Vestigial Is Required during Late-Stage Muscle Differentiation in *Drosophila melanogaster* Embryos

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The somatic muscles of *Drosophila* develop in a complex pattern that is repeated in each embryonic hemi-segment. During early development, progenitor cells fuse to form a syncytial muscle, which further differentiates via expression of muscle-specific factors that induce specific responses to external signals to regulate late-stage processes such as migration and attachment. Initial communication between somatic muscles and the epidermal tendon cells is critical for both of these processes. However, later establishment of attachments between longitudinal muscles at the segmental borders is largely independent of the muscle–epidermal attachment signals, and relatively little is known about how this event is regulated. Using a combination of null mutations and a truncated version of Sd that binds Vg but not DNA, we show that Vestigial (Vg) is required in ventral longitudinal muscles to induce formation of stable intermuscular attachments. In several muscles, this activity may be independent of Sd. Furthermore, the cell-specific differentiation events induced by Vg in two cells fated to form attachments are coordinated by *Drosophila* epidermal growth factor signaling. Thus, Vg is a key factor to induce specific changes in ventral longitudinal muscles 1–4 identity and is required for these cells to be competent to form stable intermuscular attachments with each other.

INTRODUCTION

Embryonic muscles are first specified as founder cells within the embryonic mesoderm. The specification of diversity among muscle founder cells has been linked to differences in expression of a combination of transcription factors known as muscle identity genes, including *slouch*, *apterous*, *ladybird*, *vestigial* (*vg*), *nautilus*, and *even-skipped* (Baylies *et al.*, 1998). Neighboring myoblasts are recruited to founder cells with corresponding patterns of expression of these factors and fuse with them. This initial formation of a syncytial muscle is followed by a precise series of events whereby each muscle migrates to a specific location, interacting with neighboring cells to form attachments allowing for coordinated movement.

During later stages of *Drosophila melanogaster* embryonic development, somatic muscles (SMs) organize into a complex pattern in each abdominal hemi-segment from A2 to A7

Abbreviations used: DER, *Drosophila* epidermal growth factor; DA, dorsal acute; LL, lateral longitudinal; LO, lateral oblique; LT, lateral transverse; SBM, segment border muscle; SM, somatic muscle; VA, ventral acute; VL, ventral longitudinal; VO, ventral oblique; VT, ventral transverse.

(see Figure 1, A and B; Bate, 1993). Formation and maintenance of this pattern requires both internal differentiation events and intercellular signaling to direct a precise pattern of migration, and attachments. After migration, SMs form two different types of attachments: to epidermal cells (tendon cells) and intermuscular adhesions (diagrammed in Figure 1C). Ultrastructural analysis reveals intermuscular attachments contain extensive extracellular matrix consisting of fuzzy electron-dense fibers, whereas muscle–epidermis attachments contain only a thin line of extracellular electron-dense material (Prokop *et al.*, 1998). Muscle-tendon cell interactions guide the initial stages of migration and attachment (Becker *et al.*, 1997; Yarnitzky *et al.*, 1997). Similar to its role in axon pathfinding (Kidd *et al.*, 1999), the guidance protein Slit is secreted from tendon cell precursors at the segment borders and the corresponding receptor Robo is found on the surface of ventral longitudinal (VL) muscles. In muscles, Slit has a bifunctional role, repelling myotubes during early development, but attracting them later. In *slit* mutant embryos, VL muscles aberrantly cross the midline due to the lack of a repellent Slit source along the midline. If *slit* is expressed only in midline cells, VL muscles stop crossing the midline but fail to reach their normal attachment sites due to the lack of an attractive Slit source at the segment borders (Kramer *et al.*, 2001).

Developing myotubes also secrete Vein, a ligand for the *Drosophila* epidermal growth factor receptor (DER), which activates the Ras pathway in the tendon cells, leading to the final differentiation of tendon cells through elevating expression of *stripe* (*sr*) (Yarnitzky *et al.*, 1997; Nabel-Rosen *et al.*, 1999). Sr, in turn, induces expression of the secreted protein thrombospondin (Tsp), which is required for building stable integrin-mediated junctions by binding the αPS2βPS (PS2) integrin receptor (Subramanian *et al.*, 2007). In *sr* mutant embryos, myotubes fail to make attachments with epidermis, losing their elongated morphology and becoming
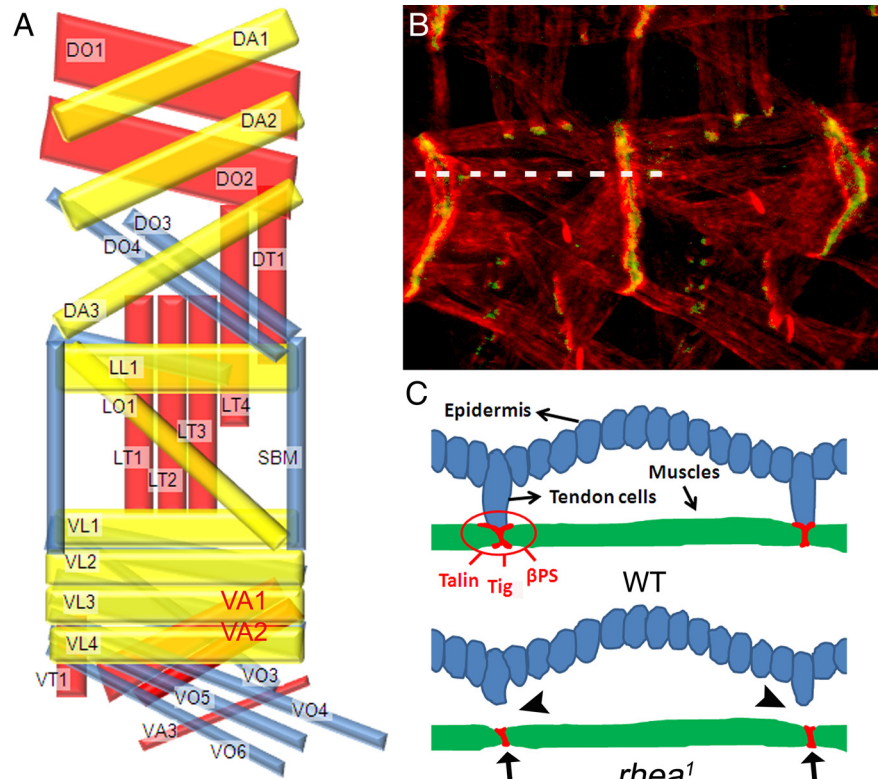
Figure 1. (A) Schematic representation of the SMs in each abdominal hemi-segment A2–A7 of the developing embryo (lateral view with anterior left and dorsal up) by using the nomenclature of Bate (1993). Inner, middle, and outer muscle layers are shown in yellow, blue, and red, respectively (Bate, 1993). Dorsal oblique (DO), DA, dorsal transverse (Swan *et al.*, 2004), LL, LO, LT (Cluzel *et al.*, 2005), SBM, VL (Lundstrom *et al.*, 2004), VA, VT, and VO. VA1 and VA2 are highlighted in red. (B) Muscle–muscle and muscle–tendon cell junctions in wild-type embryos visualized by staining developing muscle cells with actin (Tadokoro *et al.*, 2003) and βPS integrin (green). (C) Diagrams showing a cross-sectional view along the broken line in B. The adhesion proteins (talin, βPS, and Tig, etc.) all concentrate at the end of SMs and are involved in forming stable muscle–muscle or muscle–tendon cell adhesions in wild-type embryos. In *rhea¹* mutant embryos, the muscle–tendon cell connections are broken (arrowheads), but the muscle–muscle connections remain (arrows).

rounded in appearance (Volk and VijayRaghavan, 1994; Frommer *et al.*, 1996).

Formation of the junctions between muscles or muscle and tendon cells is largely mediated by integrins. Integrins are heterodimeric single-pass transmembrane receptors that mediate attachment to the extracellular matrix (ECM) (Hynes, 2002). The two major *Drosophila* integrins PS1 (αPS1βPS) and PS2 (αPS2βPS) have a complementary pattern of expression with PS2 concentrated at the ends of SMs and PS1 accumulating on the tendon cells (Brown, 1993; Brown *et al.*, 2000). PS1 cannot substitute for PS2 at the muscle attachments and vice versa (Martin-Bermudo *et al.*, 1997). The integrin-mediated myotendinous adhesions are established between muscles and tendon cells (see Figure 1, B and C; Brown *et al.*, 2000). The process of muscle specification seems to be independent of actual formation of the adhesions because absence of one or more of the adhesion proteins, including PS integrins, does not affect initial specification, fusion, and attachment of SMs. Rather, these muscles detach and round up upon first contraction due to the lack of strong adhesion.

Several lives of evidence have established that formation of muscle–muscle adhesions is a distinct process from that involved in specification of muscle–tendon attachments. Muscle–tendon cell signaling is blocked by mutation of both *engrailed* and *invected*. In these embryos, Tiggrin (Tig) and βPS remain localized at the end of muscles in contact with each other. This suggests that initial localization of the ECM component Tig at the segment borders is independent of tendon cells and integrin but requires muscle–muscle contact (Martin-Bermudo and Brown, 2000). Mutations in *rhea* (encoding Talin) cause the specific disruption of the muscle tendon cell attachments but not muscle–muscle attachments (see Figure 1C) (Prout *et al.*, 1997). Conversely, mutations in *tiggrin* (*tig*) lead to weak muscle–muscle attachments, but

muscle tendon cell attachments are not affected significantly (Bunch *et al.*, 1998). Notably, there seems to be significant redundancy in this process because embryos with mutations in either gene do not manifest a severe muscle detachment phenotype. However, in embryos with both *tig* and *rhea* mutations, SMs detach and round up due to the disruption in both types of attachments (Martin-Bermudo and Brown, 2000). Thus, embryos homozygous for *rhea* mutations are an excellent sensitized genetic background for studying the role of factors that influence establishment of intermuscular attachment.

Vg was first identified as a key "selector" gene that specifies wing identity during *Drosophila* development (Williams *et al.*, 1991). Vg does not have a DNA binding domain but contains two domains important for gene activation (MacKay *et al.*, 2003) and thus must partner with additional proteins that bring it to the DNA. In ectodermal cells of the wing imaginal disk, Vg interacts with Scalloped (Campbell *et al.*, 1991), which has a conserved DNA binding domain and a well characterized Vg interaction domain (Campbell *et al.*, 1991; Halder *et al.*, 1998; Simmonds *et al.*, 1998). These two proteins form a wing-specific transcription factor complex that directs wing development in any ectodermal cell type where it is expressed (Halder *et al.*, 1998; Simmonds *et al.*, 1998). This aspect of *vg* function is well known as many mutations in *vg* have been recovered that eliminate all adult wing formation but are otherwise viable. However, there are strong hypomorphic and dominant *vg* alleles that have phenotypes affecting other tissues. During pupal development, *vg* has been shown to be a muscle identity gene for specific flight muscles (Sudarsan *et al.*, 2001). For these muscle cell-specific activities, Vg seems to require interaction with *Dmef*2, a key myogenic gene required for specification and subsequent differentiation of all muscles (Black and Olson, 1998; Deng *et al.*, 2009).

To further clarify the role of Vg during embryonic muscle development, we performed both loss-of-*vg*-function and gain-of-*vg*-function analyses. Our results revealed a role of Vg in the establishment of stable intermuscular myotendinous junctions. Furthermore, we show DER signaling may mediate the intermuscular communication and muscles expressing Vg become competent to respond to this communication by building a stable intermuscular junctions.

MATERIALS AND METHODS

Drosophila Strains

The *rhea[1]*, *robo[1]*, and *slit[2]* mutations and *w[1118]* used as the untransformed reference strain were obtained from the Bloomington Stock Center (Department of Biology, Indiana University, Bloomington, IN); *SD[3L]* (Campbell *et al.*, 1991), *vg[null]* (Bernard *et al.*, 2003), and *dgrip[ex36]* (Swan *et al.*, 2004) have been described previously. Ectopic transgene expression was performed using the Gal4-upstream activation sequence (UAS) system (Brand and Perrimon, 1993) using the following lines: *Dmef2*-Gal4 (Ranganayakulu *et al.*, 1998), *twi*-Gal4 (Greig and Akam, 1993), *SD*-Gal4 (Roy *et al.*, 1997), C23-Gal4, and Ap-Gal4 (from Bloomington Stock Center), UAS-*robo* (Kramer *et al.*, 2001), UAS-*vg* (Deng *et al.*, 2009), UAS-*DN-egfr* (Yarnitzky *et al.*, 1997), UAS-*λ-egfr* (Queenan *et al.*, 1997), UAS-*SDΔTEA* (Garg *et al.*, 2007), and UAS-*lacZ* (Bloomington Stock Center).

Immunohistochemistry and Microscopy

Embryos were formalhyde fixed (Hughes and Krause, 1999), and the following primary antibodies were used at the indicated dilutions: mouse anti-FLAG (1:1000; Sigma-Aldrich, St. Louis, MO), rat anti-hemagglutinin (HA) (1:200; Roche Diagnostics, Indianapolis, IN), rat anti-myosin (1:500; Abcam, Cambridge, MA); mouse anti-βPS-integrin (1:500; developed by Danny Brower and obtained from the Developmental Studies Hybridoma Bank, Department of Biological Sciences, The University of Iowa, Iowa City, IA), mouse anti-β-Gal (1:500; Promega, Madison, WI), anti-muscle myosin heavy chain monoclonal antibody FMM5 (1:10; from D. Kiehart, Duke University, Durham, NC), rabbit anti-Vg (Williams *et al.*, 1991); rat anti-thrombospondin (Subramanian *et al.*, 2007); mouse anti-talin (Brown *et al.*, 2002), rabbit anti-PINCH (Clark *et al.*, 2003), and rabbit anti-Kon (Schnorrer *et al.*, 2007). Donkey Alexa488-, Alexa568-, Alexa594-, and Alexa647-conjugated secondary antibodies were used (1:4000; Invitrogen, Carlsbad, CA). Muscle actin was stained using Alexa546-conjugated phalloidin (1:25; Invitrogen). Images were obtained with a Zeiss LSM510 or UltraVIEW ERS confocal microscope (PerkinElmer-Cetus, Norwalk, CT) and assembled using Photoshop (version CS; Adobe Systems, San Jose, CA).

Tiggrin Antibody Production

A cDNA fragment encoding the C-terminal 270 amino acids including the RGD (Arg-Gly-Asp) domain of Tig (Fogerty *et al.*, 1994) was cloned into pDEST17 bacterial expression vector (Invitrogen), expressed in *Escherichia coli* [BL21(DE3); Stratagene, La Jolla, CA], and purified using nickel-nitriloacetic acid according to the manufacturer's protocol (QIAGEN, Valencia, CA). Purified fusion protein was injected into rabbits (Pocono Rabbit Farm and Laboratory, Canadensis, PA). Specificity of the rabbit polyclonal serum was determined by testing it against purified Tig and fixed *Drosophila* embryos, confirming the localization pattern was the same as published for Tig previously (Fogerty *et al.*, 1994).

RESULTS

vg[null] But Not *SD[3L]* Mutant Embryos Show Muscle Detachment in a *rhea[1]* Background

Antibody staining of wild-type embryos showed that *vg* is expressed at relatively high levels in muscles making both intermuscular and muscle–tendon cell attachments at the segment border (Figure 2A). To determine whether there is a significant role for Vg regulation of the migration or attachment function of these late-stage embryonic SMs; we examined the muscle phenotypes of embryos homozygous for the *vg[null]* mutation. Some flies with a homozygous *vg[null]* genotype do survive to adulthood, but they are invariably unable to produce progeny and have significant defects in the adult musculature (Bernard *et al.*, 2006). In homozygous *vg[null]* embryos, VL2 muscles were absent in at least 30% of segments (Figure 2, B–C[1]). This muscle-loss phenotype

seems to be VL2 cell specific because VL1 muscles were present in all segments (Figure 2C and Supplemental Figure S2, C and D). However, the loss of Vg does not seem to block initial formation of adhesions between two differentiating muscles, because similar to stage 16 wild-type embryos (Figure 2, B–B[1]), in homozygous *vg[null]*, pairs of VL muscles formed tight adhesions between their corresponding VL muscle in the next segment (Figure 2, C–C[1]).

To further dissect the requirement for Vg during formation of adhesions between SMs, we paired mutations in *vg* with those in *rhea* (talin deficient) that would make small changes in attachments induced by loss of *vg* more apparent. In *rhea[1]* mutant embryos, junction formation between pairs of VL muscles seems normal (Figure 2, D–D[1]). At early stages (15), the VL muscles showed no significant migration or attachment defects in *vg[null]*; *rhea[1]* double mutant embryos (Figure 2, E–E[1]). However, at later stages (16+) several muscles, most prevalently VL1 can be seen detaching from their normal location (Figure 2, F[1]–H[1]). This muscle detachment phenotype seems to be due solely to lack of Vg activity because it can be rescued by expression of wild-type Vg in muscle cells (Figure 2, I–I[1]). Because SD is the known binding partner for Vg function during wing development (Simmonds *et al.*, 1998), the muscle phenotype of *SD[3L]*; *rhea[1]* double mutants also was examined. Mutants homozygous for a loss-of-function *SD[3L]* allele (Campbell *et al.*, 1991; Srivastava *et al.*, 2004) and *rhea[1]* produce only a mild SM phenotype described previously (Deng *et al.*, 2009). Otherwise, they are indistinguishable from *rhea[1]* mutants (Figure 2J).

Expressing a Form of SD That Can Bind Vg, But Not DNA, Causes a Phenotype Similar to *tig* Null Mutants

In cells of the wing imaginal disk, Vg must form a complex with SD to localize to the nucleus and bind chromatin via a conserved TEA binding domain within SD (Halder *et al.*, 1998; Simmonds *et al.*, 1998). Previously, a form of SD that removes the TEA DNA binding domain (*SDΔTEA*) has been shown to bind Vg, but because the resulting complex cannot bind DNA it acts to inhibit Vg activity in imaginal discs (Garg *et al.*, 2007). During experiments to confirm that tissue-specific expression of a UAS-*SDΔTEA* transgene can specifically inhibit the gene activation function(s) of Vg in the wing disk, we noted that expression of *SDΔTEA* by *SD*-GAL4 also produced elongated pupae and adults (Figure 3, A and C). A database search produced only one other mutation that produces elongated body, muscle spacing and semilethality phenotypes, a null mutation in *tig*, required for intermuscular junction formation (Bunch *et al.*, 1998). This phenotype also was notable because *SD*-GAL4 would lead to only weak activation of *SDΔTEA* in tissues such as embryonic SMs from stage13 to stage16 (Deng *et al.*, 2009). Given the similarity of interfering with Vg function via *SDΔTEA* and *tig* mutations, we tested the effect of *SDΔTEA* in muscle cells by using a driver that is expressed at much higher levels (*Dmef2*-GAL4) (Figure 3B). This combination was semilethal, with 78.7% of pupae (n = 619) failing to eclose. The larvae hatched from embryos overexpressing *SDΔTEA* via *Dmef2*-GAL4 had obvious gaps between the dorsal acute (DA)1 muscles that were larger than wild type (Figure 3, D–D'), suggesting the elongated body type was caused by muscle defects. However, the requirement for Vg function was stage specific because expression of *SDΔTEA* in muscle progenitor cells at earlier embryo developmental stages (7–11) by using *twist*-GAL4 (Greig and Akam, 1993) did not lead to elongated adults and significant lethality (Figure 3C). Finally, to ensure that the
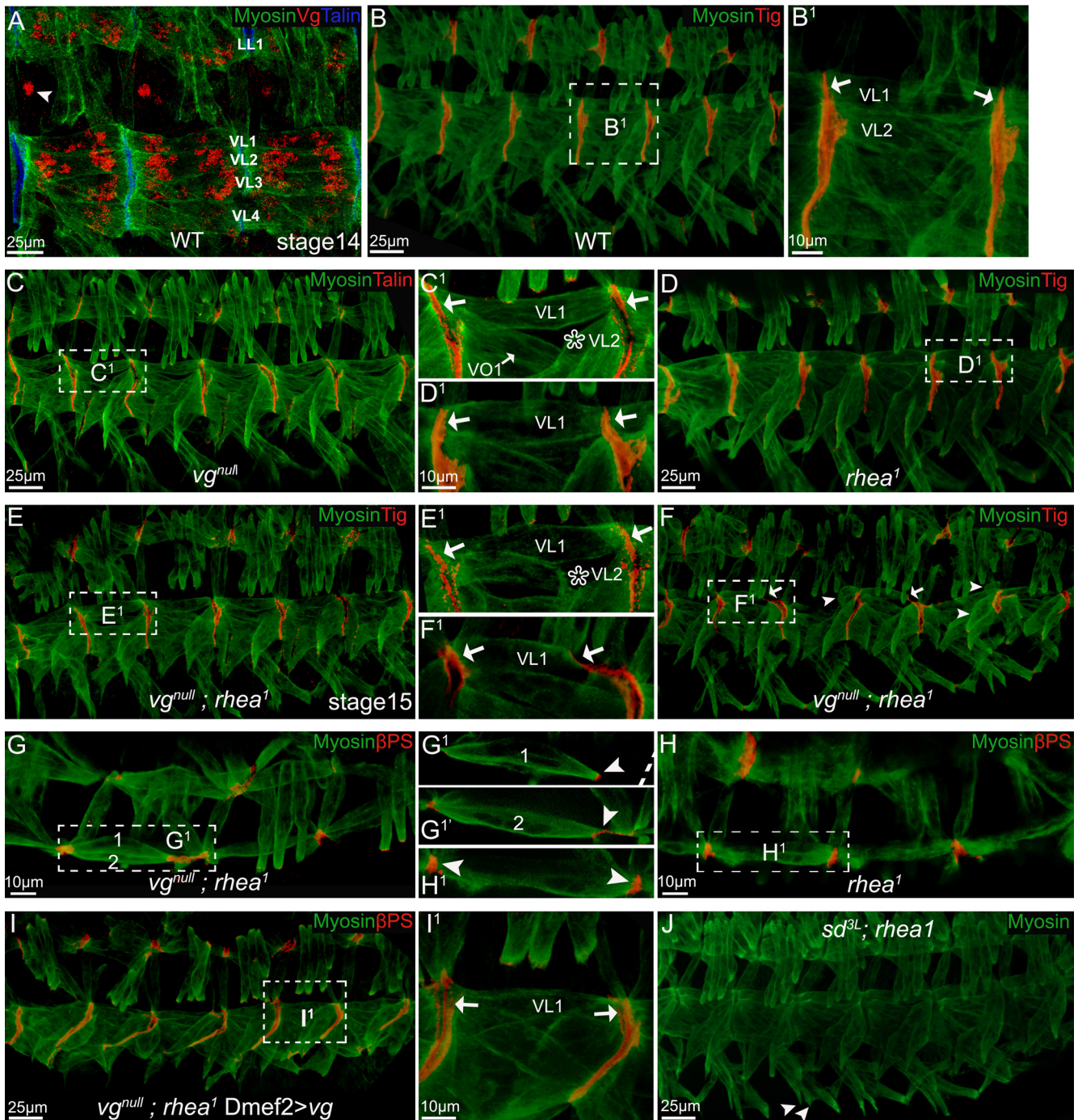
Figure 2. SMs were detached in *vg^null; rhea^1* embryos but not in *SD^3L; rhea^1* embryos. Embryos (stage 16 or a specified stage) are shown as lateral views, with dorsal up, and anterior to the left. Staining is color coded and indicated on each panel. B^1–I^1 are the close-ups of the framed area in B–I. (A) *vg* is expressed in muscle LL1 and VL1–4. The arrowhead points to a neuronal cell also expressing *vg*. Compared with wild-type embryos (B), *vg^null* (C), or *rhea^1* single mutation (D), or *vg^null; rhea^1* double mutant embryos in early stages (before stage 15; E), all produced a muscle pattern similar to wild-type embryos, except that a *vg^null* mutation caused muscle VL2 to be missing in ∼30% of segments (star in C^1 and E^1). Notice VL muscles (e.g., VL1) all formed tight adhesions between each other (arrows in B^1–E^1). (F–F^1) By late stage 16 when muscles start to contract, muscle VL1 or other VL muscles detached from the attachment sites only in the *vg^null; rhea^1* double mutant embryos (arrows in F^1). Arrows in F indicate detaching muscles and arrowheads indicate detached muscles. (G–H^1) Overview of the *vg^null; rhea^1* double mutant embryos (stage 16; G–G^1') compared with *rhea^1* embryos (H–H^1). Both VL1 and VL2 are retracting from their normal attachment sites (arrowhead in G^1–H^1). G^1 and G^1' are two different confocal sections, and the broken line in G^1 indicates the segment border. (I–I^1) The muscle detachment phenotype of *vg^null; rhea^1* embryos can be rescued by expression of Vg via *Dmef2*-GAL4. Notice VL1 muscles built tight adhesions between each other (arrows in I^1). (J) *SD^3L; rhea^1* embryos did not have a muscle detachment phenotype. Some muscles do not develop well (VO4–6; arrowheads) in these embryos, but this mirrors the phenotype seen in *SD^3L* single mutants.
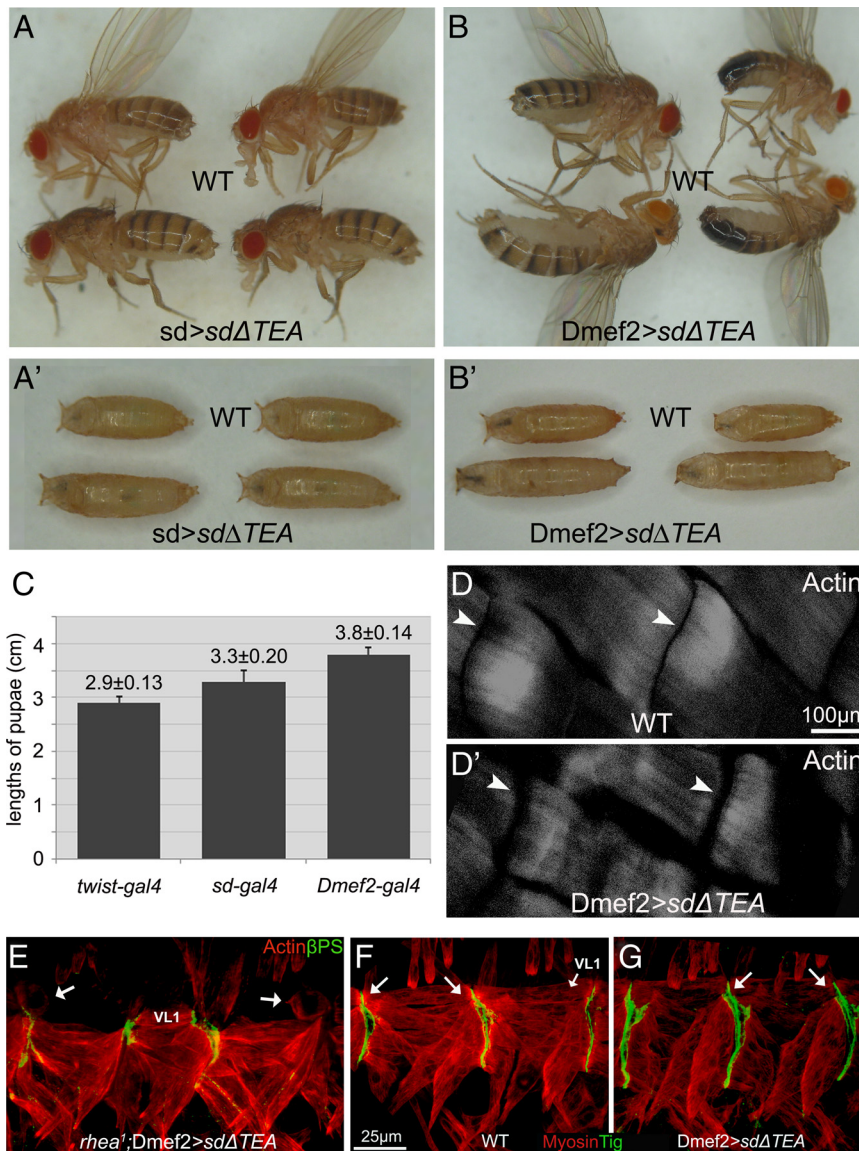
Figure 3. Tissue-specific expression of *SDΔTEA* interferes with Vg function and produced elongated larvae and adults. (A) Expression of *SDΔTEA* via *SD*-GAL4 (SD>*SDΔTEA*) in the wing disk caused loss of the adult wing by interfering with Vg . However, we also noted that the pupae (A′) and adult flies were elongated compared with wild-type (WT) siblings. (B) This phenotype was caused by interfering with Vg in the muscle cells because these effects were seen in pupae (B′) and adults when UAS-*SDΔTEA* was expressed exclusively in muscle cells via *Dmef2*-GAL4 (Dmef2>*SDΔTEA*). (C) Quantification of pupal length in animals overexpressing *SDΔTEA* by using the indicated GAL4 drivers (mean ± SD; n = 23). The pupal length of twist-GAL4>*SDΔTEA* animals was not statistically different from wild type. (D–D′) Larvae expressing *SDΔTEA* in the muscles (*Dmef2*-GAL4) (D′) had a larger gap (arrowhead) between DA1 muscles than wild type (D). (E) Expression of *SDΔTEA* in developing muscle cells in embryos that are homozygous for the *rhea¹* mutation produced a muscle detachment phenotype in which the majority of the VL1 cells became rounded (arrows). (F) Tig protein localizes to the tips of muscles forming junctions including VL1 (arrows). (G) Embryos expressing *SDΔTEA* in muscles show the same pattern of Tig localization (arrows). In all panels, ventral muscles in two or three segments are shown in embryos (stage 16) presented as lateral views, with dorsal up, and anterior to left.

phenotype we observed was caused by inhibition of Vg through formation of a nonfunctional Vg/*SDΔTEA* complex and not through overexpression of SD, we overexpressed a form of SD that cannot bind Vg (SDΔVID). This combination produced flies with no detectable defects in developing muscles.

VL Muscle Migration and Initial Adhesion Occurs Normally in the Absence of Vg

The progressive muscle detachment phenotype we observed when Vg activity is reduced could be caused by several events, including improper muscle specification, a failure of muscles to migrate to the attachment site, or select the appropriate target site, or the inability to form a strong connection that can resist the force of muscle contraction at later stages. To determine whether Vg was required for initial establishment and maintenance of the VL cell lineage, we used a marker that would be activated solely in the VL1 muscle and would persist during later development (*5053*-GAL4 and UAS-*lacZ*) (Schnorrer *et al.*, 2007). The VL1 muscle is initially specified correctly in all segments

in both *vg^null* and wild-type embryos (Supplemental Figure S2, C and D). Similarly, development of tendon cells was not affected in *vg^null*; *rhea¹* double mutants (Supplemental Figure S2, A and B).

Because early specification of VL1 was unaffected when Vg is absent, we next assayed for changes in formation of junctions between two muscles at the segment border. There are three known major components of muscle–muscle junctions (Brown *et al.*, 2000): 1) PS2 integrin; 2) ECM containing PS2 integrin ligands: Tig (Fogerty *et al.*, 1994) and Tsp (Subramanian *et al.*, 2007); and 3) talin and its associated proteins, including PINCH (Clark *et al.*, 2003). Together, they link PS2 integrin to the muscle myofiber, forming a tight adhesion junction or integrin complex holding muscles together (Figure 4, A–A³). In the *vg^null*; *rhea¹* double mutant embryos, βPS and Tig (Figure 4, B–B³), PINCH and βPS (Figure 4, D–D³), and Tsp (Figure 4, E–E²), were all concentrated at the ends of detaching VL1 muscles and connected to myofibers, similar to wild type (Figure 4, A–A³ and G–G³) or *rhea¹* single mutant embryos (Figure 4, C–C³). Thus, the affinity of PS2 integrin for its ligands did not seem to be affected and the
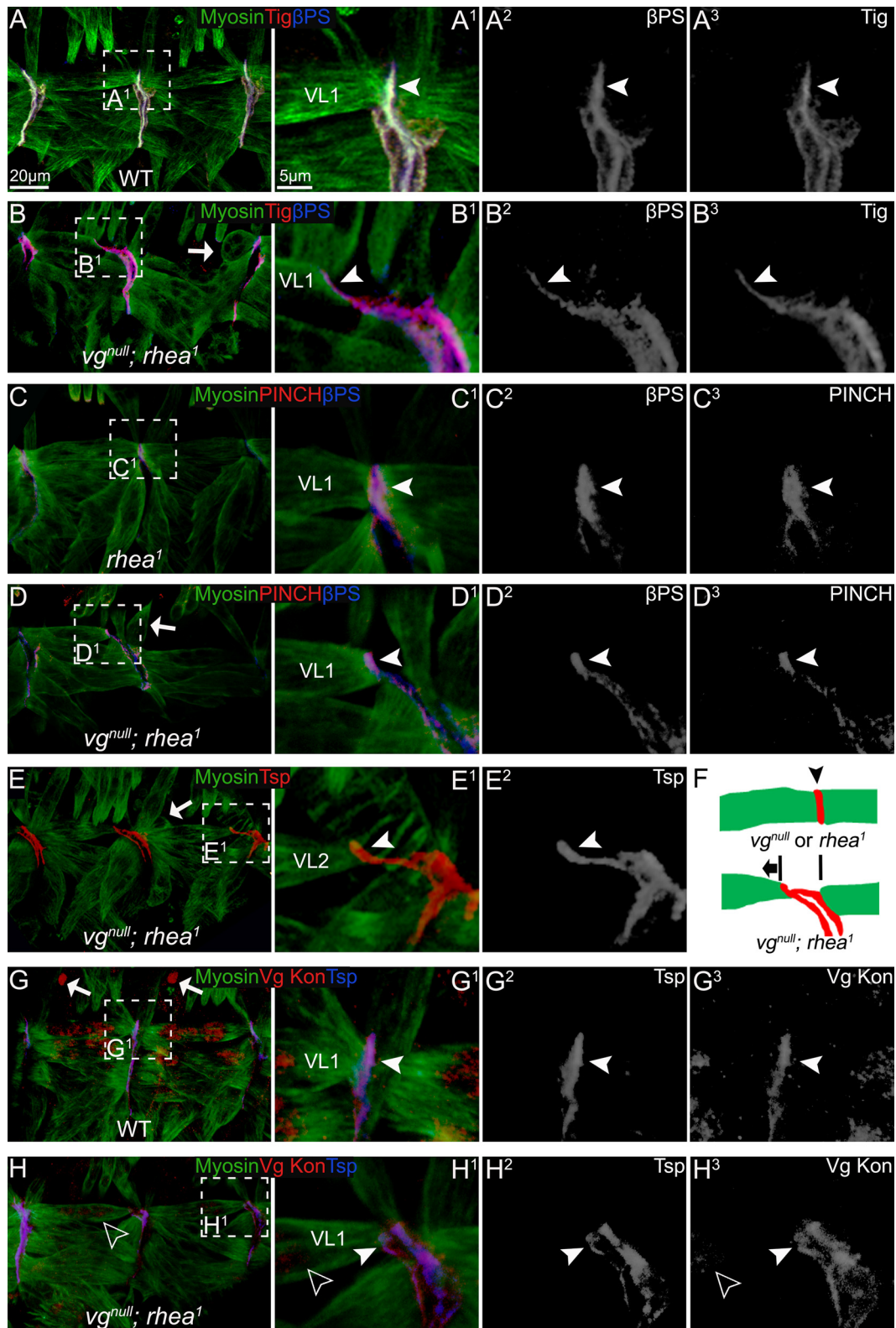
Figure 4. The muscle detachment phenotype observed in vg^{null}; $rhea^1$ embryos was not due to lack of localization of integrin or its known ligands, nor to an obvious muscle migration defect. (A) In wild-type embryos, βPS and Tig can be seen localized normally at the junctions between two VL muscles (arrowhead). (B) In vg^{null}; $rhea^1$ double mutant embryos, the VL muscles were either detaching (arrowheads) or were already detached (arrows). However, βPS and Tig remain concentrated at muscle termini and followed the detaching muscles (arrowheads). (C) In $rhea^1$ mutant embryos, the adhesion proteins PINCH and βPS formed tight junctions between VL muscles (arrowheads). (D) Similar to βPS and Tig, in detaching muscles in vg^{null}; $rhea^1$ embryos, PINCH and βPS remain concentrated at muscle termini and followed the detaching muscles (arrowheads). Many muscles seemed to be detaching from the posterior border of each segment. (E) In the vg^{null}; $rhea^1$ embryos, Tsp shows the same localization to the end of detaching muscles as PINCH, βPS, and Tig. (F) A diagram of the localization of
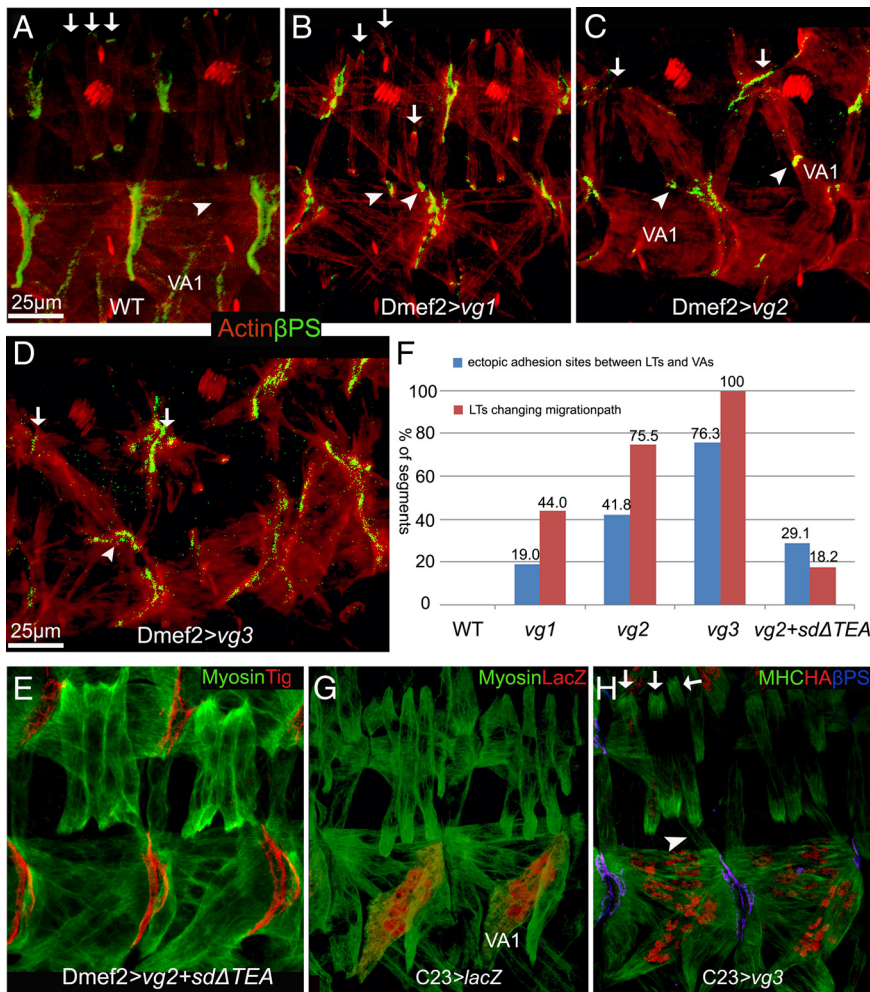
Figure 5. Ectopic expression of Vg in the developing embryonic SMs produced ectopic intermuscular attachments. The transgenic lines *vg1*, *vg2*, and *vg3* express relatively higher levels of Vg, respectively, as verified by Western blotting. (A) In wild-type embryos, LT1–4 muscles that stain brightly with muscle-specific actin (red; arrows) are seen passing left to right over the VA1 muscle and form βPS-mediated attachments (green) at intrasegmental sites. Normally, no adhesions form where the LT and VA muscles are adjacent (arrowhead). (B–D) Ectopic expression of progressively higher levels of Vg in all muscles via *Dmef2*-GAL4. (B) Ectopic expression of relatively lower levels of Vg (vg1) in SMs caused the LT muscles to abnormally form attachments at the segment borders. (C) Expression of relatively higher levels of Vg (vg2) cause the formation of abnormal attachments at the segment borders (arrows). Furthermore, ectopic muscle–muscle attachments were observed between LT and VA muscles (arrowheads). In some cases, muscle VA1 was observed deviating from its original position (arrowheads). (D) This number of abnormal and ectopic attachments becomes even more severe when a transgene (vg3) expressing relatively highest levels of Vg is used. (E) Quantification of the percentage of segments having LTs with abnormal migration (red columns) or ectopic adhesion sites between LT and VA muscle cells (blue columns) for each indicated overexpression line (n = 110). (F) Ectopic expression of Vg with *SDΔTEA* led to a partial rescue of the phenotype caused by ectopic expression of Vg from the UAS-*vg2* transgene. (G) The C23-GAL4 line induces expression at high levels in VA1 but relatively low expression in LTs as detected by an UAS-*lacZ* reporter. (H) Ectopic attachments are not formed when Vg is present at relatively high levels in VA1 cells.

integrin complex was still largely intact in muscles of the double mutant embryos, before detachment.

Because the major adhesion proteins were being localized correctly at the end of VL1 muscles in *vg^{null}*; *rhea^1* mutant embryos, we examined the process of VL1 migration. The transmembrane protein Kon-tiki (Kon) is localized to the tips of VL muscles and is required for formation of filopodia and proper migration of developing muscles (Estrada *et al.*, 2007; Schnorrer *et al.*, 2007). The expression and localization of Kon in the *vg^{null}*; *rhea^1* double mutants was indistinguishable from those of wild-type embryos (Figure 4, G–H^3). Second, the direction of VL1 muscle migration was unaffected. VL1 muscles normally migrate from the posterior border of each segment to the anterior border and then attach to both (Schnorrer *et al.*, 2007). If muscles in embryos

with *vg^{null}*; *rhea^1* mutations migrated inappropriately, then they would fail to attach to the anterior border and round up at the posterior side of each segment. However, in the double mutants, more than half of the VL1 muscles detached from the posterior borders remaining attached to the anterior (Figure 4, D–F), indicating they already reached to their attachment sites.

Ectopic Expression of Vg Induces Abnormal Muscle–Muscle Attachments

The phenotype associated with *vg^{null}* mutants strongly suggested that Vg expression induces cell-specific changes in VL muscles for them to be competent to form intermuscular attachments at the segment border. If this hypothesis is correct, then Vg should be able to induce additional attachments when expressed ectopically in muscles where it is not normally found at high levels. The lateral transverse (LT)1–4 muscles migrate vertically within each segment and normally make only muscle tendon cell attachments in wild-type embryos (Figure 5A). Ectopic-expression of Vg within the LT muscles caused them to migrate toward the segment borders and attach there, producing muscle–muscle attachments between LT and VA muscles, where they normally do not occur (Figure 5, B–D). Notably, this ectopic expression does not seem to alter the initial muscle identities of LT2, LT4, and VA2 (Supplemental Figure S2, E and F). The ectopic muscle–muscle adhesions induced by ectopic expres-

Figure 4 (cont). adhesion proteins (red; arrowhead) in *vg^{null}* or *rhea^1* mutant embryos and the direction (anterior, arrow) in which VL muscles are moving after they detach. (G) In wild-type embryos, Kon, the major migration guidance protein for VL muscles, normally found at the end of muscle cells (arrowhead). (H) In *vg^{null}*; *rhea^1* embryos, some residual (maternally supplied) Vg protein can still be seen in VL1 muscle (empty arrowheads). These muscles still had a detachment phenotype, but Kon is localized properly (arrowhead). A^1–H^1 are the close-ups of the framed area in A–H. A^2–H^2 and A^3–H^3 show each confocal channel separately.
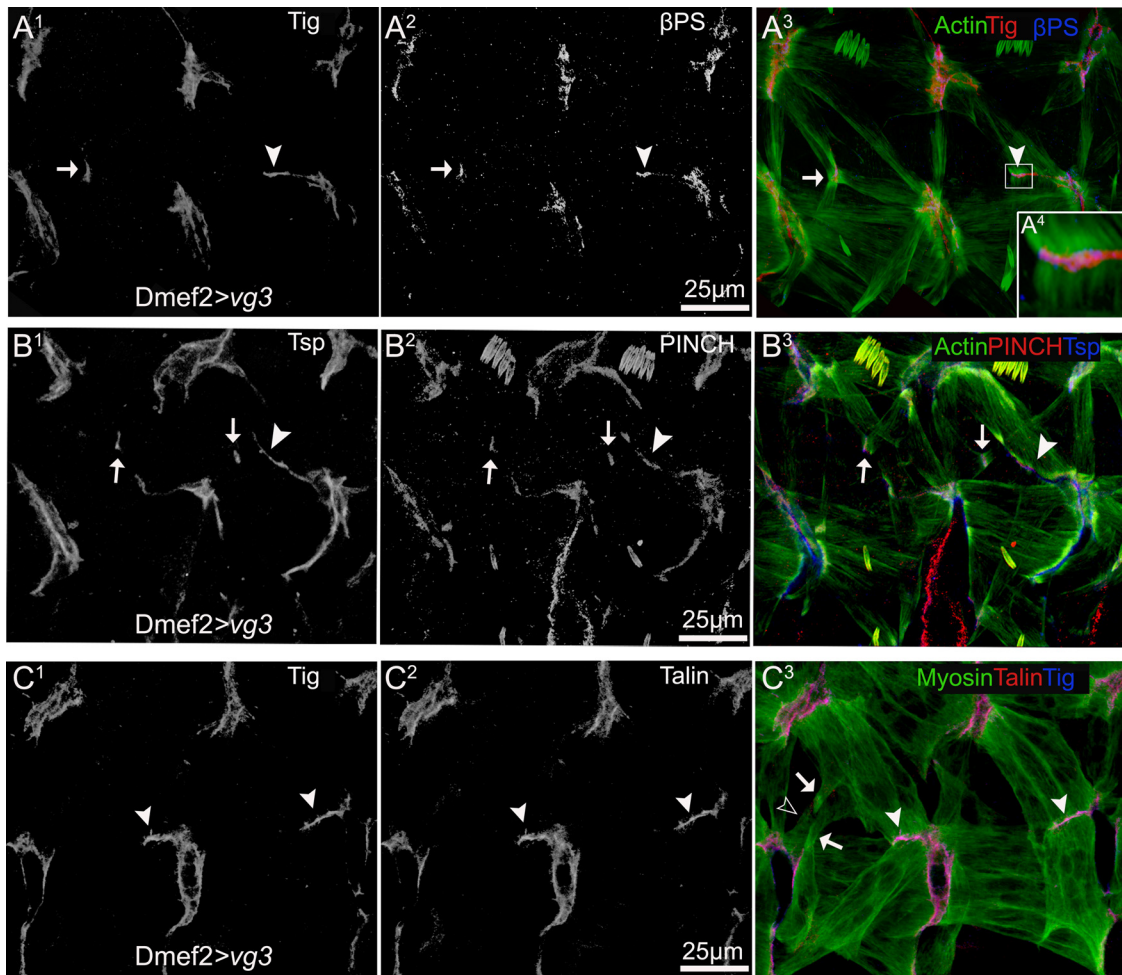
Figure 6. Muscle attachments induced by ectopic Vg include βPS integrin and its associated cytoplasmic linker proteins, PINCH and talin. (A) Ectopic expression of Vg by *Dmef2*-GAL4 caused additional attachments to form between muscles stained with muscle-specific actin (green). These ectopic attachments (arrowheads) contained Tig (red; A[1]), an extracellular ligand for PS2 integrin (blue; A[2]). Ectopic muscle attachments were also produced between muscle cells other than LTs and VAs, which also contained Tig (arrows). Individual myofibers were linked to the new adhesion sites through integrin complexes (A[4] is a close-up of the boxed area in A[3]). (B) These ectopic attachments also contain Tsp (blue; B[1]) and PINCH (red; B[2]). (C) Talin is also localized to the ectopic muscle attachments (red; C[2]). Note the processes emerged from the lateral surface of muscle LTs (arrows in C[3]) and VT1 (empty arrowhead in C[3]).

sion of Vg in the LT muscles seemed to be functional, because muscle VA1 was often located away from its normal position due to tension produced by abnormal attachment to the LT muscles (Figure 5C). We confirmed that this phenotype was Vg specific by expressing both *vg* and *SDΔTEA*. This combination showed fewer ectopic muscle attachments (24.5%, compared with 81.8% when Vg is expressed alone; Figure 5, E and F). Similarly, we saw a correlation between the severity of the ectopic LT muscle attachment phenotype and increasing the expression of ectopic Vg (Figure 5F). Finally, ectopic expression of Vg in both cells is required to induce them to form ectopic attachments, because expression of Vg in only a single muscle group failed to build ectopic adhesion sites between LTs and VAs (Figure 5, G and H). To further test this idea, expression of UAS-*vg* was also driven through *apterous* (*ap*)-GAL4 that is present only in muscle VA2 and LT1–4 starting at stage 14 when all SMs have been properly specified (Supplemental Figure S4, A–A'). This late stage expression of Vg still produced ectopic adhesions, but only between VA2 and LT muscles, not between VA1 and LT muscles although VA1 is closer to LT muscles than VA2 (Supplemental Figure S4, B–B').

Ectopic adhesion sites were also induced between ventral transverse (VT)1 and LTs (Figure 6A[3]) or between segment border muscle (SBM) and lateral oblique (LO)1 (Figure 6B[3]; see Figure 1 for legend showing muscle identities) by higher levels of ectopic Vg expression. Although expression of Vg clearly induced formation of extra attachments between muscles, it is possible that these attachments were not functional. Assembly of intermuscular junctions can be perturbed at several steps. A chimeric mimic of activated integrins can recruit talin in embryonic muscles but not other integrin-associated proteins such as PINCH (Tanentzapf *et al.*, 2006). Similarly, in mammalian cells, activation of high-affinity αVβ3 integrin produced ectopic integrin clusters that recruit talin but not other integrin-associated protein (Cluzel *et al.*, 2005). We determined whether each of these integrin-associated components (Tig, Tsp, talin, and PINCH) was recruited to the additional adhesion sites associated by ectopic/overexpression of Vg (Figure 6, A[1]–C[3]). More importantly, the internal muscle myosin–actin fiber was connected to the ectopic integrin clusters (Figure 6A[4]). This suggests that a functional intermuscular attachment was induced in cells that migrate abnormally due to ectopic Vg.
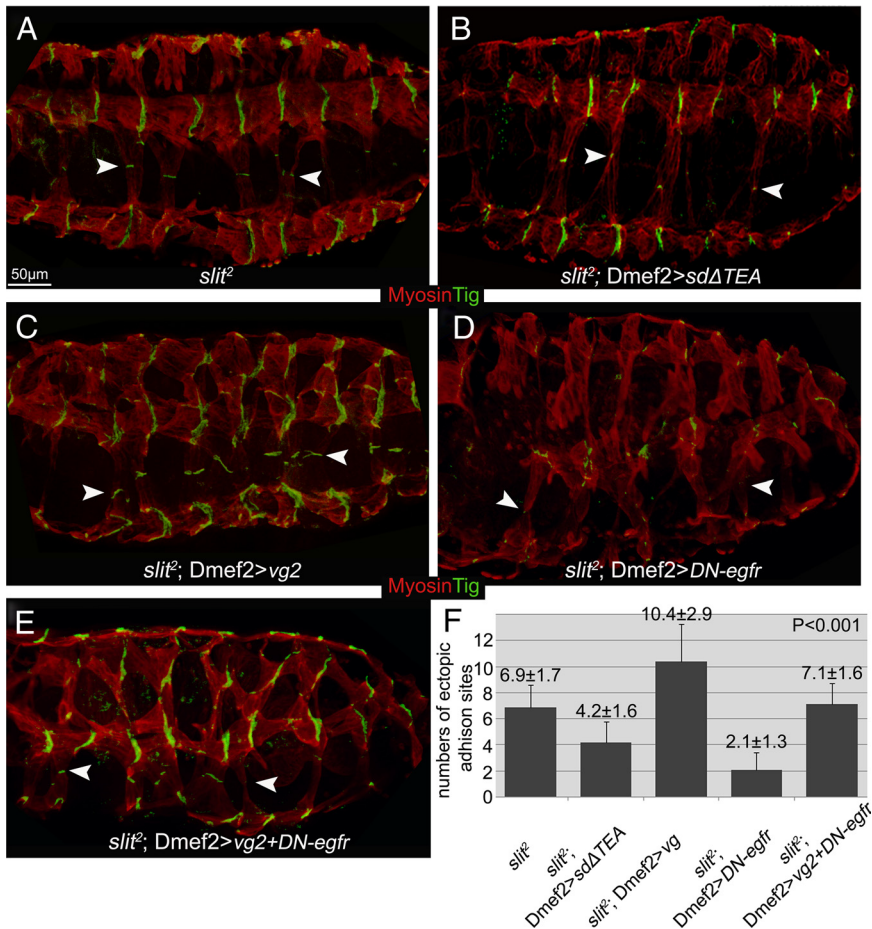
Figure 7. Altered levels of Vg function regulate ectopic intermuscular attachments independently of signaling from tendon cells in VL muscle cells, and DER mediated cell–cell communication is required for the production of intermuscular attachments between Vg expressing cells in *slit²* mutant. (A) In *slit²* mutant embryos, VL muscle cells migrate dorsally over the CNS from the lateral sides of the embryo meeting near the midline to form Tig marked muscle–muscle adhesions (arrowheads) in a region of the embryo devoid of tendon cells. (B) Interference with Vg function by expression of *SDΔTEA* in these *slit²* mutant embryos led to fewer and smaller of these midline-located adhesion sites (arrowheads). (C) Overexpression of Vg in *slit²* mutant embryos produced more and larger adhesion sites (arrowheads) in the abnormally positioned VL muscles. (D) Expression of a dominant-negative form of DER (DN-egfr) in *slit²* mutant embryos strongly reduces the overall size and number of these muscle-muscle adhesion sites (arrowheads in D). (E) The size and number of these ectopic adhesion sites (arrowheads in E) in *slit²* mutants that coexpress DN-egfr as well as Vg are reduced when compare with expressing Vg alone (compare E with C) but increased when compared with expressing DN-egfr alone (compare E with D). (F) Quantification of the number of VL cell adhesion sites formed in *slit²* mutant embryos with varying levels of Vg activity and/or changing of DER activity (mean ± SD; n ≥ 15).

Vg Is Required for the Ectopic Attachments Formed between Ventral Midline-Crossing Muscles in *slit²* Mutants

Although expression of Vg can induce the formation of muscle–muscle junctions, the contribution of signals from the surrounding tendon cells may be influencing this effect. Therefore, we examined the effect of blocking Vg function in *slit²* mutant embryos, where the VL muscles cross dorsally over the CNS meeting those from the other side to form ectopic muscle attachments along the ventral midline (Figure 7A). This allows us to test the effect of loss of Vg on muscle–muscle attachments independently, because there are no tendon cells within this region (Vorbruggen and Jackle, 1997). Blocking Vg function via *SDΔTEA* in VL muscle cells that abnormally migrate along the midline led to fewer and smaller muscle–muscle adhesions (Figure 7, B and F). Conversely, increasing the expression of Vg produced more and larger adhesion sites (Figure 7, C and F).

Embryonic Muscles Expressing Vg Require DER Signaling to Form Attachments

As a transcriptional activating "selector gene," the role of Vg is assumed to be induction of cell-specific changes in gene expression (Halder *et al.*, 1998; Simmonds *et al.*, 1998). However, because we observed that Vg expression is required in both cells forming a muscle–muscle adhesion, this would suggest additional coordination via cell–cell communication. The DER is ubiquitously expressed within the mesoderm (Zak *et al.*, 1990). Notably, one DER ligand, Vein, is

enriched at the segment borders where intermuscular junctions are formed (Yarnitzky *et al.*, 1997), making it the prime candidate for coordination of muscle–muscle adhesions in this region. Therefore, we tested whether DER signaling is required for Vg-mediated establishment of intermuscular attachments. Mesodermal expression of dominant-negative DER (*DN-egfr*; Buff *et al.*, 1998) did not affect the specification of VL muscles that were crossing the midline, although the specification of muscle lateral longitudinal (LL)1 and ventral oblique (VO)4–6 were affected, as described previously (Supplemental Figure S2, G and H; Buff *et al.*, 1998). Increasing Vg expression in the SMs of *slit²* mutants greatly enhanced the adhesion level between the midline-crossing VL muscles (Figure 7C), whereas reducing the activity of DER signaling decreased the size and/or number of adhesion sites caused by ectopic Vg (Figure 7, E and F). Mesodermal expression of DN-egfr alone in *slit²* mutants greatly decreased the size and number of the ectopic intermuscular adhesion sites along the midline (Figure 7, D and F). To remove the possibility that expression of DN-egfr via *Dmef2*-GAL4 might affect the specification of muscle cells, *ap*-GAL4 was used to drive expression of Vg as well as DN-egfr in late-stage muscle cells (Supplemental Figure S4, A–A'). *ap*-GAL4 produced a weak expression of Vg in VA2 and LT muscles, which led to production of ectopic adhesions between theses muscles in ~7% segments (Supplemental Figure S4, B–B'). However, expression of DN-egfr made these ectopic adhesions disappear (Supplemental Figure S4, C–C').
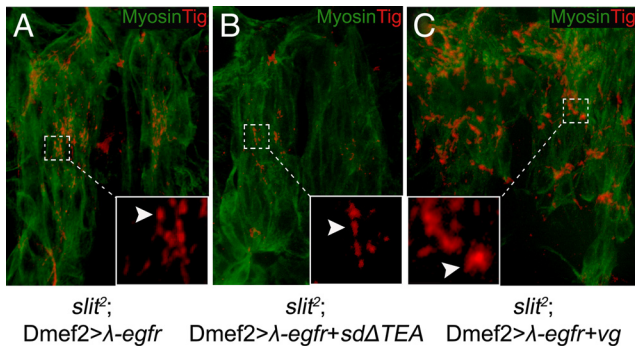
Figure 8. Changes in muscle–muscle adhesion caused by expression of a constitutively active DER (λ-egfr) are sensitive to the presence of Vg. (A) Many small adhesion sites were formed between the midline-crossing muscles in *slit²* mutant embryos (arrowhead) when λ-egfr is expressed in developing muscle cells (Dmef2>λ-egfr) (arrowhead, inset). (B) Expression of λ-egfr in developing muscle cells where Vg function was inhibited by *SDΔTEA* produced fewer of these ectopic adhesions (arrowhead; inset). (C) Relatively more and larger ectopic adhesions were formed when embryonic muscle cells were overexpressing both λ-egfr and Vg (arrowhead, inset). Insets are close-ups of the area framed by the dotted lines.

Mesodermal expression of constitutively active λ-egfr (Queenan *et al.*, 1997) in *slit²* mutants produced many small adhesion sites between midline-crossing VL muscles (Figure 8A). This ectopic attachment phenotype was shown to be Vg specific because interfering with Vg function by coexpression of *SDΔTEA* resulted in fewer of these adhesion sites (Figure 8B), whereas these adhesion sites become much bigger with increased Vg expression (Figure 8C).

Vg Induces Ectopic Adhesion between SM Cells That Make Contact with Each Other through Filopodial Extensions

Both loss of Vg and *tig* function produce a unique elongated body phenotype associated with defects in the embryonic musculature (Figure 3). One of the predicted roles of Tig is to induce formation of filopodia, and this may be required for muscle migration (Bunch *et al.*, 1998). We noted that migrating SM cells seeking attachment targets extend filopodia at their leading edges. LTs muscle extend filopodia to the segment border and filopodia from migrating SM cells can be observed contacting each other between developing LT and ventral acute (VA) muscles (Figure 9, A¹–A²). We did not observe filopodial contact between LT, VL, or VL and VA muscles. Correspondingly, ectopic adhesion sites were produced between LTs and VAs but not between LTs and VLs or between VLs and VAs when ectopically expressing Vg (Figure 9, B and D). Additional filopodia or integrin localization was not observed at the leading edge of muscles expressing elevated levels of Vg (Figure 9, C–C¹). Thus, Vg induced formation of muscle-muscle adhesions requires close filopodial contact between migrating muscles.

DISCUSSION

When two migrating somatic muscles come into close contact, there must be a cell-intrinsic mechanism to determine whether to build a stable adhesion junction. This would require coordinate regulation of this activity in each type of muscle to prevent inappropriate adhesions. We have shown that the transcriptional activator Vg is a key factor regulating this event in embryonic VL1–4 muscles. Although expression of Vg in muscle cells makes them competent to form intermuscular junctions, this process requires DER signaling to coordinate formation of attachments (Figures 7 and 8). Finally, this process is associated with contact between filopodia from each of the cells expressing Vg (Figures 5, 6, and 9).

We used three independent methods to test the requirement for Vg: null mutations, interfering with Vg function by using *SDΔTEA*, and ectopic Vg expression. When Vg function was blocked, adhesion between VL muscles was disrupted. This effect was enhanced in a *rhea¹* mutant background, whereas formation of intermuscular adhesions was
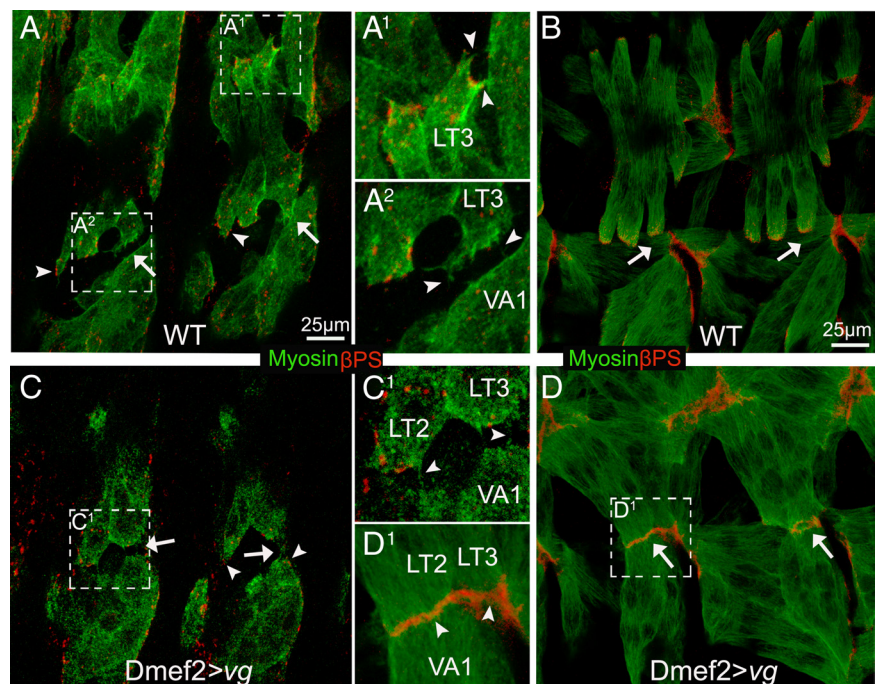

Figure 9. Filopodial contact between muscles forming junctions are not affected by changes in Vg expression. (A) In wild-type embryos (stage 14), the leading edge of LT muscles produce filopodia that contact with corresponding filopodia protruding from the lateral edge of VAs or other muscles (arrows in A and arrowheads in A¹–A²). A¹ and A² are magnified photos of the framed areas in A. βPS integrin accumulates at the leading edge of the myotube (arrowhead). (B) At developmental stage 16, LTs normally find their attachment sites and form stable adhesion inside each segment. There are no connections between LTs and VAs (arrows in B). (C) Muscles in embryos that were expressing Vg ectopically (stage 14) produced similar number of filopodia compared with wild type (arrows in C and arrowheads in C¹). Also, βPS integrin accumulated at the leading edge of muscles (arrowheads in C) in the same way as wild-type muscles. (D) At stage 16, LT muscle cells have formed stable attachments with muscle VAs (arrows in D and arrowheads in D¹).

reduced in *slit²* mutants. Conversely, adhesion between VL muscles in *slit²* mutants was enhanced when increasing *vg* expression in the muscles that normally express *vg*. It seems that formation of muscle–muscle attachments is directly related to the relative level of Vg in both cells as ectopic attachments failed to form if *vg* is expressed only in a single muscle (Figure 5, G and H, and Supplemental Figure S4, B–B′).

Blocking Vg function by using a *vg^null* mutation or overexpressing *SDΔTEA* produced similar phenotypes (Figure 2 and Supplemental Figure S1). The one paradoxical difference between these methods was that the surviving pupae and adult *vg^null* mutants were less elongated compared with those overexpressing *SDΔTEA* (Figure 2). The *SDΔTEA* transgene may block the functions of Vg-containing transcription factor complexes that do not normally include SD, which would explain the more severe phenotype. Our data support this conclusion, because *vg^null; rhea¹* double mutants have an identical phenotype to that caused by expression of *SDΔTEA* in *rhea¹* mutants (Supplemental Figure S1). We can confirm that Vg function is blocked specifically when *SDΔTEA* is expressed in SMs, because overexpressing *SDΔTEA* via *Dmef2*-GAL4 was able to significantly rescue the LT muscle rerouting phenotype caused by overexpression of Vg, whereas expression of a transgene that deleted only the Vg interaction domain produced a wild-type phenotype.

Ectopic expression of Vg in LT muscles redirects their migration to the segmental borders (Figure 5). This phenotype is similar to that caused by ectopic expression of Robo or Grip (Kramer *et al.*, 2001; Swan *et al.*, 2004). Slit-Robo signaling provides an important external cue to guide Robo-expressing muscles such as VL1–4 to the segment border (Kramer *et al.*, 2001). The postsynaptic density 95/disc-large/zona occludens domain protein Grip also plays an important role in the migration of VL muscles (Swan *et al.*, 2004). However, the aberrant muscle migration phenotype caused by ectopic Vg is independent of Slit-Robo signaling or Grip (Supplemental Figure S3). Rather, our results suggest that Vg induces cell competence to form attachments. Thus, in muscle expressing ectopic Vg, formation of extra attachments may induce abnormal migration.

The mechanical connections of muscle–muscle attachments are thought to be primarily mediated by integrin and its associated adhesion proteins (Brown *et al.*, 2000). However, there must be a corresponding cellular regulation that determines whether it is appropriate for two muscles coming into contact form specific types of attachments. Examination of the proteins representative of the three major components of the integrin complex showed that they were all present at the termini of VL muscles in *vg^null; rhea¹* mutant embryos (Figure 4), suggesting that the integrin complex was established properly. Thus, the role of Vg is clearly not during initial establishment of the junctions. However, muscular junctions are relatively dynamic and may require cellular coordination to maintain their structure. The affinity of integrin to its ligands can change under different conditions and talin binding to the integrin β integrin cytoplasmic tail represents the final common step in integrin activation (Tadokoro *et al.*, 2003). When integrin affinity to ECM is low due to the loss of interaction of talin with the β subunit cytoplasmic domains, the diffusible protein Tig does not colocalize with integrin at the end of detaching muscles (Tanentzapf and Brown, 2006), However, this phenotype is not what we observed in *vg^null; rhea¹* double mutants (Figure 4). Hyperactive PS2 integrins can be made when the cytoplasmic domain of α_{PS2} subunit is deleted, and mesodermal expression of this mutant integrin is able to produce ectopic intermuscular attachment (Martin-Bermudo *et al.*, 1998).

Even so, the phenotype produced by hyperactive PS2 integrins is far milder than what we observed when ectopic sites are induced between VA and LT muscles where Vg is over expressed. Therefore, our results suggest a role for Vg during the establishment of intermuscular attachment that is permissive rather than directly altering the affinity of integrin within the junctions.

We have shown previously that *vg* has a role in the specification of embryonic muscle VL2 together with *Dmef2* (Deng *et al.*, 2009). However, in *vg^null* mutant embryos, examination of molecular markers unique to the VL1 muscle (i.e., *Kon*) showed no apparent change in identity compared with wild type. It retained a VL1 identity and migrated to the correct location, making appropriate initial intermuscular attachments in most segments (Supplemental Figure S2, C and D). However, these same VL1 muscles detached from each other in *vg^null; rhea¹* double mutants. It would seem that *vg* is required to make a subset of muscles competent to establish intermuscular attachments. However, the cell-intrinsic role of Vg must be paired with a differential response to cell–cell communication. In cultured fibroblasts, epidermal growth factor receptor (EGFR) signaling was shown to play a role in the establishment of mature focal adhesions (Ridley and Hall, 1992). Knocking down EGFR signaling induces fast turnover of focal adhesions and produced small focal adhesions (FAs), suggesting EGFR is involved in focal adhesion stabilization (Winograd-Katz *et al.*, 2009). FAs are integrin-mediated structures closely related to the myotendinous junctions formed by skeletal muscle cells (Turner *et al.*, 1991). Although the establishment of muscle–muscle attachments is a complex process and the mechanism behind this process is not clear (Delon and Brown, 2007; Harburger and Calderwood, 2009). We observed that the relative level Vg activity directly affected the number and size of the intermuscular adhesion sites induced by ectopic λ-egfr (Figure 8). Thus, Vg might be responding to external signaling to induce as yet uncharacterized muscle-specific genes that regulate turnover of intermuscular attachment and its stabilization. Alternatively, Vg may induce expression of genes that are required for specific morphological changes in a subset of migrating muscles such as filopodia at the leading edge (Figure 9), which may be required for making initial contacts with neighboring cells to determine whether a muscle–muscle junction is to be formed.

The later stages of muscle migration and attachment are remarkably similar in both *Drosophila* and vertebrates (Schnorrer and Dickson, 2004). Our finding of a role for Vg in embryo SM development seems to make *Drosophila* Vg more analogous to the related Vestigial-like (Vgl) proteins in mammals (Maeda *et al.*, 2002; Chen *et al.*, 2004). Among them, Vestigial-like 2 (*Vgl-2*) is expressed in skeletal muscle and is able to augment myoD-induced myosin heavy chain expression in 10T1/2 cells (Maeda *et al.*, 2002). In addition to the known roles in adult wing and flight muscle development, our results reveal a novel cell-autonomous role for Vg in somatic muscle development. Two muscle cells expressing Vg communicate via DER signaling to coordinate production of intermuscular attachments.

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