Cell Reports
Article

FOXO3 Shares Common Targets with ASCL1 Genome-wide and Inhibits ASCL1-Dependent Neurogenesis

Ashley E. Webb,[1] Elizabeth A. Pollina,[1,2] Thomas Vierbuchen,[2,3,8,10] Noelia Urbán,[7,8] Duygu Ucar,[1,8] Dena S. Leeman,[1,2,8] Ben Martynoga,[7] Madhavi Sewak,[4] Thomas A. Rando,[5,6] François Guillemot,[7,9] Marius Wernig,[2,3,9] and Anne Brunet[1,2,6,*]
[1]Department of Genetics
[2]Cancer Biology Program
[3]Department of Pathology, Institute for Stem Cell Biology and Regenerative Medicine
[4]Computer Science Program
[5]Department of Neurology and Neurological Sciences
[6]Paul F. Glenn Laboratories for the Biology of Aging
Stanford University, Stanford, CA 94305, USA
[7]Division of Molecular Neurobiology, MRC-National Institute for Medical Research, London NW7 1AA, UK
[8]These authors contributed equally to this work
[9]These authors contributed equally to this work
[10]Present address: Department of Neurobiology, Harvard Medical School, Boston, MA 02115, USA
*Correspondence: abrunet1@stanford.edu
http://dx.doi.org/10.1016/j.celrep.2013.06.035

SUMMARY

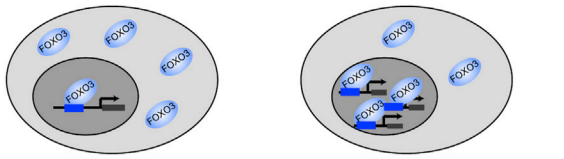
FOXO transcription factors are central regulators of longevity from worms to humans. FOXO3, the FOXO isoform associated with exceptional human longevity, preserves adult neural stem cell pools. Here, we identify FOXO3 direct targets genome-wide in primary cultures of adult neural progenitor cells (NPCs). Interestingly, FOXO3-bound sites are enriched for motifs for bHLH transcription factors, and FOXO3 shares common targets with the pro-neuronal bHLH transcription factor ASCL1/MASH1 in NPCs. Analysis of the chromatin landscape reveals that FOXO3 and ASCL1 are particularly enriched at the enhancers of genes involved in neurogenic pathways. Intriguingly, FOXO3 inhibits ASCL1-dependent neurogenesis in NPCs and direct neuronal conversion in fibroblasts. FOXO3 also restrains neurogenesis in vivo. Our study identifies a genome-wide interaction between the prolongevity transcription factor FOXO3 and the cell-fate determinant ASCL1 and raises the possibility that FOXO3's ability to restrain ASCL1-dependent neurogenesis may help preserve the neural stem cell pool.

INTRODUCTION

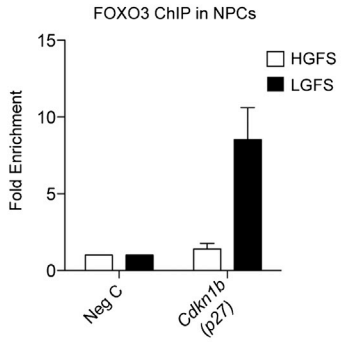
FOXO transcription factors promote longevity downstream of the insulin/IGF-1 signaling pathway from worms to mammals (Kenyon, 2010). When the insulin/insulin-like growth factor (IGF) 1 signaling pathway is inactive, FOXO transcription factors translocate to the nucleus, where they act mostly as transcriptional activators (Calnan and Brunet, 2008). Overexpression of FOXO transcription factors extends lifespan in invertebrates (Giannakou et al., 2004; Henderson and Johnson, 2001; Hwangbo et al., 2004). In humans, FOXO3, one of the four FOXO family members, has been associated with exceptional lifespan in eight cohorts of centenarians (Kenyon, 2010). Interestingly, FOXO3 has recently been found to regulate adult neural stem cells (NSCs) and hematopoietic stem cells (HSCs). FOXO3 is localized in the nucleus of NSCs and HSCs in vivo (Miyamoto et al., 2007; Paik et al., 2009; Renault et al., 2009), suggesting that FOXO3 is active in these cells. Deletion of the *Foxo3* gene in mice leads to the premature depletion of NSCs and HSCs in adult animals (Miyamoto et al., 2007; Paik et al., 2009; Renault et al., 2009; Tothova et al., 2007). Combined deletion of *Foxo1*, *Foxo3*, and *Foxo4* leads to a more pronounced depletion of NSCs and HSCs in vivo (Paik et al., 2009; Tothova et al., 2007), indicating that FOXO transcription factors have overlapping roles in maintaining adult stem cell pools in vivo. The ability of FOXO transcription factors to regulate adult stem cell homeostasis is likely to be crucial for tissue regeneration, and potentially organismal longevity.

Although the importance of FOXO3 in stem cell maintenance is well known, the role of FOXO3 in the differentiation of stem and progenitor cells has been less explored. In vivo, the loss of FOXO transcription factors causes a premature burst in neurogenesis during development (Paik et al., 2009), raising the possibility that FOXO factors inhibit neuronal differentiation. However, the importance of FOXO3 in the neuronal differentiation of neural stem and progenitor cells and the specific FOXO3 targets that may affect this neuronal differentiation have not been

A



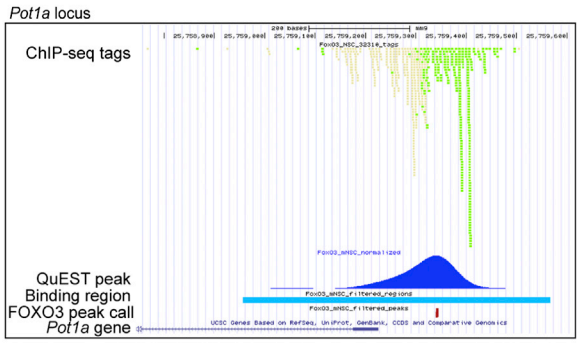
B



C

Library	Solexa Reads	QuEST Peaks	RefSeq Genes	Unique Gene Symbols	FDR
FOXO3 ChIP HGFS	5,016,116	8	2	2	<0.001
FOXO3 ChIP LGFS	12,086,257	4329	3763	2291	<0.001
No ChIP	19,984,248	0	0	0	N/A

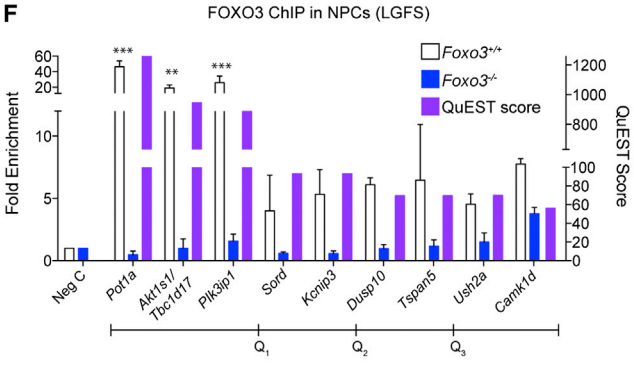
D



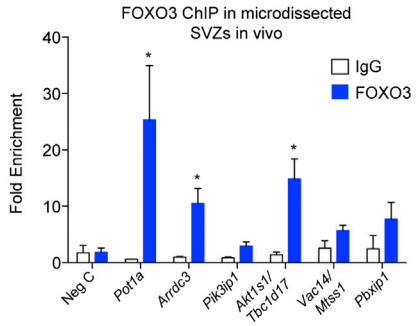
E

Symbol	Gene Name	QuEST Score	ChIP-qPCR Enrichment
Pot1a	protection of telomeres 1a	1258.58	45.2
Tbc1d17	TBC1 domain family, member 17	945.786	17.8
Akt1s1	AKT1 substrate 1	945.786	17.8
Pik3ip1	PI-3-kinase interacting protein 1	886.674	7.4
Arrdc3	arrestin domain containing 3	876.822	12.4
Pbxip1	pre-B cell leukemia TF int. prot. 1	662.543	13.9
Mtss1l	metastasis suppressor 1-like	615.746	7.5
Vac14	Vac14 homolog	615.746	7.5
Dgkg	diacylglycerol kinase gamma	613.283	ND
2610017I09Rik	non-coding RNA	608.357	ND

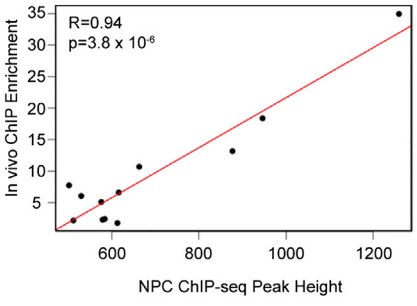
F



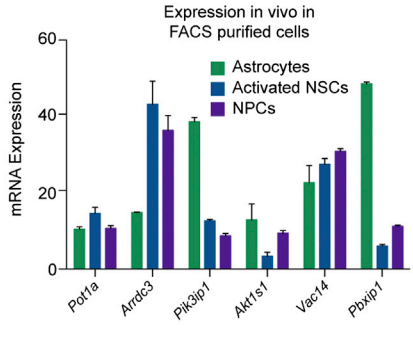
G



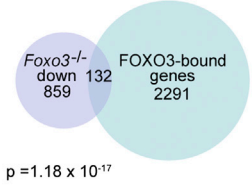
H



I



J



(legend on next page)

established. Given the importance of neural stem and progenitor cells for the generation of new neurons, and their potential use for regenerative medicine, understanding the mechanisms by which FOXO3 acts in cells from the neural lineage should provide important insight into the regulation of these cells and their ability to produce new neurons.

FOXO3 is well known to regulate gene expression programs that coordinate stress resistance, cell-cycle progression, autophagy, and apoptosis (Salih and Brunet, 2008), but the ensemble of direct target genes of FOXO3 are still largely unknown. Although recent studies have characterized FOXO1 and FOXO3 targets in a few differentiated lineages (Lin et al., 2010; Litvak et al., 2012; Ouyang et al., 2012; Shin et al., 2012), the direct FOXO3 targets have never been characterized in a stem/progenitor context. Furthermore, the mode of action of FOXO transcription factors in specific lineages is largely unknown. Finally, whether FOXO3 shares targets with other transcription factors genome-wide has never been determined in stem/progenitor cells.

Here, we use primary cultures of adult neural progenitor cells (NPCs) as a cellular system to understand FOXO3's mechanisms of action in the neural lineage. We find that FOXO3 can bind about 2,000 direct targets genome-wide in these cells. FOXO3-bound genes extensively overlap with those bound by the proneuronal bHLH transcription factor ASCL1/MASH1 in cultured NPCs. Genome-wide analysis of the chromatin landscape in these cells reveals that FOXO3 and ASCL1 are enriched at enhancer regions. Interestingly, FOXO3 represses the expression of a subset of ASCL1 neurogenic targets and restrains neurogenesis in vitro and in vivo. FOXO3 also inhibits the ability of ASCL1 to directly reprogram fibroblasts into induced neurons (iNs). These findings suggest that FOXO3 can act by repressing the transcriptional activity of a "master regulator" of cell fate. Thus, FOXO3 may help preserve an intact pool of neural stem cells at least in part by negatively regulating neuronal differentiation.

RESULTS

Genome-wide Identification of FOXO3 Binding Sites in Adult Neural Progenitor Cells

To identify FOXO3 binding sites genome-wide in a stem/progenitor context, we used primary cultures of adult NPCs. We chose these cells because they can differentiate into neurons, astrocytes, and oligodendrocytes (albeit with a bias toward astrocytes) (Pastrana et al., 2011), and they can be expanded in culture to provide cell numbers (10–100 million) that are compatible with chromatin immunoprecipitation followed by next generation sequencing (chromatin immunoprecipitation sequencing [ChIP-seq]). We performed FOXO3 ChIP-seq in these primary cultures of NPCs before and after the induction of FOXO3 nuclear localization (Figure 1A), as FOXO3 is mostly localized in the nucleus of NSCs in vivo (Figure S1A) (Paik et al., 2009; Renault et al., 2009). To induce FOXO3 nuclear localization, we transiently incubated NPCs in conditions of low growth factor signaling (Experimental Procedures). These conditions have been used previously for FOXO3 ChIP-qPCR in cultured NPCs (Renault et al., 2009) and other cellular systems (van der Vos et al., 2012). Using immunofluorescence, we verified that a tagged version of FOXO3 indeed accumulates in the nucleus of NPCs in low growth factor signaling conditions (Figures S2A and S2B). Following translocation to the nucleus, endogenous FOXO3 is indeed recruited to *Cdkn1b* (*p27*), a well-known FOXO target gene, in cultured NPCs (Figure 1B). Under these experimental conditions, greater than 90% of the NPCs still express the neural progenitor markers NESTIN and SOX2 (Figures S1B–S1F) and retain the ability to differentiate into neurons (Figure S1G). Thus, primary cultures of adult NPCs in low growth

Figure 1. Genome-wide Identification of FOXO3 Binding Sites in Adult NPCs

(A) Low-passage adult NPCs were cultured in high growth factor signaling (HGFS) conditions (FOXO3 mostly cytoplasmic) or were transiently cultured in low growth factor signaling (LGFS) conditions to induce FOXO3 nuclear accumulation.

(B) ChIP-qPCR analysis of FOXO3 binding to the known target gene *Cdkn1b (p27)* in NPCs in high growth factor signaling (HGFS) versus low growth factor signaling (LGFS) conditions. Neg C, negative control (genomic primers that amplify a region not bound by FOXO3). Mean ± SD of triplicates of one representative experiment of two independent experiments. Enrichment is calculated relative to the negative control region of the genome.

(C) Numbers of unique Solexa reads, QuEST peaks, corresponding RefSeq genes, unique gene symbols, and QuEST false discovery rates (FDRs) for FOXO3 ChIP from adult NPCs. N/A, not applicable; HGFS, high growth factor signaling; LGFS, low growth factor signaling.

(D) UCSC Genome Browser shot showing FOXO3 binding at the *Pot1a* locus. Forward and reverse ChIP-seq sequence tags are shown in yellow and green, respectively. Normalized QuEST peaks (blue), binding region (light blue), and binding site (red) are shown below the sequence tags.

(E) List of the ten FOXO3-bound genes in adult NPCs with the highest QuEST scores. Enrichment values by ChIP-qPCR are indicated on the right.

(F) FOXO3 binding sites identified by ChIP-seq are specific FOXO3 binding sites in NPCs. ChIP-qPCR in NPCs from adult $Foxo3^{+/+}$ and $Foxo3^{-/-}$ littermates in low growth factor signaling (LGFS) conditions. QuEST scores are indicated in purple. Based on QuEST enrichment, three FOXO3 targets from the top ChIP-seq quartile and two targets from each of the bottom quartiles are shown. Mean ± SD of two independent experiments. Enrichment is calculated relative to a negative control region of the genome. ***$p < 0.001$, **$p < 0.01$, Two-way ANOVA, Bonferroni correction. Neg C, negative control.

(G) FOXO3 ChIP-qPCR in adult neurogenic niches (subventricular zones [SVZs]) that were microdissected from 3- to 6-month-old mice. Mean ± SD of two independent experiments. Enrichment is calculated relative to input. *$p < 0.05$, Two-way ANOVA, Bonferroni correction. Neg C, negative control. Additional genes are presented in Figure S2C.

(H) Regression analysis comparing FOXO3 ChIP enrichments in vivo in the SVZ versus ChIP-seq peak height in cultured NPCs. Pearson correlation, $R = 0.94$, p value = 3.8×10^{-6}.

(I) Expression of top FOXO3-bound genes in vivo in FACS-purified astrocytes, activated NSCs, and NPCs freshly isolated from the adult brain. Mean ± SD of technical replicates of one representative experiment of two independent experiments. Validation of the FACS strategy and additional genes are shown in Figures S2D–S2F.

(J) Overlap between FOXO3-bound genes and genes that are downregulated in $Foxo3^{-/-}$ NPCs by microarray analysis (Fisher's exact test, $p = 1.18 \times 10^{-17}$). See also Figures S1, S2, and S3 and Tables S1, S2, and S3.

factor signaling conditions provide a cellular system to identify FOXO3 direct targets in a stem/progenitor context.

To characterize the ensemble of FOXO3 direct targets in NPCs, we performed FOXO3 ChIP-seq. FOXO3 binding sites were identified using QuEST (Valouev et al., 2008) with a stringent false discovery rate threshold (FDR < 0.001) (Figure 1C). Prior to induction of nuclear translocation, FOXO3 was, as expected, bound to very few genomic regions (eight) (Figure 1C). In contrast, following nuclear translocation, FOXO3 was bound to 4,329 genomic regions (QuEST peaks), 2,291 of which were within 5 kb of unique RefSeq genes (Figure 1C). An example of FOXO3 binding is shown in Figure 1D. The ten FOXO3-bound genes with the highest QuEST binding scores are listed in Figure 1E (complete data sets are in Tables S1 and S2). We verified that genes bound by FOXO3 in ChIP-seq experiments were also bound by ChIP-qPCR in wild-type NPCs, but not in *Foxo3*$^{-/-}$ NPCs (Figure 1F). Thus, FOXO3 binds to approximately 2,000 genes in cultured NPCs under low growth factor signaling conditions.

We next asked if FOXO3 also binds these target genes in vivo. We performed FOXO3 ChIP-qPCR on microdissected neurogenic niches (subventricular zones [SVZs]), which are enriched for neural stem and progenitor cells in the adult mouse. We found that endogenous FOXO3 was bound to several of its targets (*Pot1a*, *Arrdc3*, *Akt1s1/Tbc1d17*, *Txnip*, *Hbp1*, and *Vkorc1*) in neurogenic niches (Figures 1G and S2C; Table S1). Of the genes tested (12), half validated in vivo and are likely to represent bona fide FOXO3 targets. A regression analysis showed that FOXO3 binding enrichments in vitro and in vivo were significantly correlated (Figure 1H, Pearson correlation, R = 0.94, p = 3.8 × 10^{-06}), indicating that genes that have the highest enrichment for FOXO3 in culture are more likely to be bound by FOXO3 in vivo. Importantly, the FOXO3-bound genes that validated in vivo were indeed expressed in activated NSCs and NPCs freshly purified from the adult brain using a fluorescence-activated cell sorting (FACS) strategy (Pastrana et al., 2009) (Figures 1I and S2D–S2F).

FOXO3-bound genes in cultured NPCs significantly overlapped with genes that are downregulated in *Foxo3*$^{-/-}$ NPCs (Figure 1J, Fisher's exact test, p = 1.18 × 10^{-17}). This observation, coupled with the fact that FOXO3 is known to act as a transcriptional activator (Calnan and Brunet, 2008; Salih and Brunet, 2008), provides further support for the relevance of the FOXO3 ChIP-seq data set. FOXO3 could also act as a transcriptional repressor at some specific targets, although this was not statistically significant (Figure S2G). Interestingly, FOXO3 binding sites in NPCs significantly overlapped with FOXO1 and FOXO3 binding sites in other cell types (B cells, T cells, macrophages) (Figures S3A–S3D; Table S3), indicating that there exists a "core set" of FOXO targets as well as lineage/cell-specific FOXO targets (Figure S3D; Table S3). Collectively, these results are consistent with the notion that the FOXO3 target genes identified in cultured NPCs have physiological relevance.

In Silico Motif Analysis for FOXO3 Binding Reveals FOXO and bHLH Binding Sites

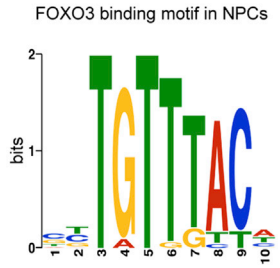
We asked whether FOXO3 co-occurs with other transcription factors genome-wide. De novo motif analysis of FOXO3 ChIP-seq data sets using the MEME motif finder (Bailey and Elkan, 1994) revealed that 90% of FOXO3-bound sites contained the FOXO consensus motif TGTTTAC (E value = 1.5 × 10^{-3175}) (Figure 2A) (Furuyama et al., 2000), confirming that FOXO3 binding sites in NPCs are bona fide recruitment sites for this transcription factor. Interestingly, analysis of the 200 bp regions surrounding the FOXO3 ChIP-seq peaks revealed two other motifs that co-occur with FOXO3 binding sites: a CAGCTG motif which co-occurs at more than 30% of the binding sites (E value = 2.5 × 10^{-406}) (Figure 2B) and a CAGGCTG motif that co-occurs at about 30% of the binding sites (E value = 3.1 × 10^{-273}) (data not shown). Analysis by STAMP (Mahony and Benos, 2007) revealed that the CAGCTG motif is a specific subclass of E-box consensus sites (Powell and Jarman, 2008). These E boxes are known to be bound by bHLH transcription factors that have been called "master regulators" of cell identity, including ASCL1/MASH1, NEUROD1, NGN1, and MYOD (Figure 2C) (Guillemot, 1999; Hu et al., 2004). Such transcription factors are sufficient to promote cell differentiation and even direct reprogramming of a differentiated cell type into another (Vierbuchen and Wernig, 2011).

We focused on the bHLH transcription factor ASCL1 because of its well-characterized function in neurogenesis (Guillemot et al., 1993; Parras et al., 2004; Vierbuchen et al., 2010) and its expression in adult NSCs/NPCs (Pastrana et al., 2009). In line with previous reports, we found that the ASCL1 protein is expressed in the nucleus of a subset of neural stem and progenitor cells in the adult brain (Figure S1A) and adult NPCs in culture (Figure 2D). *Ascl1* mRNA is highly expressed in activated NSCs and NPCs freshly purified from the brain, whereas other bHLH transcription factor mRNAs (*Neurod1*, *Neurog1*, and *Myod1*) are not expressed at detectable levels in activated NSCs and NPCs (Figure 2E). Conversely, other transcription factors whose mRNA is expressed in NPCs (*Hes1*, *E2a/Tcf3*, *Olig2*, and *Sox2*) (Figure 2E) have different binding motifs and/or are not known to be involved in neurogenesis (Figure 2C). These observations raise the possibility that ASCL1 may be the bHLH transcription factor that functionally interacts with FOXO3 in NPCs.

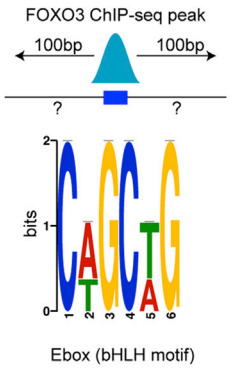
FOXO3 and ASCL1 Share Targets Genome-wide in Adult NPCs

To determine if FOXO3 and ASCL1 bind similar genes genome-wide, we performed ASCL1 ChIP-seq in NPCs (Figure 3). ASCL1 ChIP-seq revealed ~18,000 ASCL1 binding sites in NPCs, corresponding to ~6,000 unique genes (Figure 3A; Tables S4 and S5). De novo MEME motif analysis identified the presence of the ASCL1 consensus motif CAGCTG at 91.8% of the ASCL1-bound regions (E value = 2.4 × 10^{-17208}) (Figure 3B). The top ten ASCL1-bound genes are included in Figure 3C (a complete list of these genes and uploadable UCSC Genome Browser tracks are available in Tables S4 and S5). ChIP-qPCR experiments confirmed that ASCL1 is recruited to these genomic loci in NPCs (Figure 3D) and that ASCL1 is also bound to these sites in vivo in microdissected neurogenic niches (SVZs) (Figure 3E). ASCL1 binding in primary cultures of adult NPCs significantly overlaps with ASCL1 binding in NS5 cells (neural progenitor cells derived from embryonic stem cells) as well as in the ventral telencephalon in vivo (Castro et al., 2011) (Figure S3E; Table S3).

A



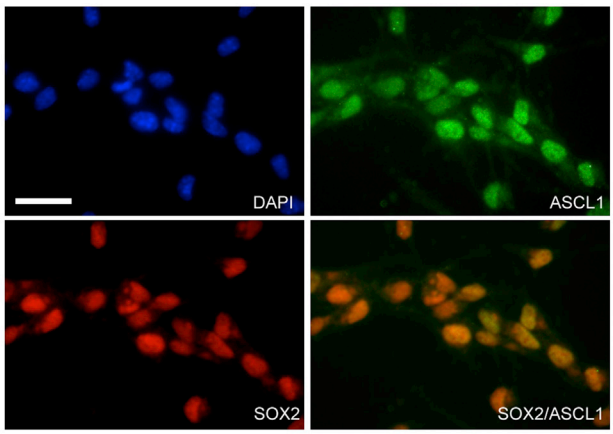
B



C

	Transcription factor	Binding Motif	Expression in NPCs	Function in NPCs
bHLH	ASCL1	CAGCTG	+	neurogenesis, NPC proliferation
bHLH	NEUROD1	CAGCTG	-	neurogenesis
bHLH	NEUROG1	CAGCTG	-	neurogenesis
bHLH	MYOD1	CAGCTG	-	none
bHLH	HES1	CACNAG	+	NSC/NPC maintenance
bHLH	E2A/TCF3	CAGCTG	+	unknown
bHLH	OLIG2	CAG/TCTG	+	glial differentiation
	SOX2	ACAAAGG	+	NSC/NPC maintenance

D



E

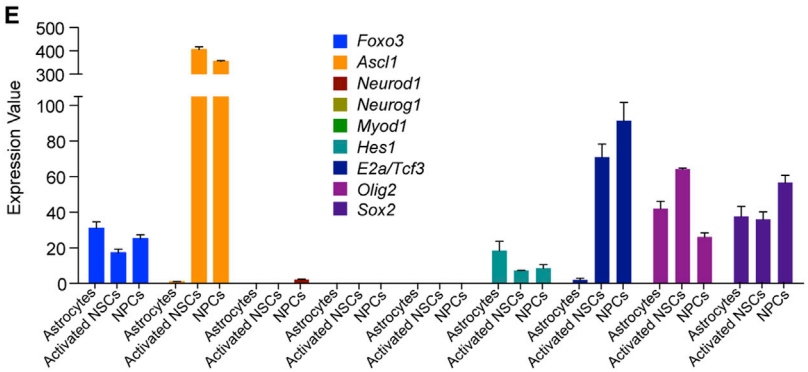


Figure 2. FOXO3 Binding Sites Are Enriched for a bHLH Transcription Factor Motif

(A) MEME motif analysis of the 100 bp regions centered around the 4,329 FOXO3 binding sites reveals a FOXO consensus motif.

(B) MEME de novo motif analysis of the 200 bp region surrounding FOXO3 ChIP-seq QuEST peaks reveals an E-box consensus motif, which is known to be bound by bHLH transcription factors.

(C) Table of transcription factors with their consensus motif, expression, and known function in NPCs.

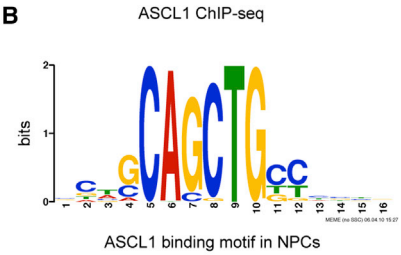
(D) ASCL1 expression in cultured NPCs. ASCL1 (green) is expressed in >80% cultured adult NPCs, with extensive overlap with SOX2 (red). ASCL1/SOX2 merge is shown in the bottom right. DAPI (blue) is shown in the upper left. Scale bar represents 25 μm.

(E) Expression of the genes encoding FOXO3, ASCL1, and other transcription factors shown in (C) in FACS-sorted populations of astrocytes, activated NSCs, and neural progenitor cells (NPCs) isolated directly from adult mouse brains. Mean ± SD of technical replicates of one representative experiment of two independent experiments.

A

Library	Unique Solexa Reads	QuEST Peaks	RefSeq Genes	Unique Gene Symbols	FDR
ASCL1 ChIP	12,343,372	18,291	7850	6271	<0.001

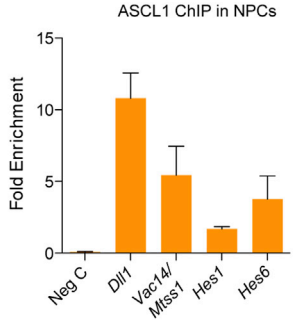
B



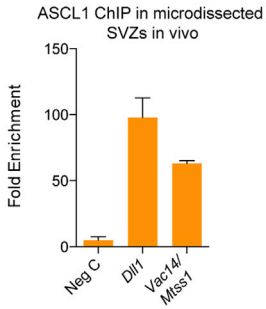
C

Symbol	Gene Name	QuEST Score
Fbxw7	F-box and WD rep dom cont 7	2406.77
Cdk2ap1	CDK2-associated protein 1	1626.39
Exosc9	exosome component 9	1516.99
Chd7	chromodomain helicase DNA BP 7	1504.23
Mnat1	menage a trois 1	1480.53
Dll1	delta-like 1	1380.24
Fam19a1	family with seq sim 19, member A1	1321.90
Nrxn2	neurexin II	1301.84
Hdac7a	histone deacetylase 7	1267.20
Afap1	actin filament associated protein 1	1232.56

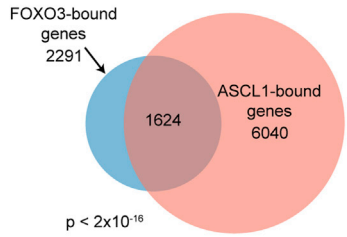
D



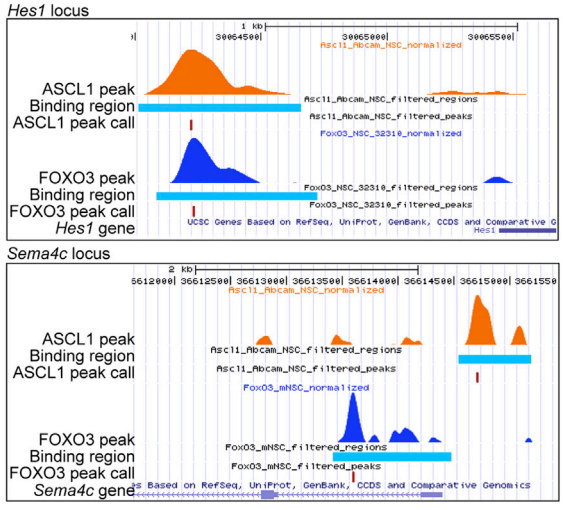
E



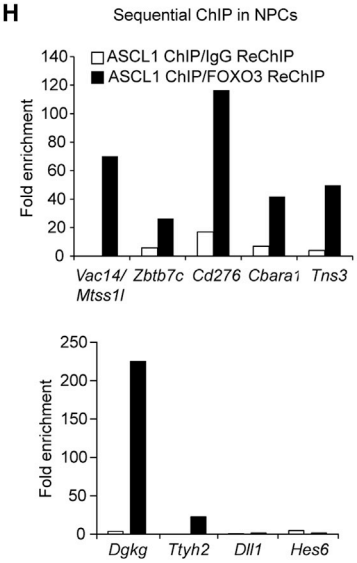
F



G



H



(legend on next page)

These data confirm that data sets obtained in primary cultures of adult NPCs are relevant in vivo.

Importantly, comparison of the FOXO3 and ASCL1 ChIP-seq data sets revealed that 70.9% of FOXO3-bound genes were also bound by ASCL1 in NPCs (Figure 3F). In about 35% of cases of common targets, FOXO3 and ASCL1 binding sites were indeed within 200 bp of each other (Figure 3G, upper panel), consistent with the percentage of E-box motif occurrence close to the FOXO motif (31.8%). FOXO3 and ASCL1 binding sites could also be more distant than 200 bp from one another (Figure 3G, lower panel). To test if FOXO3 and ASCL1 co-occur at some of their shared targets in the same cell, we performed sequential ChIP experiments (ASCL1 ChIP-FOXO3 reChIP) in NPCs in low growth factor signaling conditions (Figure 3H). Several genomic loci that were bound by ASCL1 (*Vac14/Mtss1l*, *Zbtb7c*, *Cd276*, *Cbara1*, *Tns3*, and *Dgkg*) were cobound by FOXO3, although there were also some loci at which FOXO3 and ASCL1 cobinding could not be detected (*Ttyh2*, *Dll1*, and *Hes6*) (Figure 3H). Thus, FOXO3 and ASCL1 share targets genome-wide in primary cultures of adult NPCs and can co-occur in the same cell at some genomic loci.

FOXO3 and ASCL1 Are Enriched at Enhancer Regions

FOXO3 and ASCL1 binding occurs at promoter regions, but also in intergenic regions and in introns (Figures 4A and S4), which are known to contain enhancers. These observations led us to ask if FOXO3 and ASCL1 bind to bona fide enhancers, and if regions bound by both FOXO3 and ASCL1 are different from regions bound by only one of these factors. Chromatin states of enhancers have been recently defined as enriched for H3K4me1 and depleted for H3K4me3 and H3K27me3 (Heintzman et al., 2009; Rada-Iglesias et al., 2011). To characterize enhancers in primary cultures of adult NPCs, we performed ChIP-seq for histone marks H3K4me1, H3K4me3, and H3K27me3 (Figure 4B). Both FOXO3 and ASCL1 binding sites were found in genomic regions that were enriched for H3K4me1 and depleted for H3K4me3 and H3K27me3 (Figure 4C), suggesting that FOXO3 and ASCL1 bind regions with several features of enhancers. Interestingly, FOXO3/ASCL1 cobound regions were statistically enriched for the H3K4me1 mark over regions bound by either FOXO3 or ASCL1 alone ($p = 7.37 \times 10^{-35}$ and $p = 2.08 \times 10^{-27}$, respectively) (Figure 4D). These data suggest that FOXO3 and ASCL1 are enriched at enhancers, which tend to be more lineage-specific than promoters (Heintzman et al., 2009; Rada-Iglesias et al., 2011).

FOXO3 Inhibits ASCL1-Dependent Transcription of Neurogenic Genes

To determine the functional consequence of FOXO3 and ASCL1 binding on gene expression, we asked if genes bound by both FOXO3 and ASCL1 have unique characteristics compared to genes bound by only one of these transcription factors (Figures 5A and S5). PANTHER analysis revealed that the genes bound by both FOXO3 and ASCL1 were more highly enriched for the Notch and Wnt signaling pathways than genes bound by FOXO3 or ASCL1 alone (Figure 5A; Table S6). The Notch and Wnt pathways are known to regulate the balance between NSCs and more committed progenitors (Aguirre et al., 2010; Chapouton et al., 2010; Imayoshi et al., 2010; Lie et al., 2005; Oishi et al., 2004). The Notch pathway has also been shown to be critical for ASCL1-dependent neurogenesis (Castro et al., 2006) and has been implicated downstream of FOXO1 in myoblasts (Kitamura et al., 2007). Together, these observations raise the possibility that in NPCs, FOXO3 regulates ASCL1 targets involved in Notch signaling.

We focused on *Dll1* and *Hes6* because these genes belong to the Notch pathway and are well-known targets of ASCL1 in vitro and in vivo (Castro et al., 2006). FOXO3 and ASCL1 both bind *Dll1* and *Hes6* at sites that are enriched for the enhancer mark H3K4me1 in cultured NPCs (Figure 5B). Interestingly, expression of FOXO3 in NPCs inhibits the ability of ASCL1 to upregulate *Dll1* and *Hes6* in adult NPCs (Figure 5C). FOXO3 also inhibited ASCL1-dependent expression of *Dll1* and *Hes6* in the embryonic stem cell-derived neural progenitor NS5 cell line (Figure 5D). FOXO3 does not appear to restrain ASCL1-dependent transcription by blocking the recruitment of ASCL1 to these genes: low growth factor signaling conditions, which induce the accumulation of endogenous FOXO3 into the nucleus, did not significantly affect ASCL1 binding (Figure S6A), and, vice versa, ASCL1 binding was not drastically altered in NPCs from *Foxo3*$^{-/-}$ mice (Figure S6B). Together, these data show that FOXO3 inhibits ASCL1-dependent induction of the Notch pathway genes *Dll1* and *Hes6* in cultured NPCs and are consistent with the possibility

Figure 3. Direct Targets of the Neuronal Fate Determinant ASCL1 Extensively Overlap with FOXO3 Targets

(A) Numbers of unique Solexa reads, QuEST peaks, corresponding RefSeq genes, unique gene symbols, and QuEST false discovery rates (FDR) for ASCL1 ChIP-seq from adult NPCs.

(B) MEME de novo motif analysis of ASCL1-bound genomic loci identified by ChIP-seq reveals an E-box consensus motif that is similar to that identified around FOXO3 peaks.

(C) List of the top ten ASCL1-bound genes in adult NPCs with the highest QuEST scores.

(D) ChIP-qPCR analysis of selected ASCL1 target genes in NPCs. Mean ± SD of two independent experiments. Enrichment is calculated relative to input. Neg C, negative control.

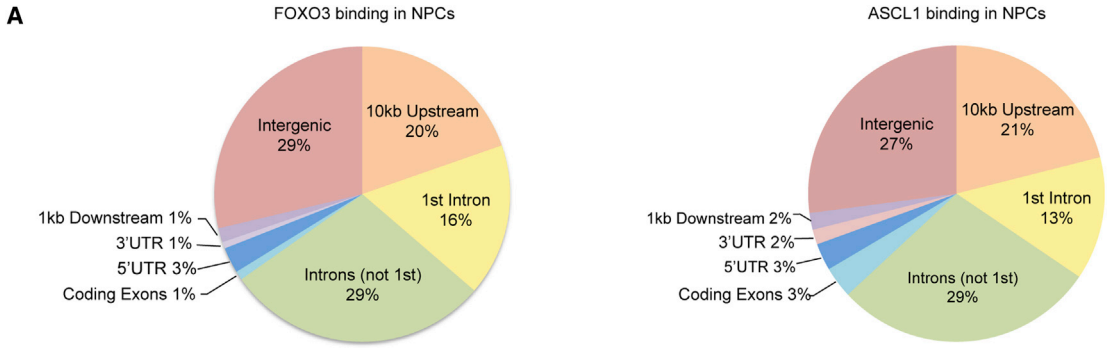
(E) ASCL1 ChIP-qPCR in chromatin extracts from microdissected SVZs from adult mice. Mean ± SD of two independent experiments, enrichment is calculated relative to input. Neg C, negative control.

(F) Genome-wide overlap between FOXO3 target genes and ASCL1 target genes.

(G) Example of genomic loci bound by both FOXO3 and ASCL1 at sites in close proximity (*Hes1* locus, upper panel) or at distant sites (*Sema4c* locus, lower panel). FOXO3 binding is shown in blue, ASCL1 binding is shown in orange.

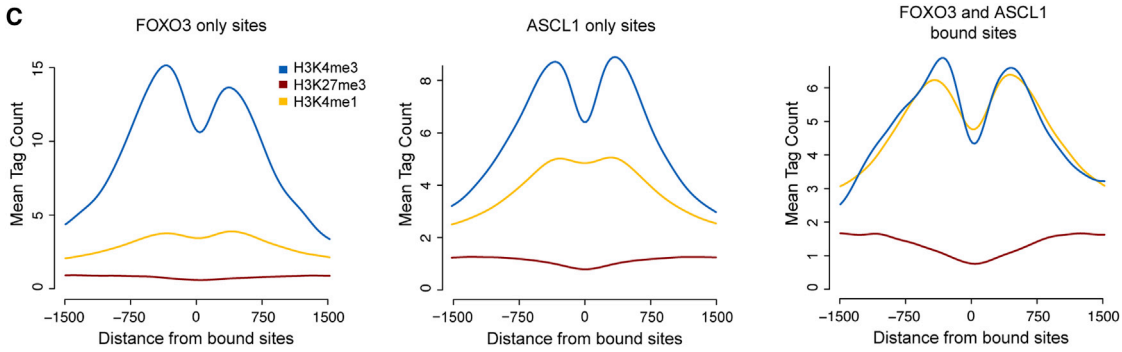
(H) FOXO3 and ASCL1 co-occupy the *Vac14/Mtss1l, Zbtb7c, Cd276, Cbara1*, *Tns3,* and *Dgkg* genomic loci in the same cell. Sequential ChIP-qPCR (ASCL1 ChIP-FOXO3 reChIP). Enrichment is normalized to a negative control region of the genome. One experiment is shown.

See also Figure S3 and Tables S4, S5, and S6.



B

Library	Unique Solexa Reads	MACS Peaks	RefSeq Genes	Unique Genes	FDR
H3K4me3 ChIP	32,724,614	21,264	17,832	12,830	0.01
H3K27me3 ChIP	33,248,015	14,219	3390	2866	0.01
H3K4me1 ChIP	32,724,614	84,701	18,202	12,967	0.01
No ChIP	36,039,121	N/A	N/A	N/A	N/A



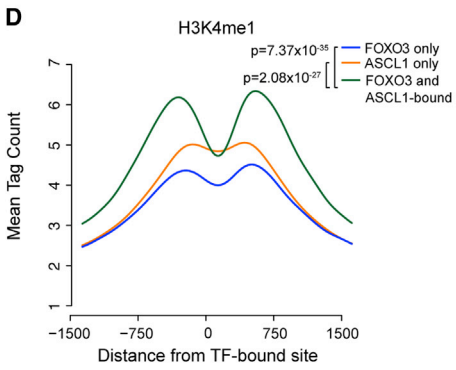


Figure 4. FOXO3 and ASCL1 Are Enriched at Enhancers

(A) FOXO3 and ASCL1 bind in intergenic and intronic regions, which contain potential enhancers.

(B) Numbers of unique Solexa reads, MACS peaks, corresponding RefSeq genes, unique gene symbols, and MACS false discovery rates (FDR) for H3K4me3, H3K27me3, and H3K4me1 ChIP from adult NPCs. N/A; not applicable.

(C) Distribution of H3K4me3, H3K27me3, and H3K4me1 at FOXO3, ASCL1 and cobound genomic regions.

(D) Genomic loci bound by both ASCL1 and FOXO3 are enriched for the H3K4me1 mark, a hallmark of enhancers.

See also Figure S4.

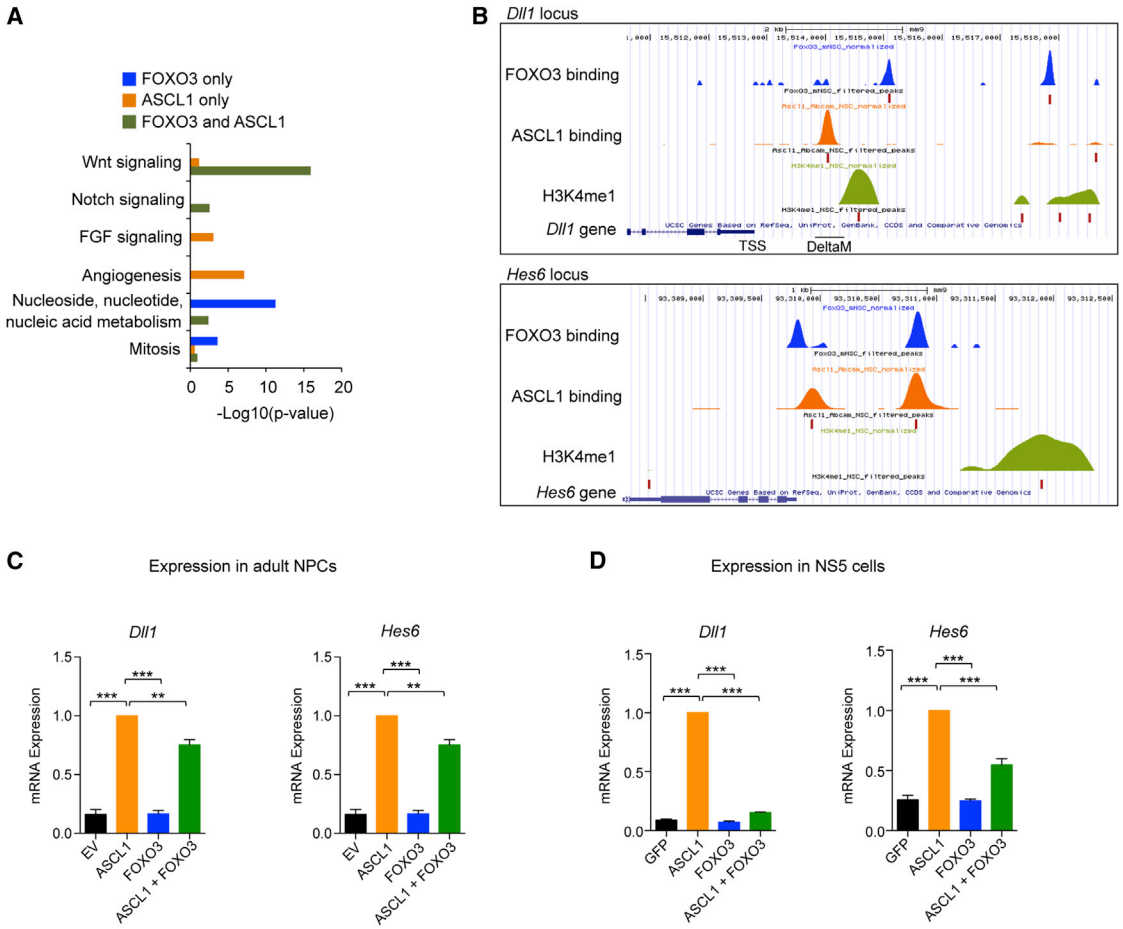


Figure 5. FOXO3 Represses ASCL1-Dependent Expression of Neurogenic Genes in NPCs

(A) PANTHER analysis (selected pathways and biological processes) on genesets bound by FOXO3 only (blue), ASCL1 only (orange), and by both FOXO3 and ASCL1 (dark green).

(B) FOXO3 binding (blue), ASCL1 binding (orange), and enrichment of the enhancer mark H3K4me1 (light green) at *Dll1* and *Hes6*, two genes of the Notch pathway. Note that the FOXO3 binding site in the *Hes6* regulatory region has a QuEST score of 46.6, which is just under the most stringent QuEST cutoff.

(C) Expression of *Dll1* and *Hes6* in primary adult NPCs expressing empty vector (EV), ASCL1, FOXO3, or a combination of ASCL1+FOXO3. Mean±SEM of three independent experiments. $^{**}p < 0.01$, $^{***}p < 0.001$. One-way ANOVA, Bonferroni correction.

(D) Expression of the Notch pathway genes *Dll1* and *Hes6* in embryonic stem cell-derived NPCs (NS5 cells) expressing GFP (negative control), ASCL1, FOXO3, or a combination of ASCL1+FOXO3. Mean ± SD of one experiment conducted in triplicate. $^{***}p < 0.001$, One-way ANOVA, Bonferroni correction.

See also Figures S5 and S6.

that FOXO3 acts by preventing ASCL1-dependent transcription rather than binding.

FOXO3 Inhibits ASCL1-Dependent Neurogenesis in Adult NPCs and Neuronal Conversion of Fibroblasts

To determine if the inhibition of ASCL1-dependent expression of neurogenic genes by FOXO3 had physiological consequences, we first tested if FOXO3 impacts ASCL1's ability to promote neurogenesis in adult NPCs. Consistent with previous reports in other cellular systems (Farah et al., 2000; Vierbuchen et al., 2010), ASCL1 expression also potently induced neurogenesis in adult NPCs (Figure 6A). Importantly, FOXO3 significantly decreased the ability of ASCL1 to induce neurogenesis (Figure 6A). However, the impact of FOXO3 on neurogenesis is more pronounced than that on gene expression, suggesting that FOXO3 could also repress neurogenesis via additional targets or mechanisms.

Expression of ASCL1 together with other proneural transcription factors (MYT1L and/or BRN2) was recently shown to directly reprogram fibroblasts into iNs (Vierbuchen et al., 2010; Pang et al., 2011). We assessed the effect of FOXO3 on the ability of ASCL1 to directly convert mouse embryonic fibroblasts (MEFs) into iNs (Figure 6B). As expected, ASCL1 and MYT1L efficiently induced the generation of TUJ1-positive iNs in MEFs (Figure 6B). Interestingly, ectopic expression of FOXO3 significantly decreased the ability of ASCL1/MYT1L to reprogram MEFs into TUJ1-positive iNs (Figure 6B). By contrast, expression of an inactive form of FOXO3 that lacks the DNA binding domain did not significantly affect ASCL1/MYT1L-dependent iN conversion (Figure 6B). These results indicate that FOXO3 binding

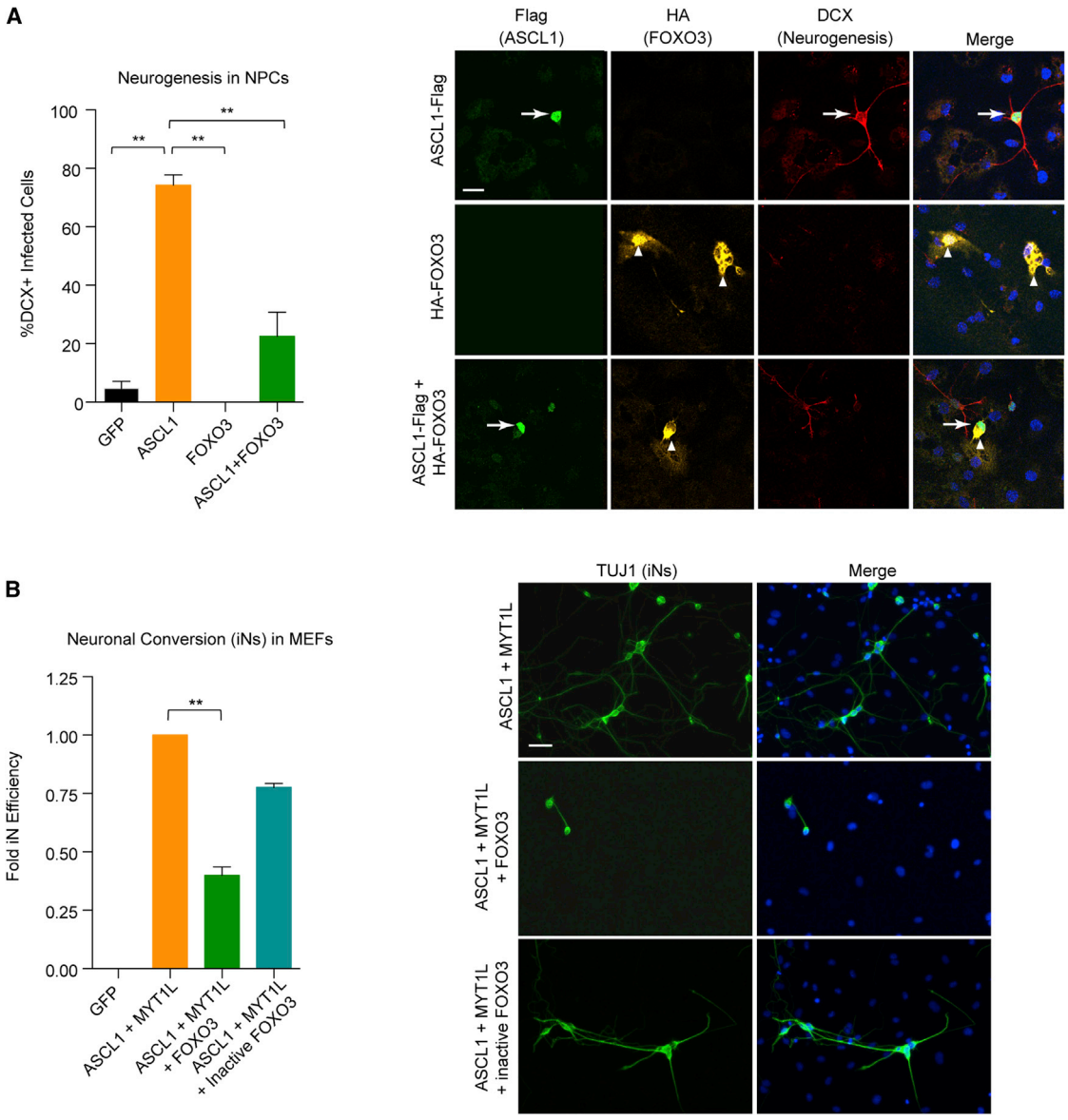


Figure 6. FOXO3 Restrains ASCL1 from Promoting Neurogenesis in Adult NPCs and Reprogramming Mouse Embryonic Fibroblasts into Neurons

(A) Percentage of DCX-positive cells (new neurons) in adult NPCs infected by lentiviruses expressing GFP, ASCL1, FOXO3, or a combination of ASCL1+FOXO3. Mean ± SEM of two independent experiments. **$p < 0.01$. One-way ANOVA, Bonferroni correction. Right panel: representative images. Scale bar represents 20 μm.

(B) Percentage of TUJ1-positive cells (iNs) in mouse embryonic fibroblasts infected by lentiviruses expressing GFP, ASCL1+MYT1L, ASCL1+MYT1L+FOXO3, or ASCL1+MYT1L+inactive FOXO3. Mean ± SEM of three independent experiments. **$p < 0.01$. One-way ANOVA, Bonferroni correction. Right panel: representative images. Scale bar represents 20 μm.

antagonizes ASCL1's ability to promote the direct neuronal conversion of fibroblasts.

Foxo3 Loss Results in Increased Neurogenesis

We asked if FOXO3 impacts neurogenesis in vivo. Costaining of adult mouse brain sections reveals that FOXO3 and ASCL1 are colocalized in neurogenic niches (SVZs) in a subset of cells that are marked with SOX2, a marker of neural stem/progenitor cells (Figures 7A and S1A). FOXO3 is localized in the nucleus in a subset of these cells, though not all (Figures 7A and S1A), indicating that active FOXO3 and ASCL1 coexist in some, but not all, neural stem and/or progenitor cells in the adult brain in vivo. We next assessed neurogenesis in adult *Foxo3*$^{-/-}$ and *Foxo3*$^{+/+}$ mice by injecting 5-ethynyl-2′-deoxyuridine (EdU) and costaining sections of the olfactory bulb, the region where new neurons generated in the SVZ migrate to, with EdU and DCX, a

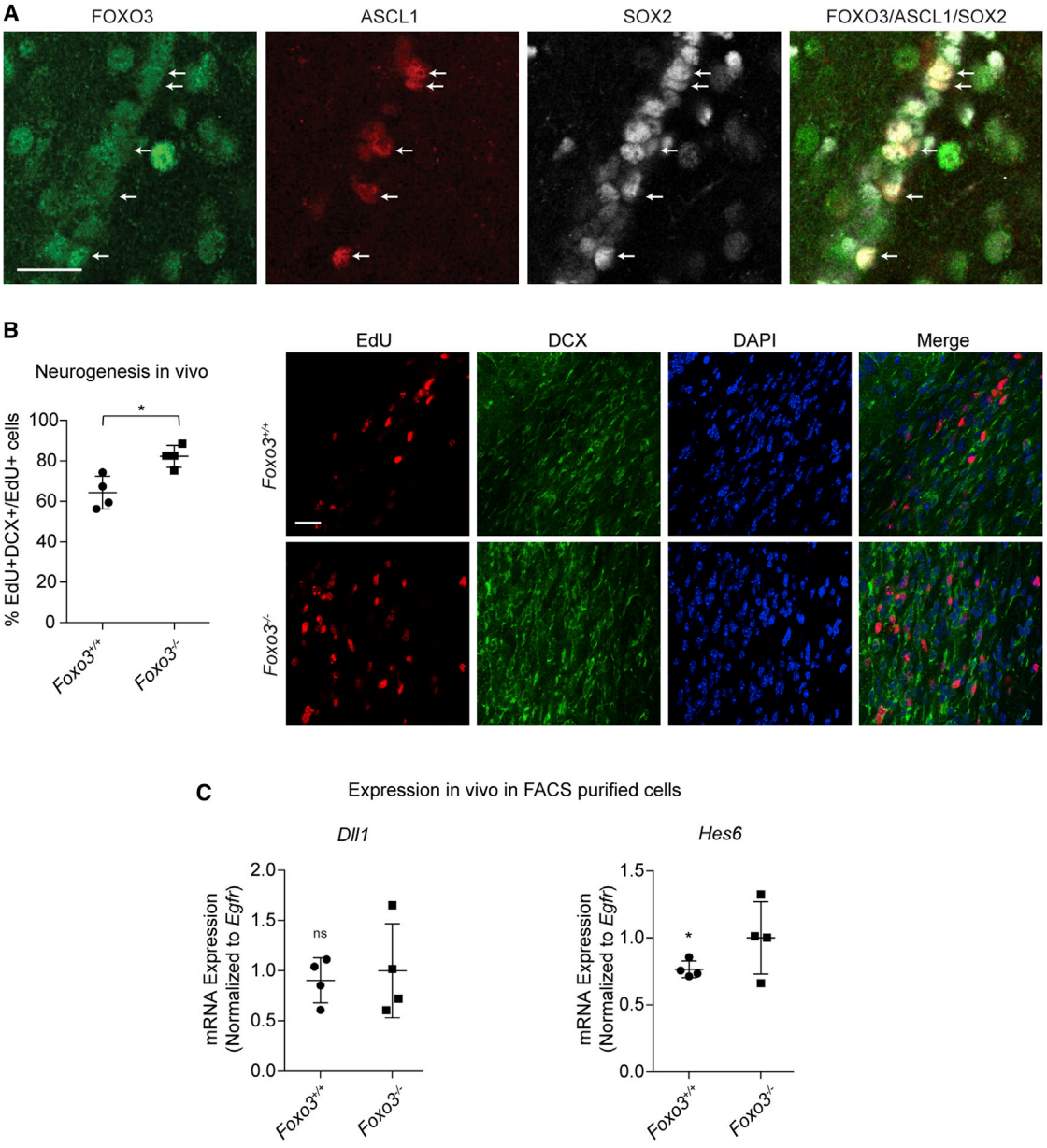


Figure 7. *Foxo3* Deficiency Increases Adult Neurogenesis

(A) FOXO3 and ASCL1 expression in the adult SVZ in vivo. Green, FOXO3; red, ASCL1; white, SOX2 (a marker of neural stem/progenitor cells). Scale bar represents 20 μm. Arrows show ASCL1-expressing cells.

(B) *Foxo3* deficiency results in increased neurogenesis in the olfactory bulb of adult mice. Percentage of EdU+DCX+ cells (new neurons) in the olfactory bulb in adult (4 months old) $Foxo3^{+/+}$ and $Foxo3^{-/-}$ mice. Mean ± SEM of n = 4 mice per group. *p < 0.05, Student's t test.

(C) *Dll1* and *Hes6* expression in $Foxo3^{+/+}$ and $Foxo3^{-/-}$ in vivo. Taqman RT-qPCR analysis of *Dll1* and *Hes6* expression in activated NSCs and NPCs freshly isolated from adult mouse brains by FACS. Mean ± SEM of n = 4 mice (two mice in two independent FACS experiments). *p < 0.05, one-sided Wilcoxon exact test.

marker of newly formed neurons. *Foxo3* deficiency led to a significant increase in the production of new neurons in the olfactory bulbs of adult mice (Figure 7B, Student's t test, $p < 0.05$). *Hes6* expression was slightly increased in activated NSCs and NPCs that were freshly purified by FACS from $Foxo3^{-/-}$ mice compared to $Foxo3^{+/+}$ mice (Figure 7C, one-sided Wilcoxon exact test, $p < 0.05$), although *Dll1* expression was not significantly affected (Figure 7C). These results are consistent with the possibility that FOXO3 may antagonize the ability of ASCL1 to induce neurogenic genes in vivo, although other targets or mechanisms may mediate the effect of FOXO3 on neurogenesis.

DISCUSSION

Interaction between a Forkhead Transcription Factor and a bHLH "Master Regulator" of Cell Fate

Our study uses ultra-high-throughput sequencing technologies to identify FOXO3 and ASCL1 targets in primary cultures of adult NPCs and reveals that FOXO3 and ASCL1 overlap genome-wide and are particularly enriched at enhancers of genes involved the Wnt and Notch signaling pathways. Our study also provides evidence that FOXO3 inhibits the ability of ASCL1 to induce neurogenesis and direct neuronal conversion. These findings have implications in other systems because bHLH master regulators are pivotal for the determination of many cell lineages. The binding of two other Forkhead transcription factors, FOXO1 and FOXH1, has been found to overlap with that of the bHLH regulator E2A in B cells and human embryonic stem cells, respectively (Lin et al., 2010; Yoon et al., 2011), although the exact bHLH transcription factor that is involved is not known. Thus, binding in close proximity to bHLH transcription factors may be a general feature of Forkhead transcription factors. As several Forkhead transcription factors are known to act as "pioneer factors" to open chromatin (Zaret and Carroll, 2011), these factors may help provide an open chromatin context for lineage-specific transcription factors to activate transcription of differentiation genes. Transcription factors of the bHLH family are particularly powerful at eliciting direct reprogramming (Vierbuchen and Wernig, 2011). Our discovery that a Forkhead transcription factor and a bHLH transcription factor genomically and functionally interact also has implications for direct reprogramming. The potential applications of direct reprogramming in cellular therapies or drug screening are limited by the relatively low efficiency of this process, especially in human cells (Pang et al., 2011). Thus, identifying molecular barriers (such as FOXO) that limit direct reprogramming should be a key step in improving the efficiency of this process.

Mechanisms of Inhibition of ASCL1-Dependent Neurogenesis by FOXO3

Our finding that FOXO3 inhibits ASCL1-dependent neurogenesis is consistent with the observation that inhibition of PDK1-AKT signaling, which results in FOXO activation, reduces neurogenesis in NPCs, whereas a constitutively active form of AKT or loss of PTEN, which leads to FOXO inhibition, enhances neurogenesis (Gregorian et al., 2009; Oishi et al., 2009). Interestingly, ASCL1/MASH1 protein stabilization has been shown to mediate at least in part the positive effect of AKT signaling on neurogenesis (Oishi et al., 2009). Thus, it is conceivable that the PI3K-AKT signaling pathway coordinates ASCL1 stabilization with ASCL1 activation (via inhibition of the repressive effect of FOXO3 on ASCL1) to achieve a more potent induction of neurogenesis.

The exact transcriptional mechanisms by which FOXO3 inhibits ASCL1-dependent gene expression remain to be established. FOXO3 and ASCL1 cobinding could not be detected by sequential ChIP at the *Dll1* and *Hes6* loci. Furthermore, FOXO3 and ASCL1 do not appear to physically interact (A.E.W. and A.B., data not shown). It remains possible that negative results in these cobinding experiments are due to technical limitations. Nevertheless, these results may also suggest a "displacement model" to explain FOXO3's ability to inhibit ASCL1 dependent transcription, in which either FOXO3 or ASCL1 could bind to the *Dll1* and *Hes6* loci, but these transcription factors could not be bound at the same time. FOXO3 may also function by inhibiting the recruitment of ASCL1 coactivators at neurogenic genes. Alternatively, FOXO3 may recruit a repressive complex in the presence of ASCL1. Part of the effects of FOXO3 on ASCL1-dependent transcription may also be indirect: for example, FOXO3 may limit neurogenesis as a consequence of promoting stem cell quiescence. Indeed, ASCL1 is required for normal NSC proliferation (Castro et al., 2011), while also functioning as a positive regulator of neurogenesis (Parras et al., 2004). Thus, FOXO3 may inhibit neurogenesis as a consequence of preventing ASCL1-dependent proliferation of committed progenitors.

How FOXO3 and ASCL1 interact in vivo, and whether FOXO3 restrains neurogenesis in the adult brain by inhibiting ASCL1-dependent transcription, is still unclear. Furthermore, the function of ASCL1 in adult neurogenesis has not been established. We note that the expression of the ASCL1 target gene *Hes6* was only slightly increased in *Foxo3*$^{-/-}$ NSCs in vivo, and that *Dll1* expression was not significantly changed in these mice. Thus, other target genes may function downstream of FOXO3 to regulate neurogenesis in vivo. It will be interesting to further dissect the genetic interactions between FOXO3 and ASCL1, as well as their target genes, in stem cell homeostasis and neurogenesis in vivo.

Stemness and Aging

Our data raise the possibility that FOXO3 maintains a stem/progenitor cell state in part by restraining master regulators of differentiated cell fates. Consistent with this model, loss of *Foxo1*, *Foxo3*, and *Foxo4* in the brain results in increased neurogenesis, followed by NSC depletion in adulthood (Paik et al., 2009; Renault et al., 2009). Recent evidence has also implicated FOXO in inhibiting premature differentiation. In hydra, FOXO depletion leads to enhanced terminal differentiation of foot cells, whereas FOXO overexpression promotes the expression of stemness genes (Boehm et al., 2012). FOXO family members have also been implicated in human embryonic stem cell (ESC) pluripotency. FOXO1 is necessary for human ESC pluripotency (Zhang et al., 2011) and FOXO4 has been shown to be required for proteasome activity, a key component of the pluripotency of these cells (Vilchez et al., 2012). FOXO4 has also been found to be necessary for the neural differentiation of human ESCs (Vilchez et al., 2013), which contrasts with our findings that adult *Foxo3*$^{-/-}$ mice exhibit increased neurogenesis and that triple *Foxo* knockout mice also show enhanced neurogenesis postnatally (Paik et al., 2009). Different FOXO family members may act at different points in cellular commitment: the competence of the progenitor pool versus differentiation per se. Alternatively, pluripotent embryonic stem cells may have different requirements from more committed lineage-specific stem cells.

FOXO transcription factors are conserved regulators of longevity (Kenyon, 2010). Our discovery of target genes shared between FOXO and bHLH factors raises the exciting possibility that bHLH transcription factors and Notch signaling may, by association, also be involved in longevity. In this context, it is interesting to note that heterochronic parabiosis, the junction

by blood circulation of an old mouse and a young mouse, restores Notch signaling and the regenerative capacity of muscle and neural stem cells in old mice (Conboy et al., 2005; Villeda et al., 2011). The concerted action of FOXO and ASCL1 in the balance between stem cell maintenance and differentiation might play an important role in tissue homeostasis and longevity.

EXPERIMENTAL PROCEDURES

Animals

FoxO3$^{+/+}$ and *FoxO3*$^{lox/lox}$ mice were a kind gift from Dr. Ron DePinho (Dana Farber Cancer Center, Boston). Wild-type FVB/N animals were purchased from Charles River. All animals were treated and housed according to the Guide for Care and Use of Laboratory Animals. All experimental procedures were approved by Stanford's Administrative Panel on Laboratory Animal Care (APLAC) and were in accordance with institutional and national guidelines.

Mouse NPC Cultures

Adult (12-week-old) mouse NPCs were isolated as previously described (Renault et al., 2009). Briefly, whole-mouse forebrains were homogenized and incubated for 30 min in HBSS (Invitrogen) with 1 U/ml Dispaself (Roche), 250 U/ml DNaseI (Sigma), and 2.5 U/ml Papain (Worthington) at 37°C. After mechanical dissociation, cells were purified by sequential 25% and 65% Percoll (Amersham) gradients. Cells were cultured in high growth factor signaling conditions: Neurobasal A (Invitrogen) medium supplemented with penicillin/streptomycin/glutamine (Invitrogen), 2% B27 (Invitrogen), and 20 ng/ml each of FGF2 (Peprotec) and EGF (Peprotec). To induce FOXO3 nuclear accumulation, cells were incubated in low growth factor signaling conditions: Neurobasal A medium supplemented with penicillin/streptomycin/glutamine and 2% B27 for 4 hr, followed by a 1.5 hr incubation with 20 μM LY294002 (LY, Calbiochem), a specific PI3K inhibitor, to inhibit residual growth factor signaling. For differentiation, NPCs were plated at 10^5 cells/ml on poly-D-lysine-coated plates and incubated in differentiation conditions (Neurobasal A medium supplemented with penicillin/streptomycin/glutamine, 2% B27 supplement, and 0.5% [unless specifically noted] fetal bovine serum [Invitrogen]) for 0, 2, or 7 days, and differentiation media was replaced every other day.

ChIP-Seq

For ChIP-seq, primary NPCs were isolated and amplified in culture for a limited number of passages (four to nine), and ChIP experiments were performed as described (Renault et al., 2009) (see Extended Experimental Procedures). For FOXO3 and ASCL1 ChIP-seq, 80–100 × 10^6 cells were used to generate Illumina single-end libraries. For H3K4me3 and H3K27me3 ChIP-seq, 10–20 × 10^6 cells were used. Libraries were generated according to the manufacturer's instructions (Illumina). Twenty-five base pair reads were generated on an Illumina Genome Analyzer II and subsequently mapped to the 2007 release of the mouse genome (mm9). Peak calling was performed using QuEST 2.4 (Valouev et al., 2008). The highest stringency QuEST parameters for transcription factors were used for FOXO3 and ASCL1 ChIP-seq (fold enrichment >50, bandwidth = 30). For histone mark ChIP-seq, Fastq files for ChIP-seq data sets were filtered using the FASTX-Tookit (http://hannonlab.cshl.edu/fastx_toolkit/index.html) and reads with phred scores less than 15 on 85% of their sequence were filtered. Histone modification sites from the filtered and aligned reads were identified using MACS (version macs2 2.0.8) (Zhang et al., 2008) at a FDR threshold 0.01 with a broad setting that enables linking nearby enriched regions (linking cutoff = 0.1). RefSeq genes, genomic features and corresponding coordinates were downloaded from the UCSC Genome Browser.

Lentiviral Infections of NPCs and Neurogenesis Assays

For lentiviral infection, NPCs were plated on poly-D-lysine at 50,000 cells/cm^2 in high growth-factor-signaling conditions. Sixteen hours after plating, cells were infected with lentiviral-conditioned supernatents at a 1:2 ratio (lentivirus-conditioned supernatent:NPC medium). Twenty-four hours postinfection, fresh proliferating medium containing 2 μg/ml doxycycline (Sigma) was added to induce expression of transduced genes. For gene expression analysis, total RNA was collected 6 hr postinduction and RT-qPCRs were performed as described in the Extended Experimental Procedures. For neurogenesis assays, cells were treated with 2 μg/ml doxycycline for 24 hr and then switched to differentiation conditions containing 2 μg/ml doxycycline. The media was changed after 2 days, and samples were collected after 4 days of differentiation. For neurogenesis assays, cells were fixed and immunocytochemistry was performed as described in the Extended Experimental Procedures with the following antibodies: DCX (Santa Cruz Biotechnology SC-8066, 1:200), Flag (Sigma F7425, 1:1,000), HA (Roche 12CA5 #11867423001, 1:200), and TUJ1 (Covance PRB-435P, 1:1,000).

ACCESSION NUMBERS

The Gene Expression Omnibus accession number for the data reported in this paper is GSE48336.

SUPPLEMENTAL INFORMATION

Supplemental Information includes Extended Experimental Procedures, six figures, and six tables and can be found with this article online at http://dx.doi.org/10.1016/j.celrep.2013.06.035.

ACKNOWLEDGMENTS

We thank A. Sidow and of the Sidow lab members A. Valouev, J. Foley, and Z. Weng for ultrahigh-throughput sequencing and for providing bioinformatic tools before publication. We particularly thank A. Valouev for assistance with high-throughput data analyses and B. Benayoun for help with bioinformatics analysis. We thank L. Attardi, J. Baker, A. Gitler, T. Palmer, J. Sage, A. Sidow, A. Valouev, and members of the Brunet lab for critically reading the manuscript. This work was supported by NIH grants R01 AG026648 (A.B.), P01 AG036695 (A.B. and T.A.R.), R01 MH092931 (M.W.), a California Institute for Regenerative Medicine New Faculty Award (A.B.), an Ellison Medical Foundation Senior Award (A.B.), an Ellison Medical Foundation Junior Award (M.W.), and a grant-in-aid U117570528 from the Medical Research Council (F.G.). M.W. is a New York Stem Cell Foundation-Robertson investigator. A.E.W. was supported by a Cancer Biology NIH/NRSA training grant (5T32 CA09302) and by an Ellison Medical Foundation/AFAR postdoctoral fellowship. E.A.P. was supported by an NSF graduate fellowship and an NIH graduate fellowship (F31 AG043232). D.S.L was supported by NDSEG and NSF graduate fellowships. D.S.L. was supported by NDSEG and NSF graduate fellowships. A.E.W. and A.B. conceived and planned the study, and A.E.W. performed experiments. E.A.P. performed the histone mark ChIP-seq studies (Figure 4). E.A.P. and D.S.L. performed the FACS studies in Figures S2D–S2F, 1I, and 2E. E.A.P. and D.S.L. performed the FACS studies in Figures S2D–S2F, 1I, and 2E. D.S.L. also performed the FACS in Figure 7C. T.V. and M.W. helped with the neurogenesis and iN studies (Figure 6). A.E.W. and A.B. wrote the paper, and all authors provided helpful comments on the manuscript.

Received: January 8, 2013
Revised: May 11, 2013
Accepted: June 25, 2013
Published: July 25, 2013

REFERENCES

Aguirre, A., Rubio, M.E., and Gallo, V. (2010). Notch and EGFR pathway interaction regulates neural stem cell number and self-renewal. Nature *467*, 323–327.

Bailey, T.L., and Elkan, C. (1994). Fitting a mixture model by expectation maximization to discover motifs in biopolymers. Proc. Int. Conf. Intell. Syst. Mol. Biol. *2*, 28–36.

Boehm, A.M., Khalturin, K., Anton-Erxleben, F., Hemmrich, G., Klostermeier, U.C., Lopez-Quintero, J.A., Oberg, H.H., Puchert, M., Rosenstiel, P., Wittlieb,

J., and Bosch, T.C. (2012). FoxO is a critical regulator of stem cell maintenance in immortal Hydra. Proc. Natl. Acad. Sci. USA *109*, 19697–19702.

Calnan, D.R., and Brunet, A. (2008). The FoxO code. Oncogene *27*, 2276–2288.

Castro, D.S., Skowronska-Krawczyk, D., Armant, O., Donaldson, I.J., Parras, C., Hunt, C., Critchley, J.A., Nguyen, L., Gossler, A., Göttgens, B., et al. (2006). Proneural bHLH and Brn proteins coregulate a neurogenic program through cooperative binding to a conserved DNA motif. Dev. Cell *11*, 831–844.

Castro, D.S., Martynoga, B., Parras, C., Ramesh, V., Pacary, E., Johnston, C., Drechsel, D., Lebel-Potter, M., Garcia, L.G., Hunt, C., et al. (2011). A novel function of the proneural factor Ascl1 in progenitor proliferation identified by genome-wide characterization of its targets. Genes Dev. *25*, 930–945.

Chapouton, P., Skupien, P., Hesl, B., Coolen, M., Moore, J.C., Madelaine, R., Kremmer, E., Faus-Kessler, T., Blader, P., Lawson, N.D., and Bally-Cuif, L. (2010). Notch activity levels control the balance between quiescence and recruitment of adult neural stem cells. J. Neurosci. *30*, 7961–7974.

Conboy, I.M., Conboy, M.J., Wagers, A.J., Girma, E.R., Weissman, I.L., and Rando, T.A. (2005). Rejuvenation of aged progenitor cells by exposure to a young systemic environment. Nature *433*, 760–764.

Farah, M.H., Olson, J.M., Sucic, H.B., Hume, R.I., Tapscott, S.J., and Turner, D.L. (2000). Generation of neurons by transient expression of neural bHLH proteins in mammalian cells. Development *127*, 693–702.

Furuyama, T., Nakazawa, T., Nakano, I., and Mori, N. (2000). Identification of the differential distribution patterns of mRNAs and consensus binding sequences for mouse DAF-16 homologues. Biochem. J. *349*, 629–634.

Giannakou, M.E., Goss, M., Jünger, M.A., Hafen, E., Leevers, S.J., and Partridge, L. (2004). Long-lived Drosophila with overexpressed dFOXO in adult fat body. Science *305*, 361.

Gregorian, C., Nakashima, J., Le Belle, J., Ohab, J., Kim, R., Liu, A., Smith, K.B., Groszer, M., Garcia, A.D., Sofroniew, M.V., et al. (2009). Pten deletion in adult neural stem/progenitor cells enhances constitutive neurogenesis. J. Neurosci. *29*, 1874–1886.

Guillemot, F. (1999). Vertebrate bHLH genes and the determination of neuronal fates. Exp. Cell Res. *253*, 357–364.

Guillemot, F., Lo, L.C., Johnson, J.E., Auerbach, A., Anderson, D.J., and Joyner, A.L. (1993). Mammalian achaete-scute homolog 1 is required for the early development of olfactory and autonomic neurons. Cell *75*, 463–476.

Heintzman, N.D., Hon, G.C., Hawkins, R.D., Kheradpour, P., Stark, A., Harp, L.F., Ye, Z., Lee, L.K., Stuart, R.K., Ching, C.W., et al. (2009). Histone modifications at human enhancers reflect global cell-type-specific gene expression. Nature *459*, 108–112.

Henderson, S.T., and Johnson, T.E. (2001). daf-16 integrates developmental and environmental inputs to mediate aging in the nematode Caenorhabditis elegans. Curr. Biol. *11*, 1975–1980.

Hu, Y., Wang, T., Stormo, G.D., and Gordon, J.I. (2004). RNA interference of achaete-scute homolog 1 in mouse prostate neuroendocrine cells reveals its gene targets and DNA binding sites. Proc. Natl. Acad. Sci. USA *101*, 5559–5564.

Hwangbo, D.S., Gershman, B., Tu, M.P., Palmer, M., and Tatar, M. (2004). Drosophila dFOXO controls lifespan and regulates insulin signalling in brain and fat body. Nature *429*, 562–566.

Imayoshi, I., Sakamoto, M., Yamaguchi, M., Mori, K., and Kageyama, R. (2010). Essential roles of Notch signaling in maintenance of neural stem cells in developing and adult brains. J. Neurosci. *30*, 3489–3498.

Kenyon, C.J. (2010). The genetics of ageing. Nature *464*, 504–512.

Kitamura, T., Kitamura, Y.I., Funahashi, Y., Shawber, C.J., Castrillon, D.H., Kollipara, R., DePinho, R.A., Kitajewski, J., and Accili, D. (2007). A Foxo/Notch pathway controls myogenic differentiation and fiber type specification. J. Clin. Invest. *117*, 2477–2485.

Lie, D.C., Colamarino, S.A., Song, H.J., Désiré, L., Mira, H., Consiglio, A., Lein, E.S., Jessberger, S., Lansford, H., Dearie, A.R., and Gage, F.H. (2005). Wnt signalling regulates adult hippocampal neurogenesis. Nature *437*, 1370–1375.

Lin, Y.C., Jhunjhunwala, S., Benner, C., Heinz, S., Welinder, E., Mansson, R., Sigvardsson, M., Hagman, J., Espinoza, C.A., Dutkowski, J., et al. (2010). A global network of transcription factors, involving E2A, EBF1 and Foxo1, that orchestrates B cell fate. Nat. Immunol. *11*, 635–643.

Litvak, V., Ratushny, A.V., Lampano, A.E., Schmitz, F., Huang, A.C., Raman, A., Rust, A.G., Bergthaler, A., Aitchison, J.D., and Aderem, A. (2012). A FOXO3-IRF7 gene regulatory circuit limits inflammatory sequelae of antiviral responses. Nature *490*, 421–425.

Mahony, S., and Benos, P.V. (2007). STAMP: a web tool for exploring DNA-binding motif similarities. Nucleic Acids Res. *35*(Web Server issue), W253–W258.

Miyamoto, K., Araki, K.Y., Naka, K., Arai, F., Takubo, K., Yamazaki, S., Matsuoka, S., Miyamoto, T., Ito, K., Ohmura, M., et al. (2007). Foxo3a is essential for maintenance of the hematopoietic stem cell pool. Cell Stem Cell *1*, 101–112.

Oishi, K., Kamakura, S., Isazawa, Y., Yoshimatsu, T., Kuida, K., Nakafuku, M., Masuyama, N., and Gotoh, Y. (2004). Notch promotes survival of neural precursor cells via mechanisms distinct from those regulating neurogenesis. Dev. Biol. *276*, 172–184.

Oishi, K., Watatani, K., Itoh, Y., Okano, H., Guillemot, F., Nakajima, K., and Gotoh, Y. (2009). Selective induction of neocortical GABAergic neurons by the PDK1-Akt pathway through activation of Mash1. Proc. Natl. Acad. Sci. USA *106*, 13064–13069.

Ouyang, W., Liao, W., Luo, C.T., Yin, N., Huse, M., Kim, M.V., Peng, M., Chan, P., Ma, Q., Mo, Y., et al. (2012). Novel Foxo1-dependent transcriptional programs control T(reg) cell function. Nature *491*, 554–559.

Paik, J.H., Ding, Z., Narurkar, R., Ramkissoon, S., Muller, F., Kamoun, W.S., Chae, S.S., Zheng, H., Ying, H., Mahoney, J., et al. (2009). FoxOs cooperatively regulate diverse pathways governing neural stem cell homeostasis. Cell Stem Cell *5*, 540–553.

Pang, Z.P., Yang, N., Vierbuchen, T., Ostermeier, A., Fuentes, D.R., Yang, T.Q., Citri, A., Sebastiano, V., Marro, S., Südhof, T.C., and Wernig, M. (2011). Induction of human neuronal cells by defined transcription factors. Nature *476*, 220–223.

Parras, C.M., Galli, R., Britz, O., Soares, S., Galichet, C., Battiste, J., Johnson, J.E., Nakafuku, M., Vescovi, A., and Guillemot, F. (2004). Mash1 specifies neurons and oligodendrocytes in the postnatal brain. EMBO J. *23*, 4495–4505.

Pastrana, E., Cheng, L.C., and Doetsch, F. (2009). Simultaneous prospective purification of adult subventricular zone neural stem cells and their progeny. Proc. Natl. Acad. Sci. USA *106*, 6387–6392.

Pastrana, E., Silva-Vargas, V., and Doetsch, F. (2011). Eyes wide open: a critical review of sphere-formation as an assay for stem cells. Cell Stem Cell *8*, 486–498.

Powell, L.M., and Jarman, A.P. (2008). Context dependence of proneural bHLH proteins. Curr. Opin. Genet. Dev. *18*, 411–417.

Rada-Iglesias, A., Bajpai, R., Swigut, T., Brugmann, S.A., Flynn, R.A., and Wysocka, J. (2011). A unique chromatin signature uncovers early developmental enhancers in humans. Nature *470*, 279–283.

Renault, V.M., Rafalski, V.A., Morgan, A.A., Salih, D.A., Brett, J.O., Webb, A.E., Villeda, S.A., Thekkat, P.U., Guillerey, C., Denko, N.C., et al. (2009). FoxO3 regulates neural stem cell homeostasis. Cell Stem Cell *5*, 527–539.

Salih, D.A., and Brunet, A. (2008). FoxO transcription factors in the maintenance of cellular homeostasis during aging. Curr. Opin. Cell Biol. *20*, 126–136.

Shin, D.J., Joshi, P., Hong, S.H., Mosure, K., Shin, D.G., and Osborne, T.F. (2012). Genome-wide analysis of FoxO1 binding in hepatic chromatin: potential involvement of FoxO1 in linking retinoid signaling to hepatic gluconeogenesis. Nucleic Acids Res. *40*, 11499–11509.

Tothova, Z., Kollipara, R., Huntly, B.J., Lee, B.H., Castrillon, D.H., Cullen, D.E., McDowell, E.P., Lazo-Kallanian, S., Williams, I.R., Sears, C., et al. (2007). FoxOs are critical mediators of hematopoietic stem cell resistance to physiologic oxidative stress. Cell *128*, 325–339.

Valouev, A., Johnson, D.S., Sundquist, A., Medina, C., Anton, E., Batzoglou, S., Myers, R.M., and Sidow, A. (2008). Genome-wide analysis of transcription factor binding sites based on ChIP-Seq data. Nat. Methods *5*, 829–834.

van der Vos, K.E., Eliasson, P., Proikas-Cezanne, T., Vervoort, S.J., van Boxtel, R., Putker, M., van Zutphen, I.J., Mauthe, M., Zellmer, S., Pals, C., et al. (2012). Modulation of glutamine metabolism by the PI(3)K-PKB-FOXO network regulates autophagy. Nat. Cell Biol. *14*, 829–837.

Vierbuchen, T., and Wernig, M. (2011). Direct lineage conversions: unnatural but useful? Nat. Biotechnol. *29*, 892–907.

Vierbuchen, T., Ostermeier, A., Pang, Z.P., Kokubu, Y., Südhof, T.C., and Wernig, M. (2010). Direct conversion of fibroblasts to functional neurons by defined factors. Nature *463*, 1035–1041.

Vilchez, D., Boyer, L., Morantte, I., Lutz, M., Merkwirth, C., Joyce, D., Spencer, B., Page, L., Masliah, E., Berggren, W.T., et al. (2012). Increased proteasome activity in human embryonic stem cells is regulated by PSMD11. Nature *489*, 304–308.

Vilchez, D., Boyer, L., Lutz, M., Merkwirth, C., Morantte, I., Tse, C., Spencer, B., Page, L., Masliah, E., Berggren, W.T., et al. (2013). FOXO4 is necessary for neural differentiation of human embryonic stem cells. Aging Cell *12*, 518–522.

Villeda, S.A., Luo, J., Mosher, K.I., Zou, B., Britschgi, M., Bieri, G., Stan, T.M., Fainberg, N., Ding, Z., Eggel, A., et al. (2011). The ageing systemic milieu negatively regulates neurogenesis and cognitive function. Nature *477*, 90–94.

Yoon, S.J., Wills, A.E., Chuong, E., Gupta, R., and Baker, J.C. (2011). HEB and E2A function as SMAD/FOXH1 cofactors. Genes Dev. *25*, 1654–1661.

Zaret, K.S., and Carroll, J.S. (2011). Pioneer transcription factors: establishing competence for gene expression. Genes Dev. *25*, 2227–2241.

Zhang, Y., Liu, T., Meyer, C.A., Eeckhoute, J., Johnson, D.S., Bernstein, B.E., Nusbaum, C., Myers, R.M., Brown, M., Li, W., and Liu, X.S. (2008). Model-based analysis of ChIP-Seq (MACS). Genome Biol. *9*, R137.

Zhang, X., Yalcin, S., Lee, D.F., Yeh, T.Y., Lee, S.M., Su, J., Mungamuri, S.K., Rimmelé, P., Kennedy, M., Sellers, R., et al. (2011). FOXO1 is an essential regulator of pluripotency in human embryonic stem cells. Nat. Cell Biol. *13*, 1092–1099.