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Competition between Blown Fuse and WASP for WIP binding regulates the dynamics of WASP-dependent actin polymerization *in vivo*

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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 co-localizes 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 and 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;

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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 also 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 paralogue Roughest that function redundantly in founder cells, and Sticks and stones (Sns) and its paralogue 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; Strunkelberg 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 is composed of 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 lifespan (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, Blow 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 stability of WASP-WIP complex and the dynamics of the WASP-mediated actin polymerization.

Results

Blow is an FCM-specific protein and co-localizes 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 Fig. 1Aa-a'' and b-b''). 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 the F-actin, both of which are FCM-specific (Fig. 1Ba-a''; Kim et al., 2007; Sens et al., 2010). Indeed, Blow-positive foci co-localize with those of Sltr, demonstrating that Blow is recruited to and enriched at sites of fusion (Fig. 1Bb-b''). 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 co-localized during their lifespan (Movie S1). Like Sltr, WASP is also enriched at sites of fusion and co-localizes with the F-actin foci in the embryo (Fig. 1Bc-c''), 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 co-localizes 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 Fig. 1Ca-a'' and b-b''; 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), *sltr* or *myoblast city* (*mbc*) (encoding a guanine nucleotide exchange factor for the small GTPase Rac) (Brugnera et al., 2002; Erickson et al., 1997) (Figure 1Cc-e''), 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* (Fig. 1Db) or in FCMs with *sns-GAL4* (Fig. 1Dc) rescued the fusion defect in *blow* mutant embryos (compare to Fig. 1. Da), whereas expressing Blow specifically in founder cells with *rP298-GAL4* did not (Fig. 1De,

compare to 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 co-immunoprecipitation 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 (Fig. 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 (Fig. 2C; S1A–B), 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 (Fig. 1Cd-d''; 4D).

We next mapped the Crk-interacting site in Blow to a single YDVP sequence (Fig. 2A and D), which matches the consensus SH2 binding motif (Pawson et al., 2001). A point 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 (Fig. 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 (Fig. 2Ha, a', b and b'), 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 co-localization 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 co-precipitated with each other (Fig. 2E, lane 3). This interaction is independent of the Blow-Crk interaction, since BlowY378F, which fails to bind Crk, still binds Sltr (Fig. 2E, lane 4). In addition, overexpressed Blow co-precipitated with endogenous Sltr (Fig. 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) (Blow Δ N99) or C-terminal 173 aa (Blow Δ C173) abolished Blow-Sltr binding (Fig. 2E, lane 5, 6) while maintaining Crk binding (Fig. S1D). Thus, both the N- and C-terminal regions of Blow are required for Sltr binding. Moreover, transgenic expression of Blow Δ C173 failed to rescue the fusion defect in *blow* mutant embryos (Fig. 2Hc and c'), 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 (Fig. S1E), and subsequently narrowed it down to a 24 aa region (Fig. 2F), which include the previously identified WASP-binding domain (WBD) in WIP (Fig. 2A, G) (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 (Fig. 2F, 2G; lane 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 co-immunoprecipitated with WASP (Fig. 3A). Likewise, increasing amount of WASP expression resulted in a gradual decrease in the amount of Sltr that co-immunoprecipitated with Blow (Fig. 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 (Fig. 3C, lane 1, 5, 6; 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 (Fig. 3C, lane 2; 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 (Fig. 3C, lane 4; 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, co-overexpression of Sltr completely suppressed the ability of overexpressed Blow to decrease the level of endogenous WASP (Fig. 3C, lane 3; 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 co-immunoprecipitated by the exogenous Sltr (Fig. 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 (Fig. S2A), and estimated the K_d of Blow-Sltr, as well as WASP-Sltr binding. The K_d between Blow-Sltr (48.1 nM) is 7.2-fold higher than that of WASP-Sltr (6.7 nM) (Fig. 3F, G), 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 (Fig. 3C, D).

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 ($3.3 \pm 0.8 \mu\text{m}^2$, n=53; Fig. 4Ab-b'' and B) compared to those in wild-type embryos ($1.7 \pm 0.6 \mu\text{m}^2$, n=55; Fig. 4Aa-a'' and B) (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 in FCMs co-localized with the phalloidin-labeled F-actin foci (Fig. 4Ca-a'''), whereas GFP-actin expressed in founder cells did not accumulate to form dense foci (Fig. 4Cb-b'''). 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 co-localized with enlarged domain of WASP and Sltr (Fig. 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 ($1.9 \pm 0.3 \mu\text{m}^2$, $n=44$; Fig. 4Ad-d'' and B) is similar to that of *sltr* ($1.9 \pm 0.3 \mu\text{m}^2$, $n=38$; Fig. 4Ac-c'' and B), but not *blow* mutant embryos ($3.3 \pm 0.8 \mu\text{m}^2$, $n=53$; Fig. 4Ab-b''' and B), 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 pre-bleaching level (Fig. 5Aa and B-D; Table S1; 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 pre-bleaching level (Fig. 5Ab and B-D; Table S1; Movie S4). These results suggest that in *blow* mutant embryos, the exchange rate of G-actin within the PLS is significantly decreased compared to 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 lifespan. Interestingly, there is a wide range of GFP-actin recovery time in both wild-type and mutant embryos, as that of the lifespan of the F-actin foci (Richardson et al., 2007), which may in part be due to 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 (Fig. 5Ae and B-D; Table S1; 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 much slower exchange rate (Fig. 5Eb, G, H; Table S1; Movie S7) compared to the wild-type embryos (Fig. 5Ea, G, H; Table S1; Movie S6). Furthermore, the intensity of the recovered fluorescent signal of GFP-WASP in *blow* mutant embryos never reached the pre-bleaching level (Fig. 5Eb, G, I; Table S1; Movie S7), whereas the fluorescent signal in wild-type embryos recovered to the pre-bleaching level (Fig. 5Ea, G, I; Table S1; 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 (Fig. 5Fb, G–I; Table S1; Movie S9) than in wild type (Fig. 5Fa, G–I; Table S1; 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 to those in wild-type embryos (Fig. 6A–B), 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 *blow* mutant embryos. 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 Blow Δ C173, (Fig. 5Ac,d and B–D), further suggesting that the ability of Blow to dissociate the WASP-Sltr complex is critical in regulating the actin polymerization dynamics *in vivo*.

Overexpressing Blow in Sltr-transfected 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 Fig. 4Eb-b''' and F), 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 (Fig. 3C, lane 2 and D), 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 change in the actin cytoskeleton (Fig. 4Ea-a''' and F), co-expression of Blow and Sltr reduced the length of Sltr-induced microspikes (Fig. 4Ec-c''' and F). This effect requires the Blow-Sltr interaction, since expression of Blow Δ C173, which does not bind Sltr, did not reduce the length of Sltr-induced microspikes (Fig. 4Ed-d''' and F). This result, together with the presence of elongated actin filaments in *blow* mutant embryos (Fig. 6A, B), support 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 (Fig. 4Ee-e''' and F). 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 the 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 were invasive and caused dimple-shaped dents on the founder cell membrane (Fig. 6Aa-a''; Table S2) (Sens et al., 2010). In contrast, only 9% (6/69) actin foci in *blow* mutant embryos appeared invasive but with reduced depth (Fig. 6Ab-b''; 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 a average of ~4.3 finger-like protrusions (with a maximum depth of 2.0 μm) into the apposing founder cell (Fig. 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) (Fig. 6Bc and d). 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 morphology (Fig. 6Bb and 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 (Fig. 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 homologue based on primary sequence, it interacted with the human WIP when the two proteins were co-expressed in *Drosophila* S2R+ cells (Fig. S3A, lane 3), albeit with a lower affinity than Blow-Sltr interaction (compare Fig. S3A, lane 3 and Fig. S3B). In contrast, no interaction was detected between human WIP and Blow Δ N99 (or Blow Δ C173) (Fig. S3A, lane 5, 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 (Fig. 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, but not merely the accumulated level of F-actin, is a critical determinant for the invasion of 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 Blow-Sltr interaction may re-bind 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 re-bind 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 (Fig. 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, should 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 (Fig. 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 (Fig. 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*, *sltr^{S1946}/CyO,actin-lacZ* (Kim et al., 2007); *kette^{J4-48}/TM6B* (Hummel et al., 2000); *lmd/TM3, ftz-lacZ* (Duan et al., 2001); *sns⁴⁰⁻⁴⁹/CyO* (Paululat et al., 1999); *sns-GAL4*(Kocherlakota 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-blow Δ C173* 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; blow¹/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.GFP3* females were crossed with either *rP298-GAL4/Y; blow¹/CyO,actin-lacZ* or *sns-GAL4,blow¹/CyO,actin-lacZ* males.

Immunohistochemistry

Embryos were fixed in 4% formaldehyde/heptane for 20 minutes, 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 hour 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 co-immunoprecipitation assays, expression constructs were transfected in S2R+ cells. Cells were harvested, washed with PBS, and incubated in NP40-Triton buffer (10mM Tris, pH 7.4, 150mM NaCl, 1mM EDTA, 1% TritonX-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 blots: 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 Procedure for protein purification). In brief, FLAG-V5-Blow or FLAG-V5-WASP was mixed with 17.6 nM FLAG-Sltr in the binding buffer (10mM Tris, pH 7.4, 150mM NaCl, 1mM EDTA and 0.2% Tween-20) containing 1 mM PMSF and protease inhibitor cocktail (Roche), incubated at room temperature for 1 hour, 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 Prism.

Confocal imaging of fixed samples

Images were obtained on a LSM 510 Meta confocal microscope with Fluor 40X, 1.3NA Oil and Plan-Apochromat 100X, 1.4NA Oil DIC objectives using Argon 458,477,488,514 nm; HeNe 543nm; and HeNe 633nm lasers, and the META detector. The pinhole was set to 1.0 AU for each channel and Z-stacks were collected at 1.0 μ m intervals for 40X magnification and 0.5 μ m intervals for 100X. 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 40X 1.3 oil lens on a LSM 700 system. The solid 488nm laser output was set to 2% to avoid photobleaching and phototoxicity. The pinhole was set to 1.0 AU and 4 frames were averaged per scan. Two pre-bleached 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 sec under the setting described above. The fluorescence intensity of the pre- and post-bleach ROI was determined using a flexible-size ROI, which was adjusted manually on every frame since the foci shape changes constantly, especially in wild-type embryos. We use an exponential decay equation $y = y_{\min} + (y_{\max} - y_{\min})(1 - e^{-kt})$ for fitting the kinetic curve (Weisswange et al., 2009). The rate constant of recovery (k) and the maximum recovery level (percentage recovery to original images) and the half-time ($t_{1/2} = \ln 2/k$) 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. Conventional chemical fixation was performed as described (Zhang and Chen, 2008). 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 (Sigma). Lead staining was done as described (Zhang and Chen, 2008) and images were acquired on a Philips CM120 TEM.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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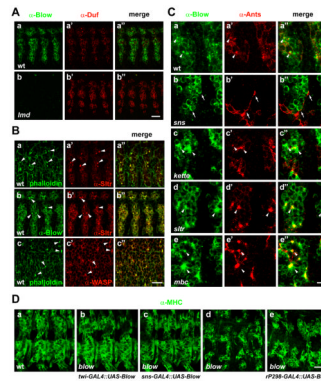


Figure 1. Blow is an FCM-specific protein that co-localizes with the WASP-Sltr complex at sites of fusion

(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 co-localization between phalloidin- and Sltr-positive foci (a-a''), 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 et al., 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 to 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 μ m; (C) 10 μ m.

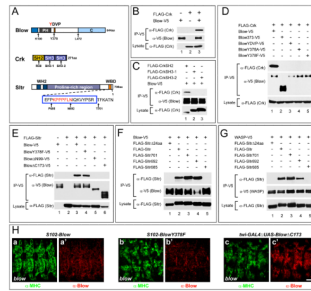


Figure 2. Blow interacts with Crk and Sltr

(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. Blow Δ N99: Blow missing N-terminal aa 1–99; Blow Δ C173: Blow missing C-terminal aa 472–644. Note that BlowY378F, which does not bind Crk, still binds Sltr (lane 4).

(F, G) Both Blow (F) and WASP (G) interact with the WASP-binding domain (WBD) of Sltr. Sltr Δ 24aa: 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 Blow Δ C173 with the *twi-GAL4* driver did not rescue the fusion defect (c-c'). Bar: 20 μ m.

See also Figure S1.

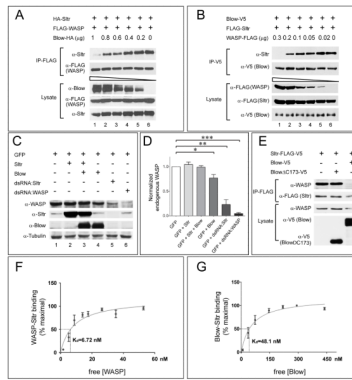


Figure 3. Blow competes with WASP for Sltr binding

(A, 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 (lane 1–6), together with Sltr (lane 2, 3) and Blow (lane 3, 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 $23.2 \pm 7.4\%$ ($n=3$) 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 Blow Δ C173, reduced the amount of endogenous WASP co-precipitated by exogenous Sltr-FLAG-V5.

(F, G) Saturation binding curves of WASP-Sltr (F) or Blow-Sltr (G) (see also Experimental Procedures, Figure S2A, Figure S3).

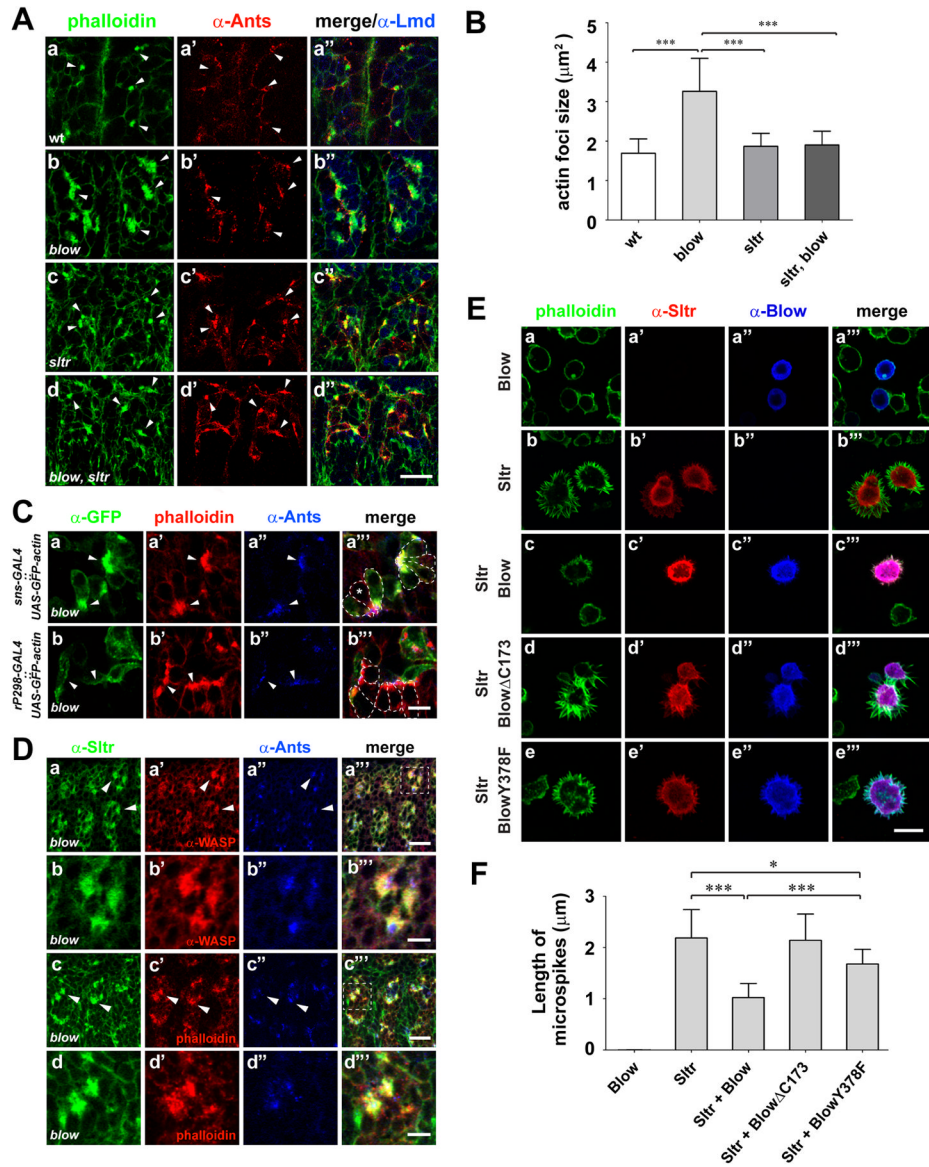


Figure 4. Blow functions through the WASP-Sltr complex to regulate actin polymerization
 (A, B) Blow regulates the formation of F-actin foci via Sltr in embryos. (A) Stage 14 embryos labeled with phalloidin (green), α -Ants (red) and α -Lmd (blue; FCMs). F-actin foci (arrowheads) are enlarged in *blow* mutant (b-b''), compared to 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'''), co-localized 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) F-actin foci are localized in FCMs of *blow* mutant embryos. Stage 14 embryos labeled with α -Sltr (green), α -Ants (blue) and merge. (E) F-actin foci are localized in FCMs of *blow* mutant embryos. Stage 14 embryos labeled with phalloidin (green), α -Sltr (red) and α -Blow (blue). (F) Mean values of length of microspikes in indicated embryos. Statistical analyses were performed by unpaired two-tail student's *t*-test (***) $p < 0.001$, (*) $p < 0.05$. Error bars: standard deviation.

(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 (red), phalloidin (red) and α -Ants (blue). Note the co-localization of F-actin foci, Sltr and WASP in enlarged domains (compared to 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, 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 co-expressed with Sltr in S2 cells, labeled with phalloidin (green), α -Sltr (red) and α -Blow (blue). Overexpression of Sltr (b-b'''), but not Blow (a-a'''), induced actin-filled microspike formation. Note that co-expression of Sltr and Blow (c-c'''), but not Sltr and Blow Δ C173 (d-d'''), reduced the length of the microspikes. Co-expression of Sltr and BlowY378F partially reduced the length of the microspikes (e-e'''). (F) Quantification of the length of the microspikes. Statistical analysis was 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 μ m; (C, D b-b''' and d-d''') 5 μ m; (D a-a''' and c-c''') 20 μ m; (E) 10 μ m.

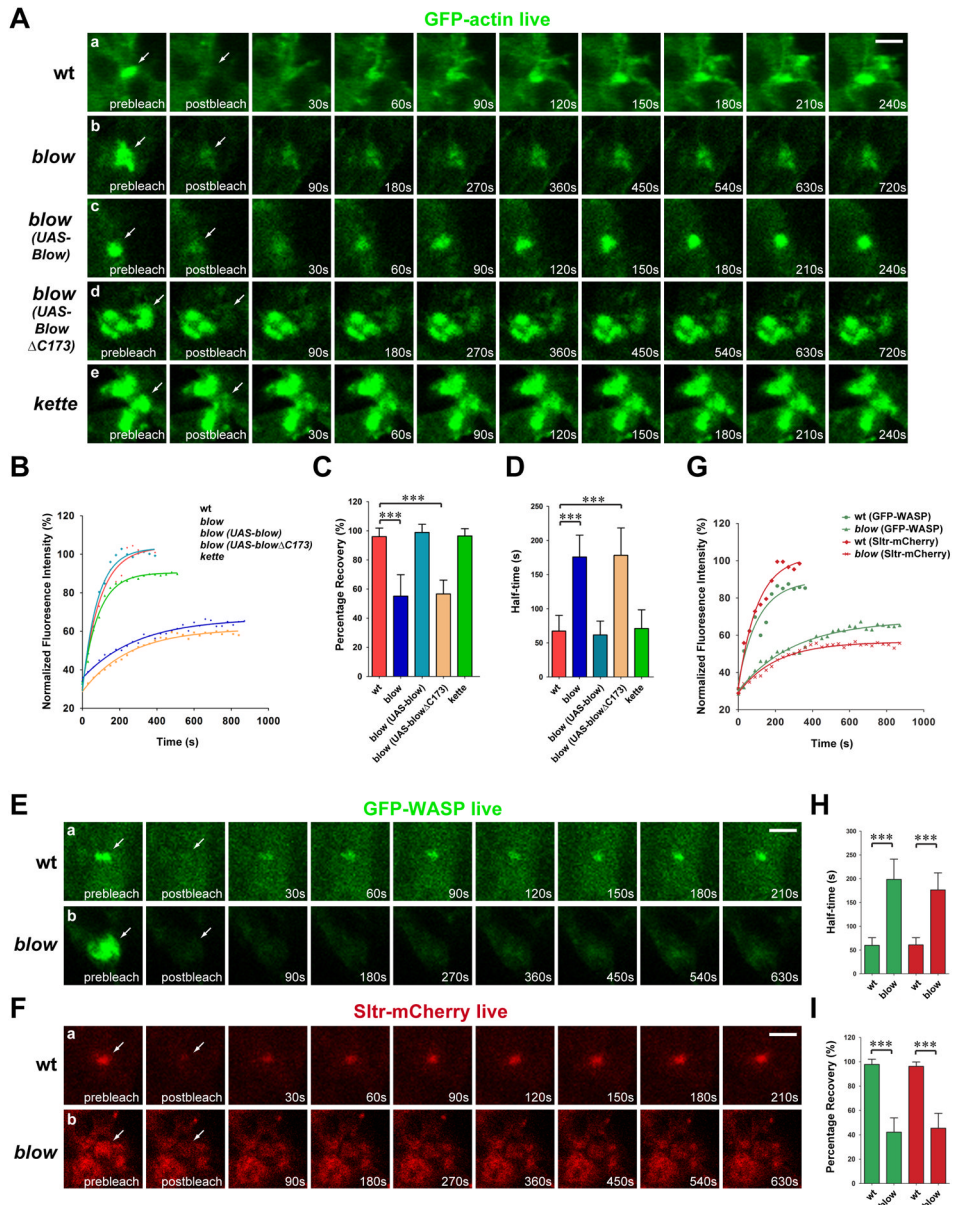


Figure 5. Blow regulates the exchange rate of G-actin, WASP and Sltr within the actin foci
Randomly selected GFP- or mCherry-positive foci were photobleached to approximately 30% of their original intensity and the fluorescence recovery was monitored live in stage 14 embryos expressing GFP-actin (A), 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 Blow Δ C173 (d), and *kette* (e) at 30 sec (a, c and e) or 90 sec (b and d) intervals.

(B) Comparison of the recovery kinetics of the GFP-positive foci shown in (A).

(C, D) Mean values of half-time (C) and the percentage recovery (D) of randomly selected GFP-actin foci photobleached in the embryos with indicated genotypes. See also Table S1.

(E, F) Stills of GFP-WASP (E) or Sltr-mCherry (F) foci in wt (a) and *blow* (b) embryos at 30 sec (a) or 90 sec (b) intervals.

(G) Comparison of the recovery kinetics of the GFP- and mCherry-positive foci as shown in E and F.

(H, I) Mean values of half-time (H) and the percentage recovery (I) of randomly selected GFP-WASP or Sltr-mCherry foci photobleached in the embryos with indicated genotypes. Green bars: GFP-WASP; Red bars: Sltr-mCherry. See also Table S1.

Statistical analysis was performed as described in Fig. 4. Error bars: standard deviations. Bars: 5 μ m.

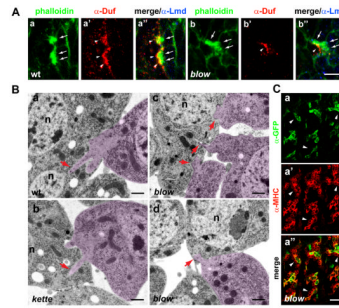


Figure 6. Defective PLS invasion and failure of fusion pore formation in embryos with decreased G-actin exchange within the PLS

(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'') Non-invasive 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 (pseudo-colored 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, d) Stage 14 *blow* mutant embryos. Each FCM projects one or two small protrusions into the opposing founder cell (determined by serial sections). 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* mutant embryos revealed by EM, indicating the presence of a loosely packed F-actin network. n: founder cell nuclei.

(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 μ m; (B) 500 nm; (C) 30 μ m.

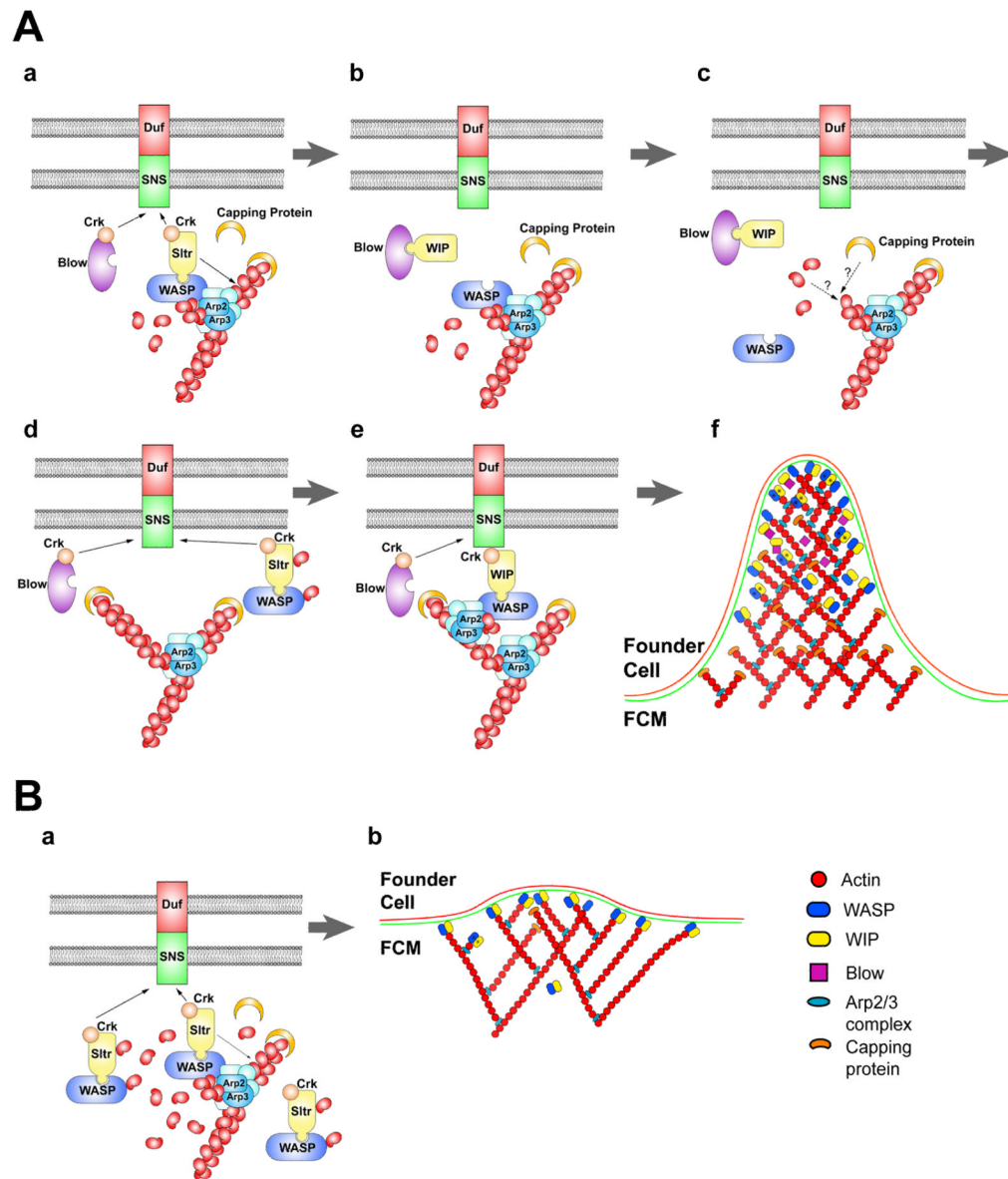


Figure 7. A model describing the regulation of actin polymerization dynamics by Blow
 (A) Blow dissociates the WASP-WIP complex resulting in the formation of a densely packed, branched F-actin network in wild-type embryo. (a) Engagement of the muscle cell type-specific adhesion molecules (Duf and Sns) leads to the independent recruitment of Blow and the WASP-WIP complex to the site of fusion. The WASP-WIP complex captures the barbed ends of F-actin, through G-actin-WASP and F-actin-WIP interactions, protecting the barbed ends from capping protein. (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 ends, leaving the ends transiently exposed to either capping protein or additional G-actin (either bound or unbound to a 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 great mechanical force to promote cell membrane protrusion. The asterisk marks the WASP-WIP complexes participating in the initiation of new branches.

(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.