Role of Pax3 acetylation in the regulation of *Hes1* and *Neurog2*

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ABSTRACT Pax3 plays a role in regulating *Hes1* and *Neurog2* activity and thereby stem cell maintenance and neurogenesis. A mechanism for Pax3 regulation of these two opposing events, during caudal neural tube development, is examined in this study. Pax3 acetylation on C-terminal lysine residues K437 and K475 may be critical for proper regulation of *Hes1* and *Neurog2*. Removal of these lysine residues increased *Hes1* but decreased *Neurog2* promoter activity. SIRT1 deacetylase may be a key component in regulating Pax3 acetylation. Chromatin immunoprecipitation assays showed that SIRT1 is associated with *Hes1* and *Neurog2* promoters during murine embryonic caudal neural tube development at E9.5, but not at E12.5. Overexpression of SIRT1 decreased Pax3 acetylation, Neurog2 and Brn3a positive staining. Conversely, siRNA-mediated silencing of SIRT1 increased these factors. These studies suggest that Pax3 acetylation results in decreased *Hes1* and increased *Neurog2* activity, thereby promoting sensory neuron differentiation.

INTRODUCTION

Pax3, a transcription factor and multifunctional regulatory protein, is expressed early in embryogenesis (Chalepakis and Gruss, 1995; Goulding et al., 1991, 1993; Epstein et al., 1991, 1993). In the nervous system, Pax3 is involved in neural tube closure (Li et al., 1999), neural crest development, and peripheral neuron differentiation (Goulding et al., 1991; Koblar et al., 1999). In muscle development, Pax3 ensures the survival of myogenic progenitor cells (Relaix et al., 2005) with Pax3-expressing progenitors giving rise to both skeletal and smooth muscle cells (Esner et al., 2006). Pax3 is also actively involved in adult regenerative myogenesis (Kuang et al., 2006).

Pax3 and its isoforms regulate downstream target genes (Mayanil et al., 2000, 2001, 2006; Lang et al., 2005; Wang et al., 2006;

Nakazaki et al., 2008). In a previous paper, we demonstrated that Pax3 regulates two basic helix-loop-helix (bHLH) transcription factor genes, *Hairy and enhancer of split homolog-1 (Hes1)* and *Neurogenin2 (Neurog2)* (Nakazaki et al., 2008), by binding to *cis*-regulatory elements on their promoters. *Hes1* and other *Hes* genes are important for neural stem cell maintenance (Shen et al., 2004; Theriault et al., 2005). Inactivation of *Hes1* accelerates early neurogenesis and decreases the number of late-born neurons (Nakamura et al., 2000). *Neurog2* plays a role in the acquisition of pan-neuronal properties (Scardigli et al., 2001; Sun et al., 2001; Lee and Paff, 2003), specification of neuronal subtypes (Ross et al., 2003, Schuurmans et al., 2004) sensory neurogenesis (Lo et al., 2002), and neural crest cell neurogenesis (Theriault et al., 2005).

In *Splotch* mutant mice, which do not express functional Pax3, neural crest cells undergo premature neurogenesis, potentially due to changes in regulation of bHLH transcription factors implicated in proliferation and differentiation (Nakazaki et al., 2008). Reeves et al. (1999) found that Pax3 expression is essential in maintaining the undifferentiated phenotype of immature ND7 cells, a rat dorsal root ganglion and mouse neuroblastoma hybrid cell line. In the absence of Pax3, these cells acquire many of the characteristics of mature neuronal cells. Overall, Pax3 appears to regulate stem cell maintenance and proliferation early in development, while initiating differentiation at a later time point. A

question that arises from these observations is how Pax3 regulates two opposing bHLH transcription factors, Hes1 and Neurog2.

Many transcription factor functions are controlled by acetylation (Bannister and Miska, 2000; Das and Kundu, 2005). For instance, GATA-1 activity is regulated by p300-mediated acetylation (Boyes et al., 1998). Other well-characterized targets of non–histone protein acetylation include important cellular factors, such as p53, nuclear factor-κB (NF-κB), p65, CBP, p300, STAT3, tubulin, PC4, nuclear receptors, c-Myc, hypoxia-inducible factor (HIF)-1a, FoxO1, heat-shock protein (Hsp)-90, HMG, E2F, MyoD, Bcr–Abl, the FLT3 kinase, and c-Raf kinase (Glozak et al., 2005; Yang and Seto 2008). Pax3 acetylation could also potentially regulate Hes1 and Neurog2, such that Hes1 levels decrease and Neurog2 levels increase when neurogenesis begins.

In this communication, we investigated Pax3 acetylation and its potential role in neuronal differentiation. We also examined whether acetylated Pax3 is a substrate for SIRT1 deacetylase. SIRT1 expression patterns in developing mouse embryos (Sakamoto et al., 2004) suggest that it may be involved in neurogenesis (Libert et al., 2008). Comparable expression patterns of Hes1 and SIRT1 suggest that the two proteins coordinate the process of neural development. SIRT1 binds to HES1 and HEY2 and enhances their transcriptional repression activities (Takata and Ishikawa, 2003). Our results support the hypothesis that acetylated Pax3 decreases Hes1 activity and promotes differentiation by increasing Neurog2 activity. Two C-terminal lysine residues, K437 and K475, may be critical in this process. Furthermore, acetylated Pax3 is a substrate for SIRT1 deacetylase, which associates with chromatin structures on Hes1 and Neurog2 promoters.

RESULTS

Acetylated Pax3 is a substrate for SIRT1
Previously, we demonstrated an interaction between acetylated Pax3 and the histone deacetylase HDAC1. We also noted occupancy by acetylated proteins on some TGFβ2 regulatory elements used by Pax3 (Mayanil et al., 2006). In the present work, we took a closer look at the relationship between Pax3 and SIRT1 at Hes1 and Neurog2 promoters. SIRT1 is involved in stem cell maintenance and differentiation during embryonic development, (Kim et al., 2007; Han et al., 2008), and it associates with HES1 (Takata and Ishikawa, 2003). Because Pax3 regulates Hes1 and Neurog2 (Nakazaki et al., 2008), we surmised that an interaction between Pax3 and SIRT1 could be relevant in stem cell maintenance and differentiation. We hypothesized that 1) Pax3 is acetylated and acetylated Pax3 is a substrate for SIRT1 deacetylase and 2) acetylated Pax3 decreases stem cell proliferation by decreasing Hes1 activity and promotes differentiation by increasing Neurog2 activity.

To examine Pax3 acetylation, DAOY cells transfected with Pax3 cDNA in pcDNA3 or pcDNA3 vector control were treated with sirtinol, a SIRT1 inhibitor (Araki et al., 2004) and curcumin (p300 acetyl transferase inhibitor) (Balasubramanyam et al., 2004). Cell lysates from treated cells were immunoprecipitated with anti–acetyl-lysine antibody and immunoblotted with Pax3 polyclonal antibody or vice versa (Figure 1, A and B). Immunoblot results showed low levels of acetylated Pax3 in nontreated controls and significant acetylated Pax3 in the presence of sirtinol but not in the presence of curcumin. These studies suggested that Pax3 is acetylated and a possible SIRT1 substrate.

To confirm that acetylated Pax3 is a SIRT1 substrate, DAOY cells were cotransfected with pcDNA3-Pax3 expression construct and SIRT1 siRNA, control siRNA, SIRT1 expression plasmid, or pcDNA3
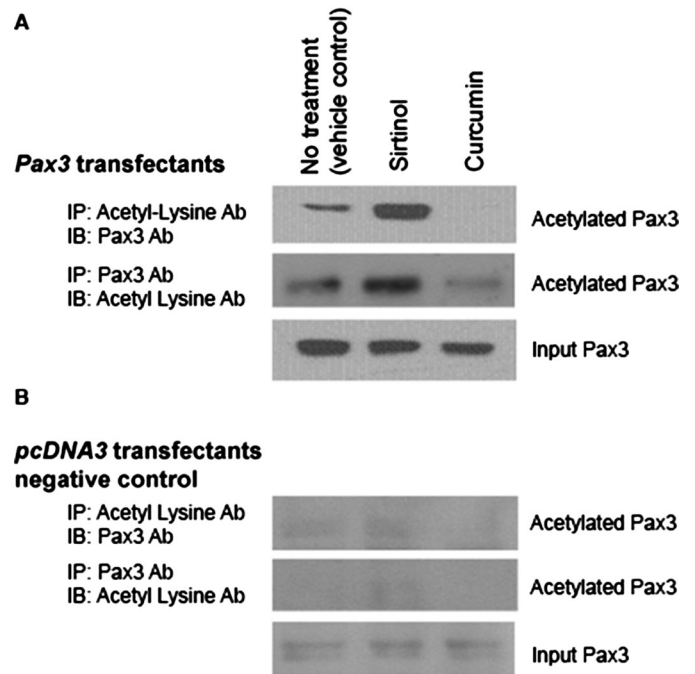

FIGURE 1: Pax3 acetylation. (A) DAOY cells transfected with Pax3-pcDNA3 expression plasmid were either not treated (vehicle control) or treated with sirtinol (100 μM) or curcumin (100 μM) for 4 h. Total cell lysates were immunoprecipitated with acetyl-lysine antibody and immunoblotted with Pax3 mAb, or immunoprecipitated with Pax3 mAb and immunoblotted with acetyl lysine polyclonal antibody. Pax3 input samples were immunoblotted to confirm the presence of Pax3. (B) DAOY cells transfected with pcDNA3 vector were used as negative Pax3 controls. These cells were treated as above. Minimal endogenous Pax3 levels, depicted in the input samples, were observed. Each experiment was performed in quadruplicate.

control plasmid (Figure 2, A and B). Cell extracts were prepared, immunoprecipitated with anti–acetyl-lysine antibody, and immunoblotted with Pax3 antibody (Figure 2A) or vice versa (Figure 2B). In the presence of SIRT1 siRNA, compared with control siRNA, Pax3 acetylation was more pronounced. When SIRT1 cDNA was cotransfected with Pax3 expression plasmid, Pax3 acetylation was reduced, compared with pcDNA3 control plasmid. This confirmed that Pax3 is acetylated and a substrate for SIRT1. SIRT1 cDNA did not completely abolish Pax3 acetylation; therefore, some acetylated lysine residues on Pax3 may not serve as SIRT1 substrates.

To show a direct interaction between Pax3 and SIRT1, coimmunoprecipitation assays were performed using ND7 cell lysates. Pax3 monoclonal antibody (mAb) was used for immunoprecipitation and SIRT1 polyclonal antibody for immunoblotting and vice versa. Mouse and rabbit immunoglobulin G (IgG) were used as negative controls. Figure 3 shows that control IgGs did not immunoprecipitate Pax3 or SIRT1. Pax3 mAb was able to immunoprecipitate SIRT1 (Figure 3A), and SIRT1 polyclonal antibody could immunoprecipitate Pax3 (Figure 3B). These studies show a direct interaction between Pax3 and SIRT1 in ND7 cells.

Acetylation of C-terminal lysine residues, K437 and K475, is SIRT1-sensitive
The C-terminal region (amino acids 298–481) of Pax3 (red brackets in Supplemental Figure S1) contains two lysine residues: K437 and K475 (underlined in Supplemental Figure S1). These lysine
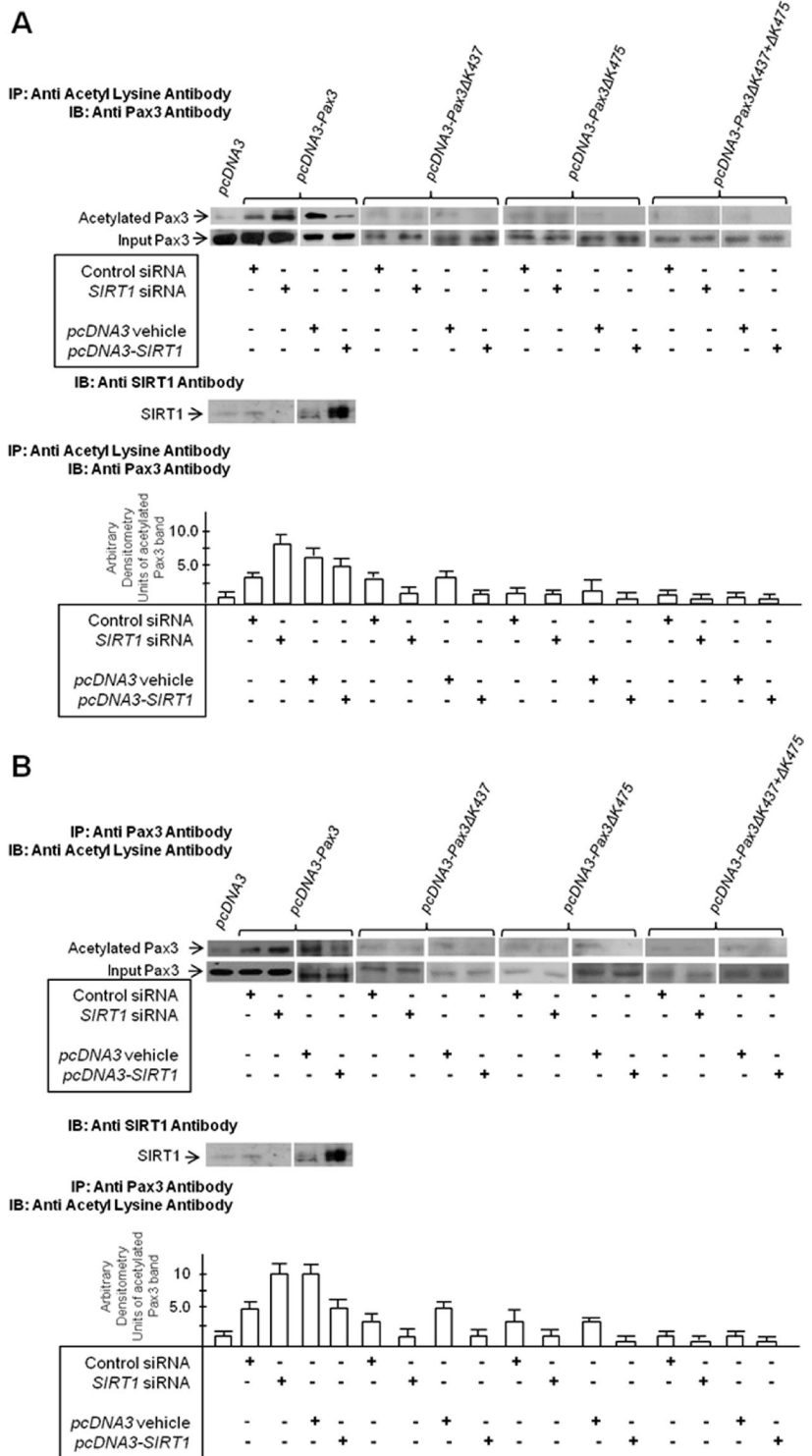
FIGURE 2: Acetylation of C-terminal lysine residues, K437 and K475, is SIRT1-sensitive. DAOY cells transfected with the following expression plasmids: *pcDNA3* vector control, Pax3-*pcDNA3*, *pcDNA3-Pax3ΔK437*, *pcDNA3-Pax3ΔK475*, or *pcDNA3-Pax3ΔK437+ΔK475*, were cotransfected with *SIRT1* cDNA in *pcDNA3*, *pcDNA3* vehicle, *SIRT1* siRNA, or control scrambled siRNA. At 48 h posttransfection, total cell lysates were tested for the presence of SIRT1. Lysates were immunoprecipitated (IP) with acetyl-lysine polyclonal antibody and immunoblotted (IB) with Pax3 polyclonal antibody as shown in (A) or immunoprecipitated with Pax3 antibody and immunoblotted with acetyl lysine antibody as shown in (B). Bands were quantified by densitometry and the band intensity (ratio of immunoprecipitated band intensity to input Pax3 band intensity) expressed as arbitrary densitometry units. Results are presented as arbitrary densitometry units + SEM. Each experiment was conducted in quadruplicate and each data point in duplicate.

residues undergo ubiquitination and are responsible for Pax3 degradation (Boutet *et al.*, 2007). Because the same lysine residues that get ubiquitinated may compete for acetylation (Benkirane *et al.*, 2010), we hypothesized that these lysine residues are acetylated and their deacetylation is SIRT1-sensitive.

To ascertain whether K437 and K475 play a role in SIRT1-sensitive Pax3 acetylation, we cotransfected wild-type *Pax3* (*pcDNA3-Pax3*) or *Pax3* lysine deletion mutants (*pcDNA3-Pax3ΔK437*, *pcDNA3-Pax3ΔK475*, or *pcDNA3-Pax3 ΔK437+ΔK475*) in combination with *SIRT1*-siRNA or *SIRT1*-cDNA into DAOY cells. Appropriate scrambled siRNA-negative controls and control cDNA were also used. At 48 h posttransfection, the cells were lysed, immunoprecipitation assays were performed with anti–acetyl lysine rabbit polyclonal antibody, and immunoblots were done with anti-Pax3 mouse mAb and vice versa (Figure 2, A and B). The results showed that K437 and K475 single-deletion mutants were not immunoprecipitated with acetyl lysine antibody or anti-Pax3 antibody. Trace amounts of Pax3 were precipitated from control siRNA and *SIRT1*-siRNA cotransfected cells. With K437 and K475 double mutants, acetyl lysine antibody did not precipitate Pax3, nor did acetyl lysine antibody detect Pax3 immunoprecipitated with Pax3 antibody. The data support the conclusion that Pax3 lysine residues K437 and K475 are acetylated and that these residues can be deacetylated by SIRT1.

C-terminal lysine residues, K437 and K475, regulate *Hes1* and *Neurog2* promoter activity

Because K437 and K475 undergo acetylation and are deacetylated by SIRT1, we hypothesized that they may be involved in regulating *Hes1* and *Neurog2* promoter activities. To test the hypothesis that K437 and K475 are important in Pax3 regulation of *Hes1* and *Neurog2*, we cotransfected *Hes1*- and *Neurog2*-promoter-luciferase reporter constructs with *pcDNA3* vector control, wild-type Pax3, Pax3 deletion mutants (*Pax3ΔK437*, *Pax3ΔK475*, *Pax3ΔK437* + *Pax3ΔK475*), or Pax3 substitution mutants (*Pax3K437→R*; *Pax3K475→R*, *Pax3K437→R+Pax3K475→R*). Vector *pgL3* was used as a negative control. The results showed that *Hes1* and *Neurog2* promoter activity significantly increased ($p < 0.001$) in the presence of wild-type Pax3, compared with *pcDNA3* transfected control (Figure 4).

Compared with wild-type Pax3, *Hes1* promoter activity increased significantly ($p < 0.001$) when K437 was deleted or substituted with arginine, but not when K475 was deleted or substituted with arginine. The double-lysine deletion or substitution mutants increased *Hes1* promoter activity similar to levels seen with the single K437 deletion mutant ($p < 0.001$). For the *Neurog2* promoter, K437 and K475 single or double deletions, or substitution mutants, decreased promoter activity compared with wild-type Pax3. These data suggest that K437 deacetylation increases *Hes1* activity, whereas K437 and K475 deacetylation decreases *Neurog2* activity.
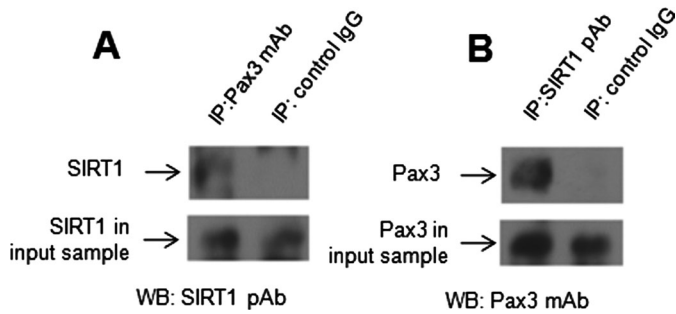
FIGURE 3: Pax3 interacts directly with SIRT1. A total of 200 μg (total protein) from undifferentiated ND7 cell lysates was used for immunoprecipitation assays. (A) Anti-Pax3 mAb and mouse IgG control were used for immunoprecipitation. The immune complex was pulled down with Protein A-sepharose and immunoblotted using anti-SIRT1 rabbit polyclonal antibody (pAb). (B) Anti-SIRT1 rabbit pAb and rabbit IgG control were used for immunoprecipitation. The immune complex was pulled down with Protein A-sepharose and immunoblotted using anti-Pax3 mAb. For panels A and B, input samples were run in separate lanes to verify SIRT1 and Pax3 levels, respectively.

SIRT1 is associated with *Hes1* and *Neurog2* promoters in the developing caudal neural tube at E9.5

If deacetylation influences *Hes1* and *Neurog2* levels in one direction, acetylation could bring about opposite outcomes. We evalu-
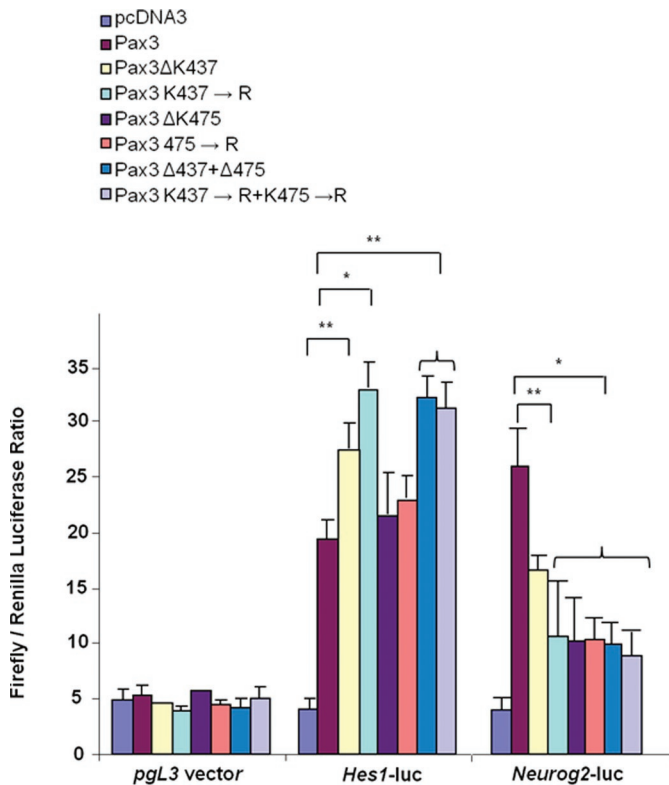

FIGURE 4: C-terminal lysine residues K437 and K475 in Pax3 regulate *Hes1* and *Neurog2* promoter activity. *Hes1*-promoter and *Neurog2*-promoter activity in a transient cotransfection experiment using *Hes1*- and *Neurog2*-promoter luciferase and *wild-type Pax3* expression construct or mutated *Pax3-pcDNA3* expression constructs as follows: *Pax3ΔK437*, *Pax3K437→R*, *Pax3ΔK475*, *Pax3K475→R*, *Pax3ΔK437+Δ475*, *Pax3K437→R+Pax3K475→R*. Each experiment was done in quadruplicate and each data point in triplicate. *, p < 0.001; **, p < 0.05 (Student's *t* test).

ated the association of deacetylases and acetylated proteins with chromatin structures on *Hes1* and *Neurog2* promoters. Chromatin immunoprecipitation assays were performed using mouse E9.5 and E12.5 caudal neural tubes. *Hes1* expression between E8.5 and E10.5 is important for neural tube closure (Ishibashi *et al.*, 1995; Nakazaki *et al.*, 2008). *Neurog2*, a marker of sensory neurogenesis, is present at E12.5 (Simmons *et al.*, 2001; Ribes *et al.*, 2008). At E9.5, there was at least threefold higher Pax3 immunoprecipitable *Hes1* than the *Neurog2* (Figure 5A). Conversely, more Pax3 immunoprecipitable *Neurog2* than *Hes1* was observed at E12.5 (Figure 5A). At E9.5, both *Hes1* and *Neurog2* promoters were associated with SIRT1 deacetylase. At E12.5, both promoters were associated with acetylated proteins. Additionally, p300 was present at E9.5, but not E12.5, on both promoters. HDAC1 was nondifferentially associated
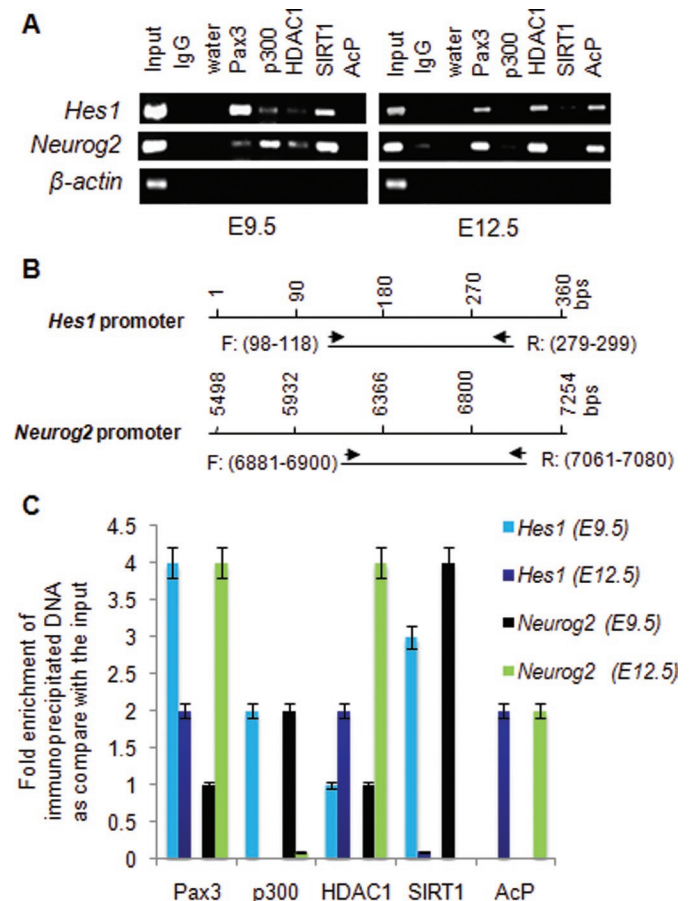

FIGURE 5: SIRT1 binds to *Hes1* and *Neurog2* promoters from E9.5, but not E12.5, mouse caudal neural tube. (A) ChIP assays were done with E9.5 and E12.5 mouse lumbar neural tube. ChIP compatible antibodies against Pax3, p300, HDAC1, SIRT1, and acetylated protein (AcP) were used to immunoprecipitate (IP) the protein–DNA complex. IgG was used as an IP-negative control. Murine *β-actin* primers were used as negative loading control. Amplified product was present only in the input and not in the control IgG or the immunoprecipitate. (B) The 200-bp amplified products using *Hes1* and *Neurog2* promoter primer sets are shown. Each ChIP experiment was performed in triplicate using one lumbar neural tube region per ChIP assay with a total of n = 4. (C) Immunoprecipitated DNA was subjected to quantitative PCR using murine primers for *Hes1* and *Neurog2* promoters. The data represent fold enrichment of immunoprecipitated DNA compared with input sample. Each ChIP experiment was performed in triplicate using one lumbar neural tube region per ChIP assay with a total n = 4.

with these promoters at either time point. These data indicate that 1) SIRT1 is associated with *Hes1* and *Neurog2* at E9.5 stage of neural tube development and 2) acetylated proteins are associated with *Hes1* and *Neurog2* promoters at E12.5. The presence of SIRT1 and the absence of acetylated proteins on the *Hes1* promoter at E9.5 may increase stem cell proliferation and maintenance at this earlier time point, whereas these conditions on the *Neurog2* promoter may decrease differentiation. The presence of acetylated proteins at a later time point, E12.5, may up-regulate *Neurog2* and influence differentiation. Western blot data showing that Hes1 and SIRT1 expression decrease in ND7 cells upon differentiation (Supplemental Figure S2) support this conclusion.

Acetylated *Pax*3 binds to *Hes1* and *Neurog2* promoter

To confirm binding of acetylated Pax3 to *Hes1* and *Neurog2* promoters, we performed electromobility shift assay (EMSA) as described in Nakazaki *et al.* (2008), using ND7 cell nuclear extracts. ND7 cells (a generous gift from M. Calissano, Institute of Child Health London) were obtained by fusing neonatal rat (E12) dorsal root ganglion neurons with mouse neuroblastoma cells (Latchman and Polak, 1995). Figure 6 shows that *Neurog2* and *Hes1* promoter oligonucleotides (30mer) bind Pax3 (control) as evident by the supershifted band in the presence of Pax3 antibody. Supershifted band intensity was stronger in nuclear extracts from ND7 cells treated with sirtinol (SIRT1 inhibitor) and minimal in nuclear extracts from ND7 cells treated with curcumin (acetyltransferase inhibitor) (Figure 6). Pax3 binding seen in control cells may be due to a mixed population of acetylated and nonacetylated Pax3. These observa-

FIGURE 6: Identification of *Neurog2* and *Hes1* promoters as biological targets of acetylated Pax3. (A) EMSA binding reactions were performed with 32P-labeled double-stranded oligonucleotides, *Neurog2* oligo: 5'- gacaccgtgctcggttccgggctgcgggga-3' and *Hes1oligo*: 5'-cattggccgccagaccttgtgcctagcggc-3', in the absence or presence of 100-fold molar excess of unlabeled oligonucleotide. Arrows indicate shifted and super-shifted bands. Nuclear extracts were from nontreated ND7 control cells in DMEM or from cells treated with curcumin (100 µM) or sirtinol (100 µM) for 4 h. Supershifted band intensity standarized to the control is shown in the histogram (average of three experiments ± SEM)

tions clearly demonstrate that acetylated Pax3 binds *Neurog2* and *Hes1* promoters.

SIRT1 overexpression decreases sensory neurogenesis in ND7 cells

To further examine the role of SIRT1 in cell proliferation and sensory neurogenesis, SIRT1 was overexpressed in ND7 cells. Proliferating ND7 cells were cultured in DMEM containing 10% fetal bovine serum and 10% donor horse serum. These hybrid cells were transfected with *pcDNA3-GFP* (vector control), or *SIRT1-flag-cDNA* in *pcDNA3-GFP* (Figure 7), or scrambled negative control siRNA-FITC or *SIRT1* siRNA (Supplemental Figure S3). At 48 h posttransfection, the cells were placed in growth media containing 20% serum and allowed to grow for 4 more days, after which they were immunostained for SIRT1, Hes1, Neurgo2, and Brn3a (Figure 7 and Supplemental Figure S3). SIRT1 overexpression increased Hes1 expression and decreased Neurog2 and Brn3a expression. Silencing SIRT1 with siRNA decreased Hes1 expression and increased Neurog2 and Brn3a immunostaining. This is in agreement with other studies showing an association between SIRT1 downregulation and neurogenesis (Prozorovski *et al.*, 2008).

SIRT1 overexpression increases Hes1 and decreases Neurog2 expression in chick embryos

To further examine SIRT1 regulation of Hes1 and Neurog2 expression in vivo, SIRT1 was overexpressed in chick embryos. Chick embryos at HH10–11 were electroporated with *SIRT1* cDNA on one side of the neural tube, with the opposite side serving as a control. Electroporated embryos were incubated at 38°C until HH15–16, at which time the neural tubes were harvested and stained for Hes1 and Neurog2. Analysis of sectioned embryos demonstrated that Hes1 expression increased, whereas Neurog2 expression decreased on the side of SIRT1 cDNA electroporation (Figure 8). These results suggest that, because Neurog2 is a proneural transcription factor, SIRT1 overexpression would affect neurogenesis adversely.

DISCUSSION

Many transcription factors are controlled by posttranslational modifications. Understanding how posttranslational modifications affect transcription factor function will answer critical questions about the mechanisms through which these molecules regulate key developmental processes (Singh *et al.*, 2010). Miller *et al.* (2008) showed that Pax3 is phosphorylated at serine 205 in proliferating cells but not in differentiating cells. In the present study, we suggest that Pax3 is a member of the group of acetylated transcription factors and that regulation of Pax3 acetylation plays a role in the switchover from stem cell proliferation to differentiation in caudal neural tube development.

Our results showed the following: 1) Pax3 is acetylated at lysine residues K437 and K475, and these residues are substrates for SIRT1 deacetylase. 2) K437 is involved in *Hes1* regulation; deletion or substitution of this residue to arginine up-regulates *Hes1* promoter-luciferase activity. 3) K437 and K475 are involved in regulating *Neurog2*; deleting or substituting these residues to arginine down-regulates *Neurog2* promoter-luciferase activity. 4) SIRT1 overexpression results in increased Hes1 expression and decreased Neurog2 in vivo (chick embryos) and also in nondifferentiating ND7 cells. Similarly, siRNA-mediated silencing of SIRT1 decreases Hes1 but increases Neurog2 and Brn3a expression in ND7 cells. 5) SIRT1 associates with *Hes1* and *Neurog2* in murine embryonic caudal neural tube at E9.5, suggesting that nonacetylated Pax3 may be responsible for regulating *Hes1* during this stage. SIRT1 does not
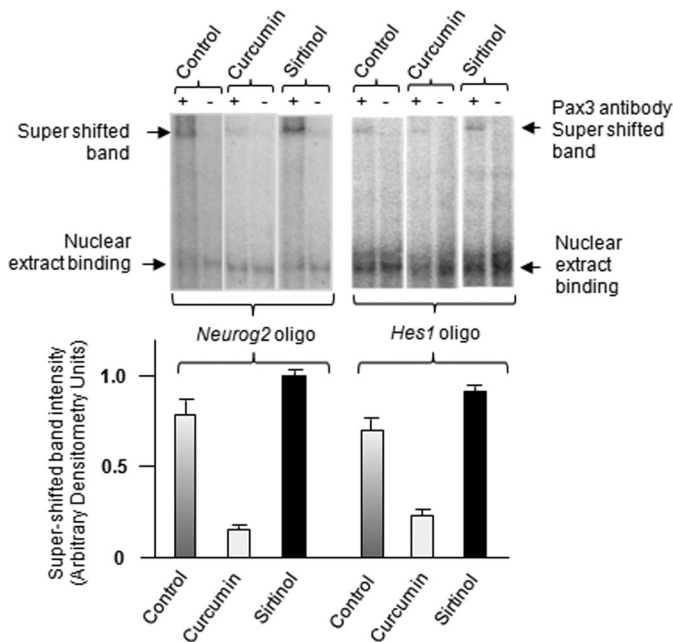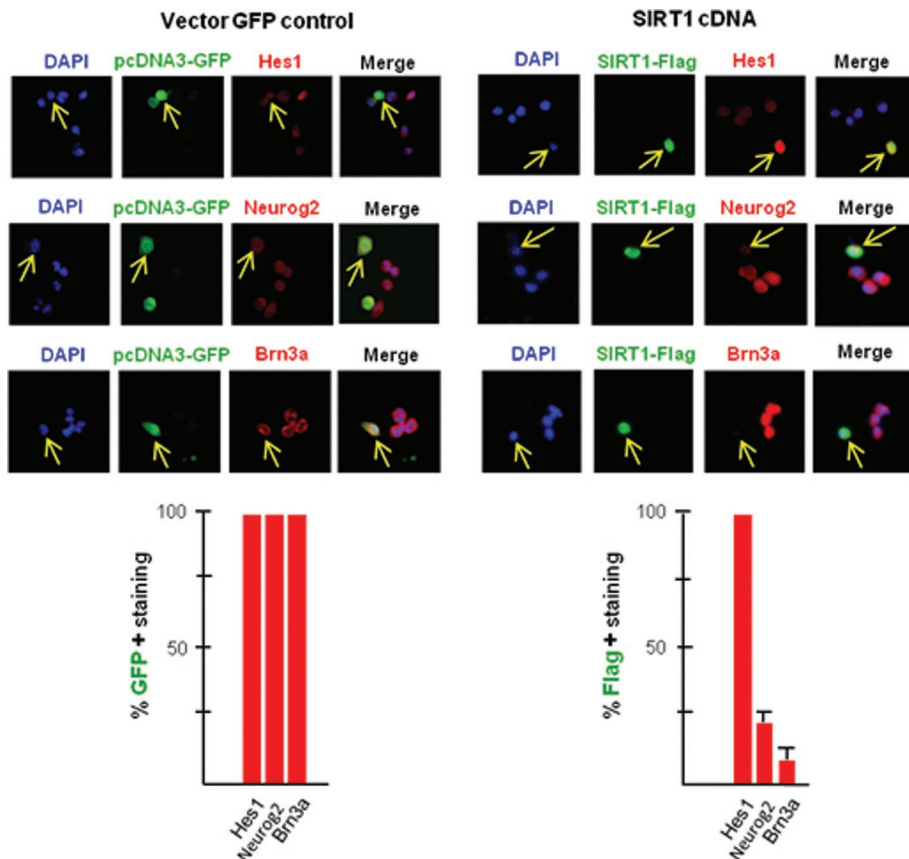
FIGURE 7: Overexpression of SIRT1 decreases Neurog2 and Brn3a expression. ND7 cells were transfected with *pcDNA3-GFP* or *SIRT1-flag-cDNA*. The cells were fixed and immunostained for Hes1, Neurog2, and Brn3a (sensory neuron marker) and Flag to ascertain SIRT1 expression in SIRT1-flag-cDNA transfected cells. Yellow arrows show that *pcDNA3-GFP* transfection did not change Hes1, Neurog2, or Brn3a expression in transfected cells. *SIRT1-flag-cDNA* transfection increased Hes1 expression and decreased Neurog2 and Brn3a expression in transfected cells. The histograms show an average ± SEM of three separate experiments.

associate with *Hes1* or *Neurog2* promoters in mouse embryonic caudal neural tube at E12.5. This suggests that acetylated protein(s) which are substrates of SIRT1 may be contributing to decreased stem cell proliferative activities and increased neurogenic activity, in particular acetylated Pax3 may down-regulate Hes1 and therefore stem cell proliferation and up-regulate Neurog2 to promote neurogenesis. Lysine residues K437 and K475 may play a role in the switch from stem cell proliferation to differentiation.

In these studies SIRT1 overexpression decreased Neurog2 and Brn3a (sensory neurogenesis markers) and increased Hes1 expression in nondifferentiated ND7 cells. Hisahara *et al.* (2008) reported that SIRT1 enhanced neuronal differentiation and decreased Hes1 expression in neural precursor cells. These results were observed under differentiating conditions during which SIRT1 transiently translocated into the nucleus from the cytoplasm. Our experiments were not performed under differentiating conditions; therefore it is unlikely that all SIRT1 translocated to the nucleus. Non-nuclear or cytoplasmic SIRT1 would deacetylate only transcription factors that are en route to the nucleus, whereas nuclear SIRT1 may deacetylate histones in addition to transcription factors. Prozorovski *et al.* (2008) suggested that SIRT1 participated in oxidation-mediated suppression of neurogenesis. It is possible that SIRT might play a distinct role on differentiation under different conditions and potentially in different cells. Hisahara *et al.* (2008) and Prozorovski *et al.* (2008) were working with murine embryonic brain cells, whereas our lab is

working with murine embryonic caudal neural cells. Further work is needed to dissect these differences.

Based on our work, a hypothetical model for the interaction of Pax3 and SIRT1 is proposed in Figure 9. In this model Pax3, acetylated by acetyltransferase, interacts with *Hes1* and *Neurog2* promoters. In the presence of SIRT1, as seen on these promoters at E9.5 (see Figure 5), Pax3 is deacetylated. This results in up-regulation of *Hes1* and promotes stem cell proliferation and maintenance. *Neurog2* is not expressed in the presence of deacetylated Pax3. At E12.5, when SIRT1 does not associate with *Hes1* and *Neurog2* promoters, acetylated Pax3 down-regulates *Hes1* and up-regulates *Neurog2* promoter activities. This promotes neurogenesis and reduces stem cell proliferation/maintenance. Although this model is overly simplistic, it highlights the possible role of acetylated Pax3 as a switch involved in regulating stem cell proliferation/maintenance and sensory neuronal differentiation.

In a recent study, we showed up-regulation of several miRNAs during early cell proliferation stages in Pax3 mutant "*Splotch*" homozygous embryos, which do not express functional Pax3 (Ichi *et al.*, 2010). Some of these miRNAs target the H3K27 histone demethylase KDM6B, decreasing KDM6B expression and increasing H3K27 methylation. We also reported that association between methylated H3K27 and *Hes1* promoter resulted in decreased *Hes1* expression and lowered stem cell proliferation. At the same time, we observed low association between methylated H3K27 and *Neurog2* promoter but high association between acetylated H3K9 and H3K18 and *Neurog2* promoter. These association patterns of methylated/acetylated histones on *Hes1* and *Neurog2* promoters could possibly result in premature neurogenesis as observed by Nakazaki *et al.* (2008). Overall, a complex model of Pax3 regulation of *Hes1* and *Neurog2* is emerging, in which Pax3 acetylation status along with H3K27 methylation and H3K9 and H3K18 acetylation may all interact to regulate these genes.

Another point at which Pax3 and SIRT1 may interact is at p53. Apoptosis is a component of neurogenesis (Nijhawan *et al.,* 2000). Pani *et al.* (2002) showed that p53 deficiency rescues not only apoptosis but also neural tube defects in *Splotch* (*Pax3* mutant) embryos. They concluded that Pax3 deficiency increased p53 protein levels. These results suggest that Pax3 regulates neural tube closure by inhibiting p53-dependent apoptosis, rather than by inducing neural tube-specific gene expression. Similarly, Gnc5 acetyltransferase is required for cranial neural tube closure in mouse embryos. Loss of Gnc5 acetyltransferase activity has been linked to neural tube closure defects and exencephaly. Deletion of p53 allows *Gnc5−/−* embryos to survive longer (Bu *et al.,* 2007). These results indicate that deletion of p53, which is a substrate of SIRT1, rescues neural tube defects in *Splotch* as well as in *Gnc5−/−* embryos. Our results showing Pax3 as a SIRT1 substrate suggest that Pax3 could potentially be a substrate for Gnc5 acetyltransferase. Thus a complex interaction may exist between p53, Pax3, SIRT1, and Gnc5 acetyltransferase.
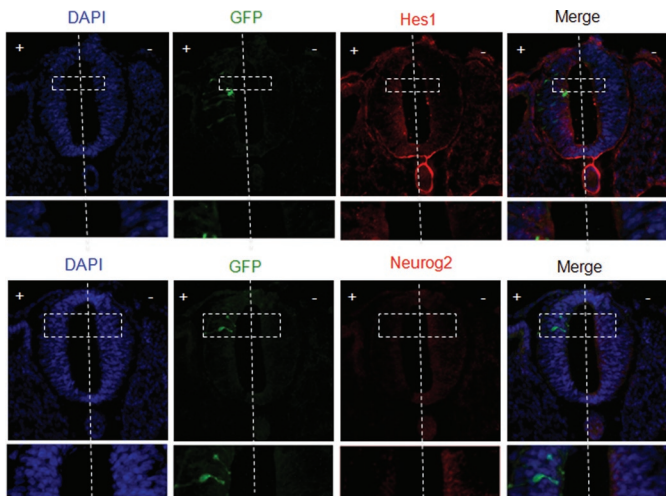
FIGURE 8: Regulation of Hes1 and Neurog2 expression by SIRT1 overexpression in vivo. (A) Murine *SIRT1* cDNA and *GFP* containing plasmids were coelectroporated into the + side of chick embryonic neural tubes, and the tubes stained with anti-Hes1 and anti-Neurog2 antibodies. GFP expression was used as a positive control for electroporation. The vertical line demarcates the two sides. *SIRT1* cDNA electroporation increased Hes1 and decreased Neurog2 immunostaining as shown in the inset (n = 6).

An anti-apoptotic role for SIRT1 has been demonstrated for apoptosis induced by p53 (Luo *et al.*, 2001; Vaziri *et al.*, 2001), and this may be another regulatory mechanism involved in neurogenesis. Overall, Pax3, SIRT1, p53, and Gnc5 acetyltransferase and the many tran-

scription factors delineated as regulators of neurogenesis (LaBonne and Bronner-Fraser, 1999) will most likely function in complex regulatory loops that have yet to be elucidated.

MATERIALS AND METHODS
Antibodies and reagents

The following antibodies were used: anti-p300/CBP rabbit polyclonal antibody (Ab2832), anti–acetylated protein rabbit polyclonal antibody (AP) (Ab193), and anti-HDAC1 rabbit polyclonal antibody (Ab7028) from Abcam (Boston, MA); anti-SIRT1 rabbit polyclonal antibody (sc-15404); anti-Hes1 rabbit polyclonal (sc-25392), anti-Neurog2 goat polyclonal (sc-19233), and anti–Lamin B goat polyclonal (sc-6217) from Santa Cruz Biotechnologies (Santa Cruz, CA); anti-Pax3 rabbit polyclonal antibody (CA1010) from EMD Biosciences (Calbiochem; San Diego, CA); anti-Tuj1 mAb from Covance (Princeton, NJ); anti-Brn3a (Ab5945) rabbit polyclonal antibody from Chemicon (Billerica, MA); anti–acetyl-lysine (9441) rabbit polyclonal antibody from Cell Signaling Technology (Beverly, MA); anti-Pax3 mouse mAb from Developmental Studies Hybridoma Bank (Iowa City, IA). Other reagents used: IgG from rabbit serum (I-5006), curcumin (C1386–5G) and EGF (E9644) from Sigma (St. Louis, MO); sirtinol (566321) from EMD Biosciences; bFGF (233-FB) from R&D Systems (Minneapolis, MN); and growth factor reduced Matrigel (35–6230) from BD Biosciences (San Diego, CA).

Removal of embryonic neural tube

The day the vaginal plug was found was noted as E0.5. Embryos were collected at E9.5 and E12.5. Caudal neural tubes were isolated as described in Bennett *et al.* (1998).
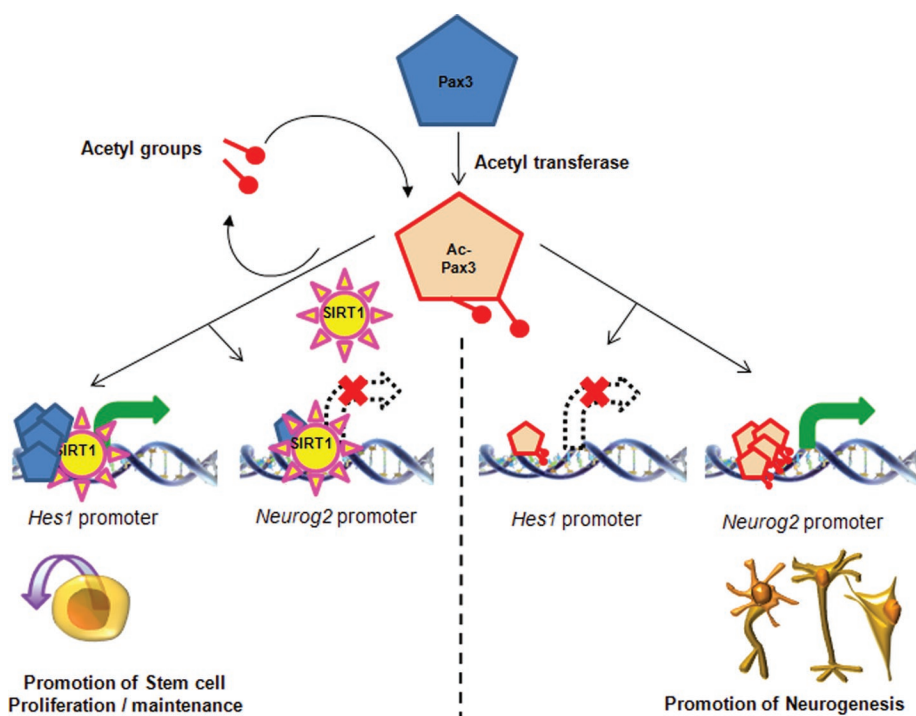
Transfection, immunoprecipitation, immunoblotting, and immunofluorescence

Lipofectamin2000 (11668–027; Invitrogen, Carlsbad, CA) reagent was used for all cotransfection as per the manufacturer's instructions. siPORT NeoFX Transfection Agent (AM4510; Ambion, Austin, TX) and MegaTran 1.0 (OriGene Technologies TT200002, Rockville, MD) were used for siRNA transfection and cDNA transfection respectively, as per the manufacturers' instructions. To lyse cells, DAOY or ND7 cells were washed and collected in cold PBS and then lysed on ice for 30 min in cell lysis buffer containing 20 mM Tris-HCl (pH 7.0), 150 mM NaCl, 1 mM EDTA, 1% Triton X-100, 2.5 mM sodium pyrophosphate, 1 mM sodium vanadate. Protease inhibitor (Sigma P2714) was added just before lysis. Immunoprecipitation was carried out with polyclonal anti–acetylated lysine antibody (9441), polyclonal anti-Pax3 antibody (CA1010), monoclonal anti-Pax3 antibody (Developmental Studies Hybridoma Bank), and polyclonal anti-SIRT1 antibody (sc-15404) as per the manufacturers' instructions. The immunoprecipitates were collected with 50 µl of 50% slurry of protein A Agarose (Sigma P7786). The precipitates were washed three times in lysis buffer, boiled in 2× SDS sample buffer for 5 min,

FIGURE 9: Hypothetical model for the regulation of *Hes1* and *Neurog2* via Pax3 and SIRT1. Pax3 is acetylated by acetyltransferase; acetylated Pax3 binds to *Hes1* and *Neurog2* promoters. When SIRT1 is associated with *Hes1* promoter, deacetylated Pax3 up-regulates *Hes1* expression. When SIRT1 is associated with *Neurog2* promoter, deacetylated Pax3 does not up-regulate *Neurog2*. The overall effect is stem cell proliferation and maintenance. When SIRT1 is not associated with these promoters, acetylated Pax3 binds the promoters resulting in downregulation of *Hes1* and up-regulation of *Neurog2*. The overall effect is promotion of neurogenesis.

and centrifuged for 1 min. Proteins in the supernatant were resolved in 8% SDS–PAGE and immunoblotted with antibodies described below. For immunofluorescence, cultured ND7 cells, both nondifferentiated and differentiated, were fixed with 4% paraformaldehyde in PBS and blocked with 10% normal donkey serum/0.1% Triton X-100/0.01% NaAzide in PBS. Cells were then incubated overnight with appropriate antibodies. The next day, cells were incubated with secondary antibodies followed by nuclear staining with DAPI (D8417; Sigma).

Primary antibodies used for immunoblotting included anti-Pax3 rabbit polyclonal (1:1000), anti-SIRT1 rabbit polyclonal (1:1000), anti-Hes1 rabbit polyclonal (1:500), anti–Lamin B goat polyclonal (1:2000), anti–acetyl-lysine rabbit polyclonal (1:500), and anti-Pax3 mouse monoclonal (1:100). Secondary antibodies included anti–rabbit and anti–goat HRP-conjugated antibodies. Antibody binding was revealed by enhanced chemiluminescence detection (Amersham Pharmacia Biotech, Piscataway, NJ). Primary antibodies for immunofluorescence included anti–rabbit SIRT1 primary polyclonal, anti–mouse Brn3a primary monoclonal, and anti–rabbit Brn3a polyclonal. Secondary antibodies were donkey anti–rabbit AlexaFluor 488 (green fluorescence) and donkey anti–mouse Cy3 (red fluorescence).

Chromatin immunoprecipitation (ChIP)

ChIP assays on caudal neural tube from E9.5 and E12.5 embryos were performed as described in Mayanil *et al.* (2006). Mouse monoclonal Pax3 antibody, rabbit polyclonal p300, HDAC1, SIRT1, and acetylated lysine antibody were used for ChIP. PCR was performed with primers for murine *Hes1* and *Neurog2* promoter regions (Nakazaki *et al.*, 2008). All ChIP samples were tested for false-positive PCR amplification by sequencing the 200-bp amplified product. False-positive PCR amplification was ruled out by amplifying a sequence from the murine *β-actin* promoter: forward 5′-gtgggc-cgccctaggcaccag-3′ and reverse 5′-ctctttgatgtcacgcacgatttc-3′. This sequence failed to give any PCR product in the immunoprecipitates. Primers for PCR amplification of murine *Hes1* promoter were as follows: forward (bp98–118) 5′-ttggctgaaagttactgtgg-3′ and reverse (bp279–299) 5′-tcttagggctacttagtgat-3′ and for murine *Neurog2* promoter: forward (bp6881–6900) 5′-ggcagatctgatt-gttttct-3′ and reverse (bp7061–7080) 5′gcagctcgcccgagtctcgt-3′. A 200-bp fragment was amplified in each case (Nakazaki *et al.*, 2008).

Electromobility shift assays

Probes were prepared for EMSA by annealing complementary oligonucleotides representing selected regions of murine *Hes1* and *Neurog2* promoters, followed by 5′-end labeling with [γ-32P]ATP by T4 polynucleotide kinase. For EMSA experiments, nondifferentiated ND7 cells were treated with 100 μM of curcumin or sirtinol for 4 h, 0.5 μg of nuclear extract protein was then purified and incubated in 20 μl of reaction mixture containing 15 mM Tris (pH 7.5), 6.5% glycerol, 90 mM KCl, 0.7 mM EDTA, 0.2 mM dithiothreitol, 1 mg/ml bovine serum albumin, 50 μM pyrophosphate, 300 ng of salmon sperm DNA as a competitor to reduce nonspecific DNA binding, and 30,000 dpm per nmol of 32P-labeled double-stranded oligonucleotide probe. EMSA was done as described (Mayanil *et al.*, 2001, 2006; Nakazaki *et al.*, 2008). Double-stranded 30-mer oligonucleotide probes were made for the following regions: *Hes1* promoter *Hes1oligo*: 5′-cattggccgccagaccttgtgcctagcggc-3′ and *Neurog2* promoter *Neurog2*oligo: 5′- gacaccgtgctcggttccgggct-gcggga-3′. For supershift assays 0.1 μg polyclonal Pax3 antibody was preincubated on ice for 30 min with nuclear extract prior to the

addition of labeled oligonucleotides. After 20-min incubation, free DNA and DNA protein complexes were resolved in 4% polyacrylamide gels using 0.25× TBE as the running buffer. Electrophoresis was performed at 4°C at 300 mV and 30 mA for 3 h. Gels were dried and subjected to PhosphorImager (Amersham Biosciences) analysis to view shifted bands.

Quantitative real-time PCR

Each 20 μl of real-time (RT)-PCR reaction included 1.25 μl forward primer, 1.25 μl reverse primer, 2.5 μl water, 5 μl immunoprecipitated DNA (1 ng/μl), and 10 μl of the double-stranded DNA binding dye SYBR Green I to detect PCR product (PerfeCTa SYBR Green FastMix, Low ROX [95074–250]; Quanta Biosciences, Gaithersburg, MD). RT-PCR cycle parameters were 95°C for 10 min and 95°C for 10 s, followed by 58°C for 50 s and 72°C for 32 s (7500 Fast Real-Time Thermal Cycler; Applied Biosystems, Foster City, CA). The following murine primers were used: *Hes1* forward 5′-ggtcctaacgcagtgtcac-ctt-3′ and reverse 5′-cagtggcctgaggctctca-3′; *Neurog2* (Accession# AF303001) forward 5′-agaggtggcccttgcaatc-3′; and reverse 5′-ca-cacgccatagtcctctttga-3′; β-actin forward 5′-acggccaggtcatcactattg-3′ and reverse 5′-tggatgccacaggattcca-3′. Primers were designed using Primer Express software (PerkinElmer Life Sciences, Waltham, MA) and synthesized by Eurofins MWG Operon (Huntsville, AL).

Analysis of *Hes1* and *Neurog2* promoter activities

To investigate the effect of Pax3 C-terminal lysine residues, K437 and K475, on *Hes1* and *Neurog2* promoter activities, plasmids containing *Hes1* promoter-luciferase (−467 to +46, 0.2 μg) or *Neurog2*-promoter luciferase (1.2-kb fragment; bp5498–7254, 0.2 μg) were transiently cotransfected with *Pax3-pcDNA3*, *pcDNA3* vector control, or different Pax3 lysine mutants into DAOY cells. Luciferase assays were done as described in Mayanil *et al.* (2006), using the Dual Luciferase kit from Promega (Madison, WI). *Renilla luciferase* plasmid, *pRL-null* (5 ng/well), was simultaneously transfected as an insertional control to assess transfection efficiency. *Hes1* and *Neurog2* promoter activities were evaluated as described in Nakazaki *et al.* (2008). *Hes1* promoter construct *Hes1* (−467 to +46) luciferase was provided by R. Kageyama (Takebayashi *et al.*, 1994), and *Neurog2* promoter constructs were kindly provided by Jane Johnson (Simmons *et al.*, 2001).

Pax3 mutants were made with the QuickChangeXL site-directed mutagenesis kit (Stratagene, La Jolla, CA). Six mutants were made in a *Pax3-pcDNA3* expression construct, three deletion mutants: *Pax3ΔK437* (lysine 437 deleted), *Pax3ΔK475* (lysine 475 deleted), and *Pax3ΔK437ΔK475* (lysine 437 and 475 deleted), and three substitution mutants, with lysine substituted by arginine: *Pax3K437→R*, *Pax3K475→R*, and *Pax3K437→R+K475→R* mutants.

Nuclear extract preparation

Nuclear extracts from ND7 cells were prepared by using NE-PER® Nuclear and Cytoplasmic Extraction Reagents (78833; Pierce Biotechnology, Rockford, IL) as per manufacturer's instructions.

Chick embryo electroporation and immunohistochemistry

White Leghorn fertilized chicken eggs were purchased from Phil's Fresh Egg (Forreston, IL) and incubated at 38°C for an appropriate period. Embryos were staged according to Hamburger and Hamilton (1951) and processed as described in Sauka-Spengler and Barembaum (2008). Embryos at HH10–11 were used for in ovo electroporation. Plasmid-encoding SIRT1 (*pcDNA3-Sir2α*, 2.0 μg/μl, a gift from Wei Gu) together with EGFP (*pcDNA3-EGFP*, 2.0 μg/μl, addgene 13031) was injected into nearly closed neural tubes, with a

PICOSPRITZERIII microinjector (Parker, Cleveland, OH). After injection, a parallel fixed and angled tip electrode (Pomona Electronics, Everett, WA) was placed on both sides of the neural tube and five 30-ms, 18-V electric pulses were applied with an electroporator (TSS20 Ovodyne Electroporator and EP21 Current Amplifier; INTRACEL, Royston, UK). Embryos were then incubated at 38°C until HH15–16, and electroporated neural tubes expressing EGFP were dissected under a Leica MZ16F microscope. Dissected neural tubes were then fixed in 4% PFA/PBS with 0.01% Triton X-100 and embedded in gelatin. Frozen neural tube sections (10 μm) were immunostained with anti–Hes1 and Neurog2 antibodies followed by staining with Cy3-conjugated donkey antibodies (711–165-152 and 705–165-147, respectively; Jackson ImmunoResearch, West Grove, PA). DAPI (Sigma; D8417) was used for counterstaining nuclei. Immunostained sections were observed and photographed with a Leica DMIRB inverted microscope.

Statistical analysis
P values were determined by Student's *t* test using the GraphPad Prism Version 5.0.

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