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A degron created by SMN2 exon 7 skipping is a principal contributor to spinal muscular atrophy severity

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Spinal muscular atrophy (SMA) is caused by homozygous survival of motor neurons 1 (SMN1) gene deletions, leaving a duplicate gene, SMN2, as the sole source of SMN protein. However, most of the mRNA produced from SMN2 pre-mRNA is exon 7-skipped (~80%), resulting in a highly unstable and almost undetectable protein (SMNΔ7). We show that this splicing defect creates a potent degradation signal (degron; SMNΔ7-DEG) at SMNΔ7’s C-terminal 15 amino acids. The S270A mutation inactivates SMNΔ7-DEG, generating a stable SMNΔ7 that rescues viability of SMN-deleted cells. These findings explain a key aspect of the SMA disease mechanism, and suggest new treatment approaches based on interference with SMNΔ7-DEG activity.

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Spinal muscular atrophy (SMA) is a common and often fatal motor neuron degenerative disease, and a leading genetic cause of infant mortality [Talbot and Davies 2001; Wirth et al. 2006a; Burnett et al. 2009a]. SMA severity corresponds to the degree of functional survival of motor neurons [SMN] protein deficiency. SMN is a ubiquitously expressed protein that plays a critical role in RNA metabolism, and is essential for viability of all cells in eukaryotes [Yong et al. 2004; Neuenkirchen et al. 2008]. As part of a large multiprotein complex, the SMN complex, SMN functions in the biogenesis of small nuclear ribonucleoproteins (snRNPs), the major subunits of the spliceosome [Fischer et al. 1997; Liu et al. 1997; Meister et al. 2001; Pellizzoni et al. 2002]. Although SMN deficiency manifests itself as a motor neuron disease, its molecular consequences are evident as profound disruptions in RNA metabolism in all tissues tested in an SMA mouse model [Gahanella et al. 2007; Zhang et al. 2008]. There are two SMN genes in humans, SMN1 and SMN2, both encoding the same ORF. The vast majority of SMA patients have homozygous SMN1 deletions and are sustained by one or more copies of SMN2. However, due to a C/T substitution at position 6 of exon 7 that does not change the encoded amino acid, the splicing of the SMN2 pre-mRNA incurs frequent (~80%) exon 7 skipping. This produces an SMN protein (SMNΔ7) that lacks the normal C-terminal 16 amino acids and acquires instead four amino acids, EMLA, encoded by exon 8 [Le et al. 2005]. Thus, SMN1 deletions expose the splicing defect of SMN2 and its ineffectiveness in producing full-length normal SMN protein [Wirth et al. 2006a; Cooper et al. 2009].

Biochemical experiments in vitro suggested that SMNΔ7 is not fully functional compared with normal SMN protein, including a diminished oligomerization and binding to protein substrates such as the snRNP Sm proteins [Lorson et al. 1998; Pellizzoni et al. 1999]. However, as SMNΔ7 is extremely unstable and is generally undetectable, a definitive measure of its functional deficit in cells has not been possible. Nevertheless, increased SMN2 copy number correlates with a milder clinical phenotype in SMA patients [Wirth et al. 2006b]. For example, severe SMA (type I) patients typically have one or two SMN2 copies, intermediate severity SMA (type II) patients usually have three SMN2 copies, and patients with mild SMA (type III) mostly have three or four SMN2 copies [Feldkotter et al. 2002; Cusco et al. 2006]. Furthermore, studies in cells [Wang and Dreyfuss 2001b] suggested—and experiments in SMN-deficient mice demonstrated—that expression of an increasing copy number of SMNΔ7 cDNA transgenes proportionately lessens SMA severity [Le et al. 2005]. This suggests that even a modest SMNΔ7 increase is beneficial in SMA. With this in mind, our experiments here were designed to determine the cause of SMNΔ7 instability.

Results and Discussion

We first established a reporter system that recapitulates the differential stability of full-length SMN and SMNΔ7 and allows quantitative assessment of SMNΔ7’s instability determinants. Luciferase (Luc) reporter proteins consisting of normal SMN or SMNΔ7 fused to the C terminus of Luc were produced by transfection of the corresponding cDNA constructs in 293T cells (Fig. 1). Forty-eight hours after transfection, cells were treated with the protein synthesis inhibitor cycloheximide (CHX), and Luc activity was measured at time intervals of up to 10 h. Consistent with previous reports [Lorson and Androphy 2000], SMN has a half-life of >8 h, whereas SMNΔ7 has a half-life of ~3 h. After 10 h of CHX chase, there was three times more SMN than SMNΔ7. Several constructs were prepared to determine the role of the C-terminal sequence of SMNΔ7 in this protein’s instability. Deletion of the C-terminal EMLA from SMNΔ7 (SMNΔ7ΔEMLA) increased the half-life of SMNΔ7 by twofold [Fig. 1B], and a further deletion of the YG box (SMNΔ7ΔYG), a conserved tyrosine/glycine-rich motif in divergent SMNs [Talbot et al. 1997] that is essential for SMN oligomerization [Pellizzoni et al. 1999], also had the same effect. These results suggest that EMLA and the YG box are major contributors to SMNΔ7’s instability. Importantly, YG + EMLA alone was sufficient to cause dramatic instability of Luc that is similar to that of SMNΔ7. Neither YG nor EMLA alone was sufficient for full destabilization activity [Fig. 1B]. N-terminal deletions...
in the YG box decreased the destabilizing activity of YG + EMLA [data not shown]. These data indicate that YG + EMLA, corresponding to SMNΔ7 amino acids 268–282, is the minimal sequence required for full SMNΔ7 destabilization, and is both necessary and sufficient to trigger rapid degradation of a heterologous protein.

As a further test of this conclusion, we fused YG + EMLA to another reporter, GFP, and expressed this protein as well as GFP as a control in 293T cells. The GFP signal from GFP-YG + EMLA, as determined by Western blots, showed a gradual decrease after treatment with CHX [Fig. 2A]. GFP-YG + EMLA protein decreased faster than GFP-NS [nonspecific sequence], and the half-life of GFP-YG + EMLA was about half that of GFP-NS. These results suggest that YG + EMLA functions as a protein degradation sequence. For comparison, we tested the destabilizing activity on the same reporter of YG + EMLA and an optimized PEST, a potent and well-characterized protein destabilizing signal [Li et al. 1998]. YG + EMLA had a similar effect to that of the genetically improved PEST [Fig. 2B]. Furthermore, SMN Exon6 + EMLA, which is the same size as the 41-amino-acid PEST sequence, conferred similar instability. As this optimized PEST sequence has about half the half-life of the natural one [Li et al. 1998], YG + EMLA could be esterified with similar or stronger destabilization activity than that of the natural PEST sequence, and Exon6 + EMLA is about twice as strong. These data demonstrate that YG + EMLA is a highly potent and transferable protein degradation signal [degron], which we term SMNΔ7-DEG, for SMNΔ7 degron. Addition of five amino acids to the C-terminal end of EMLA [SMNΔ7 + 5aa] caused SMNΔ7 stabilization, indicating that SMNΔ7-DEG must be exposed at the C terminus of the protein for activity [Fig. 2B]. This is consistent with the observation that several additional amino acids, which can be effected by aminoglycoside-forced translational read-through, enhanced SMNΔ7 stability and functionality [Mattis et al. 2008; Heier and DiDonato 2009].

SMN was shown previously to be degraded by the proteasome [Chang et al. 2004; Burnett et al. 2009b]. To determine if SMNΔ7 is also degraded by this system, cells expressing Luc-SMNΔ7 were treated with proteasome inhibitors [MG132 and Lactacystin] for 5 h in the presence of CHX. CHX treatment alone resulted in a 60% decrease in signal, but a much smaller decrease was seen in the presence of proteasome inhibitors MG132 and Lactacystin [Supplemental Fig. 1]. Inhibitors of other proteolytic activities—such as lysosomal proteases, auto-phagy, and calpain [NH4Cl, 3-methyladenine, and calpeptin, respectively]—had no effect. These data demonstrate that SMNΔ7 is degraded by the proteasome.

To identify specific residues in the SMNΔ7-DEG that are important for its activity, we performed mutagenesis of the YG box, converting every second residue to alanine in the context of full-length SMNΔ7, and determined the half-life of each in 293T cells. Of seven mutations tested, S270A produced the most striking effect, reversing the destabilizing activity of the SMNΔ7-DEG [Fig. 3A]. To confirm that S270A stabilizes SMNΔ7, HA-tagged proteins SMN, SMNΔ7, and SMNΔ7S270A were expressed in 293T cells for 24 h, and then treated with the proteasome.
inhibitor MG132 for 16 h. The levels of the tagged SMN proteins were then monitored by Western blots using anti-HA antibody (Fig. 3B). As expected, the amount of SMN\textsubscript{D7} without MG132 treatment was much lower than that of SMN. However, the amount of SMN\textsubscript{D7}S270A was similar to that of normal SMN, indicating an almost complete restoration of stability by S270A mutation. MG132 caused a dramatic increase in the amount of SMN\textsubscript{D7} (3.4-fold), but only a moderate effect on SMN and SMN\textsubscript{D7}S270A (Fig. 3C). Therefore, the S270A mutation limits the proteosome degradation of SMN\textsubscript{D7} and increases its stability very significantly. We further tested the effect of S270A in the context of SMN\textsubscript{D7}-DEG alone. S270A mutation strongly increased the stability of Luc\textsubscript{YG} + EMLA to a level similar to that of normal SMN (Fig. 3D). These data indicate that the enhancement of stability of SMN\textsubscript{D7} by the S270A mutation occurs through SMN\textsubscript{D7}-DEG.

To determine whether SMN\textsubscript{D7}S270A is a functional SMN protein, we used a previously established cell system, the S5 cell line, to ask if it could rescue the viability of SMN-depleted cells. S5 is derived from chicken DT40 cells in which the endogenous chicken SMN gene is disrupted by homologous recombination and SMN protein is expressed exogenously from a cDNA under a tetracycline-repressible promoter (Wang and Dreyfuss 2001a). Upon depletion of chicken SMN (cSMN), S5 cell growth arrests at 72 h and cell death occurs. It is therefore useful to assess the physiological functionality of SMN mutants in this cell system by monitoring cell viability after turning off cSMN cDNA expression and simultaneously expressing exogenous SMN mutants of interest. To do so, we constructed recombinant retroviruses expressing SMN, SMN\textsubscript{D7}, or SMN\textsubscript{D7}S270A and transduced S5 cells. One week after repression of cSMN expression by tetracycline (1 \(\mu\)g/mL), there was a very clear difference in viable cell number among three samples (Fig. 4A,B). As expected (Wang and Dreyfuss 2001b), while SMN rescued the viability of S5 cells, SMN\textsubscript{D7} did not. Importantly, SMN\textsubscript{D7}S270A also rescued S5 cells to a similar extent as SMN. The two rescued cell lines expressed a similar level of SMN protein (Fig. 4C). Since the deficiency in functional SMN protein is correlated directly with snRNP assembly defects in cells of SMA patients (Wan et al. 2005), we next examined whether SMN\textsubscript{D7}S270A is active in snRNP assembly as a further measure of functionality.

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**Figure 3.** S270 is critical for the activity of the SMN\textsubscript{D7}-DEG through YG + EMLA. (A) Seven residues in the YG box were each mutated to alanine as indicated. All constructs had Luc fusions, and Luc activity was assayed as described in Figure 1. (B) HA-tagged SMN, SMN\textsubscript{D7}, and SMN\textsubscript{D7}S270A were expressed in 293T cells for 24 h, and then cells were treated with DMSO (D) or 1 \(\mu\)M MG132 (MG) for 16 h. Fusion proteins were monitored by Western blots using an anti-HA tag antibody, and Magoh was used as a loading control. (C) HA-tagged proteins in B were quantified and compared with HA-SMN without MG132 treatment, which was set to 100%. The fold change of each fusion protein amount upon MG132 treatment is indicated in red above the column. (D) All constructs had Luc fusions, and Luc activity was assayed as described in Figure 1. Error bars represent SDs from three independent experiments.

**Figure 4.** SMN\textsubscript{D7}S270A rescues SMN-deficient cells and is functional in snRNP assembly. (A) S5 cells were cultured in the presence of tetracycline (1 \(\mu\)g/mL) to deplete endogenous SMN, and were infected with retroviruses expressing SMN, SMN\textsubscript{D7}, or SMN\textsubscript{D7}S270A. One week after tetracycline addition, cells were stained with Trypan blue and visualized by DIC microscopy. (B) Cell growth as in A was measured by monitoring the number of live cells at the indicated time points following tetracycline addition. (C) Western blots of SMN protein in rescued cells (10 d after tetracycline addition). (D) Cytoplasmic extracts from rescued cells were assayed for snRNP assembly on U4 snRNA in vitro, using U4\textsubscript{Sm} RNA as a control.
from cells expressing SMN and SMN\(\Delta 7^{52,70A}\) were prepared, and their snRNP assembly activity was determined by measuring the amount of Sm protein cores that form on biotinylated snRNA substrate captured on streptavidin beads [Wan et al. 2005]. Sm cores are the major constituents of snRNPs whose assembly on snRNAs depends on the SMN complex. As shown in Figure 4D, both cell lines showed similar activity. These data indicate that SMN\(\Delta 7^{52,70A}\) is a functional protein similar to normal SMN in S5 cells. We conclude that the instability of SMN\(\Delta 7\) conferred by SMN\(\Delta 7\)-DEG is a principal contributor to the deleterious phenotype of exon 7 skipping, and that S270A substitution in SMN\(\Delta 7\) abrogates the degron activity, thereby restoring the function of SMN.

Several diverse classes of degrons that target proteins to various degradation pathways have been described. Most noted are N degrons comprised of destabilizing N-terminal residues, C-terminal determinants containing relatively unstructured hydrophobic residues, and phospho-degrons that are modulated by the phosphorylation status of their serine/threonine residues in response to cell signaling [Parsell et al. 1990; Ravid and Hochstrasser 2008]. The short-lived tumor suppressor protein PTEN's oligomerization capacity and other possible deficits as a result of deletion of the peptide encoded by exon 7 may contribute to the deleterious phenotype of exon 7 skipping, and that S270A substitution in SMN\(\Delta 7\) abrogates the degron activity, thereby restoring the function of SMN.

The reduced oligomerization efficiency of SMN\(\Delta 7\) has been suggested recently to account for its instability [Burnett et al. 2009b]. Indeed, intermolecular SMN oxidative cross-linking provided direct evidence that SMN is oligomeric in cells [Wan et al. 2008]. Oligomerization is likely to be important for SMN function, and also to contribute to its stability. However, although SMN oligomerization correlated with its stability, this did not explain the intrinsic instability of SMN\(\Delta 7\). Our findings show that attachment of SMN\(\Delta 7\)-DEG to monomeric protein reporters (Luc and GFP) triggered their rapid degradation, indicating that lack of oligomerization is not the major cause of SMN\(\Delta 7\)'s instability. Loss of oligomerization capacity and other possible deficits as a result of deletion of the peptide encoded by exon 7 may result in an SMN protein that is functionally suboptimal. However, the detrimental effect of exon 7 skipping does not arise primarily from deletion of a functionally essential domain, but from the creation of a positively acting and potent degron that causes severe deficiency of SMN\(\Delta 7\) protein.

Given the ability of S270A mutation to restore SMN\(\Delta 7\)'s stability and complement SMN loss of function, it is reasonable to predict that polymorphisms that inactivate SMN\(\Delta 7\)-DEG, such as at S270, would result in a milder SMA phenotype than the genotype predicts based on SMN2 copy number in SMN1-deleted individu-als. Our finding with SMN\(\Delta 7^{52,70A}\) indicates that SMN\(\Delta 7\) is a functional SMN protein, and that its stabilization could prevent or lessen SMA severity. We suggest that interfering with SMN\(\Delta 7\)-DEG activity could be an effective approach for mitigating its deficiency as a potential treatment for SMA. Although the inhibitor studies suggest that the degradation of SMN\(\Delta 7\) likely occurs in the proteasome, general inhibition of proteasome activity would be very toxic, particularly in the long-term treatment that SMA would be expected to require. A targeted inhibition of the factors that mediate the SMN\(\Delta 7\)-DEG-dependent degradation should provide a more specific therapeutic approach, and their identification will be of great interest for SMA therapy.

SMA is thus the result of a fateful chain of events. Homozygous SMN\(1\) deletion is a cause of SMA only because it exposes the splicing defect of SMN\(2\). We argue that the splicing defect in SMN\(2\) causes SMN deficiency because it fortuitously creates a degron. The degron is a key to SMA, as it is the most direct cause of SMN deficiency, which then results in major perturbations in RNA metabolism.

Materials and methods

**Plasmid construction and generation of mutations**

To construct plasmids expressing Luc-fused proteins, the Luc gene was cloned into pcDNA3.1 vector at HindIII/KpnI sites, and then DNA fragments encoding full-length wild-type human SMN, SMN\(\Delta 7\), several deletion mutants of SMN\(\Delta 7\), and optiPEST were inserted into the KpnI/XhoI sites of SMN\(\Delta 7\) mutants with a single amino acid change were generated by mutating residues in YG + EMLA to alanine by QuickChange site-directed mutagenesis kit (Stratagene). Plasmid expressing GFP-YG + EMLA was constructed by inserting a DNA fragment encoding YG + EMLA into pEGFP vector (Clontech) at KpnI/XhoI sites. Plasmids expressing HA-SMN\(\Delta 7\)s were constructed by inserting DNA fragments encoding HA-tagged SMN\(\Delta 7\) into the BamHI/XhoI sites of pcDNA3 vector. To generate retroviral plasmids to express SMNs in S5 cells, DNA fragments encoding SMN, SMN\(\Delta 7\), and SMN\(\Delta 7^{52,70A}\) were cloned into the EcoRI/XhoI sites of pMX vector as described (Wang and Dreyfuss 2001a).

**Assays for protein stability**

Luc- and GFP-based assays were performed as described in the legends for Figures 1 and 2A, respectively. Luc activities were measured using One-Glo reagent (Promega).

**Rescue of S5 cell viability**

S5 cells were maintained and infected with retroviruses expressing SMN, SMN\(\Delta 7\), and SMN\(\Delta 7^{52,70A}\) as described [Wang and Dreyfuss 2001a].

**SMN complex activity assay**

Cytoplasmic extracts from rescued S5 cells were prepared and assayed for snRNP assembly in vitro as described [Wan et al. 2005].

**Antibodies**

Mouse monoclonal antibodies anti-SMN [62E7] and anti-Magoh [18G12] were used as described previously [Wan et al. 2005]. Rabbit polyclonal antibodies anti-HA (Santa Cruz Biotechnologies) and anti-GFP (Santa Cruz Biotechnologies) were used as recommended by the manufacturer.

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