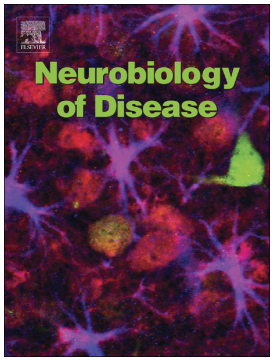

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WDR79/TCAB1 plays a conserved role in the control of locomotion and ameliorates phenotypic defects in SMA models

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HIGHLIGHTS

- WDR79/TCAB1 is required for locomotion in both *D. melanogaster* and *C. elegans*.
- The *Smn* transcript and protein product are downregulated in *WDR79* mutant flies.
- Smn overexpression rescues the *WDR79* loss-of-function phenotype in flies.
- WDR79 overexpression rescues the fly and worm phenotypes induced by Smn depletion.

ABSTRACT

SMN (Survival Motor Neuron) deficiency is the predominant cause of spinal muscular atrophy (SMA), a severe neurodegenerative disorder that can lead to progressive paralysis and death. Although SMN is required in every cell for proper RNA metabolism, the reason why its loss is especially critical in the motor system is still unclear. SMA genetic models have been employed to identify several modifiers that can ameliorate the deficits induced by SMN depletion. Here we focus on WDR79/TCAB1, a protein important for the biogenesis of several RNA species that has been shown to physically interact with SMN in human cells. We show that WDR79 depletion results in locomotion defects in both *Drosophila* and *Caenorhabditis elegans* similar to those elicited by SMN depletion. Consistent with this observation, we find that SMN overexpression rescues the *WDR79* loss-of-function phenotype in flies. Most importantly, we also found that WDR79 overexpression ameliorates the locomotion defects induced by SMN depletion in both flies and worms. Our results collectively suggest that WDR79 and SMN play evolutionarily conserved cooperative functions in the nervous system and suggest that WDR79/TCAB1 may have the potential to modify SMA pathogenesis.

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INTRODUCTION

Survival Motor Neuron protein (SMN), is an evolutionarily conserved protein required for proper locomotion behavior. Reduced SMN dosage in humans causes Spinal Muscular Atrophy (SMA), a recessive neurodegenerative disorder characterized by motor neuron loss, muscle atrophy, progressive paralysis and death. The human genome harbors two SMN genes, *SMN1* and *SMN2. SMN2* produces only a limited amount of full length SMN, which is not sufficient to compensate for homozygous *SMN1* mutations found in SMA patients. The SMN dosage in patients accounts for the severity of the neuromuscular defects and the onset age of SMA (BUTCHBACH 2016).

Although the exact mechanisms through which SMN deficiency disrupts motor function have not been fully elucidated, there is abundant evidence for a role of SMN in RNA metabolism (LI *et al.* 2014). SMN is the main component of the SMN complex, which is essential for the biogenesis of small nuclear ribonucleoproteins (snRNP), which are essential for mRNA splicing. Consistent with this notion, snRNP assembly is impaired in SMA patients, who exhibit particularly reduced snRNP levels in motor neurons (RUGGIU *et al.* 2012). In addition, SMN deficiency has been shown to induce defects in splicing in both tissue culture cells and animal models (ZHANG *et al.* 2008; LOTTI *et al.* 2012; GARCIA *et al.* 2016). However, it is currently unclear whether SMA is primarily due to splicing aberrations. It has been suggested that SMN could play splicing-independent neuronal functions (FALLINI *et al.* 2012; PRAVEEN *et al.* 2012; LI *et al.* 2014), such as axonal RNA transport (MCWHORTER *et al.* 2003), organization of the neuromuscular junction (KARIYA *et al.* 2014), and the control of proper muscle architecture (WALKER *et al.* 2008). SMN also prevents accumulation of RNA–DNA hybrids, which is thought to result in DNA damage and apoptosis (ANDERTON *et al.* 2013; ZHAO *et al.* 2016), and is required for maintenance of embryonic stem cells and neuronal differentiation (EBERT *et al.* 2009; CHANG *et al.* 2015). However, direct evidence linking defects in these processes to SMA is currently lacking.

Studies on SMA patients and vertebrate model systems have shown that overexpression of the actin-binding Plastin3 (PLS3) protein can suppress the

axon growth defects induced by *SMN1* mutations and improve the symptoms of the disease (ACKERMANN *et al.* 2013). Another potential SMA modifier is Zinc Finger Protein 1 (ZPR1), which is required for snRNP targeting to the nucleus and for SMN localization to the Cajal bodies (AHMAD *et al.* 2012).

Additional genetic modifiers of the SMN-dependent phenotypes have been identified using *Drosophila* models of SMA (CHANG *et al.* 2008; DIMITRIADI *et al.* 2010; SEN *et al.* 2011; LOTTI *et al.* 2012). Loss-of-function mutations in the *Drosophila Smn* gene result in defects in the sensory-motor neuronal network, reduced muscle growth, defective locomotion and larval lethality (IMLACH *et al.* 2012). *Drosophila Smn* mutants have reduced levels of snRNAs and defects in the splicing of a subset of U12 intron-containing RNAs, perturbing the expression of genes such as *Stasimon*, which is required for motor circuit function in both *Drosophila* and vertebrates (LOTTI *et al.* 2012). In addition, the combined use of *Drosophila* and *C. elegans* identified several genetic modifiers of the SMN-dependent phenotype (DIMITRIADI *et al.* 2010). For example, Plastin3 was shown to act as an *Smn* loss-of-function modifier in both animal models (DIMITRIADI *et al.* 2010; GALLOTTA *et al.* 2016).

Both mammalian and *Drosophila* SMN proteins accumulate in the Cajal bodies (CBs), and cells from SMA patients are defective in CB structure and abundance (COOVERT *et al.* 1997; LEFEBVRE *et al.* 1997; LIU *et al.* 2009). In humans, SMN recruitment to the CB is dependent on its interaction with WRAP53/WDR79/TCAB1 (henceforth WDR79) (MAHMOUDI *et al.* 2010), an evolutionarily conserved protein that contains repeated WD motifs rich in Tryptophan and Aspartate residues. WDR79 binds to several classes of RNAs, including small Cajal body RNAs (scaRNAs), guiding the 2'-O-methylation and pseudouridylation of snRNAs within the CB (TYCOWSKI *et al.* 2009). Here we have investigated the neuronal functions of WDR79 in both *Drosophila* and *C. elegans.* We find that WDR79 is required for normal locomotion in both animals. Furthermore, we demonstrate that increased *WDR79* expression ameliorates the *Smn* loss-of-function phenotype, suggesting a possible role of WDR79 as a SMA modifier.

MATERIALS AND METHODS

Drosophila strains

The *WDR79MB*^{19832} mutant allele (w^{1118}; Mi{ET1}WDR79^{MB10832}) and the SmnRNAi stock [P{TRiP.HMC03832}attP40 (UAS-SmnRNAi)] were obtained from the Bloomington Stock Center; The WDR79 RNAi stock [P{KK108453}(UAS-WDR79RNAi] was obtained from the Vienna Drosophila RNAi center. The construct expressing an unrelated protein used as a control (UAS CTRL) is ppGW-GFP-Mst, (PALUMBO *et al.* 2015). Silencing was achieved by combining a single copy of UAS-RNAi transgene with a single copy of the appropriate driver. A complete list of the genotypes of the strains used is reported in table S1. Either the Oregon-R or the *ywf* strain was used as a wild type control. All flies were reared according to standard procedures at 25°C. The genetic markers and special chromosomes are described in detail in FlyBase (http://www.flybase.org).

Drosophila transgenic strains

The inducible *WDR79* strain (carrying a *pUAST-ATTB-WDR79* element, here abbreviated as *UAS-WDR79*) was generated by cloning the full-length *WDR79* gene (PCR-amplified from genomic DNA and flanked by XbaI sites) into the pUAST-ATTB vector (BISCHOF *et al.* 2007). The plasmid was injected into y1 M{vas-int.Dm}ZH-2A w*; M{3xP3-RFP.attP'}ZH-86Fb embryos (Bloomington #24789). To generate the plasmids for constitutive expression of WDR79-GFP or Smn-GFP, the *EGFP* CDS fused in-frame with the 3′ end of the *WDR79* or the *Smn* CDS were cloned into the pJZ4 vector (a derivative of pCASPER4) under the control of a tubulin promoter, as described previously in (RAFFA *et al.* 2009). The RNAi resistant *Smn* gene carries synonymous substitutions in each residue of the region recognized by UAS-SmnRNAi and was synthesized by Genewiz (SIGMA-ALDRICH). The plasmid for constitutive expression of RNAi resistant Smn-FLAG (abbreviated with Smn-FLAG-res) was generated by cloning the 3XFLAG epitope CDS fused in-frame with the 3′ end of the *RNAi* resistant *Smn* CDS, into the pJZ4 vector. The *WDR79-GFP*, *Smn-GFP* or Smn-FLAG-res plasmids were injected in w^{1118} embryos; germline transformation was performed by Bestgene Inc (Chino Hills, California) using standard procedures.

Drosophila Locomotion Analyses and statistical analyses

Larval locomotor activity was measured by counting the number of peristaltic contractions of third instar larvae performed within one minute on the surface of a 1% agarose gel in a Petri dish; measurements were repeated ten times. To obtain unbiased measurement of locomotion parameters, larvae were blind-tested by three experimenters (alternatively, two of them scoring phenotypes and the third collecting and analyzing the data). At least 15 third instar larvae (with retracted anterior spiracles) per genotype were assayed. The Shapiro-Wilk Test was used to assess the Normal distribution of every group of different genotype ($P<0.001$), and the KS test was used to assess whether the H0 hypothesis could be accepted. The significance of multiple comparisons was evaluated with One Way Analysis of Variance. The Tukey's test was performed as Post-Hoc Test to determine the significance between every single group ($P<0.01$ was considered significant).

RNA extraction, reverse transcription and qRT PCR

Total RNA was extracted from *Drosophila* larvae, with the Qiagen RNeasy Plus Mini including an on column treatment with DNAse as recommended by manufacturer. The integrity of RNA samples was evaluated by gel electrophoresis. 1 µg of intact RNA (with a 28S:18S rRNA ratio = 2:1) was subjected to a second treatment with DNase (Invitrogen) as suggested by manufacturer, and reverse transcribed with the Invitrogen kit SuperScript III First-Strand Synthesis System for RT-PCR, following manufacturer's instructions. Real-time PCR reactions were performed with the QuantiTect SYBR® Green PCR Kit, with 7300 Real Time PCR System (Applied Biosystems) under the following thermal cycling conditions: an initial step of 2 minutes at 50°C, 10 min at 95°C, followed by 40 cycles of 15 s at 95°C, 1 min at 60°C and a final dissociation step. The relative quantification in gene expression was determined using the $2^{-\Delta\Delta Ct}$ method (LIVAK AND SCHMITTGEN 2001). The fold changes in gene expression were normalized to the *RP49* gene (the amplification efficiencies were not significantly different for target and reference among all samples). A total of 3 experiments were performed for three biological replicates and significance was assessed by unpaired 2-tailed Student's t tests ($P<0.05$ was considered significant). Primer pairs used in qRTPCR analyses**:** smnqF: CCAGTATCCTTCAAAGTAGGCG; smnqR: TTCTCATAGCCCAAATAGCGG; WDR79q1F:

ACGTTTTTGCGACGATTACC; WDR79q1R ACACCGCAAGTCAAAGTGC; rp49qF:CCGCTTCAAGGGACAGTATCT; rp49qR: ATCTCGCCGCAGTAAACGC

Western Blotting

Protein extracts were derived from 15 brains from third instar larvae, lysed in sample buffer, fractionated by SDS-PAGE and transferred to nitrocellulose membrane. Primary antibodies were: anti-Smn mouse 1:2000 (Imlach et al., 2012); mouse anti-tubulin (1:20000; Sigma-Aldrich). As secondary antibody we used HRP-conjugated anti-Mouse 1:5000 (GE Health Care). Detection was performed with the SuperSignal™ West Pico Chemiluminescent Substrate (Thermo); images were acquired with Chemidoc (Biorad) and quantified using the QuantityOne image analysis software (Biorad).

***C. elegans* strains**

Nematodes were grown and handled following standard procedures, under uncrowded conditions, at 20°C, on NGM (Nematode Growth Medium) agar plates seeded with *Escherichia coli* strain OP50 (BRENNER 1974). Wild-type animals used in this work were *C. elegans* variety Bristol, strain N2; mutant alleles and transgenic strains used were: *rrf-3(pk1426) II* and *vsIs48[punc-17::GFP]* provided by the *Caenorhabditis* Genetics Center (CGC), funded by NIH Office of Research Infrastructure Programs (P40 OD010440); *Is[punc-47::RFP]* kindly provided by K. Shen (Stanford University, USA). A complete list of the genotypes of the strains used is reported in table S1. The transgenes generated for this work are: *gbEx575* [GBF328 *pceWDR79::gfp*] and *gbEx587* [GBF336 *punc-119*::*ceWDR79*; *pelt-2::rfp*].

The construct GBF328 [*pceWDR79::gfp*] for analyzing the expression of *ceWDR79* was created by PCR-fusion (HOBERT 2002) of two fragments: the promoter of *ceWDR79* gene and the GFP sequence. The *ceWDR79* putative regulatory region corresponding to a fragment of 400 bp was generated by PCR using as template wild type genomic DNA. The GFP, followed by the 3'UTR of the *unc-54* gene to increase the stability of the construct, was amplified from plasmid pPD95.75, kindly provided by A. Fire (Stanford University, USA). The rescue construct GBF336 [*punc-119*:: *ceWDR79]* for pan-neuronal expression of *ceWDR79* was created by PCR fusion of two fragments: the promoter of the *unc-119* gene and the *ceWDR79* gene. The *unc-*

119 promoter has been previously used for neuronal-directed rescue of *smn-1* (BRIESE *et al.* 2009) and is considered a strong transcriptional inducer (MADURO AND PILGRIM 1995). The *ceWDR79* gene was amplified from *C. elegans* genomic DNA. All primers sequences are available on request. Germ line transformation was accomplished as described (MELLO AND FIRE 1995) by injecting, into the gonad of adult animals a DNA mixture containing a transgenic construct, obtained by PCR fusion, together with a phenotypic marker for selection of transgenic progeny. The GBF328 construct was microinjected alone at the concentration of 50 ng/µL in *vsIs48[punc-17::GFP]* animals. The GBF336 construct was microinjected at the concentration of 20 ng/µL in *wildtype* animals with the co-injection marker pJM371 [p*elt-2::NLS::RFP*] at 30 ng/µL, which drives RFP expression in the intestinal nuclei (kindly provided by J. McGhee, University of Calgary, Canada). At least three transgenic lines were examined in each experiment and the mean of the lines has been reported in the figures.

C. elegans RNA-interference

C. elegans animals were RNA-interfered by feeding (TIMMONS AND FIRE 1998) using HT115(DE3) bacterial strains harboring the *ceSmn1* and *ceWDR79* constructs from J. Ahringer Library (HGMP, Cambridge) (KAMATH *et al.* 2003). Larvae were deposited onto NGM plates containing 100 mg/ml ampicillin, 1 mM IPTG, and IPTG-induced bacteria and allowed to lay eggs. F1 individuals were assayed for phenotype. Control worms were grown on bacteria transformed with the plasmid pPD129.36(L4440) without insert. *rrf-3(pk1426)* mutants, which are more sensitive to neuronal RNAi, were used in some of the experiments to enhance the knock-down effects.

C. elegans behavioral assays

Well-fed, young adult animals were used for thrashing assay to blindly test motor neuron functionality. For thrashing assays, animals were age-synchronized using sodium hypochlorite and when adult they were picked to individual wells containing 100 µl M9 buffer and left for 10 min. Every other thrash was counted for 20 s and then multiplied by six to obtain an estimate of total thrashes per minute. A single thrash was defined as a complete change in the direction of the body down the midline. Animals that lingered on well side or were motionless for ≥10 s were discarded from the analysis. The Mann-Whitney-Wilcoxon test was used for

statistical analysis.

***C. elegans* microscopy analysis and analysis of cholinergic motor neurons**

Animals were immobilized in 0.01% tetramisole hydrochloride (Sigma-Aldrich) on 4% agar pads and visualized using Zeiss Axioskop microscope and Leica TCS SP2 AOBS laser scanning confocal microscope for colocalization experiments. All microscopes were equipped with epifluorescence and DIC Nomarski optics and images were collected with an Axiocam digital camera and with Leica digital cameras DFC 480 and 420 RGB. To assess the number of visible motor neurons, a subset of Acetylcholine releasing neurons was scored in *vsIs48 [punc-17::GFP]* transgenic strain, treated and not treated with RNA-interference. In particular 41 ACh motor neurons in the ventral cord, which span from VA2 to AS11 (WHITE *et al.* 1986) were clearly distinguishable and easily recognizable in all conditions; neurons of the DB class were not scored.

RESULTS

WDR79 is required for proper locomotion behavior in both *Drosophila* and *C. elegans.*

To explore the role of *WDR79* in *Drosophila,* we exploited *Mi{ET1}WDR79^{MB10832}*, a transposon insertion downstream of the *WDR79* transcription start site (henceforth designated as *WDR79^{MB}*). *WDR79^{MB}/WDR79^{MB}* homozygotes are viable and fertile (DERYUSHEVA AND GALL 2013). RT-PCR of RNA extracted from *WDR79^{MB}/WDR79^{MB}* larvae revealed that they produce an aberrant transcript in which the *WDR79* coding region is fused with sequences of the Minos transposon (Figure S1); this transcript is predicted to produce a truncated version of WDR79.

We first tested the locomotion ability of both *WDR79* mutant larvae and adults. A measure of larval muscle wall peristalses revealed that homozygous (*WDR79^{MB}/WDR79^{MB}*) and hemizygous (*WDR79^{MB}/Df(2L)ED385;* henceforth designated as *WDR79^{MB}/Df*) larvae exhibit a 34% (*P = 1E-09*) and 36% (*P = 7E-11*) reduction in peristalses compared to heterozygous controls, respectively (Figure 1A). Ubiquitous tubulin-GAL4- driven (abbreviated as Ub>WDR79) or nsyb-GAL4- driven pan-neuronal expression (abbreviated with neur>WDR79) of

a transgenic *UAS-WDR79* construct fully rescued locomotion defects of *WDR79* mutants (Figure 1A), confirming that these defects are due to WDR79 depletion. We also examined adult homozygous *WDR79*^{MB} mutants for climbing ability, which was reduced by 75% compared to heterozygous controls (Figure S2A). Finally, consistent with previous work (SINGER AND GALL 2011), we found that larval brain cells of *WDR79* mutants are devoid of coilin-enriched Cajal bodies. This defect was also rescued by transgenic expression of *WDR79-GFP* (Figure S2B).

To determine whether WDR79 plays an evolutionarily conserved role in locomotion in an additional model organism, we used RNA-interference to ubiquitously silence *tcab-1(Y105E8A.8)*, the *C. elegans WDR79* homologue (henceforth designed as *ceWDR79*). To assay *C. elegans* locomotion we scored the thrashing behavior, which measures the lateral swimming movements. *ceWDR79(RNAi)* animals showed 20% reduction (*P = 7,5E-06*) in locomotion compared to controls (Figure 1B). RNAi against the *C. elegans* homolog of *SMN*, *smn-1* (henceforth designed as ce*Smn1*), also led to locomotion defects (Figure 1B), as previously reported (BURT *et al.* 2006; GALLOTTA *et al.* 2016).

Thrashing behavior is mainly controlled by cholinergic motor neurons (PIERCE-SHIMOMURA *et al.* 2008). Using a genetic reporter approach, we found that ce*WDR79* is expressed in both cholinergic and GABAergic motor neurons (Figure 2A), confirming previous genome-wide microarray results (FOX *et al.* 2005). We therefore examined the morphology and viability of these neurons in *ceWDR79* and *ceSmn1* RNAi animals, using a transgenic strain that specifically expresses GFP in cholinergic motor neurons (CHASE *et al.* 2004) (Figure 2B). By counting the number of a subgroup of cholinergic motor neurons in the ventral cord, we identified on average 41 cholinergic neurons in control animals (see methods). In *ceWDR79*(*RNAi*) expressing animals there was a significant reduction of these neurons, which were on average 36 (*P< 0.0001*) (Figure 2B). Similarly, *ceSmn1*(*RNAi*) expressing animals also showed reduced numbers of cholinergic neurons that were on average 37 (*P< 0.0001*) (Figure 2B). These results reveal that in addition to the locomotion defects, *ceWDR79*(*RNAi*) and

ceSmn1(RNAi) animals, exhibit motor neuron loss. Thus, loss of WDR79 results in defective locomotion in both *C. elegans* and *Drosophila*.

Smn overexpression rescues the *WDR79* loss-of-function phenotype in flies.

The finding that *WDR79* and *Smn* mutants have similar locomotion defects in both worms and flies prompted us to investigate the levels of Smn in *Drosophila WDR79* mutants. We found that both the *Smn* transcript and protein product are downregulated in *WDR79* mutant flies (Figure 3A-B). In $WDR79^{MB}/Df$ larvae, the *Smn* transcript levels were reduced by 50% (*P = 0.004*) compared to heterozygous siblings (Figure 3A), and this reduction was fully rescued by a WDR79-GFP transgene (*P = 0.003*) (Figure 3A). Consistent with the reduction of the *Smn* transcript, the Smn protein was also reduced in $WDR79^{MB}/WDR79^{MB}$ larval brains (Figure 3B lane 2). We also observed reduced Smn levels in flies expressing an inducible *UAS-WDR79* RNAi construct (P{KK108453}VIE-260B under control of an ubiquitous actin-Gal4 driver (Figures 3B and S3A-C). However, larvae carrying two copies of the *WRD79* gene and overexpressing WDR79 did not exhibit an increase of the *Smn* transcript or protein product (Figures S4A-B).

Building on these results, we next asked if Smn overexpression could rescue the *WDR79*-dependent phenotype. We thus constructed flies carrying a *Smn-GFP* transgene under the control of the tubulin promoter (henceforth designated as *Smn-GFP)*. This transgene was capable of rescuing *Smn* mutant phenotype (data not shown). We found that expression of *Smn-GFP* in $WDR79^{MB}/WDR79^{MB}$ animals substantially improves locomotion behavior. $WDR79^{MB}/WDR79^{MB}$*; Smn-GFP/+* larvae showed 36% (*P = 7,4E-06*) more contractions per minute than $WDR79^{MB}/WDR79^{MB}$ larvae (Figure 3C). Similarly in $WDR79^{MB}/WDR79^{MB}$ adult flies the climbing rate was improved by 73% in the presence of Smn-GFP transgene (P = 0.034) (Figure S2A). Collectively these results suggest that the motility defects observed in WDR79-deficient flies are at least in part a consequence of Smn depletion.

WDR79 overexpression ameliorates the larval locomotion defects induced by Smn depletion

Previous studies have shown that RNAi-mediated depletion of Smn in flies provides a convenient hypomorphic background for functional analyses, and allows development until late larval stages (CHANG *et al.* 2008; DIMITRIADI *et al.* 2010; SEN *et al.* 2011). We thus decided to use an RNAi-based approach to investigate possible *WDR79-Smn* genetic interactions in fly locomotor behavior. To inhibit *Smn* we used an Sh RNAi strain from the TRiP collection (PERKINS *et al.* 2015), (p{TRiP.HMC03832}attp40; abbreviated with *UAS-Smn^{RNAi}*). Ubiquitous expression of this *UAS-Smn^{RNAi}* construct using an actin-GAL4 transgene resulted in a 60% reduction ($P < 0.001$) of the *Smn* transcript in third instar larvae compared to controls (Figure 4A); RNAi larvae also showed a substantial reduction of the Smn protein in brains (Figure 4A). Ubiquitous actin-GAL4-induced expression of the same *UAS-Smn^{RNAi}* construct in flies heterozygous for the *Smn^{x7}* null allele resulted in severe locomotion defects; we observed 33% decrease (*P=1,2 E-16*) in the rate of peristalses in third instar larvae compared to controls (Figure 4B). We next examined the effects of *WDR79* overexpression in Smn-depleted flies. We generated *Smn^{X7}* heterozygous larvae carrying a constitutively expressed *WRD79-GFP* construct (under control of the tubulin promoter), the *UAS-Smn^{RNAi}* construct, and the ubiquitous *actin-GAL4* driver. These larvae showed 12% increase (P=0.002) in locomotion activity compared to *UAS-Smn^{RNAi}/actin-GAL4* larvae devoid of the *WDR79-GFP* transgene (Figure 4B, box 4).

WDR79 overexpression ameliorates the post-eclosion defects induced by Smn depletion

To analyze the interaction between WDR79 and Smn in adult flies we expressed the *UAS-Smn^{RNAi}* construct only in neurons using the pan-neuronal *nsyb-GAL4* driver. Consistent with previous work, flies with a pan neuronal downregulation of *Smn* were viable (CHANG *et al.* 2008). However, 15% of these flies (n = 5540) displayed a phenotype that was not described in previous studies; they showed unexpanded wings and unretracted ptilinum, a sac-like structure in the fly head associated with temporary muscles (Figures 5A and 5B). The ptilinum extrudes from the fly head to break the anterior end of the puparium during eclosion. In wild type flies, after

eclosion is complete, the ptilinum is permanently retracted. The unretracted ptilinum phenotype was observed in nearly all *Smn* RNAi flies with unexpanded wings and was absent in their sibs with normal wings. The unexpanded wing/unretracted ptilinum phenotypes were never observed in controls bearing either the *nsyb-GAL4* driver (n = 5000) or the *UAS-Smn^{RNAi}* construct (n = 5000). The penetrance of the wing expansion/ptilinum phenotype correlates to the Smn dosage, as the percentage of flies with unexpanded wings increased to 52% (P = 4.9 *E-09*) when the *Smn^{RNAi}* transgene was expressed in neurons of *Smn^{X7}*/+ flies (Figure 5B). The coordination of the post-eclosion events is finely tuned by secretion of the bursicon neuropeptide in a well-defined set of neurons (LUAN *et al.* 2006a; LOVEALL AND DEITCHER 2010), suggesting that success in post-eclosion performance could be the readout for proper functioning of the underlying neural circuit.

To confirm that the wing expansion/ptilinum defects are a specific consequence of Smn depletion, we generated flies bearing an *Smn FLAG*-res transgene resistant to the *Smn^{RNAi}* construct (expressed under control of a tubulin promoter). This transgene contains appropriate synonymous substitutions in the *Smn* coding sequence that render it resistant to the dsRNA generated by the *Smn^{RNAi}* construct (see Materials and Methods for details). Similar to the *Smn-GFP* transgene described above, the *Smn FLAG-res* rescues the lethality of *Smn^{X7}* homozygous flies; *Smn^{X7}* homozygotes without the rescue construct die at the larval stage. We then constructed flies bearing two normal copies of the *Smn* gene, the *Smn FLAG-res gene*, the *nsyb-GAL4* driver and the *Smn^{RNAi}* construct, as well as flies carrying the same transgenes but heterozygous for the *Smn^{X7}* mutant allele. Flies of both genotypes (more than 1,000 flies counted per each genotype) showed less than 1% individuals with unexpanded wings and unretracted ptilinum (Figure 5B), indicating that this phenotype is indeed due to Smn depletion.

We next asked whether the pan-neuronal expression of a *UAS-WDR79* transgene rescues the wing/ptilinum phenotype of flies with reduced Smn expression in neuronal cells. We thus examined *Smn^{+}/Smn^{+}* and *Smn^{X7}/Smn^{+}* flies carrying the nsyb-GAL4 driver and the *Smn^{RNAi}* construct and either a *UAS-WDR79* or a UAS-control transgene (UAS-CTRL, expressing the unrelated Mst protein). In *Smn^{+}/Smn^{+}* and *Smn^{X7}/Smn^{+}* flies, the presence of the *UAS-WDR79*

transgene reduced by 52% (*P = 0,0006)* and 32% (*P = 0,0001*) the frequency of individuals with the wing/ptilinum phenotype compared to controls, respectively (Figure 5B). Thus, our analyses of both *Drosophila* larvae and adults indicate that WDR79 overexpression ameliorates the neurological phenotypes generated by pan-neuronal *Smn* silencing.

The genetic interaction between *Smn* and WDR79 genes prompted us to ask whether their protein products physically interact as occurs for their human homologues (MAHMOUDI *et al.* 2010). We therefore generated larvae expressing WDR79-GFP and Smn-FLAG and used an anti GFP antibody for co-immunoprecipitation. We performed three independent experiments and in none of them an anti FLAG antibody revealed a detectable band in precipitates (data not shown). Thus, at least under the experimental conditions used here, the *Drosophila* Smn and WDR79 proteins do not appear to interact.

ceWDR79 overexpression restores the locomotor defects induced by *ceSmn1* depletion

To test whether *ceWDR79* genetically interacts with *ceSmn1* in *C. elegans*, we ubiquitously silenced *ceSmn1* in animals overexpressing *ceWDR79* in all neurons. Compared to *ceSmn1(RNAi)* alone, locomotor thrashing was completely restored to control levels when *ceWDR79* was overexpressed (Figure 6). In addition, *ceWDR79* overexpression increased larval survival of *ceSmn1(RNAi)* animals from 84% to 100% (data not shown). These results collectively suggest that WDR79 plays a conserved role in the SMN pathway, and that WDR79 can modify the *Smn* loss-of-function phenotypes in two invertebrate models for SMA.

Discussion

Previous work has shown that *Drosophila* WDR79 localizes to the Cajal body (CB) and associates with several small CB-specific (sca) RNAs, which are known to modify snRNAs (Deryusheva and Gall, 2013). Work in human cells has shown the human WDR79 physically interacts with SMN and favors its localization in the CB (MAHMOUDI *et al.* 2010). In addition, it has been shown that human WDR79/TCAB1 has a RNA-binding activity (TYCOWSKI *et al.* 2009) (VENTEICHER AND ARTANDI 2009)

(JADY *et al.* 2012) and controls biogenesis of the telomerase RNA component (TERC) within the CB (VENTEICHER *et al.* 2009; STERN *et al.* 2012). Human SMN co-precipitates with telomerase but it is currently unknown whether SMN is required for telomerase assembly in the CB (BACHAND *et al.* 2002) and whether telomerase activity is relevant for SMA pathogenesis. Finally, several studies implicated both SMN and WDR79/TCAB1 in the maintenance of genome stability. It has been suggested that SMN prevents accumulation of DNA/RNA hybrids (Zhao et al, 2016), which have been shown to be important intermediates in DNA repair (OHLE *et al.* 2016); WDR79/TCAB1 was shown to be involved in repair of DNA double strand breaks (COUCORAVAS *et al.* 2016) (HENRIKSSON *et al.* 2014). Thus, many data suggest a strong functional link between SMN and WDR79/TCAB1 (see (HENRIKSSON *et al.* 2014) for review.

In this study, we have provided additional support to the functional connection between WDR79 and SMN. Although we were not able to show that the two proteins physically interact in *Drosophila,* we found that WDR79 deficiency lowers the SMN expression at both the RNA and protein level. We also showed that loss of WDR79 in flies results in severe locomotion defects comparable to those caused by SMN depletion, and that these defects are rescued by Smn overexpression. Importantly, this effect was reciprocal as WDR79 overexpression rescued the phenotypic defects caused by Smn depletion. We also showed WDR79 depletion in worms causes locomotion defects and loss of motor neurons similar to those elicited by Smn knockdown, and that these defects are rescued by WDR79 overexpression. Thus, our results suggest that WDR79 and Smn cooperate in subsets of neuron types to ensure correct locomotion behavior in both flies and worms, and that WDR79 plays a conserved function within the neuronal SMN pathway. Furthermore, since WDR79 binds scaRNAs within the CB, our data additionally suggest that WDR79/TCAB1 and Smn might share common RNA targets within the CB.

These results raise the question of the molecular basis of the genetic interaction between *WDR79* and *Smn*.

The motility defects observed in WDR79-depleted flies are likely to be the consequence of an Smn deficiency, as these flies exhibit a reduction in the *Smn* transcript/protein and their defects are corrected by Smn overexpression.

Hypothesizing how WDR79 overexpression ameliorates the Smn loss-of-function phenotype is more difficult. Because WDR79 is an RNA-binding protein, our current results can only suggest that it might affect the stability, the translation, or the transport/compartmentalization of the *Smn* transcript.

However, the precise functional relationships between WDR79 and Smn are currently unclear. Specifically, we have only a few data on the effects of simultaneous impairment of WDR79 and Smn functions. We found that the *WDR79* loss of function phenotype is not exacerbated by the absence of a copy of the *Smn* gene (our unpublished results). In addition, WDR79 deficiency failed to enhance the wing expansion defects in flies with reduced neuronal expression of *Smn* (our unpublished results). Thus, it would appear that the simultaneous reduction of both WDR79 and Smn does not result in additive or synergistic phenotypic effects. However, this conclusion has a very little experimental support and should be substantiated by extensive additional work.

We have shown that RNAi-mediated ubiquitous or pan-neuronal depletion of Smn provides a hypomorph *Smn* mutant background, which allows locomotion analyses to be easily performed in *Drosophila* third instar larvae. Since *Smn* RNAi larvae are viable and die only after pupariation, phenotypic analyses performed in these larvae are not complicated by pleiotropic effects due to developmental arrest caused by null Smn mutations (GARCIA *et al.* 2013). We have shown that in a wild type background neuron-targeted RNAi against *Smn* results in 15% flies with unexpanded wings and unretracted ptilinum, and that frequency of flies with these phenotypic traits was 23% when RNAi was performed in Smn^{X7}/+ background. This wing/ptilinum phenotype was suppressed by the expression of an RNAi-resistant transgene, confirming that Smn has an important role in the control of the post-eclosion program. Post-eclosion events are regulated by a well-defined set of neurons secreting the neuropeptide bursicon (LUAN *et al.* 2006a). Interestingly, the survival of these neurons requires the wild-type function of TDP-43, one of the factors responsible for Amyotrophic Lateral Sclerosis ALS (VANDEN BROECK *et al.* 2013), another neurodegenerative disease with correspondences to SMA (YAMAZAKI *et al.* 2012). Moreover, a subset of bursicon-secreting neurons lies within the expression domain of the Cha-GAL4 driver, specific for cholinergic neurons (LUAN *et al.* 2006b), in which Smn expression is necessary for the control of the sensory-motor circuit (IMLACH *et al.* 2012).

The finding that Smn depletion in neurons causes an unexpanded wing phenotype raises the possibility of exploiting this easily scorable defect in screens for chemical and genetic modifiers of this Smn-dependent phenotype. Here we have shown that WDR79 overexpression ameliorates the post-eclosion developmental defects caused by Smn deficiency in neurons. It will be of interest to test whether known modifiers of other SMN-related phenotypes will also affect the unexpanded wing/unretracted ptilinum phenotype.

In conclusion, we have clearly shown that WDR79 plays a conserved role in neurons that control locomotion in flies and worms. We have also demonstrated that WDR79 interacts genetically with Smn in both systems. Thus, given that WDR79/TCAB1 interacts with SMN in humans, our data support the hypothesis of possible involvement of WDR79/TCAB1 in SMA pathogenesis.

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FIGURE LEGENDS

Figure 1. *WDR79* deficiency results in locomotion defects in both *Drosophila* and *C. elegans*

(A) *WDR79* mutant flies exhibit locomotion defects, which are ameliorated by ubiquitous or pan-neuronal WDR79 overexpression. Box plot representation of the distribution of peristaltic contraction rates in *Drosophila* larvae of the indicated genotypes; from left to right, 16, 26, 18, 15 and 15 larvae were analyzed. The line inside the box indicates the median for each genotype and box boundaries represent the first and third quartiles; whiskers are 1.5 interquartile range (***, *P<0.001*, 1-way ANOVA with post-hoc Tukey test). Df, is a deficiency that uncovers *WDR79*; $WDR79^{MB}$ denotes $WDR79^{MB}$ homozygotes; *Ub>WDR79*, ubiquitous *UAS-WDR79* expression under control of a tubulin-GAL4 driver; neur>WDR79, neuronal-specific expression of *UAS-WDR79* under control of the nsyb-GAL4 driver.

(B) RNAi mediated depletion of *ceSmn1* and *ceWDR79* causes locomotion defects in *C. elegans*. *ceWDR79(RNAi)* and *ceSmn1(RNAi)* worms exhibit alterations in thrashing behavior; from left to right, 60, 60 and 13 worms were analyzed. ***, *P<0.001*, Mann-Whitney-Wilcoxon test.

Figure 2*. ceWDR79* is expressed in both GABAergic an cholinergic motor neurons and prevents neuron loss.

(A) *ceWDR79* is expressed in ventral cord motor neurons. Analysis of the expression pattern of *ceWDR79* using a GFP-reporter approach, shows colocalization of the GFP expressed under control of the *ceWDR79* promoter (*pceWDR79::GFP*) with RFP-labeled GABA motor neurons (*pGABA::RFP*) (white asterisks). The GFP signals not coincident with RFP signals are likely to label cholinergic neurons, as they are the only other motor neuron population present in the ventral cord (WHITE *et al.* 1986).

(B) *ceWDR79 RNAi* and *ceSmn1 RNAi* worms exhibit significant reductions in the number of cholinergic motor neurons (Ach-Mns) expressing GFP (arrows indicate

missing neurons; see methods); 40 individuals were analyzed per genotype. ***, *P<0.001*, Welch's t-test.

Figure 3. The locomotion defects of *WDR79* mutants are ameliorated by Smn overexpression.

(A) Relative levels of the Smn transcript in *WDR79* depleted larvae of the indicated genotype. The Smn transcript levels have been determined by qRTPCR in 3 independent experiments, and are relative to the Rp49 transcript (**, *P<0.01*, Student's t-test).

(B) Top panel: Representative Western blots showing the Smn protein abundance in larval brains of the indicated genotype (LC: loading control). Bottom panel: quantification of the Smn protein level relative to the loading control. Data are representative of 3 independent experiments, 15 brains per sample (*, *P* <0.05, **, *P* <0.01, Student's t-test).

(C) Distribution of peristaltic contraction rates in larvae of the indicated genotypes. From left to right, 16, 26 and 21 flies were analyzed. The lines inside the boxes indicate the median and box boundaries represent the first and third quartiles; whiskers are 1.5 interquartile range. No significant difference was observed between $WDR79^{MB}$/+ and *WDR79/Df* flies (P = 0.3). ***, *P<0.001* (1-way ANOVA with post-hoc Tukey test). $WDR79^{MB}$,denotes $WDR79^{MB}$ homozygotes; WDR79-GFP ubiquitously expresses the fusion protein under the control of a tubulin-GAL4 driver; CTRL Ub-GAL4, is a strain bearing only the actin-GAL4 driver; Ub>WDR79 RNAi, expresses the $WDR79^{RNAi}$ construct under the control of an actin-GAL4 driver; Smn-GFP, expresses the fusion protein under the control of a tubulin promoter.

Figure 4. WDR79 overexpression ameliorates the larval locomotion defects induced by Smn depletion in *Drosophila*.

(A) Relative levels of the *Smn* transcript in larvae of the indicated genotype. The *Smn* transcript levels have been determined by qRTPCR in 3 independent experiments, and are relative to the Rp49 transcript (***, P<0.001, Student's t-test). Bottom panel:

Western blotting showing the Smn abundance in larval brains of the indicated genotype (tubulin was used as loading control).

(B) Distribution of peristaltic contraction rates in larvae of the indicated genotypes. From left to right, 12, 19, 46, 25 larvae were analyzed (**, *P<0.01;* ***, *P<0.001,* 1-way ANOVA with post-hoc Tukey test). The lines inside the boxes indicate the median. CTRL SmnRNAi, a *UAS-Smn*^{RNAi} bearing strain with no driver; CTRL Ub-GAL4, a strain bearing only the actin-GAL4 driver; Ub>SmnRNAi, carries both the actin-GAL4 driver and the Smn^{RNAi} construct; WDR79-GFP, expresses WDR79-GFP under the control of a tubulin promoter. All strains are heterozygous for the *Smn*^{X7} mutant allele, which lacks most of the *Smn* coding sequence.

Figure 5. WDR79 overexpression ameliorates the post-eclosion defects induced by neuronal Smn depletion in *Drosophila*.

(A) Pan-neuronal expression of *UAS-Smn*^{RNAi} using the *nsyb-GAL4* driver (*neur>SmnRNAi*) induces defects in wing-expansion (black arrow) and abdominal clefts (white arrows), causes failures in ptilinum retraction (black arrow, right panel).

(B) Quantification of the wing expansion defects in flies expressing the *UAS-Smn*^{RNAi} (*Smn*^{RNAi}) and other indicated constructs under the control of the pan-neuronal nsyb-GAL4 driver (neur>). *UAS WDR79* that expresses an untagged WDR79 gene; UAS CTRL is a control construct expressing the Mst protein (see text); Smn FLAG-res is an Smn-FLAG transgene resistant to UAS-Smn-induced RNAi and expressed under control of the ubiquitous tubulin promoter; ΔSmn is an *Smn*^{X7} mutant allele lacking most of the Smn coding sequence (***, *P<0.001,* 1-way ANOVA with post-hoc Tukey test; n.s., non significant, *P>0.05*)

Figure 6. Pan-neuronal overexpression of *ceWDR79* rescues the phenotypes induced by *ceSmn1* depletion in *C. elegans.* *ceWDR79* overexpression in neurons under control of the *unc-119* promoter (abbreviated with neur, grey bars) rescues the thrashing locomotion defects of *ceSmn1(RNAi)*

animals; 60 worms per genotype were analyzed (***, *P<0.001*, Mann-Whitney-Wilcoxon test).

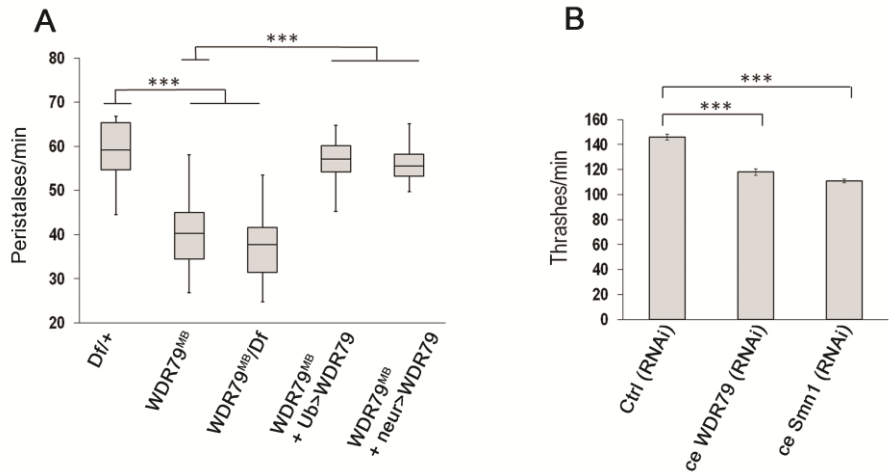

Fig. 1

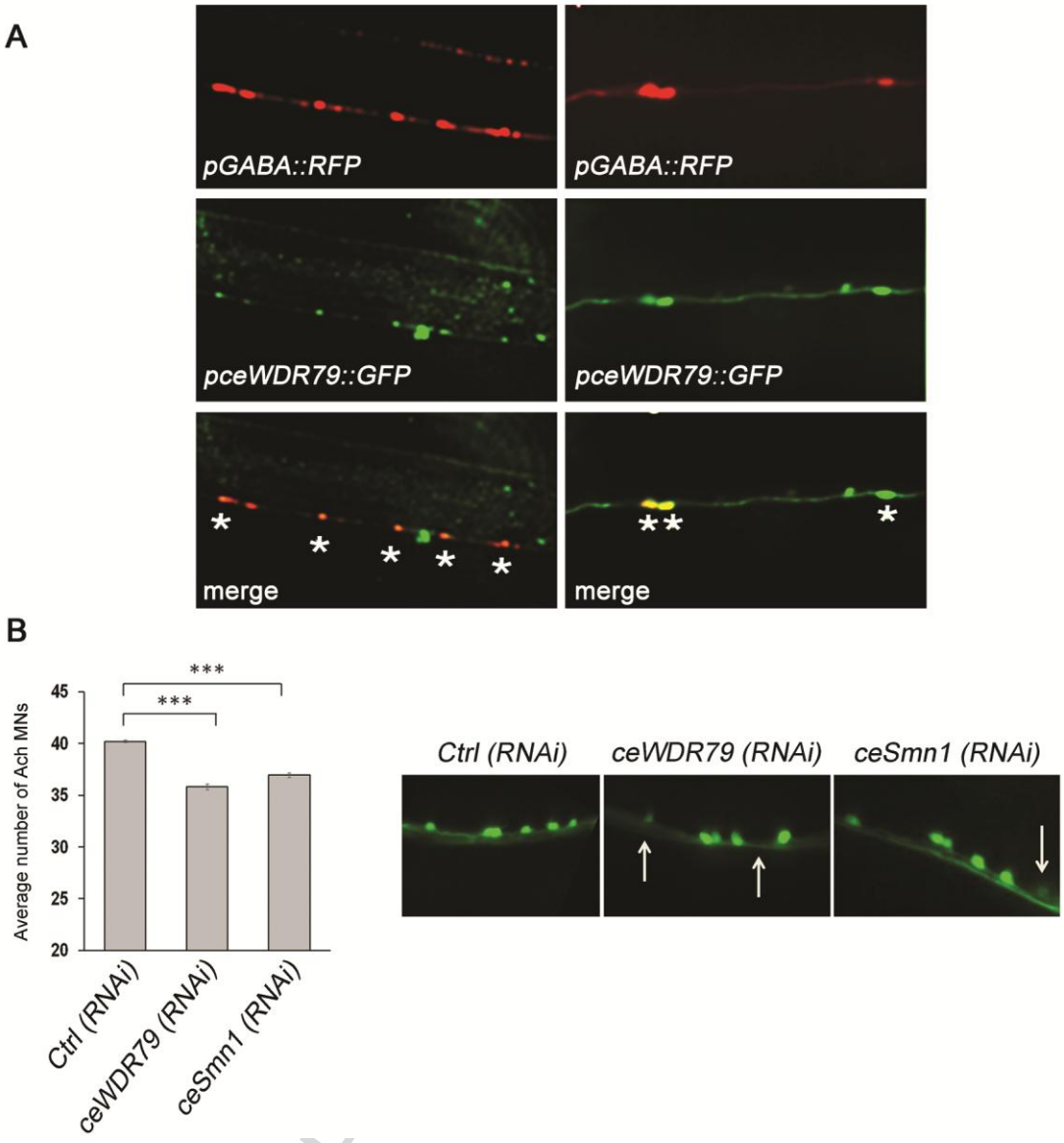

Fig. 2

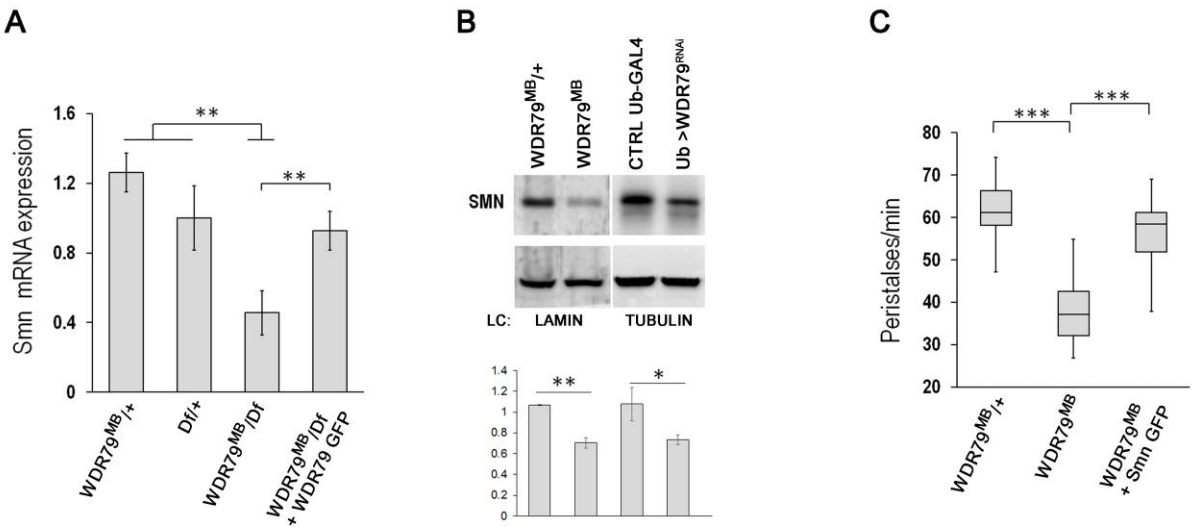

Fig. 3

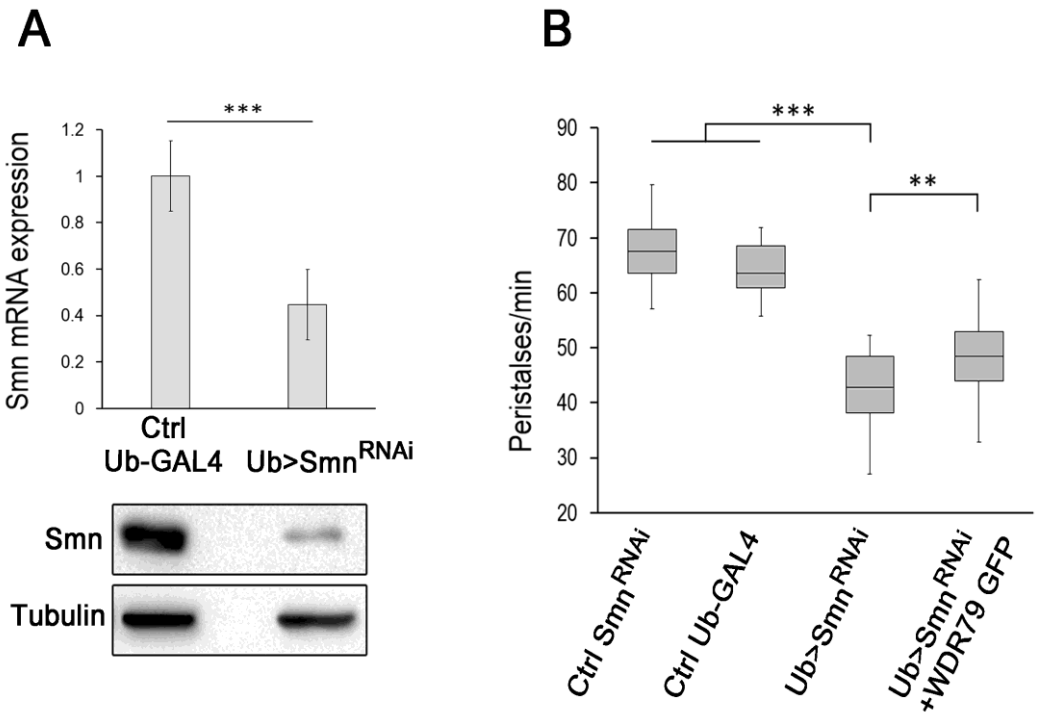

Fig. 4

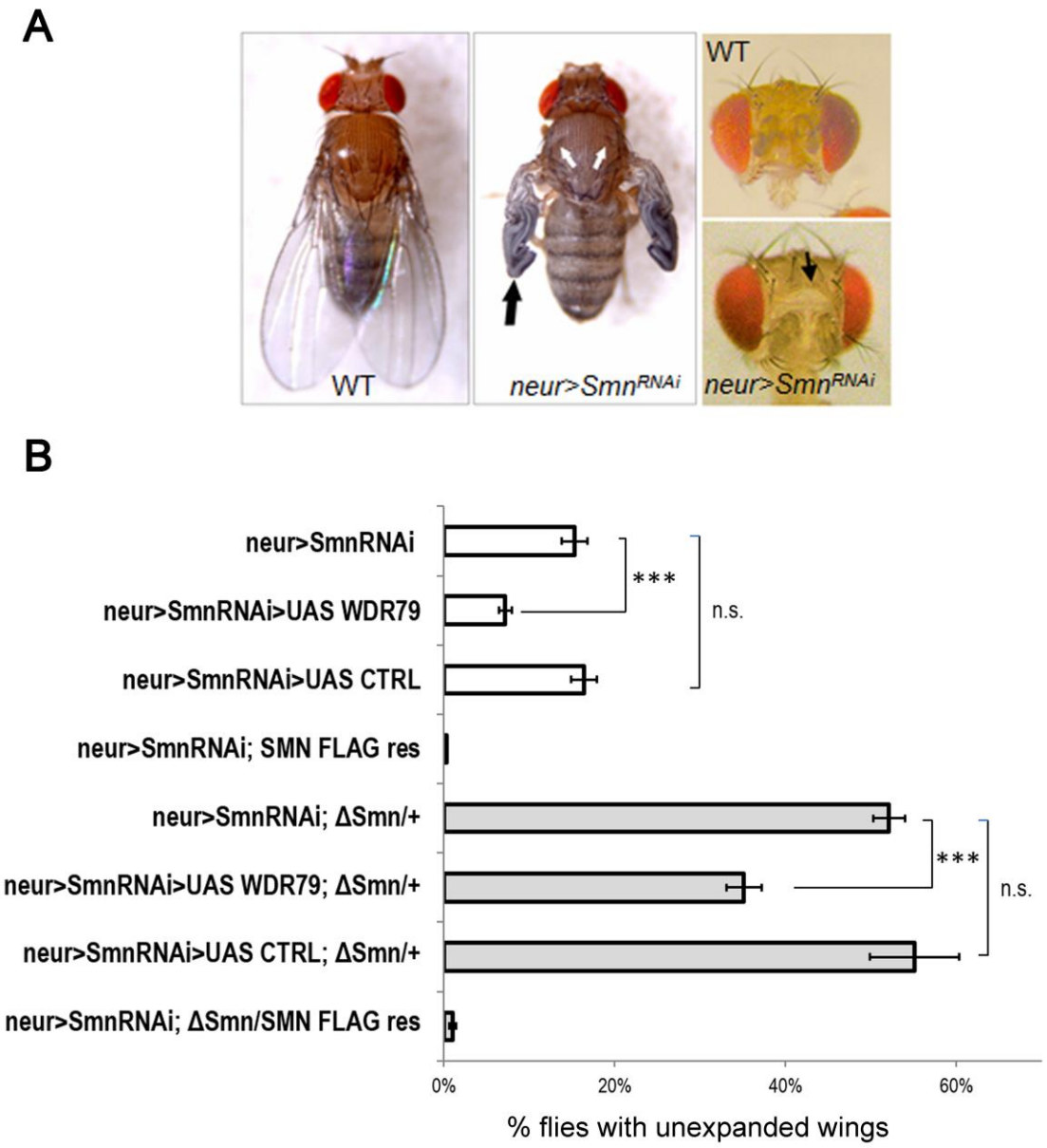

Fig. 5

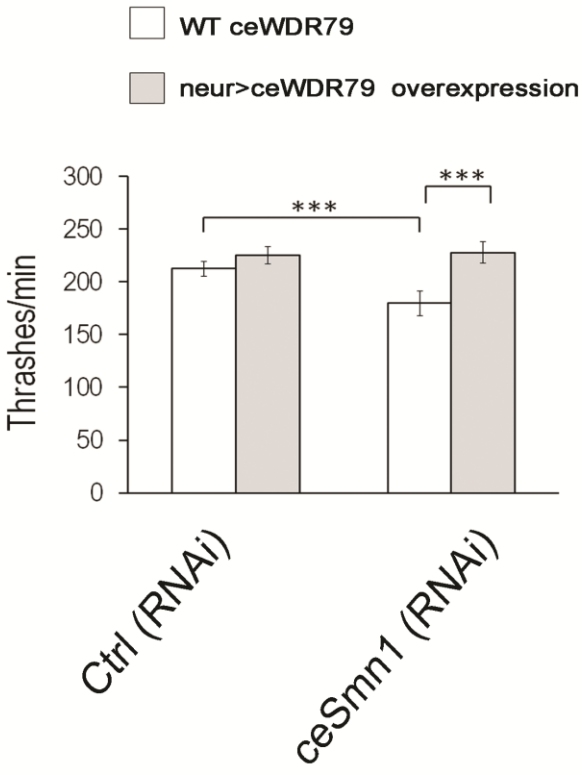

Fig. 6