Modeling spinal muscular atrophy in Drosophila links Smn to FGF signaling

Anindya Sen, Takakazu Yokokura, Mark W. Kankel, Douglas N. Dimlich, Jan Manent, Subhabrata Sanyal, and Spyros Artavanis-Tsakonas

1Department of Cell Biology, Harvard Medical School, Boston, MA 02115
2Department of Cell Biology, Emory University, Atlanta, GA 30322
3Collège de France, Paris 75005, France

Spinal muscular atrophy (SMA), a devastating neurodegenerative disorder characterized by motor neuron loss and muscle atrophy, has been linked to mutations in the Survival Motor Neuron (SMN) gene. Based on an SMA model we developed in Drosophila, which displays features that are analogous to the human pathology and vertebrate SMA models, we functionally linked the fibroblast growth factor (FGF) signaling pathway to the Drosophila homologue of SMN, Smn. Here, we characterize this relationship and demonstrate that Smn activity regulates the expression of FGF signaling components and thus FGF signaling. Furthermore, we show that alterations in FGF signaling activity are able to modify the neuromuscular junction defects caused by loss of Smn function and that muscle-specific activation of FGF is sufficient to rescue Smn-associated abnormalities.

Introduction

Spinal muscular atrophy (SMA) is an inherited neurodegenerative disease causing progressive deterioration of motor functions and loss of motor neurons (Azzouz et al., 2004). After cystic fibrosis, SMA is the most common autosomal recessive disorder in humans with an incidence of 1 in 6,000 and defines the most common genetic cause of infant mortality. SMA is caused by the loss of Survival Motor Neuron (SMN1), a ubiquitously expressed gene that encodes a key component of the SMN complex, which is essential for snRNP biogenesis. Biochemical studies established that SMN mediates the accuracy of interactions between RNA binding proteins and their target snRNAs in the cytoplasm (Massenet et al., 2002; Meister et al., 2002; Paushkin et al., 2002; Wan et al., 2005; Battle et al., 2006; Eggert et al., 2006; Zhang et al., 2008).

The human genome harbors two homologous, nearly identical genes encoding SMN1, SMN1, and SMN2. However, under normal conditions, SMN1 accounts for 90% of cellular SMN expression due to a splicing mutation in SMN2 that results in the production of only a small fraction (~10%) of full-length functional SMN (Lefebvre et al., 1997; Wolstencroft et al., 2005).

Thus, though SMA is caused by mutations that impair SMN1 function, the severity of the disease is modulated by SMN2 copy number, which varies in the human population (McAndrew et al., 1997). As SMN2 copy number increases, the amount of full-length SMN protein also increases, rendering loss of SMN1 less pathogenic. Therefore, cellular processes as well as single genes capable of augmenting SMN protein activity may be therapeutically relevant. To identify such processes/targets and gain insights into fundamental aspects of SMA, several different organisms, including Drosophila, are currently being used to model this disease (Schrank et al., 1997; Miguel-Aliaga et al., 1999, 2000; Frugier et al., 2000; Hannus et al., 2000; Hsieh-Li et al., 2000; Monani et al., 2000; Owen et al., 2000; Paushkin et al., 2000; Chan et al., 2003; McWhorter et al., 2003; Rajendr et al., 2007; Chang et al., 2008; Briese et al., 2009; Kong et al., 2009).

The Drosophila genome encodes a single orthologue of SMN, the Survival motor neuron (Smn) protein, which is ubiquitously expressed and localizes to nuclear gems (Chan et al., 2003; Liu et al., 2006; Chang et al., 2008), similar to the distribution observed in vertebrates (Monani, 2005). In Drosophila, Smn...
loss-of-function mutations result in reduced viability and decreased motility as well as muscular atrophy in the adult thorax, phenotypes analogous to the human pathology (Chan et al., 2003; Rajendra et al., 2007; Chang et al., 2008). Moreover, neuromuscular junction (NMJ) defects are associated with both vertebrate and invertebrate models (Chan et al., 2003; Chang et al., 2008; Kariya et al., 2008). In addition to its canonical subcellular distribution, Smn is also clearly concentrated in the postsynaptic region of the larval NMJ (Chang et al., 2008) and has been reported to localize to sarcomeres of adult myofibrils (Rajendra et al., 2007). Despite this, tissue-specific reduction of Smn demonstrates that normal NMJ morphology requires Smn activity in both muscles and neurons (Chang et al., 2008). Finally, an observation of critical importance to the Drosophila model is that the dosage and the physiology of the NMJ are sensitive to levels of Smn (Chang et al., 2008; unpublished data), mirroring the SMN2 dosage dependence observed in SMA patients.

Taking advantage of the dosage sensitivity of Smn loss-of-function phenotypes, we performed systematic genetic screens to identify modifiers of Smn activity (Chang et al., 2008). Among the genes identified in this manner was the breathless locus, which encodes one of the two Drosophila FGF receptors (Glazer and Shilo, 1991). In general, the FGF pathway has been demonstrated to be involved in a diverse range of cellular and developmental processes, including proliferation, migration, differentiation, and apoptosis (Itoh and Ornitz, 2004; Huang and Stern, 2005). In Drosophila, this pathway has been demonstrated to control the development of the tracheal system (Ghabrial et al., 2003) and the musculature (Shishido et al., 1993, 1997; Beiman et al., 1996; Gisselbrecht et al., 1996; Michelson et al., 1998; Vincent et al., 1998; Schulz and Gajewski, 1999; Stathopoulos et al., 2004). In contrast, the role of FGF in the Drosophila nervous system remains poorly characterized (García-Alonso et al., 2000; Forni et al., 2004).

In this study, we investigate the relationship between Smn and several components of the FGF pathway, demonstrating a clear link between Smn and FGF. Epistasis analysis reveals that Smn regulates FGF signaling output, and molecular studies indicate that Smn activity influences FGF receptor transcript levels. Furthermore, we show that activation of FGF signaling can restore Smn-associated NMJ defects, thus raising the possibility that FGF can act as a protective modifier of SMA.

Results

The FGF signaling pathway and Smn breathless (btl), which encodes one of the two known Drosophila FGF receptors, was identified in a genetic screen as a modifier of Smn-dependent lethality (Chang et al., 2008), suggesting a connection between the FGF pathway (Fig. 1 A) and Smn. We extended this finding by determining the effect of different btl mutations on Smn-dependent viability using an inducible RNAi allele of Smn, UAS-Smn-RNAiFL26B (FL26B), which displays reduced viability when ubiquitously expressed by the tubulinGAL4 (tubGAL4) driver (Chang et al., 2008). This phenotype was modified by multiple btl alleles (btlflod2s64, btlc1, and UAS-λbtl) as judged by our survival assay (Fig. S1, A and B). These genetic results confirm btl as a bona fide modifier of Smn loss-of-function mutations, thereby validating our initial observations.

If, as the above analysis of btl implies, FGF signaling can modulate Smn activity, we expect other genetic elements of the FGF pathway to behave as Smn modifiers as well. We chose to examine this relationship in the mesoderm, as the activity of the FGF signaling pathway has been shown to be important for the development and the maintenance of muscles (Shishido et al., 1993; Beiman et al., 1996; Gisselbrecht et al., 1996; Michelson et al., 1998; Vincent et al., 1998; Schulz and Gajewski, 1999; Stathopoulos et al., 2004). The mesoderm-specific how24B GAL4 driver was used to control expression of inducible RNAi transgenes that specifically target either of the two Drosophila FGF receptors, btl or heartless (htl), and a specific FGF signaling effector, stumps. We monitored the effects of these mutations on two additional Smn RNAi strains, UAS-Smn-RNAifl26B (C24) and UAS-Smn-RNAIN4 (N4), which, based on phenotypic analyses and Smn expression levels, are of increasing allelic strength with respect to the FL26B allele (Chang et al., 2008).

In control experiments, we observe an Smn-independent effect on viability in backgrounds in which Btl, Htl, or Stumps expression were reduced, whereas no effect on viability was observed upon removal of one copy of sprouty (stpy), an inhibitor of the pathway (Fig. 1, B and C). However, when Smn activity is reduced in each of these backgrounds, a further decrease in viability is detected (Fig. 1, B and C). Moreover, loss of function for the FGF pathway antagonist stpy suppresses Smn-induced lethality (Fig. 1 C). Based on these observations, we conclude that a genetic link between Smn activity and the FGF signaling cascade exists in the mesoderm.

Reduction of FGF signaling in muscles causes NMJ defects

Previous analyses established that the vast majority of mutations in genes that altered the viability of Smn loss-of-function mutations (Chang et al., 2008) were also accompanied by structural defects in the larval NMJ (Chang et al., 2008). As multiple alleles of several FGF signaling elements modify Smn-dependent lethality, we assessed the significance of this interaction at the third larval instar NMJ in several different genetic backgrounds.

An initial examination of the distribution of Htl revealed that it is specifically expressed at the NMJ during the third instar (Fig. 2, A–L; and Fig. S2). This expression appears to be primarily in the muscle, as it coincides with the postsynaptic marker Discs Large 1 (Dlg; Fig. 2 B) and does not obviously overlap with the presynaptic marker nc82 (Bruchpilot; Fig. 2 E). This is corroborated by the localization of additional presynaptic markers, Cysteine string protein (Csp; Fig. 2 H) and anti-horseradish peroxidase (anti-HRP; Fig. 2 K), which label synaptic vesicles and neuronal membranes, respectively, and appear to be distinct from Htl expression. Together, these data suggest that Htl is predominantly postsynaptic, as indeed is Smn.

The presence of the htl ligands, pyramus (pyr) and thisbe (ths) at the NMJ would suggest that Htl is active, but the localization of the ligands has not been previously described. In embryos, it is known that the ligands are expressed in the epithelia adjacent to the mesoderm, which expresses htl.
To corroborate these observations and ensure that the structural defects we observe at the larval NMJ are associated with the FGF pathway, we modulated the activities of htl and its downstream effectors, stumps (dof) and sprouty (sty) using GAL4-inducible transgenic strains to determine whether further modifications in FGF signaling result in structural defects at the larval NMJ. Muscle-specific expression of sty, a dominant inhibitor of the pathway (how24BGAL4:UAS-sty; Fig. 3, D and G; and Fig. S3 A), caused a pronounced synaptic overgrowth and an overelaboration of synaptic terminals (how24BGAL4:UAS-stumps; Fig. 3, D and G; and Fig. S3 A). Quantification of the number of synaptic boutons,
Figure 2. Heartless localizes to the postsynaptic region of the Drosophila larval NMJ. All panels show wild-type NMJs derived from larval muscle 4. (A, D, G, and J) Htl (green) expression in the NMJ boutons. (B) Dlg (red) marks the postsynaptic region of the NMJ. (C) Htl (green) and Dlg (red) expression coincide at the larval NMJ. (F) Htl (green) and the presynaptic nc82 (red) expression do not overlap at the NMJ boutons. (I) Mutually exclusive expression of Htl (green) and presynaptic marker Csp (red) at the larval NMJ. (L) Htl (green) expression does not colocalize with presynaptic HRP staining (red). (M and N) qPCR from mRNA derived from tissues extracted from third instar larvae reveals the expression of htl and its ligands, ths and pyr, in the brain (M) and muscle (N). Bar, 5 µm.
which reflects synaptic size, revealed that muscle-specific *stumps* overexpression resulted in a 15–20% increase in the number of synaptic boutons at muscle 4 (Fig. 3 G and Fig. S3 A) and a 35% increase at the muscle 6/7 A3 synapse (Fig. S4) relative to controls. Though there was a significant effect on NMJ size, there were no gross morphological defects as the localization of pre-(anti-HRP) and postsynaptic markers (anti-Dlg) were not detectably altered, and there was no major variation in muscle size or morphology. Thus, the effects of FGF loss-of-function (Fig. 3, B, C, and G; and Fig. S3 A) are opposite to those observed for FGF gain-of-function (Fig. 3, D and G; and Fig. S3 A) in the regulation of synaptic elaboration on muscle 4, a strong genetic argument in favor of a functional role for FGF signaling at the NMJ.

In contrast, presynaptic expression of RNAi transgenes for *htl* and *stumps* using the *elavGAL4* driver did not result in any measurable changes in synaptic size (*elavGAL4:UAS-htl* or *elavGAL4:UAS-stumps*; Fig. 3, E–G; and Fig. S3 A), suggesting that *htl* and *stumps* are not active presynaptically. It is important to point out, however, that we cannot exclude a presynaptic role for these elements of the FGF pathway given the possibility that RNAi in neurons may not have been effective. Despite this caveat, these experiments demonstrate that activation of the FGF pathway in the muscle is required to regulate the size of the NMJ.

**FGF signaling in muscles affects responsiveness to presynaptic transmitter release**

The preceding experiments show that altering FGF components in the muscle influences presynaptic morphology, which is likely accompanied by changes in either transmitter release or postsynaptic receptivity. To directly test this possibility, we performed electrophysiological measurements of evoked excitatory junction potentials (EJPs) under conditions in which we either increased or decreased FGF signaling selectively in the muscle using the *how24BGAL4* driver. Perturbation of FGF signaling in muscles primarily leads to altered mEJP (miniature EJP) amplitude or quantal size (Fig. 4), a phenotype most often associated with the postsynaptic compartment (Petersen et al., 1997). Thus, increasing the expression of wild-type Htl decreases the average mEJP amplitude by more than 50%, whereas inhibition of FGF signaling through expression of the dominant-negative *htl* transgene increases mEJP amplitude by 50%. Interestingly, such reciprocal regulation of quantal size by *htl* is also mirrored in our EJP measurements (Fig. 4, A and B). As a result, quantal content (defined as the number of synaptic vesicles released per action potential and estimated by dividing the mean EJP response by the mean mEJP amplitude) remains essentially unchanged across genotypes. In addition, there are no observed changes in the frequencies of spontaneous release. It is noteworthy that changes in EJP and mEJP values are contrary to those observed for presynaptic bouton number, an observation most parsimoniously explained through feedback mechanisms.
that are known to operate at the larval NMJ to control its structural and functional properties (see Discussion). Therefore, the morphological changes associated with FGF signal modulation at the NMJ are accompanied by functional abnormalities.

Synergy of Smn and FGF signaling in muscles regulates NMJ growth

Given that the effects of loss of FGF signaling on NMJ morphology are independent of Smn, it is possible that the observed interactions between this pathway and Smn arise from the cumulative, rather than synergistic, effects of these mutations. If these interactions were due to additive effects, then the introduction of a single recessive allele of either btl or htl would not be expected to significantly alter the NMJ phenotype of Smn mutants. Therefore, we assayed whether either a strong hypomorphic allele of btl (btl^{dev1}) or a null allele of htl (htl^{AB42}) could modify as heterozygotes the reduction of the NMJ size caused by the muscle-specific expression of the UAS-Smn-RNAi^{C24} allele.

Figure 4. Postsynaptic FGF signaling regulates the quantal size of transmitter release. (top) Representative recordings of EJP and mEJP at 0.5 mM extracellular Ca^{2+} are shown for control (how24BGAL4-w[iso]), muscle expression of wild-type Htl (how24BGAL4-UAS-htl), muscle expression of a Htl dominant-negative Htl (how24BGAL4-UAS-htl[DN]), and muscle expression of an RNAi targeted against Htl (how24BGAL4-UAS-htl[RNAi]). Whereas Htl expression reduces both EJP and mEJP amplitude, Htl inhibition leads to significantly larger EJP and mEJP amplitudes. Horizontal scale bar is 100 ms for EJPs and 200 ms for mEJPs. Dotted line represents magnitude of control EJP. (bottom) Quantification of EJP amplitude, mEJP amplitude, quantal content, and mEJP frequency in the four genotypes. Both EJP and mEJP amplitude are altered after experimental perturbation in Htl signaling in the muscle. Quantal content of transmitter release, however, remains unchanged. Similarly, the frequency of spontaneous release is comparable across genotypes. Asterisks denote P < 0.01 (ANOVA). The number of animals recorded for each genotype is shown within the first graph.
Examination of larvae heterozygous for either of the two FGF receptors (how24BGAL4/btldev1 or how24BGAL4/htlAB42) revealed the introduction of these mutations had no significant effect on the NMJ size of animals from the how24BGAL4 driver genetic background (how24BGAL4/+; Fig. 5, A, C, E, and G; and Fig. S3 B). In contrast, when Smn function is reduced by the introduction of the UAS-Smn-RNAiC24 allele into these genetic backgrounds (how24BGAL4 UAS-Smn-RNAiC24/btldev1 and how24BGAL4 UAS-Smn-RNAiC24/htlAB42), these heterozygous FGF receptor mutations elicited a drastic decrease in synaptic size when compared with loss of Smn alone (how24BGAL4 UAS-Smn-RNAiC24/+; Fig. 5, B, D, and G; and Fig. S3 B).

Because these results indicate the link between FGF signaling and Smn activity is synergistic rather than additive, we applied a further, more stringent assay to determine the sensitivity of the NMJ to this interaction. Examination of transheterozygous larvae carrying a combination of null alleles of Smn and htl (SmnX7/htlAB42) revealed that simultaneously reducing the dosage of each locus by half leads to a statistically significant decrease in the number of synaptic boutons per unit MSA when compared with each heterozygote alone (SmnX7/+ and +/htlAB42; Fig. 5 G and Fig. S3 B). Thus, this experiment provides formal evidence that the relationship we have uncovered is synergistic in nature and is not merely due to the additive effects of two mutations that independently affect the NMJ.

**Activation of FGF signaling in muscles rescues synaptic defects caused by Smn RNAi**

Because reduction of FGF signaling clearly exacerbates the NMJ defects caused by Smn loss, we examined whether activation of this pathway could reverse these effects. As shown in Fig. 6, muscle-specific overexpression of wild-type Htl completely rescues the NMJ phenotypes associated with both the UAS-Smn-RNAiC24 allele and the stronger UAS-Smn-RNAiN4 allele (Fig. 6, B–D and G; Fig. S3 C). In control crosses, expression of Stumps alone resulted in an expansion of synaptic branching (Fig. 6 E); however, as observed for Htl, expression of Stumps completely suppressed the NMJ defects observed in the how24BGAL4; UAS-Smn-RNAiC24 background (Fig. 6 F and G; and Fig. S3 C). These results suggest that Smn is situated upstream of the FGF pathway. Based on this genetic behavior, however, we could not distinguish whether Smn directly regulates htl or any other pathway component.

**Smn regulates expression of the FGF receptor, Htl, and its downstream effector Stumps**

To further explore the relationship between Smn and FGF signaling pathway components, we first tested whether reducing Smn number/muscle. P ≤ 0.05. Bar, 50 µm. n = 40. All preparations were stained with anti-HRP (red) and anti-Dlg (green). The muscle nucleus was labeled using DAPI.
expression impacts expression of Htl and Stumps. Using the how24B driver to direct expression of the UAS-Smn-RNAiN4 transgenic construct in the musculature resulted in a drastic postsynaptic reduction in Htl levels at the NMJ (Fig. 7, D–F) as well as a more general loss of Stumps staining throughout the muscle (Fig. S5). Conversely, we did not detect any obvious changes in the distribution or levels of the Smn protein when htl activity was reduced postsynaptically (how24BGAL4/UAS-htlDN; Fig. 7, G–I), indicating that FGF activity does not influence Smn expression. These observations corroborate the relationship observed above and indicate that Smn regulates elements of the FGF pathway.

Ideally, the aforementioned observations, which address the issue of autonomy in the Smn–FGF pathway interaction, would have included data derived from classical alleles of Smn. Unfortunately, however, it is practically impossible to generate the motor neuron or muscle mitotic clones necessary to examine this question. For this reason, we extended our observations to include the Drosophila wing imaginal disc, a tissue that is well suited to this type of experiment and where the distribution and expression levels of elements of the FGF pathway have been well characterized (Sato and Kornberg, 2002). An additional benefit of analyzing this relationship in the wing disc is that it allows us to determine whether the ability of Smn to regulate Htl is not specific for the NMJ but is conserved across tissues.

Therefore, we monitored Htl and Stumps expression in Smn loss-of-function mitotic clones that were generated using several different classical alleles of Smn. In these Smn−/− cells, both Htl and Stumps expression were lost (Fig. 8, A–F). htl expression is unaffected in stumps mutant embryos, and conversely, stumps expression is unaffected in htl mutant embryos (Vincent et al., 1998), and we thus attribute these observations in the wing imaginal disc to the loss of Smn activity. Together, these results both corroborate and extend our investigation of the epistatic relationship established between Smn and FGF signaling.

As Smn is required for basic RNA metabolism, removal of its activity might be expected to affect expression of multiple proteins. Therefore, to test the specificity of the molecular relationship between Smn and FGF, we monitored the effects of loss of Smn function on the expression of F-actin and the transcription factor, Cut, a gene whose expression overlaps that of Htl in the developing wing. Our results indicate that removal of Smn activity had no effect on the distribution or levels of either cytoskeletal actin (Fig. 8, G–I) or Cut (not depicted) in the wing imaginal disc. Consistent with this notion, reduction of Smn at the NMJ had no discernable effect on Dlg expression (Fig. 7, D–F).

RNAi-induced knockdown of Smn affects htl transcript levels

Having established that reduction of Smn at the NMJ or removal of Smn activity in the wing imaginal disc reduces or eliminates Htl expression, respectively (Fig. 7 D, Fig. 8 B), indicating that the SMN–FGF relationship is not specific for the NMJ, we were...
interested in exploring the underlying mechanism. Given the role of Smn in snRNP biogenesis, it is possible that reduction of Smn could alter levels of htl transcript, so we tested this using mRNA isolated from third larval instar brains. As shown in Fig. 8 J, knockdown of Smn (tubulinGAL4::UAS-Smn-RNAiN4) results in Smn transcript levels being reduced to 16% of wild type (tubulinGAL4). Concomitantly, htl transcript is reduced to 45% of the wild-type value. Interestingly, coexpression in the aforementioned genetic background of two independent insertions of Smn transgenes (UAS-Smn-FLAGB and UAS-Smn-FLAGC) that partially rescue Smn lethality (Chang et al., 2008) results in slight increases in Smn transcripts. It is noteworthy that, depending on the construct used, we observed significantly different changes in htl levels. Remarkably, expression of UAS-Smn-FLAGC, which led to only a slightly higher increase in Smn levels (27% of wild type) when compared with the
Figure 8. Effects of Smn on the expression of FGF signaling pathway components in the wing disc. (A–I) Smn<sup>73Ao</sup>/Smn<sup>73Ao</sup> mitotic recombination clones in third instar wing imaginal discs stained for Smn (A, D, and G), Htl (B), Stumps (E), and F-actin (H). Smn, Htl, and Stumps expression were monitored using anti-Smn (green), anti-Htl, and anti-Stumps antibodies (red). In C and F, DAPI (blue) was used to identify nuclei. Notice that Htl (B) and Stumps (E) expression is not detected in Smn<sup>73Ao</sup>/Smn<sup>73Ao</sup> clones, whereas removal of Smn activity has no effect on F-actin distribution (H). (C, F, and I) Merged images of A and B, D and E, and G and H, respectively, in which nuclei are also detected (blue). Note the presence of two Smn<sup>+</sup> cells that also express Stumps are located in the center of the clone depicted in D, E, and F. (J) Quantitative RT-PCR from third instar larval brains demonstrates that altering the level of Smn dosage
expression of UAS-Smn-FLAG\(^b\) (21% of wild type), fully restored \(htl\) transcript levels to the wild-type value, as opposed to the small rescue effect we see with UAS-Smn-FLAG\(^a\). These results demonstrate that reduction of \(Smm\) activity acts to modulate the levels of \(htl\) mRNA. Furthermore, it appears that a small increase in \(Smm\) may lead to a large change in the levels of \(htl\), which in turn influence the survival of the animal.

**Discussion**

Given the variability of the SMA phenotype and the proven relationship between the severity of the disease and small changes in wild-type SMN activity, there is a significant possibility that any modifiers of SMN activity, either direct or indirect, will have therapeutic value. To systematically explore the genome for genes that are capable of modulating SMN function in vivo, we took advantage of the existence of an SMA model offered by *Drosophila* to search for \(Smm\) genetic interactors (Chang et al., 2008). The model we developed is based on the lethality and an associated neuromuscular junction phenotype linked to loss of \(Smm\) function, a phenotype remarkably similar to the NMJ phenotype reported for human patients (Kariya et al., 2008). Though the role of SMN in biogenesis of snRNPs has been well documented, its regulators and downstream effectors have not been systematically delineated, nor has the link between mutations in SMN and the specific loss of motor neurons seen in SMA patients been uncovered. It may be the case that the specificity of this phenotype is reflective of either specialized SMN functions at the NMJ or a particular sensitivity of motor neurons to the loss of SMN activity (McWhorter et al., 2003; Carrel et al., 2006; Kariya et al., 2008; Murray et al., 2008, 2010; Kong et al., 2009). Among the genes our genetic strategy revealed as \(Smm\) loss of function modifiers was *breathless*, encoding an FGF receptor, thus establishing a link between *Smm* and the FGF pathway (Chang et al., 2008).

Importantly, in addition to this link, we also found that FGF signaling is independently involved in NMJ morphogenesis, a function demonstrated in vertebrates (Fox et al., 2007) but not previously attributed to this pathway in *Drosophila* (Chan et al., 2003). The genetic interaction we have demonstrated between FGF and *Smm* can be described as an epistatic relationship in which the FGF pathway functions downstream of *Smm* and is consistent with the observation that neuromuscular defects associated with loss of *Smm* function in muscle can be rescued by muscle-specific activation of FGF signaling. Intriguingly, the relationship we describe here between *Smm* and FGF is valid beyond the NMJ, as loss of *Smm* function affects transcript and protein levels of the FGF receptor, as well as the expression of additional elements of the FGF pathway. Whether this defines a cascade of interrelated events or whether each of these changes reflects an independent *Smm*-related regulatory event remains to be determined. Given the fact that *Smm* mutants in *Drosophila* display altered postsynaptic currents and severely compromised postsynaptic receptor clustering in muscles (Chan et al., 2003), it is conceivable that FGF signaling represents a link between *Smm* activity and postsynaptic glutamate receptor levels.

Here it should be noted that a link between SMN and the FGF pathway has been suggested by a series of studies in vertebrates where a molecular interaction between an FGF-2 isofrom and the SMN protein has been described (Claus et al., 2003, 2004; Bruns et al., 2009). These studies raise the possibility that FGF-2 may negatively interfere with SMN complex function through SMN itself. Such observations would, on first appearance, suggest that the epistatic relationship between SMN and FGF signaling in vertebrate cells may be the reverse of what we observe in *Drosophila*. In point of fact however, the differences in the experimental parameters and approaches between these studies do not allow meaningful comparisons.
An important question raised by the above phenotypic analyses is whether the abnormalities associated with FGF and/or Smn perturbations reflect developmental or maintenance issues. It may be the case that the larval system in *Drosophila* is not ideally suited to differentiate between these alternatives as larval tissue is destined to undergo programmed cell death (hastenosis) during metamorphosis. One advantage that flies do offer, however, is the ability to dissociate the development of the adult neuromuscular system from its maintenance as the entirety of its development occurs during the pupal stage, before emergence of the adult (Fernandes and Keshishian, 1998; Consoulas et al., 2002; Hebar and Fernandes, 2004). Thus, the *Drosophila* pupa/adult may provide a platform to address these issues, as *Drosophila* displays Smn-dependent adult phenotypes (unpublished data; Rajendra et al., 2007). In light of the relationship we established between Smn and FGF signaling and the known involvement of FGF signaling in the development of both the larval and adult musculature (Emori and Saigo, 1993; Shishido et al., 1993, 1997; Vincent et al., 1998; Imam et al., 1999; Schulz and Gajewski, 1999; Statkopoulos et al., 2004; Dutta et al., 2005; Wilson et al., 2005; Kadam et al., 2009), it will be particularly interesting to examine the effects of modulating FGF activity on the aforementioned processes. Such studies may be of particular relevance to SMA where it is quite difficult to discern the developmental consequences of SMN loss in humans, as neurodegenerative symptoms displayed by patients may obscure basic problems resulting from altered developmental programs such as neuronal pathfinding, initial NMJ formation, etc (Simic et al., 2008; Liu et al., 2010).

In vertebrates, synaptic development and maintenance use at least three distinct signaling mechanisms: the TGF-β, wingless, and FGF pathways. In *Drosophila*, it is noteworthy that the first two have been demonstrated to function in a similar fashion at the NMJ (Packard et al., 2002; McCabe et al., 2003). Remarkably, our genetic screens involving Smn have identified elements of all three of these pathways as modifiers of Smn-related phenotypes (Chang et al., 2008; unpublished data). We consider these observations particularly significant as they raise the possibility that Smn may serve as a node, integrating signaling events crucial for NMJ function, potentially leaving this structure particularly vulnerable to the loss of Smn. Though further correspondence between the *Drosophila* model and the human condition remains to be determined, the Smn–FGF relationship we observe in *Drosophila* raises the possibility that pharmacological manipulation of FGF signals might mitigate SMN motor neuron–related abnormalities.

**Materials and methods**

**Drosophila stocks and culture**

All fly stocks were maintained on standard fly medium at 25°C. The btlw/+, P[w+>−=UA5H1.DN[M33-B40], P[w+>−=UA5H1.DN[M33-B61 (UAS-hs*), P[w+>−=UA5H1.DN[MYDFR-F16 (UAS-hs*), sty57, and sty226 alleles were obtained from the Bloomington Drosophila Stock Center (Bloomington, IN). The btlw2884 allele was from the Exelixis collection at Harvard Medical School (Boston, MA), and the UAS-RNAi:btf1758, UAS-RNAi-hs*1758, and UAS-RNAi:stump3177 alleles were from the Vienna Drosophila RNAi Center (Vienna, Austria). The UAS-btf, UAS-sty, and UAS-stump [UAS-dof] transgenic lines were gifts from Denise Montell (Johns Hopkins University School of Medicine, Baltimore, MD; Cousins et al., 1996; Lee et al., 1996, Mark Krasnow [Stanford University School of Medicine, Stanford, CA; Sutherland et al., 1996], and Maria Leptin [European Molecular Biology Laboratory, Heidelberg, Germany; Vincent et al., 1998], respectively. UASFLAG-Smn, UAS-Smn-RNAi234, UAS-Smn-RNAi1826, and UAS-Smn-RNAi1828 transgenic strains were used to modulate the Smn expression level (Chang et al., 2008). The actGAL4 (Jao et al., 1997), elavGAL4 (Luo et al., 1994), houGAL4, P[GAL4] (Brand and Perrimon, 1993), and tubGAL4 (Lee and Luo, 1999) strains were used to drive the genes placed under the UAS promoter.

**Viability assay**

Three driver GAL4 males were crossed with four UAS transgenic females and cultured on standard fly media overnight. Flies were transferred to fresh media and allowed to fly for 2 d. Adults were subsequently discarded and the progeny were cultured at 25°C. For all crosses, the percentage of viability was calculated as (1) the total number of adults divided by the total number of pupae (defined as Adults); or (2) the total number of dead pupae 4–5 d after puparium formation plus the number of individuals that reached adult stages divided by the total number of pupae (defined as Late Pupal Viability). Viability was calculated based on four independent crosses for each genotype.

**Antibody staining**

NMJ preparation and analysis: third instar larvae were dissected in cold 1× phosphate-buffered saline (PBS) and fixed at room temperature (RT) for 20 min in 4% paraformaldehyde (PFA). The samples were washed in 0.1% Triton X-100 in PBS (PTX) and incubated overnight at 4°C with primary antibody. The primary antibody was washed off with PTX at RT. The samples were incubated at RT with secondary antibody for 90 min. This was followed by PTX wash, and the tissues were mounted in Vectashield mounting media with DAPI (Vector Laboratories). Bouton numbers were counted using a microscope (TE2000; Nikon), based on the Discs large and anti-HRP staining in the A3 segment muscle 4 as indicated. The muscle area for every animal was measured, and no significant difference was observed among different genotypes. At least 20–25 animals of each genotype were dissected for the bouton analysis. The ANOVA multiple comparison test was used for statistical analysis of the bouton number/muscle. The images were pseudo-colored using Adobe Photoshop CS2 (v9.0.2). Wing imaginal disc preparation and analysis: third instar larvae were dissected and fixed as described previously (Kankel et al., 2004). Discs were stained at RT with the following primary antibodies in PBS Triton X-100 (PBS/Tx): mouse anti-Smn (Chang et al., 2008) at 1:500; rabbit anti-HTL (Shishido et al., 1997) at 1:3,000; rabbit anti-Stumpa (Vincent et al., 1998) at 1:2,000; and mouse anti-Cut (Developmental Studies Hybridoma Bank) at 1:10; and were visualized with Alexa Fluor 488 goat anti–mouse (green) and Alexa Fluor 594 goat anti–rabbit (red), both at 1:000 (Invitrogen). Alexa Fluor 594 conjugated to phallidin was used at 1:100 (Invitrogen). Discs were mounted in Vectashield with DAPI.

**Microscopy**

All images were collected with a spectral point scanning confocal [model C1s; Nikon] connected to an inverted microscope (TE2000; Nikon) equipped with DIC, phase, and epi-fluorescence optics, 40× Plan Fluor NA 1.4 objective lens; the Perfect Focus System for continuous maintenance of focus; 100 mW mercury arc lamp illumination for viewing fluorescence by eye; and confocal scanning using solid-state diode lasers (Melles Griot): 405 nm, 488 nm (10 mW), and 561 nm (10 mW). The image acquisition software used was Nikon EZ-C1. All samples were mounted and imaged in Vectashield mounting medium with DAPI (Vector Laboratories) at room temperature. Adobe Photoshop CS5 was used to pseudocolor images.

**Electrophysiology**

Electrophysiological recordings were made from muscle 6 in segment A3 from wandering third instar larvae in modified HL3 saline (0.5 mM Ca2+) as described previously (Stewart et al., 1994; Sanayal et al., 2002; Kim et al., 2009). In brief, electrodes with tip resistances between 25–30 MΩ were used to record evoked excitatory junction potentials (EJPs) after a stimulus train delivered at 0.5 Hz to the segmental nerve such that both units were consistently recorded. Only recordings where the resting membrane potential was more polarized than ~60 mV were selected for analysis. Because EJPs were larger than 10 mV in amplitude, Martin’s correction was not ideally suited to differentiate between these alternatives as larval tissue is destined to undergo programmed cell death (hastenosis) during metamorphosis. One advantage that flies do offer, however, is the ability to dissociate the development of the adult neuromuscular system from its maintenance as the entirety of its development occurs during the pupal stage, before emergence of the adult (Fernandes and Keshishian, 1998; Consoulas et al., 2002; Hebar and Fernandes, 2004). Thus, the *Drosophila* pupa/adult may provide a platform to address these issues, as *Drosophila* displays Smn-dependent adult phenotypes (unpublished data; Rajendra et al., 2007). In light of the relationship we established between Smn and FGF signaling and the known involvement of FGF signaling in the development of both the larval and adult musculature (Emori and Saigo, 1993; Shishido et al., 1993, 1997; Vincent et al., 1998; Imam et al., 1999; Schulz and Gajewski, 1999; Statkopoulos et al., 2004; Dutta et al., 2005; Wilson et al., 2005; Kadam et al., 2009), it will be particularly interesting to examine the effects of modulating FGF activity on the aforementioned processes. Such studies may be of particular relevance to SMA where it is quite difficult to discern the developmental consequences of SMN loss in humans, as neurodegenerative symptoms displayed by patients may obscure basic problems resulting from altered developmental programs such as neuronal pathfinding, initial NMJ formation, etc (Simic et al., 2008; Liu et al., 2010).

In vertebrates, synaptic development and maintenance use at least three distinct signaling mechanisms: the TGF-β, wingless, and FGF pathways. In *Drosophila*, it is noteworthy that the first two have been demonstrated to function in a similar fashion at the NMJ (Packard et al., 2002; McCabe et al., 2003). Remarkably, our genetic screens involving Smn have identified elements of all three of these pathways as modifiers of Smn-related phenotypes (Chang et al., 2008; unpublished data). We consider these observations particularly significant as they raise the possibility that Smn may serve as a node, integrating signaling events crucial for NMJ function, potentially leaving this structure particularly vulnerable to the loss of Smn. Though further correspondence between the *Drosophila* model and the human condition remains to be determined, the Smn–FGF relationship we observe in *Drosophila* raises the possibility that pharmacological manipulation of FGF signals might mitigate SMN motor neuron–related abnormalities.
We thank Mark Krzanow, Maria Leptin, and Denise Montell for fly lines. The anti-Htl antibody was a gift from Testuya Kojima, and anti-Stumps antibody was a gift from Maria Leptin. This work was supported by a grant from the SMA Foundation to S. Artavanis-Tsakonas, Anne Hart, and Davie van Vactor, whom we thank for extensive input throughout the course of this work. A. Sen was supported by a postdoctoral fellowship from the Families of Spinal Muscular Atrophy. S. Samay acknowledges support from grant 1803DA027970 from the NDIA, National Institutes of Health. The authors thank the Nikon Imaging Center at Harvard Medical School for help with light microscopy.

Submitted: 2 April 2010
Accepted: 10 January 2011

References

Azzouz, M., T. Le, G.S. Ralph, L. Walmley, U.R. Monani, D.C. Lee, F. Wilkes, K.A. Mitrophanous, S.M. Kingsman, A.H. Burghes, and N.D. Mazarakis. 2004. Lentivector-mediated SMN replacement in a mouse model of spinal muscular atrophy. J. Clin. Invest. 114:1726–1731.

Battle, D.J., M. Kasim, J. Yong, F. Lotti, C.K. Lau, J. Mouakel, Z. Zhang, K. Han, L. Wun, and G. Dreyfuss. 2006. The SMN complex: an assembly machine for RNPs. Cold Spring Harb. Symp. Quant. Biol. 71:313–320. doi:10.1101/sqb.2006.71.001

Beiman, M., B.Z. Shilo, and T. Volk. 1996. Heartless, a Drosophila FG receptor homolog, is essential for cell migration and establishment of several mesodermal lineages. Genes Dev. 10:2933–3002. doi:10.1101/gad.10.23.2993

Brand, A.H., and N. Perrimon. 1993. Targeted gene expression as a means of altering cell fates and generating dominant phenotypes. Development. 116:401–415.

Briese, M., B. Esmaeili, S. Frabolet, E.C. Burt, S. Christodoulou, P.R. Towers, K.E. Davies, and D.B. Sattelle. 2009. Deletion of smn-1, the Caenorhabditis elegans ortholog of the spinal muscular atrophy gene, results in locomotor dysfunction and reduced lifespan. Hum. Mol. Genet. 18:97–104. doi:10.1093/hmg/ddd320

Bruns, A.F. van Bergeijk, J. Lorbeer, A. Nölle, J. Jungnickel, C. Grothe, and P. Claus. 2009. Fibroblast growth factor-2 regulates the stability of nuclear bodies. Proc. Natl. Acad. Sci. USA. 106:12747–12752. doi:10.1073/pnas.090122106

Carrel, T.L., M.L. McWhorter, E. Workman, H. Zhang, E.C. Wolstencroft, C. Lorson, G.J. Bassell, A.H. Burghes, and C.E. Beattie. 2006. Survival motor neuron function in motor axons is independent of functions required for small nuclear ribonucleoprotein biogenesis. J. Neurosci. 26:11014–11022. doi:10.1523/JNEUROSCI.1657-06.2006

Chan, Y.B., I. Miguel-Aliaga, C. Franks, N. Thomas, B. Trübsch, D.B. Sattelle, K.E. Davies, and M. van den Heuvel. 2003. Neuro muscular defects in a Drosophila survival motor neuron gene mutant. Hum. Mol. Genet. 12:1367–1376. doi:10.1093/hmg/ddg157

Chang, H.C., D.N. Dimlich, T. Yokokura, A. Mukherjee, M.W. Kankel, A. Sen, V. Sridhar, T.A. Fulga, A.C. Hart, D. Van Vactor, and S. Artavanis-Tsakonas. 2008. Modeling spinal muscular atrophy in Drosophila. PLoS One. 3:e3209. doi:10.1371/journal.pone.0003209

Claus, P., D. Doring, S. Gringel, F. Klaer, T. Le, K. Claus, and P. Claus. 2009. Fibroblast growth factor-2 isoforms and specific interaction with the survival motor neuron protein. J. Biol. Chem. 278:479–485. doi:10.1074/jbc.M206056200

Claus, P., S. Werner, M. Timmer, and C. Grothe. 2004. Expression of the fibroblast growth factor-2 isoforms and the FGF receptor-1-4 transcripts in the rat model system of Parkinson’s disease. Neurosci. Lett. 360:117–120. doi:10.1016/j.neulet.2004.01.046

Consoulas, C., L.L. Restifo, and R.B. Levine. 2002. Dendritic remodeling and altering cell fates and generating dominant phenotypes. Development.
Abnormal motoneuron migration, differentiation, and axon outgrowth in spinal muscular atrophy. *Acta Neuropathol.* 115:313–326. doi:10.1007/s00401-007-0327-1

Stathopoulos, A., B. Tam, M. Ronshaugen, M. Frasch, and M. Levine. 2004. pyramus and thibse: FGF genes that pattern the mesoderm of *Drosophila* embryos. *Genes Dev.* 18:687–699. doi:10.1101/gad.1166404

Stewart, B.A., H.L. Atwood, J.J. Renger, J. Wang, and C.F. Wu. 1994. Improved stability of *Drosophila* larval neuromuscular preparations in haemolymph-like physiological solutions. *J. Comp. Physiol. [A]*. 175:179–191. doi:10.1007/BF00215114

Sutherland, D., C. Samakovlis, and M.A. Krasnow. 1996. branchless encodes a *Drosophila* FGF homolog that controls tracheal cell migration and the pattern of branching. *Cell.* 87:1091–1101. doi:10.1016/S0092-8674(00)81803-6

Thibault, S.T., M.A. Singer, W.Y. Miyazaki, B. Milash, N.A. Dompe, C.M. Singh, R. Buchholz, M. Demsky, R. Fawcett, H.L. Francis-Lang, et al. 2004. A complementary transposon tool kit for *Drosophila melanogaster* using P and piggyBac. *Nat. Genet.* 36:283–287. doi:10.1038/ng1314

Vincent, S., R. Wilson, C. Coelho, M. Affolter, and M. Leptin. 1998. The *Drosophila* protein Dof is specifically required for FGF signaling. *Mol. Cell.* 2:515–525. doi:10.1016/S1097-2765(00)80151-3

Wan, L., D.J. Battle, J. Yong, A.K. Gubitiz, S.J. Kolb, J. Wang, and G. Dreyfuss. 2005. The survival of motor neurons protein determines the capacity for snRNP assembly: biochemical deficiency in spinal muscular atrophy. *Mol. Cell. Biol.* 25:5543–5551. doi:10.1128/MCB.25.13.5543-5551.2005

Wilson, R., E. Vogelsang, and M. Leptin. 2005. FGF signalling and the mechanism of mesoderm spreading in *Drosophila* embryos. *Development.* 132:491–501. doi:10.1242/dev.01603

Wolstencroft, E.C., V. Mattis, A.A. Bajer, P.J. Young, and C.L. Lorson. 2005. A non-sequence-specific requirement for SMN protein activity: the role of aminoglycosides in inducing elevated SMN protein levels. *Hum. Mol. Genet.* 14:1199–1210. doi:10.1093/hmg/ddi131

Xu, T., and G.M. Rubín. 1993. Analysis of genetic mosaics in developing and adult *Drosophila* tissues. *Development.* 117:1223–1237.

Zhang, Z., F. Lotti, K. Dittmar, I. Younis, L. Wan, M. Kazim, and G. Dreyfuss. 2008. SMN deficiency causes tissue-specific perturbations in the repertoire of snRNAs and widespread defects in splicing. *Cell.* 133:585–600. doi:10.1016/j.cell.2008.03.031