Disruption of SMN function by ectopic expression of the human *SMN* gene in *Drosophila*

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Abstract Spinal muscular atrophy is a neurodegenerative disorder caused by mutations or deletions in the survival motor neuron (*SMN*) gene. We have cloned the *Drosophila* ortholog of SMN (DmSMN) and disrupted its function by ectopically expressing human SMN. This leads to pupal lethality caused by a dominant-negative effect, whereby human SMN may bind endogenous DmSMN resulting in non-functional DmSMN/human SMN hetero-complexes. Ectopic expression of truncated versions of DmSMN and yeast two-hybrid analysis show that the C-terminus of SMN is necessary and sufficient to replicate this effect. We have therefore generated a system which can be utilized to carry out suppressor and high-throughput screens, and provided in vivo evidence for the importance of SMN oligomerization for SMN function at the level of an organism as a whole.

Key words: Motor neuron disease; Spinal muscular atrophy; Survival motor neuron; Transgenic; *Drosophila*

1. Introduction

Spinal muscular atrophy (SMA) is a common autosomal recessive disorder characterized by loss of lower motor neurons of the spinal cord [1]. The neuronal degeneration leads to a variable degree of muscular wasting and atrophy, and its most severe manifestation (Type I, Werdnig–Hoffmann disease) is the most common genetic cause of infant mortality [2]. SMA results from mutations or deletions in the survival motor neuron (*SMN*) gene [3]. SMN exists in two copies in a duplicated region of chromosome 5q13 [4]. The telomeric version (SMN1) generates a full-length SMN protein, whereas the centromeric counterpart (SMN2) gives rise mainly to alternatively spliced forms [5]. SMN1 is deleted or mutated in $>98\%$ of SMA patients [3]. Consistently, the absence or a reduced level of functional full-length SMN protein is the primary cause of the neuromuscular degeneration observed in SMA [3,6]. The copy number of SMN2 has been shown to determine disease severity [7], but the relative contribution of its alternatively spliced transcripts to alleviating the lack of SMN1 is unclear. It is well established that SMN is part of a multiprotein complex involved in the biogenesis of snRNPs [8,9] and pre-mRNA splicing [10]. However, these findings do not explain the absolute specificity of motor neuron death; rather they suggest, together with the ubiquitous expression of SMN, a housekeeping function for this gene.

The study of *Drosophila* homologs of many human genes has provided new insight into fundamental aspects of protein function. The creation of *Drosophila* models for human disease is a powerful tool to genetically define disease mechanism and modifier genes [11]. The evolutionary conservation of the SMN gene, together with the absence of such a genetic tool for SMA, prompted us to clone and characterize the *Drosophila melanogaster* SMN (DmSMN) gene. In this paper we disrupt SMN function in *Drosophila*, thereby generating a system which can be utilized to carry out suppressor and high-throughput screens. In addition, we provide in vivo evidence for the importance of the SMN C-terminal domain for SMN function at the level of a whole organism.

2. Materials and methods

2.1. Sequence characterization

A number of ESTs similar to human SMN were identified following a search of the EST database at BDGP (http://www.fruitfly.org/blast/). Two ESTs (LD36948, accession number AI456812 and LD38096, accession number AI518549) were obtained and fully sequenced. These sequences, together with the partially sequenced clones already available in the database, allowed the determination of the full-length DmSMN mRNA. Comparison of this assembled sequence with the *Drosophila* genomic database identified the DmSMN genomic region (accession number AC020221) and showed that the DmSMN gene contained no introns.

2.2. In situ hybridization

Antisense DmSMN RNA was synthesized from linearized template cloned into the pGEM-T vector (Promega, UK) using the SP6/T7 Transcription kit (Roche, UK). The template used was a 680 bp fragment corresponding to the entire DmSMN coding sequence. A complementary sense DmSMN RNA probe generated in a similar fashion was used as a negative control. In situ hybridization of whole mount *Drosophila* embryos was performed as previously described [12].

2.3. Drosophila genetics

Fly stocks were maintained at 25°C on standard medium. The coding sequences of human *SMN*, DmSMN and fragments of DmSMN were amplified using primers containing *Kpn*I sites. The resulting fragments were subcloned into the pUAST transformation vector [13]. For each construct, at least three independent single-insertion lines were established and used for further experiments. The GAL4 line P{GawB}how[24B] was used to drive ectopic expression of all the transgenes. P{GawB}how[24B] expression is observed in embryonic mesoderm, becoming restricted to myogenic cells and being detected in third-instar larval imaginal discs [14]. Crosses were performed by mating females homozygous for P{GawB}how[24B] with males homozygous for the UAS insertions.

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Abbreviations: DmSMN, *Drosophila melanogaster* SMN; SMA, spinal muscular atrophy; SMN, survival motor neuron

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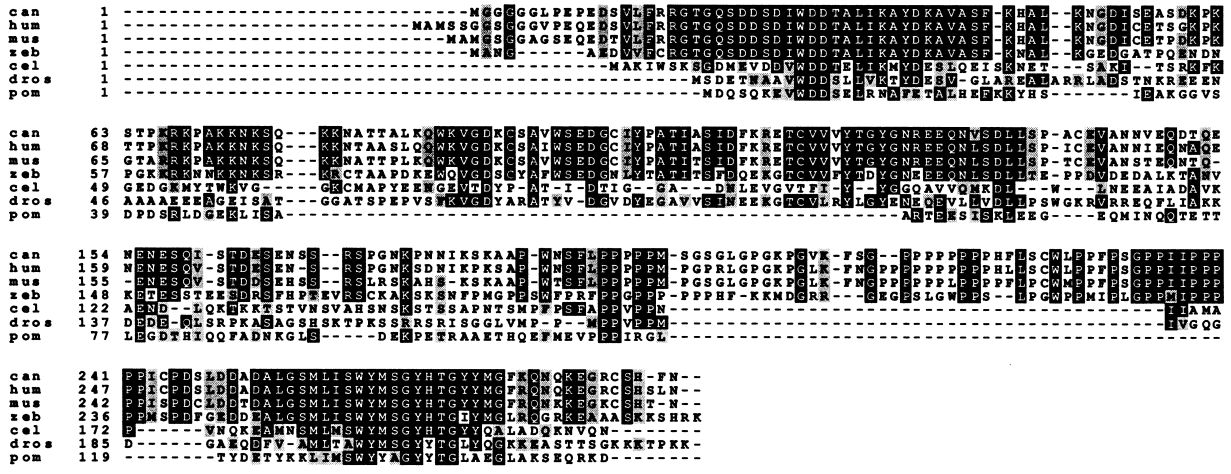


Fig. 1. Evolutionary conservation of SMN orthologs. The multiple alignment was constructed using Clustal W [28,29] and displayed using the BOXHADE server (http://www.ch.embnet.org/software/BOX_form.html). Species: can, *Canis familiaris*; hum, *Homo sapiens*; mus, *Mus musculus*; zeb, *Danio rerio*; cel, *C. elegans*; dros, *D. melanogaster* and pom, *Schizosaccharomyces pombe*. Homology is particularly significant at those regions that are thought to be functionally significant, i.e. amino acids 19–60 (SIP1 [8] and RNA [20] binding domain), amino acids 91–143 (tudor domain [23]) and amino acids 240–278 (binding to Sm proteins [8] and oligomerization [6] domain) in the human sequence. The DmSMN cDNA sequence is available from GenBank/EMBL/DDBJ under accession number AF296281.

2.4. Yeast two-hybrid analysis

Yeast two-hybrid analysis was performed using the ProQuest Two-Hybrid system (Life Technologies) as previously described [15].

3. Results and discussion

3.1. DmSMN sequence characterization and in situ analysis

A number of SMN-like sequences in several animal species have previously been reported [16], indicating SMN orthologs in vertebrates, the nematode *Caenorhabditis elegans* and yeast. Several ESTs similar to the human SMN gene were identified following a search of the *Drosophila* EST database (http://www.fruitfly.org/blast/). The full-length fly *SMN* cDNA (DmSMN) sequence was assembled from these overlapping ESTs. DmSMN exhibits a significant overall homology to other SMN orthologs (Fig. 1) and is the only SMN-like sequence in the *Drosophila* genome. In situ hybridization shows that the DmSMN mRNA is expressed ubiquitously from early, pre-transcriptional [17] stages of embryonic development (Fig. 2). This suggests that the transcript is transmitted maternally, in accord with previous observations in vertebrates and nematodes [18,19].

3.2. Ectopic expression of human SMN results in pupal lethality in Drosophila

To investigate SMN function in *Drosophila*, flies transgenic for several SMN constructs were generated using P-element-mediated transformation. Transgene expression was subsequently achieved using the GAL4-UAS system [13], a well-defined inducible system in which the gene of interest is placed under the control of a GAL4-inducible promoter. The GAL4 activator is provided by a second transgene containing GAL4 under the control of tissue-specific promoter/enhancer elements. Over-expression of full-length DmSMN under these conditions was generally benign; occasionally, a low percentage ($<5\%$) of embryonic lethality was observed, but no effects were apparent at later stages of the fly life cycle. In contrast, ectopic expression of human SMN resulted in pupal lethality (Fig. 3). Pupae developed until a relatively late stage, but failed to eclose. A variety of morphogenetic and differentiation defects could be observed instead. The penetrance was absolute for most of the independent human SMN insertions.

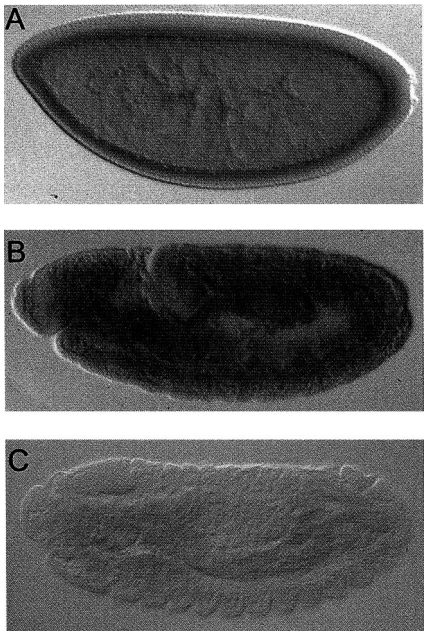


Fig. 2. In situ hybridization of DmSMN. A: A stage-4 embryo is shown in a lateral view, anterior to the left. The DmSMN transcript is already detectable at the cortical yolk-free cytoplasm underlying the blastoderm nuclei located at the periphery. The presence of DmSMN mRNA at this stage suggests that the transcript is transmitted maternally [17]. B: A stage-12 embryo is viewed from a lateral perspective, with dorsal up and anterior to the left. DmSMN expression appears widespread. C: A complementary sense DmSMN RNA probe was used as a negative control; no staining is detected in a stage-13 embryo.

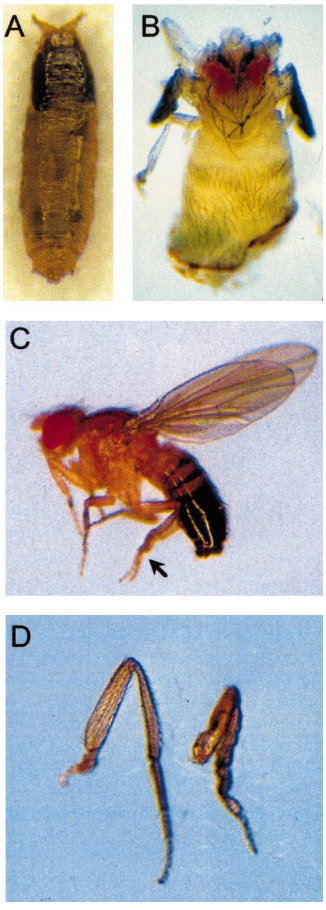


Fig. 3. Ectopic expression of human SMN. A: Typical pupal-lethal phenotype; wings and legs are abnormally positioned towards the anterior end of the pupae. Two out of five transgenic lines died at an earlier, white-pupal stage. B: Dissected dead pupae displaying morphogenesis defects in head, legs and wings. A necrotic ring terminates posterior development. C: A few pharate flies escape lethality in the milder lines (two out of five); the tarsi of the second and third pair of legs are abnormal (arrow). No pharate adults eclosed in any of the other lines. D: Detail of abnormal leg (right) versus wild-type (left). Tarsi are shorter and thicker.

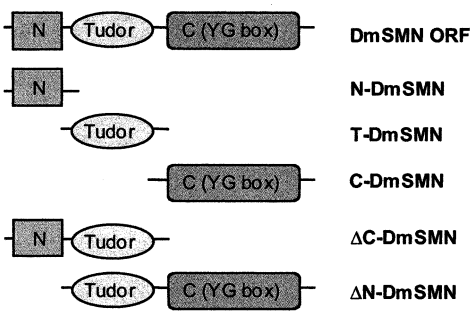
3.3. *Ectopic expression of the C-terminus of DmSMN results in identical phenotypes*

The different behavior of the two orthologs suggested that their functional similarity would be only partial; while this similarity would be sufficiently high for human SMN to somehow interfere with endogenous DmSMN metabolism, the functions of DmSMN and human SMN would not be completely interchangeable. An internal triplication of a YxxG motif within the C-terminus of the protein is highly conserved in all the SMN orthologs which have been reported so far (Fig. 1). An essential role for this domain has been proposed on the basis of this sequence similarity [16], a proposal supported by the fact that the mutations or deletions observed in SMA patients usually include this region of the protein. In vitro studies have shown that the C-terminus of SMN is necessary and sufficient for oligomerization of the protein in vertebrates [20], nematodes [19] and yeast [21]. Oligomerization facilitates binding to Sm proteins and is essential for SMN function [22]. We wondered whether our in vivo phenotypes were a consequence of a dominant-negative effect, whereby human SMN would bind endogenous DmSMN through the highly conserved C-terminal domain, but the resulting heterocomplexes human/fly SMN would not be totally functional. As a consequence, DmSMN would be titrated out, resulting in overall loss of endogenous SMN function. To address this question, several transgenic lines for truncated versions of DmSMN were generated (Fig. 4). When ectopic expression was induced, the C-terminal domain of DmSMN only (C-DmSMN) and a NH_2-terminal deletion fragment of DmSMN (ΔN-DmSMN) resulted in identical phenotypes to those observed with ectopic expression of human SMN. By contrast, none of the other truncated forms, including DmSMN lacking the C-terminus (ΔC-SMN, analogous to SMNΔ7, a natural human splice isoform [5]), replicated this effect. This suggests that the C-terminus of SMN is necessary and sufficient to generate a dominant-negative phenotype. Thus, deletion fragments of DmSMN in which essential functional domains have been removed are able to mimic the effects of ectopic human SMN expression.

3.4. *DmSMN can self-associate and bind to human SMN through its C-terminal region*

To determine the molecular nature of the genetic interactions observed, the ability of these dominant-negative forms to oligomerize was assessed using the yeast two-hybrid system (Fig. 5). The entire coding sequence of DmSMN was used as bait against prey constructs containing DmSMN, C-DmSMN, ΔN-DmSMN and human SMN. All these interactions were positive, indicating that DmSMN self-associates through its C-terminal domain. In addition, this interaction is evolutionarily conserved, and hetero-oligomerization of fly and human SMN proteins can occur.

A



B

Transgene	amino-acids	binding partners /function involved
N-DmSMN	1 – 69	SIP1, RNA/DNA
T-DmSMN	62 – 157	Sm (facilitates), Gemin3
C-DmSMN	159 – 226	oligomerization, Sm
ΔC-DmSMN	1 – 157	SIP1, RNA/DNA, Sm (facilitates), Gemin3
ΔN-DmSMN	62 – 226	Sm, Gemin3, oligomerization

Fig. 4. Deletion fragments of DmSMN. Transgenic flies for single DmSMN domains and DmSMN fragments lacking specific domains were generated. A: A schematic representation of the domains included in each transgene. B: Amino acid composition of each deletion fragment. The function of the equivalent human or mouse domains, when this has been elucidated, is indicated. These functions include binding to SIP1 [8], RNA/DNA [6], Sm proteins [8] and Gemin3 [25]. The tudor domain facilitates binding to Sm proteins [22] and the C-terminus is involved in oligomerization [20].

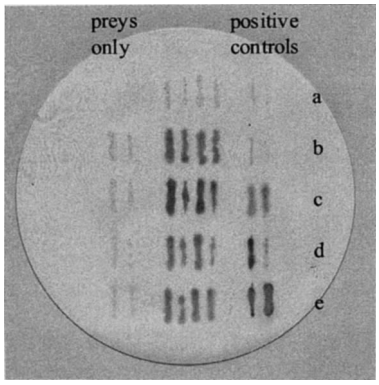


Fig. 5. Yeast two-hybrid analysis of DmSMN binding properties. DmSMN, human SMN, ΔN-DmSMN and C-DmSMN prey constructs were transformed, together with a DmSMN bait construct, into the host yeast strain MaV203 [15]. Induction of the *LacZ* gene was confirmed by assaying with X-Gal. The middle column shows positive interaction between DmSMN and (from top to bottom) itself, ΔN-DmSMN, C-DmSMN and human SMN. On the right are two patches each of five yeast control strains containing interacting proteins of varying strengths (no interaction in strain a to strong interaction in strain e). No self-activation was observed for the prey constructs (left column) or the DmSMN bait construct (four patches at the top of middle column).

3.5. Insights into SMN function and SMA etiology

We have cloned and characterized the DmSMN ortholog. Our results show that SMN function can be disrupted specifically in the fruit fly by taking advantage of the partial similarity of function among SMN orthologs. This can be achieved by ectopic expression of either human SMN or truncated forms of DmSMN encompassing its C-terminal domain. Previous experiments using more simple systems suggested a link between SMA etiology and abnormal C-terminal function [6,22]. Our results provide in vivo evidence for the role of this C-terminal region in SMN function (involving oligomerization and/or Sm protein binding) at the level of the organism as a whole. In contrast, neither the central domain of DmSMN (which corresponds to a tudor domain [23]) nor fragments lacking the C-terminus (N-DmSMN or ΔC-DmSMN) resulted in a dominant-negative phenotype. SMNΔ7, the human equivalent of ΔC-DmSMN, is the main product of *SMN2*, the centromeric copy of the human *SMN* gene [5]. It is not clear whether this alternatively spliced transcript is functional, deleterious or irrelevant to SMN function. In our system, no detrimental effect could be observed. Overall, our strategy has proved to be a useful way to dissect functional domains from an evolutionary point of view.

A growing number of SMN binding partners and, consequently, putative SMN functions are being reported [24–27] It is therefore essential to establish which of them are functionally significant, so that they can be targeted to compensate for the absence of SMN. From a therapeutic perspective one can attempt to up-regulate the small amount of full-length SMN protein generated by SMN2 [7]. This strategy is supported by the observation that over-expression of DmSMN did not result in a deleterious phenotype under our experimental conditions. Alternatively, a more informative approach is to try to discover new genes which could be targeted to compensate for the absence of SMN. In order to achieve this goal, high-throughput and suppressor screens need to be carried out. These require a simple (yet in vivo) model which combines specificity with ease of manipulation. We have generated a suitable model, thereby opening the way to the development of novel therapeutic strategies for SMA.

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