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Deletion of *smn-1*, the *Caenorhabditis elegans* ortholog of the spinal muscular atrophy gene, results in locomotor dysfunction and reduced lifespan

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INTRODUCTION

Spinal muscular atrophy is the most common genetic cause of infant mortality and is characterized by degeneration of lower motor neurons leading to muscle wasting. The causative gene has been identified as *survival motor neuron (SMN)*. The invertebrate model organism *Caenorhabditis elegans* contains *smn-1*, the ortholog of human *SMN*. *Caenorhabditis elegans smn-1* is expressed in various tissues including the nervous system and body wall muscle, and knockdown of *smn-1* by RNA interference is embryonic lethal. Here we show that the *smn-1(ok355)* deletion, which removes most of *smn-1* including the translation start site, produces a pleiotropic phenotype including late larval arrest, reduced lifespan, sterility as well as impaired locomotion and pharyngeal activity. Mutant nematodes develop to late larval stages due to maternal contribution of the *smn-1* gene product that allows to study SMN-1 functions beyond embryogenesis. Neuronal, but not muscle-directed, expression of *smn-1* partially rescues the *smn-1(ok355)* phenotype. Thus, the deletion mutant *smn-1(ok355)* provides a useful platform for functional analysis of an invertebrate ortholog of the human SMN protein.

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leading to SMA. However, since all model organisms deployed so far possess only one ortholog of the SMN1 gene, progress has been limited by the lethality resulting from complete loss of SMN. To circumvent this difficulty, various strategies have been developed. Expression of human SMN2 in Smn-/- null mice rescues their embryonic lethality and generates animals with a movement defect and motor neuron loss resembling severe SMA (9). In zebrafish, the reduction of Smn levels using antisense morpholinos produces motor axon defects such as truncations and aberrant branching (10). A Drosophila mutant containing a mis-sense mutation in the fly smn gene survives until late larval stages due to maternally contributed smn mRNA (11). This mutant exhibits reduced excitatory postsynaptic currents and structural defects at neuromuscular junctions in the absence of motor neuron loss.

A C. elegans ortholog of the human SMN1 gene has been identified and termed smn-1 (12). A green fluorescent protein (GFP) reporter construct contains full-length smn-1, and its upstream sequence produces fluorescence in various tissues including the nervous system and body wall muscles, demonstrating widespread expression of smn-1. Additionally, the SMN-1 protein was detected by immunofluorescence from the zygotic stage onwards, indicating a maternal contribution of the protein or its mRNA. So far, the functions of C. elegans smn-1 have been studied in vivo by RNA interference (RNAi) induced by injection of double-stranded RNA that produces knockdown effects in all tissues, including maternal transcripts, although silencing of neuronal genes is less efficient (13). Targeting smn-1 with RNAi gives rise to severe developmental defects and embryonic lethality, thus limiting further investigations on postembryonic roles of SMN-1 (12). Here we have characterized the smn-1(ok355) deletion allele, which removes most of smn-1 including the translation start codon, resulting in late larval arrest, a dramatic decrease in lifespan as well as impaired locomotion and pharyngeal pumping. Early larval development of smn-1(ok355) mutant animals appears normal due to maternally contributed SMN-1. As it models aspects of SMA, this smn-1 mutant lends itself to the functional analysis of an SMN ortholog and has potential future utilities in screens searching for phenotypic modifiers as a first step toward potential SMA therapies.

### RESULTS

#### The smn-1(ok355) deletion

Like all model organisms studied to date, C. elegans has only a single copy of the gene encoding the survival motor neuron protein. The smn-1(ok355) allele described here removes 975 bp comprising 246 bp of the upstream intergenic region and most of the smn-1 gene leaving only 87 bp (including the stop codon) at the 3’ end (part of exon 5; Fig. 1). The coding sequences of the adjacent genes klp-16 and F30A10.10 are unaffected by the smn-1(ok355) deletion. Absence of the smn-1 ATG start codon in the remaining sequence suggests that smn-1(ok355) is a null allele and hence the only SMN-1 present is maternal, originating from balanced smn-1(ok355)/hT2[qIs48] heterozygous mothers (strain LM99).

#### Neuronal expression of smn-1 partially rescues the smn-1(ok355) phenotype

It has recently been shown that the phenotype of severe SMA mice can be corrected by expressing full-length SMN solely in the nervous system (14). To investigate the sensitivity of different aspects of the C. elegans neuromuscular system to smn-1 loss, we sought to provide wild-type smn-1 to either muscles or neurons of smn-1(ok355) mutants. To this end, rescuing constructs were used expressing full-length smn-1 in body wall and vulval muscles through a myo-3 promoter (Pyio-3::smn-1) and in all neurons through an unc-119 promoter (Punc-119::smn-1). Promoters of unc-119 and myo-3 have been used previously for neuronal- and muscle-directed expression in C. elegans.
rescue (15,16) and are considered strong transcriptional inducers (17). The rescuing constructs were microinjected into the gonads of \textit{smn-1(ok355)/hT2[qIs48]} heterozygotes to generate heritable lines. Transgenic heterozygous mothers carry the plasmid DNA as multi-copy, extrachromosomal arrays and transmit the transgene at a certain frequency to their progeny including \textit{smn-1(ok355)} homozygotes. Extrachromosomal transgenes are frequently lost during cell division resulting in mosaic expression of the transgene (18).

Despite having injected a large number of animals, only one heritable transgenic line each was obtained carrying extrachromosomal \textit{Ex[\text{Pmyo-3::smn-1}]} or \textit{Ex[\text{Punc-119::smn-1}]} for as yet unknown reasons. As an indicator for transgene expression \textit{in vivo}, the GFP fusions \textit{Pmyo-3::GFP} and \textit{Punc-119::GFP} co-injected with the rescuing constructs showed expression in the expected tissues (body wall and vulval muscles for \textit{Pmyo-3} and nervous system for \textit{Punc-119}, data not shown).

Whereas mutant \textit{smn-1(ok355)} animals expressing muscle-directed \textit{smn-1} showed only weak phenotypic rescue in a subset of animals, pan-neuronal expression of \textit{smn-1} produced stronger rescue effects (Fig. 7A). Albeit remaining sterile, rescued \textit{smn-1(ok355); Ex[Punc-119::smn-1]} animals had a larger body size compared with \textit{smn-1(ok355)} and \textit{smn-1( ok355); Ex[\text{Pmyo-3::smn-1}]} animals (Fig. 7B) and often retained their intestinal pigmentation, thus appearing less starved. However, they did not reach the size of fully-grown adult \textit{smn-1(ok355)/hT2[qIs48]} heterozygotes, indicating partial as opposed to full rescue. We noted that the extent of the rescuing effects was variable which, most likely, reflects the mitotic instability of the extrachromosomal transgene. In agreement with the body length measurements, neuronal-expressing \textit{smn-1(ok355); Ex[Punc-119::smn-1]} animals survived longer than \textit{smn-1(ok355)} and muscle-expressing \textit{smn-1(ok355); Ex[\text{Pmyo-3::smn-1}]} animals (Fig. 7C) and showed a partial correction of the pharyngeal pumping defect (Fig. 6). Taken together, these data suggest that restoration of neuronal \textit{smn-1} activity can, at least partially, correct aspects of the \textit{smn-1(ok355)} phenotype.

The nervous system of \textit{smn-1(ok355)} animals

Having shown that neuronal-directed expression of \textit{smn-1} is much more effective than muscle-directed expression in rescuing the larval arrest phenotype of \textit{smn-1(ok355)}, we sought to test whether \textit{smn-1(ok355)} exhibits nervous system abnormalities in general and loss of cholinergic motor neurons in particular. To this end we used the pan-neuronal marker \textit{F25B3.3::GFP} (19) (strain LM111) and the marker for cholinergic neurons \textit{unc-17::GFP} (strain LM112) to facilitate neuronal identification. On the basis of their connectivity, motor neurons can be subdivided into classes that innervate dorsal

![Figure 2. Developmental comparison of \textit{smn-1(ok355)} mutants, \textit{smn-1(ok355)/hT2[qIs48]} heterozygotes and N2 wild-type \textit{C. elegans}. Age (in days post-L1) is indicated on the left. The images depict representative phenotypic appearances of synchronized animals at a given age. For all images anterior is on the left and posterior toward the right.](image-url)
muscles by sending their axons dorsally as commissures to form the dorsal nerve cord and those classes that project ventrally. Motor neurons (classes and individual cells) were identified by studying their axonal trajectories and cell body position in the dorsal/ventral nerve cord in LM111 animals (20).

We could not detect any gross abnormalities in nervous system morphology of *smn-1(ok355); F25B3.3::GFP* animals (Fig. 8A). In an attempt to test whether there is loss of neurons over time, LM112 animals were staged and the number of cholinergic neurons in the ventral nerve cord was counted using the marker *unc-17::GFP* that labels embryonic and postembryonic motor neurons (21,22). No differences were detected in the number of GFP-positive neurons in the ventral cord region between *smn-1(ok355)* and *smn-1(ok355)/hT2[qIs48]* up to 5 days post-L1 (Fig. 8B).

**DISCUSSION**

In all animal models of SMA studied to date, the ortholog of human *SMN* is ubiquitously expressed and exists as a single-copy gene. We have shown that, in the *C. elegans smn-1(ok355)* deletion mutant, the loss of SMN-1 protein leads to a progressive decline of motor function. Even though the coding regions of the neighboring genes remain intact, it remains possible that the upstream regulatory sequence of *klp-16* may be affected by the *smn-1(ok355)* deletion thus disrupting *klp-16* expression as well. However, the *klp-16(ok1505)* mutant containing a 1.1 kb deletion within the *klp-16* coding sequence has no obvious defects, with normal movement, body size and brood size (data not shown). Thus, taken together with the finding that the *smn-1(ok355)* mutant phenocopies the less affected progeny originating from *smn-1* RNAi (12), it remains unlikely that the *smn-1(ok355)* deletion phenotype described here is caused by the combined loss of *smn-1* and *klp-16* function.

The *smn-1(ok355)* mutation is essentially a null allele, albeit retaining progressively decreasing levels of maternal SMN-1. Immunofluorescence analysis of embryos has previously revealed the presence of SMN-1 at the one-cell stage (P0) prior to initiation of de novo zygotic transcription (12). Considering that RNAi-mediated knockdown of *smn-1*, which reduces the levels of both maternal and zygotic transcripts, leads to embryonic lethality, we postulate that the maternal contribution of SMN-1 allows progression of *smn-1(ok355)* animals through early development. Consistent with this notion, the thrashing rate of *smn-1(ok355)* is unaffected prior to arrest, indicating normal functioning of the neuromuscular system. Presumably, the progressive reduction of maternal SMN-1 results in developmental arrest of homozygous animals at a late larval stage reflected by a defect in gonadogenesis which is followed by paralysis and early lethality.

Closer inspection of the ventral nerve cord of neuronal GFP-expressing *smn-1(ok355)* animals failed to reveal any loss of cholinergic motor neurons. This finding indicates that embryonic and postembryonic motor neuron development proceeds normally due to sufficient levels of maternally contributed SMN-1 prior to their larval arrest. The absence of a motility defect in the early larval stages underlines the notion that, in *smn-1(ok355)* animals, motor neurons initially establish normal functional connections with their target muscles. When the movement defects first appeared, we could not detect any loss of cholinergic motor neurons. When paralysis ensues, motor neuron numbers remain unaffected. Thus, in *smn-1(ok355)* mutants, the maternally contributed SMN-1 can sustain development to late larval stages when the animals arrest in response to SMN protein depletion in the absence of any obvious loss of neuronal cell bodies.

The absence of motor neuron loss in *smn-1(ok355)* animals is in contrast with mouse models of SMA exhibiting motor defects accompanied by motor neuron degeneration (9), and a zebrafish SMA model showing axon path-finding defects in response to morpholino-induced *smn* knockout (10). Rather, the
sma-1(ok355) phenotype described here resembles that of the Drosophila SMA model bearing a missense mutation in smn similar to some of the mutations found in SMA patients (11). The mutant smn flies also survive past embryogenesis due to maternally contributed mRNA and die as late larvae following depletion of the maternal component. However, whereas homozygous smn mutant fly larvae produce both mutant and wild-type Smn protein due to the presence of both transcripts, smn-1(ok355) larvae contain only wild-type smn-1 transcript. Nevertheless, even though the smn-1(ok355) mutant relates to the vast majority of SMA cases showing deletions in the SMN1 gene, the situation in this nematode model of SMA differs from SMA in several facets. Caenorhabditis elegans produces full-length SMN-1 protein from only one gene encoding an SMN ortholog, smn-1. In contrast, in addition to SMN1, humans possess SMN2 which in SMA patients acts as a phenotypic modifier gene producing low levels of functional SMN protein. As a consequence, SMA is caused by insufficiency rather than a complete loss of SMN protein. Furthermore, unlike the case for C. elegans and Drosophila, there is no maternally contribution of SMN mRNA in mammals. For example, homozygous loss of Smn in mice is embryonic lethal (23), which has been overcome by expressing human SMN2 in Smn−/− mice (9,24). Thus, the absence of a C. elegans SMN2 ortholog combined with maternally contributed SMN-1 causes smn-1(ok355) mutants to exhibit a gradual decline in SMN-1 levels ultimately leading to a complete loss of the protein as opposed to the low remaining SMN levels seen in SMA patients. Nevertheless, even though maternal transmission may mask possible functions of SMN-1 during development, the smn-1(ok355) mutant provides the opportunity to study postembryonic roles of SMN-1 in motor neurons and at neuromuscular junctions.

To further understand the pathological events leading to SMA, it is critical to elucidate whether the SMN reduction in motor neurons is a primary defect in the disease or whether there are independent muscle defects as well. The answer to this question is particularly important for the development of therapeutic strategies aiming to up-regulate the SMN levels in selected cell types. Whether the smn-1(ok355) phenotype originates due to defects in muscles or neurons has been addressed here by rescue experiments in which smn-1 was delivered locally to either muscle cells or neurons. It emerged that neuronal-directed, but not muscle-directed, expression of smn-1 partially rescued the smn-1(ok355) phenotype. When assessing the rescue experiments, it has to be taken into account, however, that even though the unc-119 promoter used here exhibits pan-neuronal activity we cannot exclude the possibility of promoting activity levels below the detection limit in cell types other than neurons. Furthermore, we cannot rule out the possibility that the structure and composition of the extrachromosomal arrays may be different thus producing variability in the extent of mosaicism and transgene expression levels.

In conclusion, the Human SMA model bearing a mis-sense mutation in smn provides the opportunity to study postembryonic roles of SMN-1 in motor neurons and at neuromuscular junctions.
animals proceed through embryogenesis but arrest as late larvae indicating that, in *C. elegans*, SMN-1 has functions later during development and in adult life. In future studies, smn-1(ok355) mutants may serve as a starting point for the identification of modifier genes in genetic or genome-wide RNAi screens that enhance or suppress the smn-1(ok355) phenotype and for developing novel therapeutic strategies in drug screens.

**MATERIALS AND METHODS**

**Strains**

Strains were grown and maintained as described previously (25). The following strains were used in this study: N2, NW1229 [evIs111[F25B3.3::GFP; dpy-20(+)]], RM1872 [mdEx72[unc-17::GFP; pha-1(+)]], LM97 [smn-1(ok355)/+ I], LM99 [smn-1(ok355) I/hT2[bla-4(e937) let-?(q782) qIs48] (I;III)], LM111 [smn-1(ok355) I/hT2[bla-4(e937) let-?(q782) qIs48] (I;III); evIs111], LM112 [smn-1(ok355) I/hT2 [bla-4(e937) let-?(q782) qIs48] (I;III); mdEx72] and VC1005 [klp-16(ok1505) I].

**Isolation of the smn-1(ok355) deletion**

The smn-1(ok355) deletion allele of smn-1 located on chromosome I was obtained from the *C. elegans* Gene Knockout Consortium. From the genetically heterogeneous population, we cloned individual animals heterozygous for the deletion and out-crossed them six times to wild-type (N2) nematodes. The unbalanced smn-1(ok355)/+ strain (LM97) was maintained by picking heterozygous animals and confirming the presence of the deletion by PCR using primers 5'-GACTTCAGATTTGATCAACGGTC-3' and 5'-ACACCAATTACCAAACGATCAAC-3'. Stable maintenance of the deletion was achieved by introducing the genetic balancer hT2 (I;III), which is a reciprocal translocation of chromosomal segments between chromosomes I and III, generating strain LM99. To distinguish homozygous from heterozygous
animals visually, we used the $hT2[bli-4(e937)\text{ let-?}(q782)\text{ qIs48}]$ (I;III) derivative of the chromosomal balancer, where $qIs48$ is an insertion of extrachromosomal $ccEx9747$ carrying the promoter::GFP fusions $myo-2::GFP$ (expressed in the pharynx), $pex-10::GFP$ (expressed in 4–60 cell embryos) and a gut-specific enhancer fused to GFP (26,27). Heterozygous $smn-1(ok355)$ $hT2[qIs48]$ heterozygotes and arrested aneuploid progeny. Since $hT2[qIs48]$ heterozygotes and $smn-1(ok355)/hT2[qIs48]$ is homozygous lethal, all animals expressing GFP are heterozygous for $smn-1(ok355)$. Animals homozygous for the $smn-1(ok355)$ deletion can be identified due to absence of GFP fluorescence in the pharynx.

Physiological assays

Embryos were released by subjecting gravid N2 and LM99 hermaphrodites to bleach and were subsequently left in M9 overnight for obtaining hatched synchronized $smn-1(ok355)$ homozygotes, balanced $smn-1(ok355)/hT2[qIs48]$ heterozygotes and N2 wild-types at the L1 larval stage. Synchronized L1 larvae were placed on nematode growth medium (NGM) plates seeded with E. coli OP50, and left for 1 day at 22°C. Motility was then assessed every day by placing animals in M9 buffer and measuring their movement as thrashing rate in a 1 min period. A single thrash was defined as a change in the direction of bending at the mid-body. Animals were left in M9 for at least 5 min prior to measurement. For quantification of pharyngeal pumping synchronized $smn-1(ok355)$ homozygotes, $smn-1(ok355)/hT2[qIs48]$ heterozygotes, N2 wild-type and tissue-specific rescue animals (see below) aged 1 day post-L1 were placed on OP50-seeded plates at 22°C. Terminal bulb pumps were measured each day under a dissection microscope for periods of 15 s. Between 2 and 10 periods of 15 s were counted for each animal.

Lifespan and brood size assays

For the lifespan assay synchronized $smn-1(ok355)$ homozygotes, $smn-1(ok355)/hT2[qIs48]$ heterozygotes and N2 wild-types aged 1 day post-L1 were placed on OP50-seeded plates (5 cm diameter) at 22°C. Survival was monitored daily, and animals were scored as dead when they no longer responded to head or tail touch using a platinum wire pick. To avoid crowding due to progeny production and to ensure the availability of sufficient food, animals were transferred to new plates when deemed necessary. Animals that crawled off the NGM agar were excluded from the data. For brood size assays, $klp-16(ok1505)$ and wild-type N2 animals were synchronized and their brood size determined over a 5 day period post-L4 at 20°C. PCR analysis was used to confirm the presence of the $klp-16(ok1505)$ deletion using primers recommended in WormBase (http://www.wormbase.org/).

Tissue-specific rescue of the $smn-1(ok355)$ mutant

To rescue the $smn-1(ok355)$ phenotype in a tissue-specific manner, $smn-1(ok355)/hT2[qIs48]$ heterozygotes were microinjected with the promoter::$smn-1$ fusion constructs $Pmyo-3::smn-1$ or $Punc-119::smn-1$ expressing $smn-1$ in body wall muscle or neuronal tissue, respectively. These rescuing constructs contain $smn-1$ from the ATG start to the TAA stop codon (amplified from N2 genomic DNA) placed under transcriptional control of 2 kb upstream of $myo-3$ or $Pmyo-3::smn-1$ (generating muscle-directed expression) or 2.2 kb upstream of $unc-119$ in $Punc-119::smn-1$ (for pan-neuronal expression). To visualize promoter activity, $smn-1$ was replaced by GFP to generate the reporters $Punc-119::GFP$ and $Pmyo-3::GFP$. For neuronal rescue, $Punc-119::smn-1$ and $Punc-119::GFP$ were co-injected at 10 ng/μl each together with pRF4 at 200 ng/μl encoding the marker rol-6(su1006) the expression of which induces a ‘rolling’ phenotype (28). Analogously, for muscle-directed
rescue attempts, *Pmyo-3::smn-1* was co-injected with *Pmyo-3::GFP* and rpr4.

For body length measurements, photomicrographs were taken of mobile but growth-arrested *smn-1(ok355)* animals, which were identified by their starved, pale appearance, and adult *smn-1(ok355)/hT2[gal4s48]* animals from mixed-stage populations. For body length analysis of transgenic rescue nematodes, individual animals were monitored daily when deemed necessary due to variability in the extent of rescue observed. Photomicrographs of transgenic rescue animals were taken either when they were considered fully grown or when they appeared starved, i.e. growth-arrested, or immobile. Images of animals were obtained using a Nikon SMZ1000 stereomicroscope and analyzed using ImageJ software from NIH (http://rsb.info.nih.gov/ij/) to measure the body length along the midline from the tip of the head to the base of the tail. Lifespan of rescue animals was assessed by picking synchronized animals aged 3 days post-L1 and monitoring their survival as described above.

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**Conflict of Interest statement.** None declared.

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