Converging Mechanisms of p53 Activation Drive Motor Neuron Degeneration in Spinal Muscular Atrophy

Highlights

- SMN deficiency induces early p53 activation in vulnerable SMA motor neurons
- Inhibition of p53 prevents motor neuron degeneration in SMA mice
- \( p53^{S18} \) phosphorylation selectively marks degenerating SMA motor neurons
- Amino-terminal phosphorylation of p53 is required for motor neuron degeneration

Authors

Christian M. Simon, Ya Dai, Meaghan Van Alstyne, ..., Christopher E. Henderson, Livio Pellizzoni, George Z. Mentis

Correspondence

lp2284@cumc.columbia.edu (L.P.), gzmentis@columbia.edu (G.Z.M.)

In Brief

Ubiquitous SMN deficiency causes the death of specific motor neuron pools in SMA, but the mechanisms underlying this selectivity are unknown. Simon et al. identify nuclear accumulation and phosphorylation of p53 as distinct, converging mechanisms induced by SMN deficiency that trigger selective death of vulnerable SMA motor neurons.

Data and Software Availability

GSE81245

Simon et al., 2017, Cell Reports 21, 3767–3780

December 26, 2017 © 2017 The Author(s).

https://doi.org/10.1016/j.celrep.2017.12.003
Converging Mechanisms of p53 Activation Drive Motor Neuron Degeneration in Spinal Muscular Atrophy

Christian M. Simon,1,2,7 Ya Dai,1,2,7 Meaghan Van Alstyne,1,2 Charalampia Koutsioumpa,1,2 John G. Pagiazitis,1,2 Joshua I. Chalif,1,2 Xiaojian Wang,1,2 Joseph E. Rabinowitz,4 Christopher E. Henderson,1,2,3,6 Livio Pellizzoni,1,2,* and George Z. Mentis1,2,3,8,*

1Center for Motor Neuron Biology and Disease, Columbia University, New York, NY 10032, USA
2Department of Pathology and Cell Biology, Columbia University, New York, NY 10032, USA
3Department of Neurology, Columbia University, New York, NY 10032, USA
4Department of Pharmacology, Center of Translational Medicine, Temple University School of Medicine, Philadelphia, PA 19140, USA
5Present address: Carl-Ludwig-Institute for Physiology, Medical Faculty, University of Leipzig, Liebigstrasse 27, 04103 Leipzig, Germany
6Present address: Neurodegeneration and Repair, Biogen, 225 Binney Street, Cambridge, MA 02142, USA
7These authors contributed equally
8Lead Contact
*Correspondence: lp2284@cumc.columbia.edu (L.P.), gzmentis@columbia.edu (G.Z.M.)
https://doi.org/10.1016/j.celrep.2017.12.003

SUMMARY

The hallmark of spinal muscular atrophy (SMA), an inherited disease caused by ubiquitous deficiency in the SMN protein, is the selective degeneration of subsets of spinal motor neurons. Here, we show that cell-autonomous activation of p53 occurs in vulnerable but not resistant motor neurons of SMA mice at pre-symptomatic stages. Moreover, pathological or genetic inhibition of p53 prevents motor neuron death, demonstrating that induction of p53 signaling drives neurodegeneration. At late disease stages, however, nuclear accumulation of p53 extends to resistant motor neurons and spinal interneurons but is not associated with cell death. Importantly, we identify phosphorylation of serine 18 as a specific post-translational modification of p53 that exclusively marks vulnerable SMA motor neurons and provide evidence that amino-terminal phosphorylation of p53 is required for the neurodegenerative process. Our findings indicate that distinct events induced by SMN deficiency converge on p53 to trigger selective death of vulnerable SMA motor neurons.

INTRODUCTION

A major challenge in neurodegenerative diseases is to unravel the mechanisms by which mutations in ubiquitously expressed genes result in selective death of specific neuronal types, leading to the distinctive clinical manifestations of each disease (Brichta and Greengard, 2014; Kanning et al., 2010; Yaron and Schuldiner, 2016). Uncovering these mechanisms would provide insights into the molecular basis of neurodegeneration and offer clues for the development of neuroprotective therapies.

In contrast to neurodegenerative diseases such as Parkinson’s, Huntington’s, and amyotrophic lateral sclerosis (ALS), in which both cell-autonomous and non-cell-autonomous pathways contribute to degeneration of vulnerable neurons (Boillée et al., 2006; Michel et al., 2016; Sambataro and Pennuto, 2012), the cell-autonomous origin of motor neuron death in spinal muscular atrophy (SMA) is well established (Fletcher et al., 2017; Gogliotti et al., 2012; Martinez et al., 2012; McGovern et al., 2015; Simon et al., 2016). Thus, SMA provides an ideal context to identify cellular pathways and key drivers of selective neurodegeneration.

SMA is the most frequent genetic cause of infant mortality. All cases result from homozygous mutation or deletion of the survival motor neuron 1 (SMNT) gene with retention of the hypomorphic SMN2 gene (Lefebvre et al., 1995), leading to ubiquitous deficiency in the SMN protein. Yet despite the reduction of SMN in all tissues, it is selective loss of spinal motor neurons that is the hallmark of SMA in both humans and mouse models of the disease (Burghes and Beattie, 2009; Tisdale and Pellizzoni, 2015). Moreover, there is striking differential vulnerability even among individual motor neuron pools. We previously showed that, consistent with the clinical features of the disease in SMA patients, spinal motor neurons innervating proximal muscles are preferentially affected and degenerate early in the disease process compared with those that innervate distal muscles and are relatively spared (Mentis et al., 2011). To date, several effector pathways have been implicated in the process of motor neuron death in SMA, which include tau phosphorylation (Miller et al., 2015), endoplasmic reticulum (ER) stress (Ng et al., 2015), and c-Jun N-terminal kinase 3 (JNK3) activation (Genabai et al., 2015), among others. However, the events that trigger degeneration of motor neurons, but not of other spinal neurons in SMA, remain poorly defined. Even more puzzling is the reason ubiquitous SMN deficiency would induce the selective death of specific pools of vulnerable motor neurons while sparing resistant motor neurons that share similar developmental programs and functional properties.
Here, we used comparative gene expression profiling of vulnerable and resistant SMA mouse motor neurons prior to the onset of neuronal death in order to identify cellular pathways causally involved in the degenerative process. We found specific upregulation of p53 transcriptional targets in vulnerable, but not resistant, SMA motor neurons, which correlated well with early-onset nuclear accumulation of p53. We subsequently demonstrated that pharmacological or genetic inhibition of the p53 pathway rescues vulnerable motor neurons from degeneration in a well-established mouse model of SMA (Le et al., 2005), identifying p53 as a key mediator of motor neuron death induced by SMN deficiency in vivo. Intriguingly, at late stages of disease, widespread p53 activation was observed in resistant motor neurons and other spinal neurons of SMA mice, which do not degenerate in the disease. To address these seemingly conflicting observations, we identified phosphorylation of p53 serine 18 as a death-specific marker that is selectively present in vulnerable SMA motor neurons. Importantly, this specific modification was not observed in resistant motor neurons or in spinal interneurons despite nuclear accumulation of p53 at late disease stages in SMA mice. Moreover, using a newly devised in vivo replacement strategy, we provide evidence that phosphorylation of specific serines in the amino-terminal transactivation domain (TAD) of p53 is necessary to trigger death of SMA motor neurons. These findings indicate that distinct events induced by SMN deficiency converge on p53 to activate a neuronal death pathway selectively in vulnerable motor neurons.

RESULTS

SMN Deficiency Induces p53 Activation in SMA Motor Neurons

To gain insight into the death pathway of SMA motor neurons, we performed comparative profiling of gene expression changes induced by SMN deficiency in vulnerable and resistant motor neurons from SMA mice. We specifically used the well-characterized SMNΔ7 mouse model of SMA that harbors homozygous knockout of the endogenous Smn gene, two copies of the human SMN2 gene, and multiple copies of the SMNΔ7 cDNA transgene (Le et al., 2005). Using laser capture microdissection, we isolated retrogradely labeled vulnerable medial motor column (MMC) motor neurons innervating the multifidus muscle and resistant lateral motor column (LMC) motor neurons innervating the gastrocnemius and tibialis anterior muscles from wild-type (WT) and SMA mice (Figure 1A), as we described previously (Lotti et al., 2012). We focused on an early symptomatic stage (P4), preceding motor neuron death in the L5 spinal segment (Mentis et al., 2011). Microarray analysis revealed expression changes in 75 genes in vulnerable MMC SMA motor neurons, but only a single change, the Smn gene, in resistant LMC motor neurons, compared with MMC and LMC motor neurons from WT mice, respectively (Table S1). KEGG pathway analysis using the

Figure 1. SMN Deficiency Induces Early-Onset p53 Activation in Vulnerable Motor Neurons of SMA Mice
(A) ChAT+ L5 LMC and MMC motor neurons retrogradely labeled following CTb-488 injection in specific muscles (gastrocnemius and tibialis anterior for LMC; multifidus for MMC). Scale bar, 50 μm.
(B) RT-qPCR analysis of p53-regulated genes in L5-MMC motor neurons laser-captured from WT and SMA mice at P4. Statistical analyses were performed using unpaired t test.
(C) Dual fluorescence in situ hybridization for Perp and ChAT mRNAs in WT and SMA L1 motor neurons at P4. Statistical analyses were performed using unpaired t test.
(D) ChAT and p53 immunostaining of L1 and L5 spinal segments from WT and SMA mice at P4. Scale bars, 120 μm and 20 μm (for insets).
(E) Percentage of p53+ motor neurons in the L1 and L5 spinal segments from WT and SMA mice at the indicated postnatal days. Statistical analyses were performed using one-way ANOVA with Tukey’s post hoc test. Values are means ± SEM.
DAVID gene ontology platform identified p53 signaling as the top pathway altered in vulnerable SMA motor neurons (p = 1.19E-04) and the specific upregulation of ten p53 transcriptional target genes (Table S1), which we validated by RT-qPCR and fluorescence in situ hybridization (Figures 1B and 1C).

To confirm that SMN deficiency induces p53 activation, we examined p53 localization in WT and SMA motor neurons by immunohistochemistry and found strong nuclear staining of p53 in vulnerable L1 and L5-MMC SMA motor neurons but not in their WT counterparts at P4 (Figure 1D). At birth, nuclear p53 accumulation was already present in ~25% of vulnerable L1 and L5-MMC motor neurons but not in resistant L5-LMC motor neurons, and by late symptomatic stages of the disease, p53 positivity progressively increased in vulnerable motor neurons and extended to resistant motor neurons in SMA mice (Figure 1E). Taken together, these results demonstrate that SMN deficiency induces nuclear accumulation and activation of p53 in SMA motor neurons.

**Inhibition of p53 Prevents Motor Neuron Death in SMA Mice**

p53 is a transcription factor that regulates several major cellular functions, including gene transcription, DNA synthesis, DNA repair, cell cycle regulation, senescence, and cell death. p53 can be activated by many cellular stressors as well as DNA damage and, depending upon the severity of the stress and the particular cell type, may induce either adaptive responses or apoptosis (Vousden and Prives, 2009). We therefore investigated the effect of genetic and pharmacological inhibition of p53 on motor neuron survival in SMA mice. For the genetic approach, we used a self-complementary adeno-associated virus serotype 9 (AAV9) expressing GFP and a short hairpin RNA (shRNA) against mouse p53, or GFP alone as a control (Figure S1A), delivered by intracerebroventricular (ICV) injection at P0 to transduce 60%–80% of motor neurons (Figures S1B and S1C; Mende et al., 2016; Passini et al., 2010). For pharmacological p53 inhibition, we administered pifithrin-α (PFT) by daily intra-peritoneal (i.p.) injections starting at P0 with a dose (2.2 mg/kg) known to suppress p53 transcriptional activity in vivo (Komarov et al., 1999; Murphy et al., 2004). Injection of AAV9-p53shRNA led to strong reduction of nuclear p53 staining (Figures 2A, 2B, and S1D) and decreased accumulation of Perp mRNA, a p53 transcriptional target, in SMA motor neurons (Figures 2C and 2D). Furthermore, PFT treatment significantly reduced Perp mRNA expression but, as expected, did not affect p53 levels in SMA motor neurons (Figures 2A–2D). Strikingly, motor neuron counts revealed that both AAV9-p53shRNA and PFT treatment resulted in complete rescue of vulnerable L1 and L5-MMC motor neurons in SMA mice (Figures 2E and 2F). Neither treatment increased the low levels of full-length SMN2 mRNA and SMN protein expression in SMA mice at P11 (Figures S1E–S1H), indicating that the pro-survival effects of p53 inhibition are independent of SMN upregulation.

We next tested whether p53 activation in SMA motor neurons is a cell-autonomous event. To do so, we used selective restoration of SMN in motor neurons of SMA mice using Cre-dependent expression (SmnCre) under the control of the choline acetyltransferase (ChAT) promoter, as previously described (Lutz et al., 2011; Martinez et al., 2012; Fletcher et al., 2017). ChAT-driven, selective expression of SMN (SMN+SMN(ChATCre)) strongly reduced nuclear accumulation of p53 in motor neurons of SMA mice (Figures 2G and 2H) and was accompanied by rescue of motor neurons from degeneration (Figure 2H), consistent with previous studies (Fletcher et al., 2017; Martinez et al., 2012). Thus, cell-autonomous activation of p53 signaling induced by SMN deficiency drives the degeneration of vulnerable motor neurons in SMA mice.

**Functional Impact of p53 Inhibition in the SMA Motor Circuit**

In addition to motor neurons, SMN deficiency affects multiple other elements of the motor circuit (Tisdale and Pellizzoni, 2015). To investigate the circuit effects of p53 inhibition, we first assessed the L1 sensory-motor reflex physiologically and morphologically (Mentis et al., 2011). There was no effect on the loss of SMA proprioceptive (VGluT1+) synapses on L1 SMA motor neurons following pharmacological or genetic inhibition of p53 (Figures 3A and 3B). However, both treatments resulted in a significant increase in the amplitude of the monosynaptic reflex, defined as excitatory neurotransmission from proprioceptive neurons to motor neurons (Figures 3C and 3D). To determine whether this functional improvement was due to the larger number of motor neurons or to enhanced synaptic function of the remaining proprioceptive synapses, we performed repetitive stimulation of sensory synapses at high frequency (10 Hz). We found that the percentage change in the reflex amplitude following consecutive stimuli did not increase in SMA mice in which p53 was inhibited compared with control SMA mice (Figures 3E and 3F), indicating that synaptic function of proprioceptive neurons was not improved by p53 inhibition. Thus, the increase in the amplitude of the spinal reflex results from the higher number of rescued motor neurons rather than improved sensory-motor neurotransmission.

Next, we examined the effect of p53 inhibition on neuromuscular junction (NMJ) innervation and found only a modest (~15%) increase in the number of fully innervated NMs in the vulnerable axial muscle quadratus lumborum (QL) in SMA mice (Figures 3G and 3H). Behaviorally, p53 inhibition in SMA mice neither extended survival nor increased body weight (Figure 3I) but showed a mild and transient improvement in righting time that was more evident in PFT than in AAV9-p53shRNA-treated SMA mice (Figure 3J). Although off-target effects of PFT cannot be ruled out, these differences on the righting reflex might be due to pharmacological treatment with PFT reaching disease relevant cellular targets more widely and effectively than AAV9-p53shRNA.

Together these experiments reveal that vulnerable SMA motor neurons rescued from death by p53 inhibition are capable of eliciting responses following synaptic activation. However, the modest effect of p53 inhibition on other motor circuit deficits such as deafferentation and NMJ denervation likely limits the overall phenotypic benefit in SMA mice.

**Late-Onset p53 Activation in SMA Spinal Neurons Is Not Associated with Death**

We determined whether p53 might be upregulated in other tissues of SMA mice. We investigated heart, liver, and skeletal
muscle (QL) but found no nuclear p53 accumulation at any stage of the disease (Figure 4A). However, p53 positivity was detected in some neurons in the cerebellum and dorsal root ganglia of SMA mice at late symptomatic stages (Figure 4A). In the spinal cord, as the disease progressed, nuclear accumulation of p53 extended beyond motor neurons and widespread p53

Figure 2. Pharmacological and Genetic Inhibition of p53 Prevents Motor Neuron Degeneration in SMA Mice
(A) ChAT, p53, and GFP immunostaining of L1 spinal segments from WT, SMA+GFP, SMA+p53shRNA, and PFT-treated SMA mice at P11. Scale bar, 30 μm.
(B) Percentage of p53+ motor neurons in the same experimental groups shown in (A).
(C) In situ hybridization for Perp and ChAT mRNAs in the same experimental groups shown in (A). Scale bar, 30 μm.
(D) Percentage of L1 motor neurons expressing both Perp and ChAT mRNAs at P11 from the same experimental groups shown in (A).
(E and F) ChAT immunostaining (E) and total number of L1 and L5-MMC motor neurons (F) in the same experimental groups shown in (A). Scale bar, 60 μm.
(G) ChAT and p53 immunostaining in WT, SMA, and SMA+SMN (ChATCre) L1 motor neurons at P11.
(H) Number of L1 motor neurons and percentage of p53+ motor neurons in the same groups shown in (G). Scale bar, 50 μm.

Statistical analyses were performed using one-way ANOVA with Tukey’s post hoc test. Values are means ± SEM. See also Figure S1.
Figure 3. Effects of p53 Inhibition on the Sensory-Motor Circuit of SMA Mice

(A) Confocal images of immunostained VGluT1+ synapses and L1 motor neurons retrogradely labeled with Texas red dextran from WT, SMA+GFP, SMA+p53shRNA, and PFT-treated SMA mice at P11. Scale bar, 20 μm.

(B) Quantification of VGluT1+ synapses on the soma of L1 motor neurons from the same groups shown in (A).

(C) Extracellular recordings from L1 ventral root (VR) following L1 dorsal root (DR) stimulation from the same groups shown in (A). Arrows indicate the maximum amplitude of the monosynaptic reflex. Arrowheads indicate the stimulus artifact. Shaded area, monosynaptic response. Scale bars, 0.2 mV and 10 ms.

(legend continued on next page)

Cell Reports 21, 3767–3780, December 26, 2017

3771
immunoreactivity was observed in the dorsal horn and intermediate gray matter of SMA but not WT mice at P11 (Figure 4B). Moreover, although no changes in p53 mRNA expression were detectable by RT-qPCR at the whole spinal cord level throughout the disease course (Figure 4C), western blot analysis showed an increase in the levels of the p53 protein at disease end stage (Figure 4D), which reflects the greater number of p53+ spinal cells and is consistent with a post-transcriptional mechanism of p53 accumulation in SMA mice.

To address whether p53 nuclear accumulation in spinal interneurons results in neuronal death, we counted neurons in laminae I–III of the spinal cord of SMA mice at P11. We found that ~80% of the p53+ cells were NeuN+ neurons (Figures 4E and 4F), and ~20% of dorsal horn neurons were p53+ (Figure 4G). Nevertheless, the number of laminae I–III neurons did not differ between WT and SMA mice at P11 (Figure 4H), indicating that nuclear p53 accumulation alone is not sufficient to drive neuronal death in SMA.

Last, we determined whether p53 activation is a specific pathogenic event induced by SMN deficiency or a feature broadly associated with motor neuron degeneration in the context of other diseases. To do so, we tested whether p53 activation was evident in spinal motor neurons from mouse models of familial ALS associated with mutations in SOD1G93A (Peters et al., 2015) and FUSP525L (Sharma et al., 2016) at disease stages in which there is ongoing motor neuron death in these models (Figure S2) (Gurney et al., 1994; Sharma et al., 2016). We found no evidence of p53 activation in either SOD1G93A or FUSP525L mice (Figure S2). Together, these results indicate that nuclear accumulation of p53 is a specific downstream event induced by SMN deficiency that is necessary but not sufficient to cause neuronal death in SMA.

**p53S18 Phosphorylation Is a Death Marker of Vulnerable SMA Motor Neurons**

Because nuclear accumulation of p53 does not alone lead to neuronal death, we postulated that additional cues converging on p53 might be required to trigger the selective degeneration of vulnerable SMA motor neurons through activation of a specific p53-dependent death cascade. Post-translational modifications serve as a critical mode of modulation of p53 function and phosphorylation of p53 amino-terminal residues has been linked to context-dependent cell death pathways (Toledo and Wahl, 2006). In particular, we investigated the phosphorylation of serine 18 in mouse p53 (serine 15 in human p53), which is associated with apoptosis but not required for p53 stability in vivo (Chao et al., 2006; Sluss et al., 2004). Strikingly, using phospho-specific antibodies, we found increased levels of phosphorylated p53S18 (phospho-p53S18) in vulnerable L1 and L5-MMC motor neurons, but in neither resistant L5-LMC motor neurons nor spinal cord neurons that exhibit p53 nuclear accumulation at late stages of the disease in SMA mice (Figures 5A and 5B). Moreover, the onset of phospho-p53S18 accumulation precedes degeneration and occurs earlier in L1 motor neurons than in L5-MMC motor neurons, in a possible correlation with the respective timing of motor neuron death in these segments (Figures 5B and 5C). Thus, p53 phosphorylation at serine 18 is specifically restricted to vulnerable SMA motor neurons and its onset correlates with the timing of motor neuron death, making phospho-p53S18 a marker for degenerating SMA motor neurons.

### Analysis of Candidate Mediators of p53-Dependent Motor Neuron Degeneration in SMA Mice

In an effort to identify potential upstream triggers of p53 activation induced by SMN deficiency and downstream effectors of p53-mediated motor neuron death, we carried out immunohistochemistry experiments in the L1 spinal cord of WT and SMA mice at P4. We focused specifically on candidate targets that are related to p53 and have previously been implicated in the neurodegenerative process in SMA, including DNA damage (Jangi et al., 2017) and JNK activation (Genabai et al., 2015) among others. However, immunostaining with antibodies against total H2AX and phosphorylated γH2AX, which is a well-established marker of DNA damage, provided no evidence for γH2AX accumulation and induction of the DNA damage response in SMA motor neurons (Figures S3A and S3B). Similarly, there was no detectable expression of phosphorylated JNK, the activated form of this upstream kinase of p53 (Genabai et al., 2019), in vulnerable motor neurons from SMA mice. These results do not support the involvement of DNA damage or JNK signaling in the activation of p53 in SMA motor neurons. To highlight possible effectors executing the p53-dependent death process, we next looked at the expression of cleaved forms of several caspases (3, 8, and 9) but found no differences between WT and SMA motor neurons (Figures S3D–S3F). Lastly, TUNEL assay did not reveal signs of apoptotic DNA fragmentation in SMA motor neurons (Figures S3G and S3H). Taken together, these results suggest that p53 activation induced by SMN deficiency may trigger a caspase-independent, non-apoptotic form of programmed cell death in SMA motor neurons.
Amino-Terminal Phosphorylation of p53 Is Required for Motor Neuron Degeneration in SMA Mice

To test the functional relevance of p53 phosphorylation in the process of motor neuron death in SMA mice, we developed an AAV9-based strategy for in vivo replacement of p53 that combines shRNA-mediated knockdown of endogenous p53 with concomitant expression of RNAi-resistant WT or mutant mouse p53 isoforms. To validate our approach, we first injected AAV9-p53shRNA either alone or together with AAV9-p53WT in WT mice (Figure S4). Strong nuclear expression of p53 was restricted to mice injected with AAV9-p53WT and was not inhibited by p53shRNA (Figures S4A–S4D). Importantly, overexpression of p53WT, which was not phosphorylated at serine 18 (Figure S4E), did not cause death of L1 and L5-MMC motor neurons in WT mice (Figure S4F), indicating that there are no intrinsic biological features of these motor neurons making them selectively vulnerable to p53 accumulation or default pathways driving phosphorylation of serine 18 when SMN levels are normal.

Having validated the in vivo replacement approach, we then tested the effect of overexpressing WT or phosphorylation mutants of p53 in SMA mice. We generated a p53 mutant harboring a serine-to-alanine substitution at position 18 (AAV9-p53S18A) that prevents phosphorylation of this residue (Figure 6A). Previous studies showed that p53S18A mice display a mild impairment of apoptosis (Chao et al., 2003, 2006; Sluss et al., 2004) and that phosphorylation of multiple serines in the amino-terminal TAD synergistically enhances the pro-apoptotic function of p53 (Jenkins et al., 2012; Cell Reports 21, 3767–3780, December 26, 2017 3773).
Toledo and Wahl, 2006). Therefore, we also generated a mouse p53 mutant (AAV9-p53<sup>4XSA</sup>) with four serine-to-alanine substitutions at positions 18, 23, 37, and 58 (Figure 6A), which correspond to serines 15, 20, 33, and 46 of human p53, whose phosphorylation has been implicated in modulating p53 function (Bulavin et al., 1999; Chao et al., 2003, 2006; Feng et al., 2006; Lee et al., 2007; Sluss et al., 2004). The percentage of L1 and L5-MMC motor neurons that displayed nuclear accumulation of p53 in SMA mice was strongly reduced by AAV9-p53shRNA but fully restored by co-injection with each of the AAV9-p53 vectors (Figures 6B–6F). Functional analysis further revealed that AAV9-p53<sup>WT</sup> reverted the protective effect conferred by AAV9-p53<sup>shRNA</sup> in SMA mice and led to the death of vulnerable L1 and L5-MMC motor neurons (Figure 6G), further validating that

Figure 5. Phospho-p53<sup>S18</sup> Is a Selective Marker of Vulnerable Motor Neurons in SMA Mice
(A) ChAT and phospho-p53<sup>S18</sup> immunostaining in the L1 and L5 spinal segments of WT and SMA mice at P4 and P11. L1: scale bars, 100 μm for low magnification and 60 μm for higher magnification. L5: scale bars, 100 μm for lower magnification and 15 μm for insets showing MMC motor neurons.
(B) Percentage of L1, L5-MMC, and L5-LMC motor neurons expressing phospho-p53<sup>S18</sup> in WT and SMA mice at P0, P4, and P11.
(C) Total number of L1, L5-MMC, and L5-LMC motor neurons in WT and SMA mice at the indicated postnatal days.
Statistical analyses were performed using one-way ANOVA and Tukey’s post hoc test.
Values are means ± SEM. See also Figure S3.
Figure 6. Amino-Terminal Phosphorylation of p53 Is Required for Motor Neuron Degeneration in SMA Mice

(A) Schematic of the protein domains of mouse p53. The serine-to-alanine mutations in the amino-terminal TAD domain tested in this study are indicated. TAD, transactivation domain; PRD, proline-rich domain; TET, tetramerization domain; CTD, C-terminal domain.


(C) ChAT and p53 immunostaining of L1 and L5 motor neurons from the same experimental groups shown in (B). Scale bars, 50 μm and 20 μm (for insets).

(D) ChAT and phospho-p53S18 immunostaining in L5 motor neurons from the same experimental groups shown in (B). Scale bars, 50 μm and 20 μm (for insets).

(E–G) Percentage of GFP+ (E) and p53+ (F) L1 and L5-MMC motor neurons as well as total number of motor neurons (G) from the same experimental groups shown in (B).

Statistical analyses were performed using one-way ANOVA with Tukey’s post hoc test. Values are means ± SEM. See also Figure S4.
motor neuron death is mediated by p53 activation. However, whereas the p53<sup>S18A</sup> mutant was able to induce death of vulnerable SMA motor neurons, the overexpression of the p53<sup>S18A</sup> mutant did not trigger motor neuron degeneration when co-injected with AAV9-p53<sub>shRNA</sub> in SMA mice (Figure 6G). Taken together, these results provide evidence that phosphorylation of p53’s amino-terminal TAD is required to trigger degeneration of SMA motor neurons.

DISCUSSION

Selective degeneration of motor neurons is a defining characteristic of SMA, but the mechanisms and cellular pathways through which reduced SMN levels cause the loss of specific pools of motor neurons are poorly understood. Our study demonstrates that selective activation of p53 occurs early in vulnerable SMA motor neurons and is a critical driver of motor neuron death, because pharmacological and genetic inhibition of p53 fully suppresses neurodegeneration in a mouse model. At late stages of the disease, however, nuclear accumulation of p53 is also observed in resistant motor neurons and spinal interneurons of SMA mice but is not associated with neuronal death. Similarly, forced overexpression of p53 in WT mice is not sufficient to drive motor neuron death. Addressing this apparent paradox, we identify phosphorylation of serine 18 of p53 as a distinct event that selectively marks motor neurons destined to degenerate in SMA mice but is absent from resistant SMA neurons exhibiting p53 accumulation at late stages of disease. Importantly, we also provide evidence that p53 phosphorylation is required for the death of SMA motor neurons in vivo. Together, our data provide critical insights into the neurodegenerative mechanisms of SMA and support a model in which nuclear accumulation and amino-terminal phosphorylation of p53 are distinct convergent events that are necessary to drive the selective death of vulnerable motor neurons (Figure 7).

Through comparative microarray analysis and subsequent molecular validation of gene changes that occur in differentially affected neurons prior to the onset of cell death, we identified transcriptional upregulation of p53 target genes as a key signature of vulnerable SMA motor neurons in a severe mouse model of the disease. Consistent with our findings, increased expression of known p53-regulated transcripts was recently reported in vulnerable SMA motor neurons from a milder mouse model of SMA (Murray et al., 2015) and in mice in which SMN deficiency was induced postnatally using antisense oligonucleotides (Jangi et al., 2017). However, no causal link between p53 activation and motor neuron death was demonstrated in SMA mice. Here, we demonstrate that genetic or pharmacological inhibition of p53 fully rescues the survival of vulnerable SMA motor neurons in vivo. Moreover, although multiple biological activities have been ascribed to p53 (Vousden and Prives, 2009) and p53 may be associated with p53<sub>Knockout</sub> in SMA mice but is independent of p53 activation and pathogenesis. Together with early evidence for p53 immunoreactivity in motor neurons from post-mortem spinal cords of SMA patients (Simic et al., 2000), our findings establish activation of p53-dependent transcription as a major driver of motor neuron degeneration in SMA.

The death of motor neurons together with the dysfunction and loss of central excitatory synapses and NMJs are among the major pathological events leading to motor deficits and skeletal muscle atrophy in SMA (Tisdale and Pellizzoni, 2015). Our findings afforded the opportunity to evaluate more broadly the functional impact of blocking p53-dependent motor neuron death in the motor circuit of SMA mice without altering SMN expression. Interestingly, although genetic and pharmacological inhibition of p53 rescued SMA motor neurons from death, it had no benefit on the number or function of sensory afferent synapses and only moderately improved NMJ denervation and motor behavior. These results are consistent with a previous study in which p53 knockout in SMA mice was found to have no significant impact on lifespan, although analysis of the motor system was not performed (Tsai et al., 2006). Our findings indicate that the overall limited behavioral benefit of preventing motor neuron death by p53 inhibition in SMA mice reflects the persistence of remaining severe functional deficits in the motor circuit and further underscores their key functional contribution to disease pathogenesis. Moreover, they reveal that reduction of the excitatory drive and loss of central synapses on motor neurons in SMA mice are independent of p53 activation and mechanistically uncoupled from the process of motor neuron death, in agreement with the non-cell-autonomous nature of these defects we recently reported (Fletcher et al., 2017; Simon et al., 2016).
Although best known as a tumor suppressor induced by DNA damage, several studies have documented p53 induction in patient tissue and in experimental models of a variety of chronic and acute neurodegenerative disorders (Culsee and Mattson, 2005), which include Alzheimer’s disease (de la Monte et al., 1997; Kitamura et al., 1997), Huntington’s disease (Bae et al., 2003), Parkinson’s disease (Duan et al., 2002; Mogi et al., 2007; Qi et al., 2016), ALS (Martin, 2000; Ranganathan and Bowser, 2010), and traumatic brain injury (Yang et al., 2016). Interestingly, our results demonstrate that induction and nuclear accumulation of p53 are not sufficient to drive neurodegeneration \textit{in vivo}. First, we found that widespread activation of p53 occurs at late disease stages in resistant motor neurons and spinal interneurons without leading to their cell death in SMA mice. Second, overexpression of p53 in WT mice did not induce death of vulnerable motor neurons. Furthermore, challenging the direct correlation between increased p53 immunoreactivity and neuronal death, our results implicate additional regulatory events in the p53-dependent death pathway of SMA motor neurons. Post-translational modifications of p53 have key modulatory roles that finely-tune the biological responses downstream of p53 induction in a stress-specific and context-dependent manner (Jenkins et al., 2012; Toledo and Wahl, 2006). Importantly, we show that phosphorylation of serine 18 of p53, a modification previously associated with pro-apoptotic functions but not stability of p53 (Chao et al., 2003; Sluss et al., 2004), is selectively found in vulnerable motor neurons and its onset precedes and correlates with their death in SMA mice, thereby establishing phospho-p53S18 as a marker for degenerating motor neurons in this disease model.

In addition to SMA motor neurons (this study), accumulation of phospho-p53S18 has been documented in human stem cell-derived motor neurons from C9orf72-related ALS patients (Lopez-Gonzalez et al., 2019) and that of phospho-p53S18 in neonatal axotomized mouse motor neurons (Martin and Wong, 2017). However, the functional relevance of this specific modification for the neurodegenerative process was not previously investigated in any disease models. Here, we report that preventing p53S18 phosphorylation by changing serine 18 to alanine is not sufficient to block death of SMA motor neurons in SMA mice. This outcome was not entirely unexpected, because apoptosis is only mildly defective in p53G18A mice (Chao et al., 2003; Sluss et al., 2004) and phosphorylation of multiple serines in the amino-terminal TAD of p53 functionally synergize to enable full p53’s pro-apoptotic responses (Jenkins et al., 2012; Toledo and Wahl, 2006). We tested this possibility directly and found that expression of the p53S18A mutant, which harbors alanine substitutions of four serines in the amino-terminal TAD of p53 that have previously been implicated in death signaling (Bulavin et al., 1999; Chao et al., 2003, 2006; Feng et al., 2006; Lee et al., 2007; Sluss et al., 2004), failed to induce degeneration of vulnerable motor neurons in SMA mice. Although the precise phosphorylation profile of p53’s TAD in SMA motor neurons could not be assessed, because of the lack of specific antibodies, this study establishes a causal link between phosphorylation of the amino-terminal TAD of p53 and the process of neurodegeneration \textit{in vivo}. Moreover, given that each serine in the amino-terminal TAD of p53 can be phosphorylated by multiple kinases and no single kinase can phosphorylate all residues (Bode and Dong, 2004; Jenkins et al., 2012), it is conceivable that more than one kinase is implicated in the p53-dependent degeneration of SMA motor neurons.

Collectively, our findings support a model in which neurodegeneration in SMA results from distinct mechanisms that converge on p53 to induce a death-related transcriptional response selectively in vulnerable motor neurons (Figure 7). On one hand, SMN deficiency induces p53 accumulation likely through protein stabilization, because it occurs without a detectable increase in p53 mRNA levels, with earlier onset in vulnerable SMA motor neurons but extending to resistant motor neurons as well as a range of central and peripheral neurons at late disease stages. On the other hand, loss of SMN activates one or more signaling cascades leading to phosphorylation of p53’s amino-terminal TAD, of which phospho-p53S18 is a representative marker, which is restricted to a subset of SMA motor neurons. We propose that it is the unique convergence of these events that underlies the selective degeneration of vulnerable motor neurons in SMA.

The upstream triggers for the accumulation and selective phosphorylation of p53 in vulnerable SMA motor neurons as well as the downstream effectors of p53-mediated neurodegeneration remain to be identified. It is also unclear why this degenerative process would be restricted to motor neurons upon ubiquitous SMN deficiency. Recently, it has been reported that motor neurons display heterogeneity in SMN levels, and those with less SMN are more susceptible to cell death (Rodriguez-Muela et al., 2017). Together with previous evidence that motor neurons express lower levels of SMN from the SMN2 gene relative to other spinal cells (Ruggiu et al., 2012), these studies suggest that differences in the degree of SMN reduction could contribute to the selective vulnerability of specific motor neuron pools in SMA. In principle, disruption of one or more of the multiple functions of SMN in RNA regulation (Donlin-Asp et al., 2016; Li et al., 2014) may induce downstream pathogenic events that converge on p53 and its regulatory network. Interestingly, DNA damage and activation of upstream kinases such as JNK are known to induce p53 and have been implicated in the degeneration of SMA motor neurons in mouse models (Genabai et al., 2015; Jangi et al., 2017). However, we found no evidence for either DNA damage or activation of JNK that might account for p53 induction in vulnerable motor neurons of SMA mice. Additionally, our studies did not reveal accumulation of cleaved caspases (3, 8, and 9) or DNA fragmentation, suggesting that p53 may induce a caspase-independent, non-apoptotic form of programmed cell death in SMA motor neurons. Further studies will be required to identify the specific mechanisms of p53-dependent motor neuron death in SMA.

In conclusion, our study provides key insights into the mechanisms of selective neurodegeneration, pointing to stabilization and phosphorylation of p53 as distinct converging events underlying the selective death of vulnerable motor neurons in SMA. Beyond their relevance for understanding disease mechanisms, these findings have potential therapeutic implications by broadening the range of candidate targets for treating SMA. The recent approval of the first treatment for SMA patients based on antisense oligonucleotides that increase SMN expression
through SMN2 splicing modulation has been a milestone achievement (Finkel et al., 2016), and other highly promising approaches to upregulate SMN are at advanced stages of clinical development (Van Alstyne and Pellizzoni, 2016; Talbot and Tizzano, 2017). Nevertheless, these treatments are unlikely to yield a complete cure and alternative therapeutic approaches that may complement SMN-inducing drugs and provide additional clinical benefit to SMA patients are intensely sought after. In this context, further dissection of the pathways upstream and downstream of p53 activation induced by SMN deficiency may yield neuroprotective targets for SMA and possibly other neurodegenerative diseases.

EXPERIMENTAL PROCEDURES

SMA Mice and Treatments
All surgical procedures on postnatal mice were performed in accordance with the NIH guidelines and approved by the Institutional Laboratory Animal Care and Use Committee of Columbia University. SMNΔ7 mice were obtained from Jackson (stock number 005025). PFT was dissolved in DMSO and delivered daily at a concentration of 2.2 mg/kg by i.p. injection starting from P0. For AA9 gene delivery, P0 mice were anesthetized by isoflurane inhalation and injected in the right lateral ventricle of the brain with ~1 x 10^11 genome copies of AA9 vectors in a PBS solution containing a vital dye (Fast Green; Sigma-Aldrich). Approximately equal proportions of mice of both sexes were used, and aggregated data are presented because gender-specific differences were not found. Further details are provided in Supplemental Experimental Procedures.

AAV9 Production
Production and purification of AAV9 vectors was carried out as previously described (Xiao et al., 1998) with modifications that are described in Supplemental Experimental Procedures.

Laser Capture Microdissection and Microarray Profiling
LMC motor neurons and MMC motor neurons innervating the gastrocnemius/tibialis anterior and multifidus muscles, respectively, were retrogradely labeled in vivo by intramuscular injection of cholera toxin B subunit (CTb) conjugated to Alexa 488 at P2. Laser capture microdissection of motor neurons was carried out at P4 as previously described (Lotti et al., 2012), and additional details are provided in Supplemental Experimental Procedures.

For microarray analysis, total cellular RNA was isolated from laser captured motor neurons using Absolutely RNA Nanoprep Kit (Stratagene), cDNA amplification and labeling was performed using the WT-Ovation Pico RNA Amplification System (NuGen), and probes were hybridized to mouse WG-6 v2 expression beadchip (Illumina). Raw data were translated into intensities with Illumina Genome Studio. Intensities were normalized with Lumi (Du et al., 2008) using the variance-stabilizing transformation method (Lin et al., 2008) followed by robust spline normalization (Workman et al., 2002) and filtered for probes with detection p values ≤ 0.05. Differential expression was analyzed with Limma (Ritchie et al., 2015) with a significance cutoff of the Benjamini-Hochberg false discovery rate set at ≤ 0.05. Data were deposited in the Gene Expression Omnibus (GEO: GSE81245). Pathway analysis was performed using the DAVID gene ontology platform. The RT-qPCR primers used to validate expression levels of p53-regulated genes are listed in Table S2.

Immunohistochemistry and Confocal Microscopy
Experimental protocols for somatodendritic labeling of motor neurons (Mentis et al., 2005, 2008) and for immunostaining of L1 and L5 segments of the spinal cord (Mentis et al., 2011) as well as of NMJs innervating the QL muscle (Fletcher et al., 2017) have been described before. ChAT* motor neurons, VGluT1* synapses, and NMJs were counted offline using the Leica LASAF software from z-stack images acquired with SP5 or SP8 Leica confocal microscopes as previously described (Fletcher et al., 2017; Mentis et al., 2011). Further details are provided in Supplemental Experimental Procedures. The antibodies used are listed in Table S3.

Statistics
Results are expressed as mean ± SEM from at least three independent experiments using three or more animals per group. Differences between two groups were analyzed using a two-tailed Student’s t test, whereas differences among three or more groups were analyzed using one-way ANOVA followed by Tukey’s correction for multiple comparisons. GraphPad Prism 6 was used for all statistical analyses, and p values are indicated as follows: *p < 0.05, **p < 0.01, and ***p < 0.001.

DATA AND SOFTWARE AVAILABILITY
The accession number for the microarray data reported in this paper is GEO: GSE81245.

SUPPLEMENTAL INFORMATION

Supplemental Information includes Supplemental Experimental Procedures, four figures, and three tables and can be found with this article online at https://doi.org/10.1016/j.celrep.2017.12.003.

ACKNOWLEDGMENTS
We would like to thank Drs. Serge Przedborski, Lloyd Greene, Wei Gu, and Tom Jessell for critical comments; Tom Maniatis for the ChAT plasmid used in fluorescence in situ hybridization (FISH); Neil Shneider for the FUS transgenic mice; and Paolo Guarneri, Hyunjin Kim, and Richard Friedman for assistance with biomedical informatics analysis. We also would like to thank Dr. Jennifer Fuentes from Cell Signaling for providing the p-p53Ser15 antibody lots 12 and 15 from their archive. This work is supported by grants R01NS078375 (G.Z.M.), R21NS084185 (G.Z.M. and C.E.H.), R01NS102451 (L.P.), R21NS098363 (L.P.), and F30NS098551 (J.I.C.) from the National Institute of Neurological Disorders and Stroke (NINDS) and grant GR10235006 (G.Z.M.) from the U.S. Department of Defense. C.M.S. was the recipient of a Young Investigator Award from Roche.

AUTHOR CONTRIBUTIONS

DECLARATION OF INTERESTS
The authors declare no competing interests.

Received: September 4, 2017
Revised: November 9, 2017
Accepted: November 30, 2017
Published: December 26, 2017

REFERENCES

3778 Cell Reports 21, 3767–3780, December 26, 2017
p38 kinase coordinates N-terminal phosphorylation and apoptosis in response to UV radiation. EMBO J. 18, 6845–6854.


