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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

Michael Briese[1,†], Behrooz Esmaeili[1,†], Sandrine Fraboulet[1,2], Emma C. Burt[1], Stefanos Christodoulou[1], Paula R. Towers[1], Kay E. Davies[1] and David B. Sattelle[1,*]

[1]MRC Functional Genomics Unit, Department of Physiology Anatomy and Genetics, University of Oxford, South Parks Road, Oxford OX1 3QX, UK and [2]Institut des Neurosciences Grenoble, Centre de recherche Inserm U836-UJF-CEA-CHU, Université Joseph Fourier, Bâtiment Edmond J. Safra, Domaine de la Merci, 38706 La Tronche Cedex, France

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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.

INTRODUCTION

Spinal muscular atrophy (SMA) is an autosomal recessive neuromuscular disorder, mostly of childhood-onset, characterized by degeneration of lower motor neurons in the anterior horn of the spinal cord leading to proximal muscle wasting. It is the most common genetic cause of infant mortality with an incidence of around 1 in 10 000 live births (1,2). Childhood-onset SMA cases show deletions in the *survival motor neuron* (*SMN*) gene located as a telomeric copy (*SMN1*) and a centromeric copy (*SMN2*) on chromosome 5 in an inverted duplicated region (3). Owing to a single C-to-T transition in exon 7, the majority of transcripts originating from *SMN2* are alternatively spliced lacking exon 7, and give rise to a truncated, unstable protein product (4,5). Thus, *SMN1*, the SMA-determining gene, produces full-length SMN protein almost exclusively, whereas *SMN2* acts as a disease modifier gene due to the small amount of functional SMN it produces (6,7).

Even though the genetic defect underlying SMA has been shown to reside in the *SMN* gene, important questions concerning the disease etiology remain unresolved. SMN is expressed widely both within and outside the nervous system (3). Thus, the specific motor neuron defect seen in SMA cannot be explained purely in terms of the expression pattern of SMN but instead may relate to its function (8). Animal models of SMA have been indispensable for the study of physiologic roles of SMN in muscle cells and neurons as well as for the dissection of pathological events

*To whom correspondence should be addressed. Tel: +44 1865272145; Fax: +44 1865282651; Email: david.sattelle@dpag.ox.ac.uk
†M.B. and B.E. contributed equally to the work.

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).

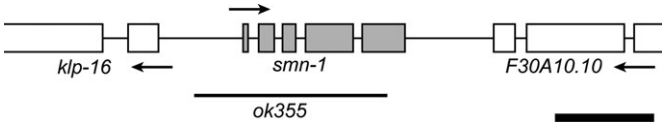

Figure 1. The *smn-1(ok355)* deletion allele, showing that only part of *smn-1* exon 5 remains. Arrows indicate direction of transcription, the open-reading frames are depicted as boxes. Scale bar: 500 bp.

Caenorhabditis elegans wild-type animals progress through four larval stages (L1 to L4) before reaching adulthood. The most striking phenotype of the *smn-1(ok355)* mutant is its late larval arrest (Fig. 2), including defects in gonadogenesis and germline differentiation (Fig. 3). Arrested *smn-1(ok355)* homozygotes become pale due to loss of intestinal pigmentation giving them a starved appearance. Their mean lifespan is 6.0 days post-L1 compared with 19.9 days for balanced *smn-1(ok355)/hT2[qIs48]* and 17.7 days for N2 wild-types (Fig. 4).

smn-1(ok355) mutants show defects in motility and pharyngeal pumping

Following larval arrest, *smn-1(ok355)* nematodes display a progressive decline in motility. To measure the locomotory activity we quantified the thrashing rate of animals in M9 buffer. During early development, i.e. before arrest, the thrashing rate of *smn-1(ok355)* homozygotes was similar to balanced *smn-1(ok355)/hT2[qIs48]* heterozygotes and N2 wild-types (Fig. 5). Thrashing of *smn-1(ok355)* animals then progressively declined and almost completely ceased after 5 days post-L1. In contrast, locomotion of *smn-1(ok355)/hT2[qIs48]* heterozygous animals was similar to N2 wild-types at all time-points throughout the assay.

Likewise, *smn-1(ok355)* mutants are unable to feed due to loss of pharyngeal function. The rate of pharyngeal pumping was measured in *smn-1(ok355)* homozygous mutants over a period of 4 days and compared with that of N2 wild-type animals and *smn-1(ok355)/hT2[qIs48]* heterozygotes (Fig. 6). On the first day of the assay (1 day after starved L1 larvae were placed on seeded NGM plates), the pharyngeal pumping rate of the mutant was indistinguishable from that of wild-type nematodes. Similar to body wall muscle activity, pharyngeal pumping rates showed a rapid and progressive decline. Taken together, these data indicate a general defect in behaviors requiring rhythmic contractions of muscle tissue in *smn-1(ok355)* animals.

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 (*Pmyo-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

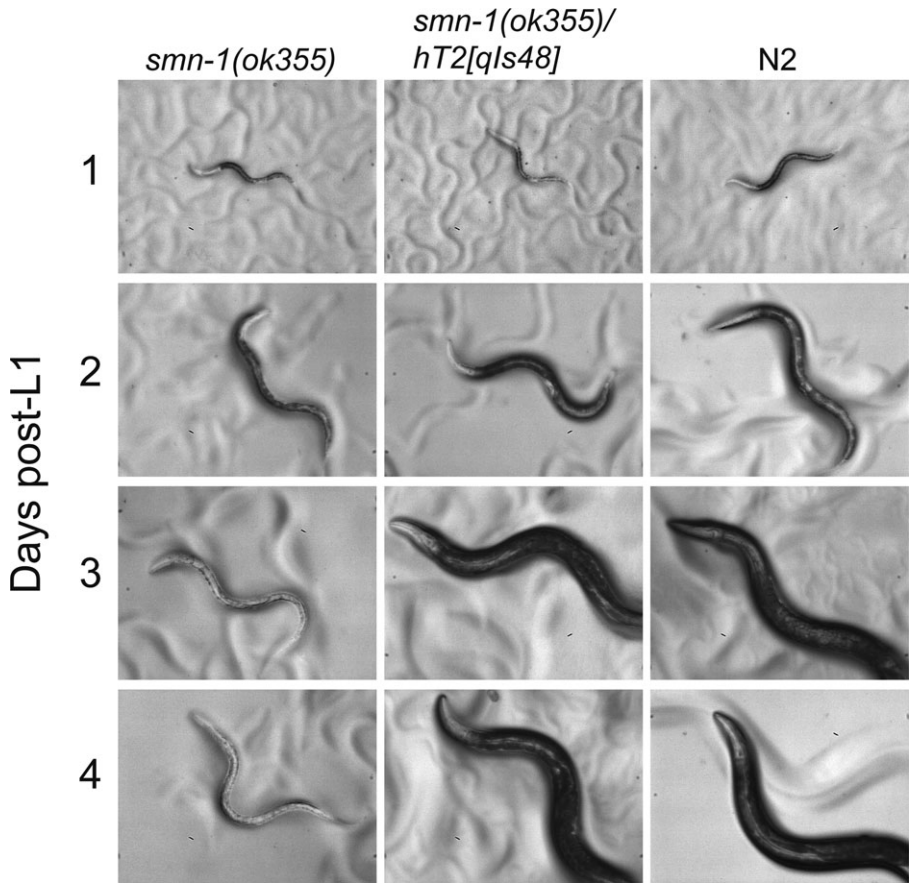

Figure 2. Developmental comparison of *smn-1(ok355)* mutants, *smn-1(ok355)/hT2[qIs48]* heterozygotes and N2 wild-type *C. elegans*. Age (in days post-L1) is indicated on the left. The images depict representative phenotypic appearances of synchronized animals at a given age. For all images anterior is on the left and posterior toward the right.

rescue (15,16) and are considered strong transcriptional inducers (17). The rescuing constructs were microinjected into the gonads of *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 *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 *Ex[Pmyo-3::smn-1]* or *Ex[Punc-119::smn-1]* for as yet unknown reasons. As an indicator for transgene expression *in vivo*, the GFP fusions *Pmyo-3::GFP* and *Punc-119::GFP* co-injected with the rescuing constructs showed expression in the expected tissues (body wall and vulval muscles for *Pmyo-3* and nervous system for *Punc-119*, data not shown).

Whereas mutant *smn-1(ok355)* animals expressing muscle-directed *smn-1* showed only weak phenotypic rescue in a subset of animals, pan-neuronal expression of *smn-1* produced stronger rescue effects (Fig. 7A). Albeit remaining sterile, rescued *smn-1(ok355); Ex[Punc-119::smn-1]* animals had a larger body size compared with *smn-1(ok355)* and *smn-1 (ok355); Ex[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 *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 *smn-1(ok355); Ex[Punc-119::smn-1]* animals survived longer than *smn-1(ok355)* and muscle-expressing *smn-1(ok355); Ex[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 *smn-1* activity can, at least partially, correct aspects of the *smn-1(ok355)* phenotype.

The nervous system of *smn-1(ok355)* animals

Having shown that neuronal-directed expression of *smn-1* is much more effective than muscle-directed expression in rescuing the larval arrest phenotype of *smn-1(ok355)*, we sought to test whether *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 *F25B3.3::GFP* (19) (strain LM111) and the marker for cholinergic neurons *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

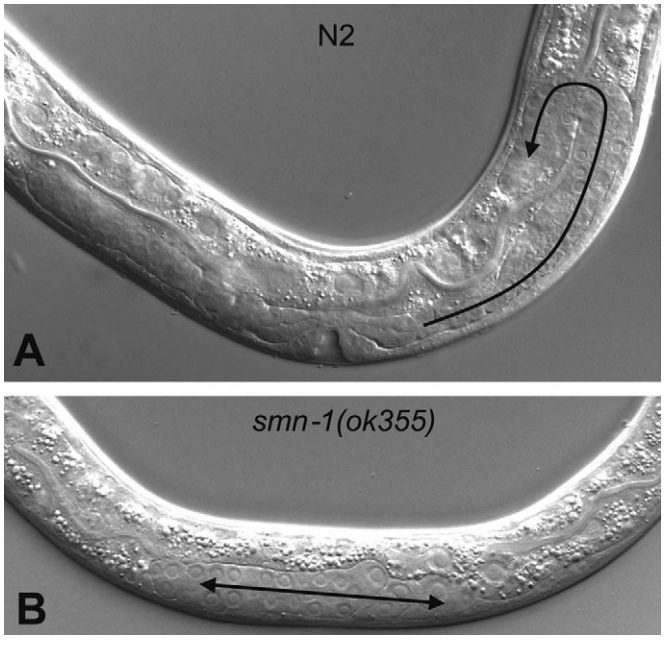

Figure 3. Gonad migration defects in *C. elegans smn-1(ok355)* mutants. (**A**) In N2 wild-type hermaphrodites the gonad folds back during the L4 stage and develops in a U-shaped manner. (**B**) In arrested *smn-1(ok355)* the gonad fails to develop properly.

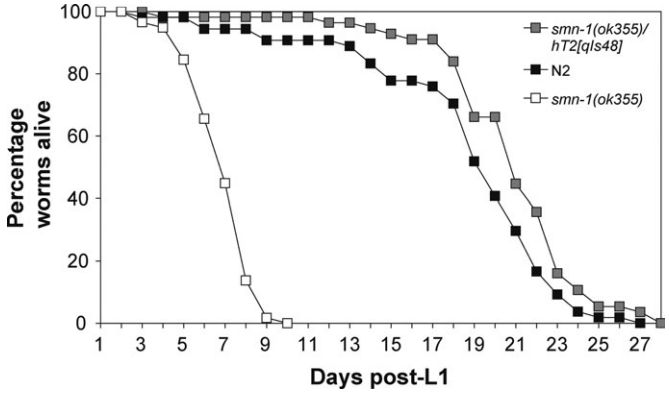

Figure 4. Survival of *smn-1(ok355)* mutants ($n = 58$) in comparison to *smn-1(ok355)/hT2[qIs48]* heterozygotes ($n = 56$) and N2 wild-type *C. elegans* ($n = 54$). Each data point represents the fraction of synchronized animals alive on a given day.

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 (P_0) 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 in the ventral nerve cord. Even 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* knockdown (10). Rather, the

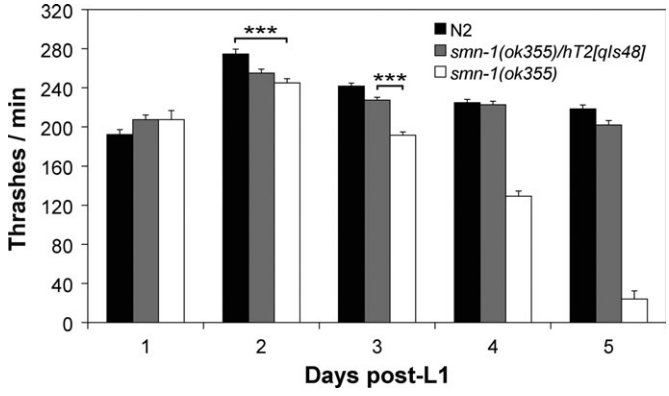

Figure 5. Progressive development of movement defects in *C. elegans smn-1(ok355)* animals, balanced *smn-1(ok355)/hT2[qIs48]* heterozygotes and N2 wild-type animals. For each group, $n = 20$. Asterisks: $P < 0.001$. The graphs depict mean ± SEM.

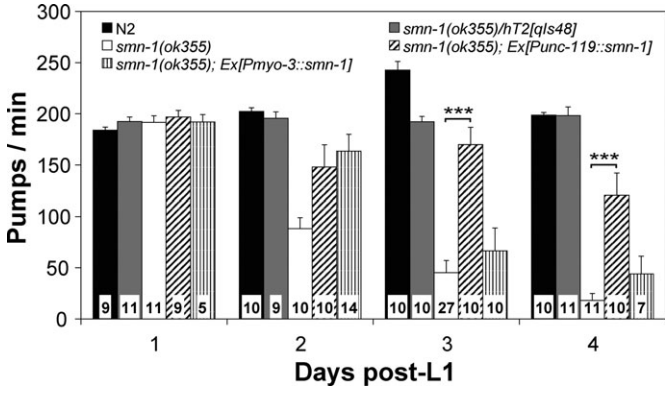

Figure 6. Pharyngeal pumping analysis of *C. elegans smn-1(ok355)* mutants, balanced *smn-1(ok355)/hT2[qIs48]* heterozygotes and N2 wild-types as well as muscle-expressing *smn-1(ok355); Ex[Pmyo-3::smn-1]* and neuronal-expressing *smn-1(ok355); Ex[Punc-119::smn-1]* animals. Asterisks: $P <$ 0.0001. Numbers at the base of each bar indicate number of animals used. The graphs depict mean ± SEM.

smn-1(ok355) phenotype described here resembles that of the *Drosophila* SMA model bearing a mis-sense 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 maternal 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 promoter 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 the light of our findings, it is of interest to note that neuronal expression of full-length human *SMN* by the Prion protein (PrP) promoter rescues severe SMA mice containing two copies of human *SMN2* and lacking mouse *Smn* ($Smn^{-/-}$; $SMN2^{+/+}$) (14). These SMA mice normally have an average lifespan of 5 days (9), which could be extended to 210 days in one line homozygous for the *PrP-SMN* transgene ($Smn^{-/-}$; $SMN2^{+/+}$; *PrP-SMN*). When SMN was targeted only to skeletal muscle using the human skeletal actin (HSA) promoter neither improvement of the SMA phenotype nor any extension of survival was seen. One *HSA–SMN* line showing low expression of SMN in the spinal cord did affect the SMA phenotype with mice living for 160 days on average. Thus, a small increase of SMN in neurons was sufficient to extend the survival of SMA mice considerably whereas high SMN levels in mature skeletal muscle alone had no effect. In comparison to these data we observed only partial as opposed to full rescue when expressing *smn-1* in the nervous system of *smn-1(ok355)* animals. This difference may be explained by the complete loss of maternally contributed SMN-1 activity in the non-neuronal tissues of rescued *smn-1(ok355); Ex[Punc-119::smn-1]* animals in contrast with the low remaining levels in rescued $Smn^{-/-}$; $SMN2^{+/+}$; *PrP-SMN* mice.

In conclusion, the *C. elegans* mutant *smn-1(ok355)* provides a disease model mimicking aspects of SMA such as reduced SMN-1 protein levels throughout the organism. Mutant

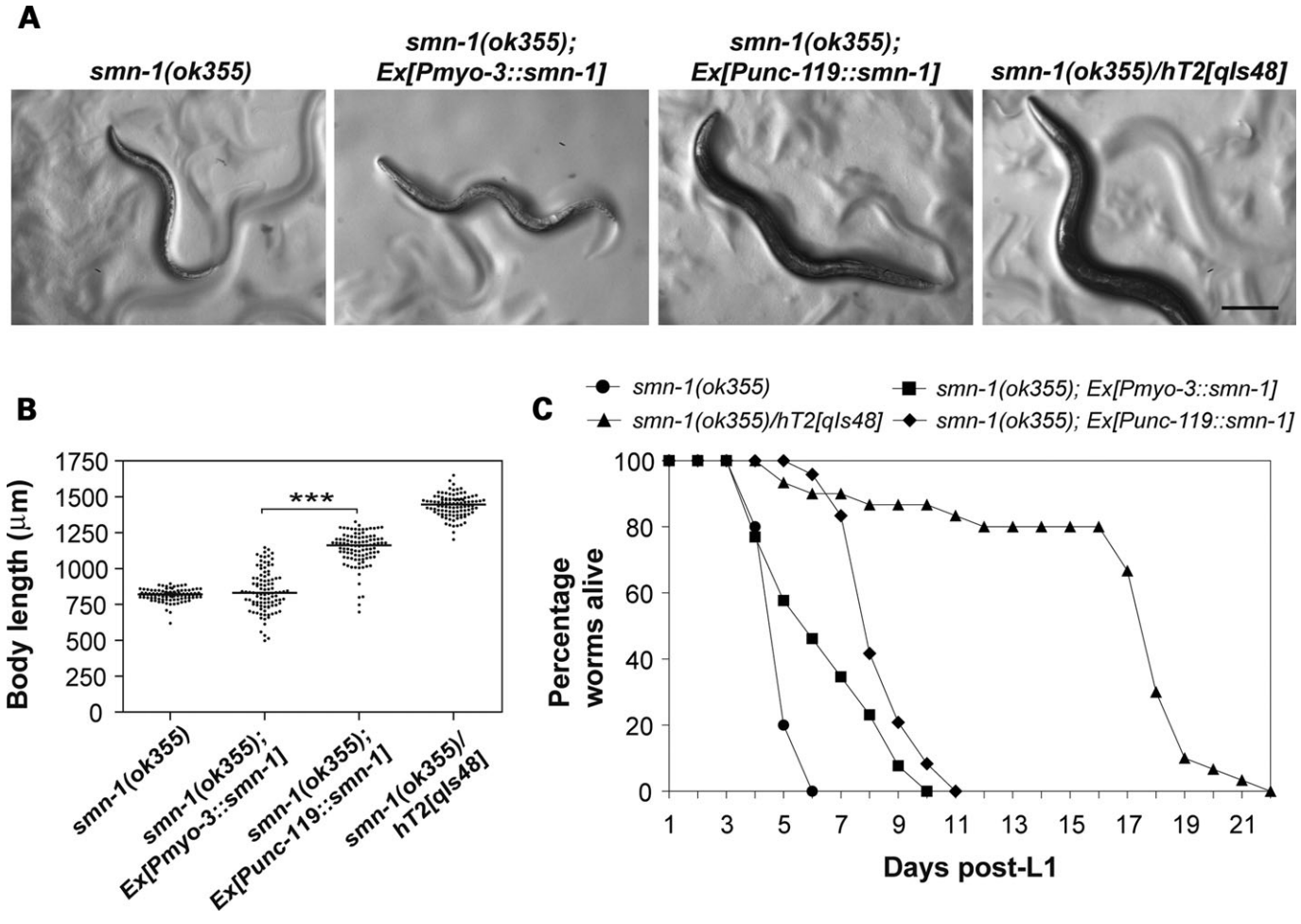

Figure 7. Neuronally expressed *smn-1* partially rescues the *C. elegans smn-1(ok355)* phenotype. (**A**) Examples of mutant animals carrying extrachromosomal *Pmyo-3::smn-1* inducing expression of *smn-1* in body wall and vulval muscles or *Punc-119::smn-1* for *smn-1* expression in all neurons. For comparison, arrested *smn-1(ok355)* and adult *smn-1(ok355)/hT2[qIs48]* animals are shown. (**B**) Comparison of body lengths of arrested *smn-1(ok355)*, adult *smn-1(ok355)/hT2[qIs48]* as well as fully-grown *smn-1(ok355); Ex[Pmyo-3::smn-1]* and *smn-1(ok355); Ex[Punc-119::smn-1]* animals. Graphs show the median, $n = 100$ for each group. Asterisks: $P < 0.001$. (**C**) Survival of *smn-1(ok355); Ex[Pmyo-3::smn-1]* ($n = 26$) and *smn-1(ok355); Ex[Punc-119::smn-1]* ($n = 24$) animals in comparison with *smn-1(ok355)/hT2[qIs48]* heterozygotes ($n = 30$) and *smn-1(ok355)* homozygotes ($n = 15$, data taken from an independent experiment). Scale bar in (A): 200 μm.

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[bli-4(e937) let-?(q782) qIs48] (I;III)]*, LM111 *[smn-1(ok355) I/hT2[bli-4(e937) let-?(q782) qIs48] (I;III); evIs111]*, LM112 *[smn-1(ok355) I/hT2 [bli-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)/+ I* strain (LM97) was maintained by picking heterozygous animals and confirming the presence of the deletion by PCR using primers 5′-GACTTCAGATTTGATCAACGGTC-3′ and 5′-ACACCA ATTACCAAACGATCAAC-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

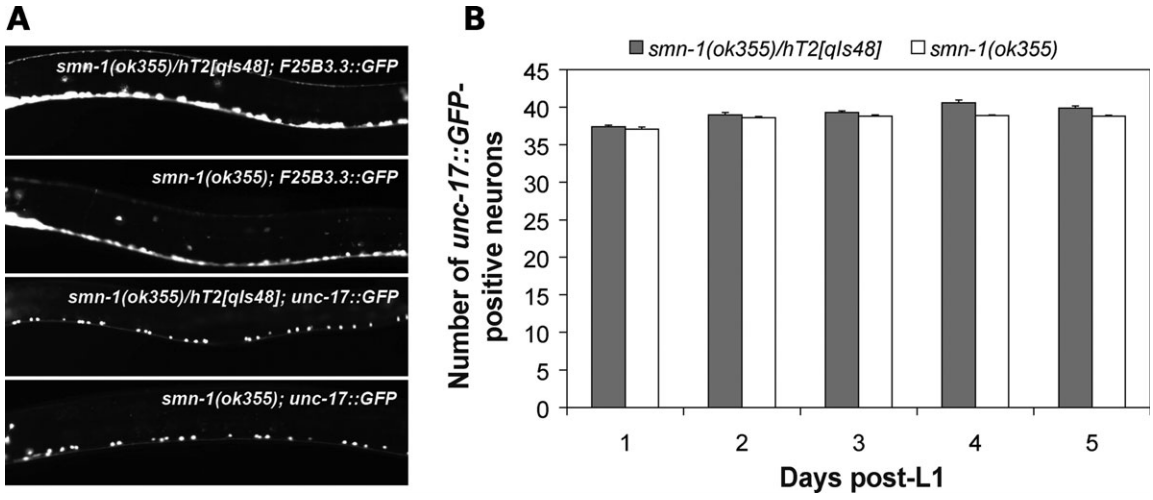

Figure 8. The nervous system of *C. elegans smn-1(ok355)* animals. (**A**) Arrangement of nerve cell bodies in the ventral nerve cord of L3 *smn-1(ok355)/hT2[qIs48]* heterozygotes and *smn-1(ok355)* homozygotes. *F25B3.3::GFP* labels all neuron types whereas *unc-17::GFP* is restricted to cholinergic neurons. (**B**) Cholinergic motor neuron number in the ventral nerve cord of *smn-1(ok355)* and *smn-1(ok355)/hT2[qIs48]* animals. GFP-positive neurons expressing the cholinergic marker *unc-17::GFP* were counted from and including the VA2 (anterior) to the VA11 neuron (posterior). For each group, $n = 10$. The graphs depict mean ± SEM.

animals visually, we used the *hT2[bli-4(e937) let-?(q782) 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), *pes-10::GFP* (expressed in 4–60 cell embryos) and a gut-specific enhancer fused to GFP (26,27). Heterozygous *smn-1(ok355) I/hT2[bli-4(e937) let-?(q782) qIs48] (I;III)* (referred to as '*smn-1(ok355)/hT2[qIs48]*') segregate *smn-1(ok355)* homozygotes, *hT2[qIs48]* homozygotes, *smn-1(ok355)/hT2[qIs48]* heterozygotes and arrested aneuploid progeny. Since *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* for *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 pRF4.

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[qIs48]* 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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REFERENCES

1. Roberts, D.F., Chavez, J. and Court, S.D. (1970) The genetic component in child mortality. *Arch. Dis. Child.*, **45**, 33–38.
2. Ogino, S. and Wilson, R.B. (2002) Genetic testing and risk assessment for spinal muscular atrophy (SMA). *Hum. Genet.*, **111**, 477–500.
3. Lefebvre, S., Burglen, L., Reboullet, S., Clermont, O., Burlet, P., Viollet, L., Benichou, B., Cruaud, C., Millasseau, P., Zeviani, M. *et al.* (1995) Identification and characterization of a spinal muscular atrophy-determining gene. *Cell*, **80**, 155–165.
4. Lorson, C.L. and Androphy, E.J. (2000) An exonic enhancer is required for inclusion of an essential exon in the SMA-determining gene, *SMN*. *Hum. Mol. Genet.*, **9**, 259–265.
5. Vitte, J., Fassier, C., Tiziano, F.D., Dalard, C., Soave, S., Roblot, N., Brahe, C., Saugier-Veber, P., Bonnefont, J.P. and Melki, J. (2007) Refined characterization of the expression and stability of the *SMN* gene products. *Am. J. Pathol.*, **171**, 1269–1280.
6. Lorson, C.L., Hahnen, E., Androphy, E.J. and Wirth, B. (1999) A single nucleotide in the SMN gene regulates splicing and is responsible for spinal muscular atrophy. *Proc. Natl Acad. Sci. USA*, **96**, 6307–6311.
7. Monani, U.R., Lorson, C.L., Parsons, D.W., Prior, T.W., Androphy, E.J., Burghes, A.H. and McPherson, J.D. (1999) A single nucleotide difference that alters splicing patterns distinguishes the SMA gene *SMN1* from the copy gene *SMN2*. *Hum. Mol. Genet.*, **8**, 1177–1183.
8. Briese, M., Esmaeili, B. and Sattelle, D.B. (2005) Is spinal muscular atrophy the result of defects in motor neuron processes? *Bioessays*, **27**, 946–957.
9. Monani, U.R., Sendtner, M., Coovert, D.D., Parsons, D.W., Andreassi, C., Le, T.T., Jablonka, S., Schrank, B., Rossol, W., Prior, T.W. *et al.* (2000) The human centromeric *survival motor neuron* gene (*SMN2*) rescues embryonic lethality in *Smn(−/−)* mice and results in a mouse with spinal muscular atrophy. *Hum. Mol. Genet.*, **9**, 333–339.
10. McWhorter, M.L., Monani, U.R., Burghes, A.H. and Beattie, C.E. (2003) Knockdown of the survival motor neuron (Smn) protein in zebrafish causes defects in motor axon outgrowth and pathfinding. *J. Cell Biol.*, **162**, 919–931.
11. Chan, Y.B., Miguel-Aliaga, I., Franks, C., Thomas, N., Trulzsch, B., Sattelle, D.B., Davies, K.E. and van den Heuvel, M. (2003) Neuromuscular defects in a *Drosophila* survival motor neuron gene mutant. *Hum. Mol. Genet.*, **12**, 1367–1376.
12. Miguel-Aliaga, I., Culetto, E., Walker, D.S., Baylis, H.A., Sattelle, D.B. and Davies, K.E. (1999) The *Caenorhabditis elegans* orthologue of the human gene responsible for spinal muscular atrophy is a maternal product critical for germline maturation and embryonic viability. *Hum. Mol. Genet.*, **8**, 2133–2143.
13. Timmons, L., Court, D.L. and Fire, A. (2001) Ingestion of bacterially expressed dsRNAs can produce specific and potent genetic interference in *Caenorhabditis elegans*. *Gene*, **263**, 103–112.
14. Gavrilina, T.O., McGovern, V.L., Workman, E., Crawford, T.O., Gogliotti, R.G., DiDonato, C.J., Monani, U.R., Morris, G.E. and Burghes, A.H. (2008) Neuronal SMN expression corrects spinal muscular atrophy in severe SMA mice while muscle-specific SMN expression has no phenotypic effect. *Hum. Mol. Genet.*, **17**, 1063–1075.
15. Feng, Z., Li, W., Ward, A., Piggott, B.J., Larkspur, E.R., Sternberg, P.W. and Xu, X.Z. (2006) A *C. elegans* model of nicotine-dependent behavior: regulation by TRP-family channels. *Cell*, **127**, 621–633.
16. Wolkow, C.A., Kimura, K.D., Lee, M.S. and Ruvkun, G. (2000) Regulation of *C. elegans* life-span by insulinlike signaling in the nervous system. *Science*, **290**, 147–150.
17. Timmons, L., Tabara, H., Mello, C.C. and Fire, A.Z. (2003) Inducible systemic RNA silencing in *Caenorhabditis elegans*. *Mol. Biol. Cell*, **14**, 2972–2983.
18. Stinchcomb, D.T., Shaw, J.E., Carr, S.H. and Hirsh, D. (1985) Extrachromosomal DNA transformation of *Caenorhabditis elegans*. *Mol. Cell Biol.*, **5**, 3484–3496.
19. Altun-Gultekin, Z., Andachi, Y., Tsalik, E.L., Pilgrim, D., Kohara, Y. and Hobert, O. (2001) A regulatory cascade of three homeobox genes, *ceh-10*, *ttx-3* and *ceh-23*, controls cell fate specification of a defined interneuron class in *C. elegans*. *Development*, **128**, 1951–1969.
20. White, J.G., Southgate, E., Thomson, J.N. and Brenner, S. (1986) The structure of the nervous system of the nematode *Caenorhabditis elegans*. *Philos. Trans. R. Soc. Lond. B Biol. Sci.*, **314**, 1–340.
21. Lickteig, K.M., Duerr, J.S., Frisby, D.L., Hall, D.H., Rand, J.B. and Miller, D.M. III (2001) Regulation of neurotransmitter vesicles by the homeodomain protein UNC-4 and its transcriptional corepressor UNC-37/groucho in Caenorhabditis elegans cholinergic motor neurons. *J. Neurosci*, **21**, 2001–2014.
22. Rand, J.B., Duerr, J.S. and Frisby, D.L. (2000) Neurogenetics of vesicular transporters in *C. elegans*. *FASEB J*, **14**, 2414–2422.
23. Schrank, B., Gotz, R., Gunnersen, J.M., Ure, J.M., Toyka, K.V., Smith, A.G. and Sendtner, M. (1997) Inactivation of the *survival motor neuron* gene, a candidate gene for human spinal muscular atrophy, leads to massive cell death in early mouse embryos. *Proc. Natl Acad. Sci. USA*, **94**, 9920–9925.
24. Hsieh-Li, H.M., Chang, J.G., Jong, Y.J., Wu, M.H., Wang, N.M., Tsai, C.H. and Li, H. (2000) A mouse model for spinal muscular atrophy. *Nat. Genet.*, **24**, 66–70.
25. Brenner, S. (1974) The genetics of *Caenorhabditis elegans*. *Genetics*, **77**, 71–94.
26. Mathies, L.D., Henderson, S.T. and Kimble, J. (2003) The *C. elegans Hand* gene controls embryogenesis and early gonadogenesis. *Development*, **130**, 2881–2892.
27. Edgley, M.L. and Riddle, D.L. (2001) LG II balancer chromosomes in *Caenorhabditis elegans*: *mT1(II;III)* and the *mIn1* set of dominantly and recessively marked inversions. *Mol. Genet. Genomics*, **266**, 385–395.
28. Mello, C.C., Kramer, J.M., Stinchcomb, D. and Ambros, V. (1991) Efficient gene transfer in *C.elegans*: extrachromosomal maintenance and integration of transforming sequences. *EMBO J*, **10**, 3959–3970.