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Rpp20 interacts with SMN and is re-distributed into SMN granules in response to stress☆

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Abstract

Spinal muscular atrophy (SMA) is a neurodegenerative disorder resulting from homozygous loss of the *SMN1* gene. To investigate SMN functions, we undertook the yeast two-hybrid screens and identified *Drosophila* Rpp20, a subunit of the RNase P and RNase MRP holoenzymes, to interact with the *Drosophila* SMN protein. Interaction between human SMN and Rpp20 was validated by in vitro binding assays and co-immunoprecipitation. The exons 3–4 of SMN are necessary and sufficient for binding to Rpp20. Binding efficiency between Rpp20 and SMNs with mutations in the Y–G domain is abrogated or reduced and correlated with severity of SMA disease. Immunofluorescence results indicate that Rpp20 is diffusely distributed throughout the cytoplasm with higher concentration observed in the nucleus. However, in response to stress, SMN forms aggregates and redistributes Rpp20 into punctuated cytoplasmic SMN granules. Our findings suggest a possible functional association of SMN with RNase P and RNase MRP complexes.

Keywords: Rpp20; RNase P; RNase MRP; Spinal muscular atrophy; The survival motor neuron

The spinal muscular atrophy (SMA) is an autosomal recessive neurodegenerative disorder caused by loss of anterior horn motor neurons. The *survival motor neuron* (*SMN1*) gene on human chromosome 5q13 was identified as the genetic cause of SMA disease [1]. The 35 kb *SMN1* gene spans 9 exons that encode a protein of 294 amino acids with a molecular weight of about 38 kDa. SMN is ubiquitously expressed with a slightly higher level detected in the central nerve system [1]. Mice with knockout of *SMN* gene die at very early stages [2], suggesting its importance during development.

The functions of SMN protein, notably its involvement in pre-mRNA splicing, have been investigated through its interactions with other cellular proteins. SMN forms multi-protein complexes with Gemin2, Gemin3 (dp103), gemin4, gemin5, gemin6, and gemin7 proteins as well as Sm and Sm-like (Lsm) proteins [3–10], suggesting its involvement in the biogenesis of the small nuclear ribonucleoproteins (snRNPs) and spliceosome [11–14]. In in vitro splicing experiments, addition or omission of SMN protein and a dominant negative form of SMN protein stimulated or inhibited splicing of pre-mRNA, strengthening the notion that SMN plays a role in mRNA processing. However, there are no reports indicating that SMN affects splicing of endogenous genes in vivo by studies of splicing patterns in normal tissues vs. patient's tissues and normal mouse tissues vs. SMN KO mouse tissues (conditional KO) as well as SMN transgenic mouse tissues. More recently, Dreyfuss group claimed that one of the key functions for SMN is to ensure the specificity of interaction of Sm proteins in the snRNP complexes and to restrict them exclusively to intended RNAs [15]. However, these

☆ *Abbreviations:* SMA, spinal muscular atrophy; dSMN, *Drosophila* survival of motor neuron protein; hSMN, human survival of motor neuron; dRpp20, *Drosophila* Rpp20; hRpp20, human Rpp20; RNP, ribonucleoprotein; GST, glutathione *S*-transferase; HA, hemagglutinin; FBS, fetal bovine serum; PBS, phosphate buffered saline; Cy3, Cy3 conjugate; BSA, bovine serum albumin; SGs, stress granules; Hua and Zhou, Cytoplasmic granules of the survival motor neuron are assembly sites for stress granules (manuscript submitted).

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observations do not explain why SMN protein interacts with other RNP proteins and why it is localized in Cajal bodies bridged through coilin in the nucleus [16]. The connections between SMN protein and rRNA processing have been also suggested previously by direct interactions of SMN protein with fibrillarin and GAR1, specific markers for two groups of small nucleolar ribonucleoprotein particles (snoRNPs) that are involved in posttranscriptional processing and modification of ribosomal RNA [17,18].

SMN is conserved across species and has been isolated from *Caenorhabditis elegans* [19], *Danio rerio* (zebrafish) [20], and *Schizosaccharomyces pombe* [21]. Biochemically, the SMN proteins from these species retain a number of properties identified in the human SMN (hSMN), including RNA binding activity and self-association. Deletions of SMN proteins in *C. elegans* and *S. pombe* affect cell viability and growth. Knockdown of SMN in zebrafish causes defects in motor axon outgrowth and pathfinding [22]. *Drosophila* SMN (dSMN) and hSMN share a 23.5% identity and 36.7% similarity. *Drosophila* SMA models have been generated by ectopic expression of domains of the hSMN protein that may function as dominant negatives to the endogenous dSMN protein, resulting in abnormally positioned wings and legs or in many cases, pupal lethality [23]. More recently, a *Drosophila* SMN mutant was isolated and zygotic SMN mutant animals show defects at the neuromuscular junctions [24]. These results suggest that SMNs are not only structurally but also functionally conserved between species.

To further investigate the functions of SMN, we screened a *Drosophila* cDNA library in a yeast two-hybrid system. We identified *Drosophila* Rpp20 (dRpp20) that specifically interacted with dSMN protein. Rpp20 is a shared component of two RNP complexes, RNase P and RNase MRP (ribonuclease mitochondrial RNA processing). RNase P plays a key role in the maturation and metabolisms of tRNA while RNase MRP is involved in rRNA processing and mitochondrial DNA replication [25,26]. Importantly, hSMN and human Rpp20 (hRpp20) also reserve strong interaction. In addition, we show that both SMN and Rpp20 are stress-responsive proteins. Under stress conditions, both proteins accumulate and co-localize in punctuated cytoplasmic structures. Our findings suggest a functional link between SMN and RNase P and RNase MRP complexes and the possible involvement of tRNA/rRNA processing in the pathogenesis of SMA.

Materials and methods

Yeast two-hybrid screens. Clontech Matchmaker Gal4 system was used in the screening. Full length of *Drosophila* SMN cDNA was amplified by RT-PCR and cloned into pGBKT7 to encode a hybrid protein containing the DNA-binding domain of Gal4 fused to dSMN protein. Yeast cells of the AH109 reporter strain were transformed with the Gal4–dSMN bait. Transformants were picked from SD-Trp medium and tested for chimera protein expression. Subsequently, cells expressing dSMN bait were transformed with *Drosophila* embryo cDNA library plasmids. Co-transformed cells were plated on SD-Ade/-His/-Leu/-Trp medium. After a few days, positive clones were selected and tested for β-galactosidase activities.

In vitro binding assays. For in vitro binding analysis between SMN and Rpp20, the dRpp20 or hRpp20 cDNA was amplified by RT-PCR, cloned into pcDNA3 (Invitrogen), and expressed in reticulocyte lysate with luciferase cDNA as a control using Promega TNT system in the presence of ^{35}S-labeled methionine. Aliquots of in vitro-translated proteins were then rocked with 2 μg of bacterially expressed GST–dSMN, GST–hSMN or GST which was bound to glutathione–Sepharose beads (Pharmacia) for 3 h at 4 °C in binding buffer (20 mM Tris–HCl, pH 7.5, 250 mM NaCl; 1 mM EDTA, 0.5% NP-40, 2 mM DTT, 3 mg/ml BSA, and protease inhibitors). The beads were then washed with binding buffer for three times. The proteins bound to GST–dSMN beads were resolved on SDS–PAGE and detected by exposure to X-ray films. For mapping of Rpp20 binding site in SMN and mutant SMN binding analysis, the format was inverted with in vitro-translated hSMN wild type, its various domains or its mutants reacting with GST chimera, a bacterially expressed hRpp20 protein. Domains of hSMN and mutant SMNs were generated by PCR with appropriate oligos and cloning of their cDNAs into pcDNA3. For examining the direct physical binding of hSMN to hRpp20, 1 μg of purified 6× His-tagged hRpp20, which was kindly provided by Dr. Sidney Altman at Yale University, was incubated at 4 °C for 3 h with 10 μL glutathione–Sepharose beads and with 2 μg GST–hSMN or GST. The beads were washed as above. The bound 6× His–hRpp20 was detected by Western blotting analysis with rabbit polyclonal antibody His-probe H-15 (Santa Cluz).

Co-immunoprecipitation. For co-immunoprecipitation of dSMN and hSMN, HA–dSMN cDNA was cloned into pcDNA3 and transfected into HeLa cells by the standard calcium phosphate method. Extracts were first reacted with mouse monoclonal antibody 12CA5 (Roche) to the HA epitope of HA–dSMN for 3 h at 4 °C in binding buffer. After the complexes were washed as above, hSMN was detected with mouse monoclonal anti-SMN antibody from Transduction Labs. For co-immunoprecipitation of dSMN and dRpp20, dRpp20 cDNA was cloned into p3×Flag–CMV (Sigma). Constructs of HA–dSMN and 3×Flag–dRpp20 were co-transfected into HeLa cells by standard calcium phosphate method. Anti-HA antibody 12CA5 was used for pulling down the immunoprecipitates, and 3×Flag–dRpp20 protein was detected by Western blot analysis with mouse monoclonal anti-Flag M2 antibody (Sigma). Rabbit polyclonal anti-hRpp20 antibody, which we obtained from Dr. Sidney Altman at Yale University, was used to precipitate endogenous hRpp20 and endogenous hSMN was then detected by Western blot analysis with rabbit polyclonal anti-SMN antibody H195 (Santa Cluz).

Immunofluorescence analysis. HeLa cells were cultured at 37 °C in DMEM with 10% FBS on glass coverslips and transfected alone with plasmid expressing 3×Flag-tagged hRpp20 or co-transfected with plasmids expressing 3×Flag-tagged hRpp20 and GFP-tagged hSMN. GFP expressing plasmid pEGFP-C1 (Clontech) was used as a control. The 3×Flag–hRPP20 and GFP–SMN constructs were generated by cloning of hRpp20 and hSMN cDNAs into p3×Flag–CMV and pEGFP-C1, respectively. Standard calcium phosphate method was used for transfection. Twenty-four hours after transfection, cells were fixed in 4% of paraformaldehyde for 20 min, washed three times with PBS, permeabilized in 0.1% of Triton X-100 in PBS for 3 min, and blocked with 3% IgG-free BSA (W/V) and 3% goat serum (V/V) in PBS for 1 h. Endogenous SMN was identified with rabbit anti-SMN H195 and highly cross-adsorbed Alexa Fluor 488 goat anti-rabbit secondary antibody (Molecular Probes). The 3×Flag–Rpp20 was identified with anti-Flag M2 and Cy3-conjugated goat anti-mouse secondary

antibody (Jackson ImmunoResearch Labs). Cells were incubated with primary antibodies for 3 h. After washing with PBS for three times, cells then were incubated with secondary antibodies for 1 h. The localization of proteins was visualized by fluorescence microscopy. For detecting the effects of various cellular stresses on the distribution of the two proteins in HeLa cells, 24 h after transfection, cells were exposed to 44 °C for 30 min (heat shock) or UV irradiation for 20 min. After treatment, cells were fixed and stained immediately as above.

Results

Identification of Rpp20 to interact with SMN by the yeast two-hybrid screens

To better understand the functions of SMN, we screened a *Drosophila* cDNA library using yeast two-hybrid assays. The full length of dSMN cDNA (678 bp) was cloned into pGBKT7. Expression of the GAL4 DNA-binding domain and dSMN fusion protein was verified by Western blot analysis (data not shown). After sequential transformation of the pGBKT7 dSMN bait and the *Drosophila* pACT2 embryo cDNA library into AH109 yeast host, approximately 500,000 transformants were plated on SD-4 (-Leu-Trp-His-Ade) selection medium, from which 14 clones that activate all three GAL4-responsive markers HIS3, Mel1, and LacZ reporter genes in AH109 were isolated. Sequence analysis revealed that one of these proteins is the homologue of hRpp20, a subunit of RNase P complex, and of RNase MRP complex. This protein, here referred to as dRpp20, interacted specifically in a yeast two-hybrid mating assay that utilized two control proteins, murine p53 and human lamin C. DRpp20 has an open reading frame of 501 bp, encoding 167 amino acids with an estimated molecular weight of 18.9 kDa, slightly larger than hRpp20 that has a 420 bps open reading frame and encodes 140 amino acids. Rpp20s are conserved between human and *Drosophila* with 32.6% identity and 47.5% similarity. These results suggest conservation of Rpp20 functions from *Drosophila* to human, while the longer c-terminus of dRpp20 (Fig. 1B) indicates possible functional diversification of Rpp20 in human and *Drosophila* through revolution.

Validation of interaction between SMN and Rpp20 by in vitro binding assays

To validate the interaction, in vitro binding assays were carried out. GST–dSMN and GST–hSMN fusion

```
A
Hum  MAMSSGGSGGGVPEQEDSVLFRRGTGQSDDSDIWDDTALIKAYDKAVASFKHAL--KNGD
Dro  ----------------------MSDETNAAVWDDSLLVKTYDESVGLAREALARRLAD
                           .:. :: :***: *:*:**::*.  :.**  : .*

Hum  ICETSGKPKTTPKRKPAKKNKSQKKNTAASLQQWKVGDKCSAIWSEDGCIYPATIASIDF
Dro  STNKREEENAAAAEEEAGEISATGGATSPEPVSFKVGDYARATYV-DGVDYEGAVVSINE
       :.  : :::. .: * : .:    *:..  .:**** . * :  **  * .::.**:

Hum  KRETCVVVYTGYGNREEQNLSDLLSPICEVANNIEQNAQENENESQVSTDESENSRSPGN
Dro  EKGTCVLRYLGYENEQEVLLVDLLPSWGKRVRREQFLIAKKDEDEQLSRPKASAG-----
     :: ***: * ** *.:*  * ***..  : ... :    :::::.*:*  ::. .

Hum  KSDNIKPKSAPWNSFLPPPPPMPGPRLGPGKPGLKFNGPPPPPPPPPPHLLSCWLPPFPS
Dro  -SHSKTPKSSRRSRI-------------------------------------------S
      *.. .***:  . :                                           *

Hum  GPPIIPPPPPICPDSLDDADALG----SMLISWYMSGYHTGYYMGFRQNQKEGRCSHSLN
Dro  GGLVMPPMPPVPPMIVGQGDGAEQDFVAMLTAWYMSGYYTGLYQGKKEASTTSGKKKTPK
     *  ::** **: *  :.:.*.      :** :******:** * * :: .. .  .:: :

Hum  -
Dro  K

B
Hum  MAENREPRGAVEAELDPVEYTLRKRLPSRLPRRP-----NDIYVNMKTDFKAQLARCQKL
Dro  MMGSNYPEHGTKPRSAKYHKQQNHRVVRKQPPRPAVSDRHNIYITSKTDFKAQQRRCEEL
     *  .. *. ..:..    .   .:*:  : * **     ::**:. *******  **::*

Hum  LDGGARGQNACSEIYIHGLGLAINRAINIALQLQAGSFGSLQVAANTSTVELVDELEPET
Dro  INSGAH------EIFLHCMGFSVTRGLNIALRLVQNSDGALSYAINTSTVQLVDELHPLC
     ::.**:      **::* :*:::.*.:****:*  .* *:*. * *****:*****.*

Hum  DTREPLTRIRNNSAIHIRVF------------------------RVTPK---
Dro  DAEDITFRQRNNSALHIKILNNSLFDIAVPQPSQSQTQAQSLGQFRGKAKARQ
     *:.:   * *****:**:::                        * ..*
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Fig. 1. SMN and Rpp20 are conserved between human and *Drosophila*. (A) Alignment of human and *Drosophila* SMNs. Boxed fragments represent exon 1 and exon 7 of hSMN, respectively. (B) Alignment of human and *Drosophila* Rpp20s. Asterisk, identical residue; colon, residue of high similarity; period, residue of low similarity. Gaps are shown as dashed lines.

proteins were expressed and purified from *E. coli*. DRpp20, dSMN, hSMN, and hRpp20 cDNAs were cloned into pcDNA3 and these plasmids as well as the luciferase control were used for in vitro translation in the presence of [^{35}S]methionine to generate radiolabeled proteins. An in vitro interaction of dSMN with dRpp20 was demonstrated by mixing equivalent aliquots of radiolabeled dRpp20 with purified GST or with GST–dSMN fusion protein bound to glutathione–agarose beads. DRpp20 was specifically retained on the beads by the GST–dSMN fusion protein but not by parental GST peptide (Fig. 2A). Although sequence analysis suggests that domains of both SMN and Rpp20 proteins are conserved between *Drosophila* and human (Fig. 1), direct interaction between hSMN and hRpp20 needs to be validated. In vitro binding results as shown in Fig. 2B demonstrates strong association of hRpp20 with GST–hSMN. Further experiment of in vitro binding between purified GST–hSMN and purified 6×His–hRpp20 indicates association between Rpp20 and SMN is physically direct (Fig. 2C). In this experiment, 6×His–hRpp20 was mixed with GST–hSMN fusion protein, and the complex was pulled down with glutathione–agarose beads and detected by Western blot analysis with an anti-6×His antibody. In addition, strong binding between hSMN and dRpp20 was also observed (Fig. 2D). These data indicate that structures and functions of Rpp20 and SMN proteins may be conserved between human and *Drosophila* through evolution and possibly partially interchangeable. Conservation of SMN structures and functions between *Drosophila* and human is also supported by functional studies of dSMN in S2 cells by RNAi [27], and self-association of dSMN protein as well as a direct interaction between dSMN and hSMN proteins by in vitro binding assays (Figs. 3A and B) and in vivo co-IP assays (Fig. 3C).

Validation of interaction between SMN and Rpp20 by co-immunoprecipitation

To test the interaction of Rpp20 with SMN in vivo, we utilized HeLa cells for co-immunoprecipitation. DRpp20 cDNA and HA-tagged dSMN cDNA were cloned into p3×Flag–CMV and pcDNA3, respectively. The 3×Flag–dRpp20 and HA–dSMN plasmids were co-transfected into HeLa cells for co-immunoprecipitation experiments. Anti-HA antibody was used to precipitate dSMN protein from HeLa cell lysates. In the precipitated complex, dRpp20 was readily detected by Western blot using anti-Flag antibody (Fig. 4A). In contrast, mouse IgG failed to co-immunoprecipitate dRpp20 from HeLa cell lysates. In a separate experiment, we employed anti-hRpp20 antibody to co-immunoprecipitate endogenous human Rpp20 and SMN proteins from HeLa cell lysates

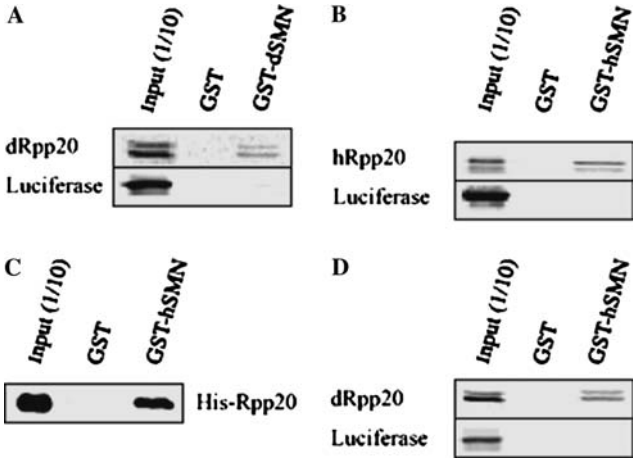

Fig. 2. Rpp20 proteins interact with SMN proteins in vitro. (A) In vitro-translated [^{35}S]methionine-labeled dRpp20 or luciferase was incubated with either GST or GST–dSMN. Bound proteins were analyzed by autoradiography. Ten percent of input is shown. (B) In vitro-translated [^{35}S]methionine-labeled hRpp20, or luciferase was incubated with either GST or GST–hSMN. Bound proteins were analyzed by autoradiography. Ten percent of input is shown. (C) Immunoblot detection of direct interaction of hSMN and hRpp20. Purified 6×His–hRpp20 was incubated with either GST or GST–hSMN. Bound hRpp20 was analyzed by Western blotting with His-probe H-15. Ten percent of input is shown. (D) In vitro-translated [^{35}S]methionine-labeled dRpp20 or luciferase was incubated with either GST or GST–hSMN. Bound protein was analyzed by autoradiography. Ten percent of input is shown.

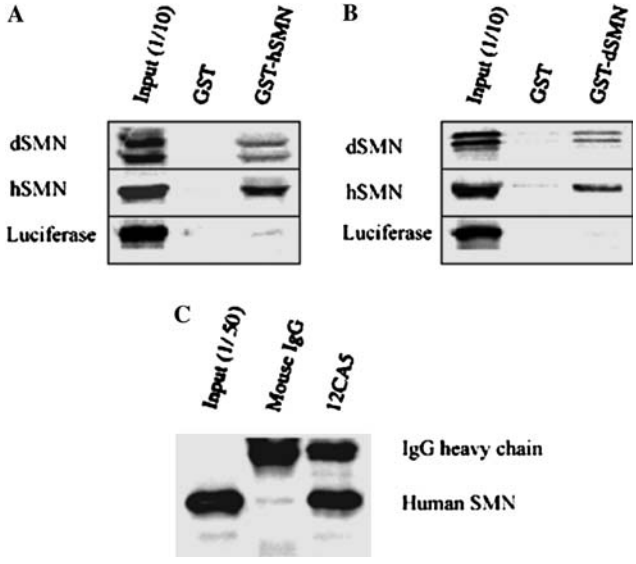

Fig. 3. *Drosophila* SMN binds to human SMN in vitro and in vivo. (A) In vitro-translated [^{35}S]methionine-labeled dSMN, hSMN, or luciferase was incubated with either GST or GST–hSMN. Bound proteins were analyzed by autoradiography. (B) In vitro-translated [^{35}S]methionine-labeled dSMN, hSMN, or luciferase was incubated with either GST or GST–hSMN. Bound proteins were analyzed by autoradiography. (C) Protein extracts were prepared from HeLa cells which were transfected with plasmid of pcDNA3-HA–dSMN. Mouse IgG or anti-HA antibody 12CA5 was used to immunoprecipitate HA–dSMN and hSMN complex. The amount of hSMN was examined by monoclonal anti-SMN antibody.

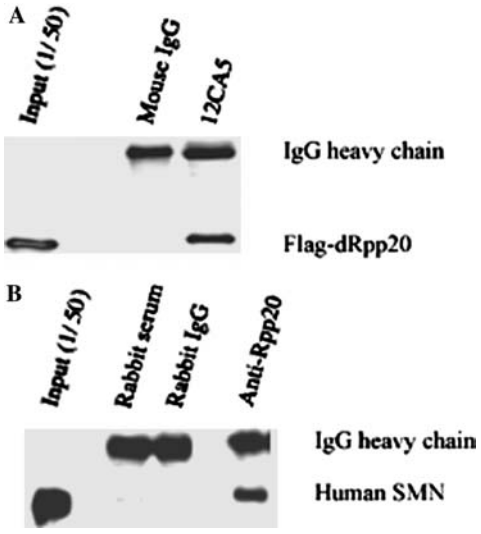

Fig. 4. Rpp20 proteins bind to SMN proteins in vivo. (A) Co-immunoprecipitation of dRpp20 with dSMN. HeLa cells were co-transfected with plasmids of HA–dSMN and 3×Flag–Rpp20. mouse IgG or anti-HA antibody 12AC5 was used to pull down the ectopic HA–dSMN protein. The 3×Flag–Rpp20 in immunoprecipitates was examined by anti-Flag antibody M2. Two percent of the input is shown. (B) Co-immunoprecipitation of hRpp20 with hSMN from HeLa cells. Rabbit serum, rabbit IgG or anti-Rpp20 antibody was used to pull down the endogenous hRpp20 and hSMN complex from HeLa cells. Immunoprecipitates were examined for the presence of SMN by using rabbit polyclonal anti-SMN antibody H195. Two percent of the input is shown.

(Fig. 4B). Negative controls of rabbit serum and rabbit IgG were unable to pull down SMN protein while a clear band of SMN was shown from anti-Rpp20 antibody precipitated beads (Fig. 4B), demonstrating that SMN is endogenously associated with Rpp20.

Exons 3–4 of SMN are essential for Rpp20 binding

SMN is a multiple-motif protein and binds to many proteins at different domains. To identify which region(s) of SMN mediate interaction between SMN and Rpp20, GST–hRpp20 protein was bacterially expressed and purified, and in vitro-translated and ^{35}S-labeled SMN domains were generated. We then performed in vitro binding experiments. As shown in Fig. 5, exons 1–4 and exons 3–4 exhibit strong binding to Rpp20 though there is a slight decrease in binding ability compared with full-length SMN, while exons 1–2, exons 1–3, exon 3, exon 4, exons 5–7, and exons 6–7 fail to bind to Rpp20. These results suggest exons 3–4 are the primary binding site for Rpp20.

Binding between Rpp20 and C-terminal mutant SMNs correlates with severity of SMA

More than 95% of SMA is caused by deletions of the SMN1 gene while point mutations in the SMN1 gene were also identified from some of SMA patients. To investigate if interaction of SMN with Rpp20 is affected by mutations in the SMN1 gene, in vitro binding assays were performed. SMN proteins with single amino acid substitutions (E134K, S262I, Y272C, T274I, and G279V) were in vitro translated in the presence of [^{35}S]methionine. In addition, we tested binding of Rpp20 to SMNΔN27, a dominant negative form of SMN protein and to SMNΔ7, the primary product of the *SMN2* gene. Our results reveal that the most severe exon 6 mutant Y272C completely abolishes the binding between SMN and Rpp20 while less severe mutant T274I

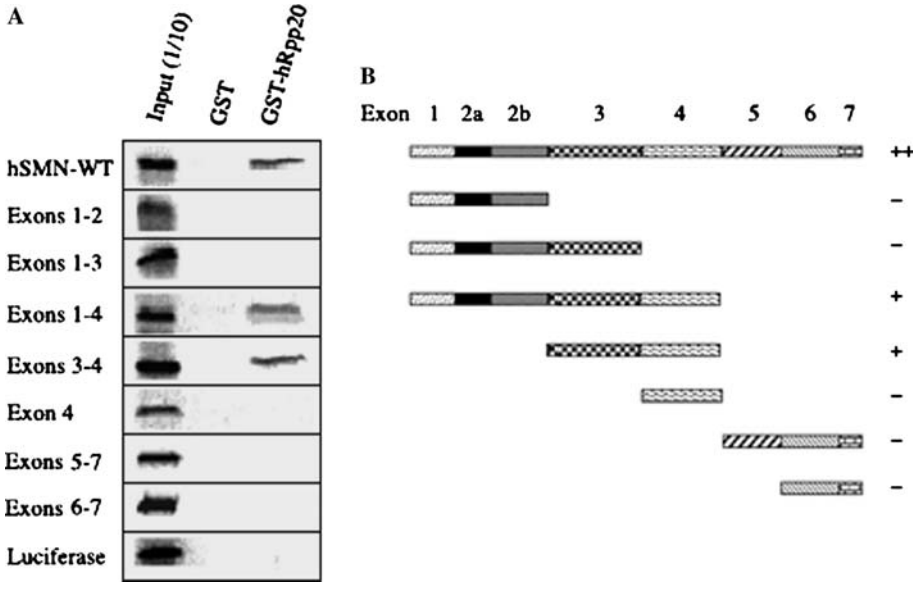

Fig. 5. Exons 3–4 of hSMN domains are essential for its interaction with hRpp20. (A) In vitro-translated [^{35}S]methionine-labeled hSMN-WT or the indicated deletion mutants were incubated with GST or GST–hRpp20. Bound proteins were analyzed by SDS–PAGE and autoradiography. Ten percent of the input is shown. (B) Schematic representation of the binding abilities of hSMN deletion mutants to hRpp20. Each hSMN domain is represented by a box filled with a different pattern. (+) Binding strength; (−) no binding.

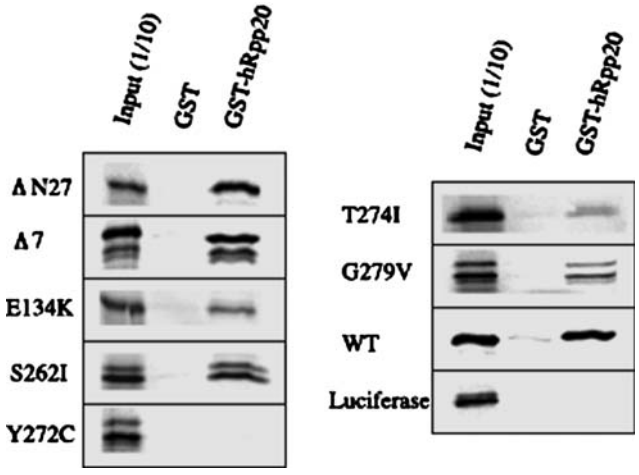

Fig. 6. hSMN mutants in the Y–G domain reduce the binding ability to hRpp20. In vitro-translated [^{35}S]methionine-labeled mutants E134K, S262I, Y272C, T274I, G279V, hSMNΔ7, and hSMNΔN27 were incubated with either GST or GST–hRpp20. Bound proteins were analyzed by autoradiography. hSMN-WT was used as a positive control and luciferase was used as a negative control.

greatly reduces the binding activity. Milder forms of S262I and G279V mutants only slightly decrease the binding of SMN to Rpp20. However, tudor domain mutant E134K only shows partial binding decrease and neither SMNΔN27 nor SMNΔ7 has any effects on their binding ability to Rpp20 compared with wild type SMN (Fig. 6). These data show that binding of mutant SMNs to Rpp20 correlates with severity of SMA disease caused by C-terminal point mutations.

Co-localization of SMN and Rpp20 by immunofluorescence

SMN protein is localized in both the cytoplasm and the nucleus where it forms complex structures termed gems [28]. SMN also interacts with coilin and may function in Cajal bodies [16]. While there is no direct evidence to suggest cellular localization of human Rpp20, it is well documented that most components of both RNase P and RNase MRP complexes are localized in Cajal bodies, the nucleolus, and the nucleoplasm [29]. To investigate where

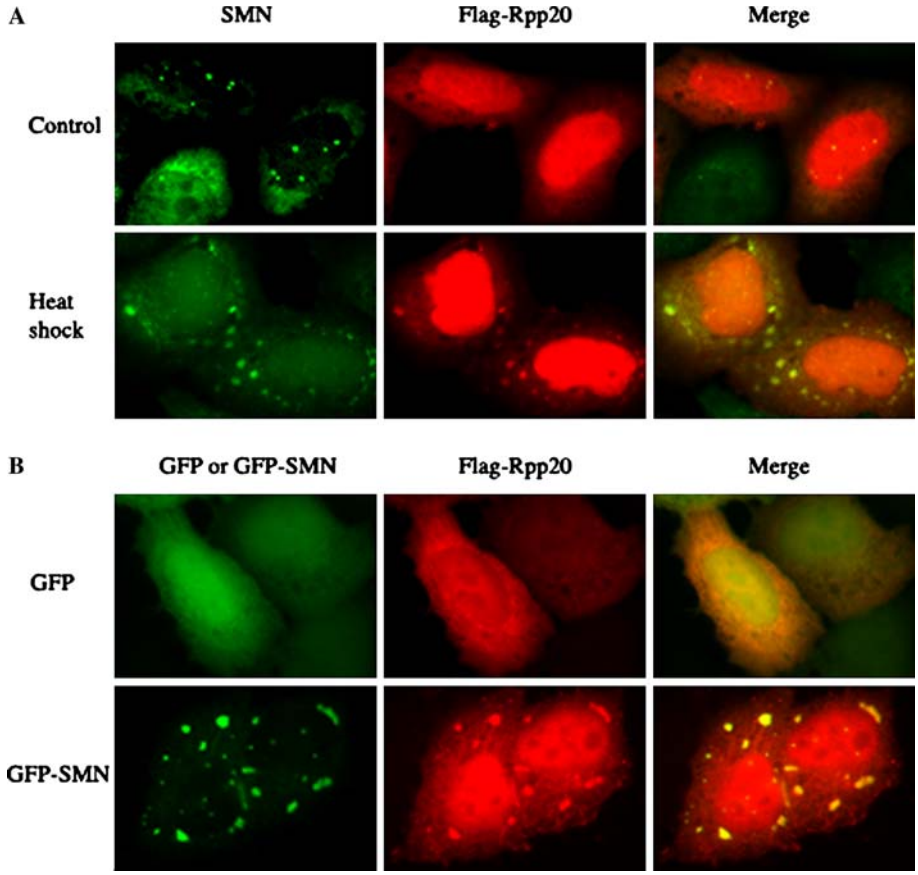

Fig. 7. hSMN and hRpp20 co-localize in punctuated cytoplasmic granules in HeLa cells. (A) hSMN and hRpp20 are co-localized into cytoplasmic granules under stresses. HeLa cells were transfected with plasmid of 3×Flag–hRpp20 and then exposed to 42 °C for 30 min. Co-immunofluorescence of endogenous SMN and Flag–hRpp20 was performed. Primary antibodies are polyclonal anti-SMN antibody H195 and monoclonal mouse anti-Flag antibody M2 with Alexa Fluor 488 goat anti-rabbit antibody and Cy3-conjugated goat anti-mouse antibody as secondary antibodies. (B) Over-expression of SMN in HeLa cells redistributes Rpp20 into cytoplasmic SMN granules. HeLa cells were co-transfected with constructs of 3×Flag–hRpp20 and GFP–SMN or vector pEGFP-C1 as a control. The 3×Flag–hRpp20 was stained with monoclonal mouse anti-Flag M2 antibody and Cy3-conjugated goat anti-mouse secondary antibody. Co-localization of SMN and Rpp20 was examined under a fluorescence microscope.

the interaction between human SMN and Rpp20 proteins occurs, we first transfected HeLa cells with plasmid that expresses 3×Flag-tagged Rpp20. Immunofluorescence analysis shows that Rpp20 is distributed in the cytoplasm and in the nucleus in a diffuse manner with higher concentration in the nucleus (Fig. 7A). Interestingly, Rpp20 has been shown to interact with heat shock protein 27 (hsp27) [30]. This prompted us to examine whether the distribution of Rpp20 is affected under cellular stress conditions. HeLa cells transfected with 3×Flag–Rpp20 expressing construct were subject to heat shock at 44 °C for 30 min, and the distributions of endogenous SMN and ectopic 3×Flag–Rpp20 were identified by double indirect immunofluorescence. We found that in most HeLa cells both SMN and 3×Flag–Rpp20 accumulated and co-localized in punctuated cytoplasmic granules. Some of these granules are of the same size as that of gems but most of them are larger than gems. Similar data were obtained when cells were exposed to other cellular stresses such as UV irradiation (data not shown), indicating both SMN and Rpp20 are stress-responsive proteins and might play protective functions under harmful cellular environment.

Over-expression of SMN partially re-distributes Rpp20

It was reported that over-expression of SMN can trigger formation of cytoplasmic SMN granules in some types of cells [31–33]. Recently, we have shown that the cytoplasmic SMN granules induced by stress and over-expression are in fact stress granules (SGs) (Hua and Zhou, submitted). To test whether these over-expression-induced SMN granules/SGs also contain Rpp20, HeLa cells were co-transfected with constructs expressing GFP–SMN and 3×Flag–Rpp20, respectively. By co-immunofluorescence, we observed in about 30% of the transfected cells GFP–SMN aggregated in the cytoplasm and formed punctuated granules and all these cytoplasmic granules exhibited positive staining of Flag tag while no HeLa cells transfected with both GFP control and 3×Flag–Rpp20 showed any granule formation of GFP or 3×Flag–Rpp20 (Fig. 7B). Our data indicate that SMN can partially re-distribute its binding partner Rpp20 into the punctuated cytoplasmic SGs triggered by over-expression of SMN.

Discussion

Interactions between Rpp20 and SMN on functions of RNase P

RNase P consists of at least 10 distinct peptide subunits and an essential RNA subunit, H1 RNA. Six of these protein subunits including Rpp20 have been shown to be also subunits of RNase MRP. In yeast, depletion of Rpp2, the homologue of Rpp20, results in defects in processing of precursor tRNA and in processing of the 35S precursor rRNA, indicating Rpp20 is essential for both RNase P and RNase MRP activities [34]. Rpp20 has ATPase activity [30]. However, ATP is not required for human RNase P to cleave pre-tRNA and Rpp20 is not a H1 RNA binding protein. One possibility is that Rpp20 serves as a physical and functional link to other RNase P-related functions [35]. The notion is supported by the finding that Hsp27 increases RNase P activity through its interaction with Rpp20 [30]. The biological significance of SMN–Rpp20 association remains to be investigated. However, since SMN has been shown to be involved in the biogenesis of snRNPs and associated with multiple other RNPs, it is logical to presume that RNase P and RNase MRP complexes are also regulated by SMN through its association with Rpp20 or other proteins. This possibility is favored by previous reports indicating that the RNA subunit of RNase P, similar to U RNAs, also interacts with Sm or Lsm proteins [36] and that the normal processing of pre-tRNA requires Lsm proteins [37], coinciding with the fact that SMN interacts with Lsm4 and Lsm6 proteins [10].

SMN–Rpp20 complex in SMN granules

Unlike other subunits in RNase P and RNase MRP that are mainly localized in the nucleus, Rpp20 is distributed in both the cytoplasm and the nucleus, suggesting Rpp20 might have additional function(s) in the cytoplasm. We have recently demonstrated that cytoplasmic SMN granules are identical to SGs and SMN serves as a SG recruiter (Hua and Zhou, submitted). In this report, we present observations for the first time that similar to SMN, Rpp20 is also a stress-responsive protein and redistributed into cytoplasmic SMN granules under harmful environment. SGs are cytoplasmic protective structures formed in response to stress. About 50% of total poly(A) + mRNA are stored in SGs when cells are under harmful condition [38]. We hypothesize that in response to stress environments, cytoplasmic SMN polymerizes and pulls Rpp20 into SGs. The function of Rpp20 in SGs is unclear. However, considering the roles of Rpp20 in maturation of tRNA and rRNA in RNase P and RNase MRP complexes, it is possible that cytoplasmic SMN granules are important for protection not only of mRNA species but also of other RNA species including tRNAs and rRNAs, especially under stressed conditions. The protection is perhaps particularly important to long stretched cells in which tightly regulated local protein synthesis is essential. The notion is supported by the observations that SMN aggregates and forms cytoplasmic granules in human fetal muscle cells and rat motor neurons [39,40], even under normal conditions. A more recent report suggests that cytoplasmic SMN granules are actively

transported to developing neurites and growth cones in a similar transport pattern as that of neuronal RNA granules [33], a structure that has been reported to deliver a subset of mRNAs to axonal growth cone for local protein synthesis [41,42]. It is reasonable to propose that deficiency of SMN may impair formation of cytoplasmic SMN granules especially in neurites of motor neurons so that these cells are sensitized for apoptosis, particularly under stressed conditions.

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