The Drosophila *wings apart* gene anchors a novel, evolutionarily-conserved pathway of neuromuscular development.

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*Running title*: Drosophila *wings apart* is required for jump muscle formation

*Key words*: Drosophila, adult muscle, jump muscle, neuromuscular development, wdr68

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Article Summary:
Mutations with pleiotropic effects frequently affect conserved developmental processes. Here, we identify and characterize a Drosophila gene named *wings apart* (*wap*), that is required for viability, muscle formation, and wing vein patterning. *wap* is highly conserved in vertebrates and involved in neuromuscular development, therefore we propose that *wap* is the pioneer molecule in an important and conserved developmental pathway.
Mutations that have pleiotropic effects often affect conserved developmental pathways in the organism. Here, we demonstrate that the Drosophila \textit{wings-apart} mutation has a pleiotropic phenotype that includes semi-lethality, wing cross-vein defects, and loss of a specific adult muscle. We show that the affected gene corresponds to the uncharacterized locus \textit{CG14614}, which encodes a WD40 repeat protein highly similar to vertebrate wdr68. We propose that wap/wdr68 represents an important pathway controlling diverse developmental processes, including muscle development in flies and craniofacial development in vertebrates.
ABSTRACT

wings apart (wap) is a recessive, semi-lethal gene located on the X chromosome in Drosophila melanogaster, that is required for normal wing vein patterning. We show that the wap mutation also results in loss of the adult jump muscle. We use complementation mapping and gene-specific RNAi, to localize the wap locus to the proximal X chromosome. We identify the annotated gene CG14614 as the gene affected by the wap mutation, since one wap allele contains a non-sense mutation in CG14614, and a genomic fragment containing only CG14614 rescues the jump muscle phenotypes of two wap mutant alleles. The wap gene lies centromere-proximal to touch-insensitive larva B, and centromere-distal to CG14619, which is tentatively assigned as the gene affected in introverted mutants. In mutant wap animals, founder cell precursors for the jump muscle are specified early in development, but are later lost. Through tissue-specific knockdowns, we demonstrate that wap function is required both in the musculature and the nervous system for normal jump muscle formation. wap/CG14614 is homologous to vertebrate wdr68 and DDB1 and CUL4 associated factor 7, which also are expressed in neuromuscular tissues. Thus, our findings provide insight into mechanisms of neuromuscular development in higher animals, and facilitate the understanding of neuromuscular diseases which may result from mis-expression of muscle-specific or neuron-specific genes.
INTRODUCTION

Understanding the genetic causes of pleiotropic diseases can provide insight into the roles of specific genes in a biological process and can also uncover a genetic pathway or framework that controls diverse developmental processes. In Drosophila, a number of genes whose mutants have a multitude of phenotypes have ultimately been proven to control centrally-important molecular pathways. Such genes include members of signaling pathways and other conserved molecular events.

The TGFβ pathway carries out a number of developmental roles during animal development, including early specification of germ layers during embryogenesis (reviewed in Watabe and Miyazono 2009). In Drosophila, this pathway also functions during pupal development, and among other roles it is required for the formation of the crossveins of the adult wing (Ralston and Blair 2005; Chen et al. 2012). Recently, we identified an additional role for TGFβ signaling during pupal development, in specification of the adult jump muscle (also called the tergal depressor of the trochanter, or TDT) (Jaramillo et al. 2009). The jump muscle arises from the leg imaginal disc of the second thoracic segment (Rivlin et al. 2000), and functions in the escape response of the animal (Nachtigall and Wilson 1968). Hypomorphic mutations affecting the TGFβ pathway cause a reduction in the number of fibers in the jump muscle, due to a failure to specify the complete number of founder cells for each fiber (Jaramillo et al. 2009).

Mutations affecting the BMP signaling pathway are one of several in Drosophila whose phenotypes include abnormal wing vein patterning, and a number of these mutants have yet to be correlated with an annotated locus (Blair 2007). With the advent of the fully-annotated Drosophila genome, it has become possible to more readily identify candidate genes for such mutations. This task is further facilitated by the several genetic tools that are available in this system, including
chromosomal aberrations that have precise molecularly-defined breakpoints (see for example Parks et al. 2004; Popodi et al. 2010).

Here, we have analyzed the wings-apart (wap) gene in Drosophila. wap has to date not been associated with an annotated gene; yet, as with mutations affecting the TGFβ pathway, wap mutant phenotypes include semi-lethality and defects in wing cross-vein formation (Lifschytz and Falk 1969; Schalet and Lefevre 1973). We demonstrate firstly that wap mutants also fail to form a jump muscle, and we localize the affected gene on the X chromosome to CG14614. One wap allele causes a premature stop in the CG14614 coding sequence, and through tissue-specific knockdown of wap/CG14614 we show that wap function is required in both the nervous system and developing musculature. Moreover, loss of wap function results in a failure of the jump muscle to form, arising from a failure to sustain the newly-developed muscle fibers. wap is homologous to the vertebrate genes, wdr68 in zebrafish and DDB1 and CUL4 associated factor 7 in mammals. These genes encode orthologous WD40 repeat proteins expressed in vertebrate muscle and nervous systems (Skurat and Dietrich, 2004), which interact physically with an ortholog of the Drosophila minibrain locus (Tejedor et al., 1995). Our findings identify a new component of the muscle development process, which is likely to be mediated by a complex of proteins conserved across species.
MATERIALS AND METHODS

Drosophila stocks and crosses

Drosophila were maintained at 25°C on Jazz food mix (Fisher Scientific) unless otherwise stated. All genotype information is according to the guidelines at flybase.org. Fly stocks were obtained from the Bloomington Drosophila Stock Center, Drosophila Genetic Resource Center, Kyoto Institute of Technology, Vienna Drosophila RNAi Center (VDRC) and the Transgenic RNAi Project (TRiP) at Harvard Medical School.

For deficiency screens, each cross was composed of equal numbers of virgin females and males and maintained at 25°C. For deficiency screening with wap alleles, each mutant allele was crossed with the deficiency lines Df(1)Exel6255 (Exelixis, Inc.), Df(1)BSC708, Df(1)LB6, Df(1)54, Df(1)DCB1-35c, Df(1)DCB1-35b, and Df(1)R8A (Schalet and Finnerty, 1968 Schalet and Lefevre 1973 and Rahman and Lindsley 1981). This was achieved by crossing female Balancer/Deficiency to males of the genotype wapx/Dp(1;Y)y+malt171, since this duplication complements the wap mutants (x refers to any or all of the three wap alleles currently available: wap2, wap3, and wap9). The number of progeny eclosed from each individual cross was counted. Comparisons between the total numbers of female progeny with Balancer/wapx genotype and female progeny with the Deficiency/wapx genotype were performed using Student's t-test. Analyses of jump muscle and wing crossvein phenotypes were performed for both Balancer/wapx and Deficiency/wapx genotypes as described below.

The previously-mentioned deficiency lines were also crossed with each other to further refine the X chromosome map. These same deficiency lines were also used to map other proximal X chromosome mutations thought to be within the region spanning 20A to 20C: l(1)G0179, eo16-2,27, eo25, intro3, uncl1, uncl10, soz1, l(1)20Cb2, l(1)20Cb6, l(1)20Ca1, l(1)20Ca2, and l(1)G1096.
Virgin females of each mutation used in the deficiency screens were also crossed with equal numbers of males from X chromosome duplication lines spanning the region deleted by the Df(1)Exel6255 deficiency. The duplication lines used in these experiments were Dp(1;3)DC382, Dp(1;3)DC383, Dp(1;3)DC384, Dp(1;3)DC562, Dp(1;3)DC386, Dp(1;3)DC387, Dp(1;3)DC388, Dp(1;3)DC389, and Dp(1;3)DC390 (Popodi et al., 2010). Not all combinations of mutations and duplications were tested against one another, since some mutations are complemented by deficiencies of the duplicated region. For those duplications tested, the number of eclosing male progeny with the genotype Balancer/Y; Duplication/+ were compared with male progeny of the mutation/Y; Duplication/+ genotype using Student’s t-test to assess the ability of each duplication tested to rescue the phenotype of the mutations.

To ensure that any phenotypic rescue observed in the duplication analysis with wap was not due to rescue of a secondary mutation, lines were also generated that had the genotype Df(1)Exel6255/FM7a; Duplication/Duplication and/or Df(1)DCB1-35c/FM7a; Duplication/Duplication for each of the following duplications: Dp(1,3)DC383, Dp(1,3)DC384, Dp(1,3)DC562, Dp(1,3)DC386, Dp(1,3)DC387, Dp(1,3)DC388, and Dp(1,3)DC389. These lines were crossed with wap^2/Dp(1;Y)y^mal^71 males. Female FM7a/wap^2; Duplication/+ progeny were compared with female Deficiency/wap^2; Duplication/+ progeny to assess whether the wap mutation can be rescued by the duplications. The jump muscle and wing crossvein phenotypes were assessed as described below for rescue by duplication.

The Gal4/UAS system was utilized for RNAi knockdown experiments (Brand and Perrimon, 1993). Equal numbers of virgin female and male flies were allowed to mate three days at 25°C, at which point crosses were transferred to 29°C to activate maximally the Gal4 drivers. tub-Gal4 and da-Gal4 were used as ubiquitous drivers for initial RNAi analysis. To determine tissue specific effects of
RNAi knockdown, *Mef2-Gal4* and *1151-Gal4* drivers were used for muscle-specific knockdown, and *elav-Gal4* was used for knockdown in the nervous system. UAS-RNAi lines were utilized for knockdown of *CG14614* (line 107076), *CG14619* (lines 37929 and 37930), *CG14618* (lines 24879 and 47451), *CG12576* (lines 51205 and 104261), and *Cp110* (lines 24874, 24875, and 101161). RNAi knockdowns were assessed for viability. Jump muscle formation was assessed in cryosections of pharate pupae of the pupal lethal knockdowns as described in the following sections.

To obtain flies carrying both the *wap* mutation and the founder cell-specific *rP298-lacZ* transgene (described in Ruiz-Gomez *et al.*, 2000), female *FM7i,GFP/wap* mutants were crossed with equal numbers of *rP298-lacZ* transgenic males. *rP298-lacZ/wap* female progeny were selected as virgins and crossed with *FM7i,GFP/Y* males. Each female of the F$_2$ generation was isolated individually as virgins and crossed with *FM7i,GFP/Y* males to establish stable stocks. Progeny from these crosses were assessed for presence of the *wap* mutation by observing the Bar-eye phenotype associated with the *FM7i, GFP* balancer. The presence of male offspring with wild-type eye morphology indicated that the lethal *wap* mutation could not be present in the generated stock. To screen for the presence of the *rP289-lacZ* transgenic marker, two adult flies from each line positive for the *wap* mutation were filleted and stained overnight at 37°C in XGAL solution [1X PBS, 100 mM K$_4$[Fe(CN)$_6$], 100 mM K$_3$[Fe(CN)$_6$], 150 mM NaCl, 1 mM MgCl$_2$, and 0.2% w/v X-Gal (Sigma)]. Stocks positive for both the *wap* mutation and *rP298-lacZ* transgene were used in pupal dissections described below. Both the *wap$^2$* and *wap$^9$* alleles were used for these crosses.

**DNA methods**

The coding sequence for *wap* was amplified from wild-type and *wap$^c$/Y* mutants, using primers of the sequence 5'-gaggagtagtggacatcagc and 5'-gtgcgctgctgcggaaagtcccg. The region amplified was
sequenced using BigDye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems Inc.). Sequences were edited, aligned, and analyzed using Sequencher (v. 4.10.1) software.

The wild-type region, after sequencing, was cloned into the pCaSpeR-4 transformation vector (Thummel et al. 1988), and injected into y w embryos for generation of transgenic rescue lines.

Paraffin sectioning, cryosectioning and immunofluorescence
Preparation, sectioning and staining for paraffin-embedded samples were as described by Cripps et al. (1998). Sections were cut at 10 µm thickness and stained with Hematoxylin and Eosin (Sigma). Stained slides were dehydrated through 100% ethanol, soaked in xylene, and mounted in Cytoseal-XYL (VWR Scientific Products).

Cryosections were prepared by removing pharate pupae from the pupal case, embedding in OCT medium and freezing. Sections were cut at 10 µm thickness at -18°C, and air dried. Samples were fixed for 10 minutes at room temperature with 3.7% v/v formaldehyde in PBTx [1XPBS, 0.2% v/v Triton-X100, 0.2% w/v Blocking Agent (Roche)], washed, and used for antibody staining as described in the following section.

For pupa l dissections to assess founder cell specification, new white prepupae were selected and aged until the appropriate time for dissection. Pupal samples were dissected in a Sylgard-coated petri dish (Dow Corning) and pinned open. Samples were fixed for 30 min on ice in 5% formaldehyde in 1X PBS, washed in PBTx, then subjected to blocking and incubated with antibody (described below).

Fixed and washed samples were stained with antibodies as described by Patel (Patel, 1994) and modified by Molina and Cripps (Molina and Cripps, 2001). Primary antibodies used for cryosections were anti-βPS-integrin 1:10 (Brower et al., 2008) (University of Iowa Developmental
Studies Hybridoma Bank). For pupal dissections, primary antibodies used were mouse anti-β-galactosidase 1:400 (Promega), and rabbit anti-MEF2 1:1000 (Lilly et al., 1995) (provided by Bruce Paterson, NIH). For immunofluorescence of sections, Alexa conjugated (Molecular Probes) secondary antibodies were mixed with Alexa-488 phalloidin at 1:500 (Molecular Probes), and with 2 µg/mL DAPI (Sigma). Alexa-conjugated secondary antibodies were diluted to 1:2000 for pupal stains.

For analysis of the crossveins of the adult wings, wings were mounted as described in Jaramillo et al. (2009). Briefly, wings were removed from adult flies and stored overnight in 70% (v/v) ethanol. Wings were transferred twice into 100% ethanol and soaked in 100% xylene prior to being mounted in Cytoseal-XYL (VWR Scientific Products) for imaging.

An Olympus BX-51 stereomicroscope with DIC or fluorescence optics was used to collect images. Adobe Photoshop was used to compile digitally collected images into figures.

RESULTS

wings apart (wap) mutants are characterized by three phenotypes

Since one signaling pathway involved in wing crossvein development is also involved in proper jump muscle development (Jaramillo et al, 2009), mutants with defects in the crossveins of the adult wings were examined to determine if these mutants also exhibited TDT defects. One such mutant was wings apart (wap), characterized by three phenotypes (Figure 1). Firstly, in the wing of an adult wild-type fly, there are five longitudinal veins and two crossveins (Figure 1A); in approximately 10% of wings apart mutants, there are supernumerary crossveins located between the second and third longitudinal veins (Lifschytz and Falk, 1969; Schalet and Lefevre, 1973; arrows in Figure 1B). Secondly, in paraffin sections of the thoracic muscles of wild-type and wap mutant flies, we observed the wild-type jump muscle organized in a rosette pattern, located between the DVM I and DVM II
muscles (Figure 1C); while in the mutant the TDT was absent, although none of the other major thoracic muscles were affected (asterisk in Figure 1D). Thirdly, *wap* mutants are semi-lethal as homozygotes or hemizygotes (Schalet, 1972), and this semi-lethal phenotype was observed in heteroallelic combinations of three *wap* alleles (Figure 1E). The rare *wap* adult escapers showed very low mobility, held their wings slightly down and to the side of the body, and died soon after eclosion. Since the phenotypes were observed in all homozygous, heteroallelic and hemizygous lines of *wap* mutants that we were able to examine, we conclude that the phenotypes all arise from mutation of the same gene.

**wap** is located in region 20C in the proximal X chromosome

Mapping of the *wap* mutations by Lifschytz and Falk suggested that *wap* is located on the proximal X chromosome in region 20A3-4 (Lifschytz and Falk, 1969). To more precisely localize the transcriptional unit affected by the *wap* mutation, each of the *wap* mutant alleles was crossed for complementation analysis with a series of deficiency lines. The deficiencies are denoted in Figure 2 as red bars. Deficiencies used in the complementation analysis were *Df(1)Exel6255* (Exelixis Inc.), *Df(1)BSC708*, *Df(1)LB6*, *Df(1)54*, *Df(1)DCB1-35c*, *Df(1)DCB1-35b*, and *Df(1)R8A* (Schalet and Finnerty, 1968, Schalet and Lefevre, 1973 and Rahman and Lindsley, 1981).

Aggregate results from complementation mapping of three *wap* alleles with the deficiency lines are shown in Table 1. The deficiencies *Df(1)BSC708*, *Df(1)LB6*, and *Df(1)R8A* complement *wap* alleles, whereas four deletions, *Df(1)Exel6255*, *Df(1)54*, *Df(1)DCB1-35c*, and *Df(1)DCB1-35b*, are semi-lethal when heterozygous with *wap* mutant alleles (Table 1). These data indicate that the region proximal to the *Df(1)BSC708* deletion, and distal to the *Df(1)R8A* deletion, is the region in which *wap* is located. To confirm that these escapers show all three previously described *wap* phenotypes, the
wings and thoraces of females heterozygous for the deficiencies and \textit{wap} were analyzed for the presence of the wing and TDT phenotypes characteristic of \textit{wap}. We found that those heterozygous females from the deficiency lines that complemented \textit{wap} had normal wing vein patterning, and the jump muscle was present (Figures 3A and 3B, results from \textit{Df(1)BSC708/wap}^2 shown). The escapers from the semi-lethal heterozygotes exhibited an additional crossvein between the second and third longitudinal vein, characteristic of \textit{wap} homozygous mutants (Figure 3C). Moreover, the jump muscle in almost all of these heterozygous females was completely absent (Figure 3D, results from \textit{Df(1)Exel6255/wap}^2 are shown). It is important to note that although almost all escapers lack the jump muscle, in a small percentage of the escapers, 3% (n=64), the TDT was present (inset in Figure 3D). However, in these cases, the muscle exhibited highly abnormal morphology and a greater than 60% reduction in the number of fibers compared to wild-type jump muscle (2-5 fibers per muscle in mutants, compared with 30-32 fibers in wild-type).

Since the annotated breakpoints (FlyBase FB2012_02) of some of the deficiency lines used in this study (\textit{Df(1)LB6}, \textit{Df(1)54}, \textit{Df(1)DCB1-35c}, \textit{Df(1)DCB1-35b}, and \textit{Df(1)R8A}) have not been mapped to exact genomic coordinates, we sought to refine our mapping by performing complementation tests of the deficiencies themselves. Since the exact breakpoints of the deficiencies are unknown, those genes estimated to be located in region 20C1 and that are deleted by \textit{Df(1)Exel6255} cannot be ruled out as candidates for \textit{wap}. Results shown in Table 2 demonstrate complementation of \textit{Df(1)LB6} with \textit{Df(1)DCB1-35c}, suggesting that the proximal breakpoint for \textit{Df(1)LB6} is to the left (i.e., centromere distal) of the distal breakpoint for \textit{Df(1)DCB1-35c}. The proximal breakpoints for \textit{Df(1)54} and \textit{Df(1)DCB1-35c} are left of the distal breakpoint for \textit{Df(1)R8A}, as both of these are complemented by the \textit{Df(1)R8A} deficiency (Table 2, last column). The distal breakpoint of \textit{Df(1)R8A} is also more proximal than the proximal breakpoint of \textit{Df(1)Exel6255}, as
inferred from complementation between these two deficiencies. The \emph{Df(1)DCB1-35b} deficiency spans the entire region tested, as previously reported (Schalet and Finnerty, 1968), and does not complement \emph{Df(1)R8A}.

While the mapping of the deficiencies resolved some of the deficiency breakpoints, it was still not adequate to completely refine the map of this genetic region. Additional mutations located within the same region of the proximal X chromosome, for which the corresponding transcriptional units have yet to be identified from among the annotated gene models, were assessed using complementation mapping. These mutations are thought to affect predominantly single genes rather than multiple genes. If the mapping has sufficient resolution, candidate genes for the different mutants could be inferred (Table 1, mutants arranged according to the predicted order on the chromosome).

The \textit{mutant/Deficiency} complementation data indicated that the locations of the mutations, relative to each of the other mutations tested, remained in the original map order, with the exception of \emph{l(1)20Ca} and \emph{l(1)20Cb}, for which the order was switched. However, the regions to which each of these mutations are located on the chromosome map did change, as discussed below.

The results presented in Table 1 indicate that the mutant \emph{l(1)G0179} was deleted by both \emph{Df(1)LB6} and \emph{Df(1)54}, but not by the other deletions. \emph{l(1)G0179} is complemented by \emph{Df(1)BSC708} suggesting its actual location on the chromosome is distal to estimated region 19E7, where the breakpoint of \emph{Df(1)BSC708} is located (based upon annotation of cytological regions relative to annotated genomic coordinates on FlyBase, FB2012_02). The results also suggest that the distal breakpoint of \emph{Df(1)54} extends even further distal to its estimated breakpoint of 19F1 and to the mapped breakpoint of \emph{Df(1)BSC708}, although the exact extent of the deletion remains unknown.
Table 1 also indicated that the lethal extra organs mutation (eo, FBgn0000580) lies between the Df(1)BSC708 and Df(1)DCB1-35c deficiencies but was not complemented by Df(1)LB6, suggesting that the proximal breakpoint for the Df(1)LB6 deficiency is to the right of the proximal breakpoint for Df(1)BSC708.

The complementation results for the introverted (intro, FBgn0001268) mutation were very similar to those obtained for the wap mutation (Table 1 and Figure 2A). For both of these mutants, the mutation was localized to the region between the proximal breakpoint of Df(1)LB6 and the between the proximal and distal breakpoints of Df(1)DCB1-35c.

At least one mutant allele of the uncoordinated-like (uncl, FBgn0003951) gene was complemented by all the deficiencies except Df(1)54 and Df(1)DCB1-35b. This suggested that uncl lies proximal to Df(1)Exel6255 and distal to Df(1)R8A. What is also suggested by these results is that one breakpoint of the Df(1)54 deficiency must also be found within the region between the breakpoints of these deficiencies. It is important to note that results were not consistent between the two uncl alleles used for the mapping. A condition for lethality in this analysis was that the deficiency tested must fail to complement all alleles tested for a specific mutant. The analysis of these mutant alleles indicated that the uncl$^{1}$ allele was lethal with all deficiencies except Df(1)LB6, suggesting that additional mutations may be present in the proximal region of the X chromosome in uncl$^{1}$ mutants.

Both the sozzled (soz, FBgn0001568) and l(1)20Ca (FBgn0001569) mutants were complemented by all the deficiencies except Df(1)DCB1-35c. This indicates that the location of these two mutations lies between the breakpoints of Df(1)Exel6255 and Df(1)R8A and proximal to uncl and the proximal breakpoint of Df(1)54. Lastly, the l(1)20Cb (FBgn0001570) mutant was complemented by all deficiencies, except for Df(1)R8A.
To provide additional rigor to the deletion mapping data, each mutation tested in the above experiments was also subjected to complementation experiments using duplication lines (blue bars in Figure 2), to determine if the duplications were capable of rescuing the phenotype observed in the mutants (Table 3). The duplication lines selected were those that span the region deleted by the *Df(1)Exel6255* deficiency, since this deletion failed to complement *wap*; moreover, the breakpoints of this deficiency have been molecularly mapped. Not all of the duplications were tested with all the mutations, since the deficiency analysis suggested that some point mutations cannot be located in the regions duplicated. The analysis showed that, consistent with our deficiency screen data, the *l(1)G0179*, *l(1)20Cb*, and *l(1)20Ca* mutations could not be rescued by any of the duplication constructs tested.

The *eo* mutation also could not be rescued by any of the tested duplications. This could be due to the presence of more than one lethal mutation on the *eo* mutant chromosome or perhaps, the entire region required for expression of the *eo* gene product or some key regulatory region is not duplicated by the duplication line being tested. It was not surprising that the *uncl* mutation could not be rescued by any of the duplications, since the results from the deficiency screen suggested that multiple sites on the X chromosome were affected in the *uncl* mutation. *soz* was rescued by the *Dp(1;3)DC390* duplication, refining the location of this gene to the region mapped in the complementation analysis.

Data presented in Table 3 also indicate the ability of *Dp(1;3)DC389* to rescue both *intro* and *wap*. This alone suggested that *intro* and *wap* are both located in the 92,593-bp region duplicated by *Dp(1;3)DC389*. When *Df(1)Exel6255/FM7a; Dp(1;3)DC389/Dp(1;3)DC389* females were crossed with *wap^2/Dp(1,Y)y^mal^{71}* males, *Df(1)Exel6255/wap^2* females, which were lethal in the absence of the duplication, were viable in the presence of the duplication (Table 3). We were also able to rescue
Df(1)DCB1-35c/wap^2 females with the Dp(1;3)DC389 duplication using the same approach; however, this result was less significant considering that the Df(1)DCB1-35c deficiency can itself be fully rescued by the Dp(1;3)DC389 duplication (data not shown). Thoraces from both the Df(1)Exel6255/wap^2; Dp(1;3)DC389/+ females (Figure 3F) and Df(1)DCB1-35c/wap^2; Dp(1;3)DC389/+ females (not shown) had a fully formed jump muscle, and these flies also exhibited normal wing crossvein development (Figure 3E). A few wap escapers eclosed from the lines with the Dp(1;3)DC383, Dp(1;3)DC386, and Dp(1;3)DC388 duplications. These flies were not considered rescued by the duplications, as all escapers died shortly after eclosion, a subset of them had extra wing veins, and all lacked the jump muscles (data not shown).

**CG14614 is the gene responsible for wap function**

Both wap and intro are found within the region defined by Df(1)Exel6255, Df(1)DCB1-35c and Dp(1;3)DC389. Six genes are found in this region: CG14614 (FBgn0031186), CG14619 (FBgn0031187), CG14613 (FBgn0031188), CG14618 (FBgn0031189), CG12576 (FBgn0031190), Cp110 (FBgn0031191), and l(1)G1096 (FBgn0027279). wap was complemented by a l(1)G0196 mutant, suggesting l(1)G0196 is not the gene responsible for the wap phenotype (data not shown). For the other genes in the region, RNAi was performed to knock down expression of the products encoded by each gene, using the well-established *Drosophila* Gal4/UAS system (Brand and Perrimon, 1993). By individually knocking down expression of each of the remaining candidate genes, the wap phenotypes should be recapitulated when the correct gene is knocked down. The constitutively expressed Gal4 lines tub-Gal4 and da-Gal4 were used to carry out the knock down experiments.
Results of the knock down experiments with both the *tub*-Gal4 (Lee and Luo, 1999) and *da*-Gal4 (Dura, 2005.12.4) drivers are summarized in Figure 4A. Knock down of *CG14613* was not tested due to unavailability of an RNAi construct, and thus we cannot assess its contribution to the wings-apart phenotype. No lethality was observed in a *CG14618* knock down, and all eclosed flies had a normal jump muscle. This gene was therefore ruled out as a candidate for *wap*. Viability was also observed when *da*-Gal4 was used to knock down *CG14619* (line 37929), *CG12576* (line 51205), and *Cp110* (all lines). Lethality was observed when *da*-Gal4 was used to drive knock down of *CG14614* and a different line targeting *CG14619* (line 37930). The *tub*-Gal4 driver resulted in lethality when used to knock down *CG14614*, *CG14619* (both lines), *CG12576* (line 104261), and *Cp110* (line 101161). All but one (*CG12576*) of the lethal *tub*-Gal4 knockdowns were lethal in the pupal stage. We were able to rule out *CG12576* as a candidate for *wap* since *wap* mutants survive until the pupal stage, leaving *CG14614*, *CG14619*, *CG14613*, and *Cp110* as the remaining candidates for the gene responsible for the *wap* phenotype.

We next determined if the *wap* TDT phenotype was reproduced in any of the pupal lethal knock down flies. Since *CG14614*, *CG14619*, and *Cp110* are all lethal in the pupal stage, pharate adults were removed from their pupal cases and cryogenically sectioned to assess jump muscle morphology. In stained cryosections of wild-type animals, the jump muscle is positioned on the lateral side of the thorax, between DVMs I and II (Figure 4B, arrow). The TDTs of *Cp110* and *CG14619* knockdown flies developed normally (Figure 4C and D, arrows), allowing these gene models to be ruled out as candidates for *wap*. Interestingly, *CG14619* knockdown pharate adults recapitulated the phenotype observed in *intro* mutants, with head structures within the thoracic area (Figure 4D, arrowhead) and a failure of both head eversion and leg and wing disc elongation (data not shown). This observation provisionally identified *CG14619* as the gene affected in *intro* mutants. It should be
noted, however, that the RNAi lines used for \textit{CG14619} knockdown each have 23 off-targets, although none of the 23 genes map to the proximal X chromosome. Thus, our assignment of \textit{CG14619} as \textit{intro} is only tentative.

Importantly, knockdown of \textit{CG14614} resulted in flies either with absent jump muscles (asterisk in Figure 4F) or with severely reduced TDT fiber numbers (Figures 4E and G, arrows). There were also some flies that exhibited both phenotypes, with a reduced jump muscle on one side of the thorax, and a missing jump muscle on the contralateral side. These results strongly suggested \textit{CG14614} is the transcriptional unit associated with the \textit{wap} phenotype.

Knockdown of \textit{CG14614} did not recapitulate the wing vein phenotypes that was observed in a subset of \textit{wap} mutants, but since the wing vein phenotype is not fully penetrant, it is most likely that we have simply yet to sample sufficient flies to observe this effect. In addition, many of the eclosed adults fail to expand their wings prior to death, making it difficult to determine if a subtle wing vein defect was present.

**Genomic duplication of \textit{CG14614} rescues the \textit{wap} jump muscle phenotype**

The data presented above provide strong evidence that \textit{wap} corresponds to \textit{CG14614}. To fully confirm this conclusion, we determined if a duplication containing only the \textit{CG14614} locus was able to rescue any of the phenotypes of \textit{wap} mutants. To achieve this, we amplified by PCR genomic DNA containing the entire \textit{CG14614} transcribed region, and cloned this DNA into a vector for generating transgenic animals. The genomic rescue fragment is indicated as a black bar in Figure 5A. Transgenic animals containing the amplified DNA were generated, and homozygous stocks were established, designated \textit{P}[\textit{CG14614}].
We next crossed FM7/wap\(^x\) females to homozygous males carrying the transgenic DNA, and analyzed the phenotypes of wap\(^x\)/Y; P[CG14614]/+ offspring. The transgene did not effectively rescue the lethality of wap\(^2\) or wap\(^9\), yet when we analyzed pharate adults for jump muscle formation, there was a significant rescue of the muscle phenotype: wap\(^2\)/Y mutants, that normally never show the presence of a jump muscle, had a TDT present in the rescued animals, although it was reduced in size (Figure 5B); more significantly, wap\(^9\)/Y males that also carried the transgene showed a complete rescue of the jump muscle phenotype (Figure 5C). It is unclear why the transgene did not rescue the lethality of the wap\(^2\) and wap\(^9\) alleles. One possibility is that each mutant has a closely linked second-site lethal mutation. Since wap\(^2\) and wap\(^9\) were isolated in separate screens, the linked mutations would each have to have independently arisen, and they must be very closely linked to wap because Dp(1;3)DC389 rescues both mutants. This could be resolved by determining if the transgene can rescue wap\(^2\)/wap\(^9\) trans-heterozygotes. Another possibility is that the genomic rescue construct does not contain enough flanking DNA to express wap in the tissues where it is required for viability. Since we have not mapped the regulatory regions of wap, this remains the most reasonable explanation. Nevertheless, the rescue of the muscle phenotype of the wap mutants overall provided further support for the hypothesis that CG14614 corresponds to wap.

In a further experiment, we sequenced the coding regions of wap\(^2\) and wap\(^9\), following PCR of genomic DNA isolated from hemizygous males. We were unable to collect sufficient DNA to sequence the coding region of the wap\(^3\) mutant, because the wap\(^3\) chromosome also carries a deletion that causes early lethality. We did not observe an alteration in the coding region of wap\(^9\), but the wap\(^2\) allele had a non-sense mutation at codon 319 of the sequence. This change is predicted to result in the formation of a product lacking the last 25 amino acids (Figure 5D). CG14614 is highly orthologous to Wdr68 in zebrafish, and murine DDB1- and CUL4-associated factor 7. These are
WD40 repeat proteins implicated in neuromuscular development (see Discussion for more details). The murine ortholog is 91% similar to CG14614, and the C-terminal amino acids missing in the \textit{wap}^2 isoform of CG14614 are conserved in the mouse isoform. Thus, the residues missing in the truncation mutant likely represent functionally important residues. Since no changes were observed in the coding region of \textit{wap}^9, we propose that this mutation arises from alteration of non-coding regulatory sequences. While we did not carry out quantitative RT-PCR to determine \textit{CG14614} expression levels in the mutants, it seems likely that transcript levels would be reduced in the mutant animals.

Altogether, we demonstrate that: RNAi knockdown of \textit{CG14614} phenocopies the \textit{wap} lethality and jump muscle phenotypes; the jump muscle phenotype of \textit{wap} was rescued using transgenic \textit{CG14614}; and a point mutation is present in the \textit{CG14614} coding region from a \textit{wap}^2 mutant. These data provide compelling evidence that \textit{CG14614} is the gene affected in \textit{wap} mutants.

**Jump muscle founder cells are specified early in development in \textit{wap} mutants but are later lost**

Founder myoblasts are required for the proper specification of individual skeletal muscles in Drosophila. Since these cells have all the genetic information to give the muscle its unique identity, and since the \textit{wap} muscle phenotype is specific to the jump muscle, we hypothesized that jump muscle-specific founder cells are not specified in \textit{wap} mutants. The \textit{wap}^2 and \textit{wap}^9 mutations were recombined with the founder cell marker \textit{duf-lacZ} (also referred to as \textit{rP298}), that shows nuclear \textit{lacZ} expression in all muscle founder cells. Wild-type and mutant pupae were dissected during early pupal formation, when the jump muscle fibers are being specified (Fernandes and VijayRaghavan, 1993). Dissected samples were stained for the myoblast nuclei marker MEF2 (red), and for β-Gal expressed
by the \textit{rP298-lacZ} marker of founder cell nuclei (green). Our results indicated that founder cells were specified early in development but were later lost (Figure 6).

As early as 16 hr APF, formation of the jump muscle was apparent between the dorsoventral indirect flight muscles DVM I and DVM II, which were used as landmarks for the location of the TDT (not shown). By 18-20 hours after puparium formation (h APF), the jump muscles of \textit{FM7i/rP298-lacZ wap} pupae had more structured musculature, and the presence of numerous TDT-specific founder cells was evident (green labeled cells, Figure 6A). By contrast, the 18-20 hr APF \textit{rP298-lacZ wap/Y} samples had largely reduced staining of jump muscle-specific founder cells, but did not exhibit a reduction in founder cell staining in the developing DVMs (Figure 6B). Additionally, the mutant jump muscle (Figure 6B) was much less organized compared with the ordered nature of the wild-type TDT (Figure 6A). By 24 hr APF, the \textit{FM7i/rP298-lacZ wap} pupal musculature had a well developed muscle pattern, with the jump muscle nestled between the DVMs (Figure 6C). By contrast, in the 24 hr APF mutant \textit{rP298-lacZ wap/Y} males, the TDT was completely absent (Figure 6D). There was not an obvious difference in the number of myoblasts contributing to the jump muscle in mutants versus wild-type, although it remains a formal possibility that myoblast number was also affected.

These data demonstrated the requirement for \textit{wap} function early in jump muscle development. Although specification and myoblast fusion were initiated in \textit{wap} mutants, development was not sustained, and the nascent jump muscles did not continue development. Clearly, \textit{wap} function is required to sustain the development of these muscles.

\textit{CG14614} is required in both neurons and muscles for normal development

Previous studies involving indirect flight muscle development have shown that disruption of neuronal restructuring during muscle development can result in degeneration of the muscle target (Fernandes
and VijayRaghavan, 1993). Due to the dependence of muscle formation and neuron developmental pathways on one another, we examined the impact of neuron-targeted knock down of wap (CG14614) on jump muscle development. In addition, we also determined if wap function in myoblasts and developing muscles was required for normal TDT formation. The results are summarized in Figure 7A.

When wap function was knocked down in myoblasts and developing muscles using the 1151-Gal4 driver (Anant et al. 1998), there was a significant reduction in the size of the jump muscle, and in the number of fibers that constituted the muscles. Whereas the TDT in control animals has >25 fibers per muscle (Jaramillo et al 2008; Figure 7A), 1151>wap RNAi animals had jump muscles containing an average of 7.88 fibers (Figure 7C). We did not jump test these knockdown animals, although our earlier studies demonstrated that a reduction in the number of jump muscle fibers compromises the ability to jump (Jaramillo et al 2008). We also used a Mef2-Gal4 driver (Ranganayakulu et al., 1997) to knock down wap function, and also saw a significant, albeit more moderate, reduction in the number of jump muscle fibers. When wap function was knocked down in the nervous system using elav-Gal4, there was also a significant, but less severe, reduction in fiber number (Figure 7D).

We also set up a cross in which both 1151-Gal4 and elav-Gal4 were used to knock down wap function. In this case, the effect on fiber number was no greater than that observed for the 1151-Gal4 driver alone (Figure 7E).

We conclude from these data that the predominant function of wap in jump muscle formation is in the myoblasts and developing muscles. Since the 1151-Gal4 driver also is active in tendon cells (Soler et al., 2004), it is also possible that wap function in these cells contributes to jump muscle formation. Interestingly, there was a significantly reduced viability in elav>wap RNAi flies (Figure 7A).
This indicates that, separate from the requirement for \textit{wap} in the muscles, there is a requirement for \textit{wap} function in the nervous system for normal viability.

**DISCUSSION**

In this paper we have identified a new function for the \textit{wings-apart} gene in controlling formation of the jump muscles in the Drosophila adult. This phenotype is in addition to the lethality and frequent wing crossvein defects that have been described in the past for this mutant (Schalet, 1972, and Schalet and Lefevre, 1973). We further demonstrate that \textit{wap} is allelic to the annotated gene \textit{CG14614}, using RNAi, sequencing of a \textit{wap} mutant allele, and genetic rescue of the jump muscle phenotype. Finally, we demonstrate that \textit{wap} functions predominantly in the developing muscles to sustain myotube survival early during the pupal stage, but that it has a vital function in the nervous system. The pleiotropic effects and the requirements for \textit{wap} function in a number of different tissues are consistent with its broad and robust expression in many Drosophila tissues throughout development (Graveley et al 2011).

What is the function of the protein generated by \textit{wap}? The \textit{wings apart} mutant affects not only the adult wing, but also the viability of the flies and the formation of the jump muscle. The observed TDT phenotype is similar to that of \textit{crossveinless} (\textit{cv}, FBgn0000394) mutants and other BMP pathway mutants (Jaramillo \textit{et al.}, 2009) but does not exhibit the same wing crossvein phenotype as in those mutants. Since \textit{cv} mutants that lacked the posterior crossvein of the adult wing also showed decreased numbers of TDT muscle fibers, and since \textit{wap} mutants have extra wing crossveins, we initially predicted the \textit{wap} mutants to have increased fiber number in the jump muscle. This was not the case. However, the observed downregulation of TDT formation by the \textit{wap} mutant can be explained by at least two different possibilities.
The first possibility is that \textit{wap} is involved in the TGF-\(\beta\) signaling pathway, like \textit{cv}, but may interact with components of the pathway that do not interact with \textit{cv}. The TGF-\(\beta\) pathway has many different ligands, different types of receptors, and a number of intracellular components that associate with each other in varying combinations in a context dependent manner (Khalsa \textit{et al}. 1998). \textit{MAN1} (FBgn0034964) protein products antagonize the TGF-\(\beta\) pathway (Wagner \textit{et al}.., 2010) and \textit{MAN1} mutation leads to the presence of ectopic wing crossveins (Pinto \textit{et al}.., 2008). These mutants do not have an observable muscle defect nor abnormal neuromuscular junctions, but affect synaptic transmission, providing further evidence for context dependence in TGF-\(\beta\) signaling activity.

A second possibility is that, although \textit{wap} acts on the same tissues as \textit{cv} and other TGF-\(\beta\) pathway components, it may be part of a different signaling pathway. The \textit{Epidermal Growth Factor receptor} (\textit{Egfr}, FBgn0003731) pathway is required for the proper formation of wing crossveins (Ralston and Blair, 2005) and also functions in the formation of muscles (Maqbool and Jagla, 2007). Angulo \textit{et al}. (2004) show that \textit{absent, small, or homeotic discs 2} (\textit{ash2}, FBgn0000139) represses EGFR signaling. Mutations in \textit{ash2} also result in ectopic wing veins (Angulo \textit{et al}.., 2004) and neural defects (Beltran \textit{et al}.., 2003).

However, \textit{MAN1} mutants do not show jump muscle defects (data not shown), and there is no observed genetic interaction between \textit{wap} mutants and null alleles of genes functioning in either the TGF-\(\beta\) or EGF pathways (data not shown). These observations suggest that \textit{wap} functions through yet another mechanism.

The predicted protein product of \textit{wap} has a WD40 repeat domain, and genes orthologous to \textit{wap} are found in organisms ranging from yeast to plants to humans. Orthologs include the \textit{TTG1} gene that regulates root, shoot, and leaf patterning in \textit{Arabidopsis} (Walker \textit{et al}.., 1999), and vertebrate craniofacial and muscle patterning genes (Nissen \textit{et al}.., 2006). WD40 repeat proteins
mediate protein-protein interactions and contain 4-10 repeating units of 44-60 residues ending in tryptophan and aspartate (WD) (reviewed in Holm et al., 2001 and Suganuma et al., 2008). These repeats form propeller-like structures, termed β-propellers, created by the folding of four antiparallel β-sheets (reviewed in Holm et al., 2001). This protein family is known to have roles in signal transduction, mRNA processing, gene regulation, vesicular trafficking, and regulation of cell cycle (reviewed in Skurat and Dietrich, 2004 and reviewed in Suganuma et al., 2008). These findings are consistent with the premise that genes whose mutants have pleiotropic effects can be members of broadly-used biological pathways.

One particular vertebrate ortholog for wap is wdr68, that is involved in craniofacial patterning in zebrafish (Nissen et al., 2006). Another orthologous gene has been isolated in rabbit skeletal muscle as DDB1 and CUL4 associated factor 7 (DCAF7) (Skurat and Dietrich, 2004). The zebrafish severe craniofacial defect observed in wdr68 mutants can be rescued by expression of Drosophila wap, suggesting that the function of the wdr68 gene is conserved in animals from invertebrates to vertebrates, even in developmental processes such as craniofacial development that are not found in invertebrates (Nissen et al., 2006).

Studies in vertebrates also demonstrate that Wdr68/DCAF7 associates with members of the Dual-specificity tyrosine phosphorylation-regulated kinase gene family, Dyrk1a and Dyrk1b, and probably functions within the nucleus. Dyrk1a plays a role in phosphorylation of glycogen synthase and is expressed at high levels in the central nervous system, heart and skeletal muscle (Skurat and Dietrich, 2004). Mutations in Dyrk1a genes in humans and mice are associated with neurological defects (Martinez de Lagran et al., 2004). Dyrk1b, which is activated by Rho-GTPase family members, has increased expression in skeletal muscles and regulates the transition from growth to differentiation. Knockdown in mouse C2C12 cell lines results in a loss of myogenin expression and
leads to failure in muscle differentiation (Deng et al., 2003). Dyrk1 also has a conserved function between vertebrates and invertebrates. The Drosophila gene minibrain (mbn) is a functional ortholog of vertebrate Dyrk1a, and is required for proper formation of post-embryonic neurons (Tejedor et al., 1995 and reviewed in Kinstrie et al., 2006). Thus, there is developing evidence that wap and mnb each function in a conserved pathway to regulate muscle and/or neuron development. In the future, it will be interesting to determine if Mnb protein co-localizes with Wap protein, and whether mnb also acts in the same pathway as wap.

ACKNOWLEDGEMENTS

We are grateful to Bruce Paterson for anti-MEF2, TyAnna Lovato for assistance with cryosectioning and generating transgenic flies, and William Gelbart for valuable discussions of this work. This research was supported by R01 GM061738 awarded by the NIH to RMC. GRM was supported by an NIH Initiatives to Maximize Student Diversity grant (R25 GM060201). CTJ was supported by a Minority Access to Research Careers award from NIH (T34 GM008751), and by a diversity supplement to GM061738 from NIH/NIGMS. This research was supported in part by the More Graduate Education at Mountain States Alliance (MGE@MSA) Alliance for Graduate Education and the Professoriate (AGEP) National Science Foundation (NSF) Cooperative Agreement No. HRD-0450137. We acknowledge technical support from the Department of Biology’s Molecular Biology Facility, supported by NIH grant number P20 GM103452 from the Institute Development Award (IDeA) Program of the National Center for Research Resources.

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FIGURE LEGENDS

Figure 1 wings apart (wap) mutants show three phenotypes. A, B: In the wings, whereas wild-type
(WT) wings have two crossveins (A), a proportion of wap mutants show additional crossveins
between L2 and L3 (B, arrows). C, D: In horizontal sections of the adult thorax, wild-type shows a
prominent tubular jump muscle, or TDT muscle (C, arrow); by contrast wap mutants do not have a detectable jump muscle (D, asterisk). DLM and DVM indicate dorsal longitudinal and dorsoventral indirect flight muscles, respectively. E: Compared to control female siblings, whose viability is normalized to 1.0, wap heteroallelic combinations show extremely low viability. n represents the total number of females scored, from a cross of FM7 / wap<sup>x</sup> females crossed to wap<sup>y</sup> / Dp(1;Y)y<sup>mal</sup> males, in which 50% of the female progeny would be expected to be of the heteroallelic genotype. Bar, 400µm for A,B; 50µm for C, D.

**Figure 2** Summary of genetic and genomic mapping of wap on the proximal X chromosome. From the top: genomic coordinates, followed by the approximate locations of genes that have yet to be annotated, based upon our deletion and duplication mapping. Annotated genes are named and shown as blue boxes, with light blue denoting genes transcribed towards the centromere, and darker blue denoting genes transcribed away from the centromere. Estimated cytology is indicated. Red boxes denote extent of indicated deficiencies; pale red regions denote uncertainties in locations of break points. Blue boxes at bottom represent the extents of named duplications.

**Figure 3** Deletion and duplication mapping of the wap wing and jump muscle phenotypes. A, B: Df(1)BSC708 does not uncover the wing and jump muscle phenotypes of wap<sup>2</sup>. C, D: Df(1)Exel6255 deletes the wap locus, since Df(1)Exel6255 / wap<sup>2</sup> combinations show ectopic wing veins (arrow in C) and a loss of the jump muscle (asterisk in D). Inset in D shows a rare residual jump muscle observed in a Df(1)54/wap<sup>2</sup> female. E, F: the Df(1)Exel6255 / wap<sup>2</sup> phenotypes can be rescued by Dp(1;3)DC389. Bar, 400µm for A,C, E; 50µm for B, D, F.
Figure 4 Effects of RNAi knockdown of gene candidates for \textit{wap}. A: Candidate genes that are annotated on the genome map were knocked down using UAS-RNAi, controlled by the ubiquitous drivers \textit{tub-Gal4} or \textit{da-Gal4}. For each gene targeted, we show: the line supplied by the Vienna Drosophila RNAi Center (VDRC); the number of off-targets predicted for the RNAi; and the viability and jump muscle phenotypes observed in the knockdown animals. B-D: Representative immunofluorescent images of horizontal sections through the thorax of knockdown animals. B: Wild-type animals show the appearance of the jump muscle. C, D: Knockdown of \textit{Cp110} or \textit{CG14619} does not affect jump muscle formation. Note the disorganized muscles in the \textit{CG14619} knockdown that results from a failure to evert the head (arrowhead) during pupal development. E-G: Knockdown of \textit{CG14614} causes a severe reduction in the number of fibers in the jump muscle, and defects in the cross-sectional shape of the muscle. Arrows in all sections denote the jump muscle; asterisk denotes absence of jump muscle. Sections were stained for F-actin (green), βPS integrin (red) and DAPI (blue). Bar, 50µm.

Figure 5 \textit{CG14614} is the gene affected in \textit{wap}. A: Higher detail of genomic region included in the \textit{Dp(1;3)DC389} duplication. Note that only five annotated genes are contained within this deficiency. Black bar denotes the wild-type rescue construct for \textit{CG14614}. B,C: Stained cryosections of \textit{wap} mutants rescued using the introduced genomic fragment described in A. Note that \textit{wap}²/Y shows partial rescue of the jump muscle (Arrow in B), while \textit{wap}³/Y shows complete rescue of the jump muscle (Arrow in C). D: Domain structures of wild-type and mutant CG14614 proteins, and the zebrafish ortholog, \textit{Wdr68}. Note the premature stop codon in the \textit{wap}² isoform. Bar, 50µm.
**Figure 6** *wap* mutants generate muscle precursors, that subsequently are lost. Control (left column) and *wap* mutant (right column) pupae were collected at the indicated pupal time points and stained for the indicated markers. A-D: Note that the founder cells for the jump muscle, visualized by βgal accumulation in the rP298 background, are specified normally in the *wap* mutants, but that the muscle is lost by 24h APF. Bar, 50µm.

**Figure 7** Tissue-specific knockdowns demonstrate that *wap* functions in both muscle and neuronal cells to sustain jump muscle development. Tissue-specific Gal4 drivers were used to knock down *wap* function in either all cells (*tub-Gal4*), adult muscles (*1151-Gal4*), neurons (*elav-Gal4*), or both muscles and neurons (*1151-Gal4* and *elav-Gal4*). A: Summary of effects of tissue-specific *wap* knockdown upon viability and jump muscle fiber number. Note that viability is more severely reduced using the neuronal driver, but a stronger muscle phenotype is apparent using the *1151-Gal4* driver. Using a combination of drivers does not enhance the jump muscle phenotype. B-E: Representative immunofluorescent images of the global and tissue-specific knockdowns. Note the reduction in (arrow), or absence of (asterisk), the jump muscle. Bar, 50µm.
E Viability of heteroallelic wap females

Proportion of eclosed females (normalized to wild-type)

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<td>wap²/wap³ n = 306</td>
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<tr>
<td>wap²/wap⁹ n = 52</td>
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A

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<td>Viable, normal TDT</td>
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B

DVM I

DVM II

C

DVM I

DVM II

D

tub>CG14619 RNAi

E

da>CG14614 RNAi

F

tub>CG14614 RNAi

G

tub>CG14614 RNAi

Actin

WT
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<th>Average Fiber number</th>
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</tr>
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<td>*18.70</td>
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<td>Adult muscle + neurons</td>
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<td><strong>8.62</strong></td>
<td>3.18</td>
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a, All adults died within two days of eclosion
*, p<0.01; **, p<0.0001
**Table 1. Deficiency mapping of proximal X chromosome point mutations.**

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<th>uncl</th>
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<td>-</td>
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<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Df(1)Exel6255</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>+</td>
<td>+</td>
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</tr>
<tr>
<td>Df(1)DCB1-35c</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Df(1)DCB1-35b</td>
<td>NT</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>+</td>
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<td>-</td>
</tr>
<tr>
<td>Df(1)R8A</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
</tbody>
</table>

a, + indicates the heteroallelic combination viable; - indicates heteroallelic combination was lethal or semi-lethal; NT indicates combination not tested.

**Table 2. Complementation tests of proximal X chromosome deficiencies.**

<table>
<thead>
<tr>
<th>Deficiency tested</th>
<th>Df(1)BSC708</th>
<th>Df(1)LB6</th>
<th>Df(1)54</th>
<th>Df(1)Exel6255</th>
<th>Df(1)DCB1-35c</th>
<th>Df(1)DCB1-35b</th>
<th>Df(1)R8A</th>
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<tbody>
<tr>
<td>Df(1)BSC708</td>
<td>-a</td>
<td>-</td>
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<td>-</td>
<td>+</td>
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<tr>
<td>Df(1)LB6</td>
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<td>-</td>
<td>+</td>
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<td>+</td>
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<tr>
<td>Df(1)54</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>Df(1)Exel6255</td>
<td>-</td>
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<td>+</td>
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</tr>
<tr>
<td>Df(1)DCB1-35c</td>
<td>-</td>
<td>-</td>
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<td>-</td>
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<tr>
<td>Df(1)DCB1-35b</td>
<td>-</td>
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<tr>
<td>Df(1)R8A</td>
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</table>

a, - indicates the heteroallelic combination was lethal; + indicates the combination was viable.
Table 3. Duplication mapping of proximal X chromosome point mutations.

<table>
<thead>
<tr>
<th>Duplication tested</th>
<th>eo</th>
<th>intro</th>
<th>wap</th>
<th>Deficiency/wap</th>
<th>uncl</th>
<th>soz</th>
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<tbody>
<tr>
<td>Dp(1;3)DC382</td>
<td>-a</td>
<td>NT</td>
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<tr>
<td>Dp(1;3)DC383</td>
<td>-</td>
<td>NT</td>
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<tr>
<td>Dp(1;3)DC384</td>
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<td>NT</td>
<td>-</td>
<td>NT</td>
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<tr>
<td>Dp(1;3)DC562</td>
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<tr>
<td>Dp(1;3)DC386</td>
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<tr>
<td>Dp(1;3)DC387</td>
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<tr>
<td>Dp(1;3)DC388</td>
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<td>-</td>
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<td>+</td>
<td>+</td>
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<tr>
<td>Dp(1;3)DC390</td>
<td>NT</td>
<td>-</td>
<td>-</td>
<td>NT</td>
<td>-</td>
<td>+</td>
</tr>
</tbody>
</table>

a, + indicates the heteroallelic combination viable; - indicates heteroallelic combination was lethal or semi-lethal; NT indicates combination not tested.