Developmental Cell
Article

The Transmembrane Protein Kon-tiki Couples to Dgrip to Mediate Myotube Targeting in *Drosophila*

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DOI 10.1016/j.devcel.2007.02.017

SUMMARY

Directed cell migration and target recognition are critical for the development of both the nervous and muscular systems. Molecular mechanisms that control these processes in the nervous system have been intensively studied, whereas those that act during muscle development are still largely uncharacterized. Here we identify a transmembrane protein, Kon-tiki (Kon), that mediates myotube target recognition in the *Drosophila* embryo. Kon is expressed in a specific subset of myotubes and is required autonomously for these myotubes to recognize their tendon cell targets and to establish a stable connection. Kon is enriched at myotube tips during targeting and signals through the intracellular adaptor Dgrip in a conserved molecular pathway. Forced overexpression of Kon stimulates muscle motility. We propose that Kon promotes directed myotube migration and transduces a target-derived signal that initiates the formation of a stable connection.

INTRODUCTION

The development of several different organ systems requires migrating precursor cells to locate, recognize, and connect to specific target cells. A striking and intensively studied example of this process occurs during nervous system development, as the axons and dendrites of differentiating neurons seek out their respective synaptic partners. Muscle cells face a similar challenge in finding, identifying, and attaching to their target cells—the tendon cells that connect to bone (in vertebrates) or epidermis (in invertebrates). Just as the establishment of correct neuronal wiring specificity is critical for neural function, so too is the precise connection specificity between muscles and tendon cells essential for normal muscular function. Yet, whereas great progress has been made over the past decade in defining the molecules and mechanisms that mediate neuronal guidance and target recognition (Dickson, 2002; Tessier-Lavigne and Goodman, 1996), those that mediate muscle guidance and target recognition are still poorly understood. Indeed, it is not even clear to what extent the superficially similar processes of neuronal and muscle targeting rely on shared or distinct molecular mechanisms.

The body muscles of the *Drosophila* embryo provide an ideal model system for a genetic approach to the problem of muscle targeting (Schnorrer and Dickson, 2004). As in vertebrates, the musculature of the *Drosophila* embryo is highly stereotyped, with uniquely identifiable muscles connecting to specific attachment sites. A total of 30 muscles form in each of the abdominal hemisegments A2–A7, with each muscle having its characteristic size, shape, and epidermal attachment sites (Bate, 1990). These muscles are single multinucleated cells, and their development can be readily followed by live imaging. Thus, this system offers the opportunity to use genetic and imaging methods to explore muscle targeting in vivo with single-cell resolution.

Drosophila muscle cells develop from two types of myoblasts: founder cells and fusion-competent myoblasts (FCMs) (Bate, 1990). Each muscle has a single founder cell, which is thought to determine the characteristic features of the muscle. These founder cells can be defined by the expression of specific combinations of transcription factors, which give each founder cell, and hence each muscle, its unique identity (Baylies et al., 1998). The FCMs, on the other hand, appear to be more generic in nature, potentially fusing with any myotube. They contribute material rather than identity to the growing myotube (Rushton et al., 1995).

Muscle migration proceeds in three phases (Schnorrer and Dickson, 2004). First, the founder cells migrate relative to each other to assume the approximate position in which the muscle will form. Second, FCMs begin to fuse with the founder cells, forming polarized myotubes with a highly dynamic leading edge at each end. These dynamic tips resemble the growth cones of neurons and appear to have a similar function in directing migration toward specific target cells. Finally, each myotube tip recognizes its specific attachment site and establishes a stable connection. These attachment sites are the tendon cells, specialized epidermal cells located along intersegmental borders and also within segments (Volk and VijayRaghavan, 1994).

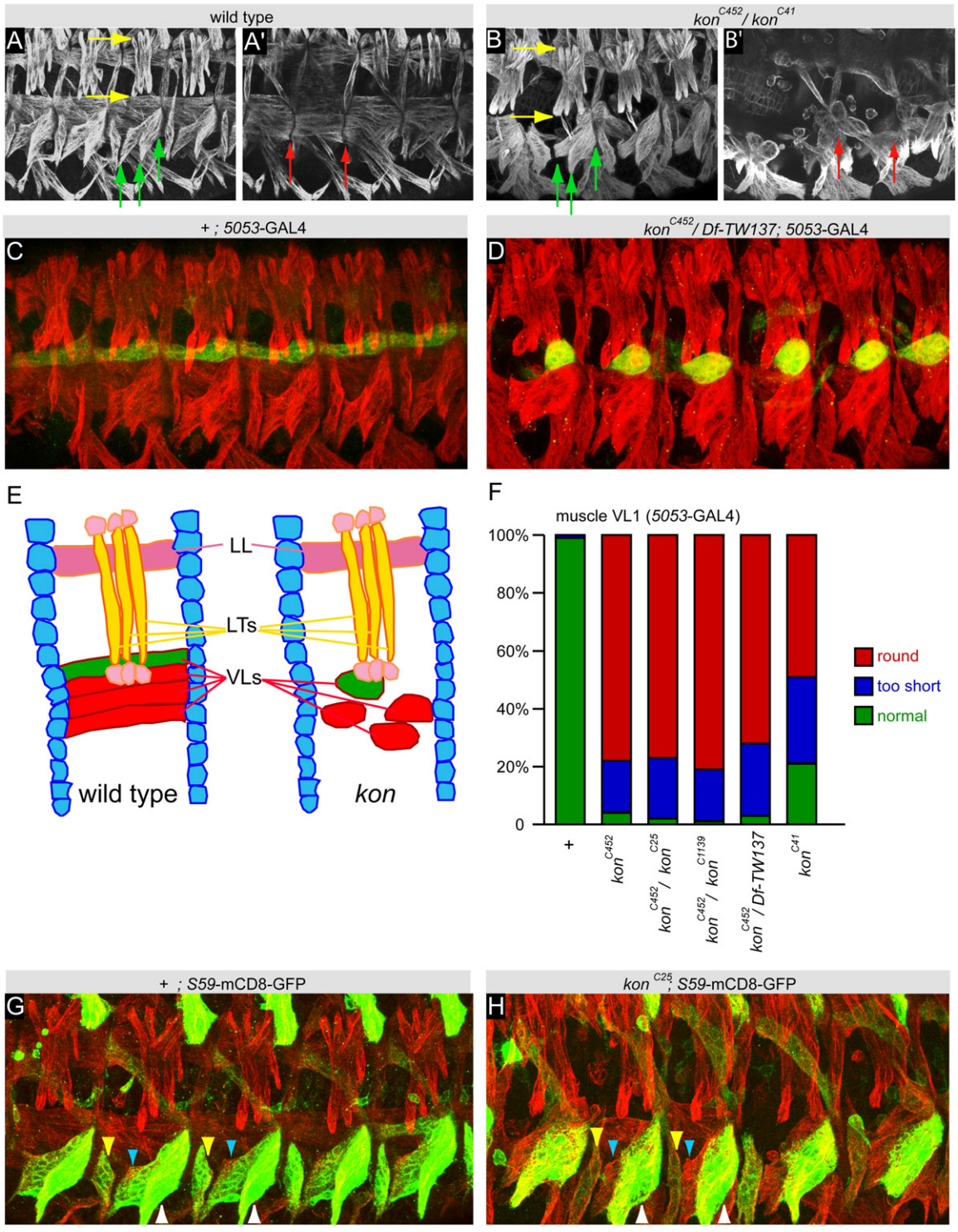


Figure 1. The *kon* Phenotype

(A–B′) Wild-type (A and A′) and kon^{C452}/kon^{C41} mutants (B and B′) stained with anti-β3-Tubulin antibody to label all muscles. Projections of all muscles (A and B) or more internal projections (A′ and B′) are shown. Note the attached VL muscles between the red arrows in (A′) which appear round in (B′). The LT muscles marked by the yellow arrows are normal, as are VA1 or VA2 marked by green arrows.

(C and D) Wild-type (C) and kon^{C452}/*Df-TW137* embryo (D) stained for all muscles in red and VL1 muscle in green (*5053*-GAL4, UAS-mCD8-GFP stained with anti-GFP). Note the round VL1 muscles in (D).

(E) Schematic of selected muscles in wild-type and *kon-tiki* mutants, with LT1–3 in yellow, VL1 in green, VL2–4 in red, LL1 in pink and intersegmental attachment sites in blue.

How do myotubes locate and recognize their specific tendon cell targets? An attractive model, based on the present understanding of neuronal wiring specificity, is that individual myotubes and tendon cells may express distinct sets of surface or secreted proteins that constitute a molecular recognition system. If this is the case, then what are these molecules, how do they function in myotube targeting, and are they the same as or different than those that operate in the nervous system? To date, only two molecular systems for muscle guidance have been identified, both initially defined by their roles in neuronal wiring. Slit and its Robo family receptors repel both axons and myotubes away from the midline (Kidd et al., 1999; Rajagopalan et al., 2000), and may later attract some of the same myotubes to their intersegmental attachment sites (Kramer et al., 2001). Similarly, the Derailed (Drl) receptor helps some commissural axons to choose the right pathway across the midline (Bonkowsky et al., 1999) and some myotubes to select the right intrasegmental attachment sites (Callahan et al., 1996). Thus, at least some of the mechanisms involved in axon guidance and targeting are also utilized during muscle development.

A less biased approach is to screen directly for genes involved in muscle development, and indeed this is the only way to identify factors that do not also act in neurons. We have conducted such a mutagenesis screen, and we report here the identification of the gene *kon-tiki* (*kon*), which is essential for promoting directed migration and target recognition of a subset of ventral myotubes. *kon* mutations do not affect embryonic nervous system development, indicating that muscles and neurons use distinct as well as shared guidance and recognition molecules. The Kon protein is a large transmembrane protein that concentrates at muscle tips. The cytoplasmic domain of Kon contains a PDZ-binding motif and interacts with the PDZ-domain protein Dgrip—a cytoplasmic adaptor previously shown to function in targeting of these myotubes (Swan et al., 2004). Both Kon and Dgrip are highly conserved, and they appear to define an ancient evolutionary molecular pathway that mediates specific muscle targeting.

RESULTS

Identification of *kon-tiki* Mutants

To identify genes required for *Drosophila* embryonic muscle morphogenesis, we performed an EMS-mutagenesis screen of the second chromosome, representing approximately 40% of the *Drosophila* genome. We used an *mhc*-Tau-GFP reporter to allow direct visualization of muscle patterns in living F2 embryos, and screened over 4000 mutant lines. We recovered a total of 140 muscle mutants in this screen, including one complementation group with nine independent alleles that we named *kon-tiki* (*kon*) after the raft on which explorer Thor Heyerdahl sailed to Polynesia.

In differentiated (stage 16–17) wild-type embryos, the four ventral-longitudinal muscles (VL1–4) are elongated and span each abdominal segment A1–A7, connecting at each end to intersegmental tendon cells (Figures 1A and 1A′). In *kon* mutants, however, all four of these muscles generally have a rounded morphology and fail to connect to any tendon cell (Figures 1B and 1B′). To examine this phenotype in more detail, we used the *5053*-GAL4 marker, which specifically labels muscle VL1 (Ritzenthaler et al., 2000). In wild-type and heterozygous embryos, VL1 spans the entire segment (99%, n = 170 segments for wild-type; Figures 1C and 1F). In contrast, in strong *kon* allelic combinations VL1 mostly exhibits a rounded morphology (79%, n = 410) or, if it is elongated, fails to span the entire segment (19%; Figures 1D and 1F). We consider the *kon* alleles kon^{C452}, kon^{C25}, and kon^{C1139} as null alleles since these alleles exhibit the same phenotype homozygous and over a *kon* deficiency (Figure 1F and data not shown). We do not have specific markers to visualize VL2–4, but in general these muscles appear to be even more strongly disrupted than VL1 (Figure 1B and Figures S1 and S2; see the Supplemental Data available with this article online).

In contrast to VL1–4, the three lateral transverse muscles (LT1–3), which are located just dorsal to VL1–4 but attach to intrasegmental sites, appear normal in *kon* mutants (Figures 1A, 1B, and 1E). We also used the marker *S59*-mCD8-GFP to label muscles VT1, VA1 and VA2, which attach with one end to intersegmental sites and with the other to intrasegmental sites (Figure 1G). All of these muscles also appear normal in *kon* mutants (Figure 1H). Thus, *kon* mutations selectively disrupt the morphogenesis of a specific subclass of muscles.

These muscle phenotypes could be a secondary consequence of defects in tendon cell differentiation. However, when we examined *kon* mutants with the tendon cell marker Stripe (Volk and VijayRaghavan, 1994), we did not observe any abnormalities in the number or position of tendon cells (Figures S1A and S1B). We conclude that the *kon* phenotype most likely reflects a defect in the muscles themselves, not their tendon cell targets.

kon-tiki Functions in Muscle Targeting

The rounded appearance of mature VL1–4 muscles in *kon* mutants could arise through several different developmental defects, such as incorrect specification of the founder cell, a failure to migrate toward the attachment site, inability to recognize and select the appropriate target site, or a failure to form a strong connection that can resist the force of muscle contraction at late stages. To distinguish between these possibilities, we performed time-lapse microscopy on intact living embryos carrying

(F) Quantification of the VL1 *kon* phenotype in different allelic combinations compared to wild-type. Green: muscle attached at segment border; blue: muscles are elongated but too short; red: muscle appear round. More than 100 A1–A7 segments from stage 16–17 were scored for each genotype. (G and H) S59-mCD8-GFP in wild-type (G) and kon^{C25} (H) labels VT1 (yellow arrowhead), VA1 (blue arrowhead) and VA2 (white arrowhead) all of which elongate and attach.

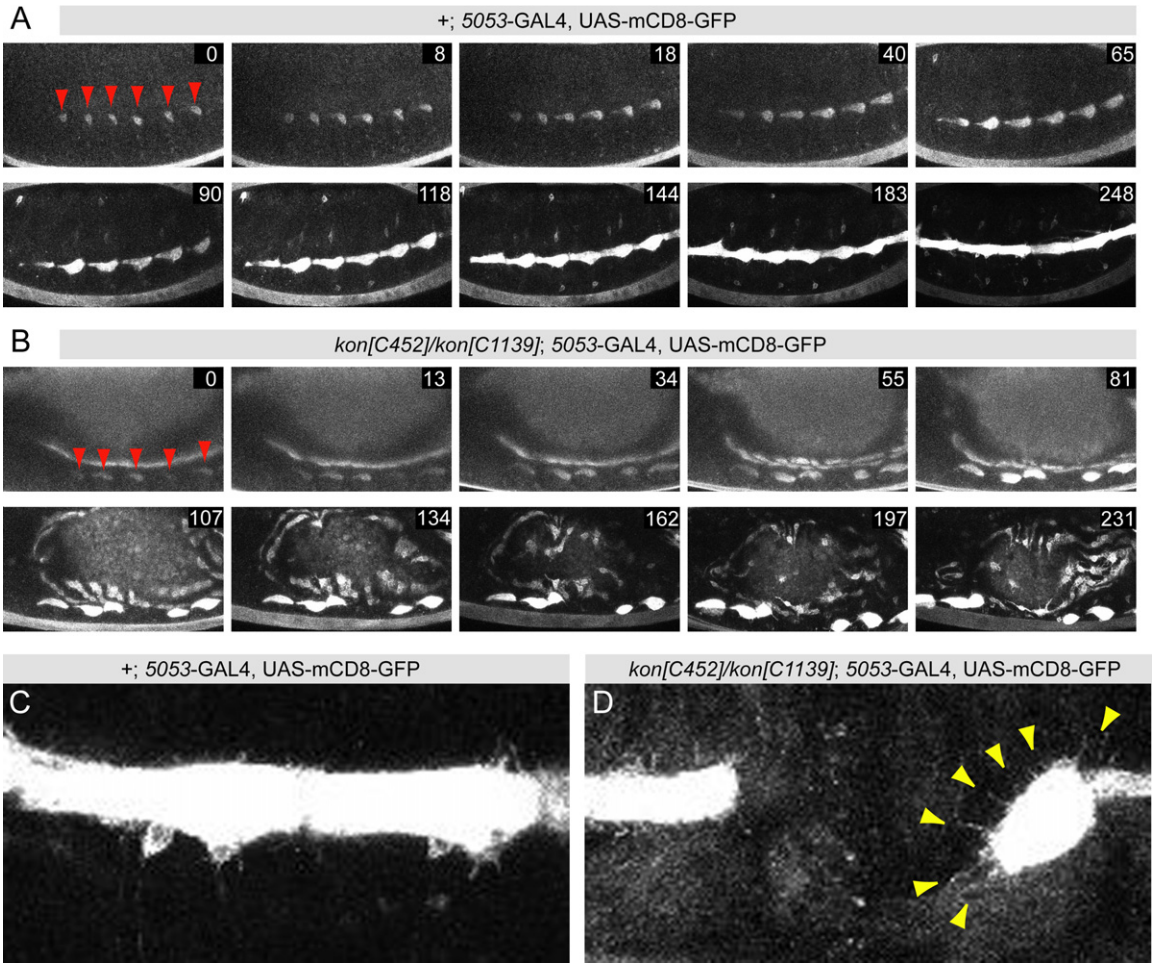


Figure 2. *kon* Affects Directed Migration and Target Selection of Muscle VL1

Time points taken from time-lapse movies using *5053*-GAL4, UAS-mCD8-GFP to label muscle VL1 in wild-type ([A], Movie S1) or kon^{C452}/kon^{C1139} ([B], Movie S2). Time is indicated in minutes. The additional cells labeled in (B) are due to a more dorsal perspective of the movie. Higher magnification of wild-type (C) and kon^{C452}/kon^{C1139} mutant VL1 (D). Note filopodia oriented in all directions in (D [arrowheads]).

the *5053*-GAL4 and UAS-mCD8-GFP transgenes to label the VL1 muscle (Figure 2).

In wild-type embryos (Figure 2A and Movie S1), the VL1 founder first becomes visible at the posterior end of each abdominal segment (t = 0 min in Figure 2A). It then polarizes (t = 8 min), and the anterior end grows directly toward the anterior segment border (t = 18–118 min) over an average distance of about 20 μm. The posterior end of VL1 does not appear to migrate a long distance, as it is already located near the posterior segment border. Finally, VL1 connects at both ends to its segment border attachment sites, and perhaps also to the VL1s in neighboring segments (t = 144 min). This results in a continuous string of VL1 muscles along the abdomen (t = 248 min). VL1 thus migrates directly to its attachment site, ignoring intrasegmental attachment sites en route as well as adjacent intersegmental attachment sites in the target region.

In *kon* mutant embryos (Figure 2B and Movie S2; see also Figure S2), the VL1 founder is first detectable at its normal posterior location (t = 0 min). Thus, *kon* does not disrupt the initial steps of founder cell specification and migration. VL1 also polarizes normally in *kon* mutants (t = 13 min), and the anterior end begins its directed migration (t = 34 min). During this anterior migration the first defects are observed. The VL1 migration appears to terminate prematurely compared to wild-type embryos (compare t = 107 min in Figure 2B and t = 90 min in Figure 2A). Although filopodial extensions of a number of VL1 myotubes do often reach the anterior segment border, they evidently fail to recognize their attachment sites, as no connection is established. These unattached myotubes continue to project filopodia in all directions, but no longer sustain a directed migration toward their target (Movie S2; Figures 2C and 2D). Similar defects also occur in muscles VL2–4, which we observed using a general muscle marker (Figure S2 and Movies S3 and S4).

From these time-lapse studies, we conclude that *kon* is essential for target recognition and the establishment of a stable connection of the VL muscles. It appears to promote migration of the VL muscles toward their targets.

Importantly, *kon* is not required for muscle motility, as VL1 muscles are highly motile in *kon* mutants, as are all other muscles. Moreover, we note that in *slit* mutants the VL muscles are redirected over the ventral midline, migrating even greater distances than normal (Kidd et al., 1999). This still occurs in *slit kon* double mutants (Figure S3), demonstrating that *kon* is not required for muscle motility itself, but for directing migration toward specific tendon cells and for recognizing these as the proper attachment sites.

kon Encodes a Conserved Single-Pass Transmembrane Protein Expressed in Migrating Myoblasts

We mapped the *kon* gene using SNP-on-chip technology (D. Chen et al., personal communication) to a position proximal to 36A10 on chromosome 2L. The location of *kon* was further refined by deficiency mapping: *Df(2L)TW137* and *Df(2L)M36-S5* delete *kon* but *Df(2L)Exel8083* and *Df(2L)Exel6041* do not. This placed *kon* within the 116 kb interval from 18,451 to 18,567 kb, a region that includes seven annotated genes (Figure 3A). Of these genes, *CG10275* seemed a strong candidate for *kon*, based on its size and its predicted product (Figures 3B and 3C). Indeed, when we sequenced all of the annotated exons of *CG10275* from *kon* heterozygous adults, we found single-nucleotide substitutions in eight of our nine alleles (Figure 3C). Five of these alleles are associated with nonsense mutations; the other three are missense mutations in conserved domains. We therefore conclude that *CG10275* corresponds to *kon*.

The computational annotation of *kon* was experimentally refined to include two additional exons which we identified by 5′ RACE on embryonic cDNA (Figure 3B). The *kon* mRNA thus includes a total of 12 exons with a total length of 8.3 kb. It is predicted to encode a transmembrane protein of 2381 amino acids, including an N-terminal signal sequence, a large extracellular region, a single membrane-spanning segment, and a cytoplasmic region of 159 amino acids (Figure 3C). The predicted extracellular region is composed of 2 laminin G domains followed by 15 chondroitin sulfate proteoglycan (CSPG) repeats, which are structurally related to cadherin domains (Staub et al., 2002). The intracellular region lacks any known protein domain, but includes at the C terminus a predicted binding site for PDZ-domain-containing proteins.

Kon is closely related to the NG2/CSPG4 and the "similar to CSPG4" family proteins in vertebrates (Figure 3D). These proteins share all of the extracellular domains present in *Drosophila* Kon, with 21%–25% identity and 39%–46% similarity in the laminin G and CSPG domains. The short cytoplasmic region is less well conserved, with the notable exception of the invariable QYWV sequence of the PDZ-binding motif. The in vivo functions of these Kon relatives in other species are unknown.

NG2/CSPG4 is linked to chondroitin sulfate (CS) in human, mouse, and rat (Bumol et al., 1984; Stallcup, 2002). However, the serine-999 residue that carries this modification in the rat homolog (Stallcup and Dahlin-Huppe, 2001) is not conserved in *Drosophila* Kon. To test whether *Drosophila* Kon contains a CS moiety linked to other residues, we immunoprecipitated endogenous or overexpressed Kon protein from embryos, and compared the electrophoretic mobility of Kon with and without prior treatment with chondroitinase ABC. In both cases, we observed a single sharp band of about 250–300 kD (Figure S4). We conclude that most *Drosophila* Kon protein is not modified by CS.

We performed in situ hybridization experiments to assess the distribution of *kon* mRNA in *Drosophila* embryos. *kon* RNA is first detected at stage 10, possibly in the longitudinal visceral muscle precursors (Figure 3E). These cells arise at the posterior of the embryo and migrate anteriorly to form muscles around the developing gut. More importantly, in the body wall muscles we detect high expression in the ventral-longitudinal myotubes in wild-type embryos at stage 14, the stage at which the first defects become apparent in these myotubes in *kon* mutant embryos. Taken together, these phenotypic, expression, and molecular data suggest that Kon might be a transmembrane receptor for a guidance or targeting cue provided by specific tendon cells—a signal that may be transduced intracellularly through Kon's PDZ-binding motif.

Kon Localizes to Specific Myotube Tips

If Kon functions as a targeting receptor, it should be localized to the plasma membrane at myotube tips. To test this, we generated polyclonal antibodies against a peptide epitope from the intracellular region of Kon (Figure 3C). These antibodies revealed a specific staining pattern in wild-type embryos (Figures 4A–4E and 4G), but not in *kon* mutants that are predicted to lack this intracellular epitope (Figures 4F, 4H, and 3C). In order to follow Kon protein distribution in a defined VL muscle during its targeting steps, we labeled the surface of muscle VL1 using *5053*-GAL4, UAS-mCD8-GFP and costained for endogenous Kon. We detect Kon at VL1 muscle tips in growth-cone-like structures approaching the target in stage 14 (Figure 4A) and during targeting slightly later (Figure 4B and 4C). Thus, Kon is localized to the correct place at the correct time in order to function directly in the targeting of the VL1 muscles.

As anticipated from the *kon* mutant phenotype, Kon is concentrated at the tips of all four VL muscles at stage 15 (not just VL1), as they start connecting to their tendon cell targets (Figure 4D). Kon remains at these sites as their attachments are strengthened during stage 16 (Figure 4E). We also detect Kon protein at the tips of muscles that attach to other intersegmental attachment sites, such as lateral longitudinal muscle 1 (LL1) (Figures 4D and 4E), or a number of dorsal muscles (Figure 4G). The amount of Kon localized at the tips of the dorsal muscles is significantly less than at the tips of the VL muscles, and we do not detect any defect of the dorsal muscles in *kon* mutants (Figures 4G and 4H). Thus, although Kon is localized the tips of most muscles that attach to intersegmental sites, it is primarily required only in the VL muscles.

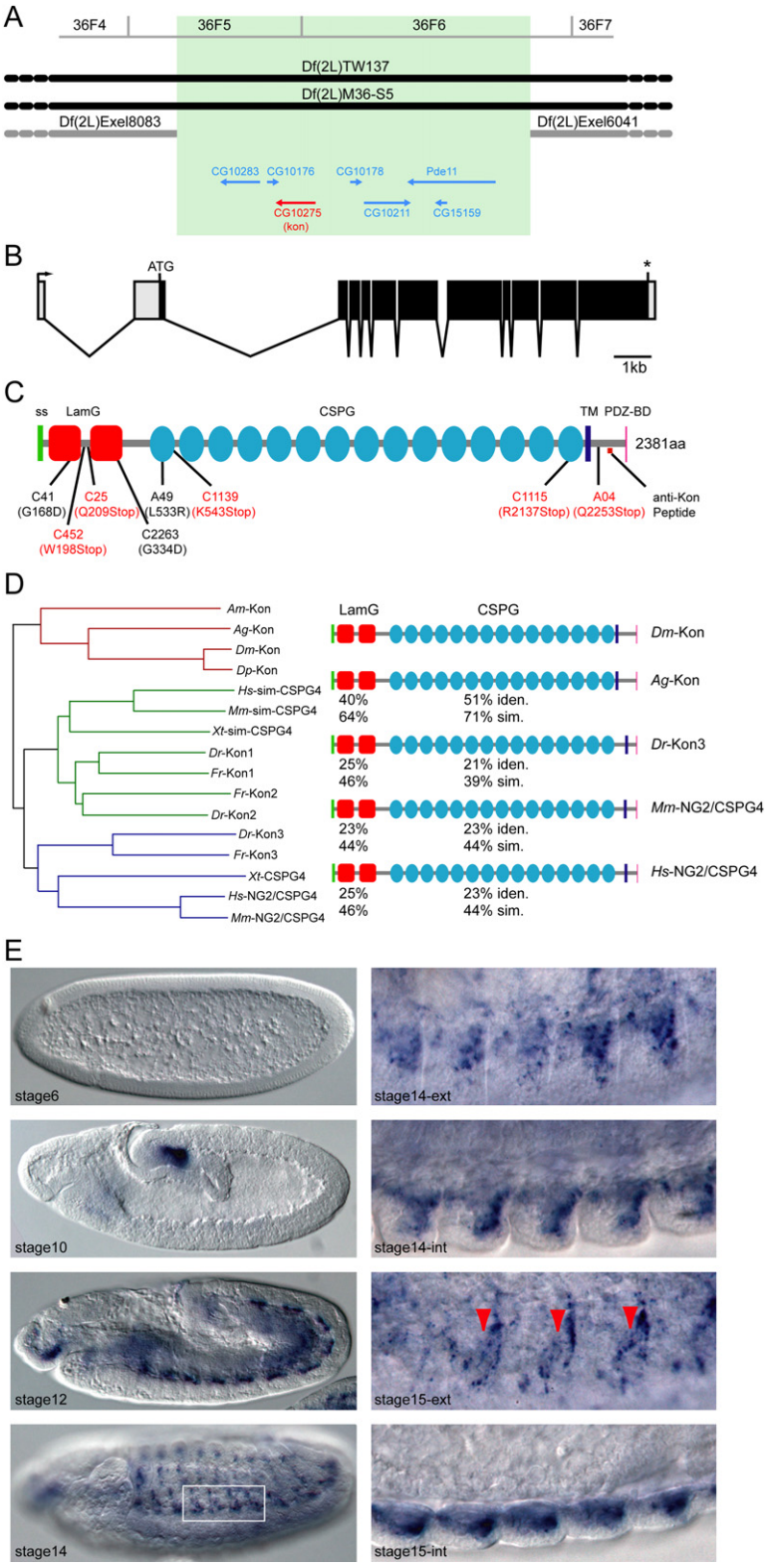


Figure 3. Molecular Organization and Expression of *kon*

(A) Genomic location of *kon* shows the extent of deficiencies that do (black) or do not (gray) delete *kon*.

(B) Exon-intron structure of *kon* genomic region: 5′ and 3′ untranslated regions in gray, coding region in black. The arrow indicates transcriptional start; the asterisk indicates the translational STOP.

(C) Schematic Kon protein domain structure. The molecular lesions of *kon* alleles and the epitope used for the anti-Kon antibody are indicated.

(D) Phylogenetic tree of Kon family members with colored branches to indicate insect Kon (red), vertebrate "similar to CSPG4" (green), and vertebrate NG2/CSPG4 (purple). The sequence identity and similarity of *Drosophila* Kon compared to *Anopheles* and vertebrate Kon homologs is shown to the right.

(E) In situ hybridization for *kon* transcripts at different developmental stages in wild-type embryos. External (ext) or more internal (int) focal planes at higher magnifications of stage 14 and stage 15 embryos of the indicated embryonic region (white box on the left) are shown to the right. Note high *kon* expression in muscle VA2 (red arrowhead).

Interestingly, we could not detect significant amounts of Kon in those muscles that attach to intrasegmental sites, such as lateral transverse muscles LT1–3 (Figures 4D and 4E). This selective localization to intersegmental attachment sites may even occur within a single muscle: VA2 expresses high levels of *kon* mRNA (Figure 3E), and although Kon appears to accumulate at its intersegmental tip, we could not detect Kon at its intrasegmental tip

(Figure 4E). Two of our mutant alleles, kon^{C41} and kon^{C2263}, are associated with missense mutations in the laminin G domains: G168D and G334D, respectively (Figure 3C). These alleles still produce Kon protein, detectable from the diffuse staining in muscle VA2, but Kon does not accumulate at muscle tips (Figure 4I). These data indicate that Kon is selectively localized to specific intersegmental attachment sites, and suggest that this localization may be required for Kon function.

To resolve whether Kon protein at VL1–4 attachment sites is localized on muscle cells or tendon cells, we used a membrane-tethered GFP to label the surface of tendon cells (*stripe*-GAL4, UAS-mCD8-GFP) and costained these embryos with anti-Kon and anti-GFP. High-resolution imaging of these embryos revealed that Kon is localized immediately adjacent to, but not within, the tendon cells (Figure 4J; see Movie S5 for 3D rendering). We therefore conclude that Kon is localized at the tips of the VL1–4 myotubes, in apposition to their tendon cell targets.

kon Acts Cell-Autonomously in Myotubes

These expression and localization data suggest that *kon* acts cell-autonomously in myotubes. It is, however, also conceivable that *kon* may have an essential role in tendon cells, even though it cannot be detected in these cells, or that it might even be involved in signaling between distinct myotubes. To distinguish between these possibilities, we asked whether restoring *kon* expression in tendon cells, in all muscles, or just in the VL1 muscle would be sufficient to rescue the *kon* mutant phenotype. For these experiments, we used *stripe*-GAL4, *mef2*-GAL4 and *5053*-GAL4 drivers, respectively, to express a UAS-HA-*kon* transgene in *kon* mutant embryos.

We do not detect any rescue when supplying Kon in the tendon cells (Figure 5A). In contrast, restoring expression of *kon* specifically in all muscles with *mef2*-GAL4 completely rescued the targeting of muscles VL1–4 (Figures 5B and 5C). Moreover, restoring *kon* function in VL1 alone with *5053*-GAL4 rescued this muscle but not the neighboring VL2–4 muscles (Figures 5D–5G). Thus, we conclude that *kon* function is neither required in tendon cells for targeting of VL1–4, nor in any other muscle for targeting of VL1. This is consistent with its proposed autonomous role as a muscle targeting receptor. Note, however, that these data do not preclude the possibility of a nonautonomous role for Kon in signaling between VL1 muscles in adjacent segments, as might occur during their attachment to a common intersegmental tendon cell.

Kon Promotes Filopodia Formation

Overexpression of Kon in all muscles does not lead to a major rerouting of muscles to intersegmental attachment sites, in particular of those muscles that normally connect to intrasegmental sites such as the LT muscles (Figure 5B). Thus Kon alone is not sufficient to guide muscles to intersegmental attachment sites from a distance. However, we do find transgenic Kon protein to be present in filopodia during muscle migration, and this ectopic Kon results in the persistence of filopodia formation even after the muscles had normally formed stable attachments (Figure S5). Thus, it appears that ectopic Kon either stabilizes filopodia for unusually long periods or, alternatively, stimulates filopodia formation. To explore this further, we performed time-lapse analysis using the live filopodial marker globular-moesin-actin-binding domain (GMA)-GFP (Bloor and Kiehart, 2001). We expressed GMA-GFP in all muscles using *mef2*-GAL4, with and without coexpression of Kon. In *mef2*-GAL4, UAS-GMA-GFP embryos we detect filopodia as muscles migrate and make their initial contact with their target (Figure 6A, t = 0–32 min, and Movie S6), but this filopodial activity gradually ceases as the muscle attachment matures (t = 69 min and later). In *mef2*-GAL4, UAS-GMA-GFP, UAS-*kon* embryos we see comparable filopodial activity during both migration and initial attachment of the ventral muscles (Figure 6B, t = 0–33 min, and Movie S7), and these filopodia do not appear to be unusually stable (compare filopodia dynamics during the migratory phases as shown in Movies S6 and S7). However, filopodia continue to form at muscle tips also at much later stages, even as the muscles begin to contract (compare Figure 6A, t = 160 min, and Figure 6B, t = 176 min). We note that the muscle contractions are much reduced in these embryos, possibly because this excessive motility interferes with the maturation of muscles and their tendon cell attachments.

Kon Signals through the PDZ-Domain Protein Dgrip

How might Kon transduce a signal intracellularly? The only obvious, conserved feature in Kon's intracellular domain is a C-terminal PDZ-binding motif. We recovered one allele, kon^{A04}, that is predicted to truncate the Kon protein intracellularly and remove the C-terminal 129 amino acids, including the PDZ-binding motif. Analysis of this allele using the VL1 marker *5053*-GAL4 revealed that it is a strong hypomorph, resulting in VL1 targeting defects similar to but weaker than those seen in the null alleles (Figures 7A and 7D). This suggest that the PDZ-binding domain is likely to be critical for full Kon function, although Kon still has a residual activity without it. We obtained a similar result when we rescued the *kon* mutant phenotype with a UAS-HA-kon^{VG} transgene which contains the same *kon* transgene as used above but the last conserved amino acid in the PDZ-binding motif is changed from a valine to a glycine. This construct rescued the *kon* null phenotype only to an extent similar to the phenotype of the kon^{A04} hypomorph, not completely as the wild-type construct does (compare Figures 7C and 7D with Figures 5C and 5F). Furthermore, when we track the filopodia formed during muscle migration and attachment in embryos overexpressing the Kon^{VG} mutant protein, we find far fewer ectopic filopodia at late stages compared to embryos overexpressing wild-type Kon (compare Figures 6B and 6C, and Movies S7 and S8). Together these data indicate a critical role for the PDZ-binding domain and imply a role for one or more PDZ-domain proteins in mediating Kon function.

A strong candidate for a Kon-interacting protein is the *Drosophila* homolog of Glutamate-receptor-interacting

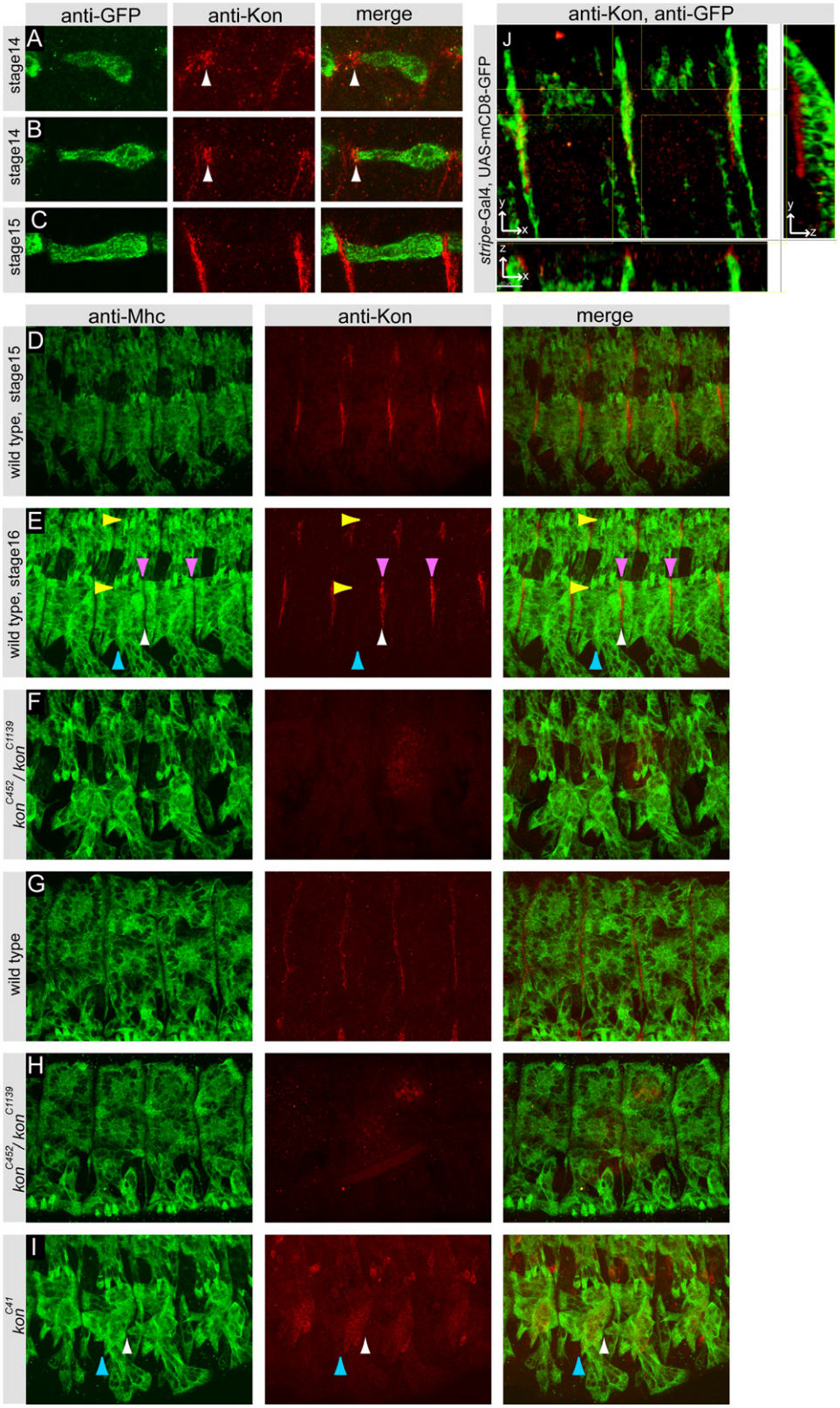


Figure 4. Kon Protein Localization to Muscle Tips

(A–I) Kon protein distribution in a 2 μm section of muscle VL1 (*5053*-GAL4, UAS-mCD8-GFP) at mid-stage 14 (A), slightly later (B), and at late stage 15 (C), stained for GFP in green and endogenous Kon in red. Note Kon localization at VL1 muscle tips before and during targeting (white arrowheads).

protein (Dgrip), which consists of seven PDZ domains. *Dgrip* mutants have been reported to have a phenotype superficially similar to the phenotype we report here for *kon* (Swan et al., 2004), and a vertebrate homolog of *kon*, NG2, binds to vertebrate Grip1 (Stegmuller et al., 2003). Indeed, when we reexamine the VL1 phenotype of $Dgrip^{ex36}$ null mutant embryos using the *5053*-GAL4 marker, we observe VL1 defects that are both qualitatively and quantitatively comparable to those found in the kon^{A04} mutant (Figures 7A, 7B, and 7D). *Dgrip* is not required for Kon localization to the myotube tips (data not shown).

In order to test if *Dgrip* functions downstream of *kon*, we chose the *Drosophila* wing, as we had found that overexpression of wild-type Kon using *MS1096*-GAL4 (Capdevila and Guerrero, 1994) results in a small and blistered wing (Figures 7E and 7F). This phenotype strictly depends on Kon's PDZ-binding motif, as overexpression of Kon^{VG} does not perturb wing development (Figure 7H). We found that the Kon overexpression phenotype is suppressed by coexpression of a *Dgrip* RNAi hairpin construct, resulting in an almost normal wing (compare Figures 7F and 7G). This indicates that Dgrip functions downstream of Kon in a signaling pathway that requires Kon's PDZ-binding motif.

To investigate whether Dgrip indeed binds directly to Kon, and if so to determine which of its seven PDZ domains are involved, we expressed several different Dgrip protein fragments in *Drosophila* S2 cells (Figure 7I). These Dgrip fragments were coexpressed with a mCD8-Kon fusion protein, consisting of the extracellular and transmembrane domains of murine CD8 and the intracellular domain of Kon. In coimmunoprecipitation experiments, we found that Dgrip fragments containing PDZ domains 5–7 or 6–7 bound to mCD8-Kon-intra, whereas fragments containing PDZ domains 1–3, 4–5, or 4–6 did not (Figure 7J). This interaction is mediated through the PDZ-binding motif in Kon, as the valine to glycine mutation completely abolished binding (Figure 7J). Together, these genetic and biochemical data suggest that Kon's PDZ-binding domain interacts specifically with the 7^{th} PDZ domain of Dgrip. We postulate that this interaction recruits Dgrip to Kon, as well as additional proteins bound to Dgrip's other PDZ domains, thereby initiating the intracellular signaling that underlies myotube target recognition.

DISCUSSION

kon Functions in Targeting of Specific Myotubes

In each abdominal hemisegment of the *Drosophila* embryo, 30 muscles connect at their ends to distinct tendon cells in the epidermis. How does each muscle find and recognize its specific tendon cell targets? Myotubes are molecularly distinct before they begin to migrate (Baylies et al., 1998; Knirr et al., 1999; Nose et al., 1998), they generally migrate directly toward their targets (Schnorrer and Dickson, 2004), and they are unperturbed if other muscles are genetically ablated (Ruiz Gomez and Bate, 1997). Thus, it appears that each myotube is endowed with the ability to independently locate and recognize its specific target cells. Here, we identify a transmembrane protein, Kon, that confers this ability on a specific subclass of myotubes.

We identified *kon* based on the aberrant muscle patterns in *kon* mutant embryos: the ventral-longitudinal muscles VL1–4 do not connect to their target cells, but most other muscles appear to attach correctly. Time-lapse analysis of these mutants indicates that *kon* function is required for targeting of the VL myotubes. The directed migration of these myotubes toward the target is also reduced. Nevertheless, filopodia do frequently touch their target cells, but seem indifferent to them. General myotube motility appears normal, and if the VL myotubes are misrouted ventrally (as in *slit* mutants), then *kon* function is not required for their migration toward the ventral midline. Hence, *kon* function is required specifically for these myotubes to recognize their targets. The VL myotubes do not connect to potential alternative targets in *kon* mutants, such as the intrasegmental attachment sites of the VA muscles. This distinguishes *kon* from both *slit* and *derailed* (*drl*). Specific myotubes are misrouted in both *slit* and *drl* mutants, yet in these mutants the misrouted myotubes are still able to connect to tendon cells (possibly selecting alternate sites according to an unknown hierarchy of preferences) (Callahan et al., 1996; Kidd et al., 1999; Kramer et al., 2001). Thus, defective guidance alone does not preclude tendon cell attachment, although it may lead to the selection of the wrong targets. We therefore conclude that *kon* function is specifically required for the VL muscles to recognize and attach to their tendon cell targets.

Misexpression of Kon in other muscles does not redirect them to other targets. This is perhaps not surprising, as misexpression of many different axon guidance receptors—such as Frazzled, Robo, and Ptp69d—similarly does not lead to axonal misrouting (Kolodziej et al., 1996; Kidd et al., 1998; Garrity et al., 1999). Presumably, in muscles as in neurons, guidance and targeting are controlled by a suite of factors acting in concert. We did however find that overexpression of Kon results in excessive filopodial activity, which persists even at the late stages during which muscles normally cease filopodial activity and establish stable contacts. This gain-of-function phenotype is consistent with our interpretation of the loss-of-function phenotype, namely, that Kon functions to promote migration until the correct connection is established.

The mCD8-GFP staining appears not to label the entire VL1 tips in (A) and (B). Wild-type stage 15 (D), stage 16 ([E]; dorsal view in [H]), kon^{C452}/kon^{C1139} ([F]; dorsal view in [H]), and kon^{C41} mutant embryos (I) with Kon protein in red, and muscle-specific myosin II in green. Note Kon at the tips of the VL muscles (pink arrowheads) but not at the tips of the LT muscles (yellow arrowheads) in (E). Intrasegmental and intersegmental attachment sites of VA2 in wild-type (E) and kon^{C41} (I) marked by blue and white arrowheads, respectively. (J) High magnification and maximum projection image of the indicated regions of *stripe*-GAL4, UAS-mCD8-GFP-expressing embryo stained for Kon in red and GFP in green. Note that there is no overlap of red and green in the z axis. See Movie S5 for a 3D rendered animation.

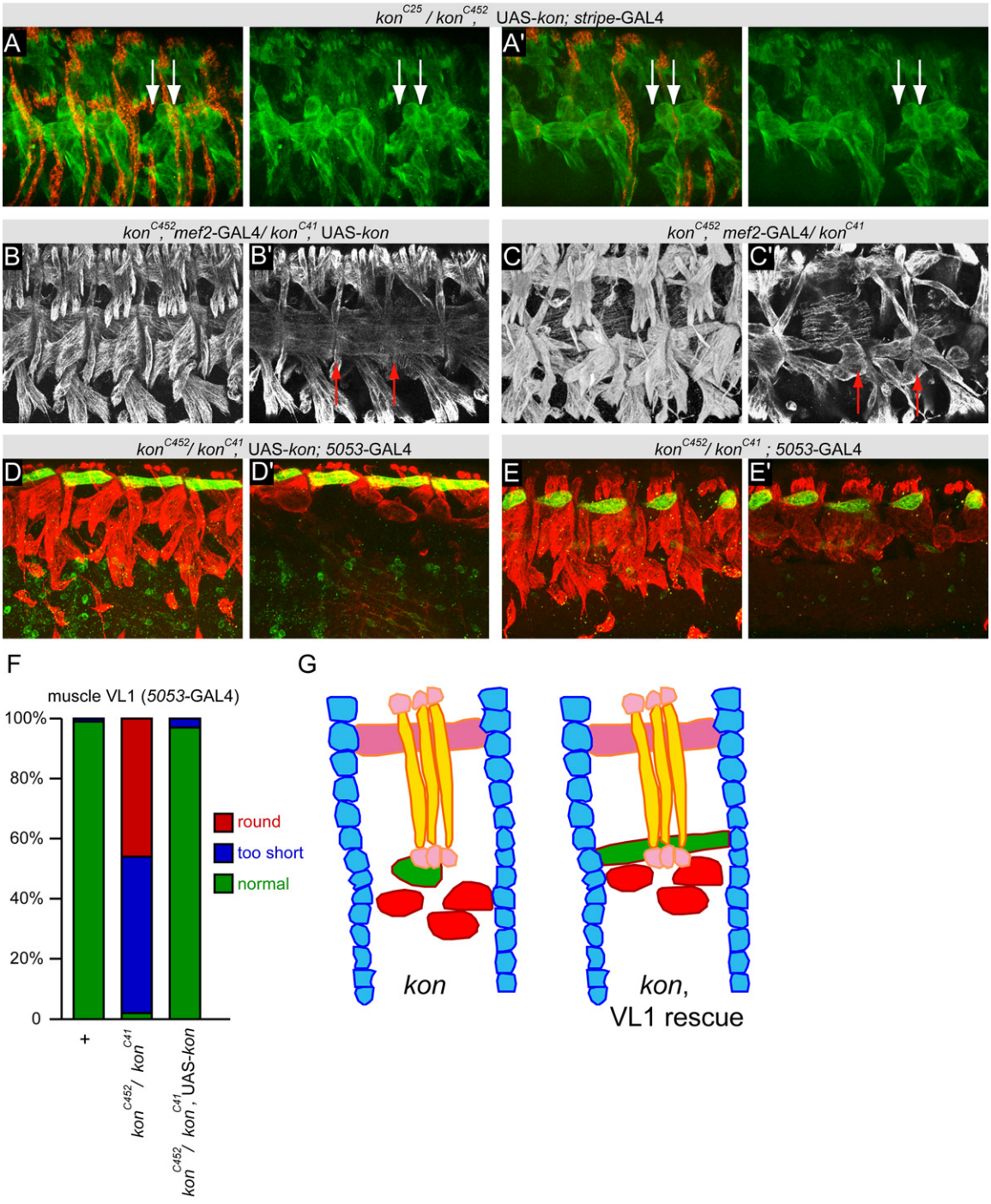


Figure 5. Kon Acts Autonomously

(A) kon^{C25}/kon^{C452} embryo expressing UAS-*kon* (red) in all tendon cells using *stripe*-GAL4. Note the rounded VL muscles indicating no rescue (white arrows).

(B–E) kon^{C452}/kon^{C41} embryos with (B and D) or without (C and E) UAS-*kon* transgene. (B) Rescue in all muscles using *mef2*-GAL4, UAS-*kon*; compare to (C). (D) Rescue only in muscle VL1 using *5053*-GAL4; UAS-*kon*, compare to (E). Muscles are stained for β3-Tubulin, and VL1 muscle is marked by GFP in green.

(F) Quantification of VL1 rescue, scored as in Figure 1F.

(G) Schematic of *kon* mutant compared to VL1 transgenic rescue.

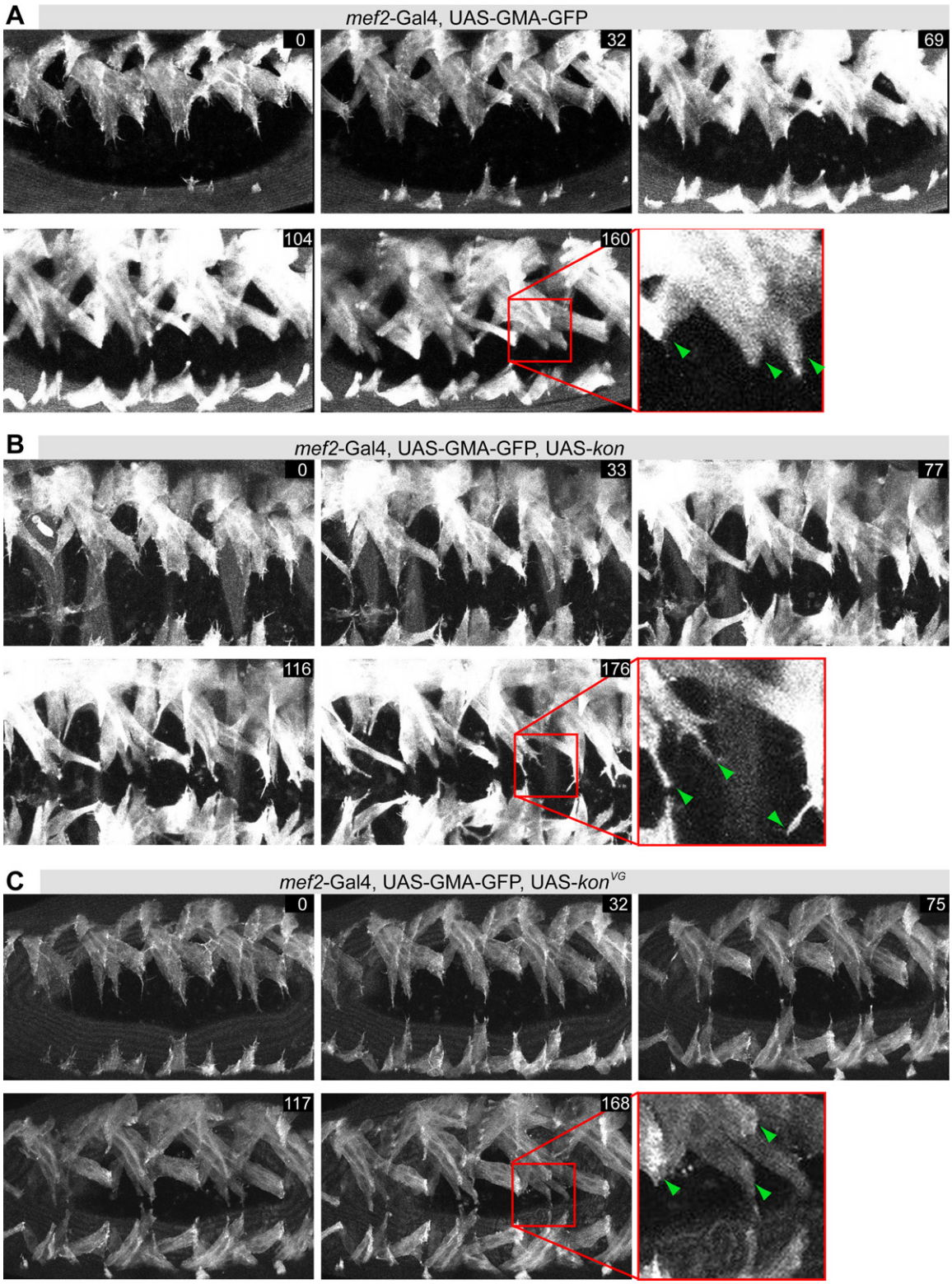
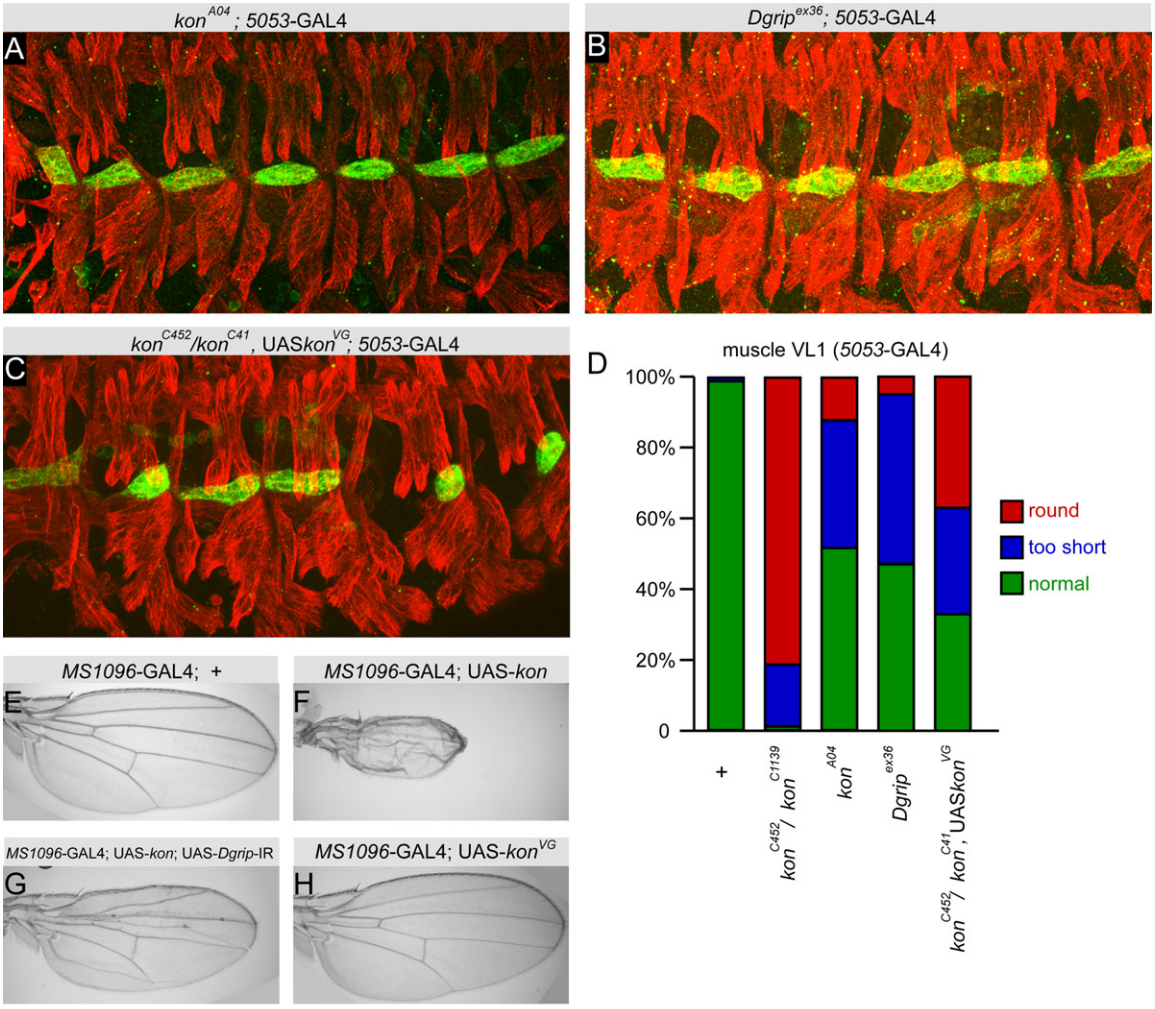
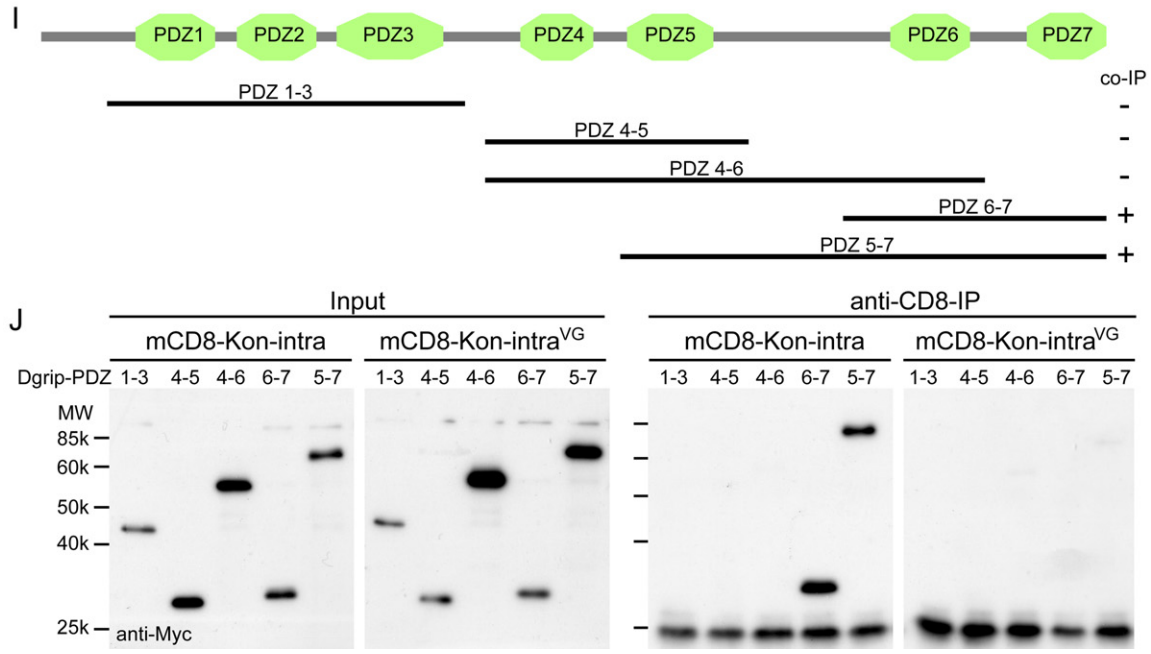


Figure 6. Kon Induces Filopodia

Time points from Movies S6, S7, and S8 of embryos expressing GMA-GFP (A) or GMA-GFP and Kon (B) or GMA-GFP and Kon^{VG} (C) in all muscles using *mef2*-GAL4. Time is indicated in minutes. Note the extensive filopodia in (B) even at late time points, compared to the smooth muscle ends in (A). This is much less dramatic in (C). The insets to the right show high magnifications of the indicated regions from time points 160 min in (A) compared to 176 min in (B) and 168 min in (C), highlighting the smooth muscle surface in (A) and (C) compared to the filopodia in (B) (arrowheads).


kon^A04; 5053-GAL4
A
Dgrip^ex36; 5053-GAL4
B
kon^C452/kon^C41, UASkon^VG; 5053-GAL4
C
muscle VL1 (5053-GAL4)
D
100%
80%
60%
40%
20%
0
round
too short
normal
+
kon^C452/kon^C1139
kon^A04
Dgrip^ex36
kon^C452/kon^C41, UASkon^VG
MS1096-GAL4; +
E
MS1096-GAL4; UAS-kon
F
MS1096-GAL4; UAS-kon; UAS-Dgrip-IR
G
MS1096-GAL4; UAS-kon^VG
H


I
PDZ1
PDZ2
PDZ3
PDZ4
PDZ5
PDZ6
PDZ7
co-IP
PDZ 1-3
-
PDZ 4-5
-
PDZ 4-6
-
PDZ 6-7
+
PDZ 5-7
+
J
Input
anti-CD8-IP
mCD8-Kon-intra
mCD8-Kon-intra^VG
Dgrip-PDZ 1-3 4-5 4-6 6-7 5-7
MW
85k
60k
50k
40k
25k
anti-Myc

If too much Kon is present, this putative "stop" or targeting signal might be overridden.

Kon and Dgrip: A Conserved Signaling Pathway

What is the molecular mechanism underlying Kon function in myotube targeting? We postulate that it is a receptor for a ligand produced by specific tendon cells, and that it transduces this signal intracellularly to modulate cytoskeletal dynamics at the myotube tip. This model is based on the following observations. First, Kon is a single-pass transmembrane protein that localizes to myotube tips during the targeting steps. Second, it functions cell-autonomously. A VL1 myotube that expresses Kon can still target correctly in an embryo that otherwise completely lacks Kon protein. Third, even if the rescued VL1 fails to attach in one segment, those in adjacent segments can still attach correctly. This argues against alternative models in which Kon might mediate homophilic adhesion across segment boundaries. Fourth, Kon protein accumulates in juxtaposition to tendon cells, as might be expected if tendon cells express a binding partner for Kon. Fifth, full Kon function requires its cytoplasmic domain, including the C-terminal PDZ-binding motif.

The identity of the putative Kon ligand is unknown. Potential ligands have however been identified for vertebrate Kon family proteins. For example, NG2 binds to PDGF-AA and bFGF (Goretzki et al., 1999), to the kringle domains of plasminogen and angiostatin (Goretzki et al., 1999), to types V and VI collagen (Burg et al., 1996; Tillet et al., 1997), and to galectin-3 (Fukushi et al., 2004; Wen et al., 2006). The interaction with galectin-3 is thought to lead to integrin activation (Fukushi et al., 2004), which could potentially modulate a cell's migratory or adhesive properties. Although at least one of the two *Drosophila* galectins is expressed in migrating myotubes (Pace et al., 2002), a similar mechanism is unlikely to apply for Kon because *Drosophila* integrins are required for stable muscle attachment, not for migration or targeting (Brown et al., 2000). Determining the nature, localization, and function of this ligand is a major goal for future genetic and biochemical studies.

Such approaches have however already allowed us to identify a cytoplasmic partner for Kon: the PDZ protein Dgrip. Biochemically, the C-terminal PDZ-binding motif in Kon interacts in vitro with the seventh PDZ domain of Dgrip. Genetically, truncating Kon before the PDZ-binding domain and completely eliminating Dgrip result in quantitatively indistinguishable muscle defects. Similarly, the seventh PDZ domain of Dgrip was recently shown to be important to mediate Dgrip function (Swan et al., 2006), presumably through Kon. This Kon-Dgrip interaction is also conserved in vertebrates, as mouse NG2 binds to the seventh PDZ domain of mouse Grip1 or Grip2 in vitro and in vivo (Stegmuller et al., 2003). If Dgrip forms homomultimers through PDZ domains 4–6, as its vertebrate counterparts do (Dong et al., 1999; Srivastava et al., 1998), then this might facilitate the clustering of Kon proteins. Alternatively, or in addition, Dgrip might function as an adaptor to recruit other signaling components to the Kon-Dgrip complex. Vertebrate Grip1 is thought to perform this function for other transmembrane proteins (Sheng and Sala, 2001), recruiting signaling proteins such as the RasGEF Grasp1 (Ye et al., 2000) or other adaptors such as Liprin-α (Wyszynski et al., 2002). Recently the broadly expressed transmembrane protein Echinoid (Ed) was identified as an binding partner for PDZ domains 1, 2, and 7 of *Drosophila* Dgrip (Swan et al., 2006). Although *ed* mutants do not display major muscle defects, the VL defects observed in Dgrip mutants are enhanced by removing one copy of *ed*, indicating that *ed* may modulate Dgrip activity, possibly by binding to PDZ domains 1 and 2 (Swan et al., 2006).

While Dgrip clearly plays a critical role in Kon signaling, we also note that the complete loss of *kon* function results in a phenotype that is significantly stronger than that which results from the loss of *Dgrip*, or the C-terminal truncation of Kon. Thus, Kon can exert at least some function that is independent of its interaction with Dgrip. This might reflect the ability of Kon to activate alternative signaling pathways, possibly involving interactions with other transmembrane proteins. It is also possible that Kon might have a dual function as both a receptor and a ligand, with only the former involving Dgrip. Our genetic data do not presently offer any insight into the molecular basis for this additional, Dgrip-independent function of Kon.

The structures of Kon and Grip proteins, as well as their physical interactions, have been preserved over more than 600 million years of evolution. We have shown here that, in *Drosophila*, this signaling pathway mediates targeting of myotubes during embryonic development. Functions of this pathway in other species are thus far unknown. In vertebrates, there are two subfamilies of Kon

Figure 7. Kon Acts via Dgrip

(A and B) kon^{A04} (A) and $Dgrip^{ex36}$ (B) mutants expressing *5053*-GAL4, UAS-mCD8-GFP stained for β3-Tubulin to label all muscles and GFP to label VL1. Projections of all muscles (A and B) are shown. Note the similar targeting defect of VL1 muscles in both mutants.

(C) $kon^{C452/C41}$ mutants expressing *5053*-GAL4, UAS-mCD8-GFP, UAS-kon^{VG}. Note the weaker rescue when compared to wild-type UAS-*kon* in Figure 5C.

(D) Quantification of the VL1 phenotype in kon^{A04} and $Dgrip^{ex36}$ compared to wild-type and a *kon* null phenotype (as also shown in Figure 1F) as well as the UAS-kon^{VG} rescue in VL1 (compare to Figure 5F for wild-type UAS-*kon*).

(E–H) Wings from females carrying *MS1096*-GAL4 (E), *MS1096*-GAL4, UAS-*kon* (F), *MS1096*-GAL4, UAS-*kon*, UAS-*Dgrip*-IR (G), and *MS1096*-GAL4, UAS-kon^{VG} (H).

(I) Schematic of Dgrip domain structure and the different Dgrip fragments.

(J) Western blot probed with anti-Myc antibody to detect Dgrip fragments expressed in S2 cells together with wild-type or Kon^{VG} mutant intracellular domain fusion to the extracellular and transmembrane domain of mCD8. Input is shown on the left, and coimmunoprecipitations with an anti-CD8 antibody are shown on the right.

proteins, equally related to *Drosophila* Kon: the NG2/CSPG4 subfamily and the "similar to CSPG4" subfamily (Figure 3D). Most research has been focused on NG2 itself. In a striking parallel to Kon, NG2 is expressed in developing skeletal muscle (Stallcup, 2002). However, NG2 is more broadly expressed than Kon, including in other migrating cells such as developing neurons, glia, mesenchymal cells, and bone (Fukushi et al., 2003; Niehaus et al., 1999; Nishiyama et al., 1991; Stallcup, 2002), as well as a variety of melanomas and cancer cell lines (Stallcup, 2002). NG2 stimulates cell migration in vitro (Fang et al., 1999; Niehaus et al., 1999), and smooth muscle cells from NG2$^{-/-}$ mice display a reduced migratory response to PDGF-AA, a putative NG2 ligand (Grako et al., 1999). Thus, Kon family proteins may have an evolutionary ancient role in the regulation of cell migration. We anticipate that further functional studies of NG2 or other Kon family proteins might reveal more specific roles for these proteins in muscle migration and targeting in vertebrates. Conversely, further genetic studies of muscle targeting in *Drosophila* may provide clues as to how this conserved signaling pathway contributes to cancer metastasis.

EXPERIMENTAL PROCEDURES

Isolation of *kon* Alleles

Males carrying an isogenic Oregon-R 2nd chromosome and a P[*myosin-heavy-chain* (*mhc*)TauGFP] transgene on the X chromosome (Chen et al., 2003) were mutagenized as described (Luschnig et al., 2004). More than 4000 stocks carrying mutagenized chromosome over CyO balancer marked with P[*Kr*-GAL4], P[UAS-GFP] were successfully established, and living F2 embryos were embedded into 3S Voltalef oil and screened under epifluorescence for defects in muscle morphology judging by the *mhc*-TauGFP pattern. Mutant lines with similar phenotypes were complemented with each other for lethality and/or muscle phenotype. All nine identified *kon* alleles are lethal in *trans* to each other and display a muscle phenotype. The *kon*^{A04} allele was used for SNP-on-chip mapping of *kon*. For this, 100 random recombinants with a Canton-S 2nd chromosome, polymorphic to the original Oregon-R chromosome, were established, phenotyped, and genotyped (D. Chen et al., personal communication). For deficiency mapping, lethality and muscle phenotype were scored in *trans* to *kon*^{A04}. DNA lesions in all *kon* alleles were determined by sequencing of PCR products amplified from *kon* heterozygous mutant adults.

Molecular Analysis of *kon* and *Dgrip*

5′ RACE (First Choice RLM-RACE, Ambion) was performed using embryonic cDNA. For the UAS-HA-*kon* rescue construct, the computer annotated *kon* genomic region (missing the first 39 amino acids of Kon) was cloned downstream of a Wg signal sequence followed by three copies of a HA tag into the pUAST transformation vector (Brand and Perrimon, 1993). For the Dgrip constructs, Dgrip fragments corresponding to amino acids 72–407 (PDZ 1–3), 464–671 (PDZ 4–5), 464–930 (PDZ 4–6), 538–1059 (PDZ 5–7), and 827–1059 (PDZ6–7) were amplified from embryonic cDNA, tagged with two copies of Myc at the N terminus, and cloned into pUAST. For mCD8-Kon-intra fusion, a *kon* fragment coding for the intracellular amino acids 2224–2381 was amplified by PCR and fused to extracellular and transmembrane domain of mouse CD8. For the mCD8-Kon-intra^{VG} the C-terminal amino acid of Kon was mutated from a Val to Gly. Both were cloned into pUAST. For the *Dgrip* RNAi hairpin 255 bp from nucleotides 2260–2514 of *Dgrip* (CG14447-RA) were cloned as inverted repeat into pUAST.

Confocal Time-Lapse Microscopy and Image Processing

All confocal pictures were obtained using a Zeiss LSM 510 Axiovert 200M. The Z sectioning and the 3D rendering for Figure 4J and Movie S5 were done with Imaris 4.0.6 (Bitplane). Other image procession was done with Photoshop or Metamorph. To acquire movies, living dechorionated embryos were oriented, glued on a coverslip, covered with 3S Voltalef oil, and imaged using a 40× lens. Usually a Z stack of 15–20 single planes covering about 20 μm was recorded every 2 to 3 min. Planes of interest were maximally projected using Zeiss software. Time stacks were converted into movies using QuickTime 7.

Generation of Kon Antibodies

Kon antibodies were obtained from rabbits immunized with a 22 amino acid peptide corresponding to amino acids 2199–2220 of Kon (Gramsch Laboratories). Sera were affinity purified using standard methods and preabsorbed against fixed 0–5 hr old wild-type embryos.

In Situ Hybridization and Immunofluorescence

In situ hybridization and immunofluorescence were performed as described (Patel, 1994; Tear et al., 1996). Kon antibodies were used 1:1000, rabbit anti-β3-Tubulin 1:5000 (Leiss et al., 1988), mouse anti-muscle-specific myosin-II (clone FMM5) 1:30 (Kiehart et al., 1990), rabbit anti-GFP 1:2000 (Torrey Pines), mouse anti-GFP 1:250 (Chemikon), mouse anti-β-Galactosidase 1:2000 (Promega), secondary antibodies conjugated to Alexa Fluor-488 or -568 were obtained from Molecular Probes. Embryos were genotyped using anti-β-Galactosidase to identify embryos carrying CyO, P[*wg*-lacZ] or anti GFP for CyO, P[*Kr*-GAL4], P[UAS-GFP].

Immunoprecipitations and Chondroitinase assay

For mCD8-Kon-intra and Dgrip coimmunoprecipitations pUAST constructs coding for the two test proteins were contransfected together with an *actin*-GAL4 construct into *Drosophila* S2 cells using Cellfectin (Invitrogen). Expression was induced for 48 hr, and cells were lysed in 0.5% Triton X-100, 100 mM NaCl, 50 mM HEPES (pH 7.5), 10% glycerol, 1 mM PMSF, and complete protease inhibitor mix (Roche), cleared, and immunoprecipitated using a rat anti-CD8 antibody (Caltag) and ProteinG Sepharose (Amersham). Westerns blots were probed with rabbit anti-Myc antibody (1:5000, Abcam) to detect coimmunoprecipitated Dgrip proteins. To test for chondroitin sulfate modification, endogenous or HA-tagged Kon was immunoprecipitated with anti-Kon or mouse anti-HA clone 16B12 (Babco) antibodies and ProteinG Sepharose. Where appropriate, Kon immunoprecipitates were treated with Chondrotinase ABC (Seikagaku) according to the manufacturer's manual and run on a western blot.

Supplemental Data

Supplemental data include five figures and eight movies and are available at http://www.developmentalcell.com/cgi/content/full/12/5/751/DC1/.

ACKNOWLEDGMENTS

We thank Hermann Aberle, Jörg Betschinger, Detlev Buttgereit, Eric Olson, Andrea Hutterer, Dan Kiehart, Stephan Sigrist, and Talila Volk for their generous gifts of flies, antibodies, and other reagents, Sebastian Krüttner for his enthusiastic help during the mutagenesis screen, Doris Chen, Annika Ahlford, and Ann Christine Syvänen for setting up the SNP-on-chip mapping and their generous help in applying it, Georg Dietzl and Michi Fellner for the *Dgrip* RNAi line, Alex Schleiffer for bioinformatic analysis, and Ruth Fuchs for technical support. We also thank Michael Kiebler, Carlos Ribeiro, and Barry Thompson for insightful comments on this manuscript. This work was supported by Boehringer Ingelheim GmbH, and by postdoctoral fellowships to F.S. from EMBO and the Human Frontier Science Program.

Received: July 14, 2006
Revised: December 6, 2006
Accepted: February 20, 2007
Published: May 7, 2007

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Accession Numbers

The GenBank accession number for full-length *kon* cDNA is EF428975.