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THE ANTISENSE TRANSCRIPT *SMN-AS1* REGULATES SMN EXPRESSION AND IS A NOVEL THERAPEUTIC TARGET FOR SPINAL MUSCULAR ATROPHY

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Summary

The neuromuscular disorder spinal muscular atrophy (SMA), the most common inherited killer of infants, is caused by insufficient expression of survival motor neuron (SMN) protein. SMA therapeutics development efforts have focused on identifying strategies to increase SMN expression. We identified a long non-coding RNA (lncRNA) that arises from the antisense strand of *SMN*, *SMN-AS1,* which is enriched in neurons and transcriptionally represses SMN expression by recruiting the epigenetic Polycomb repressive complex-2. Targeted degradation of *SMN-AS1* with antisense oligonucleotides (ASOs) increases SMN expression in patient-derived cells, cultured neurons, and the mouse central nervous system. *SMN-AS1* ASOs delivered together with *SMN2* splice-switching oligonucleotides additively increase SMN expression and improve survival of severe SMA mice. This study is the first proof-of-concept that targeting a lncRNA to

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AUTHOR CONTRIBUTIONS

Conceptualization, C.dY. and C.J.S.; Methodology, C.dY., D.M.R., and C.J.S.; Formal Analysis, C.dY., D.M.R., and N.J.P.; Investigation: C.dY., D.M.R., and N.J.P.; Resources: L.L.R., F.R., and C.F.B.; Writing – Original Draft, C.dY.; Writing – Review and Editing, C.dY., D.M.R., K.L., A.J.W., L.L.R, F.R., C.F.B. and C.J.S.; Visualization: C.dY. and D.M.R.; Supervision, C.dY. and C.J.S.; Project Administration, C.dY. and C.J.S.; Funding Acquisition, C.dY. and C.J.S.

transcriptionally activate *SMN2* can be combined with *SMN2* splicing modification to ameliorate SMA and demonstrates the promise of combinatorial ASOs for treatment of neurogenetic disorders.

Graphical abstract

eTOC blurb

d'Ydewalle et al. identify a lncRNA that regulates *SMN2* expression in a PRC2-dependent manner in neurons. Targeting *SMN-AS1* in combination with *SMN2* splicing modifiers has additive effects on SMN expression and improves the phenotype of severe SMA mice.

Keywords

spinal muscular atrophy; SMN; long non-coding RNA; antisense oligonucleotides; SMA therapeutics

INTRODUCTION

The neuromuscular disease spinal muscular atrophy (SMA) is characterized by profound muscle weakness often within weeks or months after birth. It is caused by recessive mutations of the survival motor neuron 1 (*SMN1*) gene and retention of variable copy numbers of the highly homologous *SMN2* gene (Lefebvre et al., 1995; Lorson et al., 1999). *SMN2* contains a nucleotide substitution in a splice enhancer in exon 7 resulting in alternative splicing of *SMN2*-derived pre-mRNAs (Lorson et al., 1999; Monani et al., 1999a). Most mature *SMN2* mRNAs lack exon 7 *(Δ7-SMN2*) and encode a truncated, unstable SMN protein resulting in reduced levels of functional SMN protein. All individuals affected by SMA have at least 1 copy of *SMN2* and *SMN2* copy number inversely correlates with disease severity (Lefebvre et al., 1997).

The identification of *SMN2* as a genetic modifier of SMA prompted efforts to identify therapeutics that increase *SMN2* expression (d'Ydewalle and Sumner, 2015). Splice-switching oligonucleotides (SSOs) and small molecules promote exon 7 inclusion in *SMN2* pre-mRNAs and improve disease phenotypes in SMA mice (Hua et al., 2010; Hua et al., 2011; Naryshkin et al., 2014; Palacino et al., 2015). The *SMN2* SSO Nusinersen is currently in phase III clinical trials in SMA patients (Chiriboga et al., 2016). While splicing modification is an elegant therapeutic strategy, it has a ceiling effect imposed by the amount of available *SMN2* pre-mRNAs. This is particularly disadvantageous for individuals affected by the most common and severe type I SMA who have only 1 or 2 *SMN2* copies but likely require the highest and most rapid induction of SMN. Another way to increase SMN levels is to increase *SMN2* transcription (d'Ydewalle and Sumner, 2015). We and others showed that histone deacetylase (HDAC) inhibitors can activate the *SMN2* promoter improving disease outcomes in SMA mice (Avila et al., 2007; Somers et al., 2013). HDAC inhibitors failed to show clinical efficacy in SMA patients likely due to low potency and specificity (Kissel et al., 2011; Swoboda et al., 2010). The identification of novel regulators of *SMN* transcription could be used to accentuate the effects of *SMN2* splicing modulators.

Long non-coding RNAs (lncRNAs) have emerged as key regulators of protein-coding gene expression (Faghihi and Wahlestedt, 2009; Lee, 2012) with natural antisense transcripts (NATs), in particular, often affecting expression of their associated protein-coding genes (Carrieri et al., 2012; Faghihi et al., 2008; Mahmoudi et al., 2009). One mechanism by which lncRNAs regulate protein-coding gene expression is via binding of the Polycomb repressive complex-2 (PRC2) (Margueron and Reinberg, 2011). PRC2 binds to chromatin and di- and trimethylates Histone 3 at Lysine 27 (H3K27Me2/3), an epigenetic marker of repressed transcription (Margueron and Reinberg, 2011).

Many lncRNAs are enriched in the central nervous system (CNS) where they exhibit specific temporal and spatial expression patterns (Qureshi et al., 2010). They play roles in regulating neuronal cell fate specification and neurogenesis, synapse plasticity, neuronal homeostasis and behavior (Briggs et al., 2015; Meng et al., 2015; Ng et al., 2012; Qureshi et al., 2010; Ramos et al., 2015). Dysregulation of lncRNAs has been linked to neurodevelopmental and neurodegenerative disorders (Chamberlain and Brannan, 2001; Faghihi et al., 2008; Khalil et al., 2008). LncRNAs may also be promising therapeutic targets (Meng et al., 2015).

Here, we describe a neuronally-enriched NAT, *SMN-AS1,* which arises from a central region of intron 1 of the *SMN* genes. We show that *SMN-AS1* can bind PRC2 and participate in recruiting it to the *SMN* transcription start site where it represses *SMN* expression. Knockdown of *SMN-AS1* dissociates PRC2 from the *SMN* promoter and increases transcriptional activity of *SMN* in patient-derived cells, SMA neurons and a mouse model of severe SMA. Importantly, inducing *SMN2* transcription by targeting *SMN-AS1* in combination with *SMN2* splice-switching oligonucleotides has additive effects on SMN expression and ameliorates SMA disease phenotypes in mice. Together, these studies provide proof-of-principle that *SMN-AS1* is a clinically-relevant, novel therapeutic target for SMA.

RESULTS

Characterization of *SMN-AS1*

To identify putative non-coding RNAs associated with the human *SMN1* and *SMN2* genes, we utilized the UCSC Genome Browser to identify two expressed sequence tags (ESTs) (BC045789.1 and BC037819.1 of 1,613 and 1,618 base pairs length, respectively, isolated from human hypothalamus (Strausberg et al., 2002)), which align with 100% homology to the opposite DNA strand in a central region of intron 1 of the *SMN* genes (Figure 1A). Ensembl (www.ensembl.org) and the Basic local alignment search tool (BLAST) indicate these ESTs share no homology with other genomic regions. The ESTs co-align to epigenetic markers of active transcription in human embryonic stem cells (H1-ESCs) (Figure 1A), but not in human umbilical cord endothelial cells (HUVECs) suggesting that *SMN-AS1* is expressed in a cell type-specific manner (Figure 1A). In Ensembl and AceView, these ESTs correspond to a single-exon antisense RNA, which we named *SMN-AS1* (Figure 1A). A protein coding potential calculator (Kong et al., 2007) and an open reading frame finder tool (http://www.ncbi.nlm.nih.gov/orffinder/) indicated that *SMN-AS1* has no protein-coding potential.

Since *SMN-AS1* is complementary to *SMN* pre-mRNA, we developed a strand-specific RT-qPCR assay to detect *SMN-AS1*. We measured *SMN-AS1* expression in HeLa cells (not shown) and human embryonic kidney (HEK293T) cells, which are known to express a wide variety of lncRNAs (Werner and Ruthenburg, 2015)(Figure S1A). *SMN-AS1* was expressed at low levels compared to *SMN* mRNA and other protein-coding genes, but at similar levels to other lncRNAs (Figure S1A) (Rinn et al., 2007; Smilinich et al., 1999). To confirm that *SMN-AS1* is a distinct transcript, we utilized two chimeric phosphorothioate 2′-*O*-methoxyethyl (2′MOE)/DNA ASOs targeting either exon 1 or exon 2a of the *SMN1* or *SMN2* pre-mRNAs (Figure 1C,D). Both ASOs dose-dependently reduced *SMN* mRNA levels, but did not affect *SMN-AS1* levels (Figure 1B–D). *SMN-AS1* was predominantly expressed in the nucleus like *SMN* pre-mRNA, while *SMN* mRNA was in the cytoplasm (Figures 1D **and** S1B). *SMN-AS1* does not contain sequences encoding polyadenylation signals and *SMN-AS1* was not detected in poly(A)-enriched RNA consistent with other reported lncRNAs (Figure S1C) (Quinn and Chang, 2016; Yang et al., 2011). The *SMN-AS1* locus, unlike *SMN* mRNA, is not conserved across vertebrates suggesting that *SMN-AS1* is expressed only in humans (Figure S1D).

SMN-AS1 was particularly abundant in human cerebellum and brain (Figure 2A **and** S2A,B). To investigate the correlation between *SMN-AS1* and SMN expression, we evaluated spinal cord samples (Figure 2B). SMN protein levels decreased between prenatal and early postnatal time points as previously described (Battaglia et al., 1997; Burlet et al., 1998), while *SMN-AS1* levels increased (Figure 2B). A case-by-case correlation confirmed a robust inverse relationship (Figure 2C). *SMN-AS1* was also enriched in neurons derived from induced pluripotent stem cells (iPSCs) isolated from control, type I SMA or type II SMA patients (Naryshkin et al., 2014; Ng et al., 2015). Expression levels were higher in cortical neurons compared to astrocytes and in purified Islet 1-positive motor neurons (Ng et al., 2015) compared to mixed motor neuron cultures (in which ~40% of cells are motor neurons)(Figures 2D-F **and** S2C). *SMN-AS1* abundance varied in the different cell lines perhaps due to differences in neuronal differentiation rates and/or variability in FACS efficiency (Figure S2D). Nonetheless, within a cell line *SMN-AS1* levels inversely correlated with SMN expression (Figure 2D,E).

SMN-AS1 negatively regulates *SMN* expression

To investigate whether *SMN-AS1* regulates SMN expression, we designed 78 individual chimeric 20-nucleotide phosphorothioate-modified 2′MOE/DNA ASOs complementary to *SMN-AS1* tiling >90% of the transcript. We identified 8 ASOs that reduced *SMN-AS1* levels in HeLa cells in a dose-dependent manner (Figure 3A). An ASO with a single nucleotide mismatch as well as an *SMN2* SSO did not affect *SMN-AS1* levels (Figure 3A**).** The 2 most effective ASOs dose-dependently increased full length (*FL*)-*SMN2* mRNA levels while an ASO with a scrambled sequence did not (Figures 3B–D **and** S3A–C). This increase was consistent with transcriptional activation of *SMN2* as *SMN* pre-mRNA levels also increased (Figures 3B–D **and** S3A–C). To further investigate the effects of *SMN-AS1* on *SMN*, we expressed a reporter construct containing the *SMN* minimal promoter region (−163 bp upstream of the ATG start codon) coupled to a luciferase cDNA in HeLa cells

(Figure S3D). Over-expression of *SMN-AS1* reduced luciferase activity by ~54% and knockdown of *SMN-AS1* increased luciferase activity by 55-74% (Figure S3D,E).

To verify that *SMN-AS1* regulates *SMN2* in SMA patient cells, we first confirmed *SMN-AS1* expression in multiple fibroblast lines derived from SMA patients with variable *SMN2* copy numbers (Figure S4A). We then transfected the GM03813 line with *SMN-AS1* ASO-A or *SMN2* SSO (Figure 3E-G). As expected, *SMN2* SSO did not affect *SMN-AS1* nor *SMN* pre-mRNA levels, but it increased *FL-SMN2* mRNA and SMN protein levels, while *Δ7-SMN2* mRNA levels decreased in a dose-dependent manner (Figure 3E,G). *SMN-AS1* knockdown also augmented *FL-SMN2* mRNA and SMN protein levels, but in contrast to *SMN2* SSO, it also increased *SMN* pre-mRNA levels. There was little change in *Δ7-SMN2* mRNA levels (Figure 3F) suggesting that *SMN-AS1* ASOs may cause both transcriptional activation and some modulation of splicing leading to minimal net change in *Δ7-SMN2* mRNA levels.

SMN-AS1 represses *SMN* expression in differentiating neurons

Severe SMA mice harbor the human *SMN2* gene including a ~3.4 kb portion of the human *SMN2* promoter, its exons and introns including the *SMN-AS1* locus in intron 1 (Le et al., 2005). Like in humans, *SMN-AS1* was predominantly expressed in the CNS in these mice (Figures 1 **and** 4A). We cultured primary cortical neurons with approximately 95% purity as assessed by immunocytochemical analyses of the neuronal markers β3-tubulin, Map2 and SMI32 (Figure 4B and not shown). *SMN-AS1* levels increased 2.4-fold between day 3 and day 6 *in vitro* (DIV.3 and DIV.6, respectively) correlating inversely with a 50% decrease in *FL-SMN2* mRNA and 57% decrease in SMN protein expression (Figure 4C,D). This inverse expression pattern was also observed in undifferentiated compared to differentiated human neuroblastoma cells and is consistent with the changes observed in developing human spinal cord (Figures 2 **and** S4B,C). Treatment of neurons with either *SMN-AS1* ASO-A or ASO-B by free uptake starting at DIV.3 resulted in a 65% decrease in *SMN-AS1* levels by DIV.6 and a 68% and 72% increase of *FL-SMN2* mRNA and SMN protein levels, respectively (Figures 4E,F **and** S4D). These effects were comparable to those observed with an equimolar concentration of a *SMN2* SSO (Figure 4E,F).

SMN-AS1 recruits PRC2 to the *SMN* promoter

Many nuclear lncRNAs negatively regulate expression of associated protein-coding genes by recruiting PRC2 to their promoter regions (Margueron and Reinberg, 2011; Wang and Chang, 2011). PRC2 contains the scaffolding proteins Embryonic ectoderm development (EED) and Suppressor of Zeste-12 (SUZ12) as well as the enzymatic component Enhancer of Zeste-2 (EZH2) and other proteins (Lee et al., 2006; Plath et al., 2003). To determine whether *SMN-AS1* can bind to PRC2, we performed RNA binding immunoprecipitation (RIP) experiments using HEK293T cells (Figure 5A,B). When either EED or SUZ12 were immunoprecipitated, *SMN-AS1* was detectable at levels comparable to *HOTAIR,* a lncRNA known to bind PRC2 (Figure 5A,B) (Rinn et al., 2007). We also immunoprecipitated SMN and snRNP70, another splicing protein with RNA binding capacity. *SMN-AS1* and *HOTAIR* were undetectable in these precipitated fractions, but *U1* snRNA, a small non-coding RNA known to bind to SMN and snRNP70, was present at robust levels (Figure S5A).

Knockdown of *SMN-AS1* in HEK293T cells resulted in reduced binding of *SMN-AS1* to PRC2 (Figure S5B,C).

EZH2 di- and tri-methylates Histone-3 at Lysine-27 (H3K27Me2/3), an epigenetic marker for repressed transcription found in promoter regions (Cao et al., 2002). To evaluate whether PRC2 can bind the promoter region of the *SMN* genes, we performed chromatin immunoprecipitations (ChIPs) in HEK293T cells with 16 primer sets spanning −3 kb to +700 bp of the *SMN* promoters relative to the ATG start codon (Figure 5C). We were unable to discriminate the *SMN1* and *SMN2* promoters as their sequences are nearly identical (Boda et al., 2004; Echaniz-Laguna et al., 1999; Monani et al., 1999b). EED- and SUZ12 ChIP indicated that PRC2 can bind the *SMN* promoters near the transcription start site (TSS) and this was associated with enriched H3K27Me3 marks in this region (Figures 5D **and** S4E). RNA polymerase-2 (POLR2A) ChIP showed increased occupancy of POLR2A downstream of the *SMN* TSS (Figure S4C). *SMN-AS1* knockdown reduced binding of SUZ12 and EED to the *SMN* promoters indicating that PRC2 recruitment is at least in part *SMN-AS1*-dependent (Figures 5E,F). This was also associated with decreased H3K27Me3 levels and increased POLR2A occupancy near the TSS (Figure S5F,G). PRC2 binding to and H3K27Me3 levels in the *HOXA2* proximal promoter, a known PRC2 target gene, was not affected by the ASO treatment (Figures 5E,F **and** S5F,G). Together, these data indicate that *SMN-AS1* can bind PRC2 and that PRC2 is recruited to the *SMN* promoter to repress transcription by regulating H3K27Me3 levels near the *SMN* TSS.

PRC2 represses *SMN* expression in neurons

PRC2, and EZH2 in particular, play well established roles in maintaining pluripotency of stem cells and in controlling cell proliferation (Piunti et al., 2014; Rao et al., 2015). The role of EZH2 in differentiating neurons or the developing CNS is less well established. We confirmed Ezh2 expression in the CNS by Western blot analysis of brain (not shown) and spinal cord at embryonic and early postnatal time points in wild type (WT) and SMA mice, and loss of expression in adult stages (Figure S6A). Immunohistochemical analyses of neonatal WT and SMA spinal cords indicated that Ezh2 is expressed in motor neurons (Figure 6A). Ezh2 was also expressed in primary cortical neurons isolated from SMA mice (Figure S6B). To explore if Ezh2 regulates SMN expression in neurons, we transfected SMA cortical neurons with 2 ASOs targeting mouse Ezh2. Both Ezh2 ASOs significantly reduced *Ezh2* mRNA and Ezh2 protein levels and increased SMN protein expression to similar levels achieved with *SMN1-AS1* ASO treatment (Figure 6B,C).

We confirmed that *SMN-AS1* can bind to PRC2 in SMA neurons using RIP. Knockdown of *SMN-AS1* reduced *SMN-AS1* levels and abolished PRC2:*SMN-AS1* binding (Figure 6D). *SMN-AS1* can also bind PRC2 *in vivo* in WT and SMA mouse brain (Figure S6C). To determine whether *SMN-AS1* directly binds to the *SMN* promoter in the TSS region, we performed chromatin isolation by RNA purification (ChIRP) in cultured neurons (Figure 6E). We modified our 8 lead *SMN-AS1*ASOs by replacing 10 core nucleotides with 2′MOE nucleotides and adding a 3′-end biotin label. We validated that these biotinylated ASOs pulled down *SMN-AS1* (Figure S6D). Two separate primer sets amplifying the *SMN* TSS region confirmed that *SMN-AS1* binds directly to the *SMN* proximal promoter region

(Figure 6E**)**. A DNase treatment of the precipitated material confirmed that we amplified proximal *SMN* promoter DNA (and not duplexed 5′UTR *SMN* mRNA). The *SMN-AS1* DNA locus was not amplified indicating that *SMN-AS1* does not bind its complementary DNA in this setting (Figure 6E).

We next evaluated whether Prc2 binds to *SMN2* in primary neurons. Suz12 (not shown), Ezh2 and its associated repressive marker H3K27Me3 localize to the *SMN2* proximal promoter (Figure 6F,G). Knockdown of either Ezh2 or *SMN-AS1* reduced Ezh2 binding and decreased H3K27Me3 levels in the *SMN* TSS region (Figure 6F,G). We conclude that in neurons *SMN-AS1* and Prc2 are recruited to the *SMN* proximal promoter, and that *SMN-AS1* knockdown alleviates PRC2 binding and de-represses SMN transcriptional activity.

SMN-AS1 is a therapeutic target for SMA

As a primary goal of SMA therapeutics is to increase SMN levels, targeted knockdown of *SMN-AS1* could represent a novel SMA therapeutic strategy. Severe SMA mice were injected subcutaneously with *SMN-AS1* ASO-A (400 mg/kg) at P1 and P3 as ASOs are CNS penetrant at this age (Hua et al., 2011). At P10, there was a ˜69 % and ˜51% reduction of *SMN-AS1* in brain and spinal cord, respectively and increased *SMN*2 pre-mRNA, *FL-SMN2* mRNA and SMN protein levels (Figures 7A–D **and** Figure S7A). Despite a moderate increase of SMN expression in the CNS, survival, body weight and righting reflex were not improved compared to saline-treated SMA mice (Figure 7E–G).

Severe SMA mice have dysfunction of several organ systems of uncertain relevance to the human disease (Bevan et al., 2010; Gombash et al., 2015). These systemic impairments may account for the limited phenotypic improvement previously observed with neuronal-specific genetic rescue of SMN (Gogliotti et al., 2012; Martinez et al., 2012). Given the neuronal enrichment of *SMN-AS1* and the potential to miss meaningful therapeutic effects because of systemic abnormalities in SMA mice, we evaluated the effects of combined systemic administration of a low, sub-optimal dose (50 mg/kg) of *SMN2* SSO together with a high dose (400 mg/kg) of *SMN-AS1* ASO. *SMN2* SSO alone or in combination with a control ASO increased *SMN* expression to the same magnitude as the *SMN-AS1* ASO alone in the brain and spinal cord (Figure 7B–D). *SMN2* SSO also increased *FL-SMN2* mRNA and SMN protein levels in other organs while *SMN-AS1* ASO alone did not (Figure 7B–D). When combined, *SMN2* SSO and *SMN-AS1* ASO had additive effects on *FL-SMN2* mRNA and SMN protein levels in the CNS (Figure 7C,D).

Combination therapy also improved survival of SMA mice. While saline-treated mice had a median survival of 18 days and low dose *SMN2* SSO-treated mice had a median survival time of 25 days (Figure 7E), combining *SMN2* SSO with *SMN-AS1* ASO increased median survival to 37 days with 4 out of 15 (˜25%) mice surviving more than 120 days (Figure 7E). SMA mice receiving combination ASOs were heavier than *SMN2* SSO alone treated mice (Figure 7F). Combination-treated mice also had improved motor behavior and in some tests animals receiving both *SMN-AS1* ASO and *SMN2* SSO performed as well as untreated WT control animals (Figure 7G **and** Figure S7B,C). Taken together, these data show that reducing *SMN-AS1* levels in combination with modulating *SMN2* splicing ameliorates SMA disease manifestations.

DISCUSSION

The neuromuscular disorder SMA is caused by insufficient expression levels of SMN protein resulting in profound muscle weakness and often premature death. Antisense oligonucleotides and small molecules that modulate *SMN2* splicing are in clinical trials; however, their efficacy could be accentuated by parallel induction of *SMN2* transcription. Identification of *SMN2* transcriptional activators has been limited by inadequate understanding of the mechanisms that regulate the *SMN* genes particularly in neurons. In this study, we identified a neuronally-enriched NAT, *SMN-AS1*, whose expression levels increase during neuronal differentiation inversely correlating with SMN. We show that *SMN-AS1* contributes to the recruitment of the epigenetic chromatin modifier PRC2 to the *SMN2* promoter and that *SMN-AS1* knockdown dissociates PRC2 from the *SMN* promoter thus increasing *SMN* expression in neurons and in the CNS. We also provide proof-of-principle that *SMN-AS1* is a clinically relevant, novel therapeutic target by demonstrating that *SMN-AS1* ASOs together with *SMN2* SSOs show combinatorial therapeutic effects in SMA mice. Together these data reveal a novel mechanism regulating *SMN* expression in neurons, validate a new SMA therapeutic strategy that can be combined with *SMN2* splicing modification, and highlight the potential of combinatorial ASOs targeting distinct gene regulatory mechanisms.

Dynamic expression of SMN and *SMN-AS1*

Prior studies have reported that SMN levels are developmentally regulated (Battaglia et al., 1997; Burlet et al., 1998). Our data confirms a decrease of SMN levels between prenatal and early postnatal periods in human spinal cord as well as a 2-fold reduction in SMN levels during differentiation of cultured neurons. Motor neurons may be particularly dependent on high SMN levels for normal maturation and/or maintenance during perinatal development and SMA disease onset often occurs during this critical period. In SMA mice, neonatal knockdown of SMN is required to recapitulate disease pathology (Kariya et al., 2014), while early postnatal restoration of SMN is essential to ameliorate disease manifestations (Lutz et al., 2011). These studies highlight the importance of this temporal window, yet the mechanisms that regulate SMN expression during this period have not been well defined. *SMN-AS1* is developmentally regulated in the spinal cord in an inverse pattern to SMN suggesting that it represses SMN during neuronal maturation and CNS development. Many lncRNAs have been shown to regulate the expression of protein-coding genes in a temporally and spatially restricted manner (Herriges et al., 2014). The NAT *SOX2OT*, for example, increases during aging (Barry et al., 2015) repressing the neural pluripotency marker *SOX2*. Our data raise the question of how *SMN-AS1* expression itself is controlled. In some cases, NATs and protein coding genes are inversely regulated by direct transcriptional interference (Shearwin et al., 2005). Given the active co-expression of *SMN* and *SMN-AS1,* it may be more likely that each are regulated by distinct transcription factors (Alam et al., 2014). Another question to be answered in future investigations is whether *SMN-AS1* expression levels modify SMA disease severity. These experiments are challenging, however, as they likely require determining expression levels specifically in motor neurons of multiple SMA patients.

The interaction between *SMN-AS1* and the *SMN* genes

Many nuclear lncRNAs regulate gene expression by associating with chromatin (Faghihi and Wahlestedt, 2009; Wang and Chang, 2011). Direct binding of NATs to complementary DNA is a straightforward way to explain how lncRNAs interact with their target. This mode of action has been proposed for lncRNAs including *MEG3,* which forms RNA-DNA triplexes in GA-rich sequences of TGF-β pathway genes (Mondal et al., 2015). Our ChIRP data suggest that, at least in primary cortical neurons, *SMN-AS1* does not principally bind to its DNA locus, but rather to the *SMN* TSS region corresponding to a local hotspot of PRC2 recruitment. Sequence alignment of *SMN-AS1* and the *SMN* promoters indicates ~70% sequence homology between the 3′ end of *SMN-AS1* (1376 – 1613 nt) and a region −0.6 kb upstream of the ATG start codon. This suggests that this 3′ sequence of *SMN-AS1* could mediate *SMN* promoter binding. The site of action of lncRNAs may also be determined by chromatin tertiary structure (Vance and Ponting, 2014). Although separated by 7 kb, it is plausible that the *SMN-AS1* locus and the proximal *SMN* promoter are in close vicinity due to 'DNA looping' and that *SMN-AS1* functions locally near its site of synthesis. Our studies have not determined whether *SMN-AS1* can regulate *SMN* expression only in *cis* or also in *trans* (Faghihi and Wahlestedt, 2009). *SMN-AS1* is likely transcribed from both the *SMN1* and *SMN2* genes and our data from SMA mouse neurons and tissues, which harbor only the human *SMN2* gene, indicate that *SMN-AS1* can repress the *SMN* locus from which it arises. Whether *SMN-AS1* can also regulate a neighboring *SMN* locus in *trans* will require further study.

PRC2 contributes to the regulation of *SMN* expression in neurons

PRC2 has traditionally been associated with genomic imprinting (i.e., silencing of alleles in a parent-of-origin dependent manner), X-chromosome inactivation and irreversible silencing of protein-coding genes by trimethylating H3K27 in their promoter regions (Mager et al., 2003; Margueron and Reinberg, 2011; Pinter et al., 2012; Simon et al., 2013). In contrast, our data indicate that PRC2 regulates SMN expression despite ongoing *SMN* transcriptional activity. These data are consistent with a model in which PRC2 together with the repressive H3K27Me3 mark co-exist with and are balanced by markers of active transcription resulting in a basal level of *SMN* expression, which can be further modulated. Our observations are consistent with other work demonstrating that EZH2 partially inhibits *SLIT2* expression in a variety of cancers and that *SLIT2* expression can be increased by interfering with EZH2 function (Yu et al., 2010). Furthermore in hippocampal neurons, it has been shown that neuronal stimulation can de-repress *Bdnf* expression by displacement of Ezh2 from the *Bdnf* promoters (Palomer et al., 2016) and that Ezh2 knockdown can de-repress *Runx2/p57* (Aguilar et al., 2016). Knockdown of specific lncRNAs, even in fully differentiated cells, has been shown to displace PRC2 thus de-repressing gene transcription (Gonzalez et al., 2015; Li et al., 2013).

The reversibility of PRC2-induced repression of *SMN* may also imply that both repressive and activating complexes can be recruited to the *SMN* promoter. Previous work by our group and others has implicated several other epigenetic modifiers in the regulation of SMN expression including histone deacetylases (Avila et al., 2007; Kernochan et al., 2005) and methyl-CpG binding protein 2 (MECP2) (Hauke et al., 2009). The recruitment of these

epigenetic modifiers are not mutually exclusive and can co-occupy the *SMN* promoter to maintain steady-state *SMN* expression levels. Since several chromatin modifiers likely co-regulate *SMN* transcription, this raises the question whether *SMN-AS1* recruits only PRC2, or whether it also tethers other epigenetic complexes to the *SMN2* promoter. Future ChIRP experiments followed by mass spectrometry of the precipitated proteins could identify additional interactors of *SMN-AS1,* which would provide further insights regarding the neuronal regulation of *SMN* expression.

Although knockdown of *SMN-AS1* increased *SMN* pre-mRNA and *FL-SMN* mRNA levels, it unexpectedly did not significantly increase *Δ7-SMN2* mRNA levels. This suggests that suppression of *SMN-AS1* results both in transcriptional activation of *SMN2* as well as in some modulation of *SMN2* exon 7 splicing with little net change in *Δ7-SMN2* mRNA levels. Transcriptional activation can alter RNA polymerase II elongation rates with consequent changes in splicing both *in vitro* and *in vivo* (Fong et al., 2014; de la Mata et al., 2003). Alternatively, it is possible that *SMN-AS1* knockdown not only alters the epigenetic state of the *SMN* promoter region, but also downstream regions with subsequent effects on exon 7 splicing as has been described for other genes (Ameyar-Zazoua et al., 2012; Luco et al., 2010).

SMN-AS1 as therapeutic target for SMA?

ASOs can be used to target cytoplasmic or nuclear RNA molecules thus modulating splicing, 5′ capping, polyadenylation, protein binding, or protein translation. They can also be used to direct degradation of target RNAs by RNase H (Corey, 2007; Kole et al., 2012). The potential of ASOs as a therapeutic for neurological and other genetic disorders has been studied extensively *in vitro* and *in vivo*. (Cheng et al., 2015; Donnelly et al., 2013; Lagier-Tourenne et al., 2013; Meng et al., 2015; Schoch et al., 2016). This is particularly true for SMA, in which *SMN2* SSOs are able to ameliorate disease in SMA mouse models by influencing *SMN2* splicing in the nucleus (Hua et al., 2010; 2008; Passini et al., 2011; Sahashi et al., 2013). Importantly, although penetration of ASOs into the adult CNS is low after systemic delivery as has been shown by others (Hua et al., 2008), clinical trials in patients with amyotrophic lateral sclerosis and SMA have indicated that intrathecal administration of ASOs is tolerated and safe (Chiriboga et al., 2016; Miller et al., 2013).

In an effort to determine whether *SMN-AS1* could be a novel therapeutic target, we identified ASOs that degrade *SMN-AS1* and dose-dependently activate SMN expression. When administered alone, *SMN-AS1* ASO did not improve the survival of severe SMA mice. This is not entirely unexpected as the *SMN1-AS1* ASO sequences and chemistries have not yet been optimized for *in vivo* targeting efficiency as has been done for *SMN2* SSOs (Hua et al., 2010). In addition, the expression of *SMN-AS1* and induction of SMN expression after *SMN-AS1* knockdown is quite restricted to neurons and prior neuronal-specific genetic rescue experiments in severe SMA mice showed only modest phenotypic improvements (Gogliotti et al., 2012; Martinez et al., 2012; Paez-Colasante et al., 2013). These studies highlight that in SMA mouse models there are abnormalities of multiple other organ systems (Bevan et al., 2010; Biondi et al., 2012; Gombash et al., 2015; Heier et al., 2010) which are an important determinant of SMA mouse survival, but are of uncertain

relevance to the human disease. Although systemic administration of high dose *SMN2* SSO alone substantially improves the survival of SMA mice (Hua et al., 2011), this is likely because of restoration of SMN levels peripherally (Hua et al., 2015). Given that *SMN-AS1* may specifically down-regulate SMN expression during perinatal periods, it is also possible that earlier intervention during this temporal window would be more efficacious.

Importantly, when combined with suboptimal doses of *SMN2* SSO, the disease modifying effects of *SMN-AS1* ASOs were evident with improved motor behavior, weights and survival in severe SMA mice. Our study shows that *SMN-AS1* can be a therapeutic target, and also demonstrates the potential of combinatorial ASOs targeting distinct molecular regulators of a single gene. Combining ASOs targeting two different genes with traditional chemotherapy has been proposed for cancer therapeutics (Biroccio et al., 2003; Zupi et al., 2005), but this is the first example to our knowledge of two ASOs used in combination for a neurological disorder. Like others, we found that dosing of multiple ASOs was well tolerated in SMA mice (Hua et al., 2015; Zupi et al., 2005). Although the clinical development of combination ASOs will present new challenges, they hold the promise of additive or synergistic therapeutic effects for multiple neurogenetic diseases.

EXPERIMENTAL PROCEDURES

All mice in this study were maintained according to protocols approved by the Institutional Animal Care and Use Committee at Johns Hopkins University School of Medicine. De-identified human tissue samples were collected at autopsy following parental or patient informed consent in strict observance of the legal and institutional ethical regulations. Protocols were approved by the Institutional Review Board at the Johns Hopkins University School of Medicine.

Antisense oligonucleotide synthesis

We synthesized and purified all chemically modified oligonucleotides as described in Swayze et al., 2007. The 2′-O-methoxyethyl (2′MOE) gapmer ASOs are 20 nucleotides in length, wherein the central gap segment comprising of ten 2′-deoxynucleotides are flanked on the 5′ and 3′ wings by five 2′-MOE modified nucleotides. Internucleoside linkages are purely phosphorothioate (*in vitro*) or interspersed with phosphodiester (in vivo), and all cytosine residues are 5′-methylcytosines. The sequences of the ASOs used in this study are listed in supplemental experimental procedures.

Detailed experimental procedures for cell cultures, pluripotent stem cell cultures, primary cortical neuron cultures, Western blotting, RNA isolation and RT-qPCR, ChIP, RIP and ChIRP experiments, and ASO administration and behavioral assessment of the SMA mice are described in supplemental materials.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Highlights

- *SMN-AS1* is the first long non-coding RNA identified that is associated with SMN;
- *SMN-AS1* recruits PRC2 to the *SMN2* promoter repressing its transcription in neurons;
- *SMN-AS1* knockdown increases SMN expression *in vitro* and *in vivo*;
- Combining *SMN-AS1* ASOs with *SMN2* splicing ASO attenuates disease in SMA mice.

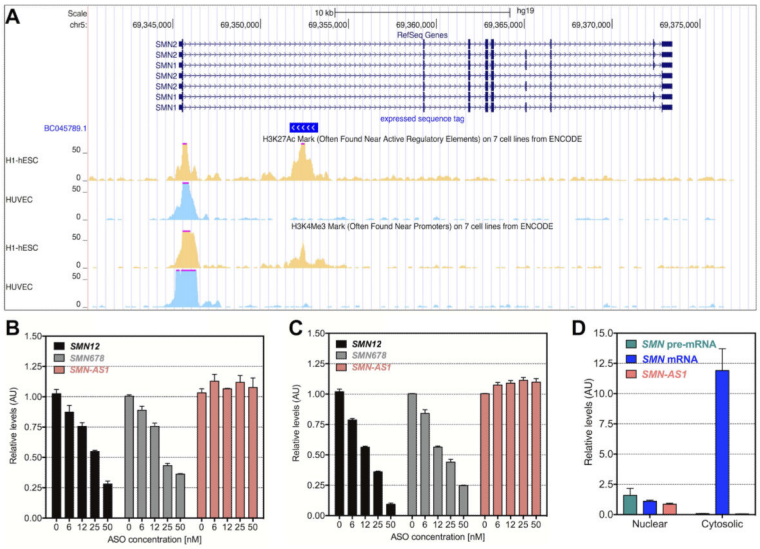

Figure 1. Identification of *SMN-AS1*

(**A**) Genome browser views of human *SMN1* and *SMN2* on chromosome 5 are shown in 5′ to 3′-end with exons shown as blue boxes and introns as horizontal blue lines. The EST BC035789.1, shown in vivid blue, is transcribed from the opposite strand. ChIP-seq of the *SMN* loci for H3K27Ac and H3K4Me3 show active transcription co-aligning to the EST in H1-ESC cells (yellow tracks), but not in HUVEC cells (light blue tracks). (**B,C**) *SMN* mRNA levels measured at exon 1/2a boundary (*SMN12*) and at exon 6/7/8 boundary (*SMN678*) and *SMN-AS1* levels in HeLa cells treated for 24 hours with ASO targeting *SMN* exon 1 (**B**) or *SMN* exon 2a (**C**). n = 4, mean + SEM. (**D**) *SMN* pre-mRNA, *SMN* mRNA and *SMN-AS1* levels measured in the nuclear and cytoplasmic fraction of HeLa cells. n = 4, mean + SEM.

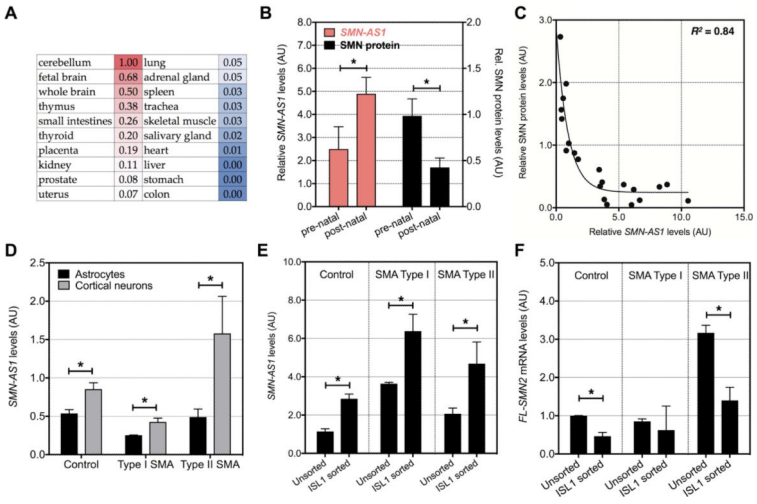

Figure 2. *SMN-AS1* expression in neuronal tissues and neurons

(**A**) Heat map indicating relative *SMN-AS1* levels in 20 different human tissues. (**B**) *SMN-AS1* and SMN protein levels measured in human thoracic spinal cords at prenatal (n=12; age: 15 to 35 weeks of gestation) and postnatal (n=10; age: 1 day to 1 year) stages. Mean + SEM, * $P < 0.05$. (**C**) One-phase decay model indicating an inverse correlation ($R^2 = 0.84$) between *SMN-AS1* and SMN protein levels in human thoracic spinal cords. (**D**) *SMN-AS1* levels in astrocytes and cortical neurons differentiated from human control and type I or type II SMA individuals. n = 3, mean + SEM, * $P < 0.05$. (**E,F**) *SMN-AS1* levels (**E**) and *FL-SMN2* mRNA levels (**F**) in mixed motor neuron cultures and sorted ISL1-positive motor neurons derived from human control and type I or type II SMA individuals. n = 3, mean + SEM, * $P < 0.05$.

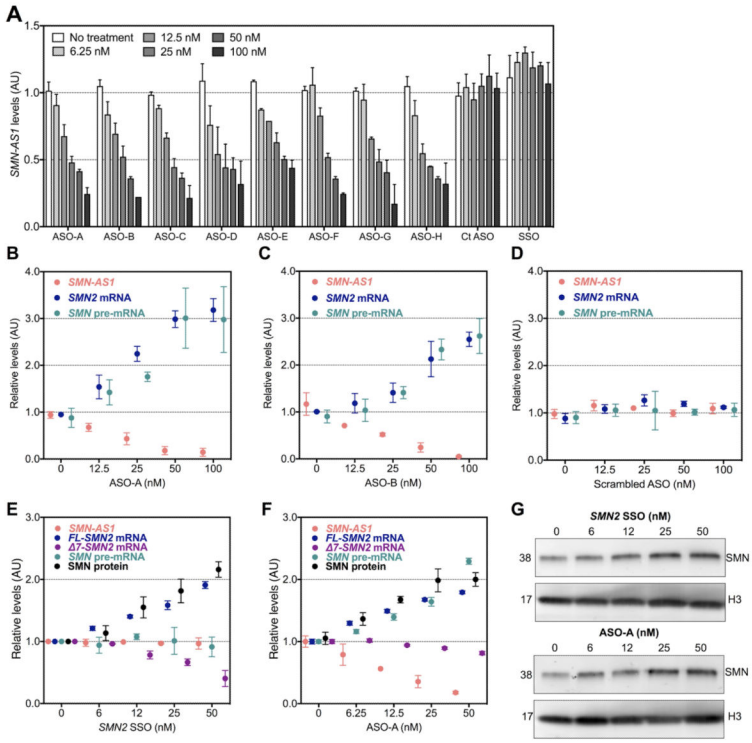

Figure 3. Antisense oligonucleotides targeting *SMN-AS1* activate SMN expression

(**A**) *SMN-AS1* levels in HeLa cells treated for 1 day with various concentrations of ASOs targeting *SMN-AS1* (ASO A-H) or control ASOs. Ct. ASO-1 = Control ASO-1 = single nucleotide mismatch ASO. SSO = *SMN2* SSO. n = 4, mean + SEM. (**B–D**) *SMN-AS1*, *SMN2* mRNA and *SMN* pre-mRNA levels in HeLa cells treated for 1 day with various concentrations of ASO-A (**B**), ASO-B (**C**), or scrambled ASO (**D**). UD = undetermined. n = 4, mean ± SEM. (**E–G**) *SMN-AS1*, *FL-SMN2* mRNA, *Δ7-SMN2* mRNA, *SMN* pre-mRNA and SMN protein levels in SMA fibroblasts treated for 3 days with various concentrations of *SMN2* SSO (**E**) or *SMN-AS1* ASO-A (**F**). n = 4, mean ± SEM. (**G**) Representative Western blots of SMN protein levels in SMA fibroblasts treated for 3 days with *SMN2* SSO or *SMN-AS1* ASO-A. The membranes were re-probed for a general loading control Histone-3 (H3). n = 3, mean ± SEM.

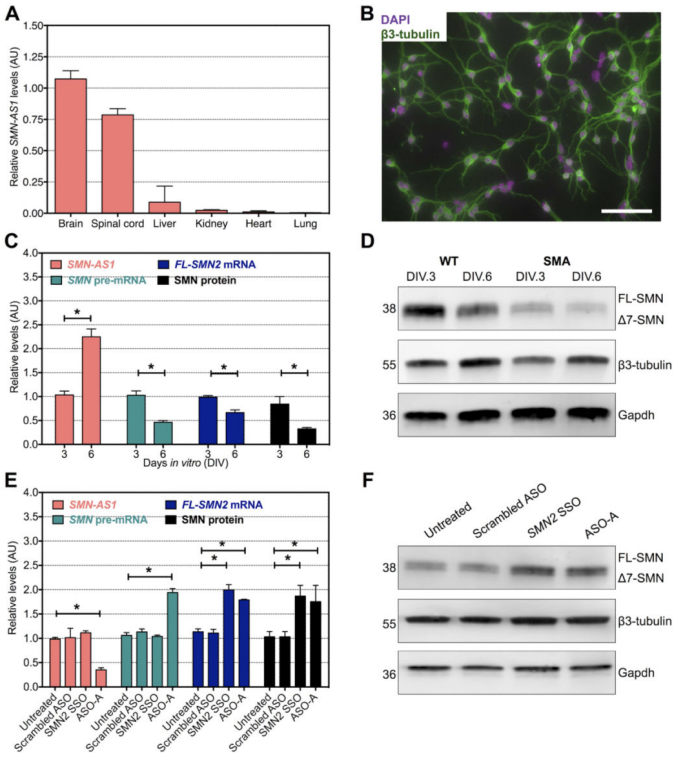

Figure 4. *SMN-AS1* regulates *SMN* expression in primary SMA neurons

(**A**) *SMN-AS1* levels in brain, spinal cord, liver, kidney, heart and lungs of P10 SMA mice. n = 3, mean + SEM. (**B**) Fluorescence micrograph of primary cortical neurons stained for the neuronal marker β3-tubulin (green) and 4′,6-diamidino-2-phenylindole (DAPI, magenta) on DIV.3. Scale bar = 20 μm. (**C**) *SMN-AS1*, *SMN* pre-mRNA, *FL-SMN2* mRNA and SMN protein levels in primary SMA cortical neurons on DIV.3 and DIV.6. n = 3, mean + SEM. * $P < 0.05$. (**D**) Representative Western blot of SMN protein levels in primary SMA cortical neurons on DIV.3 and DIV.6. The membranes were re-probed for neuronal marker β3-tubulin and a general loading control Gapdh. (**E**) SMN-AS1, SMN pre-mRNA, FL-SMN2 mRNA and SMN protein levels in primary SMA cortical neurons treated on DIV.3 for 3 days with 5 μM of scrambled ASO, *SMN2* SSO or *SMN AS1* ASO-A. (**F**). Representative Western blot of SMN protein levels in primary SMA neurons treated for 3 days with 5 μM of scrambled ASO, *SMN2* SSO or *SMN-AS1* ASO-A. The membrane was re-probed for the neuronal marker β3-tubulin and a general loading control Gapdh. n = 3, mean + SEM. * $P < 0.05$.

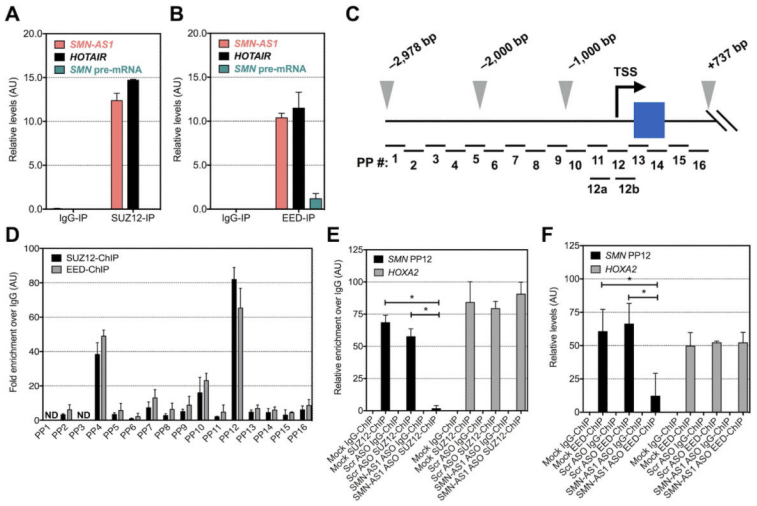

Figure 5. *SMN-AS1* recruits PRC2 to *SMN* promoter to repress transcription

(**A,B**) *SMN-AS1*, *HOTAIR* and *SMN* pre-mRNA levels following SUZ12-RIP (**A**) or EED-RIP (**B**) in HEK293T cells. The negative control IgG-RIP was set to 0. (**C**) Schematic of the *SMN* promoters spanning a region from −2,987 bp to +737 bp relative to the ATG start codon (+1 bp) of exon 1 (blue box). The TSS is indicated with a black arrow and the 2,000 and −1,000 bp marks are indicated with grey arrowheads. PP = Primer Pairs; see extended experimental procedures for details. (**D**) Relative enrichment of amplicons with indicated PP following SUZ12- and EED-ChIP in HEK293T cells. The negative control IgG-ChIP was set to 0. ND = not determined. n = 3, mean + SEM. (**E,F**) Relative enrichment of the PP12 amplicon following SUZ12-ChIP (**E**) or EED-ChIP (**F**) in HEK293T cells treated for 3 days with vehicle (mock), 250 nM scrambled (Scr) ASO or 250 nM *SMN-AS1* ASO-A. n = 3, Mean + SEM. * $P < 0.05$.

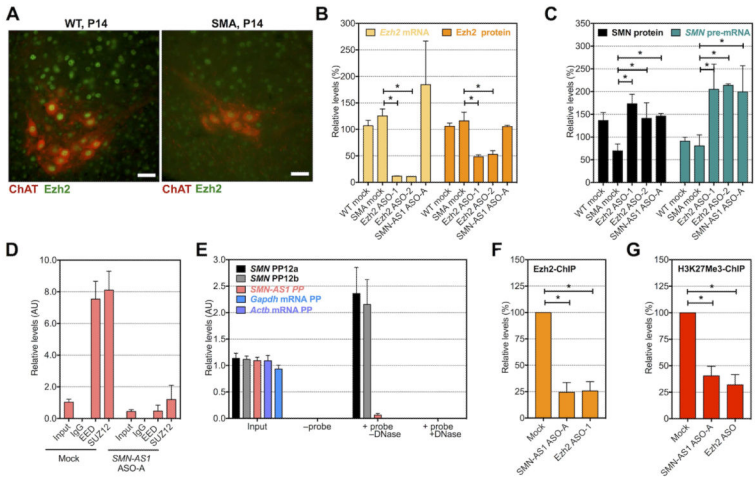

Figure 6. *SMN-AS1* recruits PRC2 to *SMN* promoter in neurons

(**A**) Fluorescence micrographs of ventral spinal cord isolated from a P14 WT (left panel) or SMA (right panel) mouse stained with ChAT (red) and Ezh2 (green). Scale bar = 50 μm. (**B**) *Ezh2* mRNA and Ezh2 protein levels in untreated WT cortical neurons or SMA cortical neurons untreated or treated for 3 days with 5 μM Ezh2 ASO-1 or Ezh2 ASO-2, or 5 μM of *SMN-AS1* ASO A. n = 3, mean + SEM. * $P < 0.05$. (**C**) SMN protein and *SMN* pre-mRNA levels in untreated WT cortical neurons or SMA cortical neurons untreated or treated on DIV.3 for 3 days with 5 μM Ezh2 ASO-1 or Ezh2 ASO-2, or 5 μM of *SMN-AS1* ASO-A. n = 3, mean + SEM. * $P < 0.05$. (**D**) *SMN-AS1* levels following SUZ12-RIP and EED-RIP in primary SMA cortical neurons treated on DIV.3 for 3 days with vehicle (mock) or 5 μM *SMN-AS1* ASO-A. The negative control IgG-RIP was set to 0. n = 3, mean + SEM. (**E**) Relative enrichment of PP12a, PP12b or *SMN-AS1* amplicons following *SMN-AS1* ChIRP with biotinylated ASOs in primary SMA cortical neurons on DIV.6. *Gapdh* and *Actb* mRNA primers were used as negative control. n = 3, mean + SEM. (**F,G**) Relative enrichment of PP12 amplicon following Ezh2-ChIP (**F**) and H3K27Me3-ChIP (**G**) in primary SMA cortical neurons treated on DIV.3 for 3 days with vehicle (mock), 5 μM *SMN-AS1* ASO-A or 5 μM Ezh2 ASO-1. n = 3, mean + SEM. * $P < 0.05$.

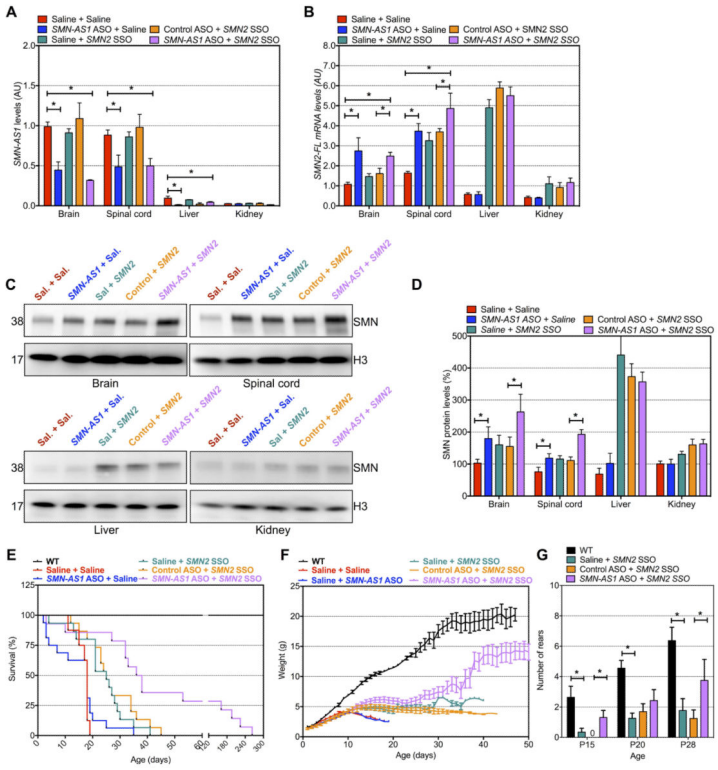

Figure 7. Targeting *SMN-AS1* in SMA mice increases SMN expression

(**A–D**) *SMN-AS1* (**A**), *FL-SMN2* mRNA (**B**) and SMN protein (**C,D**) levels in brain, spinal cord, liver and kidney at P10. n = 3, mean + SEM. * $P < 0.05$. (**C**) Representative Western blots of data presented in C, D. (**E**) Kaplan-Meier curve for treated SMA mice. WT mice are shown as a reference. n = 15 mice per group. (**F**) Daily body weight measurements of treated SMA mice. mean ± SEM. (**G**) Number of rears in 60 seconds for treated animals. Mean + SEM. * $P < 0.05$.