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Novel interactors of the *Drosophila* Survival Motor Neuron (SMN) Complex suggest its full conservation

Maia Lanfranco[1,2,3], Rebecca Cacciottolo[2,3], Rebecca M. Borg[1,2,3], Neville Vassallo[2,3], François Juge[1], Rémy Bordonné[1] and Ruben J. Cauchi[2,3]

1 Institut de Génétique Moléculaire de Montpellier, CNRS-UMR 5535, Université de Montpellier, France
2 Department of Physiology and Biochemistry, Faculty of Medicine and Surgery, University of Malta, Msida, Malta
3 Centre for Molecular Medicine and Biobanking, University of Malta, Msida, Malta

Correspondence
R. J. Cauchi, Department of Physiology and Biochemistry, Faculty of Medicine and Surgery, University of Malta, Msida, Malta
Fax: +356 21310577
Tel: +356 23402145
E-mail: ruben.cauchi@um.edu.mt

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The Spinal Muscular Atrophy disease protein Survival Motor Neuron (SMN) operates as part of a multiprotein complex whose components also include Gemins 2-8 and Unrip. The fruit fly *Drosophila melanogaster* is thought to have a slightly smaller SMN complex comprised of SMN, Gemin2/3/5 and, possibly, Unrip. Based upon *in vivo* interaction methods, we have identified novel interacting partners of the *Drosophila* SMN complex with homologies to Gemin4/6/7/8. The Gemin4 and Gemin8 orthologues are required for neuromuscular function and survival. The Gemin6/7/Unrip module can be recruited via the SMN-associated Gemin8, hence mirroring the human SMN complex architecture. Our findings lead us to propose that an elaborate SMN complex that is typical in metazoans is also present in *Drosophila*.

Keywords: Gaulos; Gemin3; Gemin7; Hezron; Sabbat; Valette

The Survival Motor Neuron (SMN) protein was identified more than two decades ago as the causative factor of the predominantly early-onset neuromuscular disorder, spinal muscular atrophy (SMA) [1]. Patients with SMA have insufficient levels of SMN, a situation triggering lower motor neuron degeneration and profound muscle weakness that restricts mobility and, in severe cases, results in respiratory failure and death [2]. SMN is indispensable for chaperoning the assembly of small nuclear ribonucleoproteins (snRNPs) which form the core components of the spliceosome. Essentially, this involves the uploading of a preorganised heptameric Sm protein ring onto a conserved uridine-rich site within small nuclear RNAs in an ordered process restricted to the cell cytoplasm [3,4]. Besides a 'housekeeping' function, SMN has also been implicated in tissue-specific roles including, chief amongst others, the assembly and axonal trafficking of messenger ribonucleoproteins (mRNPs) in motor neurons [5]. Although it remains unclear which biological process is perturbed in SMA, it is well known that SMN operates as part of a large multiprotein complex whose components also include a set of diverse proteins, namely, Gemins 2–8 and Unrip [6].

The human SMN complex has a modular composition, thus, at its centre, the SMN-Gemin8-Gemin7 module recruits the Gemin2-Gemin5 and the Gemin6-Unrip subunits via SMN and Gemin7, respectively. The remaining Gemin3-Gemin4 block clips on to the complex via both SMN and Gemin8 [7]. Additional interactions and oligomerisation of select components [7–10] generate a heavily interconnected structure that can potentially stabilise the complex in addition to facilitating its function. Despite its presence in almost

Abbreviations
Dcr-2, Dicer-2; GFP, green fluorescent protein; Gem2, Gemin2; Gem3, Gemin3; Gem4, Gemin4; Gem5, Gemin5; Gem6, Gemin6; Gem7, Gemin7; Gem8, Gemin8; Glos, Gaulos; Hez, Hezron; mRNPs, messenger ribonucleoproteins; OE, overexpression; RNAi, RNA interference; RFP, red fluorescent protein; Sbat, Sabbat; SMA, spinal muscular atrophy; SMN, Survival Motor Neuron; snRNPs, small nuclear ribonucleoproteins; UAS, upstream activation sequence; Vlet, Valette.

all eukaryotes, it is thought that the SMN complex has experienced a growth in membership throughout evolution. Hence, in contrast to the elaborate nine-membered version in vertebrates, including humans, in its simplest form found in the fission yeast *Schizosaccharomyces pombe* and the parasitic protozoan *Trypanosoma brucei*, the SMN complex is composed of only SMN and Gemin2 [11–13]. A slightly larger SMN complex comprising SMN, Gemin2, Gemin3, Gemin5, and, possibly, Unrip amongst its constituents is present in the fruit fly *Drosophila melanogaster* [14–16]. Interestingly, orthologues of most of the remaining Gemins appear to be absent in *Drosophila* although they are present in closely related species including the honey bee *Apis mellifera* and the parasitic wasp *Nasonia vitripennis* [11].

Drosophila remains an attractive model system to investigate the *in vivo* function of human orthologues [17]. Making use of this model, we and others have previously shown that loss of SMN, Gemin2, Gemin3, Gemin5, and Unrip leads to neuromuscular dysfunction [16,18–24]. Importantly, components of the *Drosophila* SMN complex were shown to interact genetically in addition to physically [15]. Attempting at furthering our understanding of the mechanics of the *Drosophila* SMN complex, here we report on the identification and initial characterisation of novel interacting partners. Through associations with known SMN complex components, we identified Gaulos and Valette, which though moderately divergent, are most probably the Gemin4 and Gemin8 *Drosophila* orthologues. Our prediction is strengthened by secondary structure conservation in addition to their role in neuromuscular survival and function. We also show that the recently characterised *Drosophila* Unrip orthologue, wmd [16], can be recruited to the SMN complex *via* Sabbat, a possible orthologue of Gemin7. The identification of Hezron, the most likely orthologue of Gemin6, which latches to the SMN complex via Sabbat/Gemin7 completes the search for the discovery of the Gemin orthologues in *Drosophila*. Our findings allow us to propose that the *Drosophila* SMN complex is fully conserved, hence, bringing this organism on a par with its metazoan counterparts.

Materials and methods

Fly stocks

Flies were cultured on standard molasses/maizemeal and agar medium in plastic vials at an incubation temperature of 25°C unless otherwise stated. Expression of inducible transgenes was performed via the GAL4/upstream activation sequence (UAS) system [25]. The chromosomal deletion (*Df [1]ED6716*) covering *Gaulos*, and the fluorescent protein transgenes (*UAS.EGFP* and *UAS.dsRed*) were obtained from the Bloomington *Drosophila* Stock Center (NIH P40OD018537) at Indiana University, USA. The RNAi transgenic constructs, $UAS.Glos\text{-}IR^{DEX}$ (*Glos RNAi*, 52356) and $UAS.Vlet\text{-}IR^{KAT}$ (*Vlet RNAi*, 15482), were obtained from the Vienna *Drosophila* Resource Center, Austria [26]. The $UAS.Gem3^{BART}$, $UAS.Gem3^{\Delta N}$, $UAS.SMN^{FL}$ (*SMN OE*), $UAS.SMN\text{-}IR^{C2}$ (*SMN RNAi*), $UAS.Gem2^{FL}$ (*Gem2 OE*), $UAS.Gem5^{FL}$ (*Gem5 OE*), *and* $UAS.Gem5\text{-}IR^{nan+sac}$ (*Gem5 RNAi*) transgenes were described and characterised previously [15,18,19,21]. The temporal and spatial expression patterns of the GAL4 driver utilised in this study are outlined in Table S1, whereas their provenance was detailed previously [18,19]. Combination of the various genetic tools including alleles, transgenes, and GAL4 lines was carried out according to standard genetic crossing schemes.

Assessment of organismal viability, adult survival and puparial axial ratios

Organismal viability in *Drosophila* was calculated as the percentage of the number of adult flies with the appropriate genotype divided by the expected number for the cross. For adult survival analysis, adult flies were maintained in vials at a density of 15–20 flies per vial. The percentage number of flies alive at each time point measured was determined by dividing the number of flies still alive by the initial number of flies in the vial and multiplying the value by 100. Puparial axial ratios were calculated by dividing the length by the width of the puparia, both of which were measured from still images.

Assessment of flight performance

In preparation for flight quantification, flies were first subjected to a 'warm-up' by inducing negative geotaxis in a new empty vial for six times. Organisms were then introduced into the top of the Droso-Drome, which consisted of a 1 L glass bottle coated with an alcohol-based sticky fluid, and divided into four sectors, of 5 cm each, spanning a total height of 20 cm. The number of flies in each sector was counted, divided by the total number of flies assessed and multiplied by 100 to generate the percentage number of flies per sector. Flight ability is determined by the height or sector in which flies are distributed.

Assessment of fly mobility

Larval mobility was determined by measuring the forward body wall contractions in 30 s exhibited by third instar larvae placed on a 0.7% agar plate. To determine climbing ability in adult flies, two empty polystyrene vials were

vertically joined by tape facing each other. A group of 10–15 adult flies were then transferred into the lower vial and allowed to acclimatise. Flies were then gently tapped down to the bottom of the vial and the time for the first fly within a group to cross an 8 cm threshold was measured. Three trials were performed for each group and a minimum of three groups were assayed for each genotype.

Co-immunoprecipitation in S2 cells

SMN, *Gaulos*/*Gemin4*, *Hezron*/*Gemin6*, *Sabbat*/*Gemin7* and *Valette*/*Gemin8* cDNA sequences were PCR-amplified and introduced into either *pAGW* or *pAHW* vectors to generate N-terminal GFP- or HA-tagged fusion proteins, respectively. Cloning was performed following *Drosophila* Gateway Vectors protocols (https://emb.carnegiescience.edu/drosophila-gateway-vector-collection). Constructs were transfected into *Drosophila* S2 cells that were cultured in Schneider's medium supplemented with 10% Foetal Bovine Serum and 5 $mL{\cdot}L^{-1}$ Penicillin-Streptomycin antibiotics. Protein extracts were prepared by re-suspending the cell pellet in lysis buffer (10 mM Tris/Cl pH 7.5; 150 mM NaCl; 0.5 mM EDTA; 0.25% NP-40; 1 mM PMSF; 1× Roche protease inhibitors cocktail) followed by bead beating and centrifugation. Co-immunoprecipitation was performed by incubating the extracts with GFP-Trap beads (Chromotek, Planegg-Martinsried, Germany) for 1.5 h at 4°C. The beads were then washed three times in wash buffer (10 mM Tris/Cl pH 7.5; 150 mM NaCl; 0.5 mM EDTA) prior to boiling and analyses by SDS/PAGE and western blot using purified rabbit anti-GFP (dilution 1/5000; TP401, AMS Biotechnology, Abingdon, UK) and rat anti-HA (dilution 1/5000; clone 3F10; Roche, Paris, France) antibodies.

Yeast two-hybrid analyses

Two-hybrid assays were performed with the CG1945 and Y187 strains [27]. Baits and preys were obtained by PCR amplification of cDNA and ligation into the *pAS*ΔΔ and *pACT2st* vectors, respectively [28]. Primer sequences and PCR regimes are available upon request. cDNA clones for *Gemin3* (LD05563), *Gemin5* (SD03652), *Valette*/*Gemin8* (IP03353) and *wmd*/*Unrip* (HL01517) were obtained from the *Drosophila* Genomics Resource Centre (Indiana University, USA). cDNAs for *Hezron*/*Gemin6*, *Sabbat*/*Gemin7*, *SmB* and *SmD2* were obtained from extracted RNA. The CG1945 strain was transformed with the bait *pAS*ΔΔ construct containing the protein sequence fused in frame with the GAL4 DNA-binding domain (GAL4-BD) and selected on –Trp plates. The Y187 strain was transformed with the prey *pACT2st* construct containing the protein sequence fused in frame with the GAL4 activation domain (GAL4-AD) and selected on –Leu plates. Bait and prey strains were mated overnight on rich YPD plates and diploids containing bait/prey combinations were selected on –Trp –Leu plates. Diploid yeast cells carrying bait/prey combinations were cultured in –Trp –Leu media and protein–protein interactions were screened by spotting serial dilutions on –Trp –Leu–His plates. Incubations were performed at 28°C for 3–5 days.

Statistical analysis

Values are presented as means ± SEM unless otherwise indicated. The unpaired *t*-test was used to compare measures between two groups whereas one-way ANOVA with Dunnett's *post hoc* test was used for multiple comparisons with the control (GRAPHPAD PRISM v7.0a, GraphPad Software, Inc., La Jolla, CA, USA). For flight assays, comparative analysis was restricted to sector 1 of the Droso-Drome in view that this is the sector that mostly determines differences between fliers and nonfliers. Differences were deemed statistically significant if $P < 0.05$, and when this is the case, the exact *P*-value is presented.

Results

Gaulos is a novel genetic interactor of *Gemin3*

We have recently reported that alterations in the levels of known SMN complex components in *Drosophila* precipitate the motor and viability phenotypes of adult flies expressing $Gem3^{BART}$, a hypomorphic Gemin3 mutant [15]. To identify novel genetic loci whose partial loss of function may strongly modify the $Gem3^{BART}$-induced viability and motor phenotype, we are performing a large-scale genetic screen for which results will be presented elsewhere. The deficiency line *Df(1)ED6716* on the first chromosome had no deleterious effect by itself but led to a marked age-dependent enhancement of motor and viability phenotypes in flies with muscle-restricted $Gem3^{BART}$ expression (Fig. 1). We performed a systematic evaluation of the candidate genes in the genomic interval (3F2-4B3) uncovered by the deficiency. This allowed us to identify the gene *CG2941* and in view of its boat-like expression pattern in wild-type *Drosophila* embryos stage 7–10 when viewed laterally (http://www.flyexpress.net), we renamed the gene *Gaulos*, a Phoenician word for a merchant boat. Whereas pan-muscular RNAi-induced knockdown of *Gaulos* – abbreviated as *Glos* – alone has no effect on motor behaviour (Fig. 1), when combined with $Gem3^{BART}$, it induced adult lethality. This is a much more drastic phenotype than that exposed by the *Df(1)ED6716* chromosome deficiency in view of the fact that the latter only induces a 50% reduction in gene copy number.

We wished to determine whether motor defects could be detected at an earlier developmental stage in flies with both *Gaulos* and *Gemin3* muscle-specific loss of function. To this end, we assessed mobility in third instar larvae. An RNAi-induced reduction in Gaulos levels leads to a marked decrease in mobility in a $Gem3^{BART}$ background but not in a wild-type background (Fig. 2A). We reveal a similar phenotype when known components of the *Drosophila* SMN complex including SMN, Gemin2 or Gemin5 levels are altered in $Gem3^{BART}$-expressing flies (Fig. 2A). No effect is observed in a wild-type genetic background. Furthermore, normal locomotor behaviour was observed in control flies in which, $Gem3^{BART}$ is combined with the inert green fluorescent protein (GFP) (Fig. 2A). Consequent to a decline in muscle power, all genotypes assessed, with the exception of the controls, failed to contract adequately during pupariation. Hence, Gaulos knockdown or disruption of any SMN complex member in a $Gem3^{BART}$ background induced a significantly elevated puparial axial ratio when compared to the control genotypes (Fig. 2B).

Considering that the nervous system is also a relevant tissue in SMA pathophysiology [29], we asked whether a genetic interaction between *Gemin3* and *SMN*, *Gemin2*, *Gemin5* or *Gaulos* can be exposed in the brain in addition to muscle. For this purpose, we made use of the stronger $Gem3^{\Delta N}$ allele, which we expressed in a pan-neuronal pattern starting from early development and disrupted each protein in this genetic background via either RNAi or overexpression. Whereas expression of the inert red fluorescent protein (RFP) had minimal effect on adult viability, a significant reduction was induced on *Gaulos* knockdown

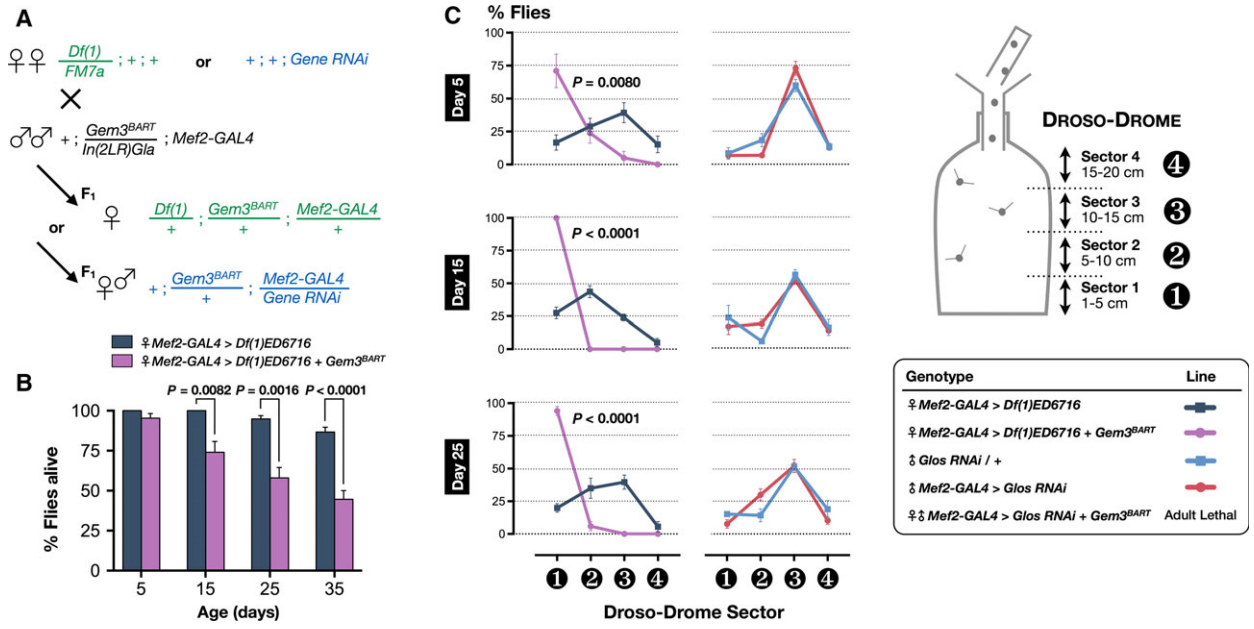

Fig. 1. Reduction in gene copy number or knockdown identify Gaulos as a strong modifier of viability and motoric ability in $Gem3^{BART}$-expressing flies. (A) Genetic crosses performed as part of the screening strategy aimed at identifying genetic enhancers of the $Gem3^{BART}$-induced viability and motor phenotype. In the first stage of the screen (green), individual first chromosome deficiencies are introduced in flies with muscle-restricted $Gem3^{BART}$ expression. Deficiencies that reduced viability and motor behaviour were defined as genomic enhancers. In the second stage (blue), candidate genes uncovered by the genomic enhancers were individually knocked down via RNAi to identify genetic enhancers. (B) Percentage number of flies, which are still alive, assessed at different time points during adulthood. Although it has minimal impact on its own, chromosome deficiency *Df(1)ED6716*, which reduces *CG2941* or *Gaulos* by a half, causes a statistically significant progressive decline in survival in adult flies with muscle-restricted expression of the $Gem3^{BART}$ hypomorphic mutant. (C) Chromosome deficiency *Df(1)ED6716* also impairs flight performance in flies with muscle-specific $Gem3^{BART}$ expression. Therefore, a significant percentage of organisms were nonfliers and fall straight to sector 1, the lowest sector of the Droso-Drome apparatus (Right). The motor phenotype is apparent on day 5 posteclosion and intensifies with age. *Gaulos*, a gene covered by the deficiency, is identified as a genetic enhancer since expression of a *Gaulos*-specific RNAi transgene (*Glos RNAi*) in the same genetic background leads to adult lethality. The deficiency or the RNAi transgene by itself has no effect on motor behaviour. In (A, B) data presented are the mean ± SEM of at least three independent experiments and $n \geq 60$ per genotype for each time point measured. Sex of the genotype assessed is indicated by symbols: ♂ = males, ♀ = females, and ♀♂ = males + females. Significance as tested by the unpaired *t*-test is indicated by the exact *P*-value.

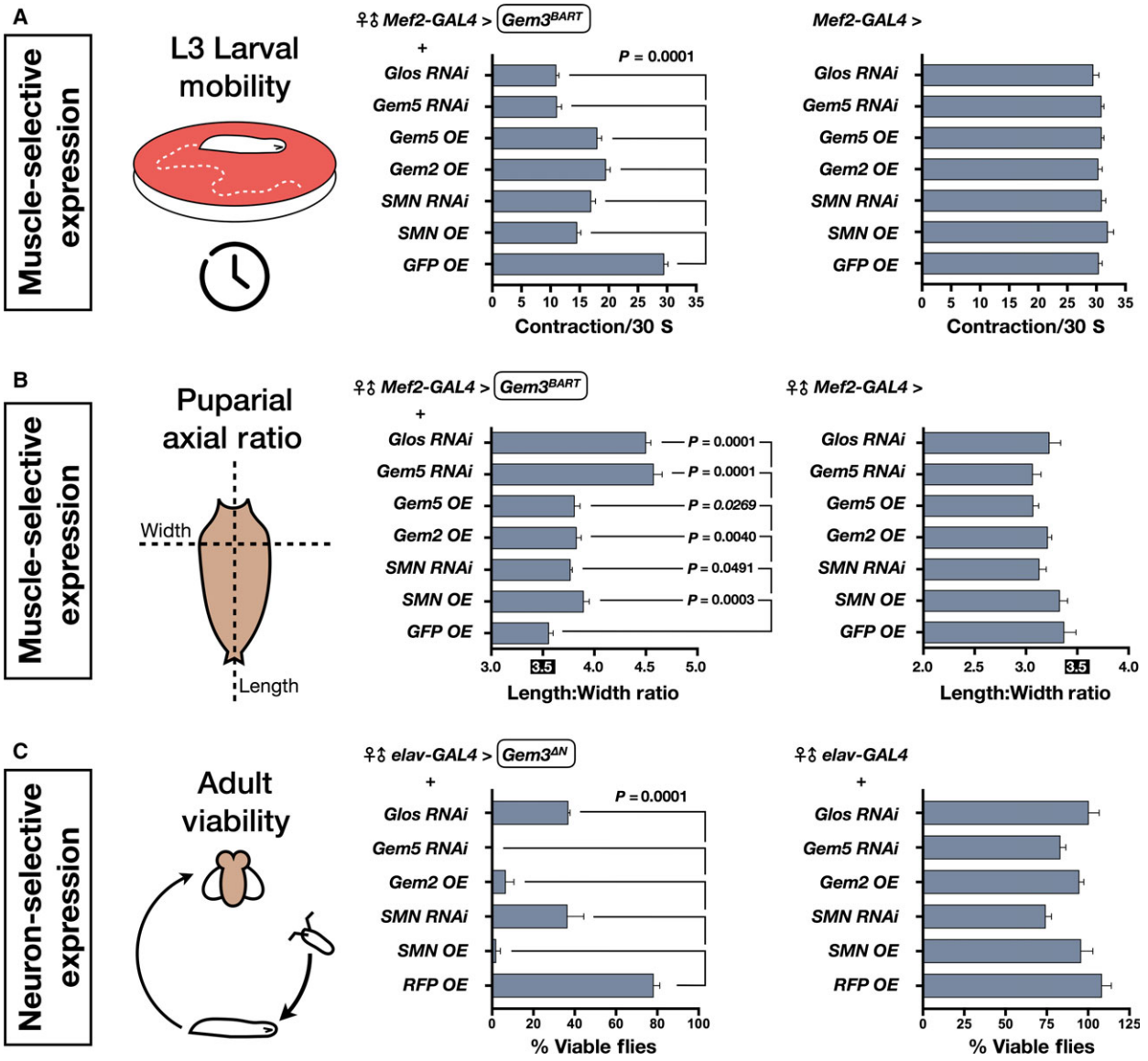

Fig. 2. Glos knockdown in *Gem3*^{BART}-expressing flies phenocopies an alteration in the levels of individual SMN complex members. (A) *Left*, Rate of body wall muscle contractions is significantly reduced in *Gem3*^{BART} third instar larvae that have muscle-specific *Gaulos* knockdown (*Glos RNAi*) compared to control flies that instead overexpress *GFP (GFP OE)*. A similar decline in larval mobility is achieved in organisms with muscle-specific *Gem3*^{BART} expression when SMN complex members are either overexpressed (OE) or reduced (RNAi). *Right*, In the absence of *Gem3*^{BART}, transgenic constructs inducing OE or RNAi do not influence larval mobility. (B) *Left*, Sluggish larval behaviour translates into puparia with significantly higher axial ratios compared to the control genotype. *Right*, In a wild-type background, on average, the transgenic constructs alone never induced puparial axial ratios greater than 3.5. (C) *Left*, Brain-specific disruption of SMN complex members or Gaulos levels via overexpression and/or knockdown leads to lethality or reduced viability in flies that co-express the antimorphic *Gem3*$^{\Delta N}$ allele. *Right*, The transgenic constructs by themselves have negligible effects on adult viability. In (A–C) data presented are the mean ± SEM of at least three independent experiments, and $n \geq 15$ (A, B) or $n \geq 80$ (C) per genotype. Sex of the genotype assessed is indicated by symbols: ♀♂ = males + females. In (C) culture was performed at 29°C instead of the standard 25°C since the former is associated with the maximal transgenic expression. Significance was tested by one-way ANOVA with Dunnett's *post hoc* test and is indicated by the exact *P*-value.

(Fig. 2C). A similar result was observed on disruption of SMN, Gemin2 or Gemin5 in flies with neuronal expression of *Gem3*$^{\Delta N}$ (Fig. 2C). As a control, adult viability was not influenced by transgenic alteration of protein levels in a wild-type genetic background (Fig. 2C). Overall, these findings reveal that similar to

well-known members of the *Drosophila* SMN complex, Gaulos is a genetic interactor of Gemin3.

Gaulos is the putative *Drosophila* orthologue of Gemin4

The findings above spurred us to investigate if Gaulos is an orthologue of the remaining unidentified Gemins in *Drosophila*. Based on amino acid length, Gaulos can potentially be the orthologue of vertebrate Gemin4. Though moderately divergent in the amino acid sequence, we found homologies between vertebrate Gemin4 and Gaulos across the whole protein (Figs 3A and S1). Considering human Gemin4 for comparison purposes, Gaulos has 13% amino acid identity, however, amino acid similarity reaches 44%. Remarkably, structure prediction reveals a high degree of overlap between Gaulos and metazoan Gemin4 sequences throughout the entire length of the protein (Figs 3A and S2). Encouraged by these results, we asked whether Gaulos can be co-immunoprecipitated with SMN as would be expected from a bona fide Gemin4 orthologue. To this end, we co-transfected *Drosophila* Schneider2 (S2) cells with plasmids containing HA-tagged SMN and GFP-tagged Gaulos or GFP alone as a control. We observed reproducible and higher than background co-purification of SMN with Gaulos (Fig. 4A). The instability or auto-activation of SMN yeast two-hybrid prey and bait constructs precluded us from testing clearly whether the physical interaction between Gaulos and SMN is direct. However, based on earlier work showing that Gaulos co-purifies with both Gemin2 and Gemin3 [30], we predict that the association between Gaulos and SMN is likely to be indirect. Overall, based on secondary structure conservation and *in vivo* association with SMN, Gaulos is most probably the putative *Drosophila* orthologue of Gemin4.

Gaulos/Gemin4 is required for neuromuscular survival and function

We wished to strengthen our case favouring Gaulos as the bona fide orthologue of Gemin4 and, hence, a core member of the *Drosophila* SMN complex. Therefore, we investigated whether Gaulos has tissue-specific requirements that are similar to those described for SMN and the known Gemins in *Drosophila* [18,20,21,24]. To this end, we utilised the *Glos RNAi* transgene, which targets the coding sequence of the *Gaulos* gene to induce a drastic reduction in the expression level of Gaulos mRNA transcripts compared to controls (Fig. S3A). First, we note that adult lethality is the endpoint of ubiquitous knockdown of Gaulos through *da*-GAL4- or *tubP*-GAL4-driven RNAi transgenic expression (Fig. 5). This result indicates that in line with that reported for the known SMN complex members [31], *Gaulos* is an essential gene. To determine whether a requirement for adult viability is due to a function within the neuromuscular system, we decreased *Gaulos* mRNA levels in either muscle or neurons starting from the earliest stages of development. Notably, we find that lethality or reduced adult viability is the consequence if Gaulos levels are depleted in muscle tissues via strong pan-muscular GAL4 drivers or when enhanced RNAi-based knockdown is restricted to the central nervous system (CNS) (Fig. 5).

We next asked whether motor dysfunction is apparent in flies with either muscle- or brain-specific Gaulos knockdown that escaped lethality through the use of less restrictive conditions. To this end, flies were subjected to a climbing assay at different stages of their adult life. Compared to the control genotypes, when knockdown was restricted to muscles, flies took significantly longer to reach a target height at late stages of adulthood (Fig. 6A). This motor defect was however obvious on all days measured in flies having reduced Gaulos levels specifically in the CNS (Fig. 6B). Nonetheless, compared to prior time points, flies with this genotype were profoundly defective on day 35 posteclosion. Furthermore, we show that flies exhibit an age-dependent progressive decline in flight performance when Gaulos expression is reduced in either the muscle or neuronal compartment of the neuromuscular system (Fig. 6D). With regards to the latter, survival of the organisms throughout adulthood was also negatively impacted (Fig. 6C). Taken together, these findings reveal that similar to SMN and the known Gemin components of the *Drosophila* SMN complex [18,21,24], Gaulos is required for viability and optimal motor behaviour in the neuromuscular system. In this regard, we are very confident that Gaulos is the *Drosophila* orthologue of Gemin4.

Valette is the putative *Drosophila* orthologue of vertebrate Gemin8

Considering our serendipitous discovery of the *Drosophila* Gemin4 orthologue, we wondered whether the composition of the *Drosophila* SMN complex is the same as that of vertebrates after all. In this regard, we attempted to identify the remaining elusive *Drosophila* Gemins. Based on amino acid and secondary structure homology to vertebrate Gemin8, we singled out *CG2371*. In view of the gene's role in

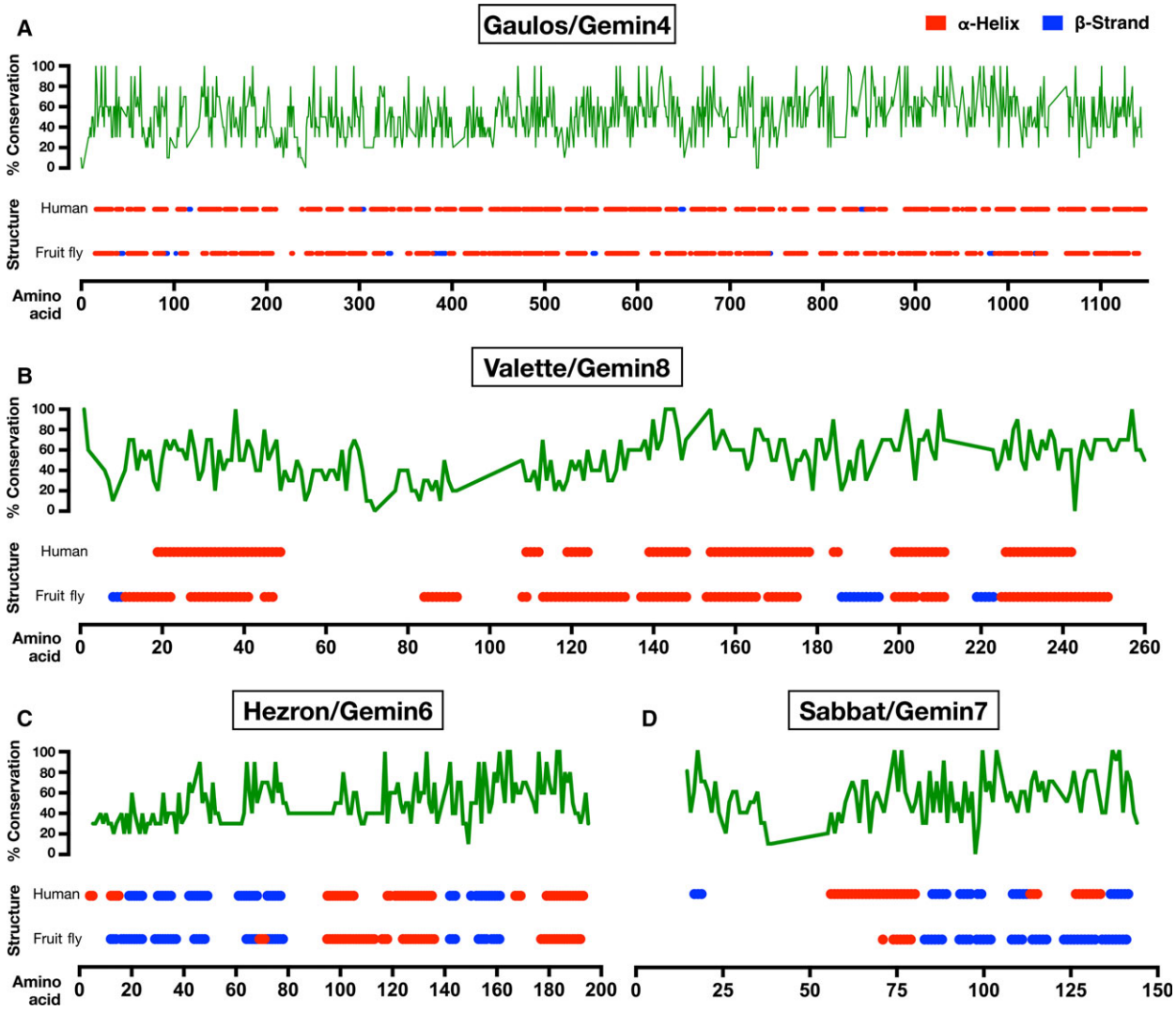

Fig. 3. Sequence and secondary structure conservation of the putative Gemin4, Gemin6, Gemin7 and Gemin8 *Drosophila* orthologues. In (A–D), *top panel* shows the percentage amino acid conservation for each respective protein when compared to its metazoan orthologue; *bottom panel* shows secondary structure prediction throughout the length of the protein. (A) On average, the degree of conservation for Gaulos/Gemin4 is higher than 50% with peaks denoting amino acid identity occurring throughout the entire length of the protein. Areas of overlap in alpha-helixes reach 65%. (B) Peaks denoting amino acid identity for Valette/Gemin8 are mostly skewed towards the C-terminus. The degree of conservation is, on average, higher than 50%. Areas of overlap in alpha-helixes reach 58%. (C, D) For both Hezron/Gemin6 and Sabbat/Gemin7, amino acid identity mostly peaks at the C-terminus. The degree of conservation is, on average, higher than 50% for both proteins. Areas of overlap in alpha-helixes and beta-strands reach 61% and 33% for Hezron/Gemin6 and Sabbat/Gemin7, respectively.

motor behaviour (described below), we renamed the gene *Valette* (abbreviated as *Vlet*), after Grand Master of the Order of Malta and distinguished warrior, Jean Parisot de Valette. Alignment of Valette with multiple metazoan Gemin8 proteins reveals that despite a low amino acid identity, Valette has a high amino acid similarity (Figs 3B and S4). Hence, when one compares Valette with human Gemin8, amino acid identity is 11% but similarity rises to 44%. The C-terminus appears to be more conserved than the N-terminus. Importantly, we show that Valette's predicted secondary structure overlaps greatly with that of metazoan Gemin8 proteins including the human version (Figs 3B and S5). Co-immunoprecipitation analyses revealed that similar to Gaulos, Valette (GFP-Vlet), but not (GFP), forms complexes with HA-SMN *in vivo* (Fig. 4A). In addition to bioinformatics analyses, this result makes us confident that Valette is the putative Gemin8 orthologue in *Drosophila*.

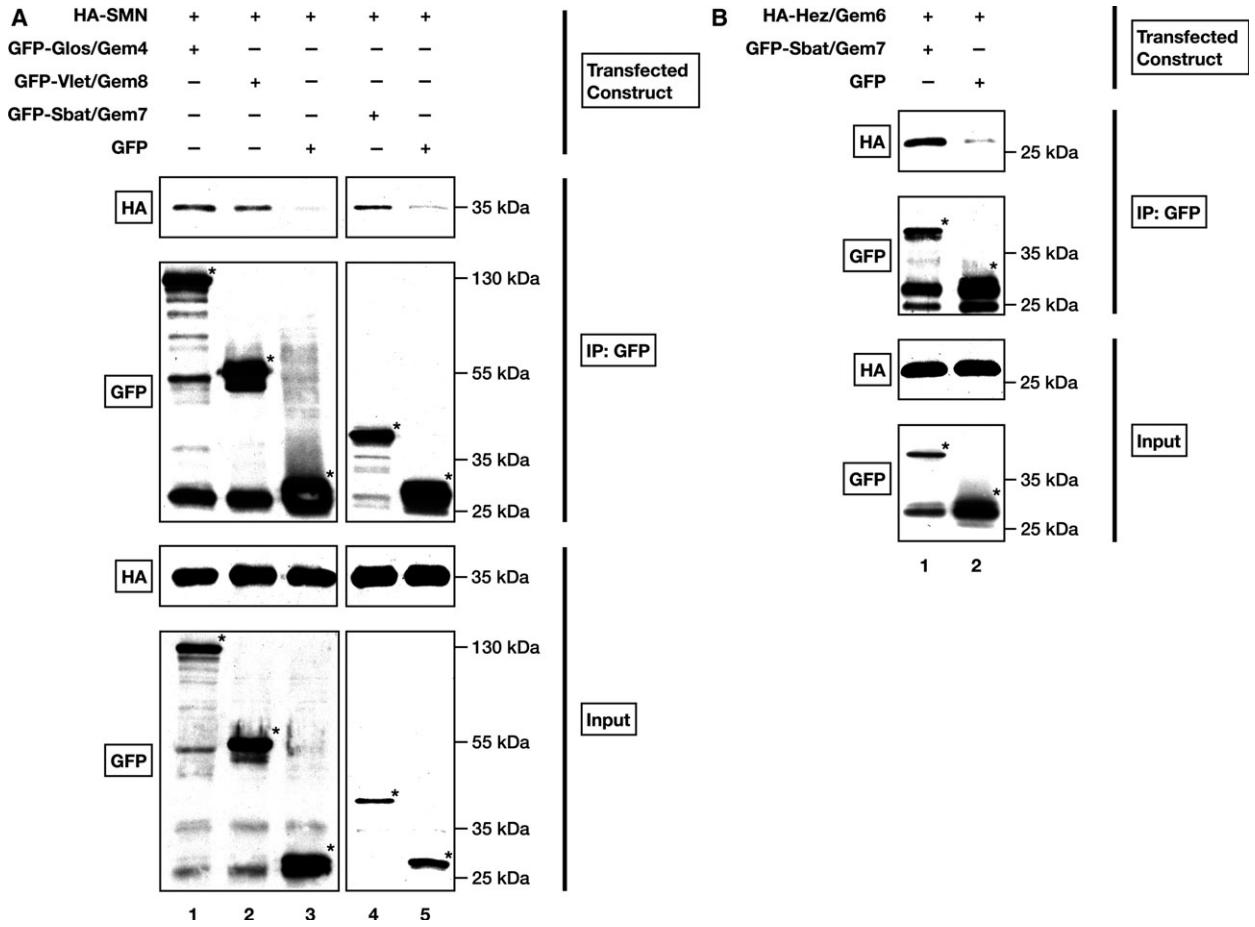

Fig. 4. Co-immunoprecipitation assay with the novel *Drosophila* Gemin4/6/7/8 orthologues. *Drosophila* S2 cells were co-transfected with the respective differentially tagged proteins indicated in the top panel. Empty GFP was used as control. Extracts were mixed with GFP-Trap beads and proteins detected by western blot using the indicated antibodies. Immunoprecipitated bait proteins are marked by asterisks. (A) HA-SMN co-immmunoprecipitates with GFP-Gaulos/Gemin4 (*lane 1*), GFP-Valette/Gemin8 (*lane 2*) and GFP-Sabbat/Gemin7 (*lane 4*). As a control, negligible amounts of HA-SMN co-immunoprecipitate with GFP (*lanes 3* and *5*). (B) HA-Hezron/Gemin6 co-immunoprecipitates strongly with GFP-Sabbat/Gemin7 (*lane 1*) while only background levels are observed with the GFP control (*lane 2*). Five percent input is shown in both (A) and (B). At least three independent experiments were performed, and the result of one representative is shown. Note that additional GFP bands found in *lanes 1* and *2* in (A) and *lane 1* in (B) correspond to degradation products of GFP-tagged Gemins.

Depletion of Valette/Gemin8 in muscle has deleterious effects on adult viability and motor behaviour

We next asked whether loss of Valette induces phenotypes that are similar to those described for Gaulos/Gemin4 (above) or the Gemin components of the SMN complex [18]. To this end, we employed an RNAi transgene (*Vlet RNAi*) targeting most of the coding sequence, hence causing a severe reduction in the expression level of *Valette* mRNA transcripts compared to controls (Fig. S3). A global reduction of Valette via strong ubiquitously expressing GAL4 drivers induces adult lethality (Fig. 5). Next, we explored the tissue-specific requirements of Valette. We find that lethality attributed to ubiquitous knockdown can be recapitulated when knockdown is restricted to muscle tissue via the strong pan-muscular *how*-GAL4 and *C179*-GAL4 drivers especially when cultured at 29°C and/or in the presence of increased Dicer-2 (Dcr-2) levels, which are associated with enhanced RNAi (Fig. 5). Under these conditions, we also observe a reduction in adult viability when Valette is specifically reduced in the nervous system or selectively in motor neurons (Fig. 5). We then assessed whether motor defects are obvious in escapees. To this end, we show muscle-specific Valette knockdown resulted in flies that had severe mobility defects and, therefore, their climbing ability was severely affected when assessed at

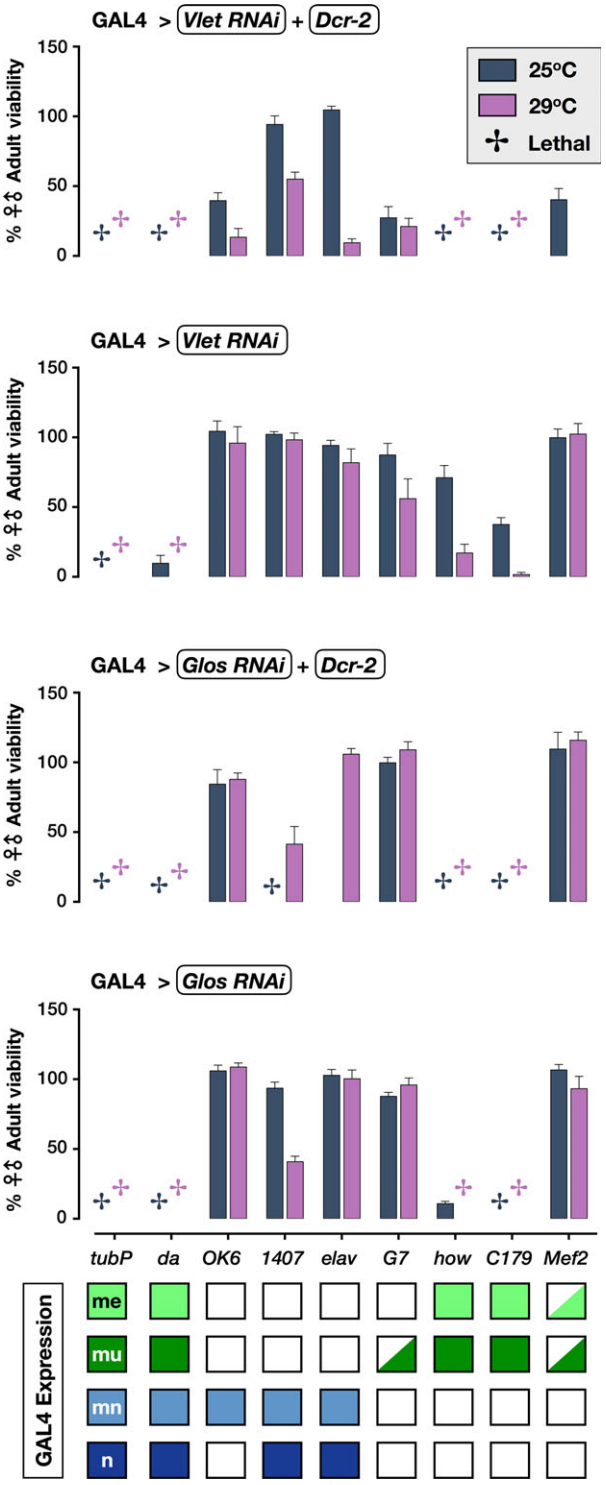

Fig. 5. Gaulos/Gemin4 and Valette/Gemin8 are indispensable for adult viability in either muscle or neurons. *Top*, bar charts showing percentage adult fly viability assayed at 25°C and 29°C on tissue-specific knockdown of *Gaulos* (*Glos*) or *Valette* (*Vlet*) in the absence or presence of increased Dicer-2 (Dcr-2) levels. Individual bars represent the mean adult viability (compared to the respective GAL4 driver control) ±SEM of at least four independent experiments ($n \geq 100$ per genotype). For each genotype, both males and females (♀♂) were assessed. *Bottom*, schematic denoting the tissue expression pattern of the GAL4 drivers utilised where *me* = mesoderm, *mu* = muscle, *mn* = motor neurons, and *n* = all CNS neurons except motor neurons. Transgenic RNAi-based knockdown of *Glos* or *Vlet* in all tissues via the ubiquitous *tubP*-GAL4 or *da*-GAL4 drivers induces adult lethality. The same genetic manipulation leads to a similar outcome or reduced viability if expression is specifically directed to either muscle or nervous tissue. Results are more pronounced at temperatures that permit maximal GAL4 activity (29°C) and/or in an enhanced Dicer-2 background.

48 hours posteclosion (Fig. 7A). At this time point, flight capacity was also profoundly disrupted (Fig. 7B). Flies never lived beyond day 5 posteclosion. Finally, we find that although flight capacity was unaffected (data not shown), an age-dependent progressive decline in adult survival was apparent in flies with muscle-specific co-expression of $Gem3^{BART}$ and Valette knockdown (Fig. 7C). Overall, these findings show that viability and motor function are significantly disrupted on loss of Valette in the muscle compartment of the neuromuscular system. These phenotypes which overlap those described for Gaulos/Gemin4 (above), the other Gemins [18] or SMN [21,24], reinforce our case favouring Valette as the bona fide Gemin8 orthologue in *Drosophila*.

Identification of the Gemin6 and Gemin7 orthologues in *Drosophila*

Gemin6 and Gemin7 are small proteins whose length is below 200 amino acids. To boost the chances of detecting remote orthologues, instead of BLAST searches, we turned to HHpred, which is a more sensitive homology search method that compares profile hidden Markov models [32,33]. A search against the HHpred *Drosophila melanogaster* proteome using the HHpred web server led to a > 99% probability match between vertebrate, wasp or bee Gemin6 and CG14164. We renamed the gene *Hezron* (abbreviated as *Hez*), a 6th generation descendent of patriarch Abraham. For Gemin7, HHpred identified CG31950 with ≥ 95% probability match to vertebrate, wasp or bee Gemin7. We renamed the gene *Sabbat* (abbreviated as *Sbat*), the 7th day of the week. Multiple sequence alignments of *CG14164*/*Hezron* and *CG31950*/*Sabbat* confirm the HHpred results, hence, large swathes of homologies are obvious if one considers the conservation of amino acids and secondary structure (Fig. 3C–D and S6–9). Overall percentage amino acid identity and similarity between Hezron and

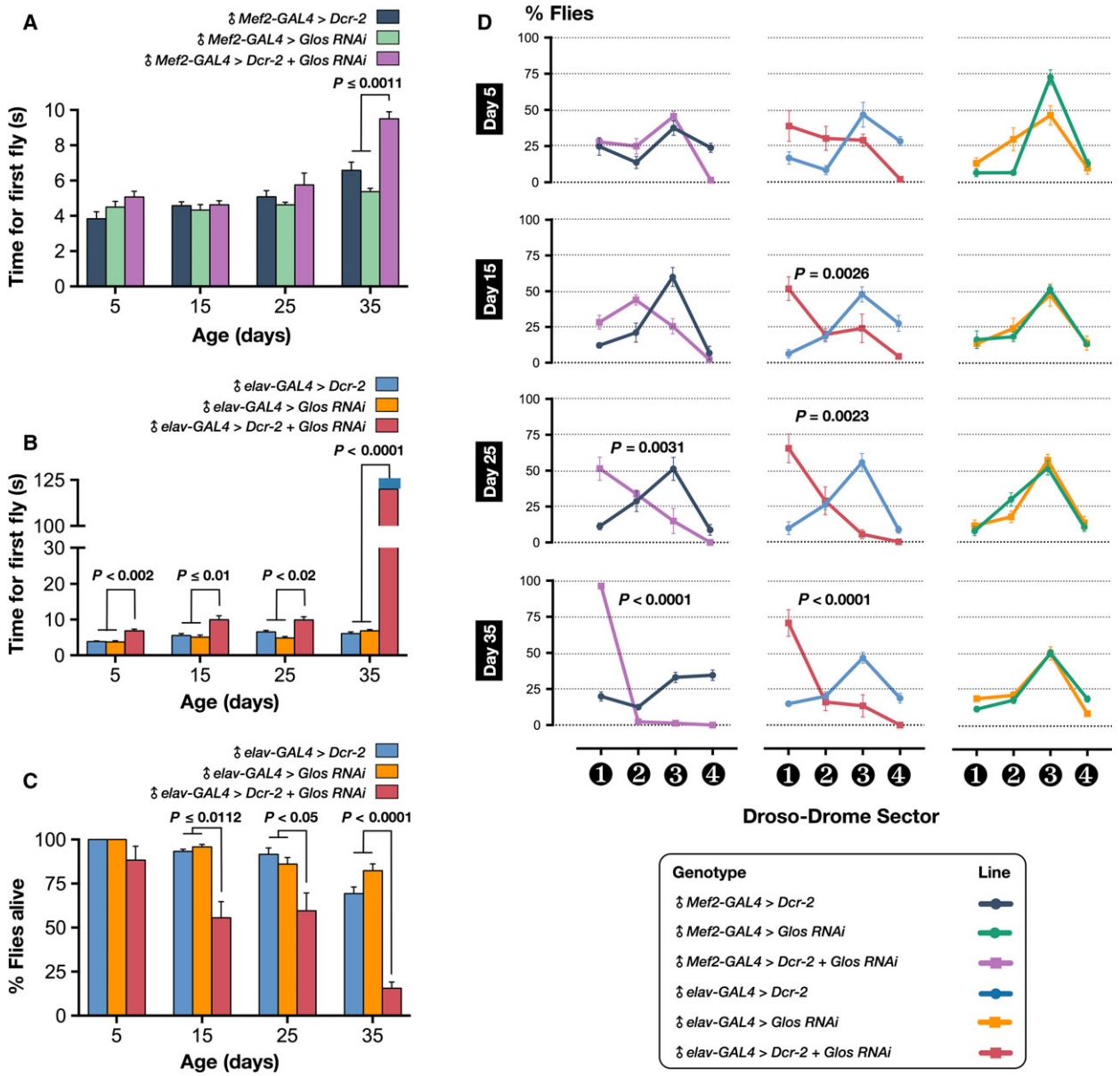

Fig. 6. Loss of Gaulos/Gemin4 function in either muscle or neurons has an age-dependent negative effect on motor behaviour. (A) Compared to the control genotypes (*Mef2-GAL4 > Dcr-2* or *Mef2-GAL4 > Glos RNAi*), the time taken for the first fly to reach a predetermined threshold is significantly increased on day 35 posteclosion in sample populations having reduced levels of Gaulos (Glos) in muscles (*Mef2-GAL4 > Dcr-2 + Glos RNAi*). (B) Similar motor defects are observed on all the days assessed in adult flies with brain-specific Glos depletion (*elav-GAL4 > Dcr-2 + Glos RNAi*) compared to the controls (*elav-GAL4 > Dcr-2* or *elav-GAL4 > Glos RNAi*). The phenotype is highly pronounced on day 35 of adult life. (C) Glos knockdown in neurons also induces an age-dependent progressive decline in adult viability. (D) Muscle- or brain-specific loss of Glos function exposes an age-dependent decrease in flight capacity. Hence, midway through adult life, a significant percentage of organisms were nonfliers and, therefore, higher numbers fell in sector 1 of the Droso-Drome apparatus. In (A–D), data presented are the mean ± SEM of at least four independent experiments, and $n \geq 60$ males (♂) per genotype for each time point measured. Significance as tested by the unpaired *t*-test is indicated by the exact *P*-value.

human Gemin6 is 14% and 47%, respectively. Similarly, compared to human Gemin7, Sabbat has an overall percentage amino acid identity of 10% whereas similarity is close to 46%. In contrast to Gaulos/Gemin4 and Valette/Gemin8 (above), Hezron/Gemin6 and Sabbat/Gemin7 are not essential genes since our preliminary evidence indicates that flies are viable and healthy on global knockdown (data not shown).

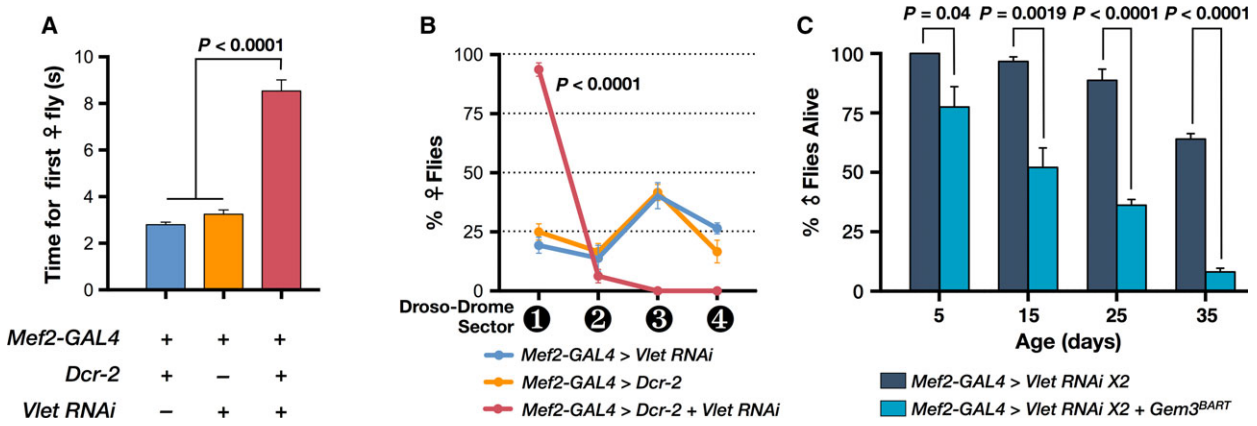

Fig. 7. Knockdown of Valette/Gemin8 selectively in muscles has a negative impact on motor behaviour. (A) When assessed at 48 h posteclosion, flies with muscle-specific *Valette* knockdown take significantly longer to reach a predetermined threshold compared to the control genotypes. (B) Moreover, the majority of organisms had a profound reduction in flight capacity, hence, a significant percentage fall straight to the lowest sector of the Droso-Drome apparatus in contrast to the control genotypes. (C) Muscle-restricted knockdown of Valette was also found to reduce survival in adult flies having the $Gem3^{BART}$ allele. In (A–C) data presented are the mean ± SEM of at least four independent experiments, and $n \geq 60$ per genotype for each time point measured. Sex of the genotype assessed is indicated by symbols: ♀ = females (A, B) and ♂ = males (C). Significance as tested by the unpaired *t*-test is indicated by the exact *P*-value.

Remarkably, through co-immunoprecipitation analyses, we show that Sabbat/Gemin7 is found in a stable complex *in vivo* with SMN (Fig. 4A) and Hezron/Gemin6 (Fig. 4B). These findings spurred us to ask whether a Gemin6-Gemin7-Unrip subunit, reported for the human SMN complex [7], is also present in *Drosophila*. Notably, we find that Sabbat/Gemin7 interacts strongly with both Hezron/Gemin6 and wmd/Unrip in a yeast two-hybrid assay (Fig. 8). Importantly, Sabbat/Gemin7 can latch to both Valette/Gemin8 and Gemin3 with comparable strength (Fig. 8). We also detect direct associations between Sabbat/Gemin7 or Hezron/Gemin6 and select Sm proteins, specifically SmB and SmD2 proteins (Fig. 8). Altogether, these findings constitute strong evidence favouring the presence of a Gemin6-Gemin7-Unrip module within the *Drosophila* SMN complex.

Discussion

The SMN complex is central to cellular metabolism as demonstrated by its presence in nearly all eukaryotes [6]. In contrast to the expanded version in metazoans including humans, *Drosophila* was reported to have a minimal SMN complex composed of only SMN, Gemin2/3/5, and, possibly, Unrip [11,14–16]. Hence, it has been suggested that the human SMN complex gained complexity in evolution through the incorporation of the remaining Gemins [11]. Our study has identified novel components of the *Drosophila* SMN complex with homologies to the elusive Gemin4/6/7/8. We speculate that similar to metazoan counterparts including *Apis* and *Nasonia* [11], an elaborate SMN complex is also present in *Drosophila*.

Previous studies have indicated that Gemin4/6/7/8 are core members of the human SMN complex [7,34–38]. Gemin4 is thought to be a co-factor for Gemin3's enzymatic activities [34]. Our work supports this hypothesis, hence, we were able to detect a strong functional relationship between Gemin3 and Gaulos, the *Drosophila* Gemin4 orthologue. Gemin8, which attaches to the human SMN complex primarily *via* SMN, is thought to recruit the Gemin6-Gemin7-Unrip trimer [7]. We show a similar interaction profile in *Drosophila*. Notably, we reveal that Gaulos/Gemin4 and Valette/Gemin8 are essential for neuromuscular survival and function in line with reports describing a similar requirement for SMN and Gemin2/3/5 [18,20,21,24,39,40]. Besides constituting evidence favouring these two proteins as the bona fide *Drosophila* orthologues of the respective Gemin counterparts in vertebrates, these findings also expose a novel *in vivo* function in the motor system for Gemin4 and Gemin8. Interestingly, corroborating our findings, a missense mutation in Gemin4 was recently found in three multiplex families with a recessive disorder encompassing global developmental delay and motor dysfunction [41]. Although Gemin8 has not been extensively characterised in vertebrates, based on our results, we predict that similar to Gemin4, it is required for developmental progression and motor function. In this context, Gemin8 might be a potential

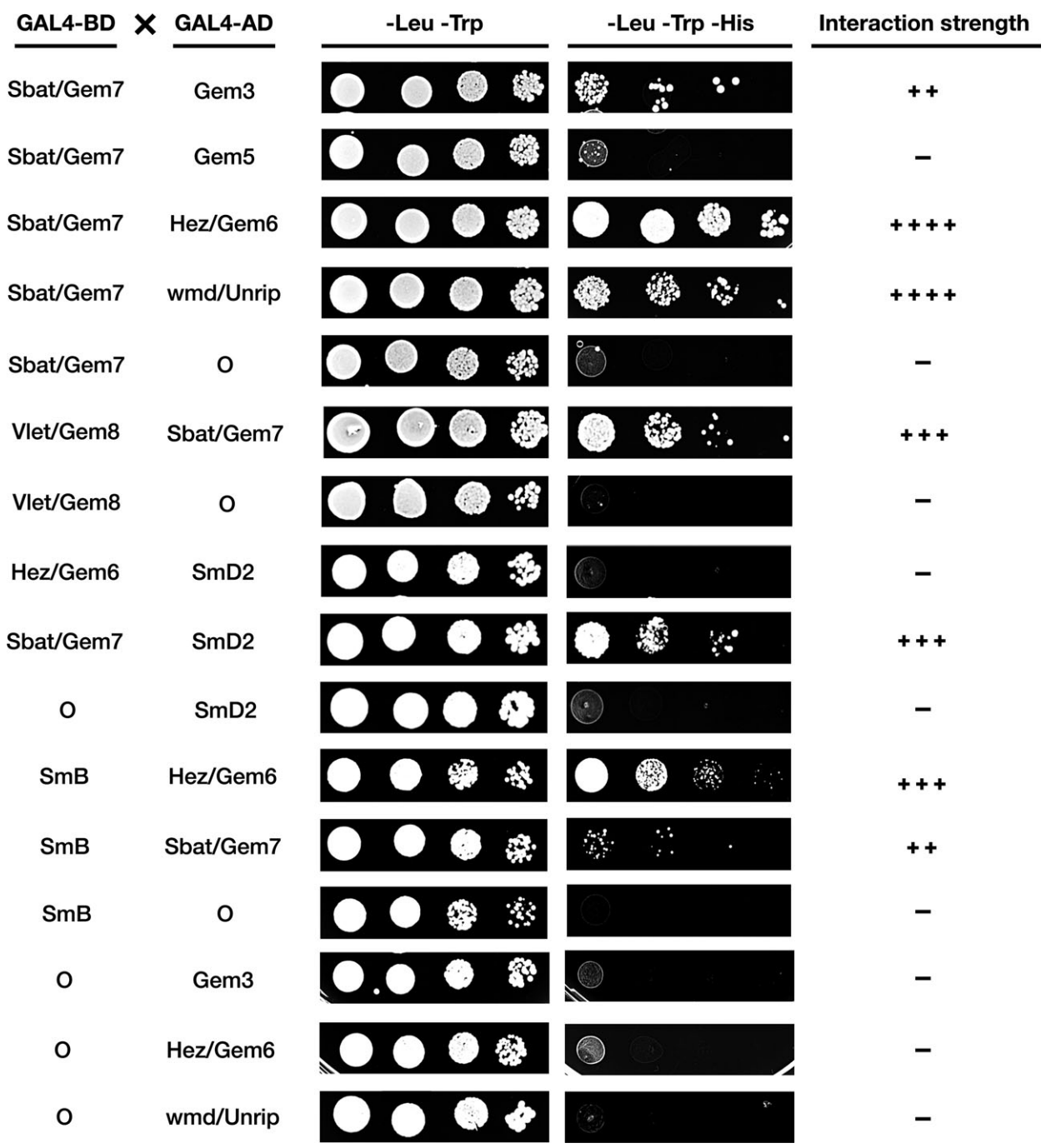

Fig. 8. Yeast two-hybrid interaction profile for Sabbat/Gemin7. The reporter strain carrying yeast two-hybrid plasmids expressing the indicated proteins was spotted in serial dilutions on –Leu–Trp or –Leu–Trp–His plates. Growth on –Leu–Trp–His plates indicates a positive interaction between Sabbat/Gemin7 and Gemin3, Hezron/Gemin6, wmd/Unrip, Valette/Gemin8 or Sm substrates D2/B. SmB was also found to associate with Hezron/Gemin6. Negative interactions are characterised by the absence of growth on –Leu–Trp–His plates. An empty vector or the unrelated Alix protein (both indicated by O) served as a negative control. At least three independent experiments were performed, and the result of one representative is shown.

target for screening in neurogenic disorders that have an as yet unidentified cause. We also report that, in contrast to the other Gemins, Hezron/Gemin6 and Sabbat/Gemin7 might not be vital for organismal survival. In view that the human counterparts have an Sm protein-like structure despite the lack of sequence

similarity with Sm proteins [42], an Sm-surrogacy role for both proteins during snRNP assembly could be redundant in *Drosophila*.

It remains unclear whether Unrip can be considered a steady member of the SMN complex in view that it associates with the complex only within the cytoplasmic compartment [43,44]. We have recently shown that similar to SMN and the Gemins, *Drosophila* wmd/Unrip is required in the neuromuscular system for adult viability and optimal motor performance [16]. Here, we show that wmd/Unrip together with Hezron/Gemin6 can be recruited to the *Drosophila* SMN complex via Sabbat/Gemin7. This interaction profile mirrors that present in the human SMN complex and together with earlier findings [16] favours the possibility that wmd/Unrip is also a core member of the *Drosophila* SMN complex.

It is noteworthy that the level of amino acid conservation of *Drosophila* Gemin4/6/7/8 is comparable to that of Gemin5, which is known to be moderately low [11]. This indicates that these orthologues are evolving significantly faster in *Drosophila* compared to other metazoans, hence, additional functions remain a likely possibility. It is with this in mind that we opted to give a synonymous gene name for the newly identified *Drosophila* Gemins. Furthermore, we do not exclude the possibility that the new *Drosophila* Gemins are morphing into different proteins and this could explain why bioinformatics prediction tools may identify a different human orthologue, albeit with modest sequence similarities, for *Hezron*/*Gemin6* (*LSM12*), *Sabbat*/*Gemin7* (*NAA38*) and *Valette*/*Gemin8* (*COMMD10*). Nonetheless, taken *in toto*, the evidence we present in this study strongly supports membership of the newly identified proteins within the *Drosophila* SMN complex.

Our identification and initial characterisation of novel SMN complex interactors lead us to conclude that we have discovered the remaining elusive Gemins in *Drosophila*. We propose that *Drosophila* has an elaborate SMN complex that is typical of metazoans. In addition to structure-based mechanistic studies, future research addressing the contribution of each member of the SMN complex to its operations, specifically within the neuromuscular system, is now possible in a genetically tractable multicellular model organism.

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Author contributions

Conceived and designed the experiments: RB and RJC. Performed the experiments: ML, RC, RMB, RB and RJC. Analysed the data: RB and RJC. Contributed reagents/materials/analysis tools: NV and FJ. Wrote the paper: RB and RJC.

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Supporting information

Additional Supporting Information may be found online in the supporting information tab for this article:
Fig. S1. Sequence conservation of Gemin4 orthologues.
Fig. S2. Secondary structure conservation of Gemin4 orthologues.
Fig. S3. Specificity of RNAi constructs utilised in the study.
Fig. S4. Sequence conservation of Gemin8 orthologues.
Fig. S5. Secondary structure conservation of Gemin8 orthologues.
Fig. S6. Sequence conservation of Gemin6 orthologues.
Fig. S7. Secondary structure conservation of Gemin6 orthologues.
Fig. S8. Sequence conservation of Gemin7 orthologues.
Fig. S9. Secondary structure conservation of Gemin7 orthologues.
Doc. S1. Materials and methods.
Table S1. Documented spatial and temporal expression patterns of GAL4 drivers utilised in the study.