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Journal
Journal of Neuroscience, 34(37)

ISSN
0270-6474

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Publication Date
2014-09-10

DOI
10.1523/jneurosci.3159-13.2014

Peer reviewed

Brief Communications

The *Ink4a/Arf* Locus Is a Barrier to Direct Neuronal Transdifferentiation

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Non-neurogenic cell types, such as cortical astroglia and fibroblasts, can be directly converted into neurons by the overexpression of defined transcription factors. Normally, the cellular phenotype of such differentiated cells is remarkably stable and resists direct cell transdifferentiation. Here we show that the *Ink4a/Arf* (also known as *Cdkn2a*) locus is a developmental barrier to direct neuronal transdifferentiation induced by transcription factor overexpression. With serial passage *in vitro*, wild-type postnatal cortical astroglia become progressively resistant to *Dlx2*-induced neuronal transdifferentiation. In contrast, the neurogenic competence of *Ink4a/Arf*-deficient astroglia is both greatly increased and does not diminish through serial cell culture passage. Electrophysiological analysis further demonstrates the neuronal identity of cells induced from *Ink4a/Arf*-null astroglia, and short hairpin RNA-mediated acute knockdown of p16Ink4a and p19Arf p16^{Ink4a} and p19^{Arf} indicates that these gene products function postnatally as a barrier to cellular transdifferentiation. Finally, we found that mouse fibroblasts deficient for *Ink4a/Arf* also exhibit greatly enhanced transcription factor-induced neuronal induction. These data indicate that *Ink4a/Arf* is a potent barrier to direct neuronal transdifferentiation and further suggest that this locus functions normally in the progressive developmental restriction of postnatal astrocytes.

Key words: **astroglia; induced neuron; *Ink4a/Arf*; transcription factor; transdifferentiation**

Introduction

Neurons can be directly converted from non-neuronal cell types via the enforced expression of specific transcription factors (Vierbuchen and Wernig, 2012). Other than tissue-specific stem cell populations, cells in the adult mammal generally do not change their cellular identities, and this inherent phenotypic stability represents a barrier to cell transdifferentiation (Zhou and Melton, 2008). Thus, discovering the molecular-genetic mechanisms that facilitate direct neuronal transdifferentiation provides insight into how cell fates normally become restricted, and may also inform strategies that enhance cell fate conversion for the purpose of disease-modeling and cell-based therapeutics.

The *Ink4a/Arf* tumor suppressor locus encodes the p16^{Ink4a} and p19^{Arf} cell cycle inhibitors, which are expressed at basal levels in differentiated cell types. In addition to their function in tumor suppression (Kim and Sharpless, 2006), *Ink4a/Arf* gene products impede the generation of induced pluripotent stem cells (iPSCs) from fibroblasts: *Ink4a/Arf* is rapidly silenced during iPSC production, and *Ink4a/Arf*-deficient fibroblasts generate iPSCs more efficiently (Banito et al., 2009; Li et al., 2009; Utikal et al., 2009). Whether *Ink4a/Arf* also constitutes a barrier to direct neuronal reprogramming is not known.

During postnatal development, astrocytes in the mouse cerebral cortex divide symmetrically, greatly expanding their numbers (Ge et al., 2012); and when isolated from postnatal day 7 (P7) mice, astroglial cells still exhibit developmental plasticity: these young astroglia can give rise to multipotent neurospheres (Palmer et al., 1999; Laywell et al., 2000) and can also be converted into mature neurons via enforced expression of neurogenic transcription factors, such as *Dlx2* (Heins et al., 2002; Berninger et al., 2007; Heinrich et al., 2010). However, cortical astrocytes from P15 mice no longer give rise to neurospheres (Laywell et al., 2000), and transcription factor-mediated neuronal transdifferentiation is greatly hindered in cells isolated from later postnatal mice (Heinrich et al., 2011; Robel et al., 2011). Thus, during this postnatal period of symmetric glial cell proliferation, cortical astrocytes appear to lose their neurogenic "competence."

Here, we used transcription factor-mediated neuronal transdifferentiation to gain insight into the molecular-genetic mechanisms that normally restrict neurogenic competence. We found that *Ink4a/Arf* is a potent barrier to direct neuronal transdiffer-

Received July 24, 2013; revised Aug. 1, 2014; accepted Aug. 6, 2014.

Author contributions: J.D.P., K.-Y.P., and D.A.L. designed research; J.D.P., K.-Y.P., J.C., R.D.S., and M.J.C. performed research; J.D.P., K.-Y.P., J.C., R.D.S., M.J.C., A.R.K., and D.A.L. analyzed data; J.D.P., K.-Y.P., and D.A.L. wrote the paper.

This work was supported by National Institutes of Health DP2-OD006505-01, the Sontag Foundation, a Northern California Institute for Research and Education/Department of Defense subaward to D.A.L., the Shurl and Kay Curci Foundation, and the San Francisco Veterans Affairs Medical Center for resources.

The authors declare no competing financial interests.

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DOI:10.1523/JNEUROSCI.3159-13.2014

 0270-6474/14/3412560-08$15.00/0

entiation of mouse astroglia isolated from non-neurogenic brain regions as well as mouse fibroblasts. Our findings suggest a generalizable approach to enhance direct cell conversion methodologies and further indicate a role for *Ink4a/Arf* in the postnatal glial-fate restriction of parenchymal astrocytes.

Materials and Methods

Animals. *Ink4a/Arf*-null mice were maintained and genotyped as in Serrano et al. (1996). All experiments were performed with mice of either sex and in accordance to protocols approved by the Institutional Animal Care and Use Committee at University of California, San Francisco.

Cell culture and neuronal transdifferentiation. Postnatal cortical astroglial cultures were established essentially as in Berninger et al. (2007). Briefly, cortical tissue above the corpus callosum was microdissected from P5–P7 mouse brain sections, dissociated by treatment with 0.25% trypsin 0.5 mM EDTA as in Lim et al. (2009), and plated into 6-well culture plates (Corning) coated with 0.1 mg/ml poly-D-lysine (Sigma-Aldrich) in astrocyte growth medium described by Heinrich et al. (2010). Glial cultures were replated 5–7 d later into 16-well poly-D-lysine-coated Nunc Lab-Tek chamber slides (Thermo Scientific) at a density of 50,000 cells per well (passage 1) or passaged into culture plates for continued culture in astrocyte growth medium. Cells in chamber slides were infected with lentivirus and switched to astrocyte differentiation medium (DMEM/F12, B27, glutamine, and penicillin/streptomycin) (Heinrich et al., 2010) 24 h later.

Mouse embryonic fibroblasts (MEFs) were isolated and cultured in N3 medium (DMEM/F12, apotransferrin, insulin, sodium selenite, progesterone, putrescine, andpenicillin/streptomycin) (Vierbuchen et al., 2010). Passage 3 MEFs were transduced with lentivirus expressing reverse tetracycline transactivator (FUW-M2rtTA (Hockemeyer et al., 2008) and the transcription factors *Myt1l* and *Brn2* (Vierbuchen et al., 2010), and rat *Ascl1* expressed from the doxycycline-inducible lentiviral vector tetO-FUW. At 16–20 h after infection, cells were switched to fresh MEF medium containing doxycycline (2 μg/ml, Sigma). At 48 h after the addition of doxycycline, cells were switched to N3 medium containing doxycycline, which was replaced every 3 d. Neuronal differentiation was analyzed by immunocytochemistry 20 d after infection.

Immunocytochemistry. Immunocytochemistry was performed as in Lim et al. (2009) with the following primary antibodies: mouse anti-Tuj1 (Covance), chicken anti-GFP (Aves), rabbit anti-dsRed (Clontech), rabbit anti-Nestin (Millipore), guinea pig anti-GLAST (Millipore), rabbit anti-S100β (Sigma), chicken anti-GFAP (Millipore), chicken anti-vimentin (Millipore), mouse anti-phospho-histone H3 (Millipore), rabbit anti-Pax6 (Covance), and rabbit anti-Sox2 (Santa Cruz Biotechnology). DAPI (Sigma) was used for nuclear staining.

Microscopy, cell counting, and statistical analysis. For quantification of cell cultures, at least 6 random, nonoverlapping fields of view were digitally acquired at 100–200× magnification (CMI 4000B, Leica), and cells were counted manually with aid of ImageJ (National Institutes of Health) or the Leica Application Suite Advanced Fluorescence software (Leica). Significance was calculated with Student's *t* test using Excel (Microsoft).

Electrophysiology. Cells were analyzed 14–30 d after infection. The patch electrodes were made from borosilicate glass capillaries (Sutter Instruments) with a resistance in the range of 5–7 MΩ. The pipettes were tip-filled with internal solution containing the following (in mM): 125 K-gluconate, 15 KCl, 10 HEPES, 4 $MgCl_2$, 4 Na_2ATP, 0.3 Na_3GTP, 10 Tris-phosphocreatine, 0.2 EGTA. The bath was constantly perfused with fresh recording medium containing the following (in mM): 145 NaCl, 3 KCl, 3 $CaCl_2$, 2 $MgCl_2$, 10 HEPES, 8 glucose, at room temperature. Recordings were made with an Axon 700B patch-clamp amplifier and 1320A interface (Molecular Devices). Signals were filtered at 2 kHz using amplifier circuitry, sampled at 10 kHz, and analyzed using Clampex 10.2 (Molecular Devices).

Microarray and qRT-PCR analysis. Analysis by qPCR was performed as in Ramos et al. (2013). For microarray analysis, samples from three replicate cultures for each cell type were prepared as in Ramos et al. (2013) and hybridized to MouseRef-8 v2.0 Expression BeadChip arrays (Illumina). Array data were processed and analyzed as in Park et al. (2014).

Lentiviral production. To acutely knock-down *Ink4a/Arf*, one short hairpin RNA (shRNA) sequence against *Ink4a/Arf* (targeting sequence: AATGGCTGGATTGTTTAAA) (Fasano et al., 2007) and one control sequence targeting luciferase described in targeting sequence: GAGCTGTTTCTGAGGAGCC (Ventura et al., 2004) was cloned into the lentiviral vector pSicoR-mCh (a gift from Dr. Miguel Ramalho-Santos). The efficiency of viral transduction ranged from 95 to 98%, and no significant differences were observed between wild-type (WT) and null glia. *Brn2*, *Ascl1*, and *Myt1l* lentiviral vectors have been described previously (Vierbuchen et al., 2010) and were obtained through Addgene. VSV-G and EnvA pseudotyped lentiviruses were produced in HEK 293T cells as in Lewis et al. (2001).

Results

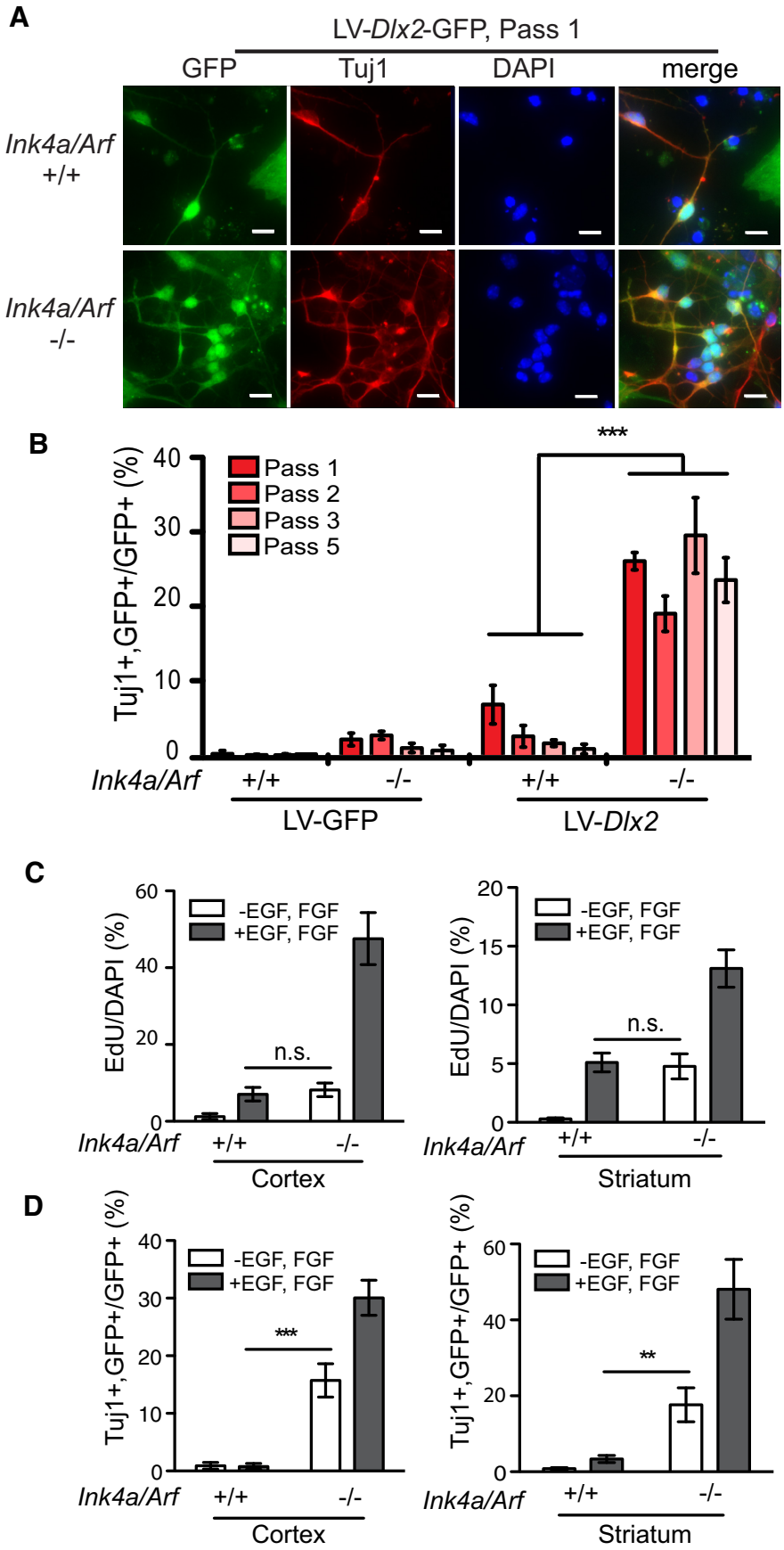
Loss of neurogenic competence of cortical astroglia through serial passage *in vitro*

P5–P7 cortical astroglial cultures infected with lentivirus expressing *Dlx2* and the GFP marker (LV-*Dlx2*-GFP) but not GFP alone (LV-GFP) produced GFP-positive neuronal cells immunopositive for βIII Tubulin (Tuj1-positive) after 7 d of differentiation (Fig. 1*A*,*B*). We investigated whether this "neurogenic competence" is maintained through serial passage *in vitro*. Cortical astroglia were serially passaged 5 times over 15 d, infected with LV-*Dlx2*-GFP or LV-GFP at passages 1, 2, 3, and 5, and neuronal differentiation was quantified 8 d after infection. While the efficiency of LV-*Dlx2*-GFP induced neuronal transdifferentiation was ~7% at passage 1 (Fig. 1*B*, Pass 1), the percentage of Tuj1-positive LV-*Dlx2*-GFP transduced cells was reduced 2.7-fold by passage 2 (Fig. 1*B*, Pass 2), becoming 8.5-fold reduced (0.8% efficiency) at passage 5 (Fig. 1*B*, Pass 5). Thus, with serial *in vitro* passage, cortical astroglia derived from P5–P7 mice progressively lose their neurogenic competence.

Ink4a/Arf deficiency enhances the neurogenic competence of postnatal astroglia

Ink4a/Arf is expressed in cortical astroglial cultures but not neurogenic neural precursors (Bachoo et al., 2002). To determine whether *Ink4a/Arf* is a barrier to neuronal transdifferentiation, we produced cultures from *Ink4a/Arf*-null mice and WT littermates. Interestingly, at passage 1, *Ink4a/Arf*-null astroglia exhibited low levels of neurogenesis even without enforced expression of *Dlx2* (Fig. 1*B*), possibly because of low levels of neural stem cell-like behavior in response to EGF signaling, as previously reported (Bachoo et al., 2002). However, by passage 3, very little of this neural stem cell-like behavior remained (Fig. 1*B*). In contrast, at passage 1, LV-*Dlx2*-GFP induced neuronal differentiation in 25.17% (SD = 1.14%, $n = 4$ independent experiments) of infected *Ink4a/Arf*-null cells (Fig. 1*B*, Pass 1), resulting in ~4-fold more neurons than in WT cultures. Furthermore, the neurogenic competence of *Ink4a/Arf*-null cells did not diminish with continued *in vitro* passage (Fig. 1*B*, Pass1–5); by passage 5, transdifferentiation of *Ink4a/Arf*-null astroglial cells was ~30-fold more efficient than WT cells (Fig. 1*B*). Thus, *Ink4a/Arf* deficiency both enhances and maintains the neurogenic competence of postnatal cortical astroglia.

In typical neural cell growth media, *Ink4a/Arf*-null astroglia grow faster than WT cultures, and cellular reprogramming, such as that of iPSCs, can be promoted by high rates of proliferation (Hanna et al., 2009). To address the possibility that the increased proliferation rate of *Ink4a/Arf*-null astroglia relates to their enhanced transdifferentiation potential, we reduced the rate of cell proliferation by omitting recombinant EGF and bFGF from the growth medium. *Ink4a/Arf*-null astroglia grown without exogenous EGF and bFGF incorporated the thymidine analog ethynyl



deoxyuridine (EdU) at a rate similar to WT astroglia cultured with EGF and bFGF (Fig. 1*C*); despite a nearly sixfold reduction in EdU incorporation, *Inka/Arf*-null astroglia cultured in growth factor-deficient conditions still exhibited 20-fold greater LV-*Dlx2*-GFP-induced neuronal transdifferentiation, compared with WT astroglia grown with EGF and bFGF (Fig. 1*D*). Thus, independent of the rate of cell proliferation, *Ink4a/Arf* deficiency promotes neuronal transdifferentiation in cortical astroglia.

Because parenchymal astrocytes can be regionally distinct (Zhang and Barres, 2010), we next asked whether *Ink4a/Arf*-null astroglia from other non-neurogenic brain regions also exhibit increased efficiencies of neuronal transdifferentiation. Whereas astroglia derived from the cerebellum and brainstem did not grow well under these culture conditions and therefore could not be evaluated, striatal astroglia did propagate efficiently. LV-*Dlx2*-GFP induced neuronal differentiation of astroglial cells in *Ink4a/Arf*-null striatal cultures with >5-fold greater efficiency than WT controls (Fig. 1*D*), even when cultured in media without EGF and bFGF, indicating that *Ink4a/Arf* deficiency enhances neuronal transdifferentiation in regionally distinct populations of astroglia.

GFAP-positive, *Ink4a/Arf*-null astroglia exhibit increased neurogenic competence

Although astroglial cultures from both *Ink4a/Arf*-null and WT cortex contained similar proportions of cells expressing Nestin, GLAST, OLIG2, and NG2 (Fig. 2*A*), *Ink4a/Arf*-null cultures contained ~20% fewer cells expressing GFAP, a marker of astrocyte identity. We therefore investigated whether *Ink4a/Arf* deficiency in GFAP-positive astroglia enhances neuronal transdifferentiation.

We restricted LV-*Dlx2*-GFP infection to cells expressing GFAP. EnvA pseudotyped lentiviruses (LV/EnvA) cannot infect mammalian cells unless they express the avian viral receptor gene, tv-a (Lewis et al., 2001). The Gtv-a mouse transgene expresses tv-a from the GFAP promoter, enabling LV/EnvA infection of

Figure 1. *Ink4a/Arf* deficiency enhances neuronal transdifferentiation. ***A***, Immunocytochemistry for GFP (green) and Tuj1 (red) of *Ink4a/Arf*-null and WT astroglia infected at passage 1 (Pass 1) with LV-*Dlx2*-GFP. ***B***, Transdifferentiation efficiencies of astroglia serially cultured from passage 1 (bright red) through 5 (light pink). ***C***, Quantification of EdU-positive cells in *Ink4a/Arf*-null and WT cortical (left) and striatal (right) astroglial cultures, with and without exogenous EGF and FGF. ***D***, Efficiencies of neuronal conversion of cells from ***C*** with and without exogenous EGF and FGF. Error bars indicate SEM. $^{**}p < 0.01$. $^{***}p < 0.001$. n.s., Not significant. Scale bars, 10 μm.

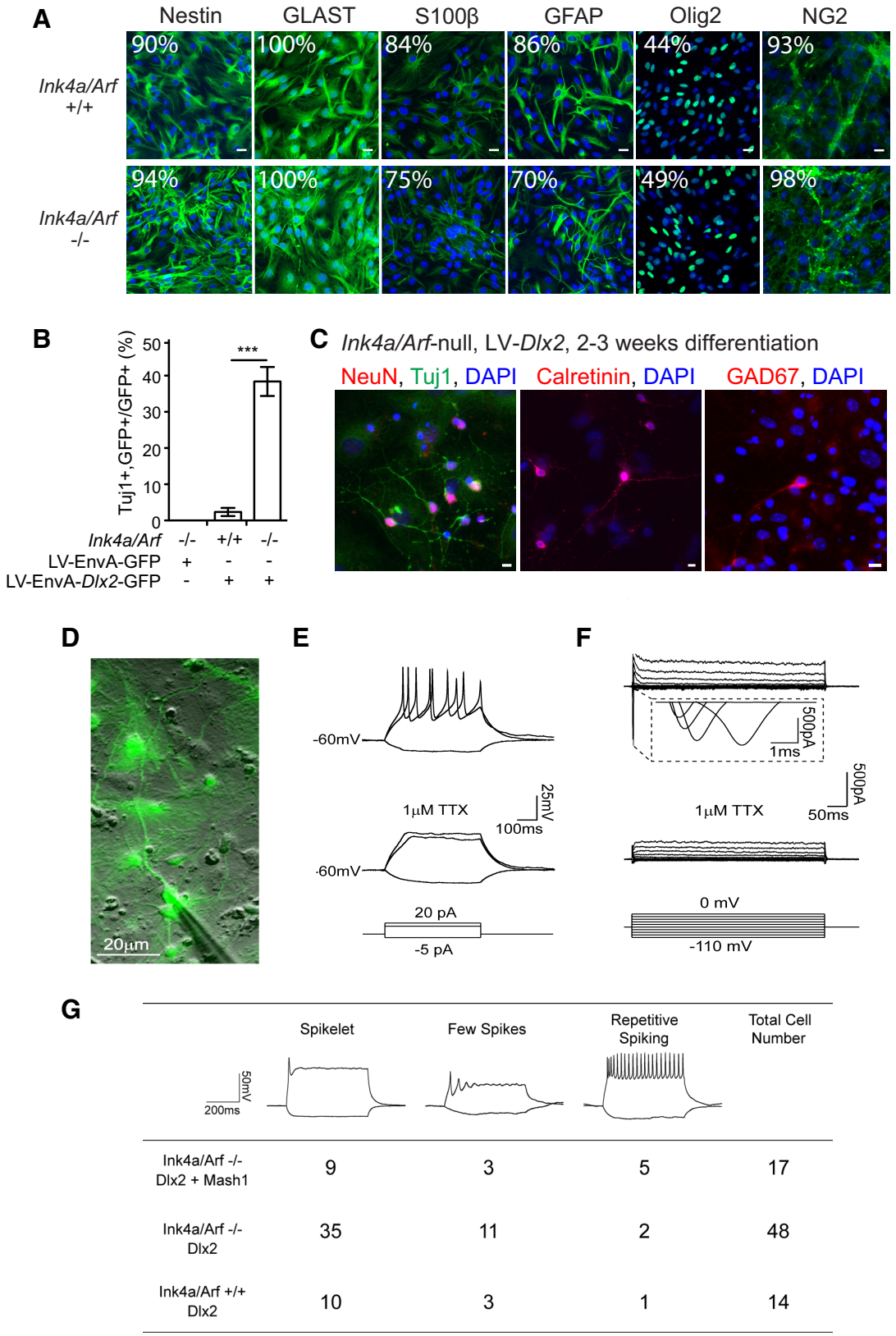
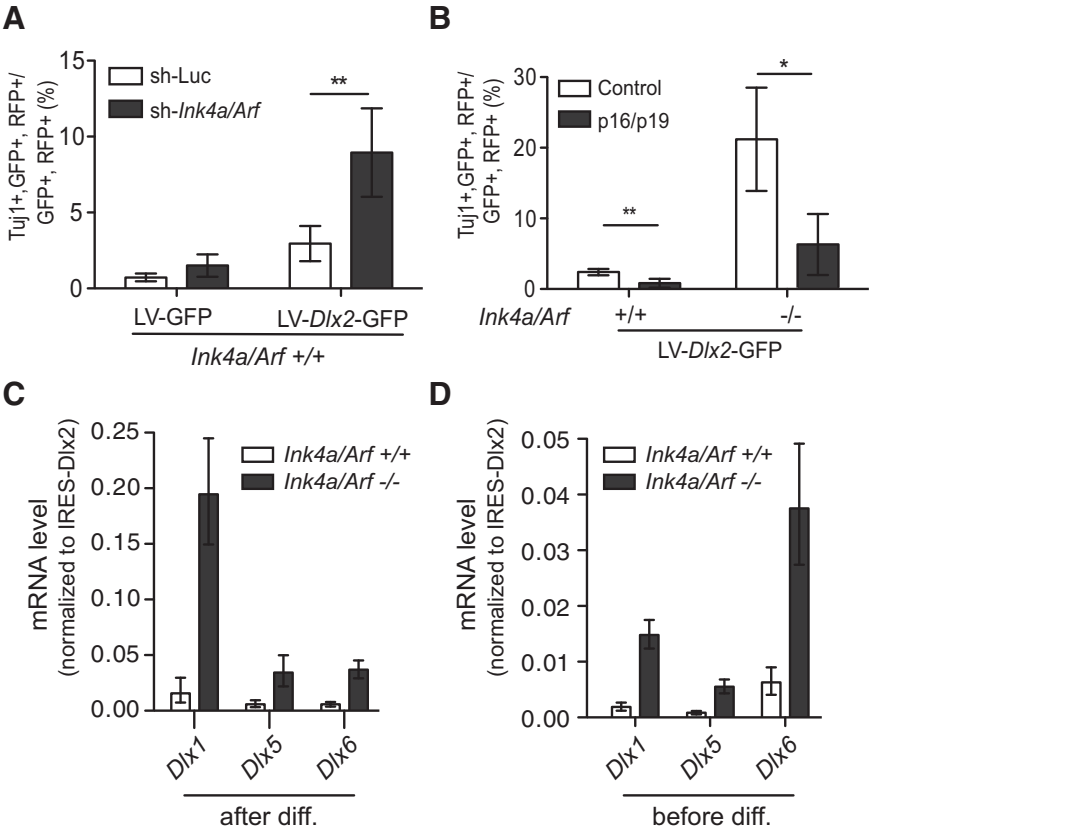


Figure 2. Transdifferentiated neuronal cells display markers of inhibitory interneuron fate and become electrophysiologically active. ***A***, Immunocytochemistry for Nestin, GLAST, S100β, Olig2, NG2, and GFAP (green) counterstained with DAPI (blue) of passage 1 cortical astroglia. Percentage of cells immunopositive for each marker is indicated (from 8 fields of view, 20×). Scale bars, 20 μm. ***B***, Transdifferentiation efficiencies of *Ink4a/Arf*-null and WT astroglia transduced with Env-A-pseudotyped virus expressing GFP alone or *Dlx2*-GFP. ***$p < 0.001$. ***C***, Immunocytochemistry for NeuN, calretinin, and GAD67 (red), Tuj1 (green), and DAPI (blue). Scale bars, 10 μm. ***D***, Merged differential interference contrast and fluorescence image showing an example of a recorded neuron. ***E***, Representative traces of *Ink4a/Arf* $^{-/-}$ neuron exhibiting repetitive action potentials (top) under stepped current injection (bottom) in current clamp. ***F***, Large inward Na^+ currents under stepped voltage (bottom) in voltage clamp (top). Both Na^+ currents and action potentials were fully abolished upon application of Na^+ channel blocker 1 μM TTX. ***G***, Summary of action potentials recorded from *Ink4a/Arf*-null and WT neurons derived by infection with *Dlx2* + *Mash1* or *Dlx2* alone.



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Gene ID	Gene Symbol	Description	Fold Change in Null	p-Value
11834	AQR	aquarius intron-binding spliceosomal factor	2.2723	1.495E-05
100042493	CCL21A	chemokine (C-C motif) ligand 21	2.1578	2.998E-04
11537	CFD	complement factor D (adipsin)	2.2943	2.489E-02
67529	FGFR1OP2	FGFR1 oncogene partner 2	2.1752	2.138E-05
66606	LRRC57	leucine rich repeat containing 57	2.7283	1.314E-05
67809	RMDN3	regulator of microtubule dynamics 3	2.0634	2.233E-06
100213	RUSC2	RUN and SH3 domain containing 2	2.2669	6.806E-06
16651	SSPN	sarcospan	2.1520	2.529E-04
21754	TESK1	testis specific protein kinase 1	2.6011	1.409E-05
12331	CAP1	CAP, adenylate cyclase-associated protein 1	-2.2045	1.916E-07
12450	CCNG1	cyclin G1	-2.4338	5.856E-05
12505	CD44	CD44 antigen	-2.1051	2.220E-08
12575	CDKN1A	cyclin-dependent kinase inhibitor 1A	-4.4689	1.762E-05
12578	CDKN2A	cyclin-dependent kinase inhibitor 2A	-3.4479	6.798E-12
333182	COX6B2	cytochrome c oxidase subunit VIb polypeptide 2	-2.4131	3.499E-05
67880	DCXR	dicarbonyl/L-xylulose reductase	-2.5716	2.829E-05
13849	EPHX1	epoxide hydrolase 1, microsomal	-2.4240	2.762E-07
14456	GAS6	growth arrest specific 6	-2.0353	1.079E-04
93695	GPNMB	glycoprotein (transmembrane) nmb	-2.0055	5.207E-05
29870	GTSE1	G-2 and S-phase expressed 1	-2.2249	2.334E-04
27280	PHLDA3	pleckstrin homology-like domain, family A, member 3	-3.6802	2.108E-06
217430	PQLC3	PQ loop repeat containing 3	-2.7262	4.491E-05
20280	SCP2	sterol carrier protein 2	-2.0279	6.908E-10
116914	SLC19A2	solute carrier family 19 (thiamine transporter), member 2	-2.0600	4.861E-05
68666	SVOP	SV2 related protein homolog	-3.4871	2.069E-04
211499	TMEM87A	transmembrane protein 87A	-2.2476	2.366E-05

Figure 3. Acute changes to *Ink4a/Arf* affect transdifferentiation efficiency. ***A***, Trandifferentiation efficiencies of *Ink4a/Arf* WT astroglia infected with lentivirus expressing RFP and an shRNA targeting *Ink4a/Arf* or control. Luc, Luciferase. ***B***, Transdifferentiation efficiencies of *Ink4a/Arf* WT or null glia infected with virus overexpressing RFP both $p16^{Ink4a}$ and $p19^{Arf}$ or control (alkaline phosphatase). ***C***, qRT-PCR expression analysis of *Dlx1*, *Dlx5*, and *Dlx6* expression after LV-*Dlx2*-GFP infection after differentiation. ***D***, qRT-PCR expression analysis of *Dlx1*, *Dlx5*, and *Dlx6* expression after LV-*Dlx2*-GFP infection before differentiation. *$p < 0.05$. **$p < 0.01$. Error bars indicate SD of triplicate reactions. ***E***, Summary of microarray expression data for genes calculated to be greater than twofold different between *Ink4a/Arf* WT or null glial cultures.

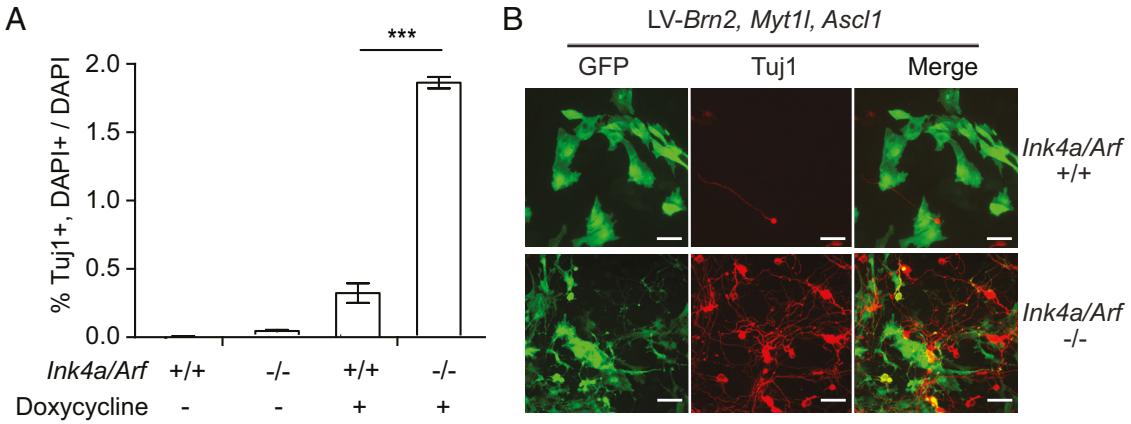

Figure 4. *Ink4a/Arf* is a barrier to fibroblast neuronal reprogramming. ***A***, Transdifferentiation efficiencies from *Ink4a/Arf*-null and WT MEFs infected with lentivirus expressing *Brn2*, *Myt1l*, and *Ascl1* with or without induction by doxycycline. ***B***, Immunocytochemistry for Tuj1 and GFP from MEF cultures 7 d after infection (***B***). ***$p < 0.001$. Scale bars, 40 μm.

GFAP-positive astroglia (Holland et al., 1998). When infected with LV/EnvA-*Dlx2*-GFP, Gtv-a;*Ink4a/Arf*-null astroglial cultures exhibited >10-fold higher neuronal differentiation efficiency compared with Gtv-a;*Ink4a/Arf*$^{+/+}$ control cultures (Fig. 2*B*). When infected with the LV/EnvA-GFP control virus, Gtv-a; *Ink4a/Arf*-null cells did not give rise to neurons, indicating that the low levels of neurogenesis observed in *Ink4a/Arf*-null cortical cultures without enforced *Dlx2* expression (Fig. 1*B*) likely originated from a GFAP-negative cell population. Together, these data indicate that *Ink4a/Arf* deficiency facilitates neuronal transdifferentiation from GFAP-positive astroglia.

Neurons induced from *Ink4a/Arf*-deficient cells fire action potentials

Neuronal cells induced from *Ink4a/Arf*-null cells by LV-*Dlx2*-GFP were immunopositive for neuronal antigen NeuN (399 of 857 Tuj1$^+$ cells; 46.6%) as well as calretinin (40 of 169 Tuj1$^+$ cells; 23.7%) and GAD67 (45 of 169 Tuj1$^+$ cells; 26.6%) (Fig. 2*C*). To further characterize their neuronal identity, we used electrophysiological analysis (Fig. 2*D–G*). As in WT astroglia, *Dlx2* alone was sufficient to generate neurons in *Ink4a/Arf*-null cultures that were able to fire action potentials; however, after 2–3 weeks of differentiation, a large proportion of transdifferentiated cells exhibited immature action potential firing patterns (few spikes, Fig. 2*E*). In WT postnatal astroglia, coexpression of proneural *Mash1 (Ascl1)* with *Dlx2* promotes further neuronal maturation (Heinrich et al., 2010). We therefore coexpressed *Mash1* and *Dlx2* from lentiviral vectors in *Ink4a/Arf*-null cells and found that GFP-positive neuronal cells exhibited repetitive action potential firing patterns under current clamp, as well as large inward Na^+ currents under voltage clamp, and both were sensitive to the Na^+ channel blocker TTX (Fig. 2*E*,*F*). Indeed, *Ink4a/Arf*-null astroglial cultures transduced with both *Mash1* and *Dlx2* lentiviral vectors produced a greater proportion of neuronal cells with repetitive spiking than *Dlx2* alone (Fig. 2*G*).

Acute changes to *Ink4a/Arf* expression regulate neurogenic competence

We next investigated whether acute inhibition of *Ink4a/Arf* expression can increase astroglial neurogenic competence. Lentiviruses encoding shRNAs targeting both *Ink4a* and *Arf* (LV-sh-*Ink4a/Arf*-mCherry) reduced their transcript levels in passage 1 cortical astroglial cells by >80% after 2 d relative to control LV-sh-luciferase-mCherry; these cultures were next infected with LV-*Dlx2*-GFP or LV-GFP, and the cellular phenotype of double-infected cells (mCherry/GFP-positive) was quantified after differentiation. Cells transduced with LV-sh-*Ink4a/Arf*-mCherry exhibited ~3-fold greater neuronal differentiation compared with LV-sh-luciferase-mCherry control (Fig. 3*A*).

To determine whether enforced expression of *Ink4a* and *Arf* would conversely inhibit neuronal transdifferentiation in *Ink4a/Arf*-null astroglia, we infected *Ink4a/Arf*-null and WT passage 1 cortical astroglial cultures with lentivirus encoding $p16^{Ink4a}$ and $p19^{Arf}$ cDNAs (LV-*Ink4a-P2A-Arf*-mCherry) or LV-mCherry control vectors and assessed the efficiency of neuronal transdifferentiation induced by LV-*Dlx2*-GFP. Reexpression of *Ink4a/Arf* reduced the number of Tuj1-positive neuronal cells by ~2.5-fold in both *Ink4a/Arf*-null and WT cells (Fig. 3*B*). Thus, acute changes to *Ink4a* and *Arf* transcript levels can rapidly alter the neurogenic competence of astroglia.

Ink4a/Arf-null glia cultures more efficiently activate Dlx2-dependent transcription

To begin to investigate why *Ink4a/Arf* deficiency enhances the neurogenic competence of postnatal astroglia, we compared the *Ink4a/Arf*-null transcriptome to that of WT controls. Surprisingly, in *Ink4a/Arf*-null cultures, only 27 genes were differentially expressed by >2-fold (Fig. 3*E*).

In normal development, DLX2 upregulates expression of both the *Dlx1/2* and *Dlx5/6* bigene clusters, which are key regulators of interneuron development (Panganiban and Rubenstein, 2002). To determine whether *Dlx2* overexpression can activate the transcription of these DLX2 target genes, we analyzed gene expression during LV-*Dlx2*-GFP-induced neuronal transdifferentiation. Three days after differentiation with LV-*Dlx2*-GFP, *Ink4a/Arf*-null cultures exhibited >10-fold higher levels of *Dlx1*, *Dlx5*, and *Dlx6* (Fig. 3*C*). To analyze gene expression related to the enforced expression of *Dlx2* rather than the resultant neuronal differentiation, we analyzed cultures before the onset of neurogenesis. Even before the emergence of Tuj1-positive cells, LV-*Dlx2*-GFP induced nearly sixfold higher levels of *Dlx1* and *Dlx5/6* expression in *Ink4a/Arf*-null cultures (Fig. 3*D*). Thus, *Ink4a/Arf*-deficient astroglia are more competent to upregulate DLX2 downstream targets.

Ink4a/Arf is a barrier to direct neuronal reprogramming of fibroblasts

To further explore whether the *Ink4a/Arf* locus is a barrier to direct neuronal transdifferentiation, we investigated direct neuronal transdifferentiation of non-neural cells. MEFs can be converted into neurons via overexpression of the transcription factors *Brn2*, *Ascl1*, and *Myt1l* (Vierbuchen et al., 2010). We infected MEFs from *Ink4a/Arf*-null and WT littermates with lentiviruses expressing the reverse tetracycline transactivator, and a pool of lentiviruses expressing *Brn2*, *Ascl1*, and *Myt1l* under the control of the tetracycline response element. As expected, 19 d after transcription factor induction with doxycycline, we observed Tuj1-positive cells with neuronal morphologies in WT MEF cultures; from *Ink4a/Arf*-null MEF cultures, neuronal transdifferentiation was nearly sixfold greater (Fig. 4*A*,*B*). Thus, *Ink4a/Arf* is a barrier to neuronal transdifferentiation in both neural and non-neural cell types.

Discussion

A major challenge to the direct conversion of one mature cell type into another is that the identity of differentiated cells is generally very stable and difficult to alter (Zhou and Melton, 2008; Vierbuchen and Wernig, 2012). How cells achieve and maintain such stable, mature phenotypes is poorly understood. We show that *Ink4a/Arf*-deficient cells exhibit greatly increased competence for direct neuronal transdifferentiation. In addition to enabling greater efficiencies of neuronal conversion for the advancement of cell reprogramming technologies, our findings suggest that *Ink4a/Arf* is a genetic requirement for the proper stabilization of mature astrocyte identity.

Astrocytes arise from radial glia, the primary multipotent embryonic neural stem cell population (Kriegstein and Alvarez-Buylla, 2009). In the postnatal mouse cortex, astrocytes proliferate through symmetric division (Ge et al., 2012). As others (Palmer et al., 1999; Laywell et al., 2000; Heins et al., 2002; Berninger et al., 2007; Heinrich et al., 2010) and we have shown here, early postnatal cortical astroglia still exhibit developmental plasticity. However, astroglia isolated from later postnatal ages have greatly diminished neurogenic competence (Laywell et al., 2000; Heinrich et al., 2011). Interestingly, cortical astroglia became increasingly resistant to neuronal conversion through serial passage, suggesting that this lineage restriction is progressive in nature and related to their proliferation. In contrast, the neurogenic competence of *Ink4a/Arf*-null astroglia did not diminish with serial passage, indicating that *Ink4a/Arf* is required for the observed restriction to astrocyte identity.

Using *Dlx2*, one of the major transcription factors that controls inhibitory interneuron fate, we were able to generate neurons that display markers of an inhibitory interneuron identity. It is feasible that the use of other lineage-specific transcription factors alone or in combination may yield other neuronal subtypes. Additionally, astroglial heterogeneity related to location or embryonic origin may play a role in the responsiveness to these factors, as transdifferentiation efficiency of striatal astrocytes appeared to be ~60% higher than those derived from the cortex.

Ink4a/Arf-null astrocytes are immortal (Bachoo et al., 2002), and this nonsenescent proliferation may also relate to their persistent neurogenic competence. *Ink4a/Arf* is a key mediator of senescence (Collado et al., 2007), and *Ink4a/Arf* as well as other senescence mediators are barriers to iPSC production (Banito et al., 2009; Li et al., 2009; Utikal et al., 2009). Reintroduction of $p16^{Ink4a}$ and $p19^{Arf}$ transcripts into *Ink4a/Arf*-null cells restores replicative senescence; and, indeed, astroglial neurogenic competence was inhibited by lentiviral expression of *Ink4a/Arf* transcripts. Although WT cortical astroglia became refractory to proliferation after Passage 5, we did not observe any classical markers of senescence (e.g., β-galactosidase activity, heterochromatin foci) in WT astrocyte cultures before this point (data not shown). Thus, we suggest that proliferation toward *Ink4a/Arf*-dependent senescence limits the neurogenic competence of astroglia. Interestingly, astroglia in the adult subventricular zone repress *Ink4a/Arf* via Polycomb-dependent mechanisms (Molofsky et al., 2003), and these subventricular zone astrocytes maintain neurogenic competence, generating large numbers of neurons throughout life (Kriegstein and Alvarez-Buylla, 2009).

Overall, our results indicate that inactivation of *Ink4a/Arf* greatly improves transcription factor-mediated neuronal transdifferentiation. This finding not only has important implications for the advancement of direct cell conversion methodologies, our data also implicate *Ink4a/Arf* as a key genetic locus in the normal developmental lineage-restriction of astroglial cells in non-neurogenic brain regions.

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