Inhibition of Apoptosis by Z-VAD-fmk in SMN-depleted S2 Cells*

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Spinal muscular atrophy is an autosomal recessive motor neuron degenerative disorder, caused by the loss of telomeric copy of the survival motor neuron gene (SMN1). To better understand how motor neurons are targeted in Spinal muscular atrophy patients, it is important to study the role of SMN protein in cell death. In this report, we employed RNA interference (RNAi) to study the loss-of-function of SMN in Drosophila S2 cells. A 601-base pair double-stranded RNA (dsRNA) of Drosophila SMN (dSMN) was used for silencing the dSMN. Our data indicate that dSMN RNAi resulted in more than 90% reduction of both RNA and protein. Further analysis of S2 cells by cell death ELISA and flow cytometry assays revealed that reduction of dSMN expression significantly increased apoptosis. The cell death mediated by SMN depletion is caspase-dependent and specifically due to the activation of the endogenous caspases, DRONC and DRICE. Significantly, the effect of dSMN RNAi was reversed by a peptide caspase inhibitor, Z-VAD-fmk. These results suggest that dSMN is involved in signal pathways of apoptotic cell death in Drosophila. Hence, the model system of reduced SMN expression by RNAi in Drosophila could be exploited for identification of therapeutic targets.

The autosomal recessive disease, spinal muscular atrophy (SMA) occurs in 1 in 10,000 live births and is one of the most common genetic causes of infant death, resulting from death of motor neurons in anterior horn cells. SMA is clinically categorized as types I, II, and III based on age of onset and clinical progression. Type I (severe) infants have onset of weakness within the first six months of life and typically die within the first two years. Type II (intermediate) children experience symptoms before age two and never walk unassisted. Type III (mild) patients exhibit less severe clinical symptoms with a highly variable age of onset. It was found that the survival of motor neuron (SMN) gene is the SMA-determining gene and is deleted, converted, or mutated in >95% of SMA patients. There are two human SMN genes, SMN1 and SMN2 (1). However, only loss of the SMN1 gene causes the disease. SMN2 is maintained in most of the SMA patients, in many cases with multiple copies. However, 80% of SMN2 products are the alternatively spliced SMNΔ7 that lacks exon 7 and is unstable, whereas the primary product of SMN1 is full-length SMN mRNA. It has been demonstrated that clinical severity of SMA disease is inversely correlated with the number of SMN2 copies in SMA patients (2), suggesting that disease progression is inversely dosage-dependent on full-length SMN proteins produced.

Mice have only a single copy of the SMN gene that does not undergo alternative splicing of exon 7 and therefore is a functional SMN1 homologue. Homozygous knockout of the mouse SMN gene is embryonic lethal at a very early developmental stage (3). By crossing mice transgenic for human SMN2 into a SMN+/− heterozygous mouse, animals with an hSMN2/− genotype were born. These mice did not die in utero, implying SMN2 can rescue the lethality of homozygous knockout. A single copy of human SMN2 was sufficient to restore mouse viability from SMN−/− lethality; however, severe weakness developed and the mice died within few days after birth. Mice with eight copies of hSMN2, on the other hand, appear normal, providing additional evidence that SMN2 functions as a disease modifying gene by producing low levels of full-length SMN, explaining the correlation between disease severity and SMN2 copy numbers (4). SMN homologues have been also isolated from Caenorhabditis elegans (5), Danio rerio (zebrafish) (6), Schizosaccharomyces pombe (7–9), and recently from Drosophila (10). Biochemically, the SMN proteins from these species retain a number of properties identified in the human SMN, including RNA binding activity and self-association. Defects of SMN proteins in C. elegans, D. rerio, and S. pombe affect cell viability and growth.

Drosophila models have been generated by ectopic over-expression of human SMN protein domains that may function as dominant negatives to disrupt the endogenous dSMN protein, resulting in abnormal positioned wings and legs or in many cases, pupal lethality (10). However, inactivation of function of a protein using dominant negative mutants can yield misleading results if the expressed protein has additional functions or if it interacts with its endogenous counterpart (11). During the last few years, RNA interference has emerged as a promising and powerful technology to investigate the loss-of-function of a protein by specifically suppressing expression of a gene in a population of cells. Thus, RNAi was used to investigate the mechanisms of SMN-deficiency-induced cell death in a cell culture model, the insect S2 cells. Our results showed that cells with SMN expression suppressed by RNAi underwent apoptosis. Apoptosis occurred via a caspase pathway-dependent process by activation of DRONC, an apical caspase in...


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*Drosophila*, which further activated its effector caspase, DRICE. Furthermore, Z-VAD-fmk (N-benzylloxycarbonyl-Val-Ala-Asp-fluoromethylketone), which is a cell-permeable caspase inhibitor, efficiently blocked cell death induced by SMN deficiency. Taken together, these results strongly suggest that investigation of factors that mediate SMN and caspase-dependent apoptosis may provide clues on functions of the SMN protein and targets for identification of small molecules/drugs for SMA therapy.

**EXPERIMENTAL PROCEDURES**

**Antibodies**—To generate antibodies specific for Drosophila SMN, a plasmid construct containing full-length of dSMN was fused to glutathione-S-transferase in pGEX-4T1 (Amersham Biosciences). Fusion protein was purified according to the manufacturer's instructions. Anti-dSMN antibodies (dSMNAb) were raised against the fusion protein in rabbits and affinity purified by the GST or GST-fusion proteins that were covalently linked to glutathione Sepharose-4B resin (12). Antibodies specific for the human SMN are from Santa Cruz Biotechnology (Santa Cruz, CA) or Transduction Laboratory (Lexington, KY). Anti-HA antibody 12CA5 is from Roche Molecular Diagnostics. The monoclonal anti-tubulin antibody is from Oncogene (San Diego, CA). The polyclonal rabbit anti-DRONC, rabbit anti-DRICE, and monoclonal mouse anti-DIAP1 (Drosophila inhibitor of apoptosis protein 1) antibodies were a generous gift from Dr. Bruce A. Hay (CalTech, Pasadena, CA).

**Cell Culture and Apoptosis Induction**—S2 cells were grown in Schneider's Drosophila media (Invitrogen) supplemented with 10% fetal bovine serum, 20 units/ml penicillin, 20 μg/ml streptomycin, and 0.434 mg/ml of GlutaMax at 27 °C. For apoptotic induction as a positive control, cells were treated with 1 μg actinomycin D for 7 h. For all the analyses, three independent experiments were conducted.

**Plasmid Constructs**—The 0.7-kilobase fragment of dSMN coding region with HA-tag was cloned into KpnI and SalI sites of a Drosophila expression vector, pRMHa-3, containing the metallothionein promoter (13) or the pcDNA3 vector. Expression of the HA-dSMN protein in pcDNA3-HA-dSMN transfected mammalian C3A cells or in the pRMHa-3-HA-dSMN transfected S2 cells that were induced by 50 μg copper sulfate for 6 h was detected by Western blot analysis with anti-HA antibody or anti-dSMN antibodies. Similarly, a construct for human SMN was generated in pRMHa-3. Expression of hSMN in S2 cells was detected by immunofluorescence with anti-hiSMN antibodies as described below.

**RNAi in S2 Cells and Z-VAD-fmk Treatment**—PCR products containing coding sequences for the dSMN (forward primer: 5'-TAA TAC GAC TCA CTA TAG GG AAG ACC TAG GAC GAG TGG-3'; and reverse primer: 5'-TAA TAC GAC TCA CTA TAG GG GTG GTG GTG GCT TCT TTC-3'; product length, 601bp; bold and italics letters represent T7 promoter sequences) and control Drosophila Prenalin (dPsn) gene (forward primer: 5’-TAA TAC GAC TCA CTA TAG GG TG GTG GCT GTC AAT TTC-3’; and reverse primer: 5’-TAA TAC GAC TCA CTA TAG GG CGA TAG CAA CGC TTC TTG-3’; product length: 543bps) were obtained and gel-purified. Double-stranded RNAs (dsRNA) were generated by *in vitro* transcription with Ribomax T7 Transcription kit (Promega) and digested with EcoRI-free DNase. The dsRNA products were ethanol precipitated and annealed by incubation at 65 °C for 30 min and then slowly allowed to cool at room temperature. The annealed dsRNA products were analyzed on a 1% agarose gel to ensure the majority of dsRNA existed as a single band. The dsRNA (2 μg) and/or plasmid DNAs (2 μg) were introduced into cells by using Cellfectin (Invitrogen) according to the manufacturer's instructions. G418 selection was achieved by using 50 μg/ml of Z-VAD-fmk (Alexis Biochemi- cal, San Diego, CA) in the culture medium.

**Semi-quantitative Multiplex RT-PCR**—Total RNA was isolated using the Trizol reagent (Invitrogen) from cells transfected with dSMN dsRNA, yeast tRNA, dPsn dsRNAs, or mock-transfected. For first-strand cDNA synthesis, 0.5 μg of total RNA was used. To amplify the endogenous dSMN and dPsn transcripts, a multiplex RT-PCR was performed using primers from dsSMN and dPsn under the following PCR condition (94 °C for 45 s, 55 °C for 1 min, and 72 °C for 1 min for 28 cycles and then 72 °C 10 min extension). The reaction gave rise to two bands (dsSMN and dPsn), each of which was used as an internal control for their respective expression levels, and they were quantified on an agarose gel by gel-documentation software (UVF, Upland, CA).

**Western Blot Analysis**—Cells were grown in 100 mm cell culture dishes and transfected with dsRNA of dSMN and controls. After 72 h cells were harvested and lysed with 500 μl of radioimmune precipitation assay buffer lysis buffer (50 mM Tris–HCl (pH 7.4), 1% Nonidet P-40, 0.25% sodium-deoxycholate, 150 mM NaCl, 1 mM EDTA, 1 mM aprotinin, and 1 μg/ml of phenylmethylsulfonyl fluoride, leupeptin, and pepstatin each), and the amount of protein was quantified by BCA assay. Equal amounts of protein from control and dsRNA of dSMN transfected cells were boiled for 5 min in Laemmli’s buffer (14) and separated on 12% and/or 15% SDS-PAGE, and the resolved gels were blotted on Immobilon P membrane (Millipore, Bedford, MA). The transferred blots were probed with antibodies against dSMN, anti-DRONC, full-length anti-DRICE, and anti-DIAP1 (15) followed by secondary horse-radish peroxidase-conjugated antibodies. In all cases, blots were stripped with stripping buffer (62.5 mM Tris (pH 6.7), 2% SDS and 90 mM 2-mercaptoethanol) and reprobed with anti-tubulin for loading controls. The signal was detected by enhanced chemiluminescence (Pierce). To generate antibodies specific for Drosophila SMN, a plasmid containing full-length coding sequences for the Drosophila SMN (dSMN) protein, two antibodies against dSMN were raised against dSMN, anti-DRONC, and anti-DIAP1, which further activated its effector caspase, DRICE. Furthermore, Z-VAD-fmk (*N*-benzyloxycarbonyl-Val-Ala-Asp-fluoromethylketone), which is a cell-permeable caspase inhibitor, efficiently blocked cell death induced by SMN deficiency. Taken together, these results strongly suggest that investigation of factors that mediate SMN and caspase-dependent apoptosis may provide clues on functions of the SMN protein and targets for identification of small molecules/drugs for SMA therapy.

**RESULTS**

**Characterization of Drosophila SMN Protein**—To investigate expression of Drosophila SMN (dSMN) protein, two antibodies against GST-dSMN protein were raised in rabbits and then affinity-purified. The dSMN gene was sub-cloned into HA-tagged pcDNA3 vector and transfected into mammalian C3A cells. Western blot analysis shows that both dSMN antibodies specifically recognized a single band in C3A cells transiently transfected with the HA epitope-tagged full-length Drosophila SMN cDNA (Fig. 1A, upper panel, lane 1), but not in cells transfected with empty vector (Fig. 1A, upper panel, lane 2). The band was also confirmed as HA-dSMN by Western blot analysis with anti-HA antibody (12CA5) (Fig. 1A, middle panel). The molecular mass of HA-dSMN vector pRMHa-3 and transfected into Drosophila S2 cells. The HA-tagged dSMN protein expression was detected in transfected cell lysates probed with anti-HA antibody (Fig. 1B, middle panel, lane 2), and the band was undetected in control cells transfected with empty vector (Fig. 1B, middle panel, lane 1). By using anti-dSMN antibodies, higher expression of dSMN was detected in HA-dSMN transfected cells (Fig. 1B, upper panel, lane 2) than in cells transfected with empty vector (Fig. 1B, upper panel, lane 1) as judged by immunoblot analysis. The antibodies clearly recog-
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Immunolocalization of SMN in S2 Cells—Because there is no report on expression of Drosophila SMN protein in cells, we sought to determine the localization of dSMN protein in S2 cells. Constructs of both dSMN and hSMN, tagged and/or untagged in the pRmHa-3 vector were generated and co-transfected into cells. After 72 h, protein expression was induced by 500 µM of copper sulfate for 6–8 h. Immunofluorescence assay revealed co-localization of dSMN and hSMN proteins to the nucleus (Fig. 3, F–H). Staining for human SMN was also detected as discrete, punctate sub-nuclear dots in co-transfected cells, reminiscent of “gems” in the nucleus of some of S2 cells (Fig. 3L). With purified anti-dSMN antibodies, we identified the localization of the endogenous Drosophila SMN in cells, and it was diffusely distributed throughout cytoplasm (Fig. 3A). However, enriched localization was detected in the nucleus when dSMN was over expressed (Fig. 3B). Only the endogenous level of the dSMN protein expression was detected in HA-dSMN transfected but non-induced cells (Fig. 3C). The localization could not be seen in cells treated with dsRNA of dSMN (Fig. 3D), indicating specificity of anti-dSMN antibodies. Staining to cytoplasm and nucleus in some cells transfected with both dsRNA and HA-dSMN may be due to incomplete suppression of HA-dSMN over-expression or those cells failed to conceive the dsRNA (Fig. 3E).

Deficiency of dSMN in S2 Cells Induces Apoptosis—To investigate how motor neurons die in SMA patients, it is important to understand the mechanisms of cell death induced by SMN deficiency. Accordingly, we used RNAi to suppress SMN expression in S2 cells. Morphological changes were observed in dSMN dsRNA-treated cells after 48 h (Fig. 3, I–K). The cells were stained with Annexin V-Cy5 and analyzed for apoptosis by flow cytometry. As a positive control for induction of apoptosis, cells were treated with a low concentration of actinomycin D (1 µM) (Fig. 4B) that has been previously shown to effectively induce apoptosis (16), whereas untreated S2 cells were used as negative controls (Fig. 4A). The percentage of Annexin-V-positive cells indicates an increase in phosphatidylserine on the cell surface of cells in response to an apoptotic stimulus. Furthermore, there was an increase in apoptosis (25.8%) in the dSMN dsRNA-treated cells (Fig. 4D) compared with the baseline percent apoptosis (9.17%) of the cells transfected with the GFP marker and CAT dsRNA (Fig. 4C). This result is representative of three separate experiments. In these three experiments, the mean percent apoptosis in SMN dsRNA/GFP co-transfectants was 18.8 ± 4.0%, whereas the mean percent apoptosis in the control-treated cells was 8.3 ±

Fig. 1. Western blot analyses demonstrating specificity of dSMN antibody to the dSMN protein. A, the mammalian C33A cells transfected with HA-tagged dSMN (in pcDNA3 vector) showing expression of dSMN protein (top panel, lane 1) (probed with anti-dSMN antibody), and the band was undetected in the empty vector transfected cell lysates (top panel, lane 2). Middle panel, the same cell lysates were immunoblotted and probed with anti-HA antibody showing the expression of dSMN (lane 1), and the band was undetected in the lysates prepared from empty vector transfected cells (lane 2). B, expression of HA-tagged dSMN (in Drosophila expression vector, pRmHa-3) in S2 cells. Top panel, the immunoblot was probed with anti-dSMN antibody showing high expression of HA-dSMN fusion protein (lane 2), and low expression of endogenous dSMN (lane 1) was detected in empty vector transfected cell lysates. Middle panel, same cell lysates were blotted and probed with anti-HA antibody showing high expression of HA-dSMN fusion protein, and it was undetected in control cell lysates (lane 1). A and B, bottom panels, the tubulin blots are shown as protein loading control.

Fig. 2. Multiplex RT-PCR and immunoblot analyses of dSMN RNAi in S2 cells. A, semi-quantitative RT-PCR analysis demonstrating the relative expression of two endogenous fly genes, Drosophila SMN (dSMN) and dPsn. S2 cells (mock-treated) and control (cells transfected with yeast tRNA) showing identical amount of expression of dSMN and dPsn. About 90% reduction of dSMN transcript was evident in dSMN RNAi experiments and the level of dPsn expression was unchanged. In contrast, dPsn expression was reduced 90% in dPsn RNAi and the level of internal control (dSMN) was not changed. B, immunoblot analysis showing the level of dSMN in control S2 cells was unaffected, and the level of dsSMN was reduced 90% in cell lysates of dSMN RNAi. C, the blot was stripped and reprobed with tubulin antibody indicating protein loading control.

nized the endogenous dSMN protein in S2 cells as a single band with a molecular mass of 27 kDa, indicating the specificity of both antibodies. The bands were stripped and reprobed with anti-tubulin antibody showing equal amounts of proteins (Fig. 1, A and B, bottom panels) were loaded.

RNAi Suppresses Expression of SMN in S2 Cells—To determine the effect of depletion of dSMN protein in cultured S2 cells, a 601-bp fragment of double-stranded RNA (dsRNA) of the dSMN gene was transfected into the cells. The semi-quantitative RT-PCR analysis demonstrated that transfection of dsRNA of dSMN in cells robustly inhibited (> 90%) its corresponding endogenous target after 72 h. The transcript of another endogenous gene, dPsn, was not affected. Similarly, cells treated with dsRNA of dPsn suppressed > 90% of its transcript, whereas the level of dSMN expression was unaffected (Fig. 2A). Similar results were confirmed in two other RNAi experiments. Silencing of dSMN expression was monitored by immunoblotting the dSMN protein. The amount of the endogenous dSMN protein was reduced to 90% in dSMN RNAi cell lysates (Fig. 2B, lane 2), and the level was unaffected in cells treated with yeast tRNA (control) (Fig. 2B, lane 1). There was no appreciable difference between the amounts of tubulin (loading control) in control and dsRNA-treated cells (Fig. 2C). Furthermore, we also analyzed the effect of RNAi after 24 and 48 h, and the level of dSMN expression was only slightly suppressed, whereas the level of dPsn expression was reduced to 90% after 24 h (data not shown), suggesting the relative stability of dSMN mRNA.

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0.9%. The association between SMN dsRNA treatment and apoptosis within each of the three experiments was found to have a p value of <0.0001 by the Chi-squared test. As controls (not shown), necrosis was induced in a subpopulation of cells using 10 μM actinomycin D. Loss of membrane integrity of necrotic cells was confirmed by Trypan Blue staining (data not shown).
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![diagram](image)

**Fig. 5. dSMN RNAi induces DRONC and DRICE activation in S2 cells.** A, cell lysates analyzed by immunoblot for DRONC protein from control (CAT dsRNA) and dSMN dsRNA treated cells. The full-length (~50 kDa) DRONC was evident in control (lane 1), and its processed, cleaved products (~40 and ~37 kDa) were detected in dSMN RNAi lysates (lane 2). B, immunoblot analysis for DRICE protein. The full-length DRICE (~36 kDa) was evident in control (lane 1) and dSMN RNAi lysates, but the processed DRICE products (Pr1 ~28 kDa, Pr2 ~26 kDa, and Pr3 ~19 kDa) were detected in cell lysates of dsRNA of dSMN-treated cells (lane 2). C, the same cell lysates were immunoblotted for DIAP1 protein, which indicated that there was no change in the level of endogenous DIAP1. D, the same blot was stripped and reprobed for tubulin antibody indicating protein loading control.

is consistent with the increased apoptosis observed with the dSMN dsRNA (Fig. 4). In three similar experiments, the mean percent apoptosis in SMNdRNA/GFP co-transfectants was 21.5 ± 6.5%, whereas the mean percent apoptosis in the Z-VAD-fmk-treated was 18.7 ± 5.4%. Thus, there was little effect of Z-VAD-fmk on Annexin V binding to apoptotic S2 cells. In the Z-VAD-fmk-treated cells, the percentage of transfected cells surviving increased from 26 to 65% (Fig. 6D), a percentage similar to that of control transfected cells (Fig. 6C). This demonstrates an appreciable effect of the caspase inhibitor, Z-VAD-fmk upon the survival of S2 cells treated with dsRNA of dSMN. Further, cell lysates were extracted from control, dSMN RNAi, and dSMN RNAi with Z-VAD-fmk-treated cells and were immunoblotted for DRONC and DRICE protein. The results confirm that there was no accumulation of processed DRONC and DRICE products in lysates prepared from Z-VAD-fmk co-treated cells (Fig. 7). However, the endogenous expression of DIAP1, a known cellular caspase inhibitor, which belongs to the family of IAPs (22) was unaffected by dSMN RNAi (Fig. 5C), and this suggests that cell death may be independent of DIAP1 pathway.

**DISCUSSION**

**Conservation and Diversification of Structures and Functions between dSMN and hSMN**—One of the characteristic features for mammalian SMN proteins is the formation of gems in the nucleus. However, it is unclear what roles, if any, the SMN protein plays in the structure. In this report, we generated specific antibodies against the Drosophila SMN protein. We demonstrated by immunofluorescence that in co-transfected insect S2 cells, hSMN and dSMN were largely co-localized in the cytoplasm. The hSMN was detected as nuclear dot-like structures in some of the S2 cells (Fig. 3L). However, only minimal dSMN in co-transfected cells but not in cells without transfection of hSMN was observed in these structures, suggesting that unlike hSMN, dSMN may not be a nuclear protein to form gems, possibly due to sequence diversification between exon 7 of dSMN and hSMN, which has been suggested to play a key role for hSMN nuclear localization (23). The minimal staining of DSMN in the nucleus from co-transfected cells may occur through a strong association between dSMN and hSMN proteins, which has been demonstrated in a separate experiment by co-immunoprecipitation assay and *in vitro* binding assay. We recently identified an interaction between SMN and Rpp20, a subunit of the RNase P complex, and we showed that the interaction between these two proteins is interchangeable between human and *Drosophila* proteins.

These data suggest that dSMN and hSMN proteins are structurally and functionally conserved in some respects but diverse in others, consistent with conservation of middle domains and diversification of C and N-terminal domains between human and *Drosophila* SMN sequences.

**SMN Involvement in Apoptosis**—The spinal muscular atrophy is caused by death of motor neurons. However, the underlying mechanisms of SMN-deficiency induced cell death are not completely understood. Disruption of *SMN* expression from *S. pombe* to mice as well as in a chicken cell line DT40 shows lethal phenotypes (3, 5, 7–9, 24), indicating a requirement of SMN for cell survival. It has been more recently demonstrated that over-expression of human SMN prolonged cell survival and inhibited apoptosis in PC12 cells deprived of trophic support by acting on mechanisms that mediate cell death through the mitochondrial release of cytochrome c, a key step in the activation of caspase-3 (25). Because the molecular mechanism of apoptosis is highly conserved throughout evolution, we used...
the insect S2 cells by RNA interference in this report to investigate how SMN depletion affected cell death. We demonstrated by both FACS and cell death ELISA assays that apoptotic cell death was induced by depletion of dSMN protein in the S2 cells.

The execution of cell death in Drosophila requires caspase activation (26). There are three pro-apoptotic genes so far cloned in the fly (rpr (27), hid (28), and grim (29)). These genes are reported to activate at least two caspases (DRICE (30) and DCP-1 (31)). DRICE is proteolytically processed during apoptosis in S2 cells (32). In flies, a total of seven caspases have been reported: DCP-1 (31), DREDD/DCP-2 (33), DRICE (30), DRONC (34), DECAY (35), DAMM (36), and STRICA (37). Of these caspases, DRONC and DREDD (apical/initiator or class I caspases) contain extensive pro-domains. They do not require other caspases for cleavage of their zymogens but are thought to interact with other regulators through their pro-domains. However, the precise molecular mechanism for the activation of initiator caspases is still unclear. DCP-1 and DRICE (effector or class II caspases) have been shown to play a role in the execution of cell death (see the review) (38). DRONC is a homolog of caspase-9 in mammals that contains a caspase recruitment domain (CARD), suggesting that it is the functional counterpart of CED-3 in C. elegans (34). DRONC is also found in embryonic tissues like eye and brain and in adult egg chambers where apoptosis occurs naturally. Up-regulation of DRONC has been reported in salivary glands and midguts before histolysis (34, 39). Recently, its interaction with DRICE, an effector caspase, has been discovered (18). Upon receipt of cell death signal, the apical or initiator caspases undergo proteolytic processing and further activate other downstream caspases that go on to cleave various target proteins causing apoptosis (40–42). Because DRONC and DRICE activation are directly involved in execution of cell death in Drosophila cells, we analyzed by immunoblotting for caspase activation using specific antibodies against DRONC and DRICE. Our results strongly suggest that apoptotic cell death induced by SMN deficiency is caspase-dependent.

Z-VAD-fmk Inhibits Caspase Activation—DRONC and DRICE are activated after various death stimuli without any significant release of cytochrome c in the cytosol (43), and retention of cytochrome c in mitochondria during apoptosis has been demonstrated in Drosophila (44). Recently, it has also been demonstrated in Drosophila cells that the central role of ARK (apoptotic protease-activating factor-1 (Apaf-1)-related killer), also known as Dark, Hac-1, or DapaF-1, in stress-induced apoptosis appears to be independent of cytochrome c (16). In contrast, release of cytochrome c has been reported in Drosophila cells during apoptosis (45). More studies are needed to elucidate whether any involvement of cytochrome c in SMN-mediated apoptotic cell death in S2 cells. In the present investigation, cells co-treated with Z-VAD-fmk and dsRNA of dSMN...
result in increased survival (Fig. 6D), a decrease in nucleosomal cleavage (Fig. 6A) and decrease in caspase cleavage (Fig. 6B). We hypothesize that the specific mediators (proteins) involved in the activation of upstream caspase DRONC to identify the SMN-mediated apoptotic pathway in Drosophila cells. These proteins may be potentially targeted for drug development for SMA disease.

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However, the rate of apoptosis as measured by Annexin V staining is unchanged by Z-VAD-fmk treatment. This data is consistent with that of previous investigators (46) who have shown that caspase inhibition by Z-VAD-fmk does not prevent phosphatidylinerine (PS) exposure on the cell surface. In contrast to these other studies (46, 47), the ultimate death of Z-VAD-fmk-treated cells was not observed here. Thus, the caspase-dependent apoptosis induced by SMN deficiency can be blocked by Z-VAD-fmk, suggesting potential targets for therapy to surpass depletion of SMN protein in SMA patients.

SMN Drosophila Models and Identification of SMA Therapeutic Targets—SMA is a disease caused by apoptotic cell death of motor neurons. However, ubiquitous expression of SMN protein in all tissues gives no explanations as to why only motor neurons are targeted in SMA patients. It has been shown previously that SMN exhibits anti-apoptotic properties and interacts with several factors including Bel-2 (48) and p53 (49), both of which play key roles in the apoptotic process but are ubiquitously expressed. In this study we demonstrated that SMN-depletion-induced apoptosis in S2 cells is caspase-dependent and could be blocked by a caspase inhibitor Z-VAD-fmk. We hypothesize that the specific mediators (proteins) between SMN and apoptotic pathways may exist and are down- or up-regulated in motor neurons, resulting in more vulnerability of these cells to low levels of SMN protein than other cell types. More studies are needed in this direction to identify scaffold molecules involved in the activation of upstream caspase DRONC to identify the SMN-mediated apoptotic pathway in Drosophila cells. These proteins may be potentially targeted for drug development for SMA disease.