Group I PAKs function downstream of Rac to promote podosome invasion during myoblast fusion in vivo

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The p21-activated kinases (PAKs) play essential roles in diverse cellular processes and are required for cell proliferation, apoptosis, polarity establishment, migration, and cell shape changes. Here, we have identified a novel function for the group I PAKs in cell–cell fusion. We show that the two Drosophila group I PAKs, DPak3 and DPak1, have partially redundant functions in myoblast fusion in vivo, with DPak3 playing a major role. DPak3 is enriched at the site of fusion colocalizing with the F-actin focus within a podosome-like structure (PLS), and promotes actin filament assembly during PLS invasion. Although the small GTPase Rac is involved in DPak3 activation and recruitment to the PLS, the kinase activity of DPak3 is required for effective PLS invasion. We propose a model whereby group I PAKs act downstream of Rac to organize the actin filaments within the PLS into a dense focus, which in turn promotes PLS invasion and fusion pore initiation during myoblast fusion.

Introduction

Myoblast fusion, the process in which mononucleated myoblasts fuse to form multinucleated muscle fibers, is essential for skeletal muscle development, maintenance, and satellite cell–based muscle regeneration and repair. Recent studies in the fruit fly Drosophila have led to the identification of evolutionarily conserved signaling pathways required for myoblast fusion (Rochlin et al., 2010; Abmayr and Pavlath, 2012). The high degree of molecular conservation between flies and vertebrates makes the relatively simple and genetically tractable Drosophila system particularly relevant to understanding the general mechanisms underlying myoblast fusion in vivo.

Myoblast fusion in Drosophila embryos commences from the recognition and adhesion between two types of muscle cells, muscle founder cells and fusion-competent myoblasts (FCMs), mediated by cell type–specific immunoglobulin (Ig) domain–containing cell adhesion molecules (Abmayr et al., 2003; Chen and Olson, 2004). The heterophilic interaction between these cell adhesion molecules, Dumbfounded (Duf)/Kirre and Roughtest in muscle founder cells (Ruiz-Gómez et al., 2000; Strümpfelberg et al., 2001) and Sticks and stones (Sns) and Hibris in FCMs (Bour et al., 2000; Artero et al., 2001; Dvorak et al., 2001; Galletta et al., 2004; Shelton et al., 2009), triggers intracellular signaling pathways leading to the formation of an asymmetric fusogenic synapse, which is comprised of these cell adhesion molecules and morphologically distinct actin-enriched structures on either side of the adherent cell membranes (Chen, 2011; Abmayr and Pavlath, 2012). In founder cells, the fusion signal from Duf and Rst is transduced to Scar/WAVE, an actin nucleation-promoting factor (NPF) for the Arp2/3 complex (Richardson et al., 2007), resulting in the formation of a thin sheath of F-actin underlying the founder cell membrane (Sens et al., 2010). In FCMs, the fusion signal from Sns and Hbs is transduced through independent signaling pathways to two Arp2/3 NPFs, WASP (Schäfer et al., 2007) and Scar. In particular, WASP is recruited to the site of fusion (Schäfer et al., 2007; Jin et al., 2011) via its binding partner Solitary (Sltr)/WASP-interacting protein (Kim et al., 2007; Massarwa et al., 2007), whereas Scar is thought to be recruited by the small GTPase Rac (Hakeda-Suzuki et al., 2002; Gildor et al., 2009), which is...
Figure 1. DPak3 and DPak1 have partially redundant functions in myoblast fusion and are enriched at sites of fusion. (A) Myoblast fusion is defective in dpak3 and dpak1 mutant embryos. Stage 15 wild-type (a, wt), Df(3R)Exel7330 (b), dpak3mut (c), dpak3mat/zyg (d), dpak1mat/zyg,dpak3mat/zyg (e), dpak1mat,dpak3mat/zyg (f), and dpak1mat/zyg,dpak3mat (g) embryos labeled with a myosin heavy chain antibody (α-MHC; green). Arrowheads indicate...

(B) Bar graph showing the number of nuclei in DA1 muscle.

(C) Immunofluorescence images of twi-GAL4::UAS-V5-DPak3 and rP298-GAL4::UAS-V5-DPak3.

(D) Bar graph showing the number of nuclei in DA1 muscle.

(E) Immunofluorescence images of phalloidin, α-Dpak3, α-Duf, and merge.
activated by the bipartite guanine nucleotide exchange factor (GEF), the Myoblast city–Elmo complex (Rushton et al., 1995; Erickson et al., 1997; Geisbrecht et al., 2008; Haralalka et al., 2011). Coordinated actions of WASP and Scar at the site of fusion promote actin polymerization, leading to the formation of an F-actin–enriched focus (Kesper et al., 2007; Kim et al., 2007; Richardson et al., 2007). Electron microscopy (EM) and cell type–specific GFP-actin expression experiments have unambiguously pinpointed the F-actin focus to the FCM and demonstrated that it is an integral part of an invasive podosome-like structure (PLS; Sens et al., 2010). The PLS dynamically invades the apposing founder cell/myocyte with multiple finger-like protrusions to promote fusion pore formation/initiation (Sens et al., 2010; Jin et al., 2011). Consistent with the observed PLS invasion in intact embryos, FCM-associated protrusions into founder cells/myotubes have also been described in cultured Drosophila primary myoblasts (Haralalka et al., 2011).

The p21-activated kinases (PAKs) comprise a family of Ser/Thr kinases conserved from yeast to human. PAKs have been shown to control cell proliferation, apoptosis, gene transcription, and cytoskeletal reorganization in various cellular processes including cell adhesion, migration, invasion, and shape changes (Bokoch, 2003; Arias-Romero and Chernoff, 2008; Eswaran et al., 2008). In mammals, there are six mammalian PAKs that belong to two groups, group I (PAK1-3) and group II (PAK4-6), based on their domain architecture and regulatory mechanisms. Both group I and II PAKs contain a C-terminal catalytic domain and an N-terminal p21-binding domain (PBD) that mediates Rac–Cdc42 interaction. In addition, group I PAKs have an N-terminal auto-inhibitory domain (AID) that partially overlaps with the PBD. Biochemical and structural studies have shown that group I PAKs form auto-inhibited homodimers, in which the AID of one PAK molecule interacts in trans with the kinase domain of a second (Parrini et al., 2002). Binding of the GTP-bound, activated Rac/Cdc42 to the PBD/AID domain of a group I PAK releases the auto-inhibition, leading to an intermediary active dimer that is trans-autophosphorylated on a Thr residue in the kinase domain and several Ser residues in the N-terminal domain (Benner et al., 1995; Thompson et al., 1998; Zhao et al., 1998; Gatti et al., 1999; Lei et al., 2000; Morreale et al., 2000; Chong et al., 2001; Pirruccello et al., 2006). Upon substrate binding, the PAK homodimer dissociates into monomers, which in turn phosphorylate downstream targets (Pirruccello et al., 2006).

Compared with mammals, the relatively simple Drosophila genome only encodes three PAKs. Two of them, DPAk1 (Harden et al., 1996) and DPAk3 (Mentzel and Raabe, 2005), are homologous to the mammalian group I PAKs and, in particular, PAK1 and PAK2, respectively. The third Drosophila PAK, Mushroom body tiny (Mbt) is a group II PAK (Schneebberger and Raabe, 2003). Although Mbt is specifically required in the nervous system, the group I DPaks have been implicated in multiple developmental and cellular processes. DPAk1 has been shown to regulate dorsal closure (Conder et al., 2004), cell polarity in the ovarian follicle epithelium (Conder et al., 2007), axon and myocyte guidance (Hing et al., 1999; Bahri et al., 2009), and maturation of postsynaptic terminals (Albin and Davis, 2004). The less studied DPAk3 has been shown to have a redundant function with DPAk1 in dorsal closure (Bahri et al., 2010) and to regulate synaptic morphology and function (Ozdowski et al., 2011).

In this study, we have identified a novel function for the Drosophila group I PAKs, DPAk3 and DPAk1, in myoblast fusion. These two PAKs are partially redundant during myoblast fusion, with DPAk3 playing a more significant role than DPAk1. We show that DPAk3 colocalizes with the F-actin focus within the PLS and that DPAk3 recruitment to the PLS is controlled by the small GTPase Rac. Moreover, the kinase activity of DPAk3 is required for PLS invasion. We propose that group I PAKs regulate actin filament assembly within the PLS to promote PLS invasion and fusion pore formation.

Results

DPAk3 is required for myoblast fusion and functions specifically in FCMs

In a deficiency screen for fusion-defective mutants, we identified a third chromosome deficiency line, Df(3R)Exel7330. Homozygous Df(3R)Exel7330 embryos contained many unfused myoblasts, as revealed by anti-myosin heavy chain (MHC) staining, indicating a fusion defect (Fig. 1A B). Of the 18 genes deleted by Df(3R)Exel7330, dpaK3 was identified as the sole candidate involved in myoblast fusion by dsRNA injection experiments in embryos. To confirm this, we generated a zygotic null allele of dpaK3 (dpaK3<sup>−/−</sup>) by deleting the entire coding sequence of DPAk3 on October 15, 2012 jcb.rupress.org Downloaded from
Despite the fusion defect, muscle cell fate specification, FCM migration, and muscle cell adhesion appeared normal in dpak3\textsuperscript{m/zyg} mutant embryos (Fig. S1), indicating that the fusion phenotype is not a secondary consequence of defects in the early steps of myogenesis. The fusion defect in dpak3\textsuperscript{m/zyg} mutant embryos could be rescued by expressing a V5-tagged DPak3 (V5-Dpak3) with the twi-GAL4 driver in mesodermal cells (Fig. 1, Ca and D; Table S1), confirming that DPak3 is required for myoblast fusion.

To determine which population of muscle cells requires DPak3 function, we performed cell type–specific transgenic rescue experiments. Although V5-Dpak3 expression driven by the founder cell–specific driver rP298-GAL4 failed to rescue the fusion defect in dpak3\textsuperscript{m/zyg} mutant embryos (5.8 ± 1.8 nuclei in DA1, n = 40; Fig. 1, Cb and D; Table S1), DPak3 expression in FCMs driven by sns-GAL4 completely rescued the fusion defect (Fig. 1, Cc and D; Table S1). Thus, DPak3 functions in the FCMs, and most likely not in founder cells, during myoblast fusion.

**DPak3 colocalizes with the F-actin focus within the PLS**

To understand the cellular function of DPak3 in myoblast fusion, we examined the localization of DPak3 in muscle cells. In situ hybridization revealed that dpak3 mRNA was expressed in a broad domain in the embryo including the mesoderm (Fig. S2). To detect the DPak3 protein, we generated an anti-DPak3 antibody, which showed specificity toward DPak3 (Fig. S3). Wild-type but not dpak3\textsuperscript{m/zyg} mutant embryos labeled with anti-DPak3 antibody revealed punctate foci that colocalized with the F-actin foci at sites of fusion (Fig. 1, Ea and b). The localization of DPak3 to the FCM-specific F-actin foci is consistent with its specific function in FCMs, and suggests a potential role of DPak3 in regulating the PLS during myoblast fusion.

**DPak1 partially compensates for DPak3 function in myoblast fusion**

The partial fusion defect in dpak3\textsuperscript{m/zyg} mutant embryos prompted us to ask whether the dpak3 mRNA and/or protein are maternally contributed. To test this possibility, we eliminated both maternal and zygotic functions of dpak3 by generating germline clones of dpak3\textsuperscript{m/zyg}. However, dpak3\textsuperscript{m/zyg} mutant embryos only showed a slight exacerbation of the fusion defect compared with dpak3\textsuperscript{m/zyg}, with 4.6 ± 1.5 nuclei (n = 37) in the DA1 muscle (Fig. 1, Ad and B; Table S1), indicating that there is no significant maternal contribution of dpak3 in the embryo.

We then asked whether the two group I PAKs in *Drosophila*, DPak3 and DPak1, have redundant functions during myoblast fusion. Although previous studies showed that the somatic musculature appeared normal in dpak1 mutant embryos (Bahri et al., 2009), we hypothesized that DPak1 may compensate for DPak3 function in the absence of the latter. To test this possibility, we made double mutants of dpak1 and dpak3. Although double zygotic mutant (dpak1\textsuperscript{m/zyg},dpak3\textsuperscript{m/zyg}) showed a similar myoblast fusion defect to that of dpak3\textsuperscript{m/zyg}, double maternal/zygotic mutant (dpak1\textsuperscript{m/zyg},dpak3\textsuperscript{m/zyg}) showed a complete lack-of-fusion phenotype (1.0 ± 0.0 nucleus in DA1, n = 11; Fig. 1, Ae and B; Table S1). Moreover, we found that the severity of the fusion defect is dependent on the amount of residual PAK proteins in the embryo. Specifically, eliminating only the maternal function of DPak1 in dpak3\textsuperscript{m/zyg} mutant (dpak1\textsuperscript{m/zyg},dpak3\textsuperscript{m/zyg}) resulted in an intermediate fusion defect (2.3 ± 0.8 nuclei in DA1, n = 42; Fig. 1, Af and B; Table S1), and eliminating only the maternal function of DPak3 in dpak1\textsuperscript{m/zyg} mutant (dpak1\textsuperscript{m/zyg},dpak3\textsuperscript{m/zyg}) resulted in a minor fusion defect (10.0 ± 1.8 nuclei in DA1, n = 55; Fig. 1, Ag and B; Table S1). These results demonstrate that DPak3 and DPak1 play redundant roles during myoblast fusion and that DPak3 has a more significant function than DPak1.

To further explore the functional compensation of DPak1 for DPak3 at the cellular level, we performed antibody labeling experiments with anti-DPak1. In wild-type embryos, DPak1 enrichment was undetectable at sites of fusion marked by the F-actin foci and founder cell–specific cell adhesion protein Duf (Fig. 1 Ec). However, in dpak3\textsuperscript{m/zyg} mutant embryos, large aggregates of DPak1 colocalized with the F-actin foci at muscle cell contact sites (Fig. 1 Ed), suggesting that DPak1 is actively recruited to these sites to promote fusion in compensation for the loss of DPak3. In addition, overexpression of DPak1 in dpak3\textsuperscript{m/zyg} mutant embryos with the mesodermal nvi-GAL4 driver resulted in a slight, but significant, phenotypic rescue (6.6 ± 1.2 nuclei in DA1, n = 59; Fig. 1, Cd and D; Table S1). Thus, DPak1 appears to compensate for the loss of DPak3 by functioning in the F-actin foci at sites of fusion.

**DPak3 genetically interacts with the Arp2/3 NPFs, WASP and Scar**

Previous studies have demonstrated that formation of the F-actin focus within the PLS requires the coordinated functions of two Arp2/3 NPFs, WASP and Scar (Sens et al., 2010). The enrichment of DPak3 and DPak1 in dpak3\textsuperscript{m/zyg} mutant embryos prompted us to ask whether DPak3 genetically interacts with WASP and Scar. Due to maternal contribution, the zygotic-null mutant of wasp does not show a fusion defect, whereas that of scar exhibits a partial loss-of-fusion phenotype (5.6 ± 1.9 nuclei in DA1, n = 36; Kim et al., 2007; Richardson et al., 2007). However, the double mutant between dpak3\textsuperscript{m/zyg} and wasp (dpak3\textsuperscript{m/zyg},wasp) showed a more severe fusion defect (1.9 ± 0.7 nuclei in DA1, n = 59; Fig. 2 A, compare a and b; Table S1) than either dpak3\textsuperscript{m/zyg} or wasp single mutant. Similarly, the double mutant between dpak3\textsuperscript{m/zyg} and scar (scar;dpak3) also showed an enhanced fusion defect (1.0 ± 0.0 nuclei in DA1, n = 40; Fig. 2 A, compare c and d; Table S1). The genetic interactions between DPak3 and the Arp2/3 NPFs strongly suggest a functional link between DPak3 and the F-actin foci at sites of fusion.

**The FCM-specific F-actin foci exhibit a dispersed morphology in dpak mutants**

To pinpoint the function of DPak3 in the F-actin foci, we analyzed the foci phenotype in dpak3\textsuperscript{m/zyg} mutant embryos. In wild-type embryos, myoblast fusion peaks at stage 14 (with ~10–12 F-actin foci per hemisegment) and is largely completed by early stage 15, indicated by a significant decrease in the foci number.
exclusively in FCMs because GFP-actin expressed in FCMs was incorporated into the F-actin foci (Fig. 2 Ca), whereas GFP-actin expressed in founder cells was not (Fig. 2 Cb). Strikingly, the majority of the FCM-specific F-actin foci in late stage 14 dpak3zyg (Fig. 3 Ab) and dpak3mat/zyg (Fig. 3 Ac) mutant embryos displayed a more dispersed morphology than wild type (Fig. 3 Aa). In wild-type embryos, the F-actin foci exhibited a dense, solid morphology with an average fluorescence and the appearance of multinucleated muscle fibers. However, in the late stage 14 dpak3zyg mutant embryos, a large number of F-actin foci (~30 foci per hemisegment) remained, indicating a failure of these foci to promote myoblast fusion (Fig. 2 B, compare a and b).

We next examined the morphology of individual F-actin foci in dpak3zyg mutant embryos. As in wild-type embryos, the F-actin foci in dpak3zyg mutant embryos were generated exclusively in FCMs because GFP-actin expressed in FCMs was incorporated into the F-actin foci (Fig. 2 Ca), whereas GFP-actin expressed in founder cells was not (Fig. 2 Cb). Strikingly, the majority of the FCM-specific F-actin foci in late stage 14 dpak3zyg (Fig. 3 Ab) and dpak3mat/zyg (Fig. 3 Ac) mutant embryos displayed a more dispersed morphology than wild type (Fig. 3 Aa). In wild-type embryos, the F-actin foci exhibited a dense, solid morphology with an average fluorescence
Figure 3. Dispersed morphology of the F-actin foci and defective PLS invasion in dpak mutant embryos. (A) Actin foci morphology visualized by confocal microscopy. Stage 14 embryos triple labeled with phalloidin (green), α-Duf (red), and α-Lmd (blue). Compared with the dense morphology in wild-type embryos (a, wt), the F-actin foci appeared fuzzy and dispersed in dpak3zyg (b), dpak3mat/zyg (c), and dpak1mat/zyg,dpak3mat/zyg (d) mutant embryos. Note that the dense wt actin focus caused a V-shaped inward curvature in the founder cell membrane (a, arrow). In contrast, the F-actin foci in dpak mutant embryos appeared dispersed and did not change the membrane curvature of the apposing founder cells (b–d, arrowheads). (B) F-actin foci visualized by EM. (a) Stage 14 wt embryo. An FCM (pseudo-colored pink) projecting multiple F-actin–enriched invasive fingers (the longest one indicated by arrowhead) into the adjacent trinucleated myotube. The protruding tip of this FCM is enlarged in (a”). Note that the F-actin–enriched area (delineated with white dashed line in a”) is almost devoid of ribosomes (small black dots) and intracellular organelles, indicating the presence of a densely packed F-actin network (also see Sens et al., 2010). (b) Stage 14 dpak3zyg mutant embryo. An FCM was in the process of invading a binucleated myotube and generated a wide, shallow dent on the myotube membrane, without projecting long, thin protrusive fingers. The tip area of the FCM is enlarged in (b”). Note that the F-actin–enriched area (delineated with white dashed line in b”) contained more ribosomes than that of wt (a”), indicating the presence of loosely organized actin filaments. Also note that the cell membranes remained intact at the muscle cell contact site. n: muscle cell nuclei. Bars: (A) 5 µm; (B) 500 nm.
intensity of 167.3 ± 15.0/focus (n = 28) in a 0–255 scale measured by the LSM software. However, the F-actin foci in late stage 14 dpak3mut embryos had an average intensity of 80.3 ± 22.9/focus (n = 43). Corresponding to the decreased intensity, the average size of the F-actin foci in dpak3mut mutant embryos (3.9 ± 2.1 µm², n = 69) was significantly larger than that of the wild-type foci (1.7 ± 0.4 µm², n = 21). Moreover, in dpak1mut, dpak3mut double mutant embryos, the F-actin enrichment at muscle cell contact sites became even more dispersed, such that they appeared as wide “thickenings,” rather than condensed foci, along the muscle cell contact zones (Fig. 3 Ad). The dispersed morphology of the F-actin foci was confirmed by EM analysis. In wild-type embryos, the area of F-actin enrichment was restricted to the protrusive tip of the FCM (Fig. 3 Ba). However, the F-actin–enriched area in the unfused FCMs of dpak3mut mutant embryos extended farther back into the cytoplasm (Fig. 3 Bb). In addition, unlike the F-actin–enriched areas in wild-type FCMs where the ribosomes were mostly excluded (Fig. 3 Ba’), these areas in dpak3mut mutant FCMs were decorated with an increased number of ribosomes (Fig. 3 Bb’), indicating the presence of loosely packed actin filaments. Consistent with the dispersed F-actin foci morphology observed in fixed embryos, live imaging of dpak3mut mutant embryos expressing GFP-actin with twi-GAL4 revealed large and fuzzy mutant F-actin foci that underwent dynamic shape changes (Fig. 4 Ab; Video 2). Unlike the wild-type F-actin foci that maintained their dense morphology throughout their lifespan (Fig. 4 Aa; Video 1), each late stage 14 dpak3mut mutant focus appeared dispersed and disorganized, without displaying an obvious “dense core,” and frequently contained clearly discernable projections and comet tail–like structures (Fig. 4 Ab). Taken together, these analyses suggest that the actin filaments within the PLS are loosely organized in dpak3mut mutant embryos.

The F-actin foci fail to invade founder cells or promote fusion pore formation in dpak3 mutant embryos

The abnormal morphology of the F-actin foci in dpak3 mutants prompted us to ask whether the mutant PLSs can invade founder cells as their wild-type counterparts. In stage 14 wild-type embryos, ~35% of the F-actin foci at a given time point appear invasive as they generate inward curvatures in the apposing founder cell/myotube membranes (Fig. 3 Aa; Sens et al., 2010). However, in late stage 14 dpak3mut, dpak3mut, and dpak1mut, dpak3mut mutant embryos, most of the F-actin foci were not associated with any inward curvature in the apposing founder cell/myotube membranes (Fig. 3 A, b–d). In the 10.5% (4/38) of the F-actin foci that appeared slightly invasive in dpak3mut mutant embryos, the maximum depth of invasion was 0.6 µm, compared with 1.9 µm of the wild-type foci (Sens et al., 2010). The defective PLS invasion in dpak3mut mutant embryos was also confirmed by EM analysis (Fig. 3, Bb and b’).

To test whether the defective PLS in dpak3mut mutant was capable of promoting fusion pore formation, we performed a GFP diffusion assay. As shown in Fig. 4 B, founder cell–expressed GFP (with rP298-GAL4) did not diffuse into the attached, mononucleated FCMs in stage 15 dpak3mut mutant embryos, suggesting that myoblast fusion is blocked before fusion pore formation and that DPak3 is required for fusion pore initiation. In addition, EM analysis also confirmed the absence of membrane openings abutting the dispersed F-actin foci in dpak3mut mutant embryos (Fig. 3, Bb and b’). These results strongly support our model that proper PLS invasion is required for fusion pore formation (Sens et al., 2010), and demonstrate a specific role for the group I PAKs in PLS invasion and fusion pore formation.

DPak3 functions downstream of Rac during myoblast fusion

To position Dpak3 in the signaling pathways controlling myoblast fusion, we first examined the localization of the known fusion proteins in dpak3mut mutant embryos. In FCMs, the fusion signal is transduced from the cell adhesion molecule, Sns, to the actin cytoskeleton via two independent pathways: Sns→Sltr−WASP and Sns→Rac→the Scar complex. Antibody labeling experiments showed that Sns, Sltr, and Rac remained localized with the F-actin foci at muscle cell contact sites in dpak3mut mutant embryos (Fig. 5 A), suggesting that Dpak3 does not function upstream of these proteins to control their localization. Conversely, we examined the localization of Dpak3 in several known fusion mutants. Not surprisingly, Dpak3’s punctate localization pattern was absent in sns mutant embryos (Fig. 5 Ba), consistent with the role of Sns in initiating the myoblast fusion signaling pathways in the FCM. Dpak3 remained localized to the F-actin foci in sltr and kette (encoding a component of the Scar complex; Schröter et al., 2004) mutant embryos (Fig. 5, Bb and b), suggesting that neither the Sltr–WASP nor the Scar complex is required for recruiting Dpak3 to the PLS. However, the Dpak3 localization to muscle cell contact sites was absent in rac1,rac2 double mutant embryos (Fig. 5 Bd), indicating that the small GTPase Rac is involved in localizing Dpak3 to the PLS. Taken together, these results suggest that Dpak3 functions downstream of the Sns→Rac pathway but in parallel with the Scar complex.

Rac binding is required for Dpak3’s subcellular localization and function in vivo

Previous biochemical and structural studies have revealed a function for the small GTPases Cdc42/Rac in activating group I PAKs’ kinase activity by binding to PAKs and releasing their transautophosphorylation (Bokoch, 2003; Arias-Romero and Chernoff, 2008; Eswaran et al., 2008). Indeed, activated Rac (RacG12V) stimulated the auto-phosphorylation and kinase activity of DPAK3 expressed in Drosophila S2R+ cells (Fig. 6, A–C). In addition to its ability to activate DPAK3, Rac may also regulate the subcellular localization of DPAK3 because DPAK3 failed to localize to muscle cell contact sites in rac1,rac2 mutant embryos (Fig. 5 Bd). Consistent with this possibility, DPAK3 colocalized with Rac and the F-actin foci at sites of fusion in wild-type embryos (Fig. 5 C). To further test whether Rac binding is directly involved in regulating DPAK3 localization, we took advantage of the DPAK3H29,31L mutant, which carried two His-to-Leu point mutations in its PBD/AID domain and failed to bind Rac in vitro (Fig. 6 D; Mentzel and Raabe, 2005). Although DPAK3H29,31L was defective in Rac binding, it was autophosphorylated when expressed in S2R+ cells (Fig. 6 B).
and had constitutive kinase activity toward an exogenous substrate in vitro (Fig. 6 C; Mentzel and Raabe, 2005). We generated a transgenic line carrying V5-DPak3H29,31L and tested its ability to rescue the dpak3zyg mutant phenotype. V5-DPak3H29,31L, whose expression in wild-type embryos did not cause any fusion defect, failed to rescue the fusion defect in dpak3zyg mutant embryos (Fig. 5 D) despite its constitutive kinase activity (Fig. 6 C). Unlike V5-DPak3 that was enriched to the F-actin foci (Fig. 5 Ea), V5-DPak3H29,31L was evenly distributed in the cytoplasm of muscle cells without aggregating to the F-actin foci in either wild-type or dpak3zyg and had constitutive kinase activity toward an exogenous substrate in vitro (Fig. 6 C; Mentzel and Raabe, 2005). We generated a transgenic line carrying V5-DPak3H29,31L and tested its ability to rescue the dpak3zyg mutant phenotype. V5-DPak3H29,31L, whose expression in wild-type embryos did not cause any fusion defect, failed to rescue the fusion defect in dpak3zyg mutant embryos (Fig. 5 D) despite its constitutive kinase activity (Fig. 6 C). Unlike V5-DPak3 that was enriched to the F-actin foci (Fig. 5 Ea), V5-DPak3H29,31L was evenly distributed in the cytoplasm of muscle cells without aggregating to the F-actin foci in either wild-type or dpak3zyg
To address this question, we first examined the presence (or absence) of phosphorylated DPak3 in stage 14 embryos in which myoblast fusion is at its peak. As shown in Fig. 6 E, V5-DPak3 expressed with twi-GAL4 in stage 14 embryos was phosphorylated, indicated by an up-shifted band on Western blot, indicating that the DPak3 kinase is activated in the mesodermal cells. Although the phosphorylation status of DPak3 within the PLS could not be assessed directly by immunohistochemistry due to the mutant embryos (Fig. 5, Eb and c). Thus, Rac binding is directly involved in DPak3 recruitment to the PLS, and the proper subcellular localization of DPak3 is critical for its function in vivo.

The kinase activity of Dpak3 is required for PLS invasion

Although the kinase activity of Dpak3 is not sufficient for its function in myoblast fusion, we asked whether it is required.
Figure 6. Biochemical characterizations of DPak3. (A) DPak3 is phosphorylated in S2R+ cells. FLAG-DPak3 was expressed in S2R+ cells and immunoprecipitated with α-FLAG, followed by CIP treatment. Note the disappearance of the up-shifted band of DPak3 (red arrowhead; lane 1) after CIP treatment (black arrowhead; lane 2), indicating that the up-shifted band was caused by phosphorylation. (B) Constitutively active Rac further activates DPak3 phosphorylation in S2R+ cells. DPak3 phosphorylation was further increased by coexpressing with activated Rac1 (Rac1G12V; red arrowhead; lane 1). The Rac-binding defective mutant DPak3H29,31L remained partially phosphorylated (lane 2), whereas the kinase-inactive mutant was unphosphorylated (black arrowhead; lane 3). (C) In vitro kinase assays for wild-type and mutant DPak3. The kinase activities of DPak3 and its mutant forms were assessed by their ability to phosphorylate myelin basic protein (MBP) in the presence of γ-[32P]ATP. Expression of MBP and different DPak3 proteins was detected by Coomassie brilliant blue staining. The kinase activities were normalized against protein expression levels and compared with that of V5-DPak3. Results from three independent experiments were quantified. Note the great increase in the DPak3 kinase activity when it was co-expressed with activated Rac1 (lane 3), the complete loss of kinase activity resulting from the K322A mutation (lane 4), and the constitutive kinase activity of DPak3H29,31L (lane 5). Error bars: standard deviations. (D) DPak3, but not DPak3H29,31L, interacts with activated Rac1 (Rac1G12V) expressed in S2R+ cells (compare lanes 2 and 3). (E) DPak3 is phosphorylated in stage 14 Drosophila embryos. Extracts prepared from stage 14 embryos expressing V5-DPak3 in the mesoderm with twi-GAL4 were subjected to SDS-PAGE (lane 2), together with an extract of S2R+ cells expressing V5-DPak3 as a control (lane 1). Note the similarly up-shifted bands in both lanes (red arrowhead), indicating phosphorylation of V5-DPak3 in vivo. Percentage of the polyacrylamide gels: 6% in A, B, and E; 15% in C; and 12% in D.

unavailability of a phospho-specific antibody against DPak3, we took advantage of a kinase-inactive mutant of DPak3 to examine the requirement of its kinase activity during myoblast fusion. Previous biochemical experiments revealed that a Lys-to-Ala substitution in the kinase domain of DPak3 (DPak3K322A) abolishes ATP binding and renders the kinase inactive without affecting Rac binding (Mentzel and Raabe, 2005). Consistent with this, DPak3K322A was unphosphorylated when expressed in S2R+ cells (Fig. 6 B) and had no kinase activity toward an exogenous substrate (Fig. 6 C). We performed a transgenic rescue experiment by expressing DPak3K322A in the mesoderm of dpak3zyg mutant embryos. Overexpressing an N-terminally tagged DPak3 K322A (V5-DPak3K322A) at 25°C, which did not cause any muscle phenotype in wild-type embryos, failed to rescue the fusion defect in dpak3-208 mutant embryos (5.4 ± 1.3 nuclei in DA1, n = 46; Fig. 7, Ab and B; Table S1). Strikingly, V5-DPak3K322A properly localized to the PLS, but failed to rescue PLS invasion into founder cells/myotubes (Fig. 7 Ca). Only 9.7% (6/62) of the F-actin foci appeared slightly invasive in these embryos, and the maximum depth of invasion was 0.5 μm. Taken together, these results demonstrate that the kinase activity of DPak3 is required for promoting PLS invasion during myoblast fusion.

Previous studies have shown that the kinase-inactive form of mammalian PAK1 behaves in a dominant-negative manner to compete with endogenous PAK1 for substrate binding by forming nonproductive kinase–substrate complexes (Morita et al., 2007; Tang et al., 1997). Interestingly, overexpressing the N-terminally tagged V5-DPak3K322A at 30°C resulted in mutant kinase accumulation in the PLS (Fig. 7 Cb) and an enhancement of the fusion defect (3.6 ± 1.3 nuclei in DA1, n = 60; Fig. 7, Ac and B; Table S1). Moreover, overexpressing a C-terminally tagged DPak3 K322A (DPak3K322A-V5) at 25°C resulted in a near complete block of myoblast fusion (1.4 ± 0.7 nuclei in DA1, n = 34; Fig. 7, Ad and B; Table S1), accompanied by an even higher accumulation of the mutant kinase in the PLS (Fig. 7 Cc).

In these embryos, the F-actin foci appeared more dispersed than those in dpak3-208 mutant embryos, with an average fluorescence intensity of 66.7 ± 22.4/focus (n = 60; Fig. 7 Cc). Correspondingly, less...
Discussion

Group I PAKs have partially redundant functions in myoblast fusion

The PAK family of Ser/Thr kinases have been implicated in many biological processes, including cell migration, invasion, proliferation, and survival, as well as regulation of neuronal outgrowth, hormone signaling, and gene transcription (Bokoch, 2003; Arias-Romero and Chernoff, 2008; Eswaran et al., 2008).
However, a role for PAKs in muscle development and cell–cell fusion has not been previously uncovered. In this study, we reveal an essential function for Drosophila group I PAKs during myoblast fusion in vivo (Fig. 8A). Specifically, we show that the two group I PAKs in Drosophila, DPak3 (a close homologue of mammalian PAK2) and DPak1 (a close homologue of mammalian PAK1), have partially redundant functions in myoblast fusion, based on the following lines of evidence. First, double and single mutants of dpak3 and dpak1 exhibited a range of fusion defects (dpak1mat/zyg, dpak3mat/zyg > dpak1mat/+, dpak1mat/zyg, dpak3mat/zyg > dpak3mat/+, dpak1mat/+, dpak1mat/zyg > dpak1mat/zyg = dpak1mat/+, dpak3mat/zyg > dpak1mat/+, dpak1mat/zyg > dpak1mat/+, dpak3mat/zyg), dependent on the residual endogenous protein level. Clearly, DPak3 plays a more significant role than DPak1, and the minor role of DPak1 can only be revealed in the context of the dpak1, dpak3 double mutant. Second, DPak3 is enriched in the F-actin foci in wild-type embryos. On the other hand, DPak1 only accumulates in the F-actin foci in the absence of DPak3, consistent with its compensatory function in the fusion process. Third, overexpression of DPak1 in the dpak3zyg mutant leads to a slight but reproducible rescue of fusion. Finally, overexpression of a kinase-inactive form of DPak3 (DPak3K322A) in dpak3zyg mutant embryos significantly enhances the fusion defect, presumably by forming nonproductive DPak3K322A–substrate complexes that exclude DPak1.

What accounts for the differential effects of DPak3 and DPak1 in myoblast fusion? One possibility is that DPak3 is recruited to the PLS at a higher level than DPak1 in wild-type embryos. However, the different recruitment levels cannot solely account for the differential effects of these two proteins because DPak1 overexpression in dpak3zyg mutant embryos does not DEM [image of Figure 8A and B].

Figure 8. Models describing the proposed function of group I PAKs in myoblast fusion. (A) Group I PAKs act directly downstream of the small GTPase Rac, and in parallel with the Scar and WASP complexes, to promote myoblast fusion. DPak3 and DPak1 are partially redundant, with DPak3 playing a more significant role, in the fusion process. PAKs do not appear to affect actin polymerization or depolymerization, but may regulate actin bundling/cross-linking proteins during myoblast fusion. (B) Group I PAKs regulate podosome invasion during myoblast fusion. In wild-type embryos, PAKs organize the Arp2/3-nucleated, branched actin filaments within the PLS into a densely packed structure devoid of ribosomes, which, in turn, efficiently invades the apposing founder cell/myotube with multiple finger-like protrusions and ultimately leads to fusion pore formation. PAKs may do so by activating actin bundling/cross-linking proteins (A), allowing the formation of highly stiff actin bundles that exert large protrusive forces against the cell membrane. In dpak mutant embryos, the actin filaments are disorganized and dispersed (the actin-enriched area decorated with ribosomes), resulting in a failure in PLS invasion and fusion pore formation.
completely rescue the fusion defect. A second possibility is that DPak3 and DPak1 may have different interacting partner(s) in the PLS, and thus may respond differently to upstream Rac signaling and/or transduce different downstream signals. In this regard, it has been reported that human PAK2, but not PAK1, can interact with MYO18A, which is involved in actin filament organization and cell migration (Hsu et al., 2010). A third possibility is that these two kinases may have intrinsic differences in substrate binding affinity and/or kinase activity. For example, DPak3 may preferentially bind and activate specific substrates in wild-type embryos and DPak1 could only access and/or inefficiently activate these substrates in the absence of DPak3. In support of this hypothesis, expressing the kinase-inactive form of DPak3 (DPak3K322A) in the dpak3^{f09} mutant abolishes the functional compensation by DPak1, suggesting that DPak3K322A may efficiently compete with DPak1 for substrate binding by forming high-affinity DPak3K322A-substrate complexes. Obviously, identification of the preferred substrates of these group I PAKs in vivo will be required to further test this hypothesis.

The subcellular localization of DPak3 is controlled directly by Rac binding

Previous studies have shown that the activity of group I PAKs is regulated by the small GTPases Rac/Cdc42. The subcellular localization of group I PAKs, on the other hand, is thought to be controlled by SH2-SH3 domain-containing small adaptor proteins Nck and Grb (Bokoch, 2003; Arias-Romero and Chernoff, 2008; Eswaran et al., 2008). Although the expression of a dominant-negative form of Rac resulted in a loss of DPak1 localization at the leading edge during dorsal closure in Drosophila embryos (Harden et al., 1996), it was unclear if Rac directly regulates DPak1 recruitment to the leading edge. Here, we provide evidence that the localization of DPak3 to a specific subcellular structure, the F-actin focus within the PLS, is directly controlled by Rac. First, Rac colocalizes with DPak3 within the F-actin foci during myoblast fusion. Second, DPak3 is no longer localized to the F-actin foci in rac1, rac2 double mutant embryos. Third, DPak3 carrying mutations in the Rac-binding domain (DPak3H29,31L) fails to localize to the F-actin foci or rescue the dpak3^{f09} mutant phenotype, despite its constitutive kinase activity. We note that although the subcellular localization of group II PAKs has been shown to be controlled by Cdc42 in cultured mammalian cells (Abo et al., 1998) and in Drosophila photoreceptor cells (Schneeberger and Raabe, 2003), our study reveals, for the first time, such a localization mechanism for a group I PAK. Moreover, our study has positioned group I PAKs in a new signaling branch downstream of the Rac GTPase during myoblast fusion, in addition to the previously known branch involving the Scar complex (Fig. 8 A).

Group I PAKs regulate podosome invasion

Mammalian group I PAKs have been implicated in regulating podosome formation, size, and number in cultured cells (Webb et al., 2005; Gringel et al., 2006; Morita et al., 2007). However, the function of PAKs in individual podosomes, especially in intact organisms, remained completely unknown. Our current study demonstrates that group I PAKs are required for regulating the invasive behavior of individual podosomes in an intact organism. We show that DPak3 is required specifically in the FCMs and colocalizes with the F-actin foci within the PLS. We also show that in dpak3^{f09}, dpak3^{mat/zyg}, and dpak1^{f09/+/mat}, dpak3^{mat/zyg} mutants, the F-actin foci persisting to late developmental stages appear dispersed and fail to invade into the apposing founder cells/myotubes. As a result, fusion pores fail to form between these defective FCMs and their apposing founder cells/myotubes. Thus, our current study not only strongly supports our model that PLS invasion is required for fusion pore formation, but also reveals, for the first time, that group I PAKs are important regulators of podosome invasion in vivo.

How do group I PAKs regulate PLS invasion? The dispersed morphology of the F-actin foci in dpak3^{f09} and dpak1^{f09/+/mat} dpak3^{mat/zyg} mutants suggests that group I PAKs may be involved in organizing branched actin filaments into a dense focal structure within the PLS. Because the kinase activity of DPak3 is required for its function during myoblast fusion, DPak3 may regulate actin cytoskeletal remodeling by phosphorylating downstream substrates associated with the actin cytoskeleton, such as regulators of actin polymerization, depolymerization, and/or actin filament bundling/cross-linking (Fig. 8 A). Our genetic and immunohistochemical analyses suggest that DPak3 is unlikely to promote actin polymerization via the Arp2/3 NFPS WASP and Scar, because DPak3 functions in parallel with the WASP and Scar complexes and the amount of F-actin in each PLS is not markedly reduced in dpak3^{f09} mutant embryos. In addition, DPak3 is unlikely to suppress actin depolymerization via PAK’s well-characterized substrate, LIM kinase (LIMK), because loss-of-function mutants of LIMK and its substrate, the actin depolymerization factor coflin, did not have a myoblast fusion defect, and DPak3 did not show genetic interactions with LIMK or coflin during myoblast fusion (unpublished data). Therefore, it is conceivable that the group I PAKs may regulate actin bundling and/or cross-linking proteins, which, in turn, organize the assembly of branched actin filaments into tightly packed bundles to promote PLS invasion (Fig. 8 B). In this regard, it has been shown that a tight intermolecular packing of the actin filaments mediated by actin cross-linkers leads to the formation of highly stiff actin bundles that exert large protrusive forces against the cell membrane (Claessens et al., 2006). Future experiments are required to identify the bona fide downstream substrate(s) of DPak3 in regulating PLS invasion during myoblast fusion.

Interestingly, mammalian group I PAKs have been associated with cellular invasion of other cell types, such as cancer cells during metastasis (Vadlamudi and Kumar, 2003; Molli et al., 2009; Whale et al., 2011). Elevated expression and hyperactivity of PAK1 and PAK2 are seen in several types of tumors (Molli et al., 2009). Overexpression of constitutively active PAK1 promotes cancer cell migration and invasion, whereas inhibiting PAK1 suppresses these phenotypes (Adam et al., 2000; Vadlamudi et al., 2000; Stofega et al., 2004; Arias-Romero et al., 2010). It is well known that cancer cell invasion is mediated by invadopodia, which are podosome-like structures with larger F-actin–enriched cores and less dynamic actin polymerization (Weaver, 2006). A role of PAK1 and PAK2 in invadopodia...
formation in an invasive metastatic human melanoma cell line has been revealed (Ayala et al., 2008). Thus, further studies of PAK function in podosome invasion in Drosophila myoblast fusion will not only provide additional insights into muscle differentiation, but also cancer cell invasion during tumorigenesis.

**Materials and methods**

**Fly genetics**

Fly stocks were obtained from the Bloomington Stock Center except for the following: w;1186 Str+;CyO, actin-lacZ (Kim et al., 2007); kett644/5; TM6B (Hummel et al., 2000); scoa+/CyO (Zallen et al., 2002); FRT92 wasp5, e/TM6B (Ben-Yaacov et al., 2001); sn-GAL4/CyO (Paululat et al., 1999); sn-GAL4 (Kocherlakota et al., 2008); rp298-GAL4 (Menon and Chia, 2001); and P(P)sak302627 and P(Bac)(RP)sak302602 (Exelixis Collection, Harvard Medical School, Boston, MA).

A null allele of dpak3, dpak3(0), was generated by deleting the entire dpak3 coding sequence between P(2)Xpap302622 and P(Bac)(RP)sak302629.

Transgenic lines containing (1) UAS-V5-Dpak3; (2) UAS-V5-Dpak320625; (3) UAS-V5-Dpak320628; (4) UAS-Dpak320628; and (5) UAS-V5-Dpak1 were generated by standard F-element-mediated transformation.

Transient rescue crosses were performed by crossing female dpak3(0) mutant flies carrying a transgene with males of (1) rp298-GAL4/Y; dpak3(0)/TM3, fzt-lacZ; (2) sn-GAL4; dpak3(0)/TM3, fzt-lacZ; or (3) twi-GAL4; dpak3(0)/TM3, fzt-lacZ.Mutant embryos were identified by the lack of anti–β-gal staining. Transgene expression was confirmed by anti-V5 staining. Two independent transgenic lines were tested for each rescue experiment.

Expressing GFP-actin in dpak3(0) mutant embryos: dpak3(0); UAS-Act5C.GFP3/TM3, fzt-lacZ females were crossed with males of (1) rp298-GAL4/Y; dpak3(0)/TM3, fzt-lacZ; (2) sn-GAL4; dpak3(0)/TM3, fzt-lacZ; or (3) twi-GAL4; dpak3(0)/TM3, fzt-lacZ. All crosses, mutant embryos were identified by the lack of β-gal expression in fixed samples or by the fusion-defective phenotype in live samples.

GFP diffusion assay: rp298-GAL4/Y; dpak3(0)/TM3, fzt-lacZ males were crossed with dpak3(0)/cytoGFP/TM3, fzt-lacZ females. Mutant embryos were identified by anti-MHC staining.

**Molecular biology**

Full-length dpak3 and dpak1 were amplified by PCR with an N-terminal or a C-terminal V5 tag from cDNA clones LD10326 and LD20767 from the Drosophila Genomics Resource Center (DGRC, Bloomington, IN). These PCR fragments were then subcloned into the pAc vector for expression in S2R+ cells or the pUAST vector for generating transgenic lines.

PCR fragments were then subcloned into the pAc vector for expression in Drosophila S2R+ cells or the pUAST vector for generating transgenic lines. These transgenic flies were generated using a standard site-directed mutagenesis protocol (Agilent Technologies) to introduce standard phenotypes into dpak3 and dpak1 in S2R+ cells and harvested as described above. The following primary antibodies were used: rabbit anti–Hsp90 (1:1,000; Kiehart and Feghali, 1986); rabbit anti-GFP (1:1,000; Molecular Probes); mouse anti–β-gal (1:1,000; Promega); mouse anti–Eve (1:30; Developmental Studies Hybridoma Bank, Iowa City, IA); mouse anti–Rac1 (1:200; BD); guinea pig anti–Duf (1:250; Sens et al., 2010); rabbit anti–DMelF2 (1:100; Nguyen et al., 1994) and rabbit anti–DPAK1 (1:2,000; Harden et al., 1996). The rat anti-Dpak3 antisera was generated against an N-terminal peptide (MSTKKWPKKKGDDGGSI; Bio synthesis) and used at 1:100. Secondary antibodies were used at 1:300: FITC, Cy3, and Cy5-conjugated antibodies made in goat (Jackson ImmunoResearch Laboratories, Inc.).

In situ hybridization

A DIG-labeled probe was prepared using a 600-bp fragment of the coding sequence of dpak1 (or dpak3) with the RNA DIG labeling kit (Roche). Fixation, post-fixation, hybridization, and development of wild-type embryos were performed according to a detailed online protocol (http://www.biology.ucsd.edu/~dave/k/).

**Biochemistry**

For communoprecipitation assays, expression constructs were transfected in S2R+ cells. Cells were harvested, washed with PBS, and incubated in NP-40–Triton buffer (10 mM Tris, pH 7.4, 150 mM NaCl, 1 mM EDTA, 1% Triton X-100, and 0.5% NP-40) containing 1 mM PMSF and protease inhibitor cocktail (Roche) for 30 min at 4°C with agitation. After centrifugation, the cleared supernatants were incubated with the indicated antibodies at 4°C for 1 h, followed by immunoprecipitation with protein G beads for 2 h. After washing, immunoprecipitated proteins were subjected to SDS-PAGE and Western blot. Antibodies used for immunoprecipitation: mouse anti–V5 (1:500; Invitrogen) and mouse anti–FLAG (1:500; Sigma-Aldrich); for Western blot: mouse anti–V5-HRP (1:5,000; Invitrogen) and mouse anti–FLAG–HRP (1:5,000; Sigma-Aldrich).

For phosphatase (CIP) treatment, FLAG-Dpak3 was expressed in S2R+ cells and harvested as described above. The FLAG-Dpak3 protein was immunoprecipitated with anti–FLAG M2 affinity gel (A2220; Sigma-Aldrich), and the immunoprecipitated protein was then incubated in phosphatase buffer (B7003; New England Biolabs, Inc.) with or without 100 U/ml CIP (M0290; New England Biolabs, Inc.) at 37°C for 30 min. Subsequently, the samples were subjected to Western blot.

For in vitro kinase assays, V5-tagged Dpak3 and its mutant forms were expressed in S2R+ cells and immunoprecipitated with anti–V5 antibody as described above. The immunoprecipitated proteins were washed three times in the NP-40–Triton buffer, once in 0.5 M NaCl, and twice in ice-cold kinase buffer (20 mM Hapes, pH 7.6, 2 mM DTT, 20 mM MgCl2, 0.5 mM Na3VO4, 10 mM β-glycerophosphate, and 50 µM ATP). After aspiring the excess kinase buffer from the protein G beads, 40 µl kinase buffer, 3 µg myelin basic protein (M-1891; Sigma-Aldrich), and 5 µCi [γ-32P]ATP (BU502A; PerkinElmer) were immediately added and mixed with the Dpak3-bound beads. The reactions were performed at 30°C for 30 min with the protein beads gently resuspended every 10 min. The proteins were eluted by 2× SDS running buffer and separated on a Bis-Tris Precast gel (Invitrogen). The phosphorylated bands were visualized by a phosphor imager and the amount of loaded proteins was determined by Coomassie brilliant blue staining.

For detecting the phosphorylation status of V5-Dpak3 in Drosophila embryos, V5-Dpak3; dpak3(0)/TM3, fzt-lacZ females were crossed with twi-GAL4; dpak3(0)/TM3, fzt-lacZ males. Stage 14 embryos were collected, homogenized, and subjected to SDS-PAGE and Western blot.

**Confocal imaging of fixed samples**

Images were obtained on a confocal microscope (LSM 700; Carl Zeiss) with a Plan-NeoFluar 40x/1.3 NA oil or Plan-Apochromat 63×/1.4 NA oil DIC objective, a pigtailed-coupled solid-state laser with polarization-preserving single-mode fiber, and a high-sensitivity PMT detector. The pinhole was set to 1.0 AU for each channel and images were collected at 1.00 µm intervals. Images were acquired with Zen 2009 software (Carl Zeiss) and processed using Adobe Photoshop CS4. All samples were mounted in ProLong Gold Antifade reagent (Invitrogen) and imaged at room temperature.

**Measurement of fluorescence intensity of F-actin foci**

Fluorescence intensity was measured by using the overlay function of Zen 2009 and Photoshop CS4 extended software. Area of interest was defined by tracing the outline of the F-actin foci labeled by phallidin as described previously (Sens et al., 2010). Specifically, to be included as part of the foci, the intensity of the phallidin signal in the pixel had to be greater than the average intensity of the cortical actin. Foci were measured if they could be clearly assigned to one FCM and were distinct from other foci to ensure that only single foci was measured. Numbers shown in the text are relative intensities based on the 0–255 scale measured by the Zen 2009 software.

**Time-lapse imaging**

Embryos were collected, dechorionated with bleach, thoroughly washed, and germinated attached to a piece of clear acid-free double-sided tape (low auto-fluorescence, 6.3 µm; Therm O Web), which keeps embryos from rolling and drifting. Embryos were covered with a few drops of halocarbon oil to keep them moist while allowing adequate oxygen exchange. Subsequently, the embryos were covered with a 22 × 40–1.5 coverslip (Thermo Fisher Scientific) and the fluorescent GFP-actin was visualized with a Plan-NeoFluar 40×/1.3 NA oil objective. The solid-state laser output was set to 2% to avoid photobleaching and phototoxicity. Other confocal settings are...
as follows: pinhole, 1 AU; z-stack, 1 μm step-wise, 4 μm total; and 4 frames averaged per scan. Zen 2009 and Imagem 1.41h (National Institutes of Health, Bethesda, MD) were used to convert confocal images to movies.

Transmission electron microscopy
High-pressure freezing/freeze substitution (HPF/FS) fixation was performed as described previously [Zhong and Chen, 2008; Sens et al., 2010]. In brief, a Bal-Tec device was used to freeze embryos. Freeze-substitution was performed using 1% osmium tetroxide and 0.1% uranyl acetate in 98% acetone and 2% methanol on dry ice. The embryos were embedded in Epon [Sigma-Aldrich]. Thin sections (70 nm) were cut with a microtome (Ultracut K; Leica), mounted on copper grids, and post-stained with 5% uranyl acetate for 10 min and Sato’s lead solution (1% Pb(NO₃)₂, 1% C₂H₁₀O₄Pb₃, 1% (CH₃COO)₂Pb, 2% C₁₂H₁₀O₄Na₆, and 0.72% NaOH) for 2 min to improve image contrast. Images were acquired on a transmission electron microscope (CM120; Philips).

Online supplemental material
Fig. S1 shows that muscle cell fate is properly specified in dpak3 mutant embryos. Fig. S2 shows in situ hybridization of dpak3 and dpak1 in wildtype embryos. Fig. S3 shows specificity of α-Dpak1 and α-Dpak3 antibodies. Video 1 shows time-lapse imaging of an actin focus in a wildtype embryo expressing GFP-actin with time-lapse imaging of an actin focus in a mutant embryo expressing GFP-actin with twi-GAL4. Video 2 shows time-lapse imaging of an actin focus in a dpak3 mutant embryo expressing GFP-actin with twi-GAL4. Table S1 shows mean numbers of nuclei in DA1 muscles of wildtype and fusion mutant embryos. Online supplemental material is available at http://www.jcb.org/cgi/content/full/jcb.201204065/DC1.

We thank Drs. S. Abmayr, H. Nguyen, N. Harden, D. Kiehart, D. Menon, B. Paterson, J. Zallen, and A. Zelhof for antibodies and fly stocks; Dr. R. Leapman for access to the HPF/FS unit; and members of the Chen laboratory B. Paterson, J. Zallen, and A. Zelhof for antibodies and fly stocks; Dr. R. Leapman for access to the HPF/FS unit; and members of the Chen laboratory for helpful discussions and comments on the manuscript.

This work was supported by a grant from the National Institutes of Health to H. Chen (RO1AR053173). P. Jin was a predoctoral fellow and R. Duan is a postdoctoral fellow of the American Heart Association.

Submitted: 12 April 2012
Accepted: 23 August 2012

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**Figure S1.** Muscle cell fate is properly specified in dpak3 mutant embryos. Late stage 14 wild-type (A, wt) and dpak3zyg mutant (B) embryos triple labeled with α-tropomyosin (green), α-DMef2 (red), and α-Duf (blue). Three hemisegments are shown. Note that the number of DMef2-positive cells in each hemisegment is comparable between wt and dpak3zyg mutant embryos. Also note that the unfused FCMs (several indicated by arrowheads) are mostly attached to the small myotubes, suggesting normal FCM migration and adhesion (with myotubes). Bar, 25 µm.
Figure S2. **In situ hybridization of dpak3 and dpak1 in wild-type embryos.** Whole-mount in situ hybridization of stage 14 wild-type embryos with anti-sense probes to dpak1 (A) or dpak3 (B). Bar, 100 µm.

Figure S3. **Specificity of the α-DPak1 and α-DPak3 antibodies.** S2R+ cells overexpressing V5-tagged DPak1 or DPak3 were lysed and subjected to Western blot analysis (6% polyacrylamide gel) with the indicated antibodies. (Lanes 1 and 2) V5-DPak1 and V5-DPak3 were detected with the α-V5 antibody. (Lanes 3 and 4) The α-DPak1 antibody specifically recognized V5-Pak1 (lane 3), but not V5-DPak3 (lane 4). Note that the band in lane 4 shows the endogenous DPak1 in S2R+ cells. (Lanes 5 and 6) The α-DPak3 antibody specifically recognized V5-DPak3 (lane 6), but not V5-DPak1 (lane 5). Note that there is little endogenous DPak3 in S2R+ cells.
Video 1. Time-lapse imaging of an actin focus in a wild-type embryo expressing GFP-actin with the mesodermal driver twi-GAL4. Time-lapse sequence from a live stage 14 wild-type embryo expressing GFP-actin in all muscle cells with twi-GAL4. Images were acquired using a laser-scanning confocal microscope (LSM 700; Carl Zeiss). A single optical slice of a confocal stack is shown. 28 frames were taken at 20-s intervals. Total sampling length was ~9 min. Note that the actin-enriched focus maintained a “dense core” throughout its lifespan.

Video 2. Time-lapse imaging of an actin focus in a dpak3 mutant embryo expressing GFP-actin with twi-GAL4. Time-lapse sequence from a live stage 14 dpak3zyg mutant embryo expressing GFP-actin in all muscle cells with twi-GAL4. Images were acquired using a laser-scanning confocal microscope (LSM 700; Carl Zeiss). A single optical slice of a confocal stack is shown. 49 frames were taken at 20-s intervals. Total sampling length was ~16 min. Note the dispersed morphology of the GFP-positive focus and its dynamic shape changes.

Table S1. Mean number of nuclei in DA1 muscles of wild-type and fusion mutants

<table>
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<th>Genotype</th>
<th>n</th>
<th>Mean ± SD</th>
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<td>1</td>
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<td>13</td>
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<td>rP298-GAL4::UAS-V5-DPak3; dpak3</td>
<td>40</td>
<td>5.8 ± 1.8</td>
<td>2</td>
<td>9</td>
</tr>
<tr>
<td>twi-GAL4::UAS-V5-DPak1; dpak3</td>
<td>59</td>
<td>6.6 ± 1.2</td>
<td>5</td>
<td>10</td>
</tr>
<tr>
<td>twi-GAL4::UAS-V5-DPak3; dpak3 (25°C)</td>
<td>46</td>
<td>5.4 ± 1.3</td>
<td>3</td>
<td>9</td>
</tr>
<tr>
<td>twi-GAL4::UAS-V5-DPak3; dpak3 (30°C)</td>
<td>60</td>
<td>3.6 ± 1.3</td>
<td>1</td>
<td>7</td>
</tr>
<tr>
<td>twi-GAL4::UAS-DPak3; dpak3 (25°C)</td>
<td>34</td>
<td>1.4 ± 0.7</td>
<td>1</td>
<td>3</td>
</tr>
<tr>
<td>twi-GAL4::UAS-DPak3; dpak3 (30°C)</td>
<td>43</td>
<td>7.4 ± 1.4</td>
<td>4</td>
<td>10</td>
</tr>
<tr>
<td>dpak3, wasp</td>
<td>27</td>
<td>1.9 ± 0.7</td>
<td>1</td>
<td>3</td>
</tr>
<tr>
<td>scar;dpak3</td>
<td>40</td>
<td>1.0 ± 0.0</td>
<td>1</td>
<td>1</td>
</tr>
</tbody>
</table>

Embryos were stained with α-Eve and α-MHC, and the numbers of Eve-positive nuclei in the DA1 muscle of abdominal hemisegments A1-A6 were counted. n: number of hemisegments counted. SD: standard deviation. Min: minimum number of nuclei. Max: maximum number of nuclei.

* Eve-positive nuclei in the DA1 muscle per hemisegment in late stage 15 embryos.