Developmental Cell
Article

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. Chen[1,*]
[1]Department of Molecular Biology and Genetics, Johns Hopkins University School of Medicine, Baltimore, MD 21205, USA
[2]Laboratory of Bioengineering and Physical Science, National Institute of Biomedical Imaging and Bioengineering, National Institute of Health, Bethesda, MD 20892, USA
[3]These authors contributed equally to this work
*Correspondence: echen@jhmi.edu
DOI 10.1016/j.devcel.2011.04.007

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.,

2001). 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 coimmunoprecipitation 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

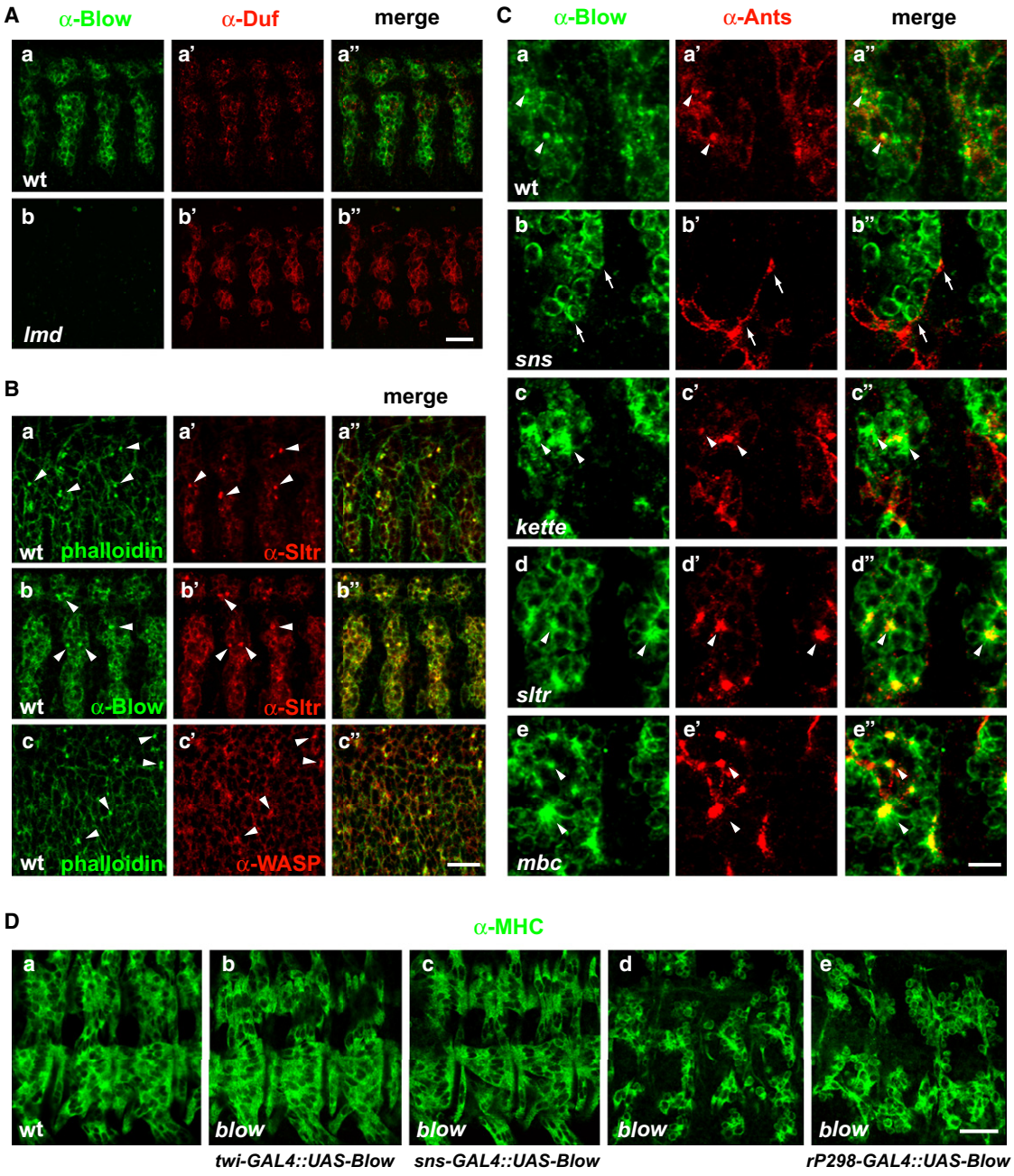


Figure 1. Blow is an FCM-Specific Protein that Colocalizes with the WASP-Sltr Complex at Sites of Fusion

(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-Antisocial/Rols7 (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.

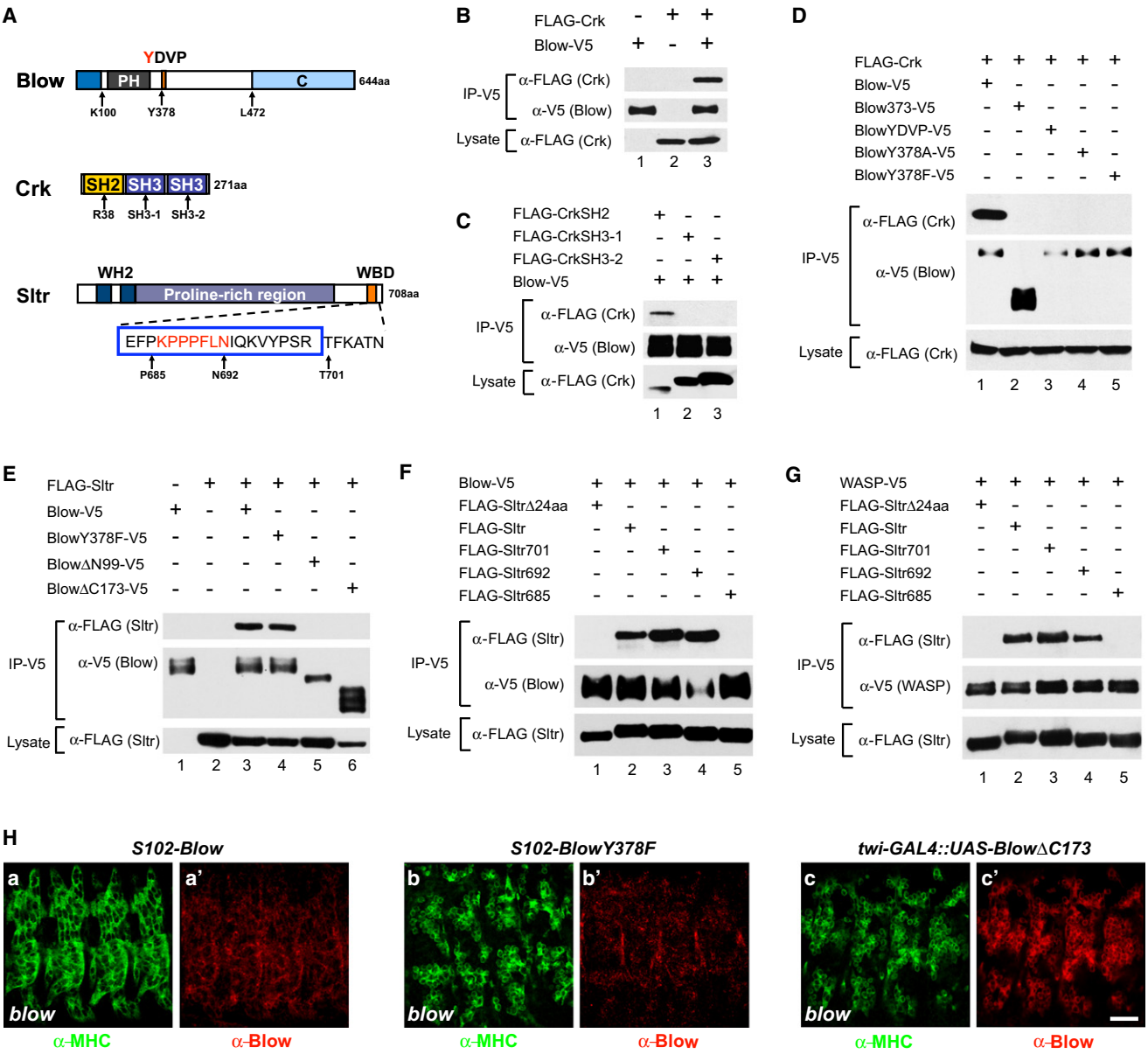


Figure 2. Blow Interacts with Crk and Sltr

(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.

(B) Interaction between Blow and Crk.

(C) Interaction between Blow and the SH2, but not the SH3, domains of Crk. Blow appears as a single band in 10% gel (B) but multiple bands in 6% gel (C).

(D) The YDVP motif of Blow mediates the Blow-Crk interaction and Y378 is required for this interaction. Blow373: Blow missing C-terminal amino acids (aa) 374-644; BlowYDVP: full length Blow carrying point mutations that changed YDVP to AAAA.

(E) Interaction between Blow and Sltr requires both the N- and C-terminal regions of Blow. BlowΔN99: Blow missing N-terminal aa 1–99; BlowΔC173: Blow missing C-terminal aa 472–644. Note that BlowY378F, which does not bind Crk, still binds Sltr (lane 4).

(F and G) Both Blow (F) and WASP (G) interact with the WASP-binding domain (WBD) of Sltr. SltrΔ24aa: Sltr carrying an internal deletion of 24 aa including the WBD (sequence shown in A); Sltr701, Sltr692 and Sltr685: Sltr missing aa C-terminal to T701, N692 and P685, respectively (marked in A).

(H) Blow-Crk and Blow-Sltr interactions are functionally required in vivo. Embryos double labeled with α-MHC (green) and α-Blow (red). Expression of wild-type Blow (a and a′), but not BlowY378F (b and b′), under the control of a tubulin promoter (S102) rescued the fusion defect in *blow* mutant embryos. Expression of BlowΔC173 with the *twi-GAL4* driver did not rescue the fusion defect (c and c′). Bar: 20 μm.

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) (BlowΔN99) or C-terminal 173 aa (BlowΔC173) 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) (Volkman 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-taggged Sltr to the co-IP mixture. We found that overexpressed Blow reduced the amount of endogenous WASP coimmunoprecipitated 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 μm^2, n = 53; Figures 4Ab–4Ab″ and 4B) compared with those in wild-type embryos (1.7 ± 0.6 μm^2, 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

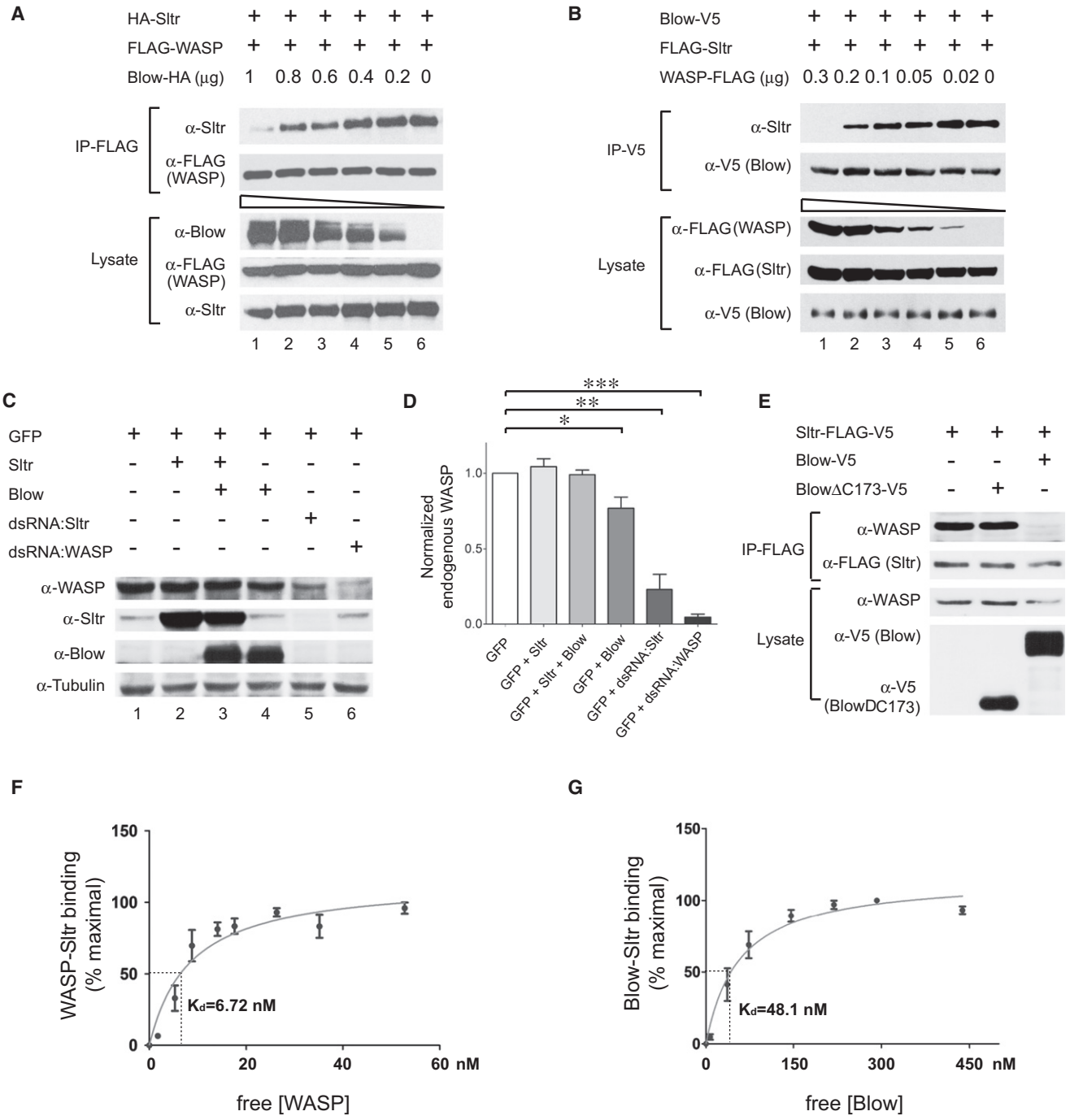


Figure 3. Blow Competes with WASP for Sltr Binding

(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).

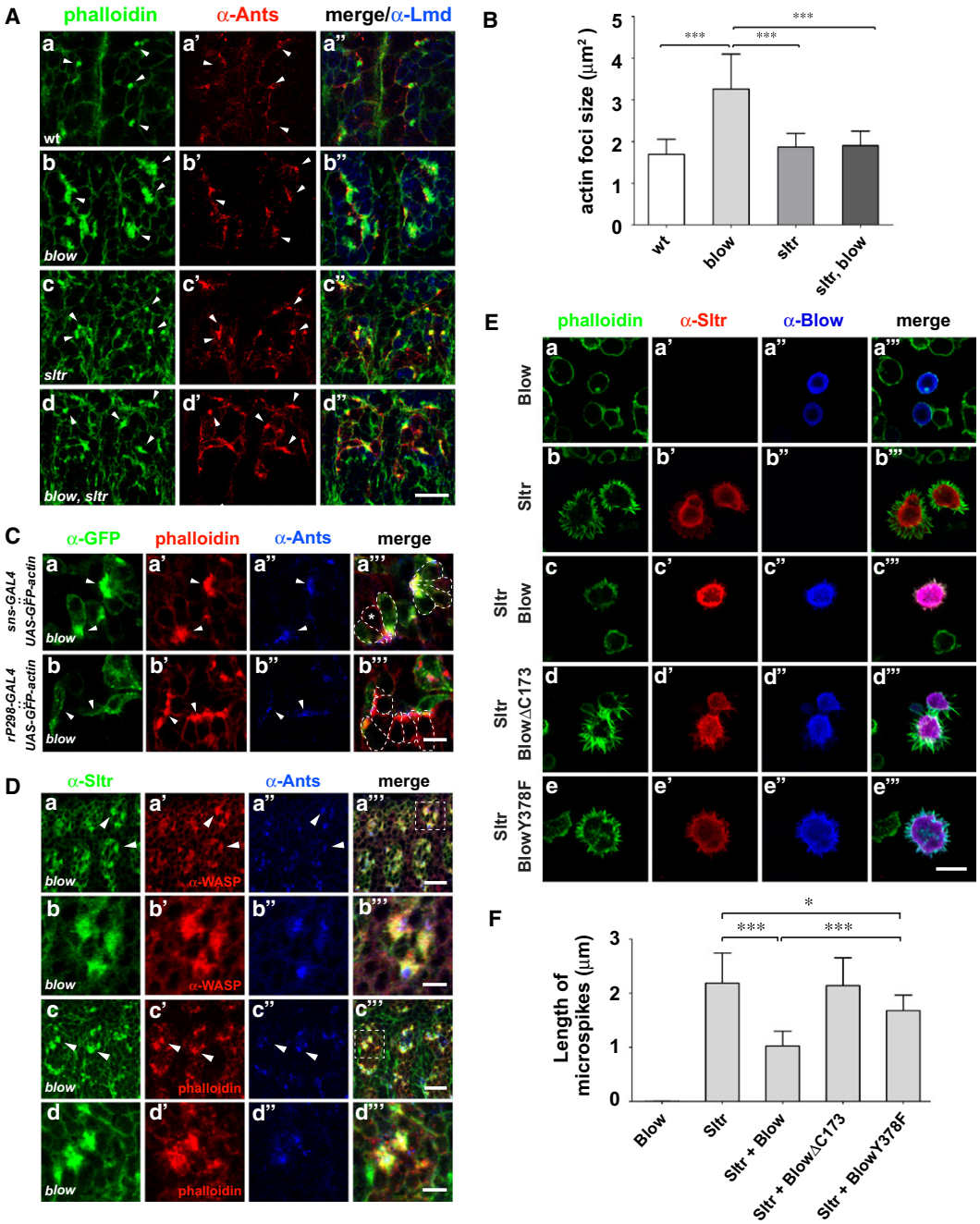


Figure 4. Blow Functions through the WASP-Sltr Complex to Regulate Actin Polymerization

(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–b″), compared with wild-type (a–a″) or *sltr* mutant (c–c″) embryos. Note that the F-actin foci in *blow,sltr* double mutant (d–d″) 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

in FCMs colocalized with the phalloidin-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-Sltr complex in promoting actin polymerization within these abnormal foci. To determine whether Blow acts through the WASP-Sltr 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 μm^2, n = 44; Figures 4Ad–4Ad″ and 4B) is similar to that of *sltr* (1.9 ± 0.3 μm^2, n = 38; Figures 4Ac–4Ac″ and 4B), but not *blow* mutant embryos (3.3 ± 0.8 μm^2, 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-Sltr complex to regulate actin polymerization in vivo.

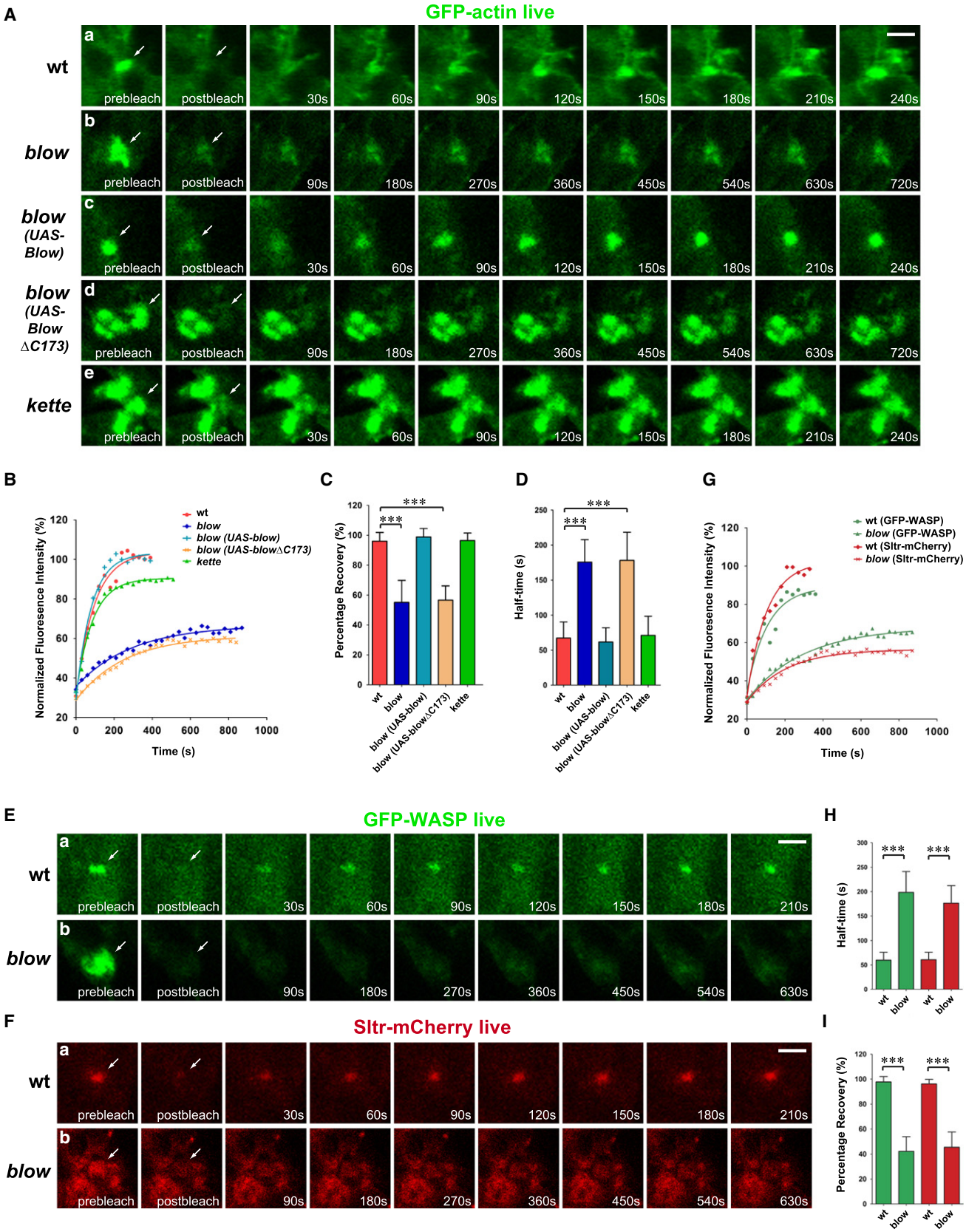
Blow Regulates the Exchange Rate of G-Actin within the PLS at Sites of Myoblast Fusion

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-positive 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 the 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, photobleaching 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 5H; 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-Sltr complex, we suggest that increased stability of the WASP-Sltr complex in *blow* mutant embryos leads to increased occupancy of barbed ends of F-actin by the WASP-Sltr 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-Sltr complex is critical in regulating the actin polymerization dynamics in vivo.

Blow (a–a‴), induced actin-filled microspike formation. Note that coexpression of Sltr and Blow (c–c‴), but not Sltr and BlowΔC173 (d–d‴), reduced the length of the microspikes. Coexpression of Sltr and BlowY378F partially reduced the length of the microspikes (e–e‴). (F) Quantification of the length of the microspikes. Statistical analyses were performed by unpaired two-tail student's t test (*$p < 0.05$; **$p < 0.01$; ***$p < 0.001$). Error bars: standard deviation.
Bars: (A), 20 μm; (C, D b–b‴ and d–d‴), 5 μm; (D a–a‴ and c–c‴), 20 μm; (E), 10 μm.


A
GFP-actin live
wt
blow
blow (UAS-Blow)
blow (UAS-Blow ΔC173)
kette
prebleach
postbleach
30s
60s
90s
120s
150s
180s
210s
240s
270s
360s
450s
540s
630s
720s
B
Normalized Fluoresence Intensity (%)
Time (s)
wt
blow
blow (UAS-blow)
blow (UAS-blowΔC173)
kette
C
Percentage Recovery (%)
***
D
Half-time (s)
G
Normalized Fluoresence Intensity (%)
Time (s)
wt (GFP-WASP)
blow (GFP-WASP)
wt (Sltr-mCherry)
blow (Sltr-mCherry)
E
GFP-WASP live
F
Sltr-mCherry live
H
Half-time (s)
I
Percentage Recovery (%)

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–4Eb‴ and 4F), 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–4Ea‴ and 4F), coexpression of Blow and Sltr reduced the length of Sltr-induced microspikes (Figures 4Ec–4Ec‴ and 4F). 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–4Ed‴ and 4F). This result, together with the presence of elongated actin filaments in *blow* mutant embryos (Figures 6A 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 4Ee–4Ee‴ and 4F). 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–6Aa″; Table S2) (Sens et al., 2010). In contrast, only 9% (6/69) of the actin foci in *blow* mutant embryos appeared invasive but with reduced depth (Figures 6Ab–6Ab″; Table S2). Based on these results, we concluded that actin foci invasion is defective in *blow* mutant embryos.

Next, we confirmed the defect in actin foci invasion by electron microscopy (EM). In contrast to wild-type embryos in which each FCM-derived actin focus extends an average of ~4.3 finger-like protrusions (with a maximum depth of 2.0 μm) into the apposing founder cell (Figure 6Ba) (Sens et al., 2010), the actin foci in *blow* mutant embryos exhibited fewer (~1.4 fingers per actin focus, n = 10), shorter and misshaped invasive fingers (with a maximum depth of 0.9 μm) (Figures 6Bc and 6Bd). However, in *kette* mutant embryos, which showed a similar G-actin exchange rate as in wild-type embryos, 40% (25/63) of the F-actin foci were invasive with similar depth as wild-type foci visualized by confocal microscopy (Table S2) (Sens et al., 2010). In addition, EM analysis also revealed invasive fingers in *kette* mutant embryos with wild-type depth and morphology (Figure 6Bb; data not shown). We conclude from these experiments that the invasiveness of the actin foci within the PLSs is dependent on the dynamics of actin polymerization, rather than the mere presence or the size of the F-actin foci.

We have shown previously that invasion of the FCM-specific PLS into the founder cells is required for fusion pore formation (Sens et al., 2010). 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 PLS 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 monitored 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 and 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.

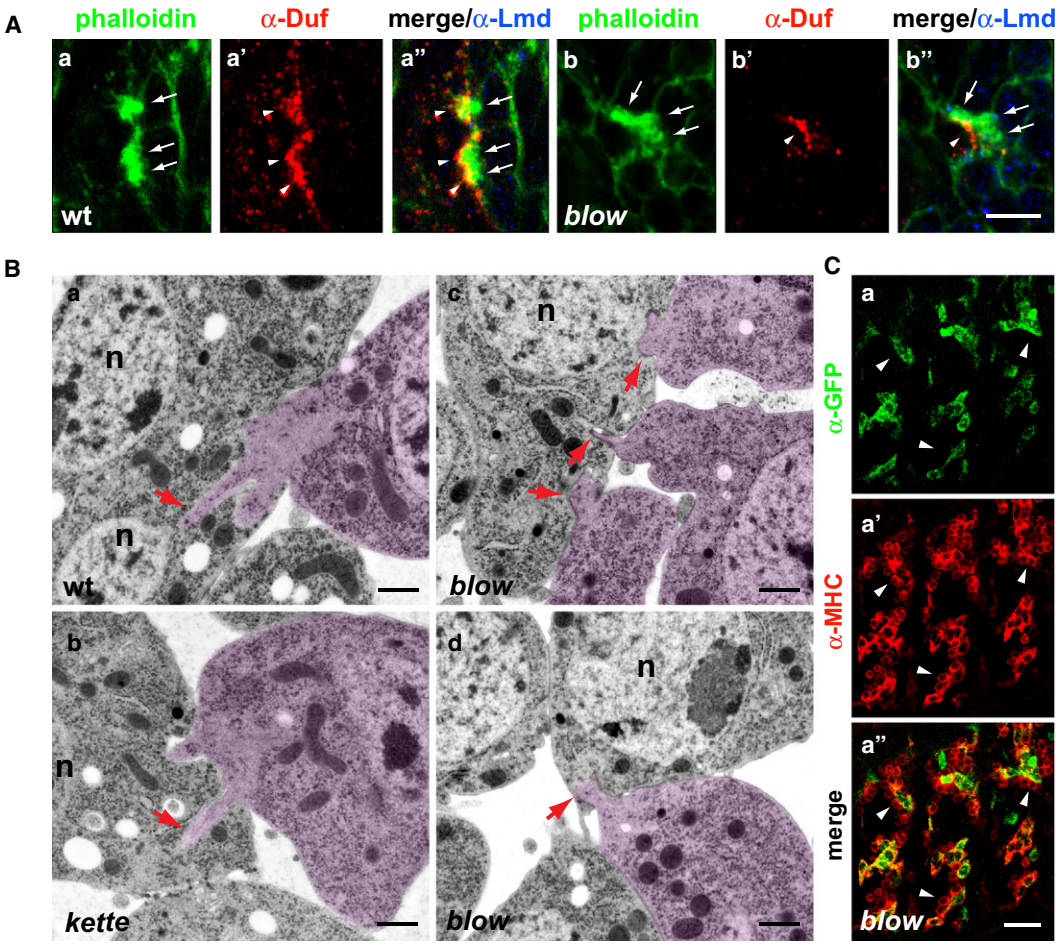


Figure 6. Defective PLS Invasion and Failure of Fusion Pore Formation in Embryos with Decreased G-Actin Exchange within the PLS

(A) F-actin foci in *blow* mutant embryos are defective in founder cell invasion. Stage 14 embryos labeled with phalloidin (green), α-Duf (red), and α-Lmd (blue). (a–a″) Wild-type (wt) embryo. Three dense F-actin foci (arrows) invading a founder cell. Arrowheads indicate the inward curvatures on the founder cell membrane. (b–b″) Noninvasive F-actin foci in a *blow* mutant embryo (arrows). Note the filamentous and elongated morphology of the F-actin structure. See also Table S2.

(B) F-actin foci invasion visualized by electron microscopy. (a) Stage 14 wild-type (wt) embryo. An FCM (pseudocolored pink) projects multiple F-actin-enriched invasive fingers (the longest one indicated by arrow) into the adjacent binucleated myotube. The F-actin-enriched area at the protruding tip of the FCM is identified by the light gray coloration and lack of ribosomes and intracellular organelles (Sens et al., 2010). (b) Stage 14 *kette* mutant embryo. Invasive fingers are morphologically similar to wild-type. (c and d) Stage 14 *blow* mutant embryos. Each FCM projects one or two small protrusions (determined by serial sections) into the opposing founder cell. Despite the overall enlarged F-actin foci size revealed by confocal microscopy, the protruding tips of FCMs contain more ribosomes than wild-type or *kette* embryos revealed by EM, indicating the presence of a loosely packed F-actin network. n: founder cell nucleus.

(C) Lack of GFP diffusion between founder cells/myotubes and adherent FCMs in *blow* mutant embryos. A cytoplasmic GFP was expressed in founder cells with *rP298-GAL4* in stage 14 *blow* mutant embryos, labeled by α-GFP (green) and α-MHC (red). Note that GFP remained in the elongated founder cells/miniature myotubes without diffusing into the adherent, mononucleated FCMs (several examples indicated by arrowheads).

Bars: (A), 5 μm; (B), 500 nm; (C), 30 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 BlowΔN99 (or BlowΔC173) (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, co-transfecting *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

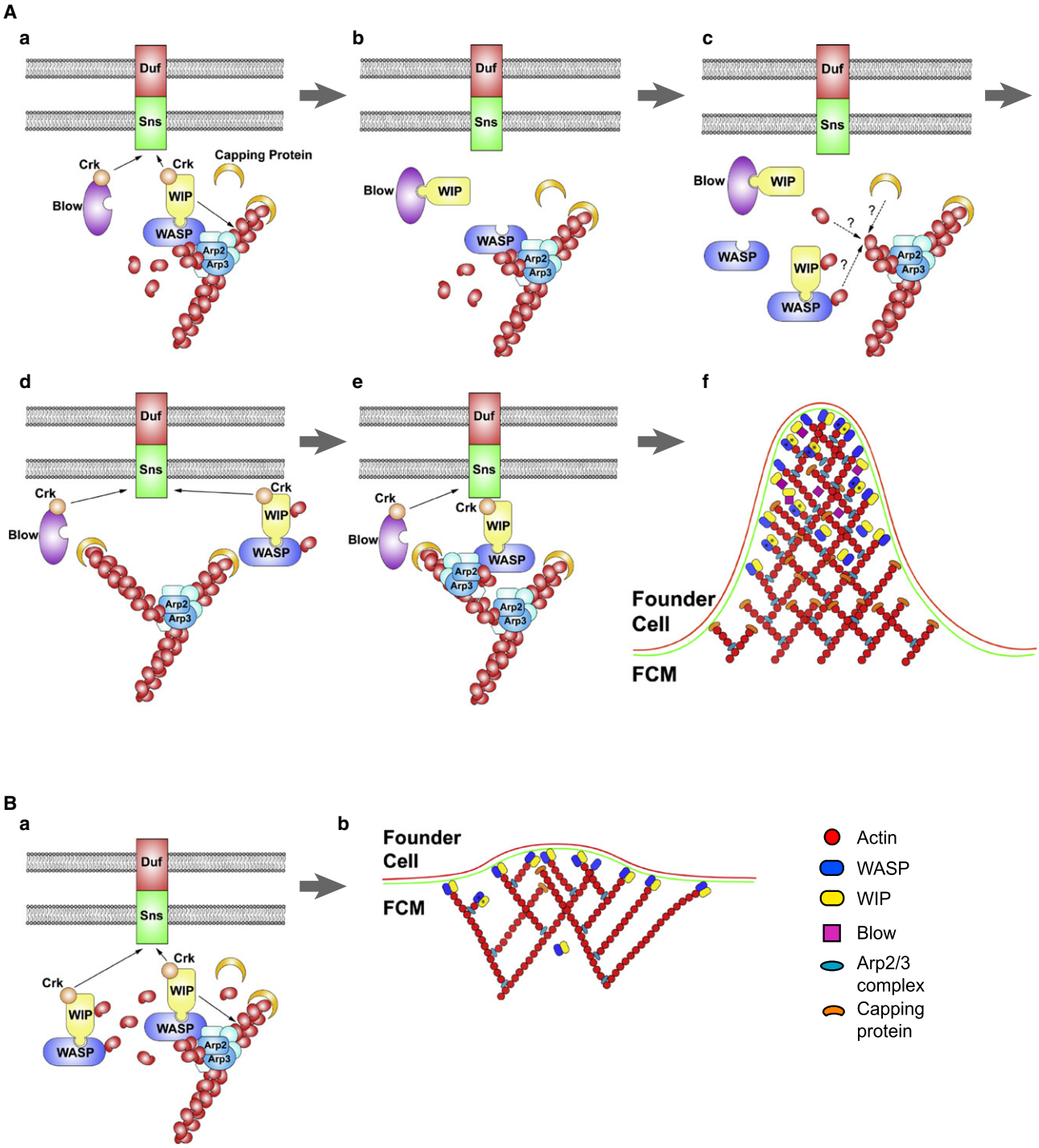


Figure 7. A Model Describing the Regulation of Actin Polymerization Dynamics by Blow

(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 Sns) 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 temporarily 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: w^{1118}, $sltr^{S1946}$/*CyO,actin-lacZ* (Kim et al., 2007); $kette^{J4\text{-}48}$/*TM6B* (Hummel et al., 2000); *lmd/TM3, ftz-lacZ* (Duan et al., 2001); $sns^{40\text{-}49}$/*CyO* (Paululat et al., 1995); *sns-GAL4* (Kocherlakota et al., 2008); *rP298-GAL4* (Menon and Chia, 2001).

Rescue crosses were performed by crossing $blow^{1}$/*CyO,actin-lacZ; UAS-blow* females to *GAL4,* $blow^{1}$/*CyO,actin-lacZ* males, in which *GAL4* represents *twi-GAL4*, *sns-GAL4*, or *rP298-GAL4*; or by crossing $blow^{1}$/*CyO,actin-lacZ; UAS-blow*Δ*C173* to *twi-GAL4,* $blow^{1}$/*CyO,actin-lacZ*. *S102-blow*, $blow^{1}$/*CyO*, *actin-lacZ*, and S102-blowY378F, $blow^{1}$/*CyO,actin-lacZ* flies were

(B) In *blow* mutant embryos, the WASP-WIP complex is stably bound to the barbed ends of F-actin, protecting them from the capping protein. The occasional dissociation of the WASP-WIP complexes from the barbed ends likely results in filament elongation rather than capping, due to the increased concentration of G-actin continuously recruited by WIP and WASP to muscle cell contact sites. Infrequent capping and scarce initiation of new F-actin branches result in the formation of longer and more sparsely packed actin filaments, which are not mechanically suitable for force generation required for membrane protrusion.

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^{1}/CyO* males were crossed with *blow^{1}, UAS-cytoGFP/CyO* females. Mutant embryos were identified by α-MHC staining. For expressing GFP-actin in muscle cells, *blow^{1}/CyO,actin-lacZ; UAS-Act5C.GFP3* females were crossed with either *rP298-GAL4/Y; blow^{1}/CyO,actin-lacZ* or *sns-GAL4,blow^{1}/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 (1:5000; Santa Cruz), mouse α-V5-HRP (1:5000; Invitrogen), mouse α-FLAG-HRP (1:5000; Sigma), rat α-Sltr (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-Sltr and WASP-Sltr 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-Sltr 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-Sltr. The relative amount of co-IPed Sltr was determined by the density of the Sltr bands on western blots measured by Adobe Photoshop CS4. The binding curves were plotted and the Kd values were calculated by the Prism software.

Confocal Imaging of Fixed Samples

Images were obtained on a LSM 510 Meta confocal microscope with Fluar 40×, 1.3NA Oil and Plan-Apochromat 100×, 1.4NA Oil DIC objectives using Argon 458,477,488,514 nm; HeNe 543nm; and HeNe 633 nm lasers, and the META detector. The pinhole was set to 1.0 AU for each channel and Z-stacks were collected at 1.0 μm intervals for 40× magnification and 0.5 μm intervals for 100×. Images were acquired with LSM software and processed using Adobe Photoshop CS4.

Time-Lapse Imaging

Time-lapse imaging was carried out as previously described (Sens et al., 2010). See Supplemental Experimental Procedures for more details.

Fluorescence Recovery after Photobleaching

Fluorescent GFP-actin, GFP-WASP and Sltr-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 $y = y_{min} + (y_{max}\text{-}y_{min})(1\text{-}e^{-kt})$ 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 prebleach 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% acetone 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 Briefly, 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.

ACKNOWLEDGMENTS

We thank Drs. S. Bogdan, D. Kiehart, H. Nguyen, D. Menon, R. Renkawitz-Pohl, B. Patterson, and the Bloomington Stock Center for antibodies and fly stocks; Dr. Richard Leapman at LBPH, NIH for access to the HPF/FS unit; Drs. Hao Zhang and Joseph Margolick at the Johns Hopkins Bloomberg School of Public Health for assistance with flow cytometry cell sorting. We thank Drs. S. Craig, D. Robinson, E. Grote, D. Pan, and members of the Chen lab for discussions and comments on the manuscript. This work was supported by grants from the National Institute of Health, American Heart Association and the Muscular Dystrophy Association. P.J. was a predoctoral fellow of the American Heart Association. E.H.C. was a Searle Scholar and a Packard Fellow.

Received: August 30, 2010
Revised: March 11, 2011
Accepted: April 19, 2011
Published: May 16, 2011

REFERENCES

Abmayr, S.M., Zhuang, S., and Geisbrecht, E.R. (2008). Myoblast fusion in *Drosophila*. Methods Mol. Biol. *475*, 75–97.

Akin, O., and Mullins, R.D. (2008). Capping protein increases the rate of actin-based motility by promoting filament nucleation by the Arp2/3 complex. Cell *133*, 841–851.

Anton, I.M., Jones, G.E., Wandosell, F., Geha, R., and Ramesh, N. (2007). WASP-interacting protein (WIP): working in polymerisation and much more. Trends Cell Biol. *17*, 555–562.

Artero, R.D., Castanon, I., and Baylies, M.K. (2001). The immunoglobulin-like protein Hibris functions as a dose-dependent regulator of myoblast fusion and is differentially controlled by Ras and Notch signaling. Development *128*, 4251–4264.

Bataille, L., Delon, I., Da Ponte, J.P., Brown, N.H., and Jagla, K. (2010). Downstream of identity genes: muscle-type-specific regulation of the fusion process. Dev. Cell *19*, 317–328.

Ben-Yaacov, S., Le Borgne, R., Abramson, I., Schweisguth, F., and Schejter, E.D. (2001). Wasp, the *Drosophila* Wiskott-Aldrich syndrome gene homologue, is required for cell fate decisions mediated by Notch signaling. J. Cell Biol. *152*, 1–13.

Bogdan, S., Stephan, R., Lobke, C., Mertens, A., and Klambt, C. (2005). Abi activates WASP to promote sensory organ development. Nat. Cell Biol. *7*, 977–984.

Bour, B.A., Chakravarti, M., West, J.M., and Abmayr, S.M. (2000). *Drosophila* SNS, a member of the immunoglobulin superfamily that is essential for myoblast fusion. Genes Dev. *14*, 1498–1511.

Brugnera, E., Haney, L., Grimsley, C., Lu, M., Walk, S.F., Tosello-Trampont, A.C., Macara, I.G., Madhani, H., Fink, G.R., and Ravichandran, K.S. (2002). Unconventional Rac-GEF activity is mediated through the Dock180-ELMO complex. Nat. Cell Biol. *4*, 574–582.

Chen, E.H., and Olson, E.N. (2001). Antisocial, an intracellular adaptor protein, is required for myoblast fusion in *Drosophila*. Dev. Cell *1*, 705–715.

Chou, H.C., Anton, I.M., Holt, M.R., Curcio, C., Lanzardo, S., Worth, A., Burns, S., Thrasher, A.J., Jones, G.E., and Calle, Y. (2006). WIP regulates the stability and localization of WASP to podosomes in migrating dendritic cells. Curr. Biol. *16*, 2337–2344.

Co, C., Wong, D.T., Gierke, S., Chang, V., and Taunton, J. (2007). Mechanism of actin network attachment to moving membranes: barbed end capture by N-WASP WH2 domains. Cell *128*, 901–913.

de la Fuente, M.A., Sasahara, Y., Calamito, M., Anton, I.M., Elkhal, A., Gallego, M.D., Suresh, K., Siminovitch, K., Ochs, H.D., Anderson, K.C., et al. (2007). WIP is a chaperone for Wiskott-Aldrich syndrome protein (WASP). Proc. Natl. Acad. Sci. USA *104*, 926–931.

Doberstein, S.K., Fetter, R.D., Mehta, A.Y., and Goodman, C.S. (1997). Genetic analysis of myoblast fusion: blown fuse is required for progression beyond the prefusion complex. J. Cell Biol. *136*, 1249–1261.

Duan, H., Skeath, J.B., and Nguyen, H.T. (2001). *Drosophila* Lame duck, a novel member of the Gli superfamily, acts as a key regulator of myogenesis by controlling fusion-competent myoblast development. Development *128*, 4489–4500.

Dworak, H.A., Charles, M.A., Pellerano, L.B., and Sink, H. (2001). Characterization of *Drosophila* hibris, a gene related to human nephrin. Development *128*, 4265–4276.

Erickson, M.R., Galletta, B.J., and Abmayr, S.M. (1997). Drosophila myoblast city encodes a conserved protein that is essential for myoblast fusion, dorsal closure, and cytoskeletal organization. J. Cell Biol. *138*, 589–603.

Frischknecht, F., Moreau, V., Rottger, S., Gonfloni, S., Reckmann, I., Superti-Furga, G., and Way, M. (1999). Actin-based motility of vaccinia virus mimics receptor tyrosine kinase signalling. Nature *401*, 926–929.

Gimona, M., Buccione, R., Courtneidge, S.A., and Linder, S. (2008). Assembly and biological role of podosomes and invadopodia. Curr. Opin. Cell Biol. *20*, 235–241.

Hummel, T., Leifker, K., and Klambt, C. (2000). The *Drosophila* HEM-2/NAP1 homolog KETTE controls axonal pathfinding and cytoskeletal organization. Genes Dev. *14*, 863–873.

Kim, S., Shilagardi, K., Zhang, S., Hong, S.N., Sens, K.L., Bo, J., Gonzalez, G.A., and Chen, E.H. (2007). A critical function for the actin cytoskeleton in targeted exocytosis of prefusion vesicles during myoblast fusion. Dev. Cell *12*, 571–586.

Kocherlakota, K.S., Wu, J.M., McDermott, J., and Abmayr, S.M. (2008). Analysis of the cell adhesion molecule sticks-and-stones reveals multiple redundant functional domains, protein-interaction motifs and phosphorylated tyrosines that direct myoblast fusion in *Drosophila* melanogaster. Genetics *178*, 1371–1383.

Konno, A., Kirby, M., Anderson, S.A., Schwartzberg, P.L., and Candotti, F. (2007). The expression of Wiskott-Aldrich syndrome protein (WASP) is dependent on WASP-interacting protein (WIP). Int. Immunol. *19*, 185–192.

Kurisu, S., and Takenawa, T. (2009). The WASP and WAVE family proteins. Genome Biol. *10*, 226.

Linder, S. (2009). Invadosomes at a glance. J. Cell Sci. *122*, 3009–3013.

Luthi, J.N., Gandhi, M.J., and Drachman, J.G. (2003). X-linked thrombocytopenia caused by a mutation in the Wiskott-Aldrich syndrome (WAS) gene that disrupts interaction with the WAS protein (WASP)-interacting protein (WIP). Exp. Hematol. *31*, 150–158.

Massarwa, R., Carmon, S., Shilo, B.Z., and Schejter, E.D. (2007). WIP/WASp-based actin-polymerization machinery is essential for myoblast fusion in *Drosophila*. Dev. Cell *12*, 557–569.

Menon, S.D., and Chia, W. (2001). *Drosophila* rolling pebbles: a multidomain protein required for myoblast fusion that recruits D-Titin in response to the myoblast attractant Dumbfounded. Dev. Cell *1*, 691–703.

Mogilner, A., and Oster, G. (1996). Cell motility driven by actin polymerization. Biophys. J. *71*, 3030–3045.

Ochs, H.D., and Thrasher, A.J. (2006). The Wiskott-Aldrich syndrome. J. Allergy Clin. Immunol. *117*, 725–738, quiz 739.

Paululat, A., Burchard, S., and Renkawitz-Pohl, R. (1995). Fusion from myoblasts to myotubes is dependent on the rolling stone gene (rost) of Drosophila. Development *121*, 2611–2620.

Pawson, T., Gish, G.D., and Nash, P. (2001). SH2 domains, interaction modules and cellular wiring. Trends Cell Biol. *11*, 504–511.

Pollard, T.D. (2007). Regulation of actin filament assembly by Arp2/3 complex and formins. Annu. Rev. Biophys. Biomol. Struct. *36*, 451–477.

Ramesh, N., and Geha, R. (2009). Recent advances in the biology of WASP and WIP. Immunol. Res. *44*, 99–111.

Ramesh, N., Anton, I.M., Hartwig, J.H., and Geha, R.S. (1997). WIP, a protein associated with wiskott-aldrich syndrome protein, induces actin polymerization and redistribution in lymphoid cells. Proc. Natl. Acad. Sci. USA *94*, 14671–14676.

Richardson, B.E., Beckett, K., Nowak, S.J., and Baylies, M.K. (2007). SCAR/WAVE and Arp2/3 are crucial for cytoskeletal remodeling at the site of myoblast fusion. Development *134*, 4357–4367.

Rochlin, K., Yu, S., Roy, S., and Baylies, M.K. (2009). Myoblast fusion: When it takes more to make one. Dev. Biol. *341*, 66–83.

Ruiz-Gomez, M., Coutts, N., Price, A., Taylor, M.V., and Bate, M. (2000). *Drosophila* dumbfounded: a myoblast attractant essential for fusion. Cell *102*, 189–198.

Schafer, G., Weber, S., Holz, A., Bogdan, S., Schumacher, S., Muller, A., Renkawitz-Pohl, R., and Onel, S.F. (2007). The Wiskott-Aldrich syndrome protein (WASP) is essential for myoblast fusion in *Drosophila*. Dev. Biol. *304*, 664–674.

Schröter, R.H., Lier, S., Holz, A., Bogdan, S., Klambt, C., Beck, L., and Renkawitz-Pohl, R. (2004). kette and blown fuse interact genetically during the second fusion step of myogenesis in *Drosophila*. Development *131*, 4501–4509.

Schröter, R.H., Buttgereit, D., Beck, L., Holz, A., and Renkawitz-Pohl, R. (2006). Blown fuse regulates stretching and outgrowth but not myoblast fusion of the circular visceral muscles in *Drosophila*. Differentiation *74*, 608–621.

Sens, K.L., Zhang, S., Jin, P., Duan, D., Zhang, G., Luo, F., Parachini, L., and Chen, E.H. (2010). An invasive podosome-like structure promotes fusion pore initiation during myoblast fusion. J. Cell Biol. *191*, 1013–1027.

Shelton, C., Kocherlakota, K.S., Zhuang, S., and Abmayr, S.M. (2009). The immunoglobulin superfamily member Hbs functions redundantly with Sns in interactions between founder and fusion-competent myoblasts. Development *136*, 1159–1168.

Stewart, D.M., Tian, L., and Nelson, D.L. (1999). Mutations that cause the Wiskott-Aldrich syndrome impair the interaction of Wiskott-Aldrich syndrome protein (WASP) with WASP interacting protein. J. Immunol. *162*, 5019–5024.

Strunkelnberg, M., Bonengel, B., Moda, L.M., Hertenstein, A., de Couet, H.G., Ramos, R.G., and Fischbach, K.F. (2001). rst and its paralogue kirre act redundantly during embryonic muscle development in *Drosophila*. Development *128*, 4229–4239.

Suzuki, T., and Sasakawa, C. (1998). N-WASP is an important protein for the actin-based motility of Shigella flexneri in the infected epithelial cells. Jpn. J. Med. Sci. Biol. Suppl. *51*, S63–S68.

Takenawa, T., and Suetsugu, S. (2007). The WASP-WAVE protein network: connecting the membrane to the cytoskeleton. Nat. Rev. Mol. Cell Biol. *8*, 37–48.

Volkman, B.F., Prehoda, K.E., Scott, J.A., Peterson, F.C., and Lim, W.A. (2002). Structure of the N-WASP EVH1 domain-WIP complex: insight into the molecular basis of Wiskott-Aldrich Syndrome. Cell *111*, 565–576.

Weisswange, I., Newsome, T.P., Schleich, S., and Way, M. (2009). The rate of N-WASP exchange limits the extent of ARP2/3-complex-dependent actin-based motility. Nature *458*, 87–91.

Zhang, S., and Chen, E.H. (2008). Ultrastructural analysis of myoblast fusion in Drosophila. In Cell Fusion: Overviews and Methods, E.H. Chen, ed. (Totowa, NJ: Humana Press), pp. 275–297.

Zallen, J.A., Cohen, Y., Hudson, A.M., Cooley, L., Wieschaus, E., and Schejter, E.D. (2002). SCAR is a primary regulator of Arp2/3-dependent morphological events in *Drosophila*. J. Cell Biol. *156*, 689–701.