HHS Public Access

Author manuscript

FEBS Lett. Author manuscript; available in PMC 2020 April 01.

Published in final edited form as:

FEBS Lett. 2019 April ; 593(7): 680–696. doi:10.1002/1873-3468.13348.

The impact of Megf10/Drpr gain-of-function on muscle development in *Drosophila*

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Abstract

Recessive mutations in multiple EGF-like domains 10 (MEGF10) underlie a rare congenital muscle disease known as MEGF10 myopathy. MEGF10 and its *Drosophila* homolog Draper (Drpr) are transmembrane receptors expressed in muscle and glia. Drpr deficiency is known to result in muscle abnormalities in flies. In the current study, flies that ubiquitously overexpress Drpr, or mouse Megf10, display developmental arrest. The phenotype is reproduced with overexpression in muscle, but not other tissues, and with overexpression during intermediate stages of myogenesis, but not in myoblasts. We find that tubular muscle subtypes are particularly sensitive to Megf10/Drpr overexpression. Complementary genetic analyses show that Megf10/Drpr and Notch may interact to regulate myogenesis. Our findings provide a basis for investigating MEGF10 in muscle development using *Drosophila*.

Keywords

Drosophila; Drpr; Megf10; Myogenesis; MEGF10 Myopathy

Introduction

MEGF10 myopathy or EMARDD (Early onset Myopathy, Areflexia, Respiratory Distress and Dysphagia) is a rare human congenital muscle disease caused by mutations in multiple

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EGF-like domains 10 (MEGF10) [1–3]. This single transmembrane receptor is expressed in skeletal muscles, the retina and in CNS glial cells, and is conserved through evolution. The *Drosophila melanogaster* (i.e., fruit fly) homolog of human MEGF10 is Draper (Drpr). Conservation of MEGF10 from insect to human, together with the versatility of fly genetics, and the identification in flies of adult muscle precursor cells (AMPs) that resemble satellite cells of higher organisms [4, 5] make *Drosophila* a useful model organism to investigate mechanisms underlying the pathogenesis of human MEGF10 myopathy. In addition to overlap in structure, similarity in reported function between human MEGF10 and fly Drpr has been described (e.g., regulation of glial engulfment of degenerating/apoptotic neurons, synapse sculpting during development) [6–17]. More recently, studies have revealed an important role for Drpr in the adult brain after axonal injury [18] as well as during aging [19]. Many gaps remain however in our understanding of the molecular functions of MEGF10/Drpr in muscle cells, and the potential efficacy and tolerance for restoration of MEGF10 in disease models has not been explored in that context. We have previously shown that loss-of-function mutations in Drpr lead to muscle alterations that recapitulate important features of the human disease [20]. We have generated and characterized a complementary model of MEGF10/Drpr gain-of-function in Drosophila muscle. Our MEGF10 loss of function and gain of function *Drosophila* models enable us to begin dissecting the conserved functional pathways regulated by this protein in muscle cells. Using our fly models, we have initiated genetic studies focused on interactions with the Notch pathway, which is an important regulator of muscle cell proliferation and differentiation.

Materials and Methods

Drosophila stocks and culture

The *UAS-drpr-I, UAS-drpr-II and UAS-drpr-III* lines [8, 9], *UAS-megf10,* and drprΔ5 mutant fly line (genotype: *w⁻; sp/CyO; drprΔ/TM6, sb, Tb, e*; where *w⁻; sp/CyO; drprΔ5/* drprΔ5 null are adult viable [12]), were generously provided by Mary Logan (Oregon Health Sciences University, Portland, OR, USA) and Marc Freeman (Vollum Institute, Oregon Health Sciences University, Portland, OR, USA). The genetic background strain w^{1118} (w allele FBal0018186) and *repo-Gal4* driver line (expression in glia) were donated by Mary Roberts (F. Rob Jackson laboratory, Tufts University School of Medicine, Boston, MA). The Gal4 driver lines listed in Table 1 were purchased from the Bloomington *Drosophila* Stock Center (Indiana University, Bloomington, IN). The *TinC-Gal4* driver line (expression in the heart) was donated by Dr. Matthew J. Wolf (Duke University, Durham, NC). All strains were raised at 25ºC in a 12 h light/12 h dark cycle on standard *Drosophila* media. To generate flies that overexpress *Mus musculus* Megf10, or *Drosophila* Drpr, transgenics carrying the corresponding *UAS-cDNA* transgene were crossed with Gal4 driver flies [21] at 29°C, 25°C, or 18°C (the strength of the *Gal4/UAS* system is temperature sensitive, and more efficient at higher temperatures). For each Gal4 driver assessed, the corresponding genetic cross was made at least twice; the number of adult progeny observed in each cross range from 10 to 120 flies.

Assessment of transcript levels

RT-PCR was used to assess the transcription level of the *Mus musculus Megf10* and *Drosophila Act5C* (housekeeping) genes. For *Drosophila* that express mouse Megf10 ubiquitously (i.e., *Act5C-Gal4/UAS-Megf10* flies), and corresponding controls (i.e., (i) *UAS-Megf10* transgene bearing flies, and (ii) w^{1118} genetic background flies), a pool of two male and three female (5-day-old) adult flies were used. Of note, a large number of Megf10 expressing flies die before reaching the adult stage. We thus used the rare Megf10 expressing mutant escapers, regardless of gender, (and a matching number of males/females control flies). RNA was extracted with the RNA STAT-60 (Tel-Test, Inc., Friendswood, TX) following the manufacturer's recommendations. DNA contamination was removed prior to the reverse transcription using a DNA-free kit (Ambion). Complementary DNA was generated using a Superscript First-Strand kit (Life Technologies) following the manufacturer's recommendations. The conditions for RT included cycles of 25ºC × 10 min, 42ºC × 20 min, and 99ºC × 5 min. Alternatively, genomic DNA was isolated from pooled adult male *UAS-Megf10* transgene-bearing flies using the QIAamo DNA Micro Kit (Qiagen), following the manufacturers' recommendations. PCR amplification was performed using the following protocol: pre-incubation at 94ºC × 10 min, followed by 35 cycles of amplification: 94ºC × 30 sec, 60ºC × 30 sec, and 72ºC × 1 min. The reaction was completed with a ten-minute incubation at 72ºC. Primers were synthesized at the Tufts University Molecular Core (Tufts University, Boston, MA). All primers were designed specifically for cDNA, (Table 1, of note, transgenic flies that express mouse Megf10 carry the corresponding cDNA transgene). The PCR reactions were analyzed on an agarose gel, and PCR band intensity was quantified using Image J software (NIH). Transcript levels were expressed as a ratio of the control (housekeeping) gene *Actin5C*. The primer pair sequences are: *Act5C* forward, 5'-CAGCCAGCAGTCGTCTAATCC-3';

Act5C reverse, 5'-CGACAACCAGAGCAGCAACTT-3';

Megf10_1 forward, 5'-CTGCCGATTCCTATCAGATC-3';

Megf10_1 reverse 5'-GCTCACTGTAGGTTCGACTT-3'

Megf10_2 forward 5'-GTTGTTCACCTGGGTACACTG-3'

Megf10_2 reverse 5'-AGAGCAGTCAGTCCCTTTGA-3'

Statistical analysis

Statistical analysis was performed using GraphPad Prism, using paired t test, or two-way ANOVA (GraphPad Software, Inc., CA).

Histological analysis

Histological analyses were performed in the Department of Pathology and Laboratory Medicine, Tufts Medical Center. Two day-old *Wg-Gal4>UAS-drpr III* mutant *Drosophila* (that escaped lethality) were immersed in Telly's fixative for seven days, embedded in paraffin, and sectioned at 5 um using standard techniques. All sections were stained with hematoxylin and eosin, then examined using standard bright-field light microscopy. All

images were reviewed by one of the authors (R.N.S.), a pathologist with extensive experience with *Drosophila* histology and histopathology.

Results

Flies that overexpress mouse Megf10 or fly drpr become arrested during development at the pupal/pharate adult stage.

Ubiquitous overexpression of Drpr isoforms I, II, III and mouse Megf10 in the w^{1118} wildtype fly background each leads to a nearly universal failure to eclose (Fig. 1), suggesting that the motif responsible for the lethality is to be found in all isoforms of Drpr, including the shortest one, Drpr-III. The few "escapers" (from progeny generated at 18oC, under minimal GAL4 activity [22]), were found to have structural abnormalities in the appendages (i.e., legs and wings). Strikingly the observed leg abnormalities, including darkened tibia/tarsus junctions and absent tarsal segments, are reminiscent of those seen in Drpr-deficient mutant flies (Supplementary Fig. 1). Molecular analysis confirmed the presence of the mouse *Megf10* transgene in the *UAS-Megf10* mutant flies (a gift from Mary Logan), as well as of *Megf10* RNA in the double *Gal4/UAS* transgenic escaper flies that express Megf10 in the entire organism (Supplementary Fig. 1, gDNA and cDNA analysis, respectively). The *UAS-Drpr I, UAS-Drpr II and UAS-Drpr III* lines (a gift from Marc Freeman), were previously characterized [9]. By using the *P{Act5C-GAL4}17bFO1* ubiquitous Gal4 driver (stock # 3954, Bloomington Drosophila stock center), it is possible to distinguish morphological differences between the experimental progeny that express the *Megf10* (or *drpr*) transgene, from control siblings that carry the transgene but do not express it. The latter carry a balancer third chromosome tagged with the dominant marker "Tubby" (*Tb*), which confers a shorter and thicker body size to the organisms, thus enabling easy quantification of experimental versus control flies throughout development (including the L1, L2, L3 larval, early and late pupal, and adult stages) (Fig. 2). We observed that flies that overexpress Megf10 or Drpr develop normally until metamorphosis then become arrested, while control siblings proceed to the adult stage (Fig. 2). Many Megf10- overexpressing flies reach the dark pupae/pharate adult stage where they die (Fig. 2). Similar results are obtained with Drpr I-, and Drpr III- overexpression, while Drpr II-overexpressing flies die as light pupae (data not shown). The number of experimental pupae is not significantly different from that of control sibling pupae (Tb) that develop simultaneously in the same vial (Fig. 2). Consistent with these findings, overexpression of Megf10/Drpr in the muscles of the embryo and larva (using the *c142-Gal4* driver) does not result in premature death (i.e., the expected number of flies emerge). These results provide a basis on which to further understand the outcome of genetic analyses using a range of tissue-specific Gal4 drivers (Table 1). While selected drivers may be active throughout *Drosophila* development, results with the ubiquitous driver indicate that high levels of Megf10/Drpr are tolerated at early stages (i.e., embryo, larvae) of development but are deleterious at later stages (i.e., pupal/pharate adult).

Overexpression of Megf10/Drpr is poorly tolerated in developing muscle, but benign in other tissues.

The lethality phenotype is reproduced with overexpression of mouse Megf10, Drpr-I or Drpr-II in muscle, using the *How-Gal4* driver. How-Gal4 also drives tendon expression,

however overexpressing Megf10 using the tendon-specific stripe *(sr)-Gal4* driver results in viable progeny (tendon overexpression of Megf10 thus does not cause the observed lethality). Similarly, targeted expression of Megf10/Drpr to the heart, glial cells, neurons, motor neurons, intestinal tract/malpighian tubules and fat body with corresponding drivers is well-tolerated (Table 1 and Fig. 3).

Drosophila are sensitive to Megf10/Drpr overexpression at a later timepoint in muscle development compared to Megf10/Drpr deficiency.

Specific Gal4 drivers were used to overexpress Megf10, Drpr-I, Drpr II or Drpr-III at different stages of myogenesis. *Drpr* is highly expressed in the adult muscle precursor (AMP)-like DmD8 cells (modENCODE Cell Lines RNA-Seq, http://flybase.org/reports/FBgn0027594.html), that express the transcription factor twist [23]. Targeted Megf10/Drpr overexpression to the AMPs/muscle precursor cells (using the *twist-Gal4* driver), as well as to muscle progenitor and founder cells (using the *kirre-Gal4* driver), is well tolerated. Targeted Megf10/Drpr expression to terminally differentiated muscle fibers (using the myosin heavy chain *(Mhc)-Gal4* driver) also result in viable progeny. Consistent with the latter observation, no deleterious effect is observed when Meg10/Drpr was expressed in the mature muscles of the adult fly, using the *DJ757* and *DJ667 Gal4* drivers (Table1). In contrast, lethality is observed when using the *Mef2-Gal4* (Mef2 induces differentiation) and *apterous-Gal4* (*apterous* is a muscle identity gene) drivers (Table 1 and Fig. 4). These results indicate that Megf10/Drpr overexpression is harmful at specific stages of myogenesis that occur later than the stage that is sensitive to Megf10/Drpr deficiency [20].

The detrimental effect of Megf10/Drpr overexpression is muscle subtype selective.

Comparative analysis of the outcomes of genetic crosses using the *apterous-Gal4*, *cut-Gal4* and *vestigial-Gal4* drivers reveals differential sensitivities of various developing muscles to increased expression of Megf10/Drpr. While crosses established with either *apterous-Gal4* or *cut-Gal4* lead to lethality in the experimental progeny, crosses with *vestigial-Gal4* result in the generation of viable adults (Table 1, Fig. 5). Intriguingly, in cut-positive cells Megf10 expression results in more severe effect than that induced by its fly counterparts, while the opposite is seen in apterous-positive cells. We cannot exclude that the subtle functional differences observed when the mouse versus the fly ortholog is expressed *in vivo* result from interspecies sequence/ domain variation, e.g., the length of the extracellular domain (857 versus 800 amino acids, respectively). Apterous (ap) and cut (ct) promote the development of tubular muscles that compose the direct flight muscles (DFMs) and jump/leg muscles, while vestigial (vg) positive fibers develop into the fibrillar indirect flight muscles (IFMs) (Fig. 5).

Sequence alignment of Drosophila and mammalian MEGF10 homologs highlights conservation in domains characteristic of Notch ligands, including a DOS motif found in the longer protein isoforms.

Drpr is the *Drosophila* homolog of human MEGF10, MEGF11, and MEGF12 (the last is also known as Jedi). Sequence alignment (CLUSTAL 2.1, Fig. 6) of Drpr-I/Drpr-PB (1031 aa, accession number NP_728660.2), Drpr-II/Drpr-PA (594 aa, accession number NP_477450.1), Drpr-III/Drpr-PC (528 aa, accession number NP_001246549.1), Mus

musculus Megf10 protein (1147 aa, accession number AAH75647.1) and Mus musculus Megf12/Jedi protein (1034 aa, accession number AF444274.1) revealed a high degree of conservation in the extracellular EMI domain (N-terminus), and in the Delta/Serrate/LAG-2 (DSL)-like domain that is found in many Notch ligands [24] (Fig. 6), as well as in four EGF-like/laminin-like domains close to the transmembrane region of the protein. Of note, a Delta and OMS-11 (DOS)-like motif highly reminiscent of the DOS motif displayed by multiple canonical Notch ligands [25] is found in the longest MEGF family members (i.e., Drpr-I, Megf10 and Jedi) but not in the shorter Drpr-II and Drpr-III (Fig. 6). Intracellularly, the NPXY motif (Fig. 1) postulated to bind the phosphotyrosine binding adapter ced-6 [9, 26] is conserved, as well as other downstream tyrosine residues (at the same position as or adjacent to Megf10 p.Y1002, p.Y1030 and p.Y1048, not shown) suggesting that these residues and their phosphorylation status may influence the observed overexpression-induced phenotype. We postulate that each of the conserved motifs plays an important role in mediating the function of this family of receptors.

Overexpression of Drpr under the control of the Serrate-Gal4 driver leads to a wing vein phenotype reminiscent of that obtained with Notch deficiency.

Targeting increased expression of Drpr in selected wing cells that express the Notch ligand Serrate (using the *Serrate-Gal4* driver) results in a split vein (extra-branching) phenotype at the wing margin (SerG4>Drpr, Fig. 7A-C), similar to that seen in flies with decreased Notch signaling activity [27]. The phenotype is 100% penetrant when overexpressing the short Drpr-II and Drpr-III isoforms, and moderately penetrant (~50%) when overexpressing the longer Drpr-I protein. The wing vein phenotype is also seen in Drpr-III overexpressing progeny generated with the *c179-Gal4* driver which targets, among other tissues, wing imaginal disc cells [28] (Fig. 7B). Notably, expressing Drpr-II in Serrate positive cells leads to exacerbation of the wing vein phenotype, and corresponding flies display an abnormal “held out wing” phenotype (i.e., the wings are held at a 45°–90°angle from the body, Fig. 7C) that is not observed with Megf10, Drpr-I or Drpr-III (data not shown). Of note, this phenotype is reminiscent of that seen in DWnt-2 mutant flies. *DWnt-2* activity is essential during pupation for proper DFM development [29]. In addition, SerG4>Drpr II flies display markedly reduced locomotor activity versus corresponding controls that do not express the Drpr-II transgene (Fig. 7D, Supplementary video).

Overexpression of Megf10/Drpr in Notch and Wingless positive cells leads to lethality. Severe muscle defects are seen in selected mutant flies that survive.

Increased expression of Drpr-I, Drpr-II, Drpr-III or Megf10 in Notch positive cells of the wing disc results death of the flies at the dark pupal/pharate adult stage (Fig. 7A and7E). In addition, increased expression of Drpr-II in Wingless (Wg)-positive cells also leads to developmental arrest at the dark pupal/pharate adult stage (Fig. 7A). Although some progeny that overexpress Megf10 or the other Drpr isoforms (i.e., Drpr-I, Drpr-III) in Wg cells can escape lethality, these flies are short-lived (data not shown). Notably, H&E staining of tissues from wgG4>Drpr-III adult flies that escape lethality show clear abnormalities in the tubular fibers that comprise the jump muscle, but not in the fibrillar fibers of the indirect flight muscles (Fig. 8, Supplementary Fig. 2).

Discussion

The current studies suggest that the mechanisms underlying Megf10/Drpr overexpression-induced damaging effects in *Drosophila* are related to somatic muscle, as only ubiquitous and muscle-specific overexpression led to lethality, whereas overexpression in multiple other tissues (including the tendons, heart, glia, neurons) was tolerated. It is well-established that two distinct waves of myogenesis produce the embryonic/larval musculature and adult musculature in *Drosophila* [30]. Our observation that Megf10/Drpr overexpressing flies develop seemingly normally until the pupal/pharate adult stages, where they die, suggest that increased levels of these proteins do not perturb embryonic/larval myogenesis, but are detrimental to adult myogenesis. Furthermore, overexpression of mouse Megf10 and the fly isoforms Drpr-I, Drpr-II and Drpr-III each leads to arrested fly development. A region common to all three Drpr isoforms and mouse Megf10 likely promotes the interactions that trigger the lethal phenotype.

Candidate regions include the N terminus of the protein (signal peptide, EMI and DSL motifs), the four distal EGF-like and transmembrane domains, and limited intracellular sequences including the NPXY signal motif (known to mediate the internalization of membrane proteins [31]) (Fig. 1B), as well as conserved tyrosine residues towards the C terminus. Among these tyrosines, MEGF10 Y1030 appears to be most closely associated with the development of EMARDD in humans, as defective phosphorylation at this residue has been linked to pathogenic mutations in the gene [1, 32]. Conversely, the intracellular ITAM domain that regulates established Megf10/Drpr-I mediated functions via interaction with Syk/Shark kinases (e.g., recruitment of macrophages to wounds [33] and engulfment/phagocytosis [15, 26]) is absent in Drpr-III (Fig. 1B) and thus not likely to underlie the lethality phenotype.

Sequence analysis and previous studies [34, 35] indicate that all three fly Drpr isoforms and mammalian Megf10 contain peptide sequences that share multiple conserved features with Notch ligands. These include (i) an N-terminus signal sequence followed by an Emilin-like domain (i.e., EMI), which participates in protein-protein interactions and can potently bind Notch1 [36], (ii) a Delta-Serrate-Lagged2 (DSL)-like domain which is a hallmark of Notch ligands, (iii) multiple extracellular Epidermal Growth Factor (EGF)-like repeats, and (iv) membrane anchoring via a single type I transmembrane domain. In addition, we show that the longest fly Drpr isoform (Drpr-I) and Megf10 both harbor a ‘Delta and OSM-11’ (DOS)-like motif located in the proximal EGF-like domains. Although DOS is found in many canonical Notch ligands and has been proposed to cooperate with the DSL motif to activate Notch [25], it is absent from Drpr-II and Drpr-III (Fig. 6), and is thus not a likely candidate region for the overexpression lethality phenotype. Of note, divergent Notch ligands that lack the DOS motif (e.g. Dll3) have been shown to play an important role in development (i.e., in somite segmentation) [37]. We postulate that at least one of the conserved extracellular domains present in the shortest isoform Drpr-III (i.e. EMI, DSL, four EGF-like domains), plays a major role in inducing fly lethality (when overexpressed in muscle) by compromising normal interactions with protein partners.

It is well established that the Notch pathway plays a central role in myogenesis. In skeletal muscle, Notch signaling regulates satellite cell self-renewal and myoblast proliferation while inhibiting myoblast differentiation [38, 39]. Similar functions have been proposed for Megf10 in myoblasts [32, 35, 40]. Further highlighting the parallels between Megf10/Drpr and Notch is the observation that developmental processes are sensitive to gene dosage in these proteins. Either haploinsufficiency or overexpression of DSL Notch ligands (wild-type or truncated forms lacking the intracellular portion) has been shown to result in morphological abnormalities [41]. Notably in *Drosophila*, both loss-of-function and gain-of-function of the Notch ligand Serrate result in similar defects at the neuromuscular junction [42]. Interestingly, we show that mutant flies that overexpress Megf10 ubiquitously and escape lethality display a tibia/tarsus leg phenotype similar to that seen in Drpr-deficient flies (Supplementary Fig. 1). It is known that Notch and its ligands, are involved in the formation of the tibial/tarsal and tarsal joints during segmentation of the insect legs [43, 44]. Our observation raises the possibility that altered levels of Megf10/Drpr affect Notch signaling during leg morphogenesis. It is unusual, however, for a gene to be poorly tolerated in both the deficient and overexpressed states [45, 46], as we observe with *Megf10/Drpr*.

It is known that complete homozygous knockouts for many genes lead to disease phenotypes, including in *Drosophila*. Haploinsufficiency phenotypes are less common but have been reported for *Drosophila* [47]. Overexpression-induced lethality, including dominant negative effects, are also less common. For example, overexpression of eighty percent of genes in yeast is well tolerated [48, 49]. In *Drosophila, C. elegans* and yeast, sensitivity to overexpression has been associated with an intrinsic lack of protein structure [50], the presence of linear binding motifs, and a high number of protein-protein interactions [51]. Megf10/Drpr may possess one or more of these properties.

Of note, flies that lack all adult muscles (as a result of Mef2 knockdown) become arrested as pharate adults [52]. Mef2 is essential to myoblast fusion. We have observed however that flies that overexpress Drpr-II in the entire organism die as light pupae; we thus hypothesize that Drpr-II may impair myogenic mechanisms prior to fusion, e.g., myoblast migration and/or adhesion, which might be more deleterious to the developing organism. Alternatively, increased Drpr-II expression may affect vital non-myogenic processes at the early pupal stage.

We previously reported that RNAi-mediated knockdown of Drpr/Megf10 in twist-positive muscle precursor cells results in a muscle/motor phenotype [20], however, overexpression in these same cells was found to be well-tolerated in the current study. Twist is a transcription factor highly expressed in AMPs [53, 54], and high levels of twist maintain AMPs in the undifferentiated state. In contrast, targeting Megf10/Drpr overexpression to downstream timepoints in myogenesis corresponding to the differentiation and specification stages (using the *Mef2-Gal4* and *apterous-Gal4* drivers, respectively) is deleterious to *Drosophila*, suggesting that Megf10/Drpr expression needs to be tightly regulated during these periods for myogenesis to conclude normally. Our analysis provides novel insights into the need for temporal control of Drpr expression *in vivo*.

In parallel, we observe that Megf10/Drpr overexpression has different effects on the two types of striated flight muscles: the indirect flight muscles (IFMs, composed of fibrillar fibers) and the direct flight muscles (DFMs, composed of tubular fibers). These muscles share the same pool of myoblasts until the late third instar larval stage, when the progenitors are specified into the two different muscle subtypes during pupation. At this stage, the adepithelial AMPs comprise cells that express either Vestigial (Vg, in distal myoblasts) or Cut (Ct, in proximal myoblasts), that are mutually exclusive transcription factors [55, 56]. Vg, a marker of the developing IFMs, induces the expression of fringe (fng), a glycosyltransferase that modifies Notch, leading to the inhibition of Notch signaling and IFM formation. Ct positive fibers give rise to the DFMs and do not express fng. During pupation, the LIM-homeodomain transcription factor apterous (Ap) is activated in Ct positive fibers and specifies DFM identity; Ap is repressed in Vg-positive muscle cells [55–58] (Fig. 5).

We show that transgene-induced Megf10/Drpr expression is well-tolerated in the developing IFM/ fibrillar fibers (using the *vestigial-Gal4* driver), but is lethal in the developing DFMs/ tubular fibers (using the *Ap-Gal4*, or *cut-Gal4*, driver). The opposite sensitivity has been described with persistent expression of Notch and twist which affects the IFMs, but not the DFMs [54]. Sensitivity of Ap positive cells to increased levels of Drpr are compatible with the results of a Beadex (Bx/dLMO) gain-of-function screen that identified Drpr loss-of-function (LOF) mutants as suppressors of the Bx-induced wing margin loss phenotype [59]. Bx is a LIM-domain protein that competes with Ap for binding to its cofactor, thus reducing Ap activity, which in turn leads to defective Notch signaling [59]. Drpr loss-of-function mutations in the Bx mutant background are also associated with loss of macrochaete on the fly notum, as seen with Notch gain-of-function mutations (Bejarano, 2008, Heitzler and Simpson 1991). In the developing IFM and DFM muscle fibers, we thus found that Notch and Drpr play divergent roles and function to delineate myoblast identity toward one muscle type or the other.

The current study shows that transgene expression of Megf10/Drpr using the *Notch-Gal4* driver leads to lethality (of note, the N^{gal4} driver is a loss-of-function allele of *N*). In addition, Drpr overexpression in wing cells that express the Notch ligand Serrate results in a distinctive wing vein phenotype (i.e., extra branching at the margin) (Fig. 7), that is also found in the setting of: (i) Serrate overexpression in the same cells, which is associated with cis-inhibition of Notch signaling induced by excess ligand [41]; (ii) Notch deficiency [27]; (iii) Delta deficiency [27, 60]; or (iv) Hairless overexpression in the same cells [61]. Hairless (H) is a canonical inhibitor of Notch signaling activity [62–64]. Previous studies have demonstrated that Serrate (Ser) and Wingless (Wg) act in concert transiently in the late larval wing disc epithelium to modulate Notch signaling-induced asymmetric division of AMPs, which in turn generates post mitotic myoblasts prone to differentiate [65]. Here we show that high levels of Megf10/Drpr in Wg cells induces developmental arrest at the pupal stage (Fig. 7). Of note, Wg in L3 wing disc cells also activates the expression of the muscle-attachment gene stripe (*sr*) [66], and is required to maintain the IFM marker vestigial (Vg) in selected AMPs [56]. It is likely however that fly death induced by increased levels of Megf10/Drpr in Wg cells is not due to disruption of Wg-mediated functions on Sr or Vg, as Megf10/Drpr overexpression using the *Sr-Gal4* and *Vg-Gal4* drivers did not reveal lethality

(Table 1). Notably, flies that survived genetic overexpression of Drpr-III in Wg cells displayed marked histological abnormalities in tubular muscle fibers (i.e., in the jump muscle), but not in fibrillar fibers (Fig. 8, Supplementary Fig. 2). Thus, the N, Ser and Wg subpopulations of wing disc cells which play an important role in the regulation of myogenesis are highly sensitive to aberrant levels of these proteins. The severe phenotypes observed with Drpr-II overexpression in these cells suggests that its intracellular ITIM domain (unique to this isoform) may potentiate the harmful effects induced by motifs common to all three Drpr isoforms. In aggregate, our genetic analyses suggest a spatial and temporal sensitivity to increased levels of Drpr during adult myogenesis, and that high Drpr affects Notch at a critical stage of *Drosophila* muscle development.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgements

We are particularly obliged to Mary Logan (OHSU) and Marc Freeman (OHSU) for their generous gift of the transgenic *UAS-drpr-I*, *UAS-drpr-II*, *UAS-drpr-III*, *UAS-Megf10* and *drpr* null *Drosophila* lines used in these studies. We appreciate the thoughtful comments of Alan S. Kopin, as well as the expert technical assistance of Paul Spindler and Kelsey Bittner at the Department of Pathology and Laboratory Medicine, Tufts Medical Center. The genetic analyses were carried out with Gal4 driver stocks obtained from the Bloomington Drosophila Stock Center at Indiana University (NIH P40OD018537). FlyBase databases were utilized throughout the project to design experiments and interpret results. This work was supported in part by NIH R01NS080929.

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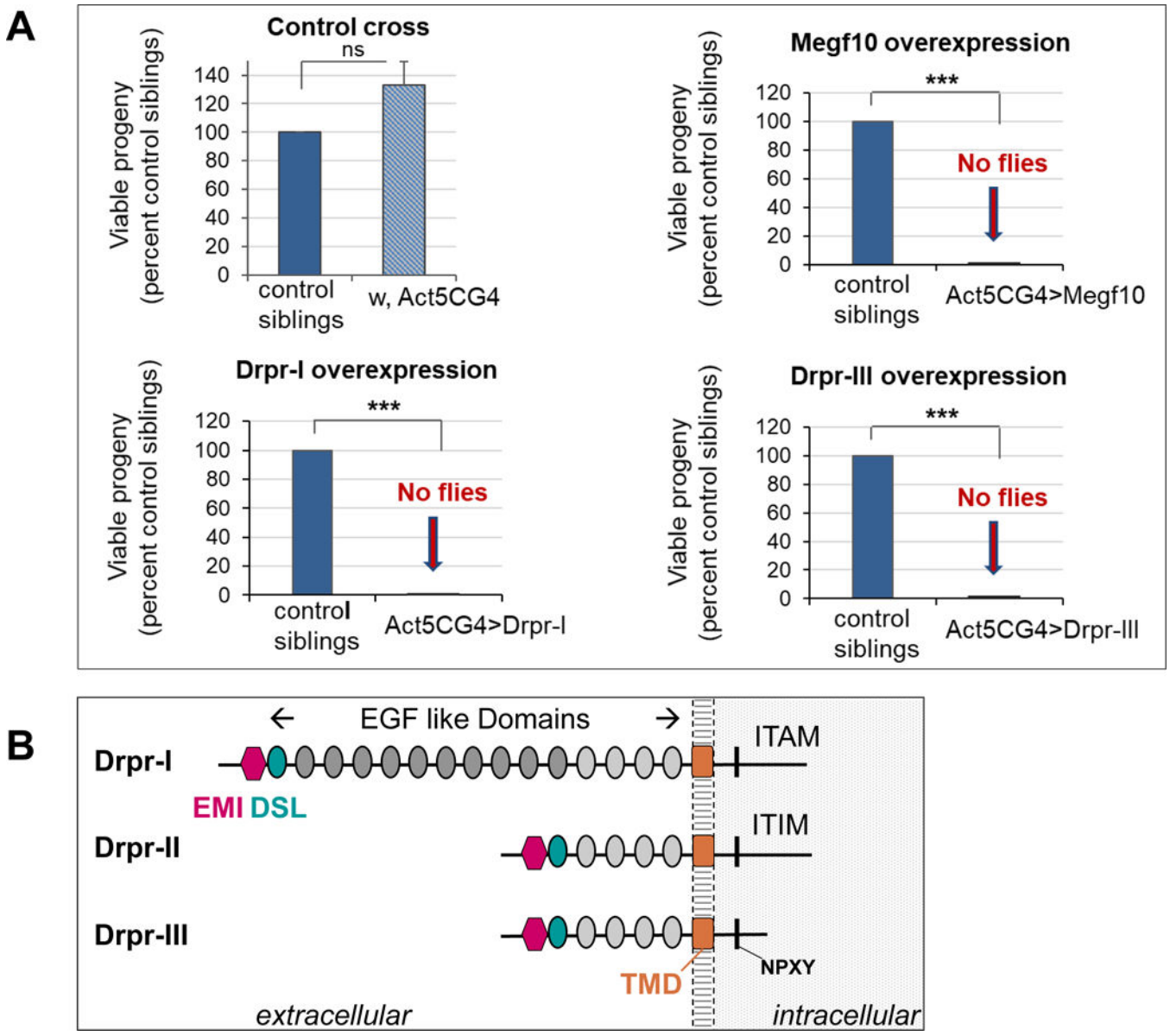


Figure 1. Ubiquitous overexpression of mouse Megf10, or fly Drpr, in Drosophila is harmful to the developing organism.

(A) Ubiquitous overexpression of mouse Megf10, or of the fly Drpr isoforms -I, or -III, results in pre-adult lethality. The *Act5C-Gal4* driver line was utilized to drive expression in the entire organism (the *actin5C* gene encodes a cytoskeletal actin). The corresponding experimental progeny is labeled Act5CG4>Megf10, Act5CG4>Drpr-I, Act5CG4>Drpr-III (abbreviated genotypes: *Act5C-Gal4/UAS-Megf10*, *Act5C-Gal4/UAS-drpr-I*, *Act5C-Gal4/UAS-drpr-III*, respectively). Control siblings that do not express the *Megf10*, or *drpr*, transgene (i.e., do not carry the *Gal4* transgene, abbreviated genotypes: *UAS-Megf10/CyO*, *UAS-drpr-I/CyO* and *UAS-drpr-III/CyO*, respectively), and emerge in the same cross, define the 100% expected progeny. Equal numbers of control siblings and experimental progeny is expected when the flies develop normally. The control cross was set up using w^{1118} (w) genetic background fly line x the *Act5C-Gal4* driver line. Two different progeny are

expected from this cross: *w*^{1118};*Act5C-Gal4* (which do not carry the *UAS* transgene), and *w*^{1118};*CyO* siblings. All progeny was generated at 29ºC. Data are expressed as means +S.D. of seven data points (control cross) and two data points (each overexpression cross); each data point/cross generates 14 to 53 viable adult flies. Similar results were obtained with crosses generated at 25ºC (not shown). The lethality phenotype was also observed with ubiquitous overexpression of Drpr isoform-II (Act5C>Drpr-II, not shown). Statistical significance, percent experimental progeny vs. control siblings, ns, not significant; ***p < 0.001 (paired t-test and 2-way ANOVA, GraphPad, respectively). (B) Drpr isoforms I, II and III share a subset of molecular domains (structures are based on [12]). Notably, overexpression of the shortest Drpr-III isoform is sufficient to induce lethality. Abbreviations: DSL, Delta/Serrate/LAG-2-like domain; EGF-like domain, Epidermal Growth Factor-like domain; TMD, transmembrane domain; ITAM, immunoreceptor tyrosine-based activation motif; ITIM, immunoreceptor tyrosine-based inhibition motif.

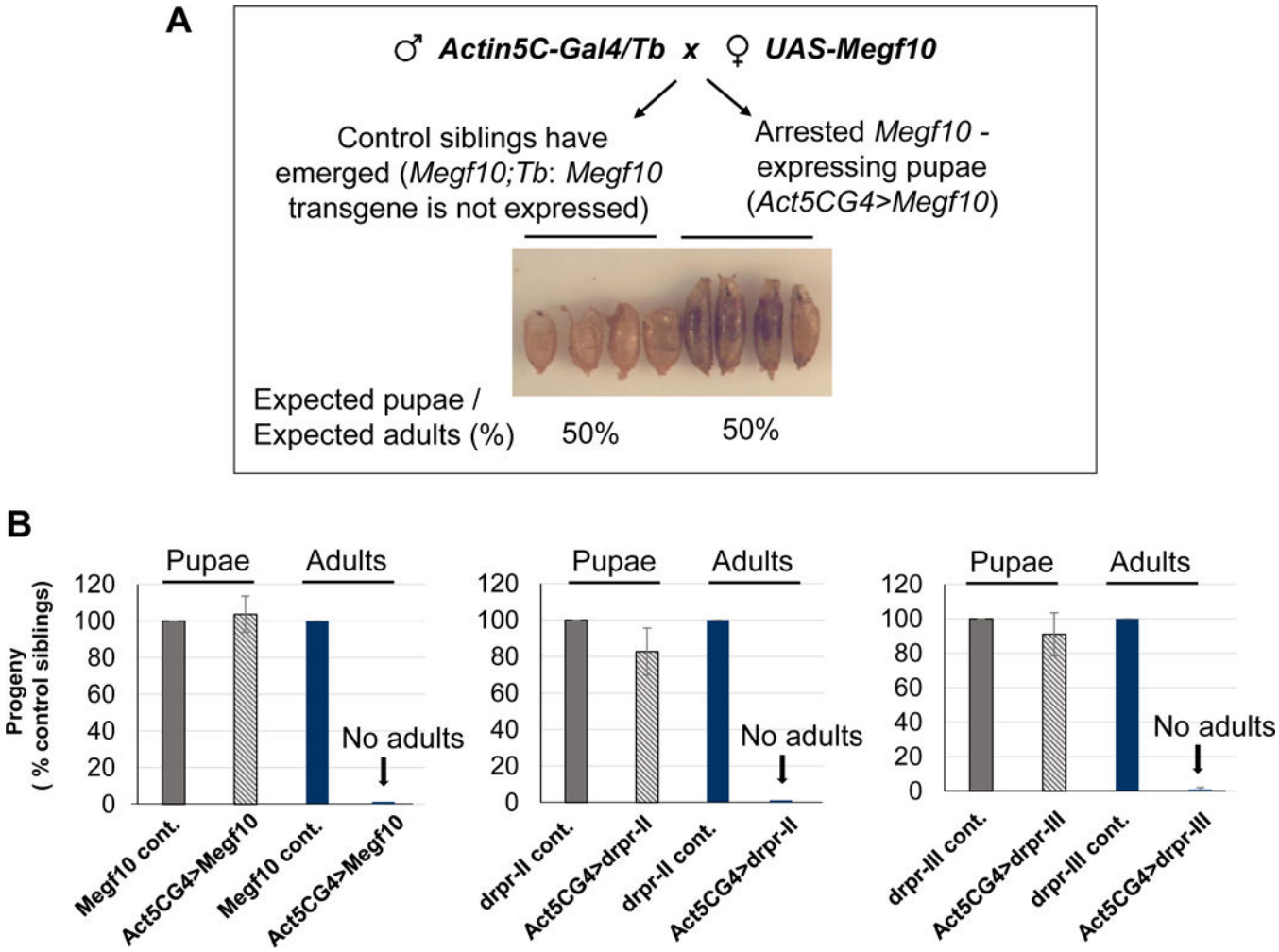


Figure 2. Drosophila that overexpress Meg10/Drpr ubiquitously become arrested late in development.

(A) Act5CG4>Megf10 progeny (abbreviated genotype *UAS-Megf10;Act5C-Gal4*) develop normally along with control siblings until the pupal stage, where the ActCG4>Megf10 flies become arrested. The control progeny (displaying a short and rounded body due to expression of the Tubby/Tb marker, abbreviated genotype *UAS-Megf10; Tb*) eclose, leaving behind empty pupal cases, while arrested Act5CG4>Megf10 flies remain in their pupal cases. Comparable results are obtained with Drpr-I, Drpr-II and Drpr-III. (B) Graphs showing quantification of Megf10/Drpr overexpressing pupae and adults (non-Tb) as a percent of corresponding control pupae and adults (Tb). 50% non-Tb animals and 50% Tb animals are expected to develop simultaneously, at each stage of development. While no significant difference in the number of experimental (i.e., Megf10/Drpr overexpressing) pupae versus control pupae is observed, the experimental flies do not eclose. Data are expressed as means +S.D. of four data points (each overexpression cross); each data point/ cross generates 35 to 144 pupae/genotype, and 26 to 90 control adult flies. Similar results are obtained with Drpr-I (data not shown).

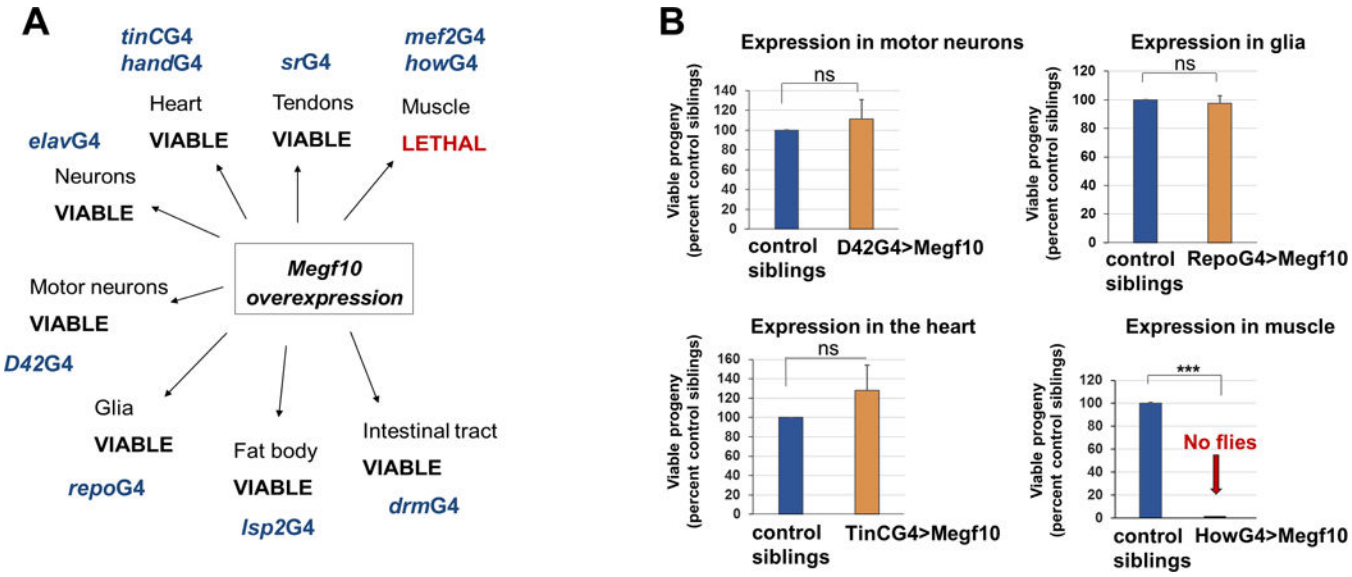
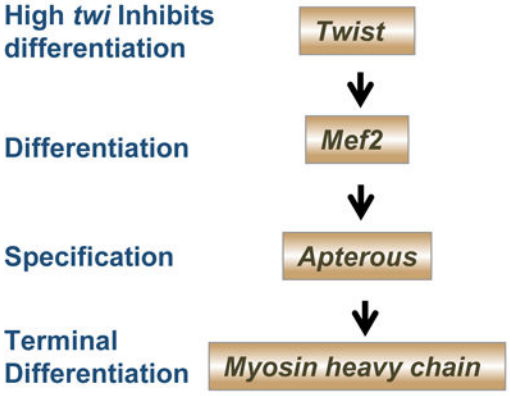


Figure 3. Expression of mouse Megf10 in Drosophila muscle but not in other tissues, leads to lethality.

In the assessed progeny, Megf10 is expressed under the control of the *Gal4/UAS* system which enables transgene expression in selected tissues and cell types. (A) Recapitulation of effects of targeted tissue-specific Megf10 expression on Drosophila viability (Table 1). The genetic driver utilized to test for lethality effect is indicated above each tissue. (B) Graph analysis of progeny obtained with the *D42-Gal4*, *Repo-Gal4*, *TinC-Gal4*, and *How-Gal4* driver lines, which target *UAS-Megf10* transgene expression specifically to motor neurons, glial cells, the heart, and muscle, respectively (the progeny is labeled D42G4>Megf10, RepoG4>Megf10, TinCG4>Megf10 and howG4>Megf10). Brown graphs correspond to the experimental progeny, and blue graphs correspond to control siblings (generated from the same cross and developing in the same vial, abbreviated genotypes: *CyO;D42-Gal4*, *CyO;Repo-Gal4*; *CyO*;*TinC-Gal4 and CyO;how-Gal4*, respectively) that define the 100% expected progeny. Equal numbers of control siblings and experimental progeny is expected when the flies develop normally. All progeny were generated at 29ºC. Data are expressed as means +S.D. of multiple data points/crosses; *D42-Gal4 n=4*, *how-Gal4 n=3*, *Repo-Gal4 n=2*, and *TinC-Gal4 n=2*; each data point/cross generates 26 to 71 viable adult flies. Similar results were obtained with crosses generated at 25ºC (not shown). Statistical significance, percent experimental progeny vs. control siblings, ns, not significant; ***$p < 0.001$ (paired t-test and 2-way ANOVA, GraphPad).

A



UAS-cDNA / Gal4 driver	Megf10	Drpr-I	Drpr-II	Drpr-III
Twist (AMPs)	Viable	Viable	Viable	Viable
Mef2	Lethal	½ Lethal	Viable	Viable
Apterous (muscle identity factor)	½ Lethal	Lethal	Lethal	Lethal
Mhc (differentiated muscles)	Viable	Viable	Viable	Viable

B

Figure 4. Overexpression of mouse Megf10 or fly Drpr at intermediate stages of myogenesis is lethal in Drosophila.

In the assessed progeny, Megf10, Drpr-I, Drpr-II or Drpr-III is expressed under the control of the *Gal4/UAS* system, which enables transgene expression at selected time points of myogenesis. (A) Overexpression of Megf10, or of Drpr, in quiescent adult muscle precursors (AMPs, that express the transcription factor twist), or in fully differentiated fibers (that express Myosin heavy chain, Mhc), does not compromise viability. Overexpression of Megf10/Drpr at intermediate steps of the myogenic process, e.g., differentiation or specification, is deleterious. 1/2 lethal = semi lethal. (B) The few flies that express Drpr-III in apterous-positive cells (apterous-G4>Drpr-III) and escape lethality display abnormalities in the wings (which remain unfolded and filled with hemolymph), as well as distorted hind legs; these flies only survive a few days (vs. a normal ~60 day-lifespan at 25ºC for wild type Drosophila). Apterous is also known to express in the developing appendages [44].

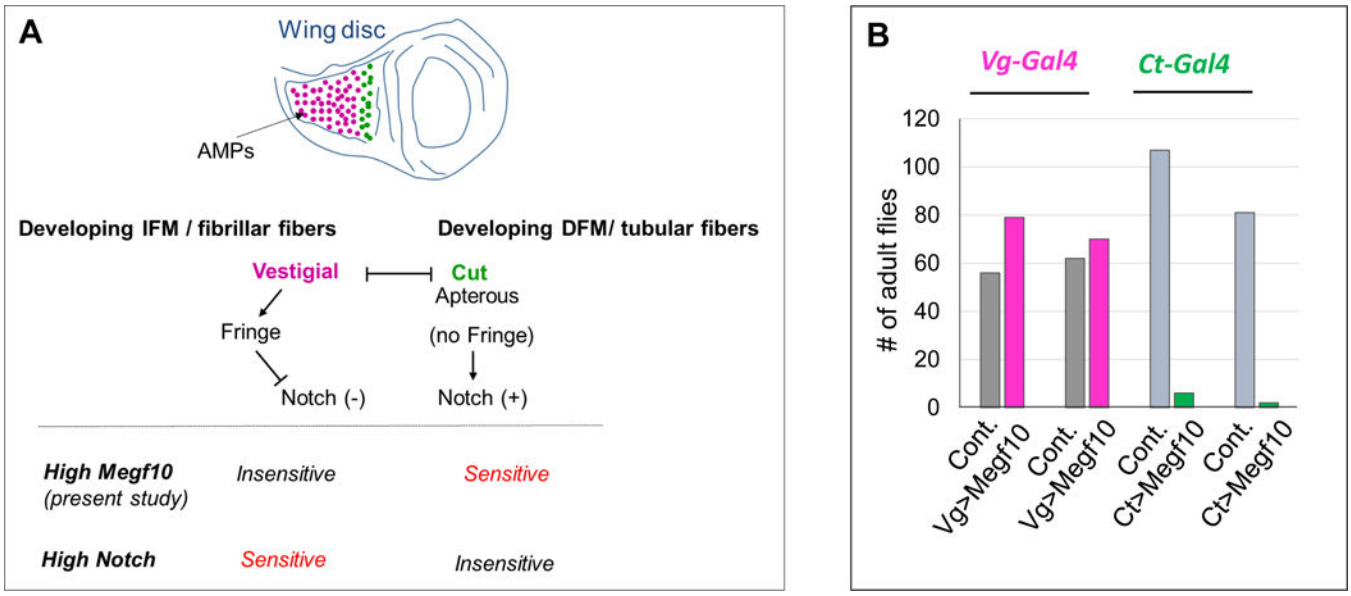


Figure 5. Overexpression of Megf10/Drpr differentially affects two subtypes of developing muscle in Drosophila and suggests inhibition of Notch by these proteins.

(A) Top: Cartoon showing Vestigial-expressing myoblasts (magenta) vs. Cut-expressing myoblasts (green) in the pool of AMPs located in the notum of an imaginal wing disc (wild type Drosophila). Vestigial fibers, which develop into the indirect flight muscle (IFMs) appear insensitive to high Megf10, while Cut/Apterous fibers which develop into the direct flight muscles (DFMs) are sensitive to high Megf10. Opposite results were previously described in the literature for high Notch and Twist [54]. A postulated inhibition of Notch by Megf10/Drpr may underlie the observed effects. (B) Graphs showing the number of Megf10-expressing adult progeny obtained from parental crosses set up with either the *vestigial-Gal4*, or *cut-Gal4*, driver (two biological replicates per Gal4 driver).

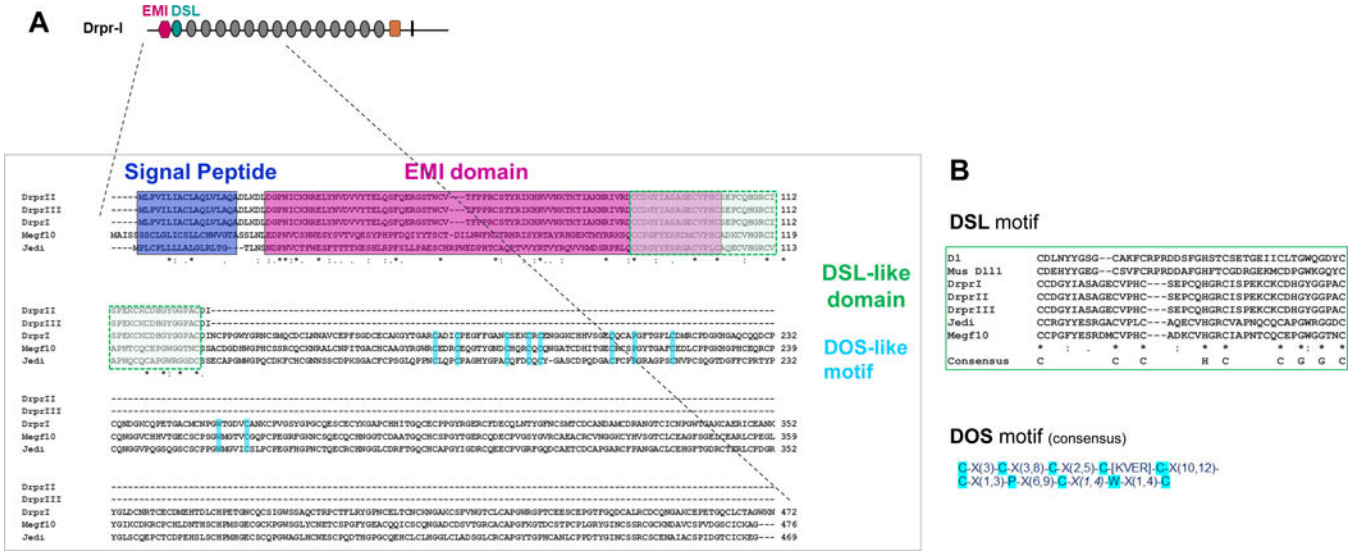


Figure 6. Sequence alignment of Drosophila Drpr protein isoforms I, II and III and the homologous proteins in mouse, Megf10 and Megf12 (Jedi), shows conservation of DSL and DOS domains.

(A) top: cartoon of full-length Drpr-I. Bottom: the highest degree of similarity is found within the N-terminal EMI domain (magenta), the DSL-like domain (green), as well as in selected distal EGF-like domains (not shown). (B) The canonical DSL domain of the Notch ligands Delta (Dl, Drosophila) and Delta-like-1 (Dll1, mouse), shows significant similarity with a comparable motif found in Drosophila Drpr isoforms -I, -II, -III, mouse Megf10 and mouse Megf12 (Jedi). In addition, a DOS-like motif is found in Drpr-I, Megf10 and Jedi (residues highlighted in blue), but not in the shorter Drpr-II and Drpr-III protein isoforms. Abbreviations: DSL: Delta/Serrate/LAG-2, DOS: Delta and OSM-11.

A **Effect of overexpression of fly Drpr or mouse Megf10 using drivers that target the Notch pathway.**

Gal4 driver (mammalian homologous gene)	Megf10	Drpr I	Drpr II	Drpr III
Notch (Notch)	Lethal	Lethal	Lethal	Lethal
Serrate (Jagged)	split vein[1]	split vein[2]	held out wings[3] and split vein[3]	split vein[3]
Wingless (Wnt)	Semi-lethal[4]	Semi-lethal[4]	Lethal	Semi-lethal[4]

The phenotype is [1] rare; [2] semi-penetrant; [3] fully penetrant
[4] Few escapers show markedly reduced lifespan

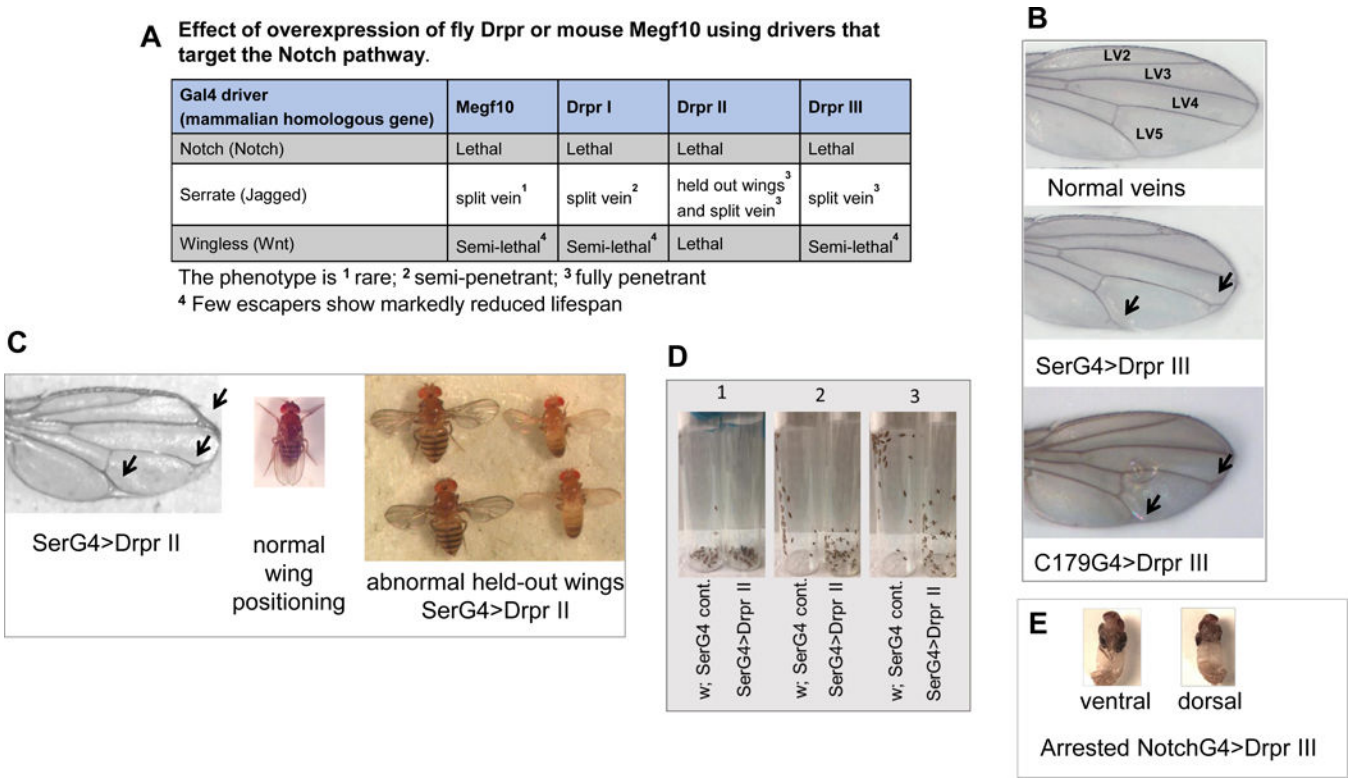


Figure 7. Expression of Megf10/Drpr in either Notch, Serrate or Wingless positive cells in flies leads to marked wing abnormalities, decreased motor function and/or lethality.

(A) Table summarizing the outcome of such genetic crosses using mouse Megf10 or fly Drpr homologs. (B-C) Overexpression of Drpr in Serrate positive cells results in a split (or 'delta') wing vein phenotype. (B) Top: normal wing from control progeny (w; SerG4). Middle: wing from progeny that express Drpr-III in serrate positive cells (SerG4>Drpr-III). The wing shows abnormal deltas in vein (arrows; the phenotype is 100% penetrant with Drpr-III, and mostly affects LV4 and LV5). Bottom: A similar phenotype is seen in progeny that express Drpr-III under the control of the *C179-Gal4* driver (SerG4>Drpr-III; Table 1). (C) Left panel: wing from progeny that express Drpr-II in serrate positive cells (SerG4>Drpr-II), a severe "split vein" phenotype is observed at the wing margin with LV3, LV4 and LV5. LV, Longitudinal Vein. Right panel: SerG4>Drpr-II progeny also display abnormally held out wings (the phenotype is 100% penetrant). (D) SerG4>Drpr-II flies also show marked defect in locomotor activity. 10 day-old control (w^{1118}; *SerG4*) and SerG4>Drpr II female flies were collected and functionally characterized. In each picture, the left vial contains the control flies (n=36), and the right vial contains the experimental flies (n=37). 1: the flies were prompted by taping both vials simultaneously once on a flat surface. 2, 3: the climbing ability of the flies was video recorded. Still pictures are shown. SerG4>Drpr II flies move markedly more slowly. The corresponding video can be accessed in the Supplementary Material. (E) Targeted expression of mouse Megf10 in wing disc cells that express Notch (using the FBst0036554 driver line) leads to lethality. The picture shows a corresponding NG4>Megf10 mutant fly which arrested at the late pupal/pharate adult stage, and was dissected out of the pupal case.

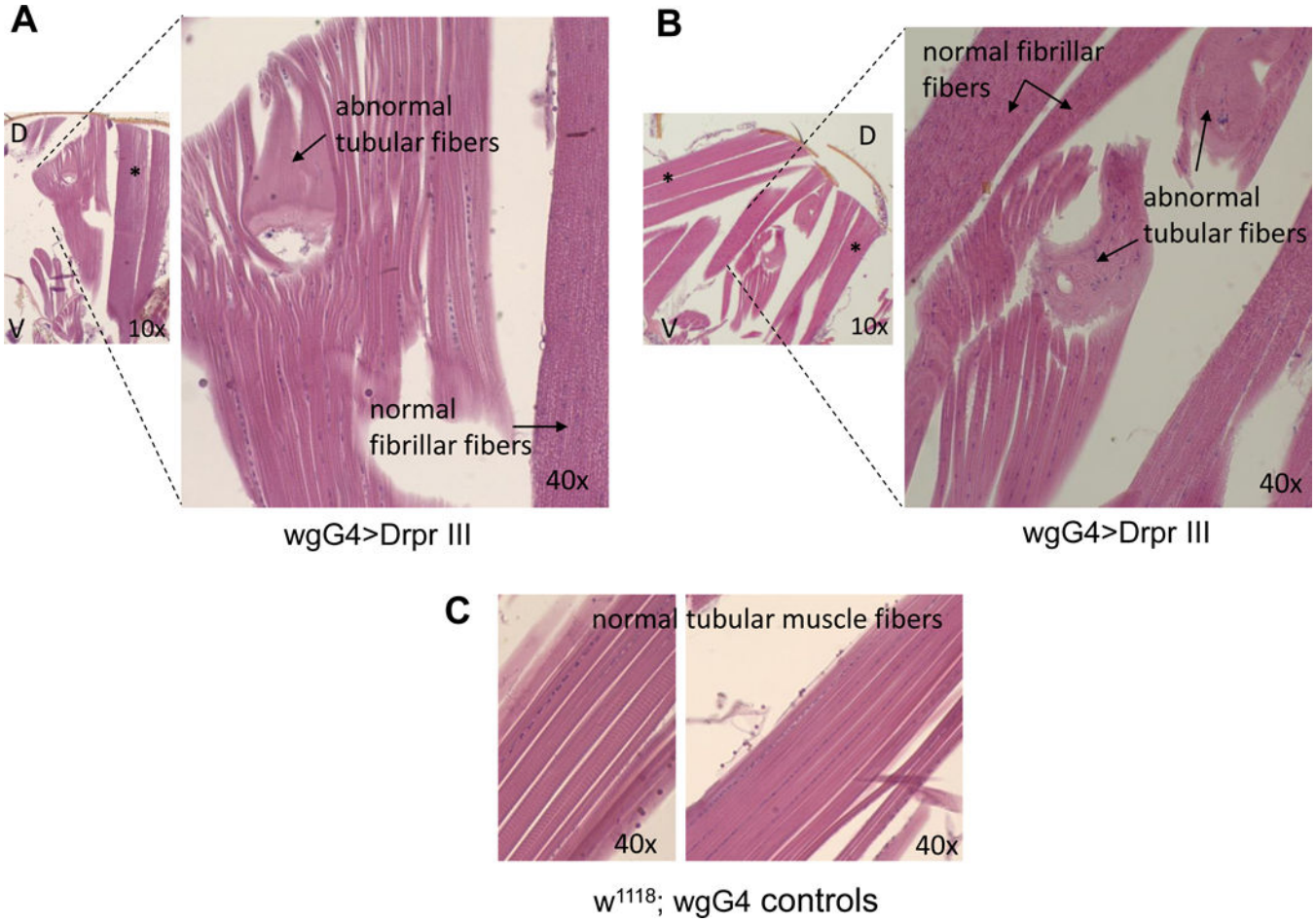


Figure 8. Overexpression of Drpr-III in Wg cells leads to severe abnormalities in tubular muscle fibers in adult flies.

Two day old *Wg-Gal4>UAS-drpr-III* (labeled wgG4>Drpr III) adult Drosophila that escaped lethality (Figure 7, Table 1), and w^{1118}*; wgG4* isogenic control flies generated in parallel, were fixed and processed for histological analysis (H&E staining). Representative longitudinal sections of fly thoraces and muscle fibers are shown. (A-B) *Wg-Gal4>UAS-drpr-III* flies. Marked abnormalities are seen in the tubular fibers that comprise the tergal depressor of the trochanter (TDT, jump muscle). The defects include marked enlargement of the fibers, hyalinization, abnormal localization/loss of nuclei, loss of striations and disruption of the characteristic bundle organization. In contrast to the tubular muscles, no gross abnormalities are detected in fibrillar fibers that comprise the large indirect flight muscles (examples are marked by asterisks at 10x magnification, as well as indicated at 40x magnification). N = 12 flies. Of note, 8 out of 12 flies where tubular fibers could be observed displayed abnormalities. Additional examples of tubular muscle defects displayed by *Wg-Gal4>UAS-drpr-III* flies are shown in Supplementary Figure 2 (from an independent replicate). (C) Age-matched w^{1118}*; wgG4* control flies show normal tubular fibers, with normal striations and well aligned central nuclei. N =15 flies. None of the control flies showed muscle abnormalities. Magnification: 10x, 40x, as indicated. Abbreviations: D, Dorsal; V, Ventral.

Table 1.

Effect of tissue-specific overexpression of fly Drpr or mouse Megf10.

A series of GAL4 drivers was used to target transgene expression in specific tissue/cell types. Lethality is observed when using ubiquitous drivers, or selected muscle drivers. Of note, adult muscles are formed from adult muscle precursors (satellite cell-like AMPs), located on the larval wing discs.

Gal4 driver Symbol	FBstock #	Expression	Ref.	Myogenesis	Megf10	Drpr I	Drpr II	Drpr III
P{***Act5C***-GAL4}25FO1	4414	Ubiquitous	[67]	All stages	Lethal	Lethal	Lethal	Lethal
P{***Act5C***-GAL4}17bFO1	3954	Ubiquitous	[67]	All stages	Lethal	Lethal	Lethal	Lethal
P{GAL4}***C142***	64267	embryonic and larval somatic muscles [strong expression]	[68], [69]		Normal	Normal	Normal	Normal
P{GawB}***c179***	6450	embryonic mesoderm and larval muscles, and wing discs	[28], [70]		Lethal	Semi-lethal	Lethal	Viable[a]
P{GawB}***kirre***[rP298-G4]	66682	Muscle progenitors and founders during early development of the mesoderm	[71]	Progenitors and founder cells	Normal	Normal	Normal	Normal
P{GAL4-***twi***.G}108.4	914	Embryonic and adult muscle precursors	[71]	Precursors (high twist inhibits differentiation	Normal	Normal	Normal	Normal
P{GawB}***how***[24B]	1767	Mesoderm/all muscles, tendons	[71]	Early differentiation Migration/ attachment	Lethal	Lethal	Lethal	Normal
P{GAL4-***Mef2***.R}3	27390	All muscles	[71]	Differentiation	Lethal	Semi-lethal	Short lived[b]	Short lived[b]
P{GawB}***ap***[md544]	3041	Apterous cells (embryo and L1: neurons, L3: wing discs)	[72]	Identity (DFM)	Short lived[b]	Lethal	Lethal	Lethal
P{***cut***-GAL4.B}3	27327	Cut cells in wing discs	[73]	Identity (DFM)	Lethal	Viable[c]	Viable[c]	Viable[c]
P{GAL4-***vg***.M}2	6819	Vestigial cells in wing discs	[74]	Identity (IFM)	Normal	Viable[d]	Normal	Normal
P{***Act88F***-GAL4.1.3}3	38459	Indirect flight muscles (IFM)	[71]	Developing IFM (after attachment) & mature IFM	Normal	Normal	Normal	Normal
P{***Mhc***-GAL4.F3–580}2	38464	All muscles	[71]	Differentiated muscles	Normal	Normal	Normal	Normal
P{GawB}***DJ667***	8171	Adult muscles (adult expression constant with age)	[75]	Differentiated muscles	Normal	Normal	Normal	Normal
P{GawB}***DJ757***	8184	Adult muscles (adult expression constant with age)	[75]	Differentiated muscles	Normal	Normal	Normal	Normal
P{GawB}***sr***[md710]	26663	Tendons	[71]	Attachment	Normal	ND	ND	Normal
tinC-GAL4	Acad.lab	Heart	[76]		Normal	Normal	Normal	Normal

Gal4 driver Symbol	FBstock #	Expression	Ref.	Myogenesis	Megf10	Drpr I	Drpr II	Drpr III
P{GMR88D05-GAL4}attP2	48396	Heart (all cardioblasts and pericardial cells), embryonic visceral muscle	[70]		Normal	Normal	Normal	Normal
P{GawB}***elav***[C155]	458	Pan neuronal	[77]		Normal	Normal	Normal	Normal
P{GawB}***D42***	8816	Motor neurons	[78]		Normal	Normal	Normal	Normal
P{GAL4}***repo***	Acad.lab.	Glia	[79]		Normal	Normal	Normal	Normal
P{***drm***-GAL4.7.1}1.1	7098	Intestinal tract / tubules	[80]		Normal	ND	Normal	Normal
P{***Lsp2***-GAL4.H}3	6357	Fat body	[81]		Normal	Normal	Normal	Normal
P{GawB}***N***[MD776] *(Notch-Gal4)*	36554	All or most cells of wing disc	***e***		Lethal	Lethal	Lethal	Lethal
P{***Ser***-GAL4.GF}1 P{***Ser***-GAL4.GF}2 *(Ser-Gal4)*	6791	Serrate cells in late L3 wing discs /future wing margin	[82]		Split vein	split vein	Held out wings/ split vein	split vein
P{GawB}***MD758*** *(Wg-Gal4)*	36525	In the pattern of the *wg* gene	[83, 84]		Semi-lethal	Semi-lethal	Lethal[f]	Semi-lethal

Normal: adult flies emerge and do not display gross abnormalities

L1: first instar larvae, L3: third instar larvae

Acad.lab.: stock obtained from an academic laboratory (details in Materials and Methods)

DFM: direct flight muscles, IFM: indirect flight muscles, ND: not determined

[a] flies display a split vein phenotype in the wing (Fig. 7)

[b] flies display reduced lifespan (~ 7 days)

[c] flies display abnormal wing epithelium and/or wing position

[d] flies show a wing phenotype as a function of age

[e] Hatton-Ellis, E., Simpson, P. (http://flybase.org/reports/FBrf0216880.html)

[f] The lethality phenotype is recapitulated with a second *wg-Gal4* driver (i.e., P{GMR16D11-GAL4}attP2, FBst0045418), from a different laboratory (GAL4 Driver Collection of Rubin Lab at Janelia Farm, http://flybase.org/reports/FBrf0213040).