The role of histone acetylation in SMN gene expression

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Increasing survival motor neuron 2 (SMN2) gene expression may be an effective strategy for the treatment of spinal muscular atrophy (SMA). Histone deacetylase (HDAC) inhibitors have been shown to increase SMN transcript and protein levels, but the specific role of histone acetylation in regulating SMN gene expression has not been explored. Using chromatin immunoprecipitation, we investigated the levels of acetylated H3 and H4 histones and HDACs associated with different regions of the human and mouse SMN genes in both cultured cells and tissues. We show that the SMN gene has a reproducible pattern of histone acetylation that is largely conserved among different tissues and species. A limited region of the promoter surrounding the transcriptional start site has relatively high levels of histone acetylation, whereas regions further upstream or downstream have lower levels. After HDAC inhibitor treatment, acetylated histone levels increased, particularly at upstream regions, correlating with a 2-fold increase in promoter activity. During development in mouse tissues, histone acetylation levels decreased and associated HDAC2 levels increased at the region closest to the transcriptional start site, correlating with a 40–60% decrease in SMN transcript and protein levels. These data indicate that histone acetylation modulates SMN gene expression and that pharmacological manipulation of this epigenetic determinant is feasible. HDAC2, in particular, may be a future therapeutic target for SMA.

INTRODUCTION

Spinal muscular atrophy (SMA) is an autosomal recessive motor neuron disease caused by mutation of the survival motor neuron (SMN1) gene (1). A highly related, centromeric form of the gene, SMN2, is retained in variable copy number in all SMA patients. Owing to a C to T base change in a splice-enhancer region of exon 7, the majority of transcripts from SMN2 lack exon 7 and encode a truncated protein (2,3). Approximately 10–25% of SMN2 transcripts are full-length. SMA is the consequence of reduced levels of functional SMN protein, with clinical severity of disease correlating inversely with SMN2 copy number and SMN protein levels (4,5).

SMN is a ubiquitously expressed protein with a well-established function in spliceosome assembly (6). SMN is present in and is necessary for the survival of all cells that are dependent on RNA splicing, evoking the question of why motor neurons are uniquely sensitive to reduced SMN levels. It has been postulated that SMN may be required for normal motor neuron development. SMN protein is highly expressed in the embryo and fetus, but decreases post-natally in mice and humans (7–10). Recently, SMN has been shown to be enriched at the leading edge of neuronal growth cones (11–13), and knockdown of SMN in a zebrafish model was found to impair motor axon outgrowth and path-finding (14).

A promising strategy for treatment of SMA is to increase SMN protein levels, which might be achieved by activating the SMN2 promoter. The human SMN1 and SMN2 gene promoters are nearly identical in sequence and activity (15,16). The principal transcriptional initiation site for the SMN genes is located 163 bp upstream of the translation initiation site. A second transcriptional initiation site has been mapped 246 bp upstream and appears to be used during fetal development (17). Mice have a single Smn gene and its promoter shares sequence homology with the human SMN promoters in an ~150 bp region upstream of the translation initiation site. This region also contains the sequences necessary for minimal promoter activity (17,18). Nevertheless, regulatory sequences relevant to SMN expression have been found as

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far as 4.6 kb upstream of the transcription initiation site in the human genes and 1.2 kb upstream in the mouse gene (15–20).

Several studies have demonstrated that compounds which inhibit histone deacetylases (HDACs) activate the human SMN2 promoter (21) and increase SMN transcript and protein levels *in vitro* (21–24). HDACs, acting in opposition to histone acetyltransferases (HATs), control the level of histone acetylation, a post-translational modification of nucleosomal histones that influences gene expression (25,26). Histone acetylation promotes gene transcription by relaxing chromatin structure and facilitating access to DNA by the transcriptional machinery, whereas histone deacetylation promotes a condensed chromatin state and transcriptional repression. HDAC inhibitors are now being studied in clinical trials in SMA patients (27); however, the mechanism by which they activate SMN2 expression remains unknown. Furthermore, the specific role of histone acetylation in regulating SMN expression has not been explored.

The aim of this study was to investigate the role of histone acetylation in mouse and human SMN gene expression. We show that the SMN gene has a characteristic pattern of histone acetylation that is altered after HDAC inhibitor treatment and during the course of development. These data suggest an important role for histone acetylation in regulating SMN gene expression and have important implications for future SMA therapeutics.

## RESULTS

### The SMN gene has a characteristic pattern of histone acetylation

Chromatin immunoprecipitation (ChIP) assays measure the interaction of protein with DNA in a given chromatin environment within the context of a cell (28,29). We used ChIP coupled with quantitative PCR to investigate the level of acetylated H3 and H4 histones and RNA polymerase II (Pol II) associated with different segments of the SMN gene (Fig. 1).

We first examined the acetylation pattern of the endogenous mouse *Smn* gene in chromatin isolated from NSC34 cells. This cell line was chosen because it is derived from motor neurons and retains motor neuron characteristics including the ability to generate action potentials and to synthesize, store and release acetylcholine (30). For comparison, we simultaneously examined, in these cells, the mouse β-globin gene in the region of the translation start site. Histones associated with this gene have been shown to be relatively hypoacetylated in tissues where the gene is transcriptionally silent, but hyperacetylated in erythroid cells where the gene is highly expressed (31). We found that regions surrounding the *Smn* transcriptional start site, designated MSP3 and MSP4, had the highest levels of acetylated H3 and H4 histones (Fig. 2). In contrast, regions ~1.3–3.5 kb upstream and a region downstream in intron 1 were associated with lower levels of acetylated histones. These low acetylation levels were comparable to the levels seen at the β-globin gene, a gene which we confirmed to be transcriptionally silent in this cell type (discussed later). The regions of *Smn* with the highest levels of acetylated histones also showed the greatest enrichment of Pol II; Pol II was present at >2-fold higher levels at MSP3 and at >5-fold higher levels at MSP4 than at the MSP0–2 or MSP5 Smn regions (Fig. 3).

We next examined the transgenic human SMN2 gene in these stably transfected NSC34 cells. A similar pattern of histone acetylation was observed with relatively high levels of acetylated histones in the regions surrounding the transcriptional start site, HuSP3 and HuSP4 (Fig. 2). In contrast, regions HuSP1 and HuSP2, 1.3–2.5 kb upstream, had lower acetylated histone levels. Pol II also showed the greatest enrichment in the HuSP3 and HuSP4 regions; ~2–3-fold higher than HuSP1 and HuSP2 (Fig. 3).

In NSC34 cells, the human SMN2 gene is a randomly inserted transgene in a mouse chromatin environment. In order to examine the human SMN2 gene in its native chromatin environment, we analyzed the levels of acetylated histones associated with the endogenous human SMN2 gene in chromatin isolated from cells derived from an SMA patient. The 3813 fibroblast cell line, derived from a type I SMA patient, is known to have two copies of the SMN2 gene and deletion of the exon 7 and 8 regions in both SMN1 genes (21). A similar pattern of histone acetylation as that observed at the mouse *Smn* gene and the transgenic human SMN2 gene was present at the endogenous human SMN2 gene in 3813 cells (Fig. 2). We also observed this pattern in preparations of chromatin isolated from the 9677 and 2906 fibroblast cell lines (derived from patients with SMA type I and type III with two and four copies of SMN2, respectively) (data not shown).

### HDAC inhibitors activate the SMN2 promoter and modify histone acetylation at the SMN2 gene

The transcriptional activity of the transgenic human SMN2 promoter was assayed in NSC34 cells that were treated with suberoyl anilide hydroxamic acid (SAHA) at doses ranging from 0 to 1 μM, valproic acid (VPA) at doses ranging from 0 to 5 mM or valpromide (VPM) at doses ranging from 0 to 5 mM for 24 h. Promoter activity showed a dose-dependent increase with SAHA and VPA treatment, with a maximum induction of ~2-fold with 1 μM of SAHA treatment and 2.5 mM of VPA treatment (Fig. 4). Some decrease in promoter activity was seen with higher doses of SAHA (data not shown) and VPA. VPM, a structural analog of VPA lacking HDAC inhibitory activity *in vitro* (32), did not increase SMN2 promoter activity. We have previously shown that VPA also increases SMN transcript and protein levels in fibroblast cell lines derived from SMA patients (21). We have observed similar effects with SAHA treatment in SMA fibroblast cell lines (unpublished data).

In order to investigate whether HDAC inhibitors might activate SMN2 by directly modifying the histone acetylation pattern at the promoter, we performed ChIP after NSC34 cells had been treated with SAHA (0, 0.5 and 1.0 μM) for 24 h. After treatment with SAHA, the transgenic human promoter in NSC34 cells showed a 2-fold increase in acetylated H3 histones and 2–3-fold increase in acetylated H4 histones at upstream promoter regions, HuSP1 and HuSP2 (Fig. 5); regions of the promoter that were relatively hypoacetylated at baseline. Downstream regions, HuSP3 and HuSP4, which
were relatively hyperacetylated at baseline, showed less marked increases in histone acetylation levels.

We next performed a time course experiment to examine histone acetylation changes with HDAC inhibitor treatment at the endogenous SMN2 gene. The 9677 fibroblast cell line was treated with VPA 2 mM for 4, 9, 24 or 72 h. All VPA-treated time points showed an \( \times 2 \)-fold increase in acetylated H3 histone levels when compared with untreated cells at the HuSP1 and HuSP2 regions, with little change in the downstream SMN2 regions (data not shown). Acetylated H4 levels increased by \( \times 2 \)-fold at the HuSP2 site with 4, 9 and 24 h of VPA treatment and at the HuSP1 site with 9 h of treatment. No change or mild decreases in H4 acetylation were seen at other time points and gene regions (data not shown).

The endogenous human SMN2 gene was next examined in the 3813 fibroblast cell line treated with VPA at doses of 1 or 5 mM for 4 h in three independent experiments. Upstream regions showed a maximum 4-fold increase in acetylated H3 histones and 5-fold increase in acetylated H4 histones with VPA treatment, with little change in histone acetylation levels observed in the downstream regions. We observed similar increases in histone acetylation in the 2906 fibroblast cell line treated with SAHA at a dose of 1 \( \mu \)M for 48 h (data not shown).

### Smn gene expression decreases during development

Previous studies have shown that SMN protein levels decrease with development (7–10); however, it was not known previously whether this was due to transcriptional or...
post-translational mechanisms. In order to characterize the expression patterns of the mouse \textit{Smn} gene in different tissues during the course of development, brain, spinal cord, kidney and liver tissues were taken from three mice at each the embryonic day 15 (E15), post-natal days 1, 7, 15 (P1, P7 and P15) and adult time points. Mouse SMN transcript levels were determined by quantitative reverse transcription–polymerase chain reaction (RT–PCR) and compared with two different endogenous RNA controls, 18S and \( \beta \)-2-microglobulin. Overall, an \( 40\% - 60\% \) decrease in SMN RNA was observed between the embryonic and the adult time points in all tissues relative to 18S RNA (Fig. 6A). An \( 85\% - 95\% \) decrease was observed relative to \( \beta \)-2-microglobulin mRNA (data not shown). At each time point, the level of SMN transcript in liver was consistently \( 30\% - 35\% \) of the other tissues. Interestingly, spinal cord showed \( 20\% \) less transcript than brain and kidney at the P1 time point, suggesting that SMN is down-regulated earlier in spinal cord than in other tissues. In both brain and spinal cord, the nadir of SMN transcript levels was the P15 time point with some recovery at the adult time point, whereas the nadir was reached at the adult time point in liver and kidney. Thus, the net change between the embryonic and the adult time points in liver, a \( 66\% \) decrease, was greater than in brain, a \( 40\% \) decrease. The level of mouse SMN transcript in NSC34 cells was \( 2.2 \pm 0.3 \)-fold higher than in E15 brain, indicating that SMN transcript is expressed at higher levels in this motor neuron cell line when compared with mouse brain, spinal cord or kidney. We also evaluated the levels of the \( \beta \)-globin transcript in these tissues and found that mouse \( \beta \)-globin was expressed at high levels in embryonic liver, a site of embryonic hematopoeisis, but was essentially transcriptionally silent \((>99\% \) decreased\) in adult liver and neural tissues. When expressed relative to embryonic liver, the \( \beta \)-globin transcript levels were as follows: embryonic liver = \( 1.01 \pm 0.05 \) (SEM), adult liver = \( 0.01 \pm 0.01 \), embryonic brain = \( 0.08 \pm 0.01 \), adult brain = \( 0.01 \pm 0.00 \) and NSC34 cells = \( 0.00 \pm 0.00 \).

Figure 2. Acetylated H3 and H4 histones are enriched in a restricted region surrounding the transcriptional start site of \textit{SMN}. Using ChIP, the baseline H3 and H4 acetylation patterns were evaluated at the endogenous \textit{Smn} gene in NSC34 cells, the transgenic human \textit{SMN2} gene in NSC34 cells and the endogenous human \textit{SMN2} gene in 3813 fibroblasts isolated from a type I SMA patient. The acetylation state of the mouse \( \beta \)-globin gene was assessed in NSC34 cells as a negative control. Each value represents the average of three independent chromatin preparations taken from cells harvested on different days (except the HuSP2 site in 3813 fibroblasts, which represents the average of two chromatin preparations). Error bars represent the SEM. The arrow indicates the region closest to the transcription initiation site of the \textit{SMN} genes.
Western blotting confirmed a developmental decrease of SMN protein, as has been previously described. Western blots were performed after quantifying total protein in triplicate in two separate protein quantification assays. In each blot, the density of the SMN band was divided by the density of either the actin or the tubulin endogenous controls. There was variability in actin and tubulin between tissues, making direct comparisons between tissues difficult. In particular, embryonic liver consistently showed lower levels of actin when compared with other tissues (Fig. 6B). Embryonic liver also showed a second, smaller SMN protein species not seen in other tissues. This band was not previously reported in human fetal liver (5). This may indicate species differences; however, it should be noted that different antibodies were used which might also explain this discrepancy. Within each tissue, an ~50–85% decrease in the SMN to actin and the SMN to tubulin ratios was observed between the embryonic and the adult time points in all tissues examined (Fig. 6C). The decrease in mouse kidney contrasts with the reported lack of developmental down-regulation in human kidney (10).

**Histone acetylation of Smn changes during development**

In order to study histone acetylation during the course of development, ChIP was used to assay liver and brain tissues from embryonic and adult time points. Spinal cord was not examined because the mass of embryonic spinal cord tissue that could be obtained at the embryonic time point was insufficient. Overall, the pattern of histone acetylation at the Smn gene in tissues was similar to that observed in cell culture, with relative hyperacetylation in regions surrounding the transcriptional start site, MSP3 and MSP4, and hypoacetylation in upstream regions, MSP0, MSP1 and MSP2 and in intron 1 of the coding region, MSP5. Hyperacetylated regions were 5–12-fold more acetylated than hypoacetylated regions in both liver and brain (Fig. 7). When comparing embryonic to adult tissues, acetylation of histones H3 and H4 decreased at the MSP4 site; the region closest to the transcriptional start site of the SMN gene. H3 acetylation decreased by 34% and H4 acetylation decreased by 38% in liver (Fig. 7), corresponding to a 66% decrease in SMN transcript (Fig. 6A). In brain, H3 acetylation decreased by 21% and H4 acetylation decreased by 7% at the MSP4 site, corresponding to a 40% decrease in SMN transcript. Generally, other upstream and downstream regions showed little change in acetylation during development, except MSP3 and MSP5 where we observed an increase in acetylation between the embryonic and the adult time points, primarily limited to liver. Histone acetylation was also measured at the mouse β-globin gene in the same samples. As expected, low
acetylation levels comparable to those observed in upstream regions of the Smn promoter were seen in both embryonic and adult brain where the β-globin gene is expressed at negligible levels (discussed previously). In liver, a major site of embryonic hematopoiesis, there was an 88% decrease in H3 acetylation and a 76% decrease in H4 acetylation between the embryonic and the adult time points (Fig. 7), corresponding to a >99% decrease in β-globin transcript levels (discussed previously).

**HDAC1 and HDAC2 association with the Smn gene increases during development**

In order to explore whether specific HDAC enzymes might be involved in SMN regulation, we performed ChIP using antibodies specific for HDACs 1–5. In three chromatin preparations prepared from each embryonic and adult brain, we observed little to no association of HDAC 4 or 5 with the Smn gene (data not shown). A low level association of HDAC3 with Smn was detected in embryonic brain, but this likely represented non-specific background as the same level of protein enrichment was seen at all sites of the Smn gene and in the β-globin gene (data not shown). In contrast, HDAC1 showed an association with the Smn gene specifically at the MSP4 site, which increased by 55% between the embryonic and the adult time points (Fig. 8). However, most striking was the association of HDAC2 with Smn. In embryonic brain, HDAC2 was enriched specifically at the MSP4 site and the binding levels increased by 311% in adult tissue (Fig. 8). There was little change in the levels of HDAC1 or HDAC2 binding to other regions of Smn. We also confirmed binding of HDAC1 and HDAC2 to the human SMN2 gene. In preparations of chromatin from 3813 cells that were untreated or treated with VPA, we observed some enrichment of HDAC1 and high levels of enrichment of HDAC2 at the HuSP3 and HuSP4 sites (Fig. 8).

**DISCUSSION**

In humans and transgenic mice, SMA disease severity correlates with SMN2 copy number and SMN protein levels. Because expression of eight copies of the SMN2 gene rescues the SMA phenotype evident in mice with reduced SMN levels, it has been postulated that increasing SMN2 expression in SMA patients could be an effective treatment for SMA. HDAC inhibitors have been shown to activate the SMN2 promoter and increase SMN transcript and protein in vitro; however, the role of histone acetylation in directly regulating the SMN gene has not been explored.

Here, we show that the SMN gene has a characteristic pattern of H3 and H4 histone acetylation that is conserved between the mouse and the human genes and in different
tissue types; regions of the gene immediately surrounding the transcriptional start site are associated with relatively high levels of histone acetylation, but regions ≥1 kb upstream and downstream within intron 1 are relatively hypoacetylated. Although few mammalian genes have been studied this way, this general pattern of histone acetylation may be common to many active genes. For example, a similar pattern has been reported in the active human interferon-β and mouse β-globin genes (31,33). Furthermore, genome wide studies of histone acetylation in yeast and in Drosophila have shown that the greatest histone acetylation is usually seen in the 5′ transcribed region of active genes (34,35).

Acetylation of nucleosomal histones is a major determinant of chromatin structure and gene activity, with the degree of histone acetylation correlating with gene transcription (35). Not surprisingly, regions of SMN that showed the highest levels of histone acetylation also showed the highest levels of Pol II binding, marking these regions as the most transcriptionally active. These regions also correspond with regions of the SMN promoter previously demonstrated to have the highest activity in promoter fragment studies (15,16,18).

Most recently, Rouget et al. (19) have refined the core sequence necessary for human SMN promoter activity to 15 bp upstream and 117 bp downstream of the transcriptional start site and for mouse Smn promoter activity to 46 bp upstream and 125 bp downstream of the transcriptional start site. These core sequences show the highest promoter activity, whereas the addition of upstream sequences represses this activity, suggesting the presence of negative regulatory elements upstream. The sequence of the core region is conserved between the mouse and the human genes and contains binding sites for the cAMP-response element binding (CREB) and the Sp families of proteins (19,36). The CREB family of transcription factors is known to recruit the highly potent HAT, p300/CREB binding protein (CBP) (37). Recruitment of p300/CBP to SMN could be responsible for the high acetylation levels observed in this region.

Although the overall pattern of SMN histone acetylation is conserved in different species and tissues, we did observe specific changes in acetylation associated with the decrease in mouse Smn gene expression that occurs during development. Using quantitative RT–PCR, we showed that Smn expression decreases by 40–60% between the embryonic and the adult time points, and using ChIP from liver and brain tissues, we showed a decrease in acetylated H3 and H4 levels specifically at the MSP4 site. The decrease in acetylation during development is modest, but small changes in acetylation can be associated with a large change in gene expression. (A) Quantitative RT–PCR was used to measure SMN transcript levels in brain, spinal cord, kidney and liver at embryonic day 15, post-natal days 1, 7, 15 and adult time points. Each data point represents the average of three mouse tissue samples for each time point. 18S RNA was used as an endogenous control. All values were normalized to a single E15 brain sample. Error bars represent the SEM. (B) A representative western blot showing SMN and actin protein levels in E15 brain, spinal cord (SC), kidney and liver tissues when compared with adult tissues. (C) Summary of quantification of SMN protein levels in tissues when controlling for actin or tubulin loading. Each data point represents the average of two sets of protein lysates. Values are normalized to the E15 sample for each set of lysates.
expression. Induction of the mouse β-globin gene by DMSO, for example, has been shown to produce only a 2-fold increase in β-globin H3 acetylation with a 150-fold increase in transcript (38). Neighboring Smn regions, MSP3 and MSP5, showed mild increases in acetylation in liver, but we believe the decrease at MSP4 is the most relevant, as this is the most transcriptionally active region of this gene. Furthermore, this developmental, regionally specific decrease in acetylation was associated with a large increase in HDAC2 and a smaller increase in HDAC1 association with the same region.

HDACs can be divided into three major classes based on sequence homology to yeast HDACs. Class I includes HDACs 1, 2, 3 and 8, which are ubiquitously expressed and found almost exclusively in the nucleus; class II includes HDACs 4, 5, 7 and 9, which show some tissue specificity and shuttle between the nucleus and the cytoplasm and class III includes the adenine dinucleotide-dependent Sir2 family of HDACs (39,40). Using the ChIP methodology, we could detect no association of HDACs 3, 4 or 5 with SMN, but we were able to demonstrate binding of HDAC1 and HDAC2 to both the human and the mouse genes, strongly suggesting that these enzymes may play important roles in regulating the expression of SMN. HDAC1 and 2 are known to be recruited to genes by the Sp1 and Sp3 proteins (40). Binding of members of the Sp family of proteins to the SMN gene has been implicated in the down-regulation of SMN gene expression that occurs with P19 cell differentiation (19). It is not surprising that HDAC1 and 2 were found together, as they often coexist in distinct multiprotein complexes that control gene expression, including the Sin3, NuRD and coREST complexes (40). HDAC1 and 2 have also been previously implicated in the developmental regulation of genes. They appear to play a role in the tissue-specific changes in gene expression that occur during mammalian epithelial differentiation, for example (41).

HDAC inhibitors have been shown to activate the SMN2 promoter, and here we show that this activation is associated with increases in histone acetylation levels at the SMN2 gene. Surprisingly, the greatest increase in acetylation was not seen in downstream regions, where we show binding of HDAC1 and 2, but rather in upstream regions. This result may imply that VPA and SAHA inhibit other HDACs that bind to upstream SMN2 regions, but have little impact on HDAC1 and HDAC2 downstream. Alternatively, it is known that HDACs not only deacetylate histones but also non-histone proteins that are transcriptional regulators (39). HDAC inhibitor treatment could result in acetylation and activation of a DNA binding factor that recruits HATs and leads to acetylation of the SMN2 gene in upstream regions.
Regardless, HDAC inhibitors appear to activate SMN2 expression, at least in part, by modifying acetylation directly at the SMN2 gene. Perhaps, drugs that specifically target HDAC1 and HDAC2 in the regions surrounding the transcriptional start site might be more effective at increasing SMN2 expression. The timing of inhibition may also be important. SMA may be due to deficiency of SMN, a critical stage in motor neuron development. Global knockdown of SMN in zebrafish during development causes a specific abnormality of motor neuron outgrowth and branching (14). Heterozygous Smn+/− mice have been shown to have motor neuron loss specifically between birth and 6 months, during a period of decreasing SMN protein levels (9). Inhibition of HDAC2 during the early post-natal period may be effective at restoring normal SMN levels.

In summary, we have shown that SMN has a reproducible pattern of histone acetylation that is modified by HDAC inhibitor treatment and during the course of development. HDAC1 and HDAC2 bind to SMN and likely modify acetylation patterns at the transcriptional initiation region. Together, these data strongly suggest that histone acetylation plays an important role in SMN regulation and that this epigenetic determinant can be manipulated pharmacologically. HDAC2, in particular, may be an important target for future SMA therapeutics.

**MATERIALS AND METHODS**

**Cell culture and treatment**

NSC34 cells, mouse motor neuron–neuroblastoma hybrid cells, stably transfected with a 3.4 kb fragment of the human SMN promoter linked to a β-lactamase reporter gene (21) were maintained in Dulbecco’s minimal essential medium containing 5% heat-inactivated fetal bovine serum, 1% penicillin–streptomycin–gentamicin and 1% geneticin (Gibco, Carlsbad, CA, USA). Human type I SMA (GM03813 and GM09677) fibroblast cell lines with two copies of the SMN2 gene (Coriell Cell Repositories, Camden, NJ, USA) and human type III SMA 2906 cell line (provided by T. Crawford at Johns Hopkins University) with four copies of the SMN2 gene were maintained in minimal essential medium containing 15% fetal bovine serum and 1% penicillin–streptomycin–gentamicin. For drug treatment experiments, cells were treated with media alone or with media containing VPA (Sigma, St Louis, MO, USA), VPM (Katwijk Chemie BV, The Netherlands) or suberoylanilide hydroxamic acid (SAHA, Calbiochem, San Diego, CA, USA).

**Mouse tissue**

C57/Bl6 mice (Charles River Labs, Wilmington, MA, USA) were anesthetized with isoflurane then sacrificed by cervical
dislocation (P7, P15 and adult) or decapitation (E15 and P1). Brain, spinal cord, kidney and liver tissues were dissected from mice at ages E15, P1, P7, P15 and 3 months and were flash frozen in liquid nitrogen. Tissue was thawed on ice immediately before use for protein extraction, RNA extraction or ChIP.

Promoter activity

β-Lactamase activity (SMN2 promoter activity) was determined by measuring the cleavage of the CCF-2AM β-lactamase fluorescent substrate (Panvera, Invitrogen, Carlsbad, CA, USA) as previously described (21). Cells were plated 500 000 cells per well in 96-well plates and incubated in serum-free media with or without drug for 24 h. The Cytoflour 4000 fluorescent plate reader (Applied Biosystems, Foster City, CA, USA) was used to determine the ratio of cleaved (emission, 450 nm) to uncleaved (emission, 520 nm) substrate. Values were normalized to the untreated sample.

RNA extraction and quantification

Tissue was homogenized in 1 ml TRIzol/50–100 mg tissue (Invitrogen), and cells were suspended in 1 ml TRIzol/100 mm plate. RNA was extracted as previously described (21) and then purified with the RNeasy clean-up kit, according to the manufacturer’s protocol (Qiagen, Valencia, CA, USA). Total RNA was quantified by absorption at 260 nm and 450 ng of each RNA sample was converted to cDNA using the High Capacity cDNA Archive Kit (Applied Biosystems). Aliquots of 20 µl quantitative PCR reactions were run in triplicate using the ABI Prism 7900 sequence detection system (Applied Biosystems). The level of SMN transcript in triplicate using the ABI Prism 7900 sequence detection system (Applied Biosystems). The level of SMN transcript was quantified by the ΔΔCt method using 18S or β-2-microglobulin as endogenous RNA controls (Applied Biosystems) (42). Values were normalized to one of the E15 mouse brain samples. Mouse SMN primers and probe (spanning exons 2 and 3) and β-globin primers and probe were used (listed 5’–3’): SMN forward: GAATGCCCAACTCCCTTGG; SMN probe: FAM-CAGTGGAAAGTTGGTGAC-BHQ; SMN reverse: GCAGGCGCTTTCTGACCA; β-globin forward: GGAAAGGTGAACCTCCGATGAAAT; β-globin probe: FAM-ACAACCGACGCCTTC-BHQ and β-globin reverse: CAAAGTGATCGGTGGTCCAA (Applied Biosystems).

Protein extraction and western blotting

Samples were homogenized in 1 ml of lysis buffer containing 0.1% Nonident P-40 and 0.5% sodium deoxycholate, and protein was extracted as previously described (21). Total protein concentration was determined by the BCA Protein Assay Kit (Pierce, Rockford, IL, USA) according to the manufacturer’s instructions, and lysates were separated on 12% Tris–glycine gels then transferred to nitrocellulose membranes. Membranes were probed with mouse monoclonal anti-SMN primary antibody at 1:5000 (Transduction Laboratories, San Diego, CA, USA), then goat anti-mouse-HRP secondary antibody at 1:2000 (Santa Cruz Laboratories, Santa Cruz, CA, USA), and developed with the Western Lightning chemiluminescence system (Perkin–Elmer Life Sciences, Oak Brook, IL, USA). Blots were stripped and re-probed with the endogenous controls: anti-β actin at 1:2000 (Sigma) and anti-α-tubulin at 1:4000 (Sigma). Density of bands was determined using NIH Image software. For quantification of SMN protein within a tissue during development, the SMN value was divided by either the actin or the tubulin value. Values were normalized to the embryonic SMN/endogenous control sample for each set of lyses.

Chromatin immunoprecipitation

ChIP was performed on cells and tissue homogenates as previously described (28,29). In brief, the protocol was as follows. For cells, chromatin was prepared from three 150 mm plates of confluent NSC34 cells or from 12 plates of confluent fibroblasts for each condition. For tissues, chromatin was prepared from 100 to 150 mg of tissue. Proteins and DNA in each sample were reversibly cross-linked with 1% formaldehyde (Sigma), 10 ml/150 mm plate of cells or 10 µl/mg of tissue, for 10 min at 37°C. DNA was sheared by sonication on ice (eight times for 15 s), yielding 0.5–1 kb fragments of DNA. Sonication efficiency was verified in each experiment by running the samples on an agarose gel. After preclearing, 50 µl of chromatin was removed to use as an input control. For each immunoprecipitation, 1.9 ml of chromatin was incubated with anti-acetyl-histone H3 (no. 06–599), anti-acetyl-histone H4 (no. 06–599), anti-HDAC-4 (no. 07–040), anti-HDAC5 (no. 07–045, Upstate, Lake Placid, NY, USA), anti-HDAC1 (no. ab7028), anti-HDAC3 (no. ab2379), anti-RNA Pol II (no. ab817–100, Abcam Cambridge MA, USA), anti-HDAC2 (Zymed Laboratories, South San Francisco, CA, USA), non-specific IgG (no. sc-2027, Santa Cruz Biotechnology) or no antibody. After immunoprecipitation, DNA–protein crosslinks were reversed with protease K, and DNA was isolated using phenol chloroform extraction.

Quantitative PCR was used to measure relative amounts of DNA. Sequence specific PCR primer/probe sets were designed to amplify specific regions of the mouse and human SMN genes (Fig. 1). It should be noted that because of the high homology between the human SMN1 and the SMN2 genes, our PCR primers do not distinguish between the two genes. If any of the fibroblast cell lines retain an analyzed portion of the SMN1 gene, the ChIP result represents the cumulative enrichment of protein at both the SMN1 and the SMN2 genes. This is unlikely in type I SMA patient-derived fibroblast cell lines, as deletions in these patients are typically large scale encompassing the entire SMN1 gene (43,44). Primers specific for the translational start region of the mouse β-globin gene were 5’–3’ as follows: forward: AGGT GAGTGGATGCTGTTGCT; probe: ACTATGTCAGAAG CAATGT and reverse: GTGCACCAGATGCTGTTTC TG. Standard curves were run on each PCR plate. A standard curve for the human gene was generated from 10-fold dilutions of plasmid containing a single copy of the 3.4 kb fragment of the human SMN2 promoter. A standard curve for the mouse gene was generated from 10-fold dilutions of whole mouse genomic DNA (Santa Cruz Biotechnology). PCR reactions in triplicate were carried out in 384 well plates using the ABI Prism 7900 sequence detection system.
(Applied Biosystems). PCR product quantities were calculated from the standard curve. For anti-acetyl-histone H3 and H4, relative protein enrichment (ratio of bound/input) was determined by subtracting the no antibody control value from the immunoprecipitated value and dividing by the input value. For all other antibodies, relative protein enrichment (ratio of bound/input) was determined by subtracting the non-specific IgG value from the immunoprecipitated value and dividing by 1/25th of the input value.

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