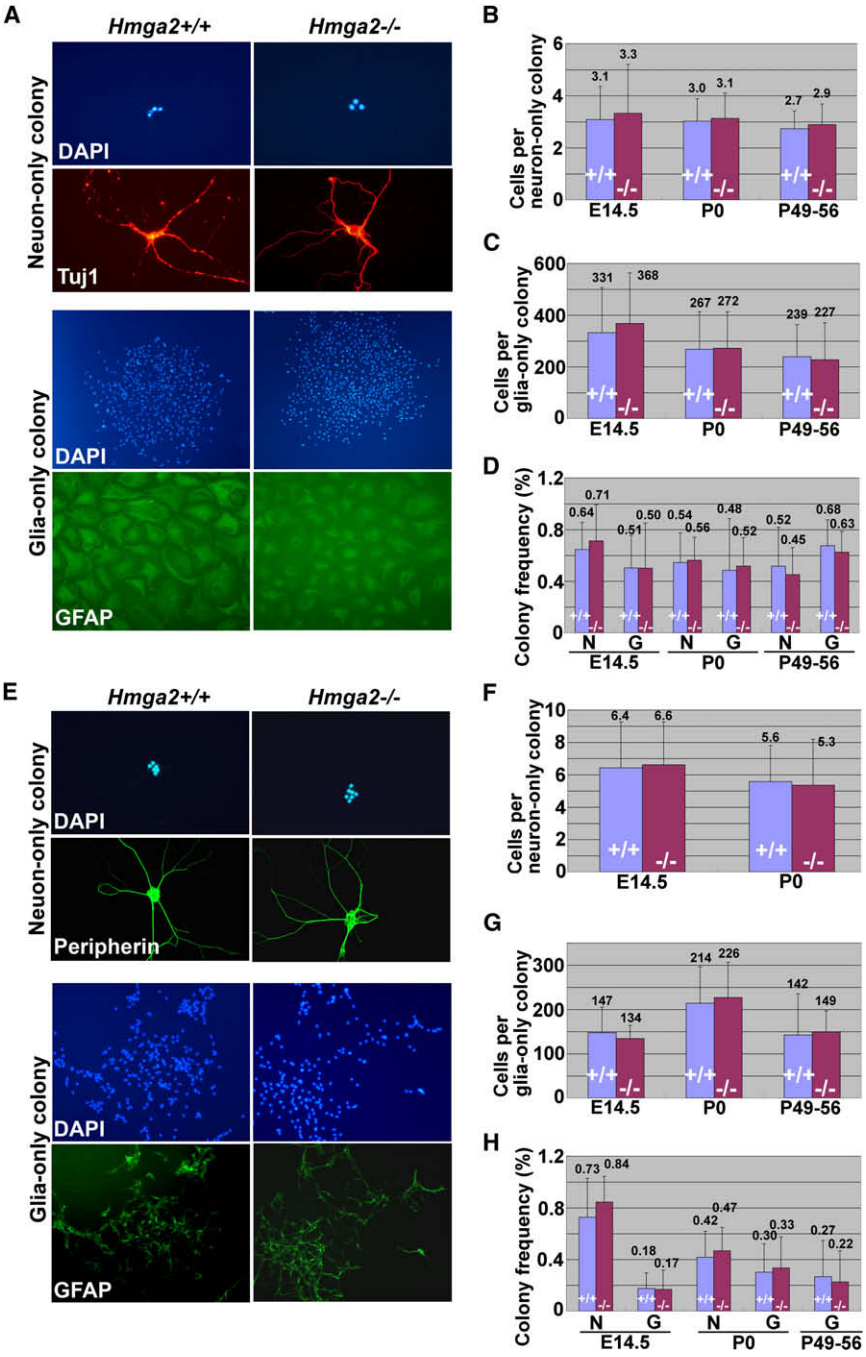


**Figure 4. Restricted Neural Progenitors from the CNS and PNS Proliferate Normally in the Absence of *Hmga2***

(A–D) E14.5 telencephalon cells, P0 VZ cells, or P49–56 SVZ cells were dissociated and cultured at clonal density for 12 days.

(E–H) E14.5 gut cells or P0 or P49–56 outer muscle layer gut cells were dissociated and cultured at clonal density for 12 days.

Typical neuron-only or astrocyte-only colonies formed by E14.5 telencephalon cells are shown (A). Typical neuron-only or glia-only colonies formed by E14.5 gut cells are shown(E). The number of cells per neuron-only colony, the number of cells per glia-only colony, and the frequency of cells that formed these colonies did not differ between wild-type (+/+; blue) and *Hmga2*$^{-/-}$ (–/–; burgundy) mice in the CNS (B–D) or PNS (F–H) (three to four independent experiments per stage; error bars always represent SD). All t tests were paired.

colonies) and restricted glial progenitors (that make glia-only colonies) in adherent cultures at clonal density. Whether these restricted progenitors were cultured from the E14.5 telencephalon, the P0 VZ, or the P49–56 SVZ, the size and frequency of CNS neuron-only colonies and glia-only colonies were unaffected by *Hmga2* deficiency (Figures 4A–4D). The same was true in the PNS (Figures 4E–4H). These results contrast with the reduced size of multilineage stem cell colonies from the CNS (Figure 2) and PNS (Figure S2) in the absence of Hmga2 in both adherent and nonadherent cultures. Hmga2 therefore does not promote the proliferation of all cells but rather is preferentially required for the self-renewal of stem cells.

## Hmga2 Negatively Regulates p16$^{Ink4a}$ and p19$^{Arf}$ Expression

Since Hmga2 expression declines in SVZ cells as p16$^{Ink4a}$ and p19$^{Arf}$ expression increase during aging (Figure 1C), we wondered whether Hmga2 might promote neural stem cell self-renewal by negatively regulating p16$^{Ink4a}$ and p19$^{Arf}$ expression. We examined *p16$^{Ink4a}$* and *p19$^{Arf}$* expression by qPCR in CNS and PNS neurospheres cultured from *Hmga2*$^{-/-}$ mice and littermate controls. *p16$^{Ink4a}$* and *p19$^{Arf}$* expression increased significantly in CNS (Figure 5A) and PNS (Figure 5B) neurospheres from E14.5, P0, and P49–56 *Hmga2*$^{-/-}$ mice as compared to littermate controls. Fetal neurospheres showed the greatest increase in *p16$^{Ink4a}$* and *p19$^{Arf}$* expression in the absence of *Hmga2*. Importantly, there was no effect of *Hmga2* deficiency on *p16$^{Ink4a}$* or *p19$^{Arf}$* expression by neurospheres cultured from old (P570–600) mice, consistent with our failure to detect Hmga2 expression in neural stem and progenitor cells from old mice (Figure 1). The same trends were evident at the protein level by western blot in CNS (Figure 5C) and PNS (Figure 5D) neurospheres. p16$^{Ink4a}$ and p19$^{Arf}$ expression levels therefore increase in *Hmga2*$^{-/-}$ CNS stem and progenitor cells from fetal and young-adult but not old-adult mice.

To ensure that Hmga2 also negatively regulates p16$^{Ink4a}$ and p19$^{Arf}$ expression in CNS stem and progenitor cells in vivo, we performed western blots on E14.5 telencephalon cells, P49–56

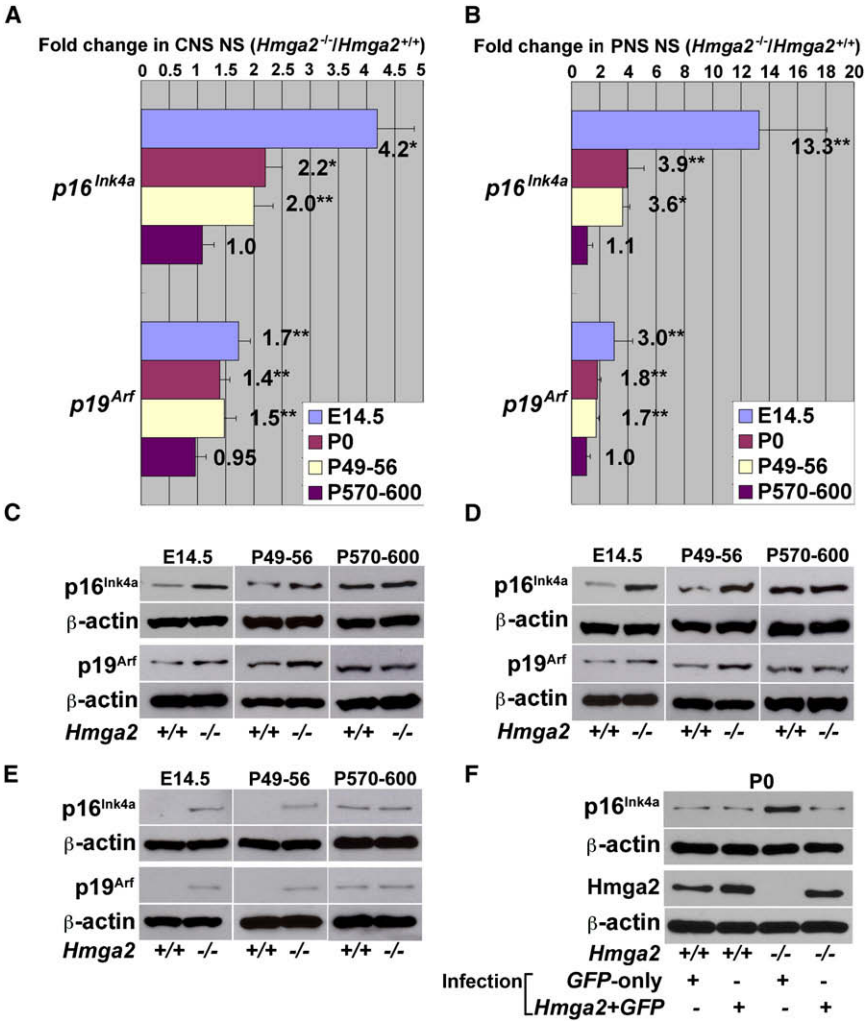


**Figure 5. Hmga2 Negatively Regulates p16$^{Ink4a}$ and p19$^{Arf}$ Expression in CNS and PNS Stem and Progenitor Cells from Fetal and Young-Adult Mice but Not from Old-Adult Mice**

(A and C) CNS neurospheres were cultured from wild-type or *Hmga2*$^{-/-}$ mice of different ages (E14.5 telencephalon, P0 VZ, P49–56 SVZ, or P570–600 SVZ).

(B and D) PNS neurospheres were cultured from wild-type or *Hmga2*$^{-/-}$ mice of different ages (E14.5 gut, or P0, P49–56, or P570–600 outer muscle layer).

*p16*$^{Ink4a}$ and *p19*$^{Arf}$ transcript levels were determined by qPCR in primary CNS (A) or PNS (B) neurospheres. Each bar shows the fold increase in *Hmga2*$^{-/-}$ as compared to wild-type neurospheres (error bars represent SD, five to six independent experiments per stage; *$p < 0.01$, **$p < 0.05$). Western blot for p16$^{Ink4a}$, p19$^{Arf}$, or β-actin (loading control) in CNS (C) or PNS (D) neurospheres is shown.

(E) Western blot for p16$^{Ink4a}$, p19$^{Arf}$, or β-actin in freshly dissected E14.5 telencephalon, P49–56 SVZ, and P570–600 SVZ cells. In vitro and in vivo, p16$^{Ink4a}$ and p19$^{Arf}$ expression levels were elevated in *Hmga2*$^{-/-}$ cells at the fetal and young-adult, but not old-adult, stages.

(F) P0 SVZ cells were dissociated from *Hmga2*$^{+/+}$ (+/+) or *Hmga2*$^{-/-}$ animals (−/−), infected with *GFP-only* control virus or *Hmga2+GFP* virus, and allowed to form neurospheres. Hmga2 overexpression in *Hmga2*$^{-/-}$ cells was sufficient to reduce p16$^{Ink4a}$ to approximately wild-type levels by western blot.

All t tests were unpaired.

SVZ cells, and P570–600 SVZ cells from *Hmga2*$^{-/-}$ mice and littermate controls. In wild-type mice, p16$^{Ink4a}$ and p19$^{Arf}$ were not detectable in cells from fetal telencephalon or young-adult SVZ but became detectable in old-adult SVZ (Figure 5E), consistent with a prior study (Molofsky et al., 2006). In contrast, p16$^{Ink4a}$ and p19$^{Arf}$ were expressed in fetal telencephalon and young-adult SVZ of *Hmga2*$^{-/-}$ mice, though the levels in old-adult SVZ were not affected by *Hmga2* deficiency (Figure 5E). Hmga2 is thus required to repress p16$^{Ink4a}$ and p19$^{Arf}$ expression by CNS stem and progenitor cells from fetal and young-adult mice, but not old-adult mice, in vitro and in vivo.

Overexpression of *Hmga2* in neurospheres cultured from the P0 VZ of *Hmga2*$^{-/-}$ mice also rescued the p16$^{Ink4a}$ expression phenotype (p19$^{Arf}$ was not assessed in this experiment because of the limited protein available). Overexpression of *Hmga2* in wild-type neurospheres only slightly increased Hmga2 protein levels and did not affect p16$^{Ink4a}$ levels (Figure 5F); however, overexpression of *Hmga2* in *Hmga2*$^{-/-}$ neurospheres restored approximately normal Hmga2 and p16$^{Ink4a}$ protein levels (Figure 5F).

These results were also confirmed with an shRNA against *Hmga2,* which partially knocked down Hmga2 protein expression within CNS neurospheres, increasing p16$^{Ink4a}$ expression and reducing self-renewal potential (Figure S4).

## Hmga2 Promotes Self-Renewal by Negatively Regulating p16$^{Ink4a}$ and p19$^{Arf}$ Expression

To test whether Hmga2 promotes neural stem cell self-renewal by negatively regulating p16$^{Ink4a}$ and p19$^{Arf}$ expression, we cultured neurospheres from young-adult *Hmga2/p16*$^{Ink4a}$*/p19*$^{Arf}$ compound mutant mice. *p16*$^{Ink4a}$*/p19*$^{Arf}$ deficiency did not significantly affect the percentage of wild-type SVZ cells that gave rise to multipotent neurospheres in culture, the diameter of these neurospheres, or their self-renewal potential (number or percentage of cells from individual primary neurospheres that gave rise to multipotent secondary neurospheres upon subcloning; Figure 6A). In contrast, *p16*$^{Ink4a}$*/p19*$^{Arf}$ deficiency significantly ($p < 0.05$) but partially rescued the reductions in the percentage of *Hmga2*$^{-/-}$ SVZ cells that gave rise to multipotent neurospheres in culture, the diameter of *Hmga2*$^{-/-}$ neurospheres, and their self-renewal potential (Figure 6A). *p16*$^{Ink4a}$*/p19*$^{Arf}$ deficiency also significantly but partially restored the frequency and self-renewal potential of *Hmga2*$^{-/-}$ NCSCs (Figure S5A).

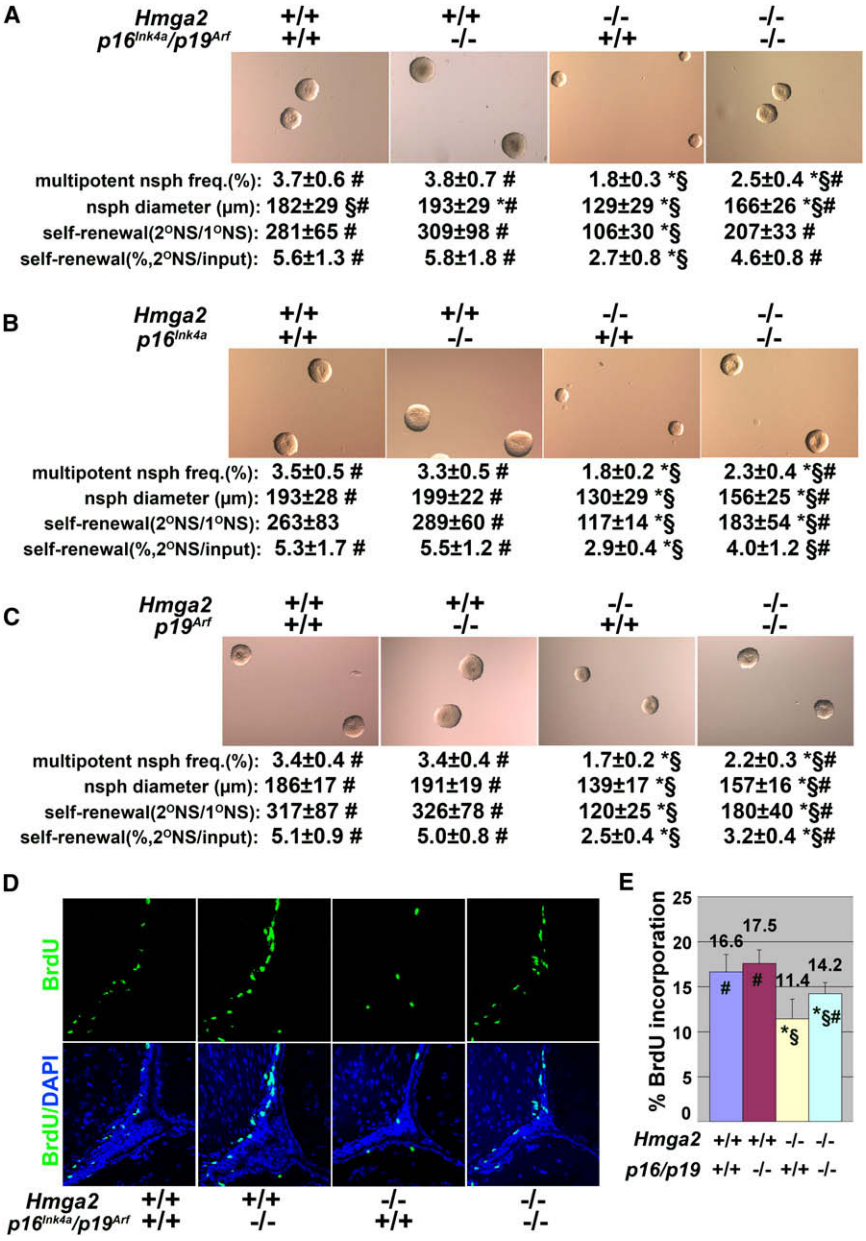


**Figure 6. Deletion of $p16^{Ink4a}$ and/or $p19^{Arf}$ Partially Rescues the Defects in Neural Stem Cell Frequency and Self-Renewal Potential as well as SVZ Proliferation in $Hmga2^{-/-}$ Mice**

(A–C) Images show typical CNS neurospheres after 9 days culture of P49–56 SVZ cells at clonal density. $p16^{Ink4a}/p19^{Arf}$ deficiency ([A]; four to six mice/genotype in four independent experiments), $p16^{Ink4a}$ deficiency ([B]; four to five mice/genotype in three independent experiments), or $p19^{Arf}$ deficiency ([C]; three to five mice/genotype in three independent experiments) did not affect the percentage of wild-type SVZ cells that formed multipotent neurospheres, the diameter of these neurospheres, or their self-renewal potential (absolute number or percentage of primary neurosphere cells that gave rise to multipotent secondary neurospheres upon subcloning of individual neurospheres) but did significantly increase the frequency, diameter, and self-renewal of $Hmga2^{-/-}$ neurospheres. All data represent mean ± SD (*, significantly different [$p < 0.05$] from wild-type; §, significantly different from $Hmga2^{+/+}p16^{Ink4a}/p19^{Arf-/-}$ mice [A], $Hmga2^{+/+}p16^{Ink4a-/-}$ mice [B], or $Hmga2^{+/+}p19^{Arf-/-}$ mice [C]; #, significantly different from $Hmga2^{-/-}p16^{Ink4a}/p19^{Arf+/+}$ mice [A], $Hmga2^{-/-}p16^{Ink4a+/+}$ mice [B], or $Hmga2^{-/-}p19^{Arf+/+}$ mice [C]).

(D) $BrdU^+$ cells in the SVZ of $Hmga2/p16^{Ink4a}/p19^{Arf}$ compound mutant mice.

(E) $p16^{Ink4a}/p19^{Arf}$ deficiency partially rescued the reduction in SVZ proliferation within young-adult $Hmga2^{-/-}$ mice without affecting proliferation in wild-type littermates (three mice per genotype, six to ten sections per mouse).

All t tests were unpaired; error bars represent the SD.

We also cultured neurospheres from young-adult $Hmga2^{-/-}p16^{Ink4a-/-}$ mice and $Hmga2^{-/-}p19^{Arf-/-}$ mice and littermate controls. $p16^{Ink4a}$ deficiency (Figure 6B) or $p19^{Arf}$ deficiency (Figure 6C) did not affect the percentage of wild-type SVZ cells that gave rise to multipotent neurospheres in culture, the diameter of these neurospheres, or their self-renewal potential. In contrast, $p16^{Ink4a}$ deficiency (Figure 6B) or $p19^{Arf}$ deficiency (Figure 6C) significantly but partially rescued the reductions in the percentage of $Hmga2^{-/-}$ SVZ cells that gave rise to multipotent neurospheres in culture, the diameter of $Hmga2^{-/-}$ neurospheres, and their self-renewal potential in the CNS. Each of $p16^{Ink4a}$ and $p19^{Arf}$ also partially rescued NCSC frequency and self-renewal potential in $Hmga2^{-/-}$ mice (Figures S5B and S5C). Therefore, $p16^{Ink4a}$ and $p19^{Arf}$ both contribute to the reduced neural stem cell frequency and self-renewal in the CNS and PNS of $Hmga2^{-/-}$ mice.

To test whether $p16^{Ink4a}/p19^{Arf}$ deficiency can also rescue neural stem and progenitor cell defects observed in $Hmga2^{-/-}$ mice in vivo, we examined the rate of proliferation in the SVZ of young-adult mice by administering a 2 hr pulse of BrdU immediately before sacrifice. $p16^{Ink4a}/p19^{Arf}$ deficiency did not affect the percentage of SVZ cells that incorporated BrdU in an otherwise wild-type background (Figures 6D and 6E). However, the percentage of SVZ cells that incorporated BrdU was significantly ($p < 0.01$) reduced in the absence of *Hmga2*, and this reduction was partially rescued by $p16^{Ink4a}/p19^{Arf}$ deficiency (Figures 6D and 6E). In the PNS, $p16^{Ink4a}/p19^{Arf}$ deficiency did not affect the number of myenteric neurons per transverse section through the distal ileum in wild-type mice (Figures S5D and S5E). However, the number of myenteric neurons per transverse section through the distal ileum was significantly ($p < 0.01$) reduced in the absence of *Hmga2*, and this reduction was significantly but partially rescued by $p16^{Ink4a}/p19^{Arf}$ deficiency (Figures S5D

and S5E). *p16*$^{Ink4a}$ and/or *p19*$^{Arf}$ deficiency also partially rescued the reduction in brain mass within *Hmga2*$^{-/-}$ mice but not the reduction in overall body mass in *Hmga2*$^{-/-}$ mice (Figure S6). *p16*$^{Ink4a}$/*p19*$^{Arf}$ deficiency therefore partially rescues the neural stem and progenitor cell defects in the CNS and PNS of *Hmga2*$^{-/-}$ mice in vivo.

## Hmga2 Does Not Regulate the Self-Renewal of Neural Stem Cells from Old-Adult Mice

Hmga2 expression declined with age and no longer affected p16$^{Ink4a}$ or p19$^{Arf}$ expression in the SVZ of old-adult mice (Figure 5E). To test whether Hmga2 regulates neural stem cell function in old mice, we aged *Hmga2*$^{-/-}$ mice and cultured neurospheres from P570–600 mice and littermate controls. The frequency of SVZ or gut cells that formed multipotent neurospheres in culture was significantly ($p < 0.05$) reduced with age in wild-type mice (compare Figure 7A to Figure 2B and Figure S2B to Figure S7A) as previously reported (Molofsky et al., 2006). *Hmga2* deficiency did not significantly affect the percentage of SVZ cells or outer muscle layer gut cells from old mice that formed multipotent neurospheres in culture, the diameter of these neurospheres, or their self-renewal potential (Figure 7A; Figure S7A). Interestingly, the frequencies of CNS and PNS cells that could form stem cell colonies in culture recovered to near wild-type levels in old *Hmga2*$^{-/-}$ mice, suggesting that homeostatic mechanisms are able to restore normal neural stem cell frequencies in old mice, when *Hmga2* is no longer required for self-renewal. *Hmga2* deficiency also did not significantly affect the number of cells per colony or the rate of BrdU incorporation within CNS (Figures S7B and S7C) or PNS (Figures S7D and S7E) stem cell colonies cultured from old mice. Finally, *Hmga2* deficiency did not significantly affect the overall rate of proliferation within the SVZ of old mice (Figure 7B). These data demonstrate that *Hmga2* is not required for the self-renewal of neural stem cells in old mice, in contrast to its role in fetal and young-adult mice.

## Increasing *let-7* Expression Contributes to the Decline in Hmga2 Expression with Age

We compared the expression of seven members of the *let-7* family by qPCR in E14.5 telencephalon, P0 VZ, P360 SVZ, and P720 SVZ. Only *let-7b* showed progressive increases in expression with age that inversely correlated with the declines in Hmga2 expression (Figure 7C). To test whether elevated expression of *let-7b* in neural stem cells could reduce their self-renewal potential, we overexpressed *let-7b* and *GFP* in wild-type cells cultured from P0 VZ using a dual promoter lentivirus (Rubinson et al., 2003). The *GFP-only* control virus did not significantly affect the diameter of neurospheres or the self-renewal of multipotent neurospheres as compared to uninfected neurospheres within the same cultures (Figure 7D). However, the *let-7b+GFP* virus did significantly ($p < 0.01$) reduce the diameter of neurospheres and the self-renewal of multipotent neurospheres compared to uninfected neurospheres within the same cultures, as well as compared to *GFP-only* infected neurospheres in sister cultures (Figure 7D). *let-7b* overexpression in these neurospheres also reduced Hmga2 expression and increased p16$^{Ink4a}$ expression (p19$^{Arf}$ was not assessed in these experiments because of the limited protein) (Figure 7F, first panel). These data indicate that increasing *let-7b* expression can reduce Hmga2 expression, increase p16$^{Ink4a}$ expression, and reduce self-renewal, potentially explaining the changes that are observed in aging neural stem cells.

We also tested whether *Hmga2* overexpression could increase the self-renewal of neural stem cells from old mice. Interestingly, overexpression of wild-type *Hmga2* in neural stem cells did not significantly affect neurosphere diameter or the self-renewal of multipotent neurospheres from old wild-type (Figure 7E) or *Hmga2*$^{-/-}$ mice (Figure S7F). Moreover, in contrast to what was observed in neurospheres from the P0 VZ (Figure 5F), overexpression of wild-type *Hmga2* in neurospheres from the P600 SVZ only marginally increased Hmga2 protein levels, irrespective of whether the cells were from wild-type or *Hmga2*$^{-/-}$ mice (Figure 7F). Since we had observed significantly increased *let-7b* expression in P720 SVZ (Figure 7C), we hypothesized that Hmga2 expression in old neural stem cells is limited by increased *let-7b* expression. To test this, we overexpressed a form of *Hmga2* with a wild-type coding sequence but a truncated 3′ untranslated region that lacks the *let-7* binding sites. This *let-7*-insensitive form of *Hmga2* increased Hmga2 protein levels and reduced p16$^{Ink4a}$ protein levels in neurospheres from old mice similar to those observed in neurospheres from newborn mice (Figure 7F). Overexpression of *let-7*-insensitive *Hmga2* also significantly ($p < 0.05$) increased the diameter and self-renewal potential of multipotent neurospheres from P600 wild-type mice (Figure 7E) and *Hmga2*$^{-/-}$ mice (Figure S7F). These data suggest that increasing let-7 expression within aging neural stem and progenitor cells contributes to the decline in *Hmga2* expression in these cells.

## Hmga2 May Indirectly Regulate *p16*$^{Ink4a}$/*p19*$^{Arf}$ Expression

We have not so far been able to detect Hmga2 binding to the *p16*$^{Ink4a}$/*p19*$^{Arf}$ locus by chromatin immunoprecipitation (Figure S8A). In contrast, we have been able to detect Hmga2 binding to the *JunB* locus (Figure S8A). JunB promotes *p16*$^{Ink4a}$/*p19*$^{Arf}$ expression in stem cells (Passegue et al., 2004). To test whether Hmga2 regulates *JunB* expression, we compared *JunB* transcript levels by qPCR in neurospheres cultured from E14.5 telencephalon, P0 VZ, P49–56 SVZ, and P600 SVZ from *Hmga2*$^{-/-}$ mice and littermate controls. We observed significantly increased *JunB* expression in *Hmga2*$^{-/-}$ neurospheres as compared to wild-type neurospheres cultured from the fetal and young-adult but not the old-adult stage in both the CNS (Figure S8B) and PNS (Figure S8C). Finally, *JunB* expression levels increased with age in wild-type SVZ cells (Figure S8D). These data are all consistent with the possibility that Hmga2 negatively regulates *JunB* expression by a mechanism that involves binding to the *JunB* locus and that JunB promotes *p16*$^{Ink4a}$/*p19*$^{Arf}$ expression in the absence of Hmga2. Although Hmga2 has generally appeared to be a transcriptional activator, there are some contexts in which it has appeared to have repressive activity (Reeves, 2001). We consider this model for Hmga2 function preliminary because it will be necessary to generate and age *Hmga2*/*JunB* compound mutant mice to functionally evaluate the model.

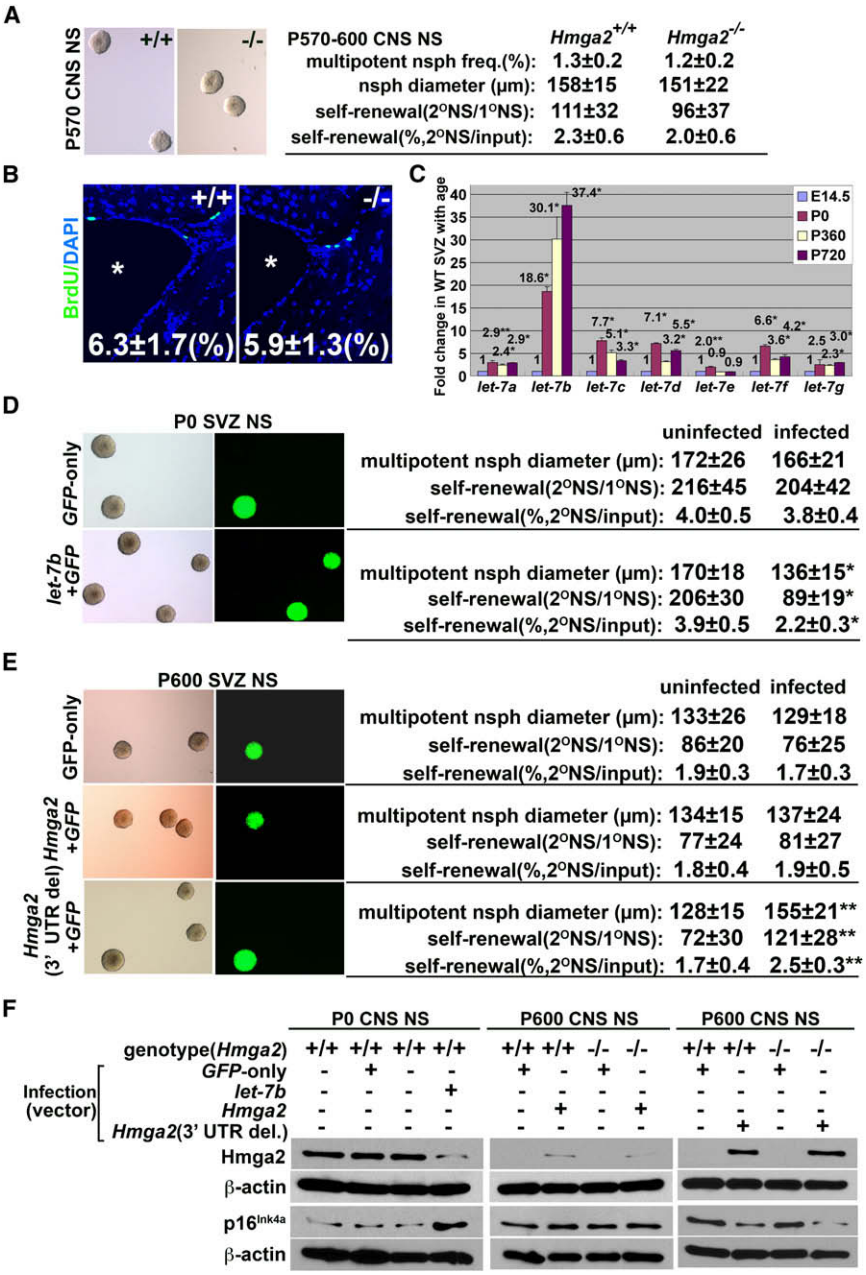


**Figure 7. *Hmga2* Is Not Required for the Self-Renewal of CNS Stem Cells from Old Mice, and Increasing *let-7* MicroRNA Expression with Age Contributes to the Decline in Hmga2 Expression**

(A) Cells were isolated from the SVZ of P570–600 wild-type (+/+) or *Hmga2*$^{-/-}$ (−/−) mice and cultured 9 days for the generation of neurospheres. The frequency of SVZ cells that formed multipotent neurospheres, their diameter, and their self-renewal potential were unaffected by *Hmga2* deficiency (mean ± SD from three independent experiments).

(B) Typical photos of P570 SVZ showing infrequent BrdU$^{+}$ cells in the SVZ, irrespective of genotype (* indicates lateral ventricle). A pulse of BrdU was incorporated by 6.3% ± 1.7% of wild-type SVZ cells and 5.9% ± 1.3% of *Hmga2*$^{-/-}$ SVZ cells from old mice (mean ± SD for three brains, seven to ten sections per brain).

(C) *let-7* family microRNA expression was compared by qPCR in freshly dissected E14.5 telencephalon, P0 VZ, P360 SVZ, and P720 SVZ (expressed as fold change relative to E14.5 telencephalon; each bar represents mean ± SD for three to four mice per stage). *let-7b* expression significantly increased with age (*$p < 0.01$, **$p < 0.05$).

(D) P0 wild-type SVZ cells were infected with *GFP-only* control lentivirus or with *let-7b*+*GFP* lentivirus. *let-7b* overexpression significantly decreased the diameter of the neurospheres and their self-renewal (three experiments; *$p < 0.01$).

(E) P600 wild-type SVZ cells were infected with *GFP-only* virus, *Hmga2*+*GFP* virus, or 3′ UTR-truncated *Hmga2*+*GFP* virus. Truncated Hmga2 lacked *let-7* binding sites in the 3′ UTR. Only 3′ UTR-truncated *Hmga2* overexpression significantly increased the diameter and self-renewal of neurospheres (three experiments; **$p < 0.05$).

(F) Protein from neurospheres examined in (D) and (E) was subjected to western blot. Overexpression of *let-7b* reduced Hmga2 expression and increased p16$^{Ink4a}$ expression. Overexpression of 3′ UTR-truncated *Hmga2* increased Hmga2 expression in P600 CNS neurospheres and reduced p16$^{Ink4a}$ expression. Overexpression of wild-type *Hmga2* had little effect.

All t tests were paired, except in (C).

## DISCUSSION

Neural stem cell frequency, self-renewal potential, mitotic activity, and neurogenesis all decline with age (Kruger et al., 2002; Enwere et al., 2004; Maslov et al., 2004; Molofsky et al., 2006). However, the mechanisms that increase the frequency and function of fetal and young-adult stem cells relative to old-adult stem cells have remained unclear. One important insight was the discovery that p16$^{Ink4a}$ expression is induced in aging tissues (Zindy et al., 1997; Krishnamurthy et al., 2004) and that this contributes to the decline in stem and progenitor cell function with age (Janzen et al., 2006; Krishnamurthy et al., 2006; Molofsky et al., 2006). p19$^{Arf}$ expression also increases in aging tissues, though it has not yet been tested whether this contributes to the decline in stem cell function or tissue regenerative capacity with age. The recent discovery of polymorphisms near the *p16*$^{Ink4a}$/*p19*$^{Arf}$ locus that are associated with age-related diseases suggests that this locus may be broadly involved in age-related morbidity (Sharpless and DePinho, 2007 and references therein). For these reasons, it is important to understand the mechanisms that regulate changes in p16$^{Ink4a}$/p19$^{Arf}$ expression and stem cell function with age.

In this study, we demonstrate that Hmga2 increases the frequency and self-renewal of fetal and young-adult stem cells as compared to old-adult stem cells (Figure 2 and Figure S2B), partly by negatively regulating the expression of p16$^{Ink4a}$ and

p19$^{Arf}$ in fetal and young-adult but not old-adult stem cells (Figure 5). This demonstrates that *p16$^{Ink4a}$*/*p19$^{Arf}$* can be expressed by fetal stem cells in vivo and that specific mechanisms are required to prevent this. Together with the fact that Bmi-1 represses *p16$^{Ink4a}$* and *p19$^{Arf}$* postnatally in stem cells (Jacobs et al., 1999; Molofsky et al., 2003; Bruggeman et al., 2005; Molofsky et al., 2005), our results demonstrate there are overlapping transcriptional mechanisms that maintain stem cells throughout life by preventing the expression of *p16$^{Ink4a}$* and *p19$^{Arf}$*.

Our results are consistent with a number of prior studies that found *Hmga2* to be a proto-oncogene (Fusco and Fedele, 2007) and raise the possibility that the tumorigenic effects of Hmga2 may partly reflect its ability to negatively regulate the expression of the p16$^{Ink4a}$ and p19$^{Arf}$ tumor suppressors. Another recent study found that HMGA1 and HMGA2 can promote cellular senescence by cooperating with p16$^{Ink4a}$ to promote the formation of senescence-associated heterochromatin foci (SAHF) in cultured human fibroblasts (Narita et al., 2006). Our results are not inconsistent with those of Narita et al. for two reasons. First, Narita et al. found that *HMGA1* was necessary for the formation of SAHF but that *HMGA2* had much less effect (Narita et al., 2006). This raises the possibility that the main physiological function of Hmga2 may be to promote stem cell function in fetal and young-adult mice, whereas Hmga1 may be the main regulator of cellular senescence. Second, the ability of HMGAs to promote senescence is context dependent: HMGA function may be different in mouse neural stem cells in vivo than in human fibroblasts in culture.

Additional mechanisms likely play important roles in regulating the change in p16$^{Ink4a}$/p19$^{Arf}$ expression with age, either by acting upstream or downstream of Hmga2 or by acting in parallel. For example, it is unclear whether polycomb complexes function as part of the same pathway or as part of a different pathway as compared to Hmga2 to regulate p16$^{Ink4a}$/p19$^{Arf}$ expression. Recent data raised the possibility that loss of Ezh2 function in aging stem cells may contribute to increasing p16$^{Ink4a}$/p19$^{Arf}$ expression (Bracken et al., 2007).

Together, our data suggest the existence of a novel pathway that regulates stem cell aging: *let-7b* expression increases with age in neural stem cells, decreasing Hmga2 expression, which in turn increases JunB and p16$^{Ink4a}$/p19$^{Arf}$ expression, reducing stem cell frequency and function. Future studies will be required to further evaluate the roles of *let-7* family members and JunB in this pathway, but the data in this study clearly demonstrate an important role for Hmga2 in regulating age-related changes in stem cell function and *p16$^{Ink4a}$*/*p19$^{Arf}$* expression.

## EXPERIMENTAL PROCEDURES

*Hmga2$^{+/-}$* (Zhou et al., 1995), *p16$^{Ink4a}$*/*p19$^{Arf+/-}$* (Serrano et al., 1996), *p16$^{Ink4a+/-}$* (Sharpless et al., 2001), and *p19$^{Arf+/-}$* (Kamijo et al., 1997) mice were housed at the University of Michigan Unit for Laboratory Animal Medicine and backcrossed at least six times onto C57BL/Ka background. All mice were genotyped by PCR as described in the Supplemental Data.

### Cell Culture and Self-Renewal Assay

CNS and PNS progenitors were isolated as described in prior studies (Molofsky et al., 2003, 2005) (see the Supplemental Data for details). For adherent cultures, CNS and PNS progenitors were plated at a clonal density of 0.33 cells/μl (500 cells per 35 mm well), in 6-well plates (Corning) that had been sequentially coated with 150 μg/ml poly-d-lysine (Biomedical Technologies, Stoughton, MA) and 20 μg/ml laminin (Sigma). For the nonadherent culture of neurospheres, CNS and PNS progenitors were plated at a density of 0.67–1.33 cells/μl (1000–2000 cells per 35 mm well) in ultra-low binding 6-well plates (Corning). The medium was a 5:3 mixture of Dulbecco's modified Eagle's medium (DMEM)-low:neurobasal medium, supplemented with 20 ng/ml recombinant human bFGF (R&D Systems, Minneapolis, MN), 1% N2 supplement (GIBCO), 2% B27 supplement (GIBCO), 50 mM 2-mercaptoethanol, and penicillin and streptomycin (Biowhittaker). "Self-renewal medium" for CNS cultures (designed to promote the expansion of undifferentiated cells) also contained 20 ng/ml epidermal growth factor (EGF) (R&D Systems) and 10% chick embryo extract (CEE). "Self-renewal medium" for PNS cultures contained 15% CEE, 35 mg/ml (110 nM) retinoic acid (Sigma), and 20 ng/ml IGF1 (R&D Systems). "Differentiation medium" for CNS cultures contained 10 ng/ml (instead of 20 ng/ml) bFGF, no EGF, no CEE, and 5% fetal bovine serum (GIBCO). Differentiation medium for PNS cultures was the same except that retinoic acid was also added. All cultures were maintained at 37°C in 6% $CO_2$, balance air.

For the measurement of self-renewal, individual primary CNS neurospheres were dissociated by trituration and then replated at clonal density in nonadherent secondary cultures. Individual PNS neurospheres were replated for 72 hr into adherent plates then treated with trypsin and collagenase (four parts 0.05% trypsin-ethylenediaminetetraacetic acid (EDTA) plus one part 10 mg/mL collagenase IV) for 3 min at 37°C followed by trituration. Secondary neurospheres were counted 7–9 days later and then transferred to adherent cultures containing differentiation medium for assessment of the percentage of secondary neurospheres that could undergo multilineage differentiation.

For viral infection experiments, CNS progenitors were plated at a high density of 10 cells/μl and cultured adherently in CNS self-renewal medium. After 48 hr, viral supernatant was added for 24 hr and then switched to CNS self-renewal medium for a further 24 hr. Cells were harvested by incubating for 1.5 min at 37°C in trypsin/EDTA and transferred to nonadherent cultures to form neurospheres for 6–7 days. See the Supplemental Data for details regarding retroviral vector construction and packaging.

### Immunocytochemistry

Neurospheres were tested for multipotency by replating of one neurosphere per well of 48-well plates coated with poly-d-lysine and laminin (as described above) and then culturing adherently in differentiation medium for 5–10 days prior to immunohistochemical staining (see the Supplemental Data). For proliferation studies, cells were pulsed with 10μM BrdU (Sigma) for 20 min (CNS cells) or 60 min (PNS cells), fixed in 70% ethanol for 30 min at −20°C, and stained with anti-BrdU antibody (1:100, Caltag, Burlingame, CA). For caspase-3 staining, plates were fixed for 10 min at room temperature in 4% paraformaldehyde, blocked, and then stained with anti-activated caspase-3 antibody (1:1000, Pharmigen, San Diego, CA). In all cases, cells were counterstained for 10 min at room temperature with 10 μg/ml DAPI (Sigma D-8417).

### Western Blots

Cells or tissues were resuspended in ice-cold cell lysis buffer (Cell Signaling Technology) with protease inhibitor cocktail (complete mini-tablet, Roche) and incubated for 15 to 30 min on ice. SDS-PAGE was done in 4%–20% Tris-Glycine Gels (Invitrogen) and transferred to platelet-derived growth factor (PVDF) membranes (Millipore). The membranes were blocked in Tris-buffered saline with 0.05% Tween-20 and 5% milk, incubated with primary and secondary antibodies, and washed according to standard procedures. Primary antibodies were rabbit polyclonal anti-Hmga2 (a generous gift from M. Narita and S. Lowe), rabbit polyclonal anti-p16$^{Ink4a}$ (M-156; Santa Cruz Biotechnology), rabbit polyclonal anti-p19$^{ARF}$ (ab80, Abcam), and mouse monoclonal anti-β-actin (sc-47778; Santa Cruz).

### Quantitative RT-PCR

For *let7* family expression experiments, small RNAs (<200 nt) were extracted with the *mir*Vana miRNA isolation kit (Ambion). Quantitative RT-PCR was then performed with specific primers and probes supplied in Taqman

MicroRNA Assay kits (Applied Biosystems). See the Supplemental Data for other details.

### Chromatin Immunoprecipitation

Chromatin Immunoprecipitation (ChIP) was performed with the EZ ChIP Chromatin Immunoprecipitation Kit (Upstate) according to the manufacturer's instructions. CNS neurospheres from P0 VZ cells infected with *Hmga2-FLAG*X3 vector were fixed with 1% paraformaldehyde (PFA) for 10 min at room temperature, washed with ice-cold PBS, and lysed in SDS lysis buffer supplemented with protein inhibitor cocktail. See the Supplemental Data for details.

## SUPPLEMENTAL DATA

Supplemental Data include Supplemental Experimental Procedures and eight figures and can be found with this article online at http://www.cell.com/cgi/content/full/135/2/227/DC1/.

## ACKNOWLEDGMENTS

This work was supported by the Howard Hughes Medical Institute, the National Institute of Neurological Disorder and Stroke (R01 NS040750), and the National Institute on Aging (R01 AG024945-01). Flow cytometry was partially supported by the University of Michigan (UM) Comprehensive Cancer National Institutes of Health (NIH) CA46592. Antibody production was supported in part by National Institute of Diabetes, Digestive, and Kidney Diseases grant NIH5P60-DK20572 to the Michigan Diabetes Research and Training Center. J.N. was supported by a postdoctoral fellowship from the Japan Society for the Promotion of Science. Thanks to David Adams and Martin White for flow cytometry and to Elizabeth Smith (UM Hybridoma Core) for antibody production. Thanks to Charles Sherr for providing $p19^{Arf-/-}$ mice and to Ron DePinho for providing $p16^{Ink4a}/p19^{Arf-/-}$ and $p16^{Ink4a-/-}$ mice. Thanks to Scott Lowe and to Masashi Narita for providing anti-Hmga2 antibody and to Tom Lanigan in the UM Vector Core for generating virus.

Received: March 24, 2008
Revised: July 13, 2008
Accepted: September 8, 2008
Published: October 16, 2008

## REFERENCES

Arlotta, P., Tai, A.K., Manfioletti, G., Clifford, C., Jay, G., and Ono, S.J. (2000). Transgenic mice expressing a truncated form of the high mobility group I-C protein develop adiposity and an abnormally high prevalence of lipomas. J. Biol. Chem. *275*, 14394–14400.

Benson, K.F., and Chada, K. (1994). Mini-mouse: Phenotypic characterization of a transgenic insertional mutant allelic to pygmy. Genet. Res. *64*, 27–33.

Bracken, A.P., Kleine-Kohlbrecher, D., Dietrich, N., Pasini, D., Gargiulo, G., Beekman, C., Theilgaard-Monch, K., Minucci, S., Porse, B.T., Marine, J.C., et al. (2007). The Polycomb group proteins bind throughout the INK4A-ARF locus and are disassociated in senescent cells. Genes Dev. *21*, 525–530.

Bruggeman, S.W., Valk-Lingbeek, M.E., van der Stoop, P.P., Jacobs, J.J., Kieboom, K., Tanger, E., Hulsman, D., Leung, C., Arsenijevic, Y., Marino, S., et al. (2005). Ink4a and Arf differentially affect cell proliferation and neural stem cell self-renewal in Bmi1-deficient mice. Genes Dev. *19*, 1438–1443.

Enwere, E., Shingo, T., Gregg, C., Fujikawa, H., Ohta, S., and Weiss, S. (2004). Aging results in reduced epidermal growth factor receptor signaling, diminished olfactory neurogenesis, and deficits in fine olfactory discrimination. J. Neurosci. *24*, 8354–8365.

Fedele, M., Visone, R., De Martino, I., Troncone, G., Palmieri, D., Battista, S., Ciarmiello, A., Pallante, P., Arra, C., Melillo, R.M., et al. (2006). HMGA2 induces pituitary tumorigenesis by enhancing E2F1 activity. Cancer Cell *9*, 459–471.

Fusco, A., and Fedele, M. (2007). Roles of HMGA proteins in cancer. Nat. Rev. Cancer *7*, 899–910.

Hirning-Folz, U., Wilda, M., Rippe, V., Bullerdiek, J., and Hameister, H. (1998). The expression pattern of the Hmgic gene during development. Genes Chromosomes Cancer *23*, 350–357.

Hock, H., Hamblen, M.J., Rooke, H.M., Schindler, J.W., Saleque, S., Fujiwara, Y., and Orkin, S.H. (2004a). Gfi-1 restricts proliferation and preserves functional integrity of haematopoietic stem cells. Nature *431*, 1002–1007.

Hock, H., Meade, E., Medeiros, S., Schindler, J.W., Valk, P.J., Fujiwara, Y., and Orkin, S.H. (2004b). Tel/Etv6 is an essential and selective regulator of adult hematopoietic stem cell survival. Genes Dev. *18*, 2336–2341.

Jacobs, J.J.L., Kieboom, K., Marino, S., DePinho, R.A., and v Lohuizen, M. (1999). The oncogene and polycomb-group gene bmi-1 regulates cell proliferation and senescence through the ink4a locus. Nature *397*, 164–168.

Janzen, V., Forkert, R., Fleming, H.E., Saito, Y., Waring, M.T., Dombkowski, D.M., Cheng, T., DePinho, R.A., Sharpless, N.E., and Scadden, D.T. (2006). Stem-cell ageing modified by the cyclin-dependent kinase inhibitor p16INK4a. Nature *443*, 421–426.

Kamijo, T., Zindy, F., Roussel, M.F., Quelle, D.E., Downing, J.R., Ashmun, R.A., Grosveld, G., and Sherr, C.J. (1997). Tumor suppression at the mouse INK4a locus mediated by the alternative reading frame product p19ARF. Cell *91*, 649–659.

Kiel, M.J., Iwashita, T., Yilmaz, O.H., and Morrison, S.J. (2005). Spatial differences in hematopoiesis but not in stem cells indicate a lack of regional patterning in definitive hematopoietic stem cells. Dev. Biol. *283*, 29–39.

Kim, I., Saunders, T.L., and Morrison, S.J. (2007). Sox17 dependence distinguishes the transcriptional regulation of fetal from adult hematopoietic stem cells. Cell *130*, 470–483.

Krishnamurthy, J., Torrice, C., Ramsey, M.R., Kovalev, G.I., Al-Regaiey, K., Su, L., and Sharpless, N.E. (2004). Ink4a/Arf expression is a biomarker of aging. J. Clin. Invest. *114*, 1299–1307.

Krishnamurthy, J., Ramsey, M.R., Ligon, K.L., Torrice, C., Koh, A., Bonner-Weir, S., and Sharpless, N.E. (2006). p16INK4a induces an age-dependent decline in islet regenerative potential. Nature *443*, 453–457.

Kruger, G.M., Mosher, J.T., Bixby, S., Joseph, N., Iwashita, T., and Morrison, S.J. (2002). Neural crest stem cells persist in the adult gut but undergo changes in self-renewal, neuronal subtype potential, and factor responsiveness. Neuron *35*, 657–669.

Lee, Y.S., and Dutta, A. (2007). The tumor suppressor microRNA let-7 represses the HMGA2 oncogene. Genes Dev. *21*, 1025–1030.

Lessard, J., and Sauvageau, G. (2003). Bmi-1 determines the proliferative capacity of normal and leukaemic stem cells. Nature *423*, 255–260.

Lowe, S.W., and Sherr, C.J. (2003). Tumor suppression by Ink4a-Arf: Progress and puzzles. Curr. Opin. Genet. Dev. *13*, 77–83.

Maslov, A.Y., Barone, T.A., Plunkett, R.J., and Pruitt, S.C. (2004). Neural stem cell detection, characterization, and age-related changes in the subventricular zone of mice. J. Neurosci. *24*, 1726–1733.

Mayr, C., Hemann, M.T., and Bartel, D.P. (2007). Disrupting the pairing between let-7 and Hmga2 enhances oncogenic transformation. Science *315*, 1576–1579.

Molofsky, A.V., Pardal, R., Iwashita, T., Park, I.K., Clarke, M.F., and Morrison, S.J. (2003). Bmi-1 dependence distinguishes neural stem cell self-renewal from progenitor proliferation. Nature *425*, 962–967.

Molofsky, A.V., He, S., Bydon, M., Morrison, S.J., and Pardal, R. (2005). Bmi-1 promotes neural stem cell self-renewal and neural development but not mouse growth and survival by repressing the p16Ink4a and p19Arf senescence pathways. Genes Dev. *19*, 1432–1437.

Molofsky, A.V., Slutsky, S.G., Joseph, N.M., He, S., Pardal, R., Krishnamurthy, J., Sharpless, N.E., and Morrison, S.J. (2006). Increasing p16INK4a expression decreases forebrain progenitors and neurogenesis during ageing. Nature *443*, 448–452.

Narita, M., Narita, M., Krizhanovsky, V., Nunez, S., Chicas, A., Hearn, S.A., Myers, M.P., and Lowe, S.W. (2006). A novel role for high-mobility group a proteins in cellular senescence and heterochromatin formation. Cell *126*, 503–514.

Park, I.-K., Qian, D., Kiel, M., Becker, M., Pihalja, M., Weissman, I.L., Morrison, S.J., and Clarke, M. (2003). Bmi-1 is required for the maintenance of adult self-renewing hematopoietic stem cells. Nature *423*, 302–305.

Passegue, E., Wagner, E.F., and Weissman, I.L. (2004). JunB deficiency leads to a myeloproliferative disorder arising from hematopoietic stem cells. Cell *119*, 431–443.

Reeves, R. (2001). Molecular biology of HMGA proteins: hubs of nuclear function. Gene *277*, 63–81.

Rubinson, D.A., Dillon, C.P., Kwiatkowski, A.V., Sievers, C., Yang, L., Kopinja, J., Rooney, D.L., Zhang, M., Ihrig, M.M., McManus, M.T., et al. (2003). A lentivirus-based system to functionally silence genes in primary mammalian cells, stem cells and transgenic mice by RNA interference. Nat. Genet. *33*, 401–406.

Schoenmakers, E.F., Wanschura, S., Mols, R., Bullerdiek, J., Van den Berghe, H., and Van de Ven, W.J. (1995). Recurrent rearrangements in the high mobility group protein gene, HMGI-C, in benign mesenchymal tumours. Nat. Genet. *10*, 436–444.

Serrano, M., Lee, H., Chin, L., Cordon-Cardo, C., Beach, D., and DePinho, R.A. (1996). Role of the INK4a locus in tumor suppression and cell mortality. Cell *85*, 27–37.

Sharpless, N.E., Bardeesy, N., Lee, K.-H., Carrasco, D., Castrillon, D.H., Aguirre, A.J., Wu, E.A., Horner, J.W., and DePinho, R.A. (2001). Loss of p16Ink4a with retention of p19Arf predisposes mice to tumorigenesis. Nature *413*, 86–91.

Sharpless, N.E., and DePinho, R.A. (2007). How stem cells age and why this makes us grow old. Nat. Rev. Mol. Cell Biol. *8*, 703–713.

Shi, Y., Chichung Lie, D., Taupin, P., Nakashima, K., Ray, J., Yu, R.T., Gage, F.H., and Evans, R.M. (2004). Expression and function of orphan nuclear receptor TLX in adult neural stem cells. Nature *427*, 78–83.

Yu, F., Yao, H., Zhu, P., Zhang, X., Pan, Q., Gong, C., Huang, Y., Hu, X., Su, F., Lieberman, J., et al. (2007). let-7 regulates self renewal and tumorigenicity of breast cancer cells. Cell *131*, 1109–1123.

Zhou, X., Benson, K.F., Ashar, H.R., and Chada, K. (1995). Mutation responsible for the mouse pygmy phenotype in the developmentally regulated factor HMGI-C. Nature *376*, 771–774.

Zindy, F., Quelle, D.E., Roussel, M.F., and Sherr, C.J. (1997). Expression of the p16(INK4a) tumor suppressor versus other INK4 family members during mouse development and aging. Oncogene *15*, 203–211.