Role of Pax3 Acetylation in the Regulation of Hes1 and Neurog2

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Abbreviations used: SIRT1, sirtuin (silent mating type information regulation 2 homolog) 1; Hes1, hairy and enhancer of split 1; Neurog2, Neurogenin2; ChIP, Chromatin Immunoprecipitation.

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. Over-expression 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; Nakazaki et al., 2008; Wang et al., 2006; Lang et al., 2005). 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 (Theriault et al., 2005; Shen et al., 2004). 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 (Lee and Paff, 2003; Scardigli et al., 2001; Sun et al., 2001), specification of neuronal subtypes (Schuurmans et al., 2004; Ross et al., 2003) 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 basic helix-loop-helix 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 does Pax3 regulate 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-kB (NF-kB), 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 if acetylated Pax3 is a substrate for SIRT1
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). Since 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: a) Pax3 is acetylated and acetylated Pax3 is a substrate for SIRT1 deacetylase; b) 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 1A and B). Immunoblot results showed low levels of acetylated Pax3 in non-treated 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 co-transfected with pcDNA3-Pax3 expression construct and SIRT1 siRNA, control siRNA, SIRT1 expression plasmid or pcDNA3 control plasmid (Figure 2A and B). Cell extracts were prepared, immunoprecipitated with anti-acetyl-lysine antibody and immunoblotted with Pax3 polyclonal antibody or vice versa (Figure 1A and B). Immunoblot results showed low levels of acetylated Pax3 in non-treated 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 show a direct interaction between Pax3 and SIRT1, co-immunoprecipitation assays were performed using ND7 cell lysates. Pax3 monoclonal antibody was used
for immunoprecipitation and SIRT1 polyclonal antibody for immunoblotting and vice versa. Mouse and rabbit IgG were used as negative controls. Figure 3 shows that control IgGs did not immunoprecipitate Pax3 or SIRT1. Pax3 monoclonal antibody 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 residues undergo ubiquitination and are responsible for Pax3 degradation (Boutet et al., 2007). Since 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 co-transfected 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. 48 hrs post-transfection, the cells were lysed, immunoprecipitation assays were performed with anti-acetyl lysine rabbit polyclonal antibody and immunoblots were done with anti-Pax3 mouse monoclonal antibody and immunoblots were done with anti-Pax3 mouse monoclonal antibody and vice versa (Figure 2A 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 co-transfected 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 supports 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**

Since 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 co-transfected 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. This data suggests that K437 deacetylation increases Hes1 activity, whereas K437 and K475 deacetylation decreases Neurog2 activity.

**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 evaluated 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 three-fold 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 non-differentially associated with these promoters at either time point. This data indicates that (i) SIRT1 is associated with Hes1 and Neurog2 at E9.5 stage of neural tube development; (ii) 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, while 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 Pax3 Binds to Hes1 and Neurog2 Promoter**

To confirm binding of acetylated Pax3 to Hes1 and Neurog2 promoters we performed EMSA as described in Nakazaki et al. (2008), using ND7 cell nuclear extracts. ND7 cells (a generous gift from Dr. 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 non acetylated Pax3. These observations clearly demonstrate that acetylated Pax3 binds Neurog2 and Hes1 promoters.

**SIRT1 Over-Expression Decreases Sensory Neurogenesis in ND7 Cells**

To further examine the role of SIRT1 in cell proliferation and sensory
neurogenesis SIRT1 was over-expressed in ND7 cells. Proliferating ND7 cells were cultured in DMEM containing 10% FBS and 10% donor horse serum. These hybrid cells were transfected with pcDNA3-GFP (vector control), or SIRT1-flag-cDNA in pcDNA3-GFP (Fig 7), or scrambled negative control siRNA-FITC or SIRT1 siRNA (Supplemental Fig S3). 48 hours post transfection 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, Neurog2 and Brn3a (Figure 7 and Supplemental Figure S3). SIRT1 over-expression 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 Over-Expression Increases Hes1 and Decreases Neurog2 expression in Chick Embryos**

To further examine SIRT1 regulation of Hes1 and Neurog2 expression in vivo, SIRT1 was over-expressed 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 since Neurog2 is a proneural transcription factor, SIRT1 overexpression would affect neurogenesis adversely.

**DISCUSSION**

Many transcription factors are controlled by post-translational modifications. Understanding how post-translational 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 switch-over from stem cell proliferation to differentiation in caudal neural tube development.

Our results showed that (i) Pax3 is acetylated at lysine residues K437 and K475, and these residues are substrates for SIRT1 deacetylase. (ii) K437 is involved in Hes1 regulation; deletion or substitution of this residue to arginine up-regulates Hes1 promoter-luciferase activity. (iii) K437 and K475 are involved in regulating Neurog2; deleting or substituting these residues to arginine down-regulates Neurog2 promoter-luciferase activity. (iv) SIRT1 over-expression results in increased Hes1 expression and decreased Neurog2 in vivo (chick embryos) and also in non-differentiating ND7 cells. Similarly, siRNA mediated silencing of SIRT1 decreases Hes1, but increases Neurog2 and Brn3a expression in ND7 cells. (v) SIRT1 associates with Hes1 and Neurog2 in murine embryonic caudal neural tube at E9.5, suggesting that non-acetylated Pax3 may be responsible for regulating Hes1 during this stage. SIRT1 does not 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 over-expression decreased Neurog2 and Brn3a (sensory neurogenesis markers) and increased Hes1 expression in non-differentiated 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 only deacetylate 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 out 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 over-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, where 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. 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 transcription 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; 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; anti-Pax3 rabbit polyclonal antibody (CA1010) from EMD Biosciences (Calbiochem); anti-Tuj1 monoclonal antibody from Covance; anti-Brn3a (Ab5945) rabbit polyclonal antibody from Chemicon; anti-acetyl-lysine (9441) rabbit polyclonal antibody from Cell Signaling Technology; anti-Pax3 mouse monoclonal antibody from Developmental Studies Hybridoma Bank. Other reagents used: IgG from rabbit serum (I-5006), curcumin (C1386-5G) and EGF (E9644), from Sigma; sirtinol (566321) from EMD Biosciences; bFGF (233-FB) from R&D Systems; and growth factor reduced Matrigel (35-6230) from BD Biosciences.

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 (Invitrogen 11668-027) reagent was used for all co-transfection as per the manufacturer's instructions. siPORT™ NeoFX™ Transfection Agent (Ambion AM4510) and MegaTran 1.0 (OriGene TT200002) were used for siRNA transfection and cDNA transfection respectively, as per the manufacturer's 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 (Cell Signaling 9441), polyclonal anti-Pax3 antibody (Calbiochem CA1010), monoclonal anti-Pax3 antibody (Developmental Studies Hybridoma Bank) and polyclonal anti-SIRT1 antibody (sc-15404) as per the manufacturer's 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 2x SDS sample buffer for 5 min, 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 non-differentiated 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 (Sigma, D8417).

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 (ECL) detection (Amersham Pharmacia Biotech). Primary antibodies for immunofluorescence included: anti-rabbit SIRT1 primary polyclonal, anti-mouse Brn3a primary monoclonal, 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'gtggcgcctaggcaccag-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: forward (bp98–118) 5'-ttggctgaagttactgtgg-3' and reverse (bp279–299) 5'-tcttaggctacttagtga-3’ and for murine Ngn2 promoter: forward (bp6881–6900) 5'gacgatctctctggctctc-3' and reverse (bp7061–7080) 5'gcagctcgccgggtctgat-3’. A 200-bp fragment was amplified in each case (Nakazaki et al., 2008).

Electro Mobility 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, non-differentiated ND7 cells were treated with 100μM of curcumin or sirtinol for 4 hours, 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 and Nakazaki et al., 2008). Double-stranded 30-mer oligonucleotide probes were made for the following regions: Hes1 promoter Hes1oligo: 5’-cattggccgacgatgtgctacgggc-3’ and Neurog2 promoter Neurog2oligo: 5’-gacaccgtgctgtccgggtgcgga-3’. For supershift assays 0.1 μg polyclonal Pax3 antibody was preincubated on ice for 30 min with nuclear extract prior to addition of labeled oligonucleotides. After 20-min incubation, free DNA and DNA protein complexes were resolved in 4%, polyacrylamide gels using 0.25X 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 RT-PCR reaction included 1.25 μl forward primer, 1.25 μl reverse primer, 2.5 μl water, 5 μl immunoprecipitated DNA (1ng/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). RT-PCR cycle parameters were 95°C for 10 min and 95°C for 10 sec. followed by 58°C for 50 sec and 72°C for 32 sec (Applied Biosystem 7500 Fast Real-Time Thermal Cycler). The following murine primers were used: Hes1 forward 5’-ggcttcaacgcgtcagctc-3’ and reverse 5’-cagtggcctgaggctctca-3’; Neurog2 (Accession# AF303001) forward 5’-agaggtgcccctgcaatc-3’; and reverse 5’-acacgcccatagtcctttga-3’; β-actin forward 5’-acggccaggtcatcactattg-3’ and reverse 5’-tgatggccacacgattcca-3’; Primers were designed using Primer Express software (PerkinElmer Life Sciences) and synthesized by Eurofins MWG Operon.

**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 co-transfected with Pax3-pcDNA3, pcDNA3 vector control or different Pax3 lysine mutants into DAOY cells and luciferase assays were done as described in Mayanil et al. (2006), using the Dual Luciferase kit from Promega. Renilla luciferase plasmid, pRL-null (5 ng/well), was simultaneously transfected as an insertional control to asses 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 Dr. R. Kageyama (Takebayashi et al., 1994) and Neurog2 promoter constructs were kindly provided by Dr Jane Johnson (Simmons et al., 2001)

Pax3 mutants were made with the QuickChangeXL site-directed mutagenesis kit (Stratagene): 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 (Pierce Biotechnology 78833) 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 (HH) (1951) and processed as described in Sauka-Spengler and Barenbaum (2008). Embryos at HH10-11 were used for in-ovo electroporation. Plasmid-encoding SIRT1 (pcDNA3-Sir2α, 2.0 µg/µl, a gift from Dr 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 30msec 18V 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 M216F microscope. Dissected neural tubes were then fixed in 4%PFA/PBS with 0.01% Triton X-100 and embedded in Gelatin. 10µm frozen neural tube sections were immunostained with anti-Hes1 and Neurog2 antibodies followed by staining with Cy3- conjugated donkey antibodies (Jackson ImmunoResearch, 711-165-152 and 705-165-147 respectively). DAPI (Sigma, D8417) was used for counter staining 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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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 hrs. Total cell lysates were immunoprecipitated with acetyl-lysine antibody and immunoblotted with Pax3 monoclonal antibody, or immunoprecipitated with Pax3 monoclonal antibody and immunoblotted with acetyl lysine polyclonal antibody. Pax3 input samples were immuno-blotted 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.
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 co-transfected with SIRT1 cDNA in pcDNA3, pcDNA3 vehicle, SIRT1 siRNA or control scrambled siRNA. 48 hrs post-transfection 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.
Figure 3: Pax3 interacts directly with SIRT1. 200 µg (total protein) from undifferentiated ND7 cell lysates was used for immunoprecipitation assays. A. Anti-Pax3 monoclonal antibody (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 polyclonal antibody (pAb) and rabbit IgG control were used for immunoprecipitation. The immune complex was pulled down with Protein A-sepharose and immunoblotted using anti-Pax3 monoclonal antibody (mAb). For A and B input samples were run in separate lanes to verify SIRT1 and Pax3 levels, respectively.
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 co-transfection 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 int in triplicate. p values: * is <0.001 and ** is <0.05 (Student T test).
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 represents 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.
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'-gacaccgtgcgtcggctgcgggga-3' and Hes1 oligo: 5'-cattggccgccagacctgtgcctagcggc-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 non-treated ND7 control cells in DMEM, or from cells treated with curcumin (100 µM), or sirtinol (100 µM) for 4 hrs. Supershifted band intensity standardized to the control is shown in the histogram (Average of 3 experiments ± SEM)
**Figure 7:** Over-expression 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 3 separate experiments.
Figure 8: Regulation of *Hes1* and *Neurog2* expression by SIRT1 over-expression *in vivo*. A. Murine *SIRT1* cDNA and *GFP* containing plasmids were co-electroporated 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).
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 down-regulation of Hes1 and up-regulation of Neurog2. The overall effect is promotion of neurogenesis.
**Supplemental Figure S1:** C-terminal lysine residues of Pax3. The C-terminal region (amino acids 298-481) of Pax3 (in red brackets) contains two lysine residues K437 and K475 (underlined). These lysine residues undergo ubiquitination and are responsible for Pax3 degradation (Boutet *et al.*, 2007).

**Supplemental Figure S2:** Down-regulation of Pax3, SIRT1 and Hes1 in differentiated ND7 cells. Immunoblots were done with Pax3, SIRT1 and Hes1 antibodies on undifferentiated and differentiated ND7 cell nuclear extracts. Lamin B was a loading control for nuclear extracts. UD= undifferentiated; D=differentiated.

**Supplemental Figure S3:** siRNA mediated silencing of SIRT1 in ND7 cells decreases Hes1, but increases Neurog2 and Brn3a expression. ND7 cells were transfected with a negative control FITC conjugated siRNA or with SIRT1 specific siRNA (Santa Cruz Biotechnology). The cells were fixed and stained for SIRT1, Hes1, Neurog2 and Brn3a. There was no change in the expression of Hes1, Neurog2 and Brn3a in FITC positive cells. Yellow arrows indicate that silencing SIRT decreased Hes1, but increased Neurog2 and Brn3a expression.