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Competition between Blown Fuse and WASP for WIP Binding Regulates the Dynamics of WASP-Dependent Actin Polymerization In Vivo

Peng Jin,1,3 Rui Duan,1,3 Fengbao Luo,1 Guofeng Zhang,2 Sabrina N. Hong,1 and Elizabeth H. Chen1,*

1Department of Molecular Biology and Genetics, Johns Hopkins University School of Medicine, Baltimore, MD 21205, USA
2Laboratory of Bioengineering and Physical Science, National Institute of Biomedical Imaging and Bioengineering, National Institute of Health, Bethesda, MD 20892, USA
3These authors contributed equally to this work
*Correspondence: echen@jhmi.edu
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SUMMARY

Dynamic rearrangements of the actin cytoskeleton play a key role in numerous cellular processes. In Drosophila, fusion between a muscle founder cell and a fusion competent myoblast (FCM) is mediated by an invasive, F-actin-enriched podosome-like structure (PLS). Here, we show that the dynamics of the PLS is controlled by Blown fuse (Blow), a cytoplasmic protein required for myoblast fusion but whose molecular function has been elusive. We demonstrate that Blow is an FCM-specific protein that colocalizes with WASP, WIP/Solitary, and the actin focus within the PLS. Biochemically, Blow modulates the stability of the WASP-WIP complex by competing with WASP for WIP binding, leading to a rapid exchange of WASP, WIP and G-actin within the PLS, which, in turn, actively invades the adjacent founder cell to promote fusion pore formation. These studies identify a regulatory protein that modulates the actin cytoskeletal dynamics by controlling the stability of the WASP-WIP complex.

INTRODUCTION

Actin cytoskeletal remodeling is involved in numerous cellular processes such as cell migration, polarization, division, and fusion. Despite the discovery of many factors required for the actin filament assembly, the regulation of actin cytoskeletal dynamics is not well understood. Actin assembly occurs by adding G-actin to the barbed ends of F-actin, and this process is mediated by several classes of actin nucleators, among which the actin-related protein 2 and 3 (Arp2/3) complex is the only one that promotes the formation of branched actin networks instead of linear F-actin (reviewed by Pollard, 2007). The Arp2/3 complex is activated by the Wiskott-Aldrich syndrome protein (WASP) family of nucleation promoting factors (NPFs), which contains two WASPs (WASP an N-WASP) and three WAVEs/Scars (WAVE/Scar1, 2, and 3) in mammals (reviewed by Kurisu and Takenawa, 2009; Takenawa and Suetsugu, 2007), but a single WASP and Scar in Drosophila (Ben-Yaacov et al., 2001; Zallen et al., 2002). These Arp2/3 NPFs are scaffolds that link upstream signals to dynamic rearrangements of the actin cytoskeleton.

Among the many proteins that bind WASPs are the WASP-interacting protein (WIP) family proteins. WASP or N-WASP binds WIP in a 1:1 molar ratio through an interaction between the WASP-homology-1 (WH1) domain in WASPs and the WASP-binding domain (WBD) in WIP (reviewed by Anton et al., 2007; Ramesh and Geha, 2009). The WASP-WIP interaction maintains the stability of WASP, as the WASP protein level is severely diminished in WIP-knockdown cells and T cells from WIP−/− mice (Chou et al., 2006; de la Fuente et al., 2007; Konno et al., 2007). In addition, the WASP-WIP interaction is also required for recruiting WASP to specific subcellular locations (Chou et al., 2006). The importance of the WASP-WIP interaction is highlighted by the clustering of missense mutations in the WH1 domain of WASP in patients with Wiskott-Aldrich syndrome (reviewed by Ochs and Thrasher, 2006), some of which have been shown to disrupt WASP-WIP binding (Luthi et al., 2003; Stewart et al., 1999). At the cellular level, the WASP-WIP complex is known to promote the formation of podosome/invadopodium (collectively called invadosomes), which are actin-rich cell adhesive structures involved in extracellular matrix digestion, cell migration and invasion (reviewed by Gimona et al., 2008; Linder, 2009). In addition, the N-WASP-WIP complex can be harnessed by pathogens, such as vaccinia virus and Shigella, to activate the formation of actin tails that propel the movement of these infectious particles/cells in host cells (Frischknecht et al., 1999; Suzuki and Sasakawa, 1998).

Recent studies have demonstrated an essential role of the Arp2/3 NPFs in Drosophila myoblast fusion (Kim et al., 2007; Massarwa et al., 2007; Richardson et al., 2007; Schafer et al., 2007; Sens et al., 2010). Myoblast fusion in Drosophila occurs between two populations of muscle cells, muscle founder cells and fusion competent myoblasts (FCMs). The recognition and adhesion between founder cells and FCMs are mediated by two pairs of Ig domain-containing cell adhesion molecules, including Dumbfounded (Duf) and its paralog Roughest that function redundantly in founder cells, and Sticks and stones (Sns) and its paralog Hibris that are partially redundant in FCMs (Artero et al., 2001; Bour et al., 2000; Dworak et al., 2001; Ruiz-Gomez et al., 2000; Shelton et al., 2009; Strunkelnberg et al., 2008).
The engagement of the cell type-specific adhesion molecules in trans triggers distinct signal transduction cascades within the founder cell and FCM, leading to the recruitment of the Arp2/3 NPF Scar in the founder cell, and both Scar and the WASP-WIP complexes in the FCM, to the site of cell adhesion (reviewed by Abmayr et al., 2008 and Rochlin et al., 2009; Sens et al., 2010). The combinatorial functions of these NPFs result in the formation of an asymmetric fusogenic synapse, which consists of an invasive podosome-like structure (PLS) at the tip of the FCM and a corresponding thin sheath of F-actin underlying the apposing founder cell membrane (Sens et al., 2010). The FCM-specific PLS contains a dense F-actin focus encircled by overlapping adhesive rings formed by the cell adhesion molecules, Sns and Duf. While both Scar and WASP play a role in the formation of the actin focus within the PLS, the WASP-WIP complex, but not the pentameric Scar complex, is required for the efficient invasion of the PLS into the apposing founder cell. The invasiveness of the PLS is required for fusion pore formation, as fusion pores fail to form in solitary (sltr) (the Drosophila ortholog of WIP) mutant embryos where invasion is impaired (Kim et al., 2007; Sens et al., 2010). Electron microscopy and live imaging studies show that the FCM-specific PLS extends multiple invasive fingers into the founder cell and that the PLS is a dynamic structure, which undergoes constant shape changes during its life span (Sens et al., 2010). However, it is unclear how the dynamics of the PLS is modulated prior to fusion pore formation.

In this study, we demonstrate that a cytoplasmic protein, Blown fuse (Blow), regulates the dynamics of the WASP-mediated actin polymerization via a competition mechanism. Although blow was identified as a gene required for myoblast fusion over a decade ago (Doberstein et al., 1997), the molecular mechanism of Blow function during myoblast fusion has been elusive. We show here that Blow is biochemically linked to the WASP-WIP complex and functions exclusively in FCMs. Blow regulates the dynamics of the PLS by modulating the stability of the WASP-WIP complex via a competitive binding mechanism. Moreover, the dynamics, but not the mere presence, of the PLS is critical for its invasiveness and ultimately fusion pore formation. Thus, we have uncovered a mechanism that modulates the dynamics of the WASP-mediated actin polymerization in vivo.

RESULTS

Blow Is an FCM-Specific Protein and Colocalizes with WASP, Sltr, and the F-actin Focus at the Site of Fusion

To investigate Blow function in myoblast fusion, we first clarified the localization of Blow in the two populations of muscle cells, since there have been conflicting reports placing Blow either in both populations or specifically in FCMs (Richardson et al., 2007; Schröter et al., 2006). We examined Blow expression in lame duck (lmd) mutant embryos, in which the fate of FCMs is not specified due to the absence of the FCM-specific transcription factor Lmd (Duan et al., 2001). Blow protein is absent in lmd mutant embryos, whereas the expression of the founder cell-specific Duf persists (compare Figures 1Aa–1Aa” and 1Ab–1Ab”). Thus, Blow is not expressed in founder cells and likely to be specifically expressed in FCMs.

Interestingly, Blow is not ubiquitously distributed in the cytoplasm of muscle cells, but rather aggregates to distinct foci. The punctate Blow-positive foci are reminiscent of the dense foci at sites of fusion formed by Sltr (Drosophila WIP) and F-actin, both of which are FCM-specific (Figures 1Ba–1Ba”) (Kim et al., 2007; Sens et al., 2010). Indeed, Blow-positive foci colocalize with those of Sltr, demonstrating that Blow is recruited to and enriched at sites of fusion (Figures 1Bb–1Bb”). Consistent with this, live imaging experiments with embryos expressing Blow-mCherry and GFP-actin in muscle cells showed that the mCherry- and GFP-positive foci colocalized during their life span (see Movie S1 available online). Like Sltr, WASP is also enriched at sites of fusion and colocalizes with the F-actin foci in the embryo (Figures 1Bc–1Bc”), consistent with our previous biochemical analysis demonstrating an interaction between Sltr and WASP in transfected cells (Kim et al., 2007). Taken together, we conclude that Blow colocalizes with the WASP-Sltr complex, as well as the F-actin focus of the PLS at sites of fusion.

The recruitment of Blow to sites of fusion is dependent on the FCM-specific adhesion molecule, Sns, as Blow no longer aggregates to specific foci at muscle cell contact sites in sns mutant embryos (compare Figures 1Ca–1Ca”) and 1Cb–1Cb”; Richardson et al., 2007). In contrast, Blow enriched foci are present in other fusion mutants, such as kette (encoding a member of the pentameric Scar complex) (Hummel et al., 2000; Schröter et al., 2004), sltr, or myoblast city (mbc) (encoding a guanine nucleotide exchange factor for the small GTPase Rac) (Brugnera et al., 2002; Erickson et al., 1997) (Figures 1Cc–1Ce”), suggesting that the recruitment of Blow to sites of fusion is independent of these cytoplasmic proteins.

We then asked whether Blow is functionally required in a cell type-specific manner. Expressing Blow in all muscle cells with twi-GAL4 (Figure 1Db) or in FCMs with sns-GAL4 (Figure 1Dc) rescued the fusion defect in blow mutant embryos (compare with Figure 1Da), whereas expressing Blow specifically in founder cells with rP298-GAL4 did not (Figure 1De, compare to Figure 1Dd). These results demonstrate that Blow is functionally required in FCMs during myoblast fusion.

Blow Interacts with the SH2 and SH3 Domain-Containing Adaptor Protein Crk

To determine how Blow is recruited to the site of fusion by Sns, we performed communoprecipitation experiments using Drosophila S2R+ cells. While we did not detect any interaction between Blow and Sns (data not shown), we found that Blow interacted with Crk (Figure 2B), an SH2–SH3 domain-containing adaptor protein that has been proposed to recruit Sltr to sites of fusion (Kim et al., 2007). Domain mapping experiments revealed that Blow and Sltr interact with the SH2 domain and the first SH3 domain (SH3-1) of Crk, respectively (Figure 2C; Figures S1A and S1B), suggesting that Blow and Sltr may be recruited to sites of fusion independently via Crk binding. Consistent with this hypothesis, Blow and Sltr remain enriched at muscle cell contact sites in the absence of each other (Figures 1Cd–1Cd” and 4D).

We next mapped the Crk-interacting site in Blow to a single YDVP sequence (Figures 2A and 2D), which matches the consensus SH2 binding motif (Pawson et al., 2001). A point...
Figure 1. Blow is an FCM-Specific Protein that Colocalizes with the WASP-Sltr Complex at Sites of Fusion

(A) Expression of Blow in wild-type and lmd mutant embryos. Stage 14 wild-type (a–a") or lmd (b–b") embryos double labeled with α-Blow (green) and α-Duf (red; founder cells). Note the absence of Blow but the presence of Duf in lmd mutant embryo.

(B) Enrichment of Blow at sites of fusion. Stage 14 wild-type embryos labeled with phalloidin (green), α-Blow (green), α-Sltr (red), and/or α-WASP (red). Arrowheads indicate the colocalization between phalloidin- and Sltr-positive foci (a–a"; Kim et al., 2007), Blow- and Sltr-positive foci (b–b"), and phalloidin- and WASP-positive foci (c–c").

(C) Localization of Blow in wild-type and several fusion mutants. Stage 14 embryos double labeled with α-Blow (green) and α-Ants (Ants) (red; founder cells) (Chen and Olson, 2001). Blow is recruited to muscle cell contact sites (arrowheads) marked by Ants, which shows similar enrichment at sites of fusion as Duf (Sens et al., 2010), in wild-type (a–a"), kette (c–c"), sltr (d–d"), and mbc (e–e") mutant embryos. Note that Blow is not enriched to muscle cell contact sites (arrows) in the sns mutant embryo (b–b").

(D) Transgenic rescue of blow mutant embryos. Stage 15 embryos labeled with α-myosin heavy chain (MHC). Full-length blow (UAS-blow) driven by a pan-mesodermal driver twi-GAL4 (b) or an FCM-specific driver sns-GAL4 (c) rescued the fusion defects (compare with wild-type [a] and blow mutant [d]). However, a founder cell-specific driver rP298-GAL4 did not rescue the fusion defect (e).

Bars: (A, B, D), 20 μm; (C), 10 μm.
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Figure 2. Blow Interacts with Crk and Sltr
(A) Schematic diagrams of the domain organization of Blow, Crk, and Sltr.
(B–G) In all experiments, extracts from S2R+ cell transfected with the indicated plasmids (listed above the gels) were immunoprecipitated (IP) and probed with the indicated antibodies.

See also Figure S1.
mutation of the tyrosine residue in this motif to non-phosphorylatable alanine (BlowY378A) or to phenylalanine (BlowY378F) in the context of the full-length Blow protein completely abolished Crk binding (Figure 2D), demonstrating that Y378 is critical for the Blow-Crk interaction. To test the functional importance of the SH2-binding motif in vivo, we performed transgenic rescue experiments. Wild-type Blow, but not BlowY378F, rescued the fusion defects in blow mutant embryos (Figures 2Ha, 2Ha′, 2Hb, and 2Hb′), suggesting that SH2 binding is critical for Blow function in vivo. Taken together, these results suggest that SH2 domain-containing adaptor proteins such as Crk may recruit Blow to sites of fusion.

Physical Interaction between Blow and Sltr Is Required for Blow Function In Vivo
The colocalization of Blow and Sltr at sites of fusion prompted us to investigate whether these two proteins may form a complex. Indeed, Blow and Sltr overexpressed in S2R+ cells coprecipitated with each other (Figure 2E, lane 3). This interaction is independent of the Blow-Crk interaction, since BlowY378F, which fails to bind Crk, still binds Sltr (Figure 2E, lane 4). In addition, overexpressed Blow coprecipitated with endogenous Sltr (Figure S1C). To map the Sltr-binding domain in Blow, we made N- or C-terminal deletion constructs of Blow and found that constructs carrying a deletion of either the N-terminal 99 amino acid (aa) (BlowD N99) or C-terminal 173 aa (BlowD C173) abolished Blow-Sltr binding (Figure 2E, lanes 5 and 6) while maintaining Crk binding (Figure S1D). Thus, both the N- and C-terminal regions of Blow are required for Sltr binding. Moreover, transgenic expression of BlowΔC173 failed to rescue the fusion defect in blow mutant embryos (Figures 2Hc and 2Hc′), suggesting that the Blow-Sltr interaction is required for Blow function during myoblast fusion in vivo.

Blow Interacts with the WASP-Binding Domain of Sltr and Competes with WASP for Sltr Binding
The Blow-Sltr interaction described above, along with the well-established WASP-Sltr binding (Kim et al., 2007), prompted us to investigate how Blow may affect the stability of the WASP-Sltr complex. We first mapped the Blow-interacting domain in Sltr to a large C-terminal region (Figure S1E), and subsequently narrowed it down to a 24 aa region (Figure 2F), which includes the previously identified WASP-binding domain (WBD) in WIP (Figures 2A and 2G) (Volkmann et al., 2002). Further deletion analysis within this WBD revealed a 7 aa region (K686-N692) that is absolutely required for both Blow and WASP binding (Figures 2F and 2G, lanes 5). That both WASP and Blow bind to an identical region within Sltr raised the possibility that WASP and Blow may compete with each other for Sltr binding. Indeed, in S2R+ cells, increasing amount of Blow expression led to a gradual decrease in the amount of Sltr that coimmunoprecipitated with WASP (Figure 3A). Likewise, increasing amount of WASP expression resulted in a gradual decrease in the amount of Sltr that coimmunoprecipitated with Blow (Figure 3B).

Next, we tested whether overexpressed Blow competes with endogenous WASP for Sltr binding. S2 cells contain a significant amount of endogenous WASP, the level of which was greatly reduced by RNAi knockdown of WASP or Sltr (Figures 3C, lanes 1, 5, and 6, and Figure 3D). Thus, Sltr functions as a chaperone for WASP and its absence leads to WASP destabilization, consistent with previous findings in T cells (de la Fuente et al., 2007). Overexpression of Sltr did not increase the level of endogenous WASP (Figures 3C, lane 2, and 3D), indicating that endogenous WASP (at a steady state level) is protected by Sltr from degradation and that overexpression of Sltr does not induce new synthesis of WASP in S2 cells. Interestingly, overexpressing Blow in S2 cells caused a ~23% decrease in the level of endogenous WASP (Figures 3C, lane 4, and 3D), indicating that WASP is partially degraded in the presence of an excess amount of Blow. This is likely due to the sequestration of endogenous Sltr into the Blow-Sltr complex, thereby leaving some endogenous WASP in an uncomplexed state and thus prone to degradation. Consistent with this hypothesis, cooverexpression of Sltr completely suppressed the ability of overexpressed Blow to decrease the level of endogenous WASP (Figures 3C, lane 3, and 3D). To directly test whether Blow competes with endogenous WASP for Sltr binding, we examined WASP-Sltr interaction in S2 cells overexpressing Blow. Since the available Sltr antibody could not efficiently pull down the endogenous Sltr, we added a small amount of cell lysate containing exogenously expressed FLAG-V5-tagged Sltr to the co-IP mixture. We found that overexpressed Blow reduced the amount of endogenous WASP communoprecipitated by the exogenous Sltr (Figure 3E), demonstrating that Blow can compete with endogenous WASP for Sltr binding.

Blow Binds to Sltr with a Lower Affinity Than WASP-Sltr Binding
To better understand the competition between Blow and WASP for Sltr binding, we evaluated the affinity of both Blow-Sltr and WASP-Sltr binding. We purified epitope-tagged Blow, Sltr and WASP from Drosophila S2R+ cells (Figure S2A), and estimated the Kd of Blow–Sltr, as well as WASP–Sltr binding. The Kd between Blow-Sltr (48.1 nM) is 7.2-fold higher than that of WASP-Sltr (6.7 nM) (Figures 3F and 3G), suggesting that Sltr binds to Blow with a 7.2-fold lower affinity than it does to WASP. The relatively lower affinity of Blow-Sltr binding likely accounts for the partial degradation of endogenous WASP observed in cells overexpressing Blow (Figures 3C and 3D).

Blow acts through the WASP-Sltr Complex to Regulate Actin Polymerization In Vivo
Since Blow is localized to sites of fusion and regulates the stability of the WASP-Sltr complex, we examined F-actin foci in blow mutant embryos. F-actin foci persist till late embryogenesis in blow mutant embryos and their sizes are enlarged (3.3 ± 0.8 μm²; n = 53; Figures 4Ab–4Ab′) compared with those in wild-type embryos (1.7 ± 0.6 μm²; n = 55; Figures 4Aa–4Aa′ and 4B) (Richardson et al., 2007). Consistent with this finding, live imaging of blow mutant embryos expressing GFP-actin revealed accumulation of GFP-actin and prolonged persistence of actin foci (Movie S2).

In wild-type embryos, the F-actin foci of the PLSS reside exclusively within the FCMs (Sens et al., 2010). To examine whether the abnormally enlarged F-actin foci in blow mutant embryos also reside in FCMs, we expressed GFP-actin in either founder cells or FCMs in blow mutant embryos. GFP-actin expressed...
**Figure 3. Blow Competes with WASP for Sltr Binding**

(A and B) S2R+ cells were transfected with epitope-tagged Sltr, WASP, and Blow. Expression of increasing amounts of Blow (A) or WASP (B) decreased WASP-Sltr or Blow-Sltr interactions, respectively.

(C) Sltr functions as a chaperone for endogenous WASP in S2 cells. In all experiments, S2 cells were transfected with GFP (lanes 1–6), together with Sltr (lanes 2 and 3) and/or Blow (lanes 3 and 4), or treated with dsRNA against Sltr (lane 5) or WASP (lane 6). GFP-positive cells were FACS sorted and endogenous WASP in these cells was examined by western blot.

(D) Quantification of the endogenous WASP protein level shown in (C). Note that Blow expression caused a 23.2 ± 7.4% (n = 3) decrease in the endogenous WASP level, which is statistically significant (p < 0.05) (determined by paired student’s t test) compared to the control. *p < 0.05; **p < 0.01; ***p < 0.001. Error bars: standard deviations.

(E) Blow competes with the endogenous WASP for Sltr binding. Overexpression of Blow, but not BlowΔC173, reduced the amount of endogenous WASP coprecipitated by exogenous Sltr-FLAG-V5. See also Figure S3.

(F and G) Saturation binding curves of WASP-Sltr (F) or Blow-Sltr (G) (see also Experimental Procedures and Figure S2A).
Figure 4. Blow Functions through the WASP-Sltr Complex to Regulate Actin Polymerization
(A and B) Blow regulates the formation of F-actin foci via Sltr in embryos. (A) Stage 14 embryos labeled with phalloidin (green), α-Ants (red), and α-Lmd (blue; FCMs). F-actin foci (arrowheads) are enlarged in blow mutant (b–b00), compared with wild-type (a–a00) or sltr mutant (c–c00) embryos. Note that the F-actin foci in blow, sltr double mutant (d–d00) are similar in size compared to those in sltr mutant. (B) Mean values of actin foci sizes in indicated embryos. Statistical analyses were performed by unpaired two-tail student’s t test (***p < 0.001). Error bars: standard deviation.

(C) F-actin foci are localized in FCMs of blow mutant embryos. Stage 14 embryos labeled with α-GFP (green), phalloidin (red), and α-Ants (blue). GFP-actin expressed in FCMs (sns-GAL4) (a–a%), but not founder cells (rP298-GAL4) (b–b%), colocalized with F-actin foci (arrowheads). Selected FCMs outlined in a% and b%, based on the phalloidin staining that labels the cortical F-actin. The FCM marked by an asterisk had not yet expressed GFP-actin at the time of fixation, thus is phalloidin-positive but GFP negative.

(D) Enlarged F-actin foci at muscle cell contact sites correspond to increased accumulation of Sltr and WASP in blow mutant embryos. Stage 14 embryos labeled with α-Sltr (green), α-WASP, or phalloidin (red), and α-Ants (blue). Note the colocalization of F-actin foci, Sltr, and WASP in enlarged domains (compared with Figure 1B) at muscle cell contact sites marked by elevated accumulation of Ants (arrowheads). Boxed areas in a” and c” enlarged in b–b” and d–d”, respectively.

(E and F) Overexpression of Blow decreased the length of actin-filled microspikes induced by the WASP-Sltr complex in S2 cells. (E) Wild-type and indicated mutant Blow proteins were coexpressed with Sltr in S2 cells, labeled with phalloidin (green), α-Sltr (red), and α-Blow (blue). Overexpression of Sltr (b–b”), but not

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in FCMs colocalized with the phallloidin-labeled F-actin foci (Figures 4Ca–4Ca”), whereas GFP-actin expressed in founder cells did not accumulate to form dense foci (Figures 4Cb–4Cb”). Thus, as in wild-type embryos, the enlarged F-actin foci in blow mutant embryos reside in FCMs.

Interestingly, the enlarged F-actin foci in blow mutant embryos colocalized with enlarged domain of WASP and Sltr (Figure 4D), consistent with a role for the WASP-Str complex in promoting actin polymerization within these abnormal foci. To determine whether Blow acts through the WASP-Str complex to regulate actin polymerization, we examined the F-actin foci in blow, sltr double mutant embryos. The size of the F-actin foci in blow, sltr double mutant (1.9 ± 0.3 μm², n = 44; Figures 4Ad–4Ad”) is similar to that of sltr (1.9 ± 0.3 μm², n = 38; Figures 4Ac–4Ac” and 4B), but not blow mutant embryos (3.3 ± 0.8 μm², n = 53; Figures 4Ab–4Ab” and 4B), demonstrating that sltr is genetically epistatic to blow. This result supports a model that Blow functions through the WASP-Str complex to regulate actin polymerization in vivo.

**Blow Regulates the Exchange Rate of G-Actin within the PLS at Sites of Myoblast Fusion**

The persistence of actin foci in the fusion-defective blow mutant embryos suggests that myoblast fusion requires not only the formation of actin foci, but also additional attributes such as the dynamics of actin polymerization within these foci. To investigate this possibility, we examined actin dynamics in wild-type and blow mutant embryos using fluorescence recovery after photobleaching (FRAP). Photobleaching of individual GFP-positve actin foci in wild-type embryos expressing GFP-actin in all muscle cells resulted in a rapid recovery of the fluorescent signal to the prebleaching level (Figures 5Aa and 5B–5D; Table S1 and Movie S3). Strikingly, parallel analysis of photobleaching in blow mutant embryos resulted in a slower fluorescence recovery, and the intensity of the recovered fluorescent signal never reached the prebleaching level (Figures 5Ab and 5B–5D; Table S1 and Movie S4). These results suggest that in blow mutant embryos, the exchange rate of G-actin within the PLS is significantly decreased compared with the wild-type embryos. Therefore, the enlarged F-actin foci in blow mutant embryos is not due to increased rate of actin polymerization, but rather the gradual accumulation of F-actin during their long life span. Interestingly, there is a wide range of GFP-actin recovery time in both wild-type and mutant embryos, as that of the life span of the F-actin foci (Richardson et al., 2007), which may in part be due to an different kinetics of myoblast fusion in muscles of different sizes (Bataille et al., 2010). Of note, the decreased actin dynamics in blow mutant embryos is not simply due to a block in myoblast fusion, since in the kette mutant embryos, which are also characterized by a lack of fusion and the presence of persistent and enlarged F-actin foci (Richardson et al., 2007), GFP-actin showed a wild-type-like recovery rate and level after photobleaching (Figures 5Ae and 5B–5D; Table S1 and Movie S5).

**Blow Regulates the Exchange Rate of WASP and Sltr at Sites of Myoblast Fusion**

Actin polymerization occurs primarily on the barbed ends of F-actin. The decreased exchange rate of G-actin in blow mutant embryos suggests that there may be fewer free barbed ends within the blow mutant actin foci. Since barbed ends are known to be captured by N-WASP (Co et al., 2007) and transient dissociation between N-WASP and the barbed ends allows the addition of new G-actin, we speculated that the interaction between WASP and the barbed ends may be more stable in blow mutant embryos, thus leaving fewer free barbed ends for G-actin addition. This model predicted that the exchange rate of WASP, like that of G-actin, may be reduced in blow mutant actin foci. To test this hypothesis, we performed FRAP experiments in embryos expressing GFP-WASP in muscle cells. Indeed, photo-bleaching of individual GFP-WASP foci in blow mutant embryos resulted in a slower exchange rate (Figures 5Eb, 5G, and 5H; Table S1 and Movie S7) compared with wild-type embryos (Figures 5Ea, 5G, and 5I; Table S1 and Movie S6). Furthermore, the intensity of the recovered fluorescent signal of GFP-WASP in blow mutant embryos never reached the prebleaching level (Figures 5Eb, 5G, and 5I; Table S1 and Movie S7), whereas the fluorescent signal in wild-type embryos recovered to the prebleaching level (Figures 5Ea, 5G, and 5I; Table S1 and Movie S6). Thus, WASP is more stably associated with the actin foci in blow mutant embryos. Similarly, the WASP-interacting protein Sltr also exhibits a slower exchange rate and a lower fluorescence recovery level in blow mutant embryos (Figures 5Fb and 5G–5I; Table S1 and Movie S9) than in wild-type (Figures 5Fa and 5G–5I; Table S1 and Movie S8), consistent with these two proteins (WASP and Sltr) functioning as a complex to regulate actin polymerization. Interestingly, decreased exchange rate of WASP and G-actin in blow mutant embryos resulted in the formation of less densely packed and elongated actin filaments compared with those in wild-type embryos (Figures 6A and 6B), suggesting a defect in initiating additional branched actin polymerization. In light of the biochemical activity of Blow in dissociating the WASP-Str complex, we suggest that increased stability of the WASP-Str complex in blow mutant embryos leads to increased occupancy of barbed ends of F-actin by the WASP-Str complex, thus accounting for the slower exchange rate of WASP, Sltr, and G-actin, as well as defects in initiating new branched actin polymerization. In support of this, the slow and incomplete G-actin recovery in blow mutant embryos was rescued by transgenic expression of full-length Blow, but not BlowΔC173, (Figures 5Ac, 5Ad, and 5B–5D), further suggesting that the ability of Blow to dissociate the WASP-Str complex is critical in regulating the actin polymerization dynamics in vivo.
Overexpressing Blow in S2 Cells Reduces the Length of the F-Actin-Filled Microspikes Induced by Sltr

The function of Blow in dissociating the WASP-Sltr complex and regulating actin polymerization dynamics was further tested in cultured Drosophila S2 cells. We have previously shown that overexpression of Sltr in S2 cells leads to the formation of actin-filled microspikes (Kim et al., 2007; also see Figures 4Eb–4Fb), an effect that is mediated by the WASP-Sltr complex (Kim et al., 2007). Since the endogenous WASP level remains the same in Sltr-expressed cells compared to untransfected cells (Figures 3C, lane 2, and 3D), the microspikes induced by Sltr likely result from a redistribution of the WASP proteins to the cell periphery upon Sltr overexpression. We found that while transfection of Blow alone in S2 cells did not lead to any detectable changes in the actin cytoskeleton (Figures 4Ea–4Ec), coexpression of Blow and Sltr reduced the length of Sltr-induced microspikes (Figures 4Ed–4Ec). This effect requires the Blow-Sltr interaction, since expression of BlowΔC173, which does not bind Sltr, did not reduce the length of Sltr-induced microspikes (Figures 4Ed–4Ec). This result, together with the presence of elongated actin filaments in blow mutant embryos (Figures 6Aa and 6B), supports the model that Blow promotes the initiation of branched actin polymerization. We note that overexpression of BlowY378F, which does not bind the adaptor protein Crk, caused a mild reduction of the length of microspikes (Figures 4Ed–4Ec). Thus, expression of an excessive amount of BlowY378F in S2 cells can partially bypass the requirement of Crk to recruit Blow to the plasma membrane.

Blow Is Required for PLS Invasion and Fusion Pore Formation

The results presented above demonstrate that Blow regulates the dynamics of actin polymerization through the WASP-Sltr complex and that loss of Blow results in less dynamic actin foci. To understand how decreased actin dynamics leads to a defect in myoblast fusion, we used light and electron microscopy to examine the invasiveness of the FCM-specific PLS, since such invasion has been shown to be required for fusion pore formation (Sens et al., 2010). Using confocal microscopy, we found that 35% (18/52) of the wild-type actin foci within the PLSs at a given developmental time point caused dimple-shaped dents on the founder cell membrane (Figures 6Aa–6Ab); Table S2). To test whether the defect in actin foci invasion in the blow mutant embryos leads to a failure of fusion pore formation, we performed a GFP diffusion assay by expressing a cytoplasmic GFP (cytoGFP) in founder cells. The cytoGFP was retained in founder cells/miniature myotubes without diffusing into the adherent FCMs (Figure 6C), indicating a failure in fusion pore formation between founder cells and the attached FCMs. Taken together, dynamic actin polymerization is required for the invasiveness of the FCM-specific PLSs into the founder cells as well as the formation of fusion pores between these two types of muscle cells.

Blow Competes with WASP for Binding to the Human WIP

The ability of Blow to modulate actin polymerization by dissociating the WASP-Sltr complex in Drosophila raised the question of whether a similar mechanism may be used to regulate actin dynamics in mammals. Although Blow does not have an obvious mammalian homolog based on its primary sequence, it interacted with the human WIP when the two proteins were

**Figure 5. Blow Regulates the Exchange Rate of G-Actin, WASP, and Sltr within the Actin Foci**

Randomly selected GFP- or mCherry-positive foci were photobleached to approximately 30% of their original intensity and the fluorescence recovery was measured live in stage 14 embryos expressing GFP-actin (A), GFP-WASP (E), and Sltr-mCherry (F), respectively, in all muscle cells (twi-GAL4). (A) Stills of GFP-actin foci in wild-type (wt) (a), blow (b), blow embryo expressing full-length Blow (c) or BlowΔC173 (d), and kette (e) at 30 s (a, c, e) or 90 s (b and d) intervals. (B) Comparison of the recovery kinetics of the GFP-positive foci shown in (A). (C and D) Mean values of the half-time (C) and percentage recovery (D) of randomly selected GFP-actin foci photobleached in embryos with indicated genotypes. See also Table S1. (E and F) Stills of GFP-WASP (E) or Sltr-mCherry (F) foci in wt (a) and blow (b) embryos at 30 s (a) or 90 s (b) intervals. (G) Comparison of the recovery kinetics of the GFP- and mCherry-positive foci as shown in (E) and (F). (H and I) Mean values of the half-time (H) and percentage recovery (I) of randomly selected GFP-WASP or Sltr-mCherry foci photobleached in embryos with indicated genotypes. Green bars, GFP-WASP; Red bars, Sltr-mCherry. See also Table S1. Statistical analyses were performed as described in Figure 4. Error bars: standard deviations. Bars: 5 μm.
coexpressed in Drosophila S2R+ cells (Figure S3A, lane 3), albeit with a lower affinity than Blow-Sltr interaction (compare Figure S3A, lane 3, and Figure S3B). In contrast, no interaction was detected between human WIP and Blow/DN99 (or Blow/DG173) (Figure S3A, lanes 5 and 6), neither of which binds Sltr, suggesting that human WIP may interact with Blow in a similar manner as its Drosophila counterpart. Furthermore, cotransfecting Drosophila WASP, which also interacted with human WIP (data not shown), with Blow and human WIP in S2R+ cells decreased the interaction between Blow and human WIP (Figure S3A, lane 4). Thus, Blow competes with WASP for binding to human WIP and the stability of the mammalian WASP-WIP complex may be modulated by a similar competitive binding mechanism.

**DISCUSSION**

In this study, we identify a molecular mechanism by which the dynamics of WASP-mediated actin polymerization is regulated in vivo. We show that a cytoplasmic protein Blow controls the dynamics of actin polymerization by modulating the stability of the WASP-WIP complex. Our study further reveals that the dynamics of actin polymerization, instead of merely the accumulated level of F-actin, is a critical determinant for the invasion of...
Figure 7. A Model Describing the Regulation of Actin Polymerization Dynamics by Blow

(A) Blow dissociates the WASP-WIP complex resulting in the formation of a densely packed, branched F-actin network in wild-type embryos. (a) Engagement of the muscle cell type-specific adhesion molecules (Duf and Sna) leads to the independent recruitment of Blow and the WASP-WIP complex to the site of fusion. The WASP-WIP complex captures the barbed end of F-actin, through G-actin-WASP and F-actin-WIP interactions, protecting the barbed end from capping proteins. (b) Blow competes with WASP for WIP binding and displaces WIP from WASP. (c) Without the F-actin-WIP interaction, WASP is prone to dissociate from the barbed end, leaving the end transiently exposed to either capping proteins or additional G-actin (either bound or unbound) to the WASP-WIP complex. (d) Continuous displacement of WIP from WASP (bound to the barbed ends) by Blow eventually results in capping of the actin filament. (e) Filament capping frees more WASP-WIP complexes to participate in the initiation of new F-actin branches mediated by the Arp2/3 complex. (f) Frequent capping and new branch initiation will lead to the formation of densely packed, shorter, and stiffer actin filaments, which, in turn, generate sufficient mechanical force to promote cell membrane protrusion. The asterisk marks the WASP-WIP complexes participating in the initiation of new branches.
the FCM-specific PLS and fusion pore formation during myoblast fusion.

**Blow Regulates the Stability of the WASP-Sltr Complex**

Although blow was identified over a decade ago as a gene required for myoblast fusion (Doberstein et al., 1997), its molecular mechanism has been elusive. A previous study linked Blow with Kette based on their genetic interactions during myoblast fusion (Schröter et al., 2004). However, since both the WASP and Scar complexes are required for PLS formation (Sens et al., 2010), genetic interactions between members of the two complexes do not necessarily imply a biochemical link. Here, we demonstrate that Blow functions as a regulator of the WASP-Sltr complex. Our biochemical analyses demonstrate that Blow interacts with Sltr and competes with WASP for Sltr binding, which results in the dissociation of the WASP-Sltr complex. Interestingly, Blow binds to Sltr with a 7.2-fold lower affinity than the WASP-Sltr interaction. Thus, Blow functions to fine-tune the stability of the WASP-Sltr complex without causing an overall degradation of the WASP protein and reduction of actin polymerization. The transiently dissociated WASP protein (from the WASP-Sltr complex) by the Blow-Sltr interaction may rebind to a free Sltr protein if there is a high concentration of Sltr in the cytoplasm. Since Sltr, WASP, and Blow are all concentrated within the actin foci in FCMs of Drosophila embryos, it is conceivable that the WASP proteins dissociated from the WASP-Sltr complexes by Blow within the PLS will rapidly rebind to free Sltr proteins in the vicinity and promote additional rounds of branched actin polymerization.

**Blow Regulates Actin Dynamics by Modulating the Exchange Rate of WASP**

Previous studies of actin polymerization induced by vaccinia virus have demonstrated that the binding affinity between N-WASP and the barbed ends of F-actin is inversely correlated to the exchange rate of N-WASP and the motility of the virus (Weisswange et al., 2009). Thus, modulating the binding affinity between WASP and the barbed ends of F-actin directly regulates the dynamics of actin polymerization, likely by antagonizing filament capping. Our studies show that the transient dissociation of the WASP-Sltr complexes by Blow leads to increased dynamics of WASP-mediated actin polymerization within the PLSs during myoblast fusion in Drosophila. In blow mutant embryos, stabilized WASP-Sltr complexes appear to bind to the barbed ends of F-actin with a high affinity, which is likely to be mediated by a pair of protein-protein interactions – interaction between the WASP and G-actin, as well as between Sltr and F-actin (both the mammalian WIP and Drosophila Sltr have been shown to bind F-actin (Kim et al., 2007; Ramesh et al., 1997). Although Blow does not directly modulate either Sltr-F-actin interaction (Figure S2B) or WASP-G-actin interaction, by displacing Sltr from the WASP-Sltr complex in wild-type embryos, Blow indirectly decreases the binding affinity between WASP and the barbed ends of F-actin and increases the dynamics of actin polymerization. Thus, dissociation of the WASP-Sltr complex by a Sltr-binding protein represents a previously unrecognized mechanism by which dynamics of actin polymerization can be regulated in vivo. Although Blow does not have an apparent mammalian homolog based on its protein sequence, the interaction between Blow and human WIP, as well as the competitive binding between Blow and WASP to human WIP, raises the interesting possibility that an unidentified WIP-binding protein in mammals may regulate the dynamics of actin polymerization using a similar competitive binding mechanism to dissociate WASP-WIP complex.

**Actin Dynamics Is Required for the Invasiveness of the PLS**

Our FRAP analyses suggest that the dynamics of WASP exchange and actin polymerization, rather than the accumulated level of F-actin per se, is critical for the invasiveness of the FCM-specific PLSs during myoblast fusion. But how does the dynamics of WASP exchange impact the invasiveness of PLSs during myoblast fusion? Previous studies have revealed that an increased exchange rate of N-WASP at the barbed ends lead to increased filament capping, which makes available more free N-WASP to nucleate new branched actin filaments together with the Arp2/3 complex (Akin and Mullins, 2008; Weisswange et al., 2009). Increased F-actin branching, in turn, would produce shorter and stiffer filaments that have been proposed to be better suited to harness the free energy of actin polymerization to perform mechanical work (Mogilner and Oster, 1996), such as increasing the motility of virus particles in a host cell or promoting lamellipodia extension in a migratory cell. We suggest that in the case of myoblast fusion, an appropriate level of F-actin branching generates shorter and stiffer filaments that are required to promote the protrusion of the FCM plasma membrane into the founder cells in wild-type embryos (Figure 7A). In contrast, decreased exchange rate of WASP in fusion mutants, such as blow, results in the formation of longer and less densely packed actin filaments and a defect in FCM protrusion (Figure 7B). Thus, modulation of the stability of the WASP-WIP complex may represent a general mechanism in creating plasma membrane protrusions in a variety of cellular processes.

**EXPERIMENTAL PROCEDURES**

**Fly Genetics**

Fly stocks were obtained from the Bloomington Stock Center except for the following: w1118, strbr34/CyO,actin-lacZ (Kim et al., 2007); ketteJ4-48/TM6B; (Hummel et al., 2000); mid/TM3, fts-lacZ (Duan et al., 2001); sns40-49/CyO (Paululat et al., 1995); sns-GAL4 (Kocherakotak et al., 2008); rP298-GAL4 (Menon and Chia, 2001). Rescue crosses were performed by crossing blow1/CyO,actin-lacZ; UAS-blow females to GAL4, blow1/CyO,actin-lacZ males, in which GAL4 represents twi-GAL4, sns-GAL4, or rP298-GAL4; or by crossing blow1/CyO,actin-lacZ; UAS-blowJ173 to twi-GAL4, blow1/CyO,actin-lacZ, S102-blow, blow1/CyO, actin-lacZ, and S102-blowY378F, blow1/CyO,actin-lacZ flies were
generated by standard P-element mediated transformation. Mutant embryos were identified by the lack of α-β-gal staining. Transgene expression was confirmed by α-Blow staining. Two independent transgenes were tested for each rescue experiment. For the GFP diffusion assay, rP298-GAL4/Y; blow/+; CyO males were crossed with blow1, UAS-cytoGFP/CyO females. Mutant embryos were identified by α-MHC staining. For expressing GFP-actin in muscle cells, blow1/CyO;actin-lacZ; UAS-Act5C.GFP females were crossed with either rP298-GAL4/Y; blow/+;CyO;actin-lacZ or snr-GAL4;blow/+;CyO, actin-lacZ males.

Immunohistochemistry
Embryos were fixed in 4% formaldehyde/heptane for 20 min, devitellinized, and stored in methanol as described (Kim et al., 2007). Primary and secondary antibodies were added and incubated overnight at 4°C. For phalloidin staining, embryos were fixed in formaldehyde-saturated heptane (50/50 mix of 37% formaldehyde/heptane, shaken well and left overnight) for 1 hr at room temperature, then hand-devitellinized in PBST as described (Sens et al., 2010). FITC- or Alexa 568-conjugated phalloidin was added with both primary and secondary antibodies. See Supplemental Experimental Procedures for more details.

Molecular Biology
Constructs for S2 and S2R+ cell transfection and transgenic rescue experiments are described in Supplemental Experimental Procedures.

Cell Culture and Transfection
S2 cells were grown in Express Five SFM serum-free medium (GIBCO), and S2R+ cells were grown in Schneider’s Drosophila medium (GIBCO) containing 10% fetal bovine serum (GIBCO). Cells were transfected using Effectene (QIAGEN) according to the manufacturer’s instructions.

Biochemistry
For coimmunoprecipitation assays, expression constructs were transfected in S2R+ cells. Cells were harvested, washed with PBS, and incubated in NP40-Triton buffer (10 mM Tris [pH 7.4], 150 mM NaCl, 1 mM EDTA, 1% Triton X-100, and 0.5% NP40) containing 1 mM PMSF and protease inhibitor cocktail (Roche) for 30 min at 4°C with agitation. After centrifugation, the cleared supernatants were subjected to immunoprecipitation (IP) followed by western blot. Antibodies used for IP: mouse α-v5 (1:500; Invitrogen) and mouse α-FLAG (1:500; Sigma); for western blot: mouse α-HA-HRP (1:500; Santa Cruz), mouse α-FLAG-HRP (1:5000; Invitrogen), mouse α-FLAG-HRP (1:5000; Sigma), rat α-Str (1:2000; Kim et al., 2007), rabbit α-tubulin (1:1000; Cell Signaling), rabbit α-Blow (1:2000; this study), and guinea pig α-WASP (1:5000; Bogdan et al., 2005).

Binding affinities between Blow-Str and WASP-Str were measured by co-IP using purified proteins (see Supplemental Experimental Procedures for protein purification). In brief, FLAG-V5-Blow or FLAG-V5-WASP was mixed with 17.6 nM FLAG-Str in the binding buffer (10 mM Tris [pH 7.4, 150 mM NaCl, 1 mM EDTA, and 0.2% Tween-20) containing 1 mM PMSF and protease inhibitor cocktail (Roche), incubated at room temperature for 1 hr, followed by overnight incubation with agitation at 4°C. The solutions were then subjected to IP with α-v5 and western blot with α-Str. The relative amount of co-IPed Str was determined by the density of the Str bands on western blots measured by Adobe Photoshop CS4. The binding curves were plotted and the Kd values were calculated by the Prism software.

Fluorescence Recovery after Photobleaching
Fluorescent GFP-actin, GFP-WASP and Str-mCherry foci were visualized by a Zeiss EC Plan-Neofluar 40× 1.3 oil lens on a LSM 700 system. The solid 488 nm laser output was set to 2% to avoid photobleaching and phototoxicity. The pinhole was set to 1.0 AU and four frames were averaged per scan. Two prebleached images were first acquired to record the original intensity of the foci. Regions of interest (ROI) (randomly selected actin foci) were identified manually and quickly bleached to around 30% of its original intensity. Subsequently, images were acquired every 30 s using the settings described above. The fluorescence intensity of the pre- and postbleach ROI was determined using a flexible-size ROI, which was adjusted manually on every frame since the foci shape changed constantly, especially in wild-type embryos. An exponential decay equation $y = y_{\text{max}} + \left(y_{\text{max}} - y_{\text{min}}\right)e^{-kt}$ was used to fit the kinetic curve (Weisswange et al., 2009). The rate constant of recovery (k), the maximum recovery level (percentage recovery to the pre-bleach level), and the half-time ($t_{1/2} = \ln2/k$) were calculated from the fitted curves by the Prism software. Zeiss LSM Image Browser 4.2 (Carl Zeiss Microimaging) and Image J 1.41h (Wayne Rasband, NIH) were used to convert confocal images to movies (see Supplemental Experimental Procedures for additional information).

Transmission Electron Microscopy
HPF/FS fixation was performed as described (Zhang and Chen, 2008). A Bal-Tec device was used to freeze embryos. Freeze-substitution was performed using 1% osmium tetroxide and 0.1% uranyl acetate in 98% acetic acid and 2% methanol on dry ice. The embryos were embedded in EPON (Sigma). Conventional chemical fixation was performed as described (Zhang and Chen, 2008). In Brief, embryos were fixed in heptane equilibrated with 25% glutaraldehyde/10% acrolein in 0.1 M sodium-cacodylate buffer (pH 7.4). Postfixation was performed with osmium tetroxide and embryos were stained with 1% uranyl acetate before embedding in EPON. Lead staining was done as described (Zhang and Chen, 2008) and images were acquired on a Philips CM120 TEM.

SUPPLEMENTAL INFORMATION
Supplemental Information includes Supplemental Experimental Procedures, three figures, two tables, and nine movies and can be found online at doi:10.1016/j.devcel.2011.04.007

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REFERENCES


Supplemental Information

Competition between Blown Fuse and WASP for WIP Binding Regulates the Dynamics of WASP-Dependent Actin Polymerization In Vivo

Peng Jin, Rui Duan, Fengbao Luo, Guofeng Zhang, Sabrina N. Hong, and Elizabeth H. Chen

Inventory of Supplemental Information

**Figure S1, related to Figure 2.** Additional characterization of Blow-Crk and Blow-Sltr interactions.

**Figure S2, related to Figure 3.** Purification of Sltr, WASP and Blow and their interaction with F-actin.

**Figure S3, related to Figure 3.** Blow interacts with human WIP (hWIP).

**Table S1, related to Figure 5.** Exchange rate and recovery percentage of GFP-actin, GFP-WASP and Sltr-mCherry in wild-type and mutant embryos.

**Table S2, related to Figure 6.** F-actin foci invasion in wild-type and mutant embryos.

**Movie S1, related to Figure 1.** Time-lapse imaging of Blow-mCherry and GFP-actin in wild-type embryo.

**Movie S2, related to Figure 4.** Time-lapse imaging of GFP-actin in blow mutant embryo.

**Movie S3-5, related to Figure 5.** FRAP of GFP-actin in wild-type, blow and kette mutant embryos.

**Movie S6 and S7, related to Figure 5.** FRAP of GFP-WASP in wild-type and blow mutant embryos.

**Movie S8 and S9, related to Figure 5.** FRAP of Sltr-mCherry in wild-type and blow mutant embryos.

**Supplemental Experimental Procedures**

**Supplemental References**
Figure S1
Figure S2
Figure S3
Supplemental Figure Legends

Figure S1, related to Figure 2. Additional characterization of Blow-Crk and Blow-Sltr interactions.
In all experiments, extracts from S2R+ cell transfected with the indicated plasmids (listed above the gels) were immunoprecipitated (IP) and probed with the indicated antibodies.
(A) Blow specifically interacts with the SH2 domain of Crk. CrkR38K: full length Crk carrying a point mutation in the SH2 domain (unable to bind phosphotyrosine; Tanaka et al., 1995). Note that no interaction was detected between Blow and CrkR38K, further demonstrating that Blow specifically binds the SH2 domain of Crk.
(B) Sltr interacts with the full length, as well as the SH3-1 domain, of Crk.
(C) Blow interacts with endogenous Sltr in S2R+ cells.
(D) Blow carrying either an N- (BlowΔN99) or a C-terminus deletion (BlowΔC173), neither of which interacts with Sltr, binds Crk normally.
(E) Blow interacts with a C-terminal fragment of Sltr (SltrC176), which contains the last 176 amino acids of the Sltr protein.

Figure S2, related to Figure 3. Purification of Sltr, WASP and Blow and their interaction with F-actin.
(A) Affinity-purified Sltr, WASP and Blow proteins were analyzed by SDS-PAGE and the gel was stained with Coomassie Blue. See Figure 3F and G for the saturation binding curves of WASP-Sltr and Blow-Sltr obtained using these purified proteins, respectively.
(B) Blow does not interfere with the Sltr-F-actin interaction. Purified proteins as indicated above the gels were incubated with F-actin and subjected to co-sedimentation assays. Note that Blow does not bind to F-actin (lane 1), whereas Sltr binds to F-actin with a high affinity (lane 3). Although Blow can be co-precipitated by Sltr (lane 5), it does not affect the Sltr-F-actin interaction (compare lane 3 and 5). Similarly, WASP does not affect the Sltr-F-actin interaction (compare lane 3 and 4).

Figure S3, related to Figure 3. Blow interacts with human WIP (hWIP).
(A) Extracts from S2R+ cell transfected with the indicated plasmids (listed above the gels) were immunoprecipitated (IP) and probed with the indicated antibodies. Note the interaction between hWIP and the full-length Blow (lane 3), as well as the lack of interaction between hWIP and either the N- (BlowΔN99) (lane 5) or C-terminal truncation (BlowΔC173) (lane 6) of Blow, both of which abolish Blow-Sltr binding. Also note that the addition of Drosophila WASP interferes with the Blow-hWIP interaction (lane 4).
(B) Blow interacts with Sltr with a higher affinity than it does with hWIP. In both (A) and (B), 10% of the cell lysate was loaded to examine the input of hWIP (A) or Sltr (B). The rest of the cell lysate was used for co-IP. Note that Sltr was expressed at a lower level compared to hWIP, but Blow (expressed at a similar level in A and B) could co-IP a larger amount of Sltr than hWIP.
Table S1, related to Figure 5. Recovery percentage and exchange rate and recovery percentage of GFP-actin, GFP-WASP and Sltr-mCherry in wild-type and mutant embryos.

### FRAP of GFP-actin

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<th>Half-time (s)</th>
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<td>Max</td>
<td>Average</td>
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<tr>
<td>w1118</td>
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<td></td>
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<td>blowa</td>
<td>31.1</td>
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<td>blow (twi-GAL4::UAS-Blow)</td>
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<td>41.3</td>
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<tr>
<td>kette</td>
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<td>100.5</td>
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### FRAP of GFP-WASP

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<tr>
<td>blowb</td>
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### FRAP of Sltr-mCherry

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<td>blowb</td>
<td>31.6</td>
<td>64.1</td>
<td>45.5 ± 12.1</td>
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a There is a significant (p<0.0001) difference in the average percentage recovery and recovery half-time of GFP-actin between wild-type embryos and blow mutant (or blow mutant over-expressing BlowΔC173), determined by the two-tailed unpaired student’s t test.

b There is a significant (p<0.0001) difference in the average percentage recovery and recovery half-time of GFP-WASP or Sltr-mCherry between wild-type and blow mutant embryos, determined by the two-tailed unpaired student’s t test.
Table S2, related to Figure 6. F-actin foci invasion in wild-type and mutant embryos.

<table>
<thead>
<tr>
<th>Genotype</th>
<th>Percentage (%)</th>
<th>Mean ± SD (µm)(^a)</th>
<th>Min (µm)</th>
<th>Max (µm)</th>
<th>p-value</th>
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<tr>
<td>wt</td>
<td>35 (18/52)</td>
<td>1.0 ± 0.4</td>
<td>0.4</td>
<td>1.9</td>
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<td>17</td>
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<tr>
<td>kette</td>
<td>40 (25/63)</td>
<td>0.9 ± 0.3</td>
<td>0.4</td>
<td>2.0</td>
<td>0.42</td>
<td>30</td>
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<tr>
<td>blow</td>
<td>9 (6/69)(^b)</td>
<td>0.6 ± 0.1</td>
<td>0.5</td>
<td>0.8</td>
<td>0.036</td>
<td>6</td>
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</table>

the smallest depth that could be reliably measured by confocal microscopy was 0.3 µm.

\(^a\) The average depth of invasion in stage 14 embryos. Statistical comparisons were made between wild type (wt) and fusion mutants.

\(^b\) The percentage of invasive F-actin foci in blow mutant embryos is much lower than that of wild-type embryos, indicating that the PLSs in blow mutant are less invasive than wild type.
Supplemental Movie Legends

Movie S1, related to Figure 1. Time-lapse imaging of Blow-mCherry and GFP-actin in wild-type embryo.
Time lapse sequence of a stage 14 wild-type embryo in which Blow-mCherry and GFP-actin were co-expressed in all muscle cells (driven by twi-GAL4). Left: GFP-actin; middle: Blow-mCherry; right: merge. Note the colocalization of mCherry- and GFP-positive foci (indicated by arrow and arrowhead) during their lifespan. 24 frames were taken at 1 min intervals.

Movie S2, related to Figure 4. Time-lapse imaging of GFP-actin in a blow mutant embryo.
Time lapse sequence of a stage 14 blow mutant embryo in which GFP-actin was expressed in all muscle cells (driven by twi-GAL4). Note the persistence of the GFP-positive foci (one is indicated by arrow) and their gradual increase in size. 20 frames were taken at 3 min intervals.

Movie S3-5, related to Figure 5. FRAP of GFP-actin in wild-type, blow and kette mutant embryos.
GFP-actin was expressed in all muscle cells (driven by twi-GAL4) in wild-type (Movie S3), blow (Movie S4) and kette (Movie S5) embryos. In each movie, a GFP-actin-positive focus (arrow) in a stage 14 embryo was photobleached to approximately 30% of its original intensity and subsequently imaged every 30 sec. Four frames were averaged per scan. Note the rapid recovery of the GFP signal to the prebleach level in wild-type (Movie S3) and kette (Movie S5) embryos, and the slow recovery of the GFP signal in the blow mutant embryo (Movie S4), which never reached the prebleach level. Arrowhead (Movie S4) and star (Movie S5) indicate control actin foci that were not photobleached, respectively.

Movie S6 and S7, related to Figure 5. FRAP of GFP-WASP in wild-type and blow mutant embryos.
GFP-WASP was expressed in all muscle cells (driven by twi-GAL4) in wild-type (Movie S6) and blow (Movie S7) embryos (stage 14). Note the rapid recovery of the GFP signal to the prebleach level in the wild-type embryo (Movie S6) (similar to GFP-actin in Movie S3), and the slow recovery of the GFP signal in the blow mutant embryo (Movie S7), which never reached the prebleach level (similar to GFP-actin in Movie S4).

Movie S8 and S9, related to Figure 5. FRAP of Sltr-mCherry in wild-type and blow mutant embryos.
Sltr-mCherry was expressed in all muscle cells (driven by twi-GAL4) in wild-type (Movie S8) and blow mutant (Movie S9) embryos (stage 14). Note the rapid recovery of the mCherry signal to the prebleach level in the wild-type embryo (Movie S8) (similar to GFP-actin in Movie S3 and GFP-WASP in Movie S6), and the slow recovery of the mCherry signal in blow mutant embryo (Movie S9), which never reached the prebleach level (similar to GFP-actin in Movie S4 and GFP-WASP in Movie S7).
Supplemental Experimental Procedures

Immunohistochemistry
The following antibodies were used for immuno-staining of embryos: rabbit α-MHC (1:1000) (Kiehart and Feghali, 1986), rabbit α-Lmd (1:800) (Duan et al., 2001), guinea pig α-WASP (1:200) (Bogdan et al., 2005), guinea pig α-Ants (1:1000) and guinea pig α-Duf (1:250) (Sens et al., 2010), rat α-Sltr (1:30) (Kim et al., 2007), mouse α-GFP (1:500) (Invitrogen), and mouse α-β-gal (Promega, 1:1000). A rabbit α-Blow antibody was generated against a C-terminal peptide, GCGPSPDRLKKAAKKSYSN (Bio-Synthesis), and used at 1:250. This antibody did not detect any signal in blow mutant embryos (data not shown). Secondary antibodies used at 1:300 were: FITC-, Cy3-, and Cy5-conjugated (Jackson Immunoresearch) and biotinylated antibodies (Vector Laboratories) made in goat. The Vectastain ABC kit (Vector Laboratories) and the TSA system (Perkin Elmer) were used to amplify the fluorescent signals. FITC-phalloidin (Sigma, 1 mg/ml) was diluted to 20 μM in ethanol and used at 1:250. Alexa 568-phalloidin (Molecular Probes) was used at 1:300.

S2 cells were fixed with 4% paraformaldehyde, washed, and stained with the following reagents diluted in PBSBT: rat α-Sltr (1:500) (Kim et al., 2007), rabbit α-Blow (1:500; this study), and FITC-conjugated phalloidin (Sigma). Secondary FITC- or Cy5-conjugated antibodies were used at 1:500 (Jackson Immunoresearch).

Molecular biology
Constructs for S2R+ cell transfection: full-length blow cDNA was subcloned in-frame into the pAc-V5 His expression vector (Invitrogen). Full-length sltr, wasp, and crk were amplified by PCR with the 5’ primers containing a FLAG tag and subcloned into a pAc vector.

Constructs for protein purification: full-length blow and wasp were amplified by PCR with the 5’ primers containing FLAG and V5 tags and subcloned into a pAc vector.

Constructs for transgenic rescue experiments: full-length blow was subcloned into the transformation vector pUAST. Constructs containing deletions or point mutations were prepared using standard PCR procedures (Stratagene) to introduce changes into the full-length clone before being subcloned into pUAST.

Constructs for time-lapse imaging:
Blow-mCherry: full-length blow was C-terminally fused to mCherry and subcloned into the pUAST vector.

GFP-WASP: full-length wasp was N-terminally fused to GFP, and the GFP-WASP fusion protein was subcloned into the pUAST vector.

All constructs were verified by sequencing analysis.

Protein purification
FLAG-Sltr, FLAG-V5-WASP and FLAG-V5-Blow were expressed in S2R+ cells. Cells were harvested 48 hours after transfection. Following centrifugation, the cleared supernatants were incubated with α-FLAG M2 beads (Sigma) at 4°C for 2 hours. Subsequently, the beads were washed with the NP40-Triton buffer for 5 times and the tagged proteins were eluted by elution buffer (500 mM NaCl and 50mM Tris buffer, pH=7.6) containing the FLAG peptide (200 ng/ul).

F-actin co-sedimentation assay
F-actin was polymerized from G-actin according to the manufacturer’s protocol (Cytoskeleton, Inc.). F-actin (20 μl) was incubated with purified Sltr, WASP and Blow proteins at the final concentrations of 110 nM, 110 nM and 730 nM, respectively, in the buffer containing 5 mM Tris pH 8.0, 0.2 mM CaCl2, 5 mM KCl, 0.2 mM MgCl2, and 0.2 mM ATP for 30 min at room temperature. The incubation mix was then centrifuged at 21,000g for 1hr at 4°C. After carefully removing the supernatants, the pellets were subjected to SDS–PAGE and western blot with specific antibodies indicated in Figure S2.

FACS
To examine whether overexpression of Blow and Sltr affects endogenous WASP, S2 cells were transfected with plasmids indicated in Fig. 3C and sorted using a MoFlo MLS flow cytometer (Beckman Coulter). The GFP positive cells were collected and lysed for western analysis.
**Time-lapse imaging**

Embryos were collected, dechorionated with 50% bleach, thoroughly washed, and gently aligned onto the acid-free double-sided tape (Therm O Web), which keeps embryos from rolling and drifting. Subsequently, embryos were covered with a thin layer of Halocarbon oil 700/27 (2:1; Sigma), which allows oxygen exchange and keeps embryos alive. Fluorescent foci were imaged and tracked by confocal microscope (Zeiss LSM510). Image J 1.41h (Wayne Rasband, NIH) was used to convert confocal images to movies. For time-lapse imaging with Blow-mCherry and GFP-actin simultaneously in wild-type embryos, twi-GAL4, UAS-GFP-actin males were crossed to UAS-Blow-mCherry females.

**Fly genetics for FRAP**

FRAP of GFP-actin foci –

(1) in wild-type: twi-GAL4 X UAS-GFP.Act5C (III)
(2) in blow mutant: blow¹/Cyo,actin-lacZ; UAS-GFP.Act5C (III) X twi-GAL4, blow¹/Cyo,actin-lacZ
(3) in kette mutant: UAS-GFP.Act5C (II); kette/+ X twi-GAL4/++; kette/+ 
(4) in blow mutant overexpressing BlowΔC173: blow¹, UAS-BlowΔC173/Cyo; UAS-GFP.Act5C (III) X twi-GAL4, blow¹/Cyo, actin-lacZ

FRAP of GFP-WASP foci –

(1) in wild-type: twi-GAL4 X UAS-GFP-WASP
(2) in blow mutant: blow¹, UAS-GFP-WASP/Cyo X twi-GAL4, blow¹/Cyo, actin-lacZ

FRAP of Sltr-mCherry foci –

(1) in wild-type: twi-GAL4 X UAS-Sltr-mCherry
(2) in blow mutant: blow¹, UAS-Sltr-mCherry/Cyo X twi-GAL4, blow¹/Cyo, actin-lacZ

Mutant embryos were identified by the presence of a large number of mononucleated myoblasts visualized by fluorescent signal in the embryo.
Supplemental References