The *Drosophila wings apart* Gene Anchors a Novel, Evolutionarily Conserved Pathway of Neuromuscular Development

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ABSTRACT *wings apart* (*wap*) is a recessive, semilethal gene located on the X chromosome in *Drosophila melanogaster*, which 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 RNA interference 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 in both the musculature and the nervous system for normal jump-muscle formation. *wap/CG14614* is homologous to vertebrate *wdr68*, *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 that may result from mis-expression of muscle-specific or neuron-specific genes.

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 TGF β 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). Re-

cently, we identified an additional role for TGF β 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 1967). Hypomorphic mutations affecting the TGF β 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 Bone Morphogenetic Protein (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 and Popodi *et al.* 2010).

Here, we have analyzed the *wings-apart* (*wap*) gene in *Drosophila*. *wap* to date has not been associated with an annotated gene; yet, as with mutations affecting the TGF β pathway, *wap* mutant phenotypes include semilethality and defects in wing crossvein formation (Lifschytz and Falk 1969; Schalet and Lefevre 1973). We demonstrate first 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 the 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° on Jazz food mix (Fisher Scientific) unless otherwise stated. All genotype information is according to the guidelines at <http://www.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 at Harvard Medical School.

For deficiency screens, each cross was composed of equal numbers of virgin females and males and maintained at 25°. 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; Rahman and Lindsley 1981). This was achieved by crossing female *Balancer/Deficiency* to males of the genotype *wap^x/Dp(1;Y)y⁺mal¹⁷¹*, 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/wap^x* genotype and female progeny with the *Deficiency/wap^x* genotype were performed using Student’s *t*-test. Analyses of jump-muscle and wing crossvein phenotypes were performed for both *Balancer/wap^x* and *Deficiency/wap^x* 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 *wap²/Dp(1;Y)y⁺mal¹⁷¹* males. Female *FM7a/wap²; Duplication/+* progeny were compared with female *Deficiency/wap²; 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 RNA interference (RNAi) knockdown experiments (Brand and Perrimon 1993). Equal numbers of virgin female and male flies were allowed to mate 3 days at 25°, at which point crosses were transferred to 29° to activate maximally the Gal4 drivers. *tub-Gal4* and *da-Gal4* were used as ubiquitous drivers for initial RNAi analysis. To determine the 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 paragraphs.

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^x* mutants were crossed with equal numbers of *rP298-lacZ* transgenic males. *rP298-lacZ/wap^x* female progeny were selected as virgins and crossed with *FM7i,GFP/Y* males. Each female of the F₂ generation was isolated individually as a virgin 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 *rP298-lacZ* transgenic marker, two adult flies from each line positive for the *wap* mutation were filleted and stained overnight at 37° in XGAL solution [1× PBS, 100 mM K₄[Fe(CN)₆], 100 mM K₃[Fe(CN)₆], 150 mM NaCl, 1 mM MgCl₂, and 0.2% w/v X-Gal (Sigma)]. Stocks positive for both the *wap* mutation and the *rP298-lacZ* transgene were used in the pupal dissections described below. Both the *wap²* and *wap⁹* alleles were used for these crosses.

DNA methods

The coding sequence for *wap* was amplified from wild-type and *wap^x/Y* mutants, using primers of the sequences 5'-gag gtagtagtgacatcagc and 5'-gtcgcgtgccgaaagtcccg. The region amplified was sequenced using BigDye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems Inc.). Sequences were edited, aligned, and analyzed using Sequencer (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 Optimal Cutting Temperature (OCT) medium, and freezing. Sections were cut at 10-μm thickness at -18° and air dried. Samples were fixed for 10 min at room temperature with 3.7% v/v formaldehyde in PBTx [1× PBS, 0.2% v/v Triton-X100, 0.2% w/v Blocking Agent (Roche)], washed, and used for antibody staining as described below.

For pupal 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 1× PBS, washed in PBTx, then sub-

jected to blocking, and incubated with antibody (described below).

Fixed and washed samples were stained with antibodies as described by Patel (1994) and modified by Molina and Cripps (2001). Primary antibodies used for cryosections were anti-βPS-integrin 1:10 (Brower *et al.* 1984) (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, National Institutes of Health). 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

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 *wap*, characterized by three phenotypes (Figure 1). First, in the wing of an adult wild-type fly, there are five longitudinal veins and two crossveins (Figure 1A); in ~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). Second, 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). Third, *wap* mutants are semilethal as homozygotes or hemizygotes (Schalet 1972), and this semilethal 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,

Figure 1 *wap* mutants show three phenotypes. (A and 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 and D) In horizontal sections of the adult thorax, wild type shows a prominent tubular jump muscle, the TDT muscle (C, arrow); by contrast, *wap* mutants do not have a detectable jump muscle (D, asterisk). DLM, dorsal longitudinal indirect flight muscle; DVM, dorsoventral indirect flight muscle. (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^x* females crossed to *wap^y/Dp(1;Y)y⁺mal¹⁷¹* males, in which 50% of the female progeny would be expected to be of the heteroallelic genotype. Bar, 400 μ m for A and B; 50 μ m for C and D.

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 (1969) suggested that *wap* is located on the proximal X chromosome in region 20A3-4. 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; 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 semilethal 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 thoraxes of females heterozygous for the deficiencies and *wap* were analyzed for the presence of the wing and TDT phenotypes characteristic of *wap*. We found that those heterozygous females from the deficiency lines that complemented *wap* had normal wing-vein patterning and that the jump muscle was present (Figure 3, A and B, results from *Df(1)BSC708/wap²* shown). The escapers from the semilethal heterozygotes exhibited an

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 toward the centromere and darker blue denoting genes transcribed away from the centromere. Estimated cytology is indicated. Red boxes denote extent of indicated deficiencies; light red regions denote uncertainties in locations of breakpoints. Blue boxes at bottom represent the extents of named duplications.

additional crossvein between the second and third longitudinal vein, characteristic of *wap* homozygous mutants (Figure 3C). Moreover, the jump muscle in almost all of these heterozygous females was completely absent (Figure 3D, results from *Df(1)Exel6255/wap²* 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 >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 [*Df(1)LB6*, *Df(1)54*, *Df(1)DCB1-35c*, *Df(1)DCB1-35b*, and *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 *Df(1)Exel6255* cannot be ruled out as candidates for *wap*. Results shown in Table 2 demonstrate complementation of *Df(1)LB6* with *Df(1)DCB1-35c*, suggesting that the proximal breakpoint for *Df(1)LB6* is to the left (i.e., centromere distal) of the distal breakpoint for *Df(1)DCB1-35c*. The proximal breakpoints for *Df(1)54* and *Df(1)DCB1-35c* are left of the

distal breakpoint for *Df(1)R8A*, as both of these are complemented by the *Df(1)R8A* deficiency (Table 2, last column). The distal breakpoint of *Df(1)R8A* is also more proximal than the proximal breakpoint of *Df(1)Exel6255*, as inferred from complementation between these two deficiencies. The *Df(1)DCB1-35b* deficiency spans the entire region tested, as previously reported (Schalet and Finnerty 1968), and does not complement *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 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 *l(1)20Ca* and *l(1)20Cb*, for which the order was switched. However, the regions to which each

Table 1 Deficiency mapping of proximal X-chromosome point mutations

Deficiency tested	Mutation mapped							
	<i>l(1)G0179</i>	<i>eo</i>	<i>intro</i>	<i>wap</i>	<i>uncl</i>	<i>soz</i>	<i>l(1)20Cb</i>	<i>l(1)20Ca</i>
<i>Df(1)BSC708</i>	+	+	+	+	–	+	+	+
<i>Df(1)LB6</i>	–	–	+	+	+	+	+	+
<i>Df(1)54</i>	–	–	–	–	–	+	+	+
<i>Df(1)Exel6255</i>	+	–	–	–	–	+	+	+
<i>Df(1)DCB1-35c</i>	+	+	–	–	+	+	+	+
<i>Df(1)DCB1-35b</i>	NT	–	–	–	–	–	+	–
<i>Df(1)R8A</i>	+	+	+	+	+	+	–	+

“+” indicates that the heteroallelic combination was viable; “–” indicates that the heteroallelic combination was lethal or semilethal; NT indicates that the combination was not tested.

these mutations are located on the chromosome map did change, as discussed below.

The results presented in Table 1 indicate that the mutant *l(1)G0179* was deleted by both *Df(1)LB6* and *Df(1)54*, but not by the other deletions. *l(1)G0179* is complemented by *Df(1)BSC708*, suggesting that its actual location on the chromosome is distal to estimated region 19E7, where the breakpoint of *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 *Df(1)54* extends even further distal to its estimated breakpoint of 19F1 and to the mapped breakpoint of *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 2). For both of these mutants, the mutation was localized to the region between the proximal breakpoint of *Df(1)LB6* and 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 *uncl¹* allele was lethal with all deficiencies except *Df(1)LB6*, suggesting that additional

Figure 3 Deletion and duplication mapping of the *wap* wing and jump-muscle phenotypes. (A and B) *Df(1)BSC708* does not uncover the wing and jump-muscle phenotypes of *wap²*. (C and D) *Df(1)Exel6255* deletes the *wap* locus, since *Df(1)Exel6255/wap²* 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/wap²* female. (E and F) *Df(1)Exel6255/wap²* phenotypes can be rescued by *Dp(1;3)DC389*. Bar, 400 µm for A, C, and E; 50 µm for B, D, and F.

mutations may be present in the proximal region of the X chromosome in *uncl¹* 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*. Finally, 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

Table 2 Complementation tests of proximal X-chromosome deficiencies

Deficiency tested	Deficiency mapped						
	<i>Df(1)BSC708</i>	<i>Df(1)LB6</i>	<i>Df(1)54</i>	<i>Df(1)Exel6255</i>	<i>Df(1)DCB1-35c</i>	<i>Df(1)DCB1-35b</i>	<i>Df(1)R8A</i>
<i>Df(1)BSC708</i>	–	–	–	–	–	–	+
<i>Df(1)LB6</i>		–	–	–	+	–	+
<i>Df(1)54</i>			–	–	–	–	+
<i>Df(1)Exel6255</i>				–	–	–	+
<i>Df(1)DCB1-35c</i>					–	–	+
<i>Df(1)DCB1-35b</i>						–	–
<i>Df(1)R8A</i>							–

“–” indicates that the heteroallelic combination was lethal; “+” indicates that the combination was viable.

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 both *intro* and *wap* are 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 *wap²/Dp(1,Y)⁺mal¹⁷¹* males, *Df(1)Exel6255/wap²* 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/wap²* 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). Thoraxes from both the *Df(1)Exel6255/wap²*; *Dp(1;3)DC389/+* females (Figure 3F) and the *Df(1)DCB1-35c/wap²*; *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*. Seven genes are found in this region: *CG14614* (FBgn0031186), *CG14619* (FBgn0031187), *CG14613* (FBgn0031188), *CG14618* (FBgn0031189), *CG12576* (FBgn0031190), *Cp110* (FBgn0031191), and *l(1)G0196* (FBgn0027279). *wap* was complemented by a *l(1)G0196* mutant, suggesting that *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 knockdown experiments.

Results of the knockdown experiments with both the *tub-Gal4* (Lee and Luo 1999) and *da-Gal4* (Dura 2005) drivers are summarized in Figure 4A. Knockdown 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* knockdown, 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 knockdown 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 knockdown 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 DVM I and DVM II (Figure 4B, arrow). The TDTs of *Cp110* and *CG14619* knockdown flies developed normally (Figure 4, C 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

Table 3 Duplication mapping of proximal X-chromosome point mutations

Duplication tested	Mutation mapped					
	<i>eo</i>	<i>Intro</i>	<i>wap</i>	<i>Deficiency/wap</i>	<i>uncl</i>	<i>soz</i>
<i>Dp(1;3)DC382</i>	–	NT	–	NT	–	–
<i>Dp(1;3)DC383</i>	–	NT	–	–	–	–
<i>Dp(1;3)DC384</i>	–	NT	–	NT	–	–
<i>Dp(1;3)DC562</i>	–	–	–	–	–	–
<i>Dp(1;3)DC386</i>	–	–	–	–	–	–
<i>Dp(1;3)DC387</i>	–	–	–	–	–	–
<i>Dp(1;3)DC388</i>	–	–	–	–	–	–
<i>Dp(1;3)DC389</i>	–	+	+	+	–	–
<i>Dp(1;3)DC390</i>	NT	–	–	NT	–	+

“+” indicates that the heteroallelic combination was viable; “–” indicates that the heteroallelic combination was lethal or semilethal; NT indicates that the combination was not tested.

shown). This observation provisionally identified *CG14619* as the gene affected in *intro* mutants. It should be noted, however, that the RNAi lines used for *CG14619* knockdown each have 23 off-targets, although none of the 23 genes map to the proximal X chromosome. Thus, our assignment of *CG14619* as *intro* is only tentative.

Importantly, knockdown of *CG14614* resulted in flies either with absent jump muscles (asterisk in Figure 4F) or with severely reduced TDT fiber numbers (Figure 4, E 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 suggest that *CG14614* is the transcriptional unit associated with the *wap* phenotype.

Knockdown of *CG14614* did not recapitulate the wing-vein phenotypes that was observed in a subset of *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 *CG14614* rescues the *wap* jump-muscle phenotype

The data presented above provide strong evidence that *wap* corresponds to *CG14614*. To fully confirm this conclusion, we determined if a duplication containing only the *CG14614* locus was able to rescue any of the phenotypes of *wap* mutants. To achieve this, we amplified by PCR genomic DNA containing the entire *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 and designated as *P[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 *wap²* or *wap⁹*, yet when

we analyzed pharate adults for jump-muscle formation, there was a significant rescue of the muscle phenotype: *wap²/Y* mutants, which 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, *wap⁹/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 *wap²* and *wap⁹* alleles. One possibility is that each mutant has a closely linked second-site lethal mutation. Since *wap²* and *wap⁹* 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 *wap²/wap⁹* 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 *wap²* and *wap⁹*, following PCR of genomic DNA isolated from hemizygous males. We were unable to collect sufficient DNA to sequence the coding region of the *wap³* mutant because the *wap³* chromosome also carries a deletion that causes early lethality. We did not observe an alteration in the coding region of *wap⁹*, but the *wap²* 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 *wap²* 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 *wap⁹*, we propose that this mutation arises from alteration of noncoding regulatory sequences. While we did not carry out quantitative RT-PCR to determine *CG14614* expression levels in the mutants, it seems likely that transcript levels would be reduced in the mutant animals.

Altogether, we demonstrate the following: that RNAi knockdown of *CG14614* phenocopies the *wap* lethality and jump-muscle phenotypes; that the jump-muscle phenotype of *wap* was rescued using transgenic *CG14614*; and that a point mutation is present in the *CG14614* coding region from a *wap²* mutant. These data provide compelling evidence that *CG14614* is the gene affected in *wap* mutants.

Figure 4 Effects of RNAi knockdown of gene candidates for *wap*. (A) Candidate genes that are annotated on the genome map were knocked down using UAS-RNAi, controlled by the ubiquitous drivers *tub-Gal4* or *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 and D) Knockdown of *Cp110* or *CG14619* does not affect jump-muscle formation. Note the disorganized muscles in the *CG14619* knockdown that results from a failure to evert the head (arrowhead in D) during pupal development. (E–G) Knockdown of *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 panels denote the jump muscle; asterisk denotes absence of jump muscle. Sections were stained for F-actin (green), β PS integrin (red), and DAPI (blue). Bar, 50 μ m.

Jump-muscle founder cells are specified early in development in *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 *wap* muscle phenotype is specific to the jump muscle, we hypothesized that jump-muscle-specific founder cells are not specified in *wap* mutants. The *wap²* and *wap⁹* mutations were recombined with the founder cell marker *duf-lacZ* (also referred to as *rP298*), which shows nuclear *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 *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 after puparium formation (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 hr APF, the jump muscles of *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- to 20-hr APF *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 *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 *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 vs. wild type, although it remains a formal possibility that myoblast number was also affected.

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

Figure 5 CG14614 is the gene affected in *wap*. (A) Higher detail of genomic region included in the *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 CG14614. (B and C) Stained cryosections of *wap* mutants rescued using the introduced genomic fragment described in A. Note that *wap²/Y* shows partial rescue of the jump muscle (arrow in B), while *wap⁹/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, Wdr68. Note the premature stop codon in the *wap²* isoform. Bar, 50 μ m.

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 knockdown 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.* 2009) (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 num-

ber of jump-muscle fibers compromises the ability to jump (Jaramillo *et al.* 2009). We also used a *Mef2-Gal4* driver (Ranganayakulu *et al.* 1998) 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 *wap* in the muscles, there is a requirement for *wap* function in the nervous system for normal viability.

Figure 6 *wap* mutants generate muscle precursors that subsequently are lost. Control (left) and *wap* mutant (right) 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 β -gal accumulation in the *rP298* background, are specified normally in the *wap* mutants, but that the muscle is lost by 24 h APF. Bar, 50 μ m.

Discussion

In this article we have identified a new function for the *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; Schalet and Lefevre 1973). We further demonstrate that *wap* is allelic to the annotated gene *CG14614*, using RNAi, sequencing of a *wap* mutant allele, and genetic rescue of the jump-muscle phenotype. Finally, we demonstrate that *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 *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 *wap*? The *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 *crossveinless* (*cv*, FBgn0000394) mutants and other BMP pathway mutants (Jaramillo *et al.* 2009) but does not exhibit the same wing crossvein phenotype as in those mutants. Since *cv* mutants that lacked the posterior crossvein of the adult wing also showed decreased numbers of TDT muscle fibers, and since *wap* mutants have extra wing crossveins, we initially predicted the *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 *wap* mutant can be explained by at least two different possibilities.

The first possibility is that *wap* is involved in the TGF β -signaling pathway, like *cv*, but may interact with components of the pathway that do not interact with *cv*. The TGF β 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 *et al.* 1998). *MAN1* (FBgn0034964) protein products antagonize the TGF β pathway (Wagner *et al.* 2010) and *MAN1* mutation leads to the presence of ectopic wing crossveins (Pinto *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 β -signaling activity.

A second possibility is that, although *wap* acts on the same tissues as *cv* and other TGF β pathway components, it may be part of a different signaling pathway. The *Epidermal Growth Factor receptor* (*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 *et al.* (2004) show that *absent*, *small*, or *homeotic discs 2* (*ash2*, FBgn0000139) represses EGFR signaling. Mutations in *ash2* also result in ectopic wing veins (Angulo *et al.* 2004) and neural defects (Beltran *et al.* 2003).

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

The predicted protein product of *wap* has a WD40 repeat domain, and genes orthologous to *wap* are found in organisms ranging from yeast to plants to humans. Orthologs include the *TTG1* gene that regulates root, shoot, and leaf patterning in *Arabidopsis* (Walker *et al.* 1999) and vertebrate craniofacial and muscle patterning genes (Nissen *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, messenger RNA processing, gene regulation, vesicular trafficking, and regulation of the cell cycle (reviewed in Skurat and Dietrich 2004 and 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*, which is involved in craniofacial patterning in zebrafish

Genotype	Tissue type	Viability	Average Fiber number	Std. Dev.
1151-Gal4	Adult muscle	100%	28.00	0.00
elav-Gal4	Neurons	100%	23.00	3.00
tub-Gal4>wap RNAi	All tissues	0%	2.00	2.00
1151-Gal4>wap RNAi	Adult muscle	100%	**7.88	2.75
Mef2-Gal4>wap RNAi	All muscle	100%	*18.70	3.53
elav-Gal4>wap RNAi	Neurons	^a 51%	*18.70	2.41
1151-Gal4 + elav-Gal4>wap RNAi	Adult muscle + neurons	^a 19%	**8.62	3.18

a, All adults died within two days of eclosion
*, p<0.01; **, p<0.0001

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, 50 μ m.

(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* (*mnbr*) is a functional ortholog of vertebrate *Dyrk1a* and is required for proper formation of post-embryonic neurons (Tejedor *et al.* 1995;

reviewed in Kinstrie *et al.* 2006). Thus, there is developing evidence that *wap* and *mnbr* 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 colocalizes with *Wap* protein and whether *mnbr* also acts in the same pathway as *wap*.

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