Drosophila Fascin is a novel downstream target of prostaglandin signaling during actin remodeling

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ABSTRACT **Although prostaglandins (PGs)—lipid signals produced downstream of cyclooxygenase (COX) enzymes—regulate actin cytoskeletal dynamics, their mechanisms of action are unknown. We previously established *Drosophila* oogenesis, in particular nurse cell dumping, as a new model to determine how PGs regulate actin remodeling. PGs, and thus the *Drosophila* COX-like enzyme Pxt, are required for both the parallel actin filament bundle formation and the cortical actin strengthening required for dumping. Here we provide the first link between Fascin (*Drosophila* Singed, Sn), an actin-bundling protein, and PGs. Loss of either *pxt* or *fascin* results in similar actin defects. Fascin interacts, both pharmacologically and genetically, with PGs, as reduced Fascin levels enhance the effects of COX inhibition and synergize with reduced Pxt levels to cause both parallel bundle and cortical actin defects. Conversely, overexpression of Fascin in the germline suppresses the effects of COX inhibition and genetic loss of Pxt. These data lead to the conclusion that PGs regulate Fascin to control actin remodeling. This novel interaction has implications beyond *Drosophila*, as both PGs and Fascin-1, in mammalian systems, contribute to cancer cell migration and invasion.**

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INTRODUCTION

Prostaglandins (PGs) are locally acting, transient lipid signaling molecules responsible for mediating a wide range of biological activities, including pain, inflammation, fertility, and cancer development and progression (reviewed in Funk, 2001). PGs are synthesized from the conversion of free arachidonic acid into the PG precursor PGH_2 by the cyclooxygenase (COX) enzymes COX1 and COX2, which are the targets of nonsteroidal anti-inflammatory drugs such as aspirin. Biologically active prostaglandins, including PGE_2 and $PGF_{2\alpha}$, signal through specific G protein–coupled receptors to mediate PG and cell type–specific effects (Bos *et al.*, 2004). One known target of PGs is the actin cytoskeleton, where PGs can promote filament formation and stability or depolymerization, depending on the cell type and the specific PG (Peppelenbosch *et al.*, 1993; Tamma *et al.*, 2003; Bulin *et al.*, 2005). The mechanisms by which PGs regulate actin cytoskeletal dynamics (Pierce *et al.*, 1999; Dormond *et al.*, 2002; Birukova *et al.*, 2007), however, are largely unknown.

To elucidate the mechanisms by which PGs regulate the actin cytoskeleton, we use *Drosophila* oogenesis as a model system. *Drosophila* oogenesis consists of 14 distinct morphological stages through which egg chambers or follicles mature (Spradling, 1993). During stages 10 and 11 (S10 and S11), dynamic rearrangements of the actin cytoskeleton occur in the nurse cells, facilitating a process called nurse cell dumping. At stage 10B (S10B), parallel bundles of actin filaments (hereafter referred to as bundles) extend from the plasma membrane to form a cage-like structure around the nucleus, and the ring of cortical actin is strengthened just inside the plasma membrane (Guild *et al.*, 1997). During S11, the cortical actin contracts, squeezing the nurse cells in order to transfer their cytoplasmic contents into the growing oocyte; this is referred to as nurse cell dumping (Wheatley *et al.*, 1995). At the same time, bundles hold the nurse cell nuclei in place to prevent the ring canals—the structures through which the cytoplasm is dumped—from being blocked

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Abbreviations used: COX, cyclooxygenase; F-actin, filamentous actin; PG, prostaglandin; S10A, S10B, S11, S12, S13, S14, specific stages of oogenesis.

(Cooley *et al.*, 1992; Mahajan-Miklos and Cooley, 1994a,b). PG signaling is required for this process to occur, as pharmacological (via COX inhibitors) or genetic loss of Pxt, the *Drosophila* COX-like enzyme, results in impaired bundle formation and a failure of the nurse cells to dump (Tootle and Spradling, 2008). Furthermore, *pxt* mutant females are sterile. Numerous actin-binding proteins are also required for the process of nurse cell dumping (reviewed in Hudson and Cooley, 2002b); however, no connection has been established that links Pxt or PGs to specific actin-binding proteins.

To identify downstream targets of Pxt and PG signaling that mediate actin remodeling, we undertook a pharmacogenetic interaction screen using a previously described in vitro follicle maturation assay (Tootle and Spradling, 2008) to identify dominant modifiers that enhance or suppress sensitivity to COX inhibition (Spracklen, Meyer, and Tootle, unpublished data). We found that reduced levels of Fascin (*Drosophila* Singed, Sn) enhance COX inhibition.

Fascin is an actin-bundling or cross-linking protein (reviewed in Edwards and Bryan, 1995). In *Drosophila*, Fascin is encoded by *singed* (*sn*) and mediates nurse cell dumping (Cant *et al.*, 1994). Specifically, Fascin is required in the nurse cells during S10B for the extension of bundles toward the nucleus. Similar to *pxt* mutant flies, *fascin* mutants fail to undergo nurse cell dumping, and strong alleles result in female sterility. Of interest, it was recently suggested that *Drosophila* Fascin exhibits both bundling and bundling-independent roles in actin remodeling during nurse cell dumping (Zanet *et al.*, 2012). Fascin is also required for cell motility in both *Drosophila* (Zanet *et al.*, 2009) and mammalian systems, as Fascin 1–dependent actin bundles are key to the formation of filopodia (Vignjevic *et al.*, 2006; Hashimoto *et al.*, 2007) and invadopodia (Li *et al.*, 2010; Schoumacher *et al.*, 2010). In addition, increased Fascin-1 levels positively correlate with invasion, migration, and metastasis of tumor cells in human cancer cell lines and mouse models (Hashimoto *et al.*, 2005; Minn *et al.*, 2005; Hwang *et al.*, 2008; Chen *et al.*, 2010; Xing *et al.*, 2011) and a poor prognosis in patients (Hashimoto *et al.*, 2004; Yoder *et al.*, 2005; Lee *et al.*, 2007; Okada *et al.*, 2007; Li *et al.*, 2008; Chan *et al.*, 2010). Although it is clear that Fascin plays critical roles in cytoskeletal dynamics in diverse systems, few upstream regulatory mechanisms controlling Fascin activity are known.

Here we find that *Drosophila* Fascin interacts, genetically and pharmacologically, with PG signaling. Fascin mutants are phenotypically similar to *pxt* mutants and enhance the sensitivity of follicles to COX inhibitor treatment. Although a mild reduction in either Fascin or Pxt has little effect on nurse cell dumping, reduction of both is synergistic, resulting in actin-remodeling defects and a block in nurse cell dumping. Overexpression of Fascin suppresses the defects due to either COX inhibition or loss of Pxt. Taken together, these data support the model that PGs regulate Fascin during *Drosophila* nurse cell dumping to mediate rearrangement of the actin cytoskeleton. Of importance, this is the first evidence linking PGs to Fascin, a critical regulator of bundle formation in many systems.

RESULTS

Loss of *fascin* and *pxt* result in similar actin defects

Loss of Pxt results in actin-remodeling defects during *Drosophila* nurse cell dumping (S10B/11). The most severe *pxt*-mutant phenotype, found most often in pxt^{f01000} follicles (referred to as pxt^{f} in the figures), consists of little to no bundle formation in the nurse cells (Figure 1, B–B′ compared with A–A′). Of note, nurse cell nuclei do not plug the ring canals in *pxt* mutants, indicating that contraction is also defective. When bundles are present in *pxt* mutants (pxt^{f01000}, $pxt^{EY03052}$ [referred to as pxt^{EY} in the figures], and $pxt^{f01000}/pxt^{EY03052}$), the bundles are variable in length and are abnormally distributed along the membranes compared with wild-type follicles (unpublished data). In addition, the bundles in the mutant follicles often exhibit extreme bends not seen in wild-type bundles (unpublished data). Together these actin-remodeling defects lead to a failure to complete nurse cell dumping during S11, resulting in S12 follicles where the nurse cells have failed to completely transfer their cytoplasmic contents to the elongating oocyte (Figure 1, E–E′ compared with D–D′). This is most obvious in *pxt*-mutant S14 follicles, as they retain nurse cell remnants and are shorter in length than wild-type (*yw*) follicles (Figure 1H compared with G). The diversity of actin-remodeling defects observed in *pxt* mutants and the previously characterized role of Pxt in PG signaling (Tootle and Spradling, 2008) suggest that PGs regulate multiple aspects of actin remodeling, from filament/bundle formation and/or stability to bundle structure.

Given that actin remodeling and nurse cell dumping are mediated by a diverse array of actin-binding proteins, we hypothesized that Pxt leads to the production of PG or PG-like signals that modulate the activity of specific actin-binding proteins, thereby regulating cytoskeletal remodeling to promote nurse cell dumping. To test this hypothesis, we performed a pharmaco-interaction screen to identify the actin-binding proteins that act downstream of PG signaling during nurse cell dumping (Spracklen, Meyer, and Tootle, unpublished data). Briefly, the screen was performed using the previously developed in vitro follicle maturation assay in which S10B follicles are matured in culture. Loss of PG synthesis via pharmacological COX inhibition blocks nurse cell dumping in cultured S10B follicles in a dose-dependent manner (Tootle and Spradling, 2008). Previously we determined the IC_{50}, or concentration that blocks 50% of the follicles from undergoing dumping, for numerous COX inhibitors. We used the IC_{50} concentration for the COX inhibitor aspirin to generate a sensitized state to identify actin-binding proteins that may mediate PG signaling during dumping. If an actin-binding protein is required downstream of PG synthesis to promote cytoskeletal remodeling, then heterozygosity for a strong allele or homozygosity for a weak allele of the actin-binding proteins is predicted to enhance the effect of COX inhibition, that is, increase the number of follicles that fail to undergo nurse cell dumping. Specifically, S10B follicles from flies heterozygous for mutations in actin-binding proteins were incubated in control media versus media containing the IC_{50} concentration of aspirin and compared with those from wild-type controls. Any genotype exhibiting an effect greater than three SDs away from the wild-type values was considered an interactor. We found that reduced Fascin (Singed) levels (sn^{36a}/+) enhance the effects of aspirin.

Fascin is an actin-bundling protein (reviewed in Edwards and Bryan, 1995). Loss of Fascin results in severe actin defects during bristle formation and nurse cell dumping (Cant *et al.*, 1994). Transheterozygous *fascin*-mutant follicles (sn^{2}/sn^{36a}, shown in the figures; and sn^{34e}/sn^{36a}, data not shown) exhibit *pxt* mutant–like actin defects during nurse cell dumping. Specifically, loss of *fascin* results in reduced bundle formation in S10B nurse cells compared with heterozygous *fascin* controls (sn^{36a}/+; Figure 1, C–C′, and data not shown). Like *pxt* mutants, these defects result in incomplete nurse cell dumping at S11, resulting in S12 follicles in which the nurse cells have failed to completely dump their cytoplasmic contents (Figure 1, F–F′ compared with D–D′) and the subsequent retention of nurse cell remnants and a short egg phenotype at S14 (Figure 1, I compared with G).

In addition to bundle defects, loss of either Pxt or Fascin also results in cortical actin abnormalities (Figure 2). Specifically, the

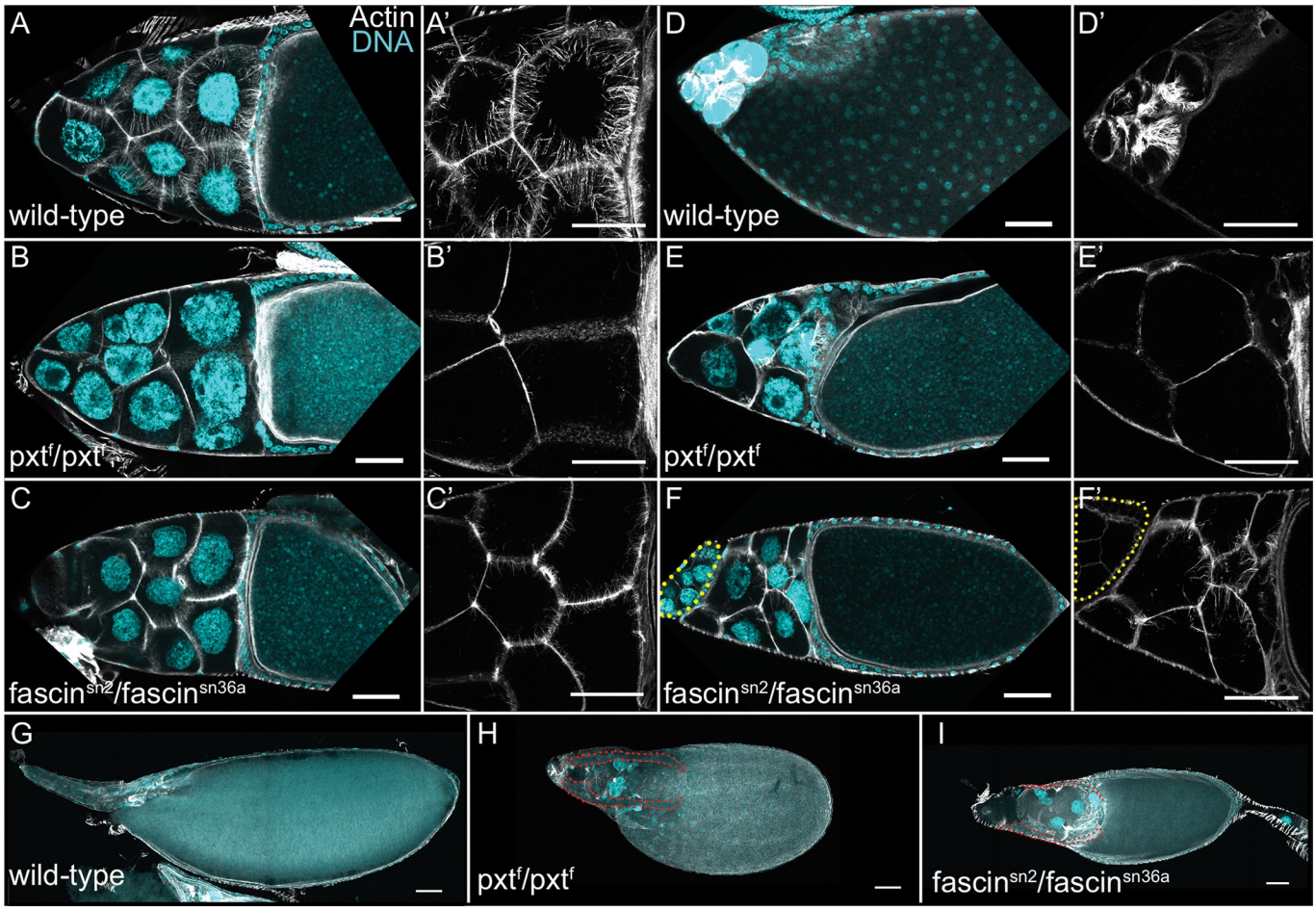

FIGURE 1: The actin bundle defects during late-stage follicle development are similar in *fascin* and *pxt* mutants. (A–F) Maximum projections of three confocal slices of S10B (A–C) and S12 (D–F) follicles taken at 20× magnification. (A′–F′) Maximum projections of four or five confocal slices of posterior nurse cells of the follicles in A–F acquired at 40× magnification. (G–I) Maximum projections of six or seven confocal slices of S14 follicles acquired by tiling at 20× magnification. (A–I) Merged images: F-actin (phalloidin), white; DNA (DAPI), cyan. (A′–F′) F-actin (phalloidin), white. (A–A′, D–D′, G) Wild-type (*yw*). (B–B′, E–E′, H) pxt^{f}. (C–C′, F–F′, I) $fascin^{sn2}/fascin^{sn36a}$. Wild-type S10B follicles possess a robust network of parallel actin filament bundles extending from the nurse cell membranes toward the nuclei in all of the nurse cells (A, A′). At the completion of nurse cell dumping (S12), the nurse cells have completely transferred their cytoplasmic contents to the elongating oocyte and the bundles have condensed (D, D′). *pxt*-mutant S10B follicles exhibit a spectrum of actin-remodeling defects, ranging from reduced bundle formation to a complete loss of bundles (B, B′). Similarly, *fascin* transheterozygotes show a substantial reduction in elongated bundles at S10B (C, C′). As a result, the nurse cells in both mutants fail to complete dumping at S12 (E–E′ *pxt*; F-F′ *fascin*, earlier-stage follicle outlined by dotted yellow line). Unlike in wild-type S14 follicles (G), this results in a short-egg phenotype and the persistence of nurse cells at S14 in both *pxt* and *fascin* mutants (H–I, dorsal appendages outlined by dotted red line). Scale bars, 50 μm.

cortical actin integrity is lost in mid-to-late oogenesis, resulting in multinucleate nurse cells. Cortical actin breakdown is observed in ~88% of *pxt* mutant (Figure 2, B–B′ compared with A–A′ and D, $n = 42$) and ~44-49% of *fascin* transheterozygous S10–11 follicles (Figure 2, C–C′ and D, $n = 45$ for each allelic combination) from 4- to 5-d-old flies. To assess whether the nurse cell membranes are also disrupted, we used an antibody to phosphotyrosine to mark the membranes. We found that in both *pxt* and *fascin* mutants, when the cortical actin is disrupted the membrane is also broken (Figure 2, B″–C″ compared with A″, yellow arrows). Thus, on the basis of the pharmaco-interaction screen results and the phenotypic similarity between loss of Fascin and loss of Pxt, we hypothesize that Fascin is a downstream target of PG signaling, regulating bundle formation and strengthening cortical actin, two events critical for *Drosophila* nurse cell dumping and female fertility.

Loss of Fascin enhances the effects of COX inhibitors

To test the foregoing hypothesis, we assessed whether reduced levels of Fascin, using multiple alleles, enhance the effect on nurse cell dumping of pharmacologically reducing PG signaling. We found that a reduction in Fascin levels, by either heterozygosity for a strong allele ($sn^{36a}/+$) or homozygosity for weak alleles (sn^{34e}/sn^{34e} and sn^{2}/sn^{2}), enhances the dumping defects due to two COX inhibitors, aspirin and NS-398 (Figure 3A and Supplemental Figure S1). Wild-type follicles were treated with either control media or media containing the IC_{50} concentration of the COX inhibitor, and the ratio of the percentage of follicles completing dumping in the inhibitor treated to the percentage of follicles completing dumping in control media was determined. This ratio is reduced when Fascin levels ($sn^{36a}/+$, sn^{34e}/sn^{34e}, and sn^{2}/sn^{2}; paired *t* tests <0.05) are decreased for both aspirin and NS-398 compared with wild-type follicles. It is

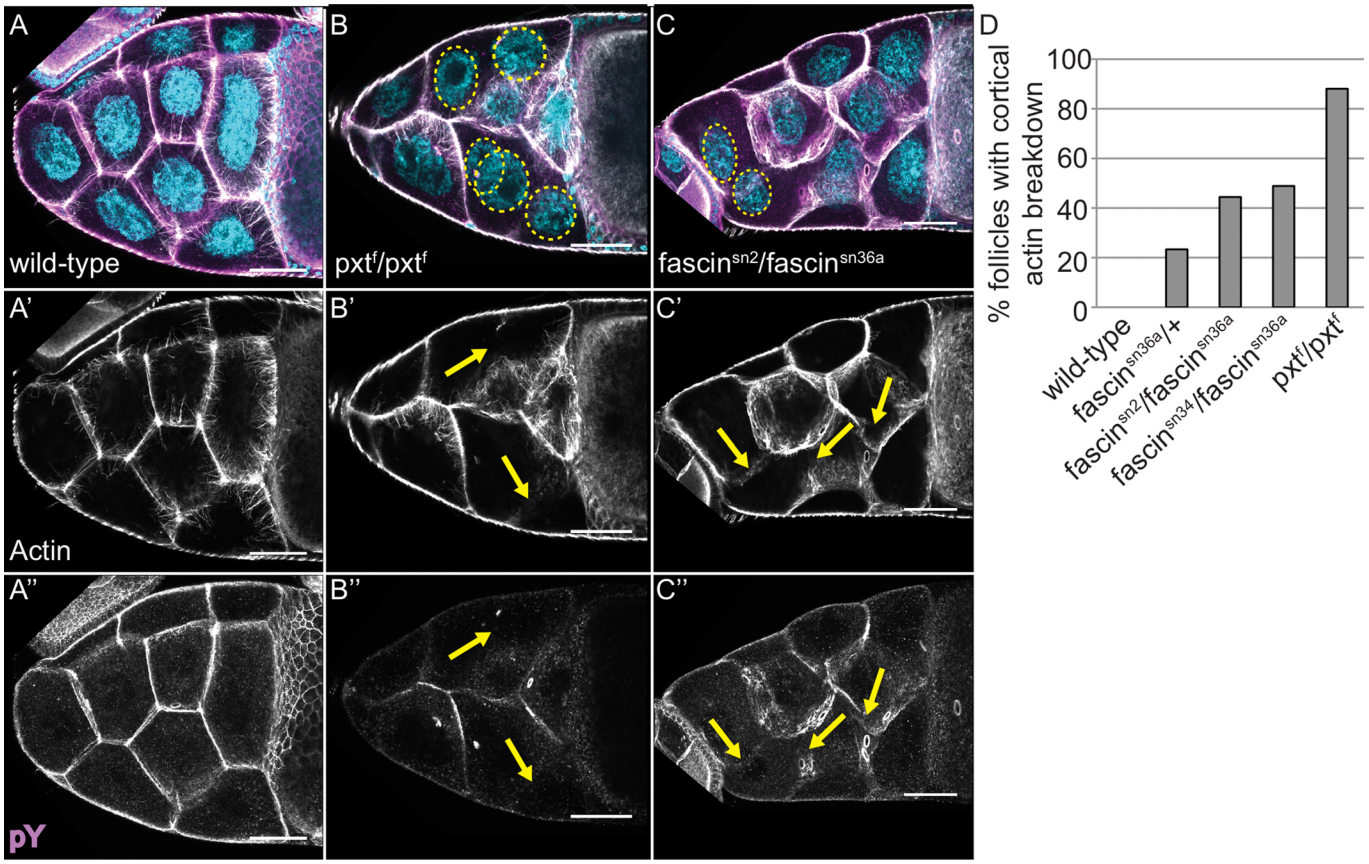

FIGURE 2: Cortical actin and membrane integrity are lost in *fascin* and *pxt* mutants. (A–C″) Maximum projections of three confocal slices of S10B follicles taken at 20× magnification. (A–C) Merged images: F-actin (phalloidin), white; membrane (anti-phosphotyrosine), magenta; DNA (DAPI), cyan. (A′–C′) F-actin (phalloidin), white. (A″–C″) Membrane (anti-phosphotyrosine), white. (A–A″) Wild-type (*yw*). (B–B″) pxt^{f}. (C–C″) $fascin^{sn2}/fascin^{sn36a}$. (D) Chart quantifying the cortical actin defects observed ($n = 20$ for wild type, $n = 30$ for *fascin*/+, $n = 42$ for pxt^{f}, and $n = 45$ for each allelic combination of *fascin*). Wild-type follicles display robust cortical actin underlying nurse cell membranes (A–A″, D) whereas *pxt* and *fascin* mutants exhibit cortical actin and membrane breakdown (B–B″ and C–C″, respectively, yellow arrows; D). This results in multinucleate nurse cells (B and C, nuclei outlined by dotted yellow line). Scale bars, 50 μm.

important to note that follicles from the *fascin*-mutant backgrounds used develop normally in control media (Supplemental Figure S1A). In addition, the IC_{50} for both of the COX inhibitors is lowered in the *fascin*-mutant backgrounds. The IC_{50} of aspirin is reduced from 1.34 mM in wild type to 0.737 mM in $fascin^{sn36a}$/+ and 0.735 mM in $fascin^{sn34e}/fascin^{sn34e}$ (Figure 3B and Supplemental Figure S1B-D); similarly the IC_{50} of NS-398 is reduced from 122 μM in wild type to 70 μM in $fascin^{sn36a}$/+ and 84 μM in $fascin^{sn34e}/fascin^{sn34e}$ (Figure 3B and Supplemental Figure S1, E–G). Thus reduced Fascin levels enhance the nurse cell dumping defects and thus actin abnormalities, due to reduced PG synthesis and signaling.

$PGF_{2\alpha}$ blocks the effects of reduced Fascin levels on COX inhibition

Although our finding that reduced levels of Fascin enhance the dumping defects of two COX inhibitors strongly suggests that this interaction is due to reduced PG synthesis, it remains possible that the interaction is due to off-target effects of these inhibitors. To address this, we tested whether exogenous PGs can restore dumping in the presence of both COX inhibition and decreased Fascin levels. We previously showed that an exogenous $PGF_{2\alpha}$ analogue, fluprostenol, restores nurse cell dumping in the presence of COX inhibition (Tootle and Spradling, 2008). Therefore we wanted to determine whether Fascin enhances the effect of the loss of this specific PG. We found that exogenous $PGF_{2\alpha}$ rescues the dumping defects due to aspirin and heterozygosity for *fascin* (Figure 3C). Therefore the interaction between reduced Fascin levels and inhibition of COX enzymes is due to reduced $PGF_{2\alpha}$ signaling.

Fascin genetically interacts with Pxt

All of the data up to this point could support two models: 1) PGs and Fascin act in parallel pathways to regulate bundle formation, or 2) PGs and Fascin act in a linear pathway to control bundle formation. To distinguish between these models, we next assessed the genetic interactions between Fascin and Pxt, the *Drosophila* COX-like enzyme. Follicles that have reduced levels of either Fascin (*fascin*/+) or Pxt (*pxt*/+) alone dump normally in culture, whereas follicles with reduced levels of both (*fascin*/+; *pxt*/+) exhibit a significant decrease in their ability to dump in culture (Figure 3D). We went on to quantitatively assess in vivo actin remodeling by phalloidin staining. Heterozygosity for either *fascin* or *pxt* alone has little effect on actin remodeling during nurse cell dumping (Figure 4, A, E, and F, and data not shown), whereas follicles from *fascin*/+; *pxt*/+ mutants exhibit a range of S10B actin phenotypes from wild-type (Figure 4, B–B″ compared with A–A″) to abnormal bundles (Figure 4, C–C″) to severely decreased bundle formation (Figure 4, D–D″).

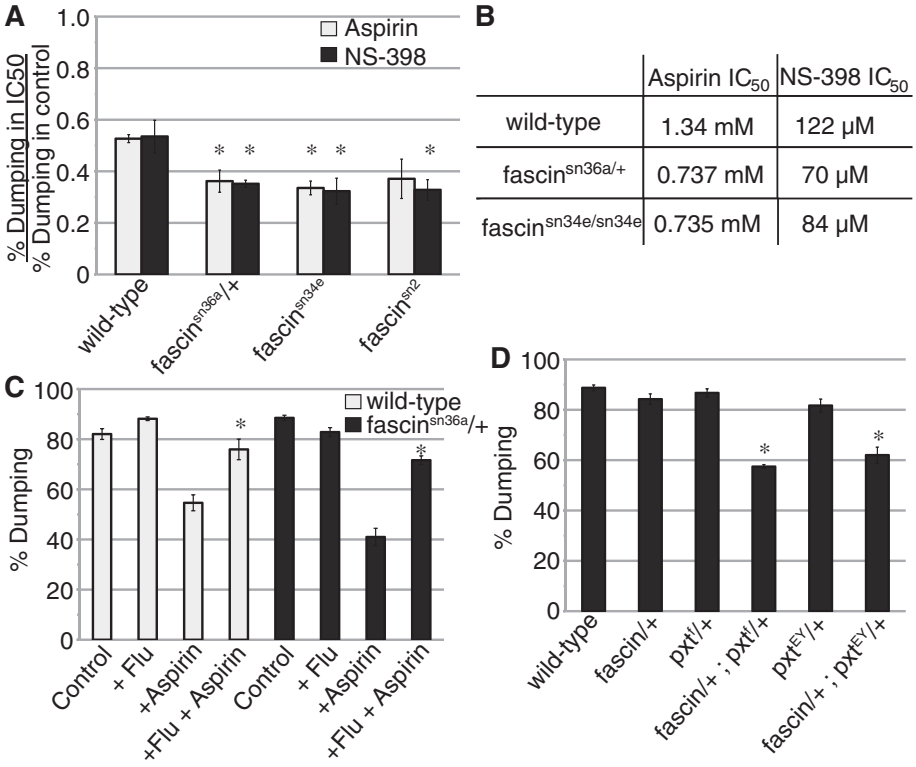

FIGURE 3. Loss of Fascin enhances the effects of COX inhibition. (A–D) In vitro follicle maturation assays. (A) IC_{50} treatments of wild-type and *fascin* alleles with aspirin (1.5 mM) or NS-398 (125 μM). Data are shown as the ratio of the percentage of follicles dumping in IC_{50} of the COX inhibitor to the percentage of follicles (of the same genotype) dumping in control media. (B) Table of IC_{50} of aspirin and NS-398 for wild-type, *fascin*$^{sn36a/+}$, and *fascin*$^{sn34e/sn34e}$ follicles. (C) Treatment of follicles with control media, IC_{50} aspirin, 10 nM fluprostenol (Flu, $PGF_{2\alpha}$ analogue), or aspirin and fluprostenol. (D) Wild-type, *fascin*^{sn36a} heterozygote, *pxt* heterozygote, or *fascin/+;;pxt/+* double heterozygote follicle development in control media. (C, D) Data are presented as the percentage of follicles that completed dumping. Heterozygosity for a strong *fascin* allele (*sn*^{36a}, $p = 0.0038$ for aspirin and $p = 0.023$ for NS-398) or homozygosity for a weak allele (*sn*^{34e}, $p = 0.00008$ for aspirin and $p = 0.031$ for NS-398; or *sn*^{2}, $p = 0.089$ for aspirin and $p = 0.021$ for NS-398) enhances sensitivity to COX inhibition compared with wild-type controls (A, B). Meanwhile, treatment of follicles with fluprostenol shows that the exogenous $PGF_{2\alpha}$ analogue suppresses sensitivity to COX inhibition in wild-type and *fascin*$^{sn36a/+}$ flies (C, $p = 0.0047$ compared with aspirin-only treatment). Follicles that are heterozygous for either a *fascin* or *pxt* mutation alone undergo dumping normally in culture, whereas reduced levels of both significantly inhibits dumping (D; $p = 0.00005$ for *fascin/+; pxt*^{f}*/+*and $p = 0.002$ for *fascin/+; pxt*^{EY}*/+* compared with their respective *pxt/+*). All data represent a minimum of three independent experiments. *$p < 0.05$ using a paired *t* test, unequal variance.

Abnormal bundles are defined by decreased length, irregular distribution (red arrowhead in Figure 4C″), and/or actin aggregates (red arrows in Figure 4, C′–C″). These defects remain throughout the later stages of follicle development (Supplemental Figure S2). Quantification of these actin defects (Figure 4E) in S10B follicles was performed using confocal microscopy (see *Materials and Methods*) and revealed that follicles from 3- to 4-d-old *fascin/+* exhibit actin defects (combining both lack of bundles and abnormal actin bundles into one category) in ~15% of the follicles (~5% with no bundles and ~10% with defective bundles, $n = 22$), *pxt*^{f}*/+* exhibit defects in ~10% of the follicles (all with defective bundles, $n = 29$), and *pxt*^{EY}*/+* exhibit defects in ~21% of the follicles (~3% with no bundles and 18% with defective bundles, $n = 28$). Reduced levels of both Fascin and Pxt synergize to result in actin defects in ~76% (~31% with no bundles and ~45% with defective bundles, $n = 29$) and ~67% (~22% with no bundles and ~45% with defective bundles, $n = 27$) in *fascin/+; pxt*^{f}*/+* and *fascin/+; pxt*^{EY}*/+*, respectively. This interaction is significantly higher than an additive effect (~8% with no bundles and 28% with defective bundles); therefore these data indicate that PGs and Fascin act in a linear pathway. Furthermore, the percentage of S10B follicles that exhibit cortical actin breakdown (Figure 4, F and G) is significantly increased in *fascin/+; pxt/+* (27 and 51%, depending on the *pxt* allele) compared with either heterozygote alone (~3–8%, depending on genotype). The prevalence of this defect is ~2.5- to 4.6-fold higher than the additive effect, which would be 11%. It is important to note that this phenotype gets more severe with age in both *pxt* and *fascin* mutants; this is reflected in the higher level of cortical actin breakdown observed in *fascin/+* in Figure 2D. Taken together, these data support the model that PGs and Fascin act in a linear pathway to regulate actin remodeling and bundle formation.

Villin, another actin-bundling protein, does not interact with PG signaling

Villin (Quail) is an actin-bundling protein that is also required for actin remodeling during *Drosophila* nurse cell dumping. Loss of Villin causes decreased bundle formation, which results in nurse cell nuclei plugging the ring canals, thus blocking dumping (Mahajan-Miklos and Cooley, 1994b). Because both Villin and Fascin are actin-bundling proteins that are required for nurse cell dumping and we found that Fascin enhances the effects of reduced PG synthesis during dumping, we wanted to determine whether PG signaling controls actin remodeling by regulating bundling in general or acts more specifically through Fascin. To address this, we examined Villin localization and expression in *pxt* mutants and used our in vitro follicle maturation assay to determine the effects of reduced Villin levels on COX inhibition, reduced Pxt levels, and reduced Fascin levels.

Villin localizes to both the cortical actin and the actin bundles that are formed in the nurse cell cytoplasm preceding dumping (Figure 5, A–A″). Although *pxt* mutants (both *pxt*^{f} and *pxt*^{EY}) exhibit numerous actin-remodeling defects (see Figure 1), Villin localizes normally to the bundles and cortical actin that are present in these mutants (Figure 5, B–C″). Similarly, Villin protein levels are normal in *pxt* mutants (Figure 5D). This indicates that PG signaling does not regulate Villin expression level or localization.

We next wanted to determine whether a reduction in Villin level alters the effects of COX inhibitor treatment or reduced Pxt levels. Using our in vitro follicle maturation assay, we found that heterozygosity for multiple *villin* mutant alleles (*qua*$^{6\text{-}396}$*/+*, *qua*$^{8\text{-}1062}$*/+*, and *qua*^{EY03072}*/+*) has no effect on the defects due to treatment with the IC_{50} of aspirin or NS-398 (Figure 5E); similarly, double heterozygotes for *villin* and *pxt* mutants dump normally in culture (Figure 5F).

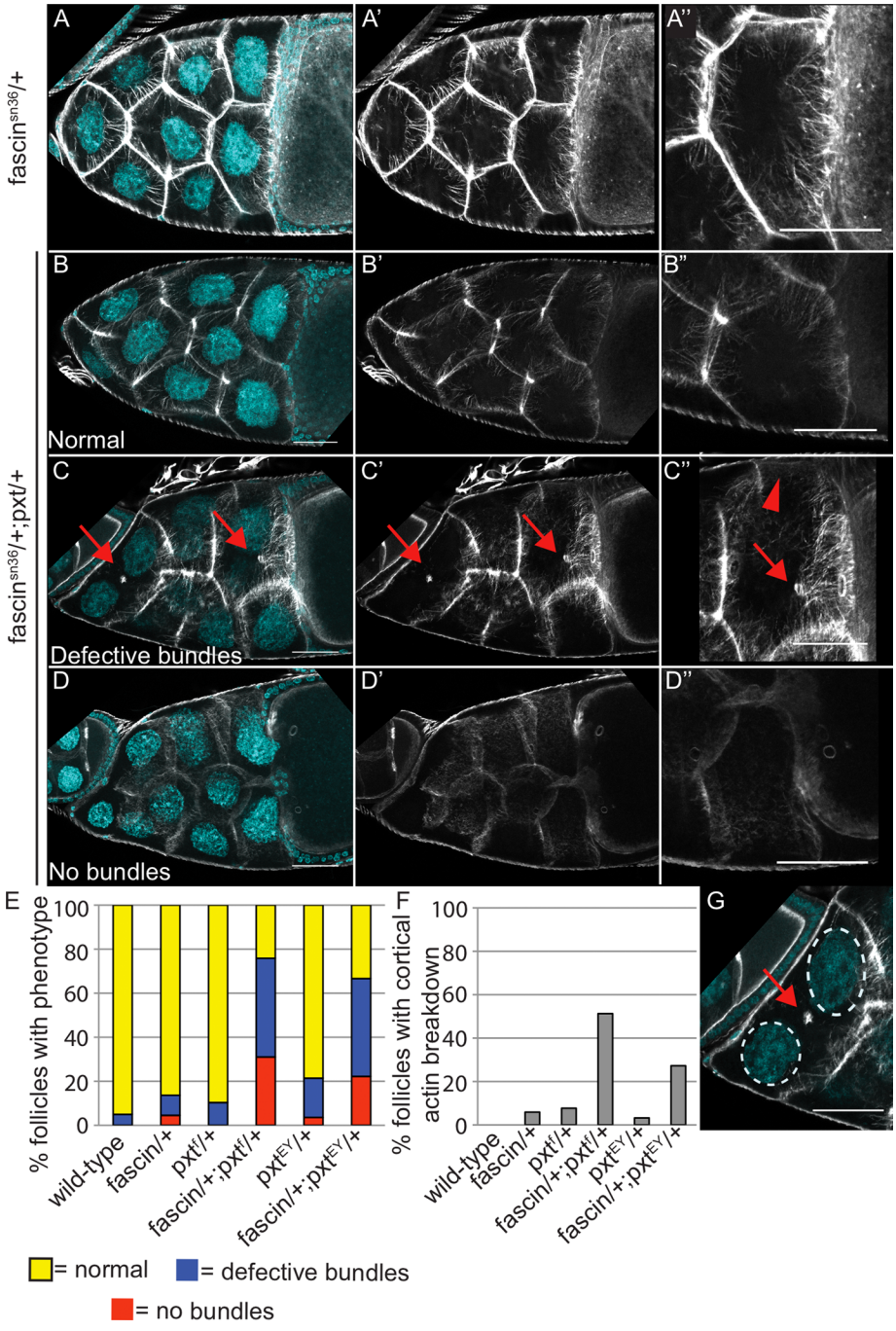

FIGURE 4: Reduced Fascin and PG levels synergize to cause actin-remodeling defects. (A–D″, G) Maximum projections of three to five confocal slices of S10B follicles. (A–D, G) Merged images: F-actin (phalloidin), white; DNA (DAPI), cyan. (A′–D′) F-actin (phalloidin), white. (A″–D″) Zoomed-in image of posterior nurse cells, actin channel only. (E, F) Charts quantifying the observed actin-remodeling defects. (A–A″) *fascin*^{sn36a}/+. (B–D″, G) *fascin*^{sn36a}/+; *pxt*^{f}/+or *fascin*^{sn36a}/+; *pxt*^{EY}/+ follicles. Immunofluorescence analyses reveal that *fascin*/+ follicles undergo normal actin remodeling and dumping (A–A″, E, and F), whereas *fascin*/+; *pxt*/+ follicles exhibit a range of phenotypes, including wild-type/normal remodeling (B–B″); defective bundles (C–C″), which include abnormal bundles/aggregates (red arrow) and altered bundle length and/or density (red arrowhead); and complete inhibition of bundle formation (D–D″). Quantification of these actin-remodeling defects reveals that the majority of *fascin*/+; *pxt*/+ follicles exhibit bundle defects (E; $n = 20$ for wild-type, $n = 22$ for *fascin*/+, $n = 29$ for *pxt*^{f}/+, $n = 28$ for *pxt*^{EY}/+, $n = 29$ for *fascin*^{sn36a}/+; *pxt*^{f}/+, and $n = 27$ for *fascin*^{sn36a}/+; *pxt*^{EY}/+). In addition, such follicles also exhibit increased cortical actin breakdown (C–C′, F, and G, red arrow; $n = 23$ for wild type, $n = 34$ for *fascin*/+, $n = 39$ for *pxt*^{f}/+, $n = 31$ for *pxt*^{EY}/+, $n = 39$ for *fascin*^{sn36a}/+; *pxt*^{f}/+, and $n = 33$ for *fascin*^{sn36a}/+; *pxt*^{EY}/+). Scale bar, 50 μm.

Furthermore, a coreduction in Villin and Fascin levels does not affect dumping in the in vitro follicle maturation assay (Figure 5G). These data suggest that Villin is not a downstream target of PG signaling during nurse cell dumping.

Overexpression of Fascin suppresses the dumping defects due to reduced PG signaling

Our data indicate that PGs and Fascin act in a linear pathway to regulate actin remodeling, particularly bundle formation and cortical actin integrity, during nurse cell dumping. Given that PGs are signaling molecules and Fascin physically mediates actin bundling, we hypothesize that PGs act upstream of Fascin during nurse cell dumping. If PGs function by increasing either the level or activity of Fascin, then overexpression of Fascin should suppress the effects of COX inhibition. To test this, we used the UAS/Gal4 system (Brand and Perrimon, 1993) to overexpress Fascin specifically in the germline. It is well established that because the UAS/GAL4 system was taken from yeast, it exhibits temperature-dependent levels of expression (i.e., there is more expression at higher temperatures). Follicles from flies overexpressing Fascin in the germline (UAS Fascin/+; matαGal4 [second or third chromosome]/+) were analyzed by the in vitro follicle maturation assay, comparing development when treated with control versus the IC_{50} concentration of aspirin. Compared to the controls (UAS Fascin/+, matαGal4(2)/+, and matαGal4(3)/+), flies overexpressing Fascin at high levels (25 and 27.5°C) suppress the defects due to COX inhibition (IC_{50}) during nurse cell dumping (Figure 6A). Lower levels of Fascin expression, achieved by maintaining the flies at 21°C, fail to suppress COX inhibition (Figure 6A); this suggests that the level and/or activity of Fascin is tightly regulated during nurse cell dumping. To further assess the role of Fascin downstream of PGs, we asked whether overexpression of Fascin could suppress a high level of COX inhibition (3 mM aspirin). We found that overexpression of Fascin weakly suppressed the effects of a strong loss of PG synthesis (Figure 6B). This suggests that although Fascin is a downstream effector of PGs, PG signaling is likely regulating additional actin-binding proteins to control actin remodeling during nurse cell dumping. Indeed, our pharmaco-interaction screen revealed several additional putative targets of PGs (Spracklen, Meyer, and Tootle, unpublished data).

The UAS/Gal4 system was also used to overexpress Fascin in the germline in a *pxt*-mutant background. Similar to the

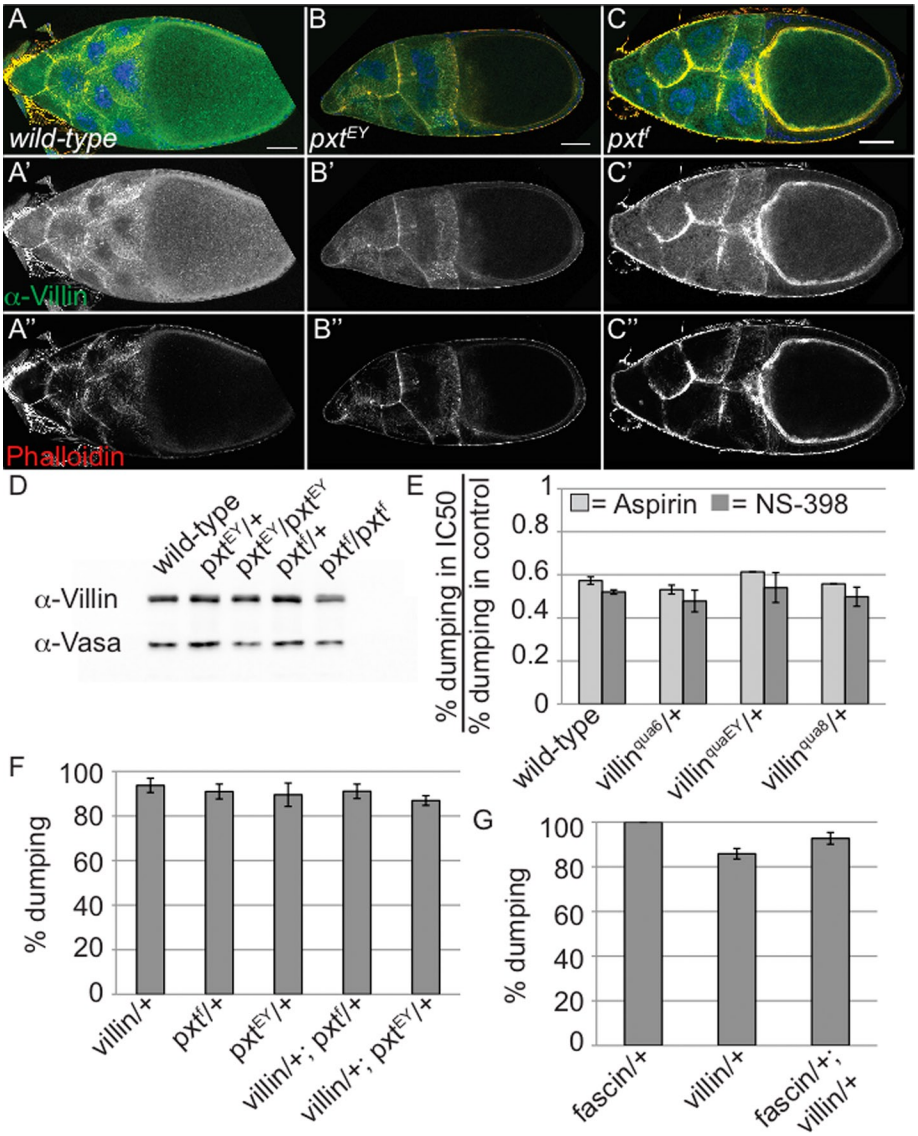

FIGURE 5: Villin is not a downstream target of PG signaling. (A–C″) Maximum projections of three to five confocal slices of S10B follicles. (A–A″) Wild type (*yw*). (B–B″) pxt^{EY}. (C–C″) pxt^{f}. (A–C) Merged images: Villin, green; F-actin (phalloidin), red; DNA (DAPI), blue. (A′–C′) Villin, white. (A″–C″) F-actin (phalloidin), white. (D) Western blot. Vasa serves as the loading control. (E–G) In vitro follicle maturation assays. Villin localizes to the membranes and the bundles within the nurse cells (A–A′). Whereas *pxt* mutants exhibit numerous actin defects (B″, C″), Villin localizes normally to the actin structures within the mutant nurse cells (B–C′), and Villin protein levels are unchanged (D). Reduced Villin levels have no effect on COX inhibitor (aspirin and NS-398) sensitivity (E) or reduced *pxt* levels (F) by the in vitro follicle maturation assay. Similarly, coreduction of both Villin and Fascin does not affect nurse cell dumping (G). Scale bar, 50 μm.

pharmacological experiments, in the in vitro follicle maturation assay, overexpression of Fascin suppresses the dumping defects due to reduced Pxt levels (Figure 6C). This suppression could occur if loss of PG signaling results in reduced Fascin expression. Previously microarray analysis revealed that *fascin* mRNA levels are not changed in *pxt* mutants compared with wild-type follicles throughout the end of oogenesis (Tootle *et al.*, 2011). In addition, Western blot analysis of either whole-ovary or S10B follicle lysates reveals that Fascin levels are not reduced in *pxt* mutants (Figure 6D and data not shown). Thus PGs do not regulate Fascin at the level of expression.

We next assessed the extent to which increased Fascin levels rescue the in vivo actin defects in *pxt*-mutant follicles (S10B–S11). Control genotypes (*UAS Fascin; pxt^{f}/+* or *UAS Fascin; matαGal4; pxt^{f}/bal*) exhibit few actin-remodeling defects (Figure 7, A–A′, D, and E), whereas *pxt* mutants (*UAS Fascin; pxt^{f}/pxt^{f}*) exhibit severe defects, from abnormal bundles to a substantial reduction in bundle formation and cortical actin breakdown (Figure 7, B–B′, D, and E). Overexpression of Fascin in *pxt* mutants (*UAS Fascin; matαGal4; pxt^{f}/pxt^{f}*) partially rescues these defects (Figure 7, C–C′, D, and E). Quantification of these actin defects (Figure 7D) in S10B–S11 was performed using confocal microscopy (see *Materials and Methods*) and reveals that *UAS Fascin; pxt^{f}/+* exhibits actin defects in ~15% of the follicles (all with defective bundles, $n = 13$) and *UAS Fascin; matαGal4; pxt^{f}/+* exhibits defects in ~20% of the follicles (all with defective bundles, $n = 15$), whereas *UAS Fascin; pxt^{f}/pxt^{f}* exhibits defects in 100% of the follicles (~28% with no bundles and 72% with defective bundles, $n = 18$). It is important to note that based on our extensive characterization of *pxt*-mutant phenotypes, the percentage of follicles with no bundles appears reduced when UAS Fascin is in the background; this suggests that low expression of Fascin may occur from this construct in the absence of a Gal4 driver. The actin defects due to loss of Pxt are suppressed by overexpression of Fascin in the germline (*UAS Fascin; matαGal4; pxt^{f}/pxt^{f}*), because only ~42% of the follicles exhibit actin defects (~11% with no bundles and ~31% with defective bundles, $n = 19$). These data are consistent with Fascin being a downstream target of PGs during parallel bundle formation during nurse cell dumping. Furthermore, the percentage of S10B–11 follicles that exhibit cortical actin breakdown (Figure 7, B–B′ and E) is significantly decreased in *UAS Fascin; matαGal4; pxt^{f}/pxt^{f}* (~13%, $n = 23$) compared with *UAS Fascin; pxt^{f}/pxt^{f}* (~77%, $n = 22$). Taken together, these data support the model that PGs regulate Fascin to control bundle formation and cortical actin integrity during late *Drosophila* follicle development.

DISCUSSION

PGs regulate actin remodeling during *Drosophila* nurse cell dumping (Tootle and Spradling, 2008). Dumping has been widely used to identify and characterize the conserved functions of actin-binding proteins (reviewed in Hudson and Cooley, 2002b), making it an ideal system with which to determine the mechanisms by which PGs regulate actin dynamics. Here we provide the first evidence that *Drosophila* Fascin (Singed, Sn), an actin-bundling protein, is a downstream target of PGs and is required for PG-dependent formation of parallel actin filament bundles and maintenance of cortical actin integrity.

Mutations in *fascin* and *pxt* exhibit similar phenotypes, including the lack of or a reduction in bundles, breakdown of cortical actin,

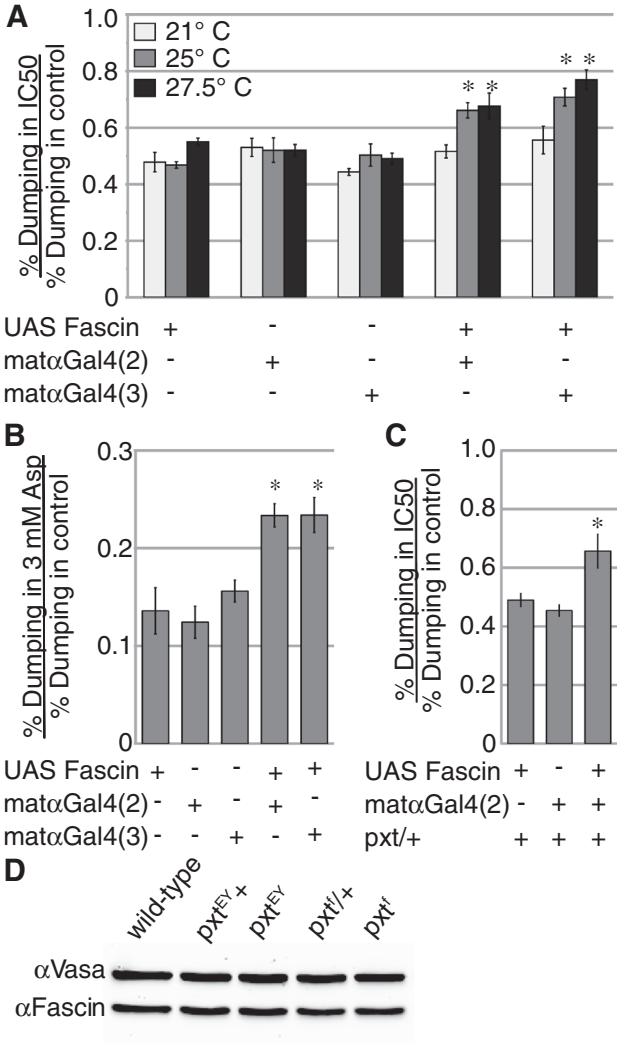

FIGURE 6: Overexpression of Fascin suppresses the effects of COX inhibition. (A–C) In vitro follicle maturation assays. (A, C) IC_{50} aspirin (1.5 mM) treatment of follicles from the indicated genotypes and temperatures. Data are shown as the percentage of follicles dumping in IC_{50} of the COX inhibitor divided by the percentage of follicles dumping in control media. (B) Data are shown as the percentage of follicles dumping in 3 mM aspirin divided by the percentage of follicles dumping in control media; the flies were maintained at 27.5°C. (D) Western blot of whole-ovary lysates; Vasa serves as the loading control. Overexpression of Fascin in the germline using the UAS/Gal4 system suppresses the effects of COX inhibition in a temperature-dependent manner (A, *UAS Fascin/+; matαGal4(2)/+*: $p = 0.038$ at 25°C and $p = 0.022$ at 27.5°C compared with driver, and $p = 0.0009$ at 25°C and $p = 0.047$ at 27.5°C compared with UAS. *UAS Fascin/+;; matαGal4(3)/+*: $p = 0.006$ at 25°C and $p = 0.001$ at 27.5°C compared with driver, and $p = 0.0007$ at 25°C and $p = 0.0046$ at 27.5°C compared with UAS). In addition, overexpression of Fascin at 27.5°C suppresses the effects of high-dose aspirin treatment (B, *UAS Fascin/+; matαGal4(2)/+*: $p = 0.0017$ compared with driver and $p = 0.0071$ compared with UAS; *UAS Fascin/+;; matαGal4(3)/+*: $p = 0.0023$ compared with driver and $p = 0.0074$ compared with UAS). Overexpression of Fascin (25°C) also suppresses the effects of reduced Pxt levels (C, $p = 0.015$ compared with driver and $p = 0.032$ compared with UAS). Each bar in A–C represents a minimum of three independent experiments from at least two separate crosses. $^*p < 0.05$ compared with controls using a paired *t* test, unequal variance. Fascin protein levels are not reduced in *pxt*-mutant ovaries (D).

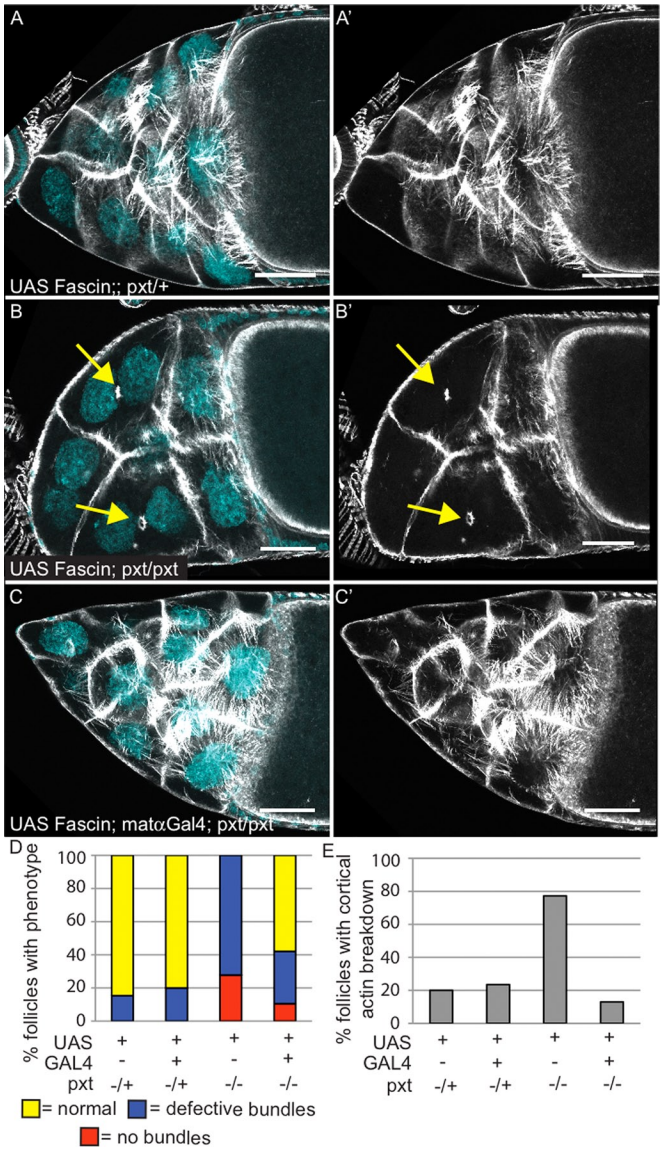

FIGURE 7: Overexpression of Fascin suppresses the actin-remodeling defects in *pxt* mutants. (A, C′) Maximum projections of three to five confocal slices of S10B follicles. (A–C) Merged images: F-actin (phalloidin), white; DNA (DAPI), cyan. (A′–C′) F-actin (phalloidin), white. (D–E) Charts quantifying the observed actin-remodeling defects. (A–A′) *UAS Fascin; pxt^f/+*. (B–B′) *UAS Fascin; pxt^f/pxt^f*. (C–C′) *UAS Fascin; matα-Gal4; pxt^f/pxt^f*. Immunofluorescence analyses reveal that *UAS Fascin; pxt^f/pxt^f* follicles exhibit *pxt*-like actin defects, including reduced to no bundle formation, and cortical actin breakdown (B–B′ compared with A–A′; yellow arrow, actin aggregate at site of cortical actin breakdown). Overexpression of Fascin in the germline of *pxt* mutants (*UAS Fascin; matα-Gal4; pxt^f/pxt^f*) suppresses the actin defects in *pxt* mutants, partially restoring bundle formation (C–C′). Quantification of these actin-remodeling defects reveals that that whereas 100% of the *UAS Fascin; pxt^f/pxt^f* follicles exhibit bundle defects, overexpression of Fascin in the germline rescues this, as 42% of the *UAS Fascin; matα-Gal4; pxt^f/pxt^f* follicles exhibit bundle defects (D; $n = 13$ for *UAS Fascin; pxt^f/+*, $n = 15$ for *UAS Fascin; matα-Gal4; pxt^f/+*, $n = 18$ for *UAS Fascin; pxt^f/pxt^f*, and $n = 19$ for *UAS Fascin; matα-Gal4; pxt^f/pxt^f*). In addition, overexpression of Fascin suppresses the cortical actin breakdown defects in *pxt* mutants (C–C′, E; $n = 20$ for *UAS Fascin; pxt^f/+*, $n = 17$ for *UAS Fascin; matα-Gal4; pxt^f/+*, $n = 23$ for *UAS Fascin; matα-Gal4; pxt^f/pxt^f*, and $n = 22$ for *UAS Fascin; pxt^f/pxt^f*). Scale bar, 50 μm.

inhibition of dumping, and, ultimately, female sterility. Reduced Fascin levels enhance the effects of COX inhibitor treatment and synergize with reduced Pxt levels to cause actin-remodeling defects and a block in dumping. This supports the model that *Drosophila* Fascin is a downstream target of PGs. Additional evidence for this model comes from overexpression experiments in which increased Fascin levels suppress the defects due to the loss of PG synthesis.

Although there are many similarities in the actin defects in *pxt* and *fascin* mutants, there are also differences. The bundles in *pxt*-mutant follicles are variable in length and are not evenly distributed along membranes, whereas those in *fascin* mutant follicles exhibit uniform length and distribution but fail to elongate. We interpret these differences as evidence that whereas Fascin is a downstream target of PGs, PGs regulate additional factors to control actin remodeling. Indeed, we identified a number of other putative PG effectors in our pharmaco-interaction screen (Spracklen, Meyer, and Tootle, unpublished data).

The integrity of the cortical actin in the nurse cells is not maintained in either *pxt* or *fascin* mutants. The breakdown of the cortical actin occurs starting in S8 in *pxt* mutants (Tootle and Spradling, 2008). This breakdown is suppressed by overexpression of Fascin. These data indicate that Fascin plays an important, and previously undescribed, role in cortical actin. Supporting this idea, Fascin levels appear enriched in the subcortical region (Cant *et al.*, 1994) and green fluorescent protein–Fascin localizes to the cortical actin (data not shown and Zanet *et al.*, 2009). At first glance, it seems surprising that Fascin regulates the cortical actin, given that it is believed to be a branched network of actin filaments. Supporting that this is indeed the structure of the cortical actin in the nurse cells are the findings that cortical actin integrity requires Arp2/3 subunits (Hudson and Cooley, 2002a) and Wash (Liu *et al.*, 2009), a regulator of Arp2/3 activity. Other factors regulating nurse cell cortical actin include Profilin (Cooley *et al.*, 1992), an actin monomer–binding protein, Enabled (Gates *et al.*, 2009), an actin filament elongation factor, and Capping (Gates *et al.*, 2009), a barbed end–binding protein and antagonist of Enabled. All of these actin regulators are implicated in the formation of branched actin networks. Fascin, however, has not been widely implicated in the formation of such a network because Fascin is generally found in parallel bundles (reviewed in Edwards and Bryan, 1995). Of interest, a few mammalian cell culture studies have revealed a role of Fascin-1 in lamellipodia, which are composed of branched actin networks. Specifically, Rac and Cdc42 trigger the localization of Fascin-1 to the lamellopodia, where it contributes to the formation of microspikes necessary for cell motility (Adams, 1997; Adams *et al.*, 1999; Adams and Schwartz, 2000). In addition, in fish fibroblasts it has been shown that Fascin 1–dependent bundles are folded into the lamella network (Nemethova *et al.*, 2008). These previous findings, along with our evidence that Fascin regulates nurse cell cortical actin, suggest that Fascin may generate microspikes or short filopodia that are required to strengthen the likely branched network of this cortical actin. Furthermore, there is evidence that, in vitro, Arp2/3 and Fascin-1 activity must be balanced to regulate the type and extent of actin polymerization (Ideses *et al.*, 2008). Such an Arp2/3-dependent network can lead to Fascin-1 recruitment and bundle formation (Vignjevic *et al.*, 2003). These data make it tempting to speculate that Arp2/3 and Fascin activity must be balanced in order to generate properly structured nurse cell cortical actin. If there is too much Arp2/3 or too little Fascin activity, then the cortical actin structure is altered such that integrity is lost. Thus, in *pxt* mutants, where Fascin activity is likely reduced, cortical actin breaks downs; this is rescued by overexpression of Fascin. It will be interesting to determine whether reduced Arp2/3 activity also suppresses the cortical actin defects in *pxt* mutants. Another possibility for the role of Fascin in cortical actin is that bundle elongation is required to maintain the cortical actin. Therefore the bundle defects in *pxt* and *fascin* mutants would cause the breakdown of the cortical actin, and because overexpression of Fascin in *pxt* mutants restores bundle formation/elongation, the cortical actin defects are also suppressed. We think that this is unlikely because cortical actin defects are apparent in S8 *pxt*-mutant follicles, well before the onset of bundle formation (Tootle and Spradling, 2008). Further characterization of the structure of the nurse cell cortical actin and the interplay between the factors required for its integrity is required to determine the role of Fascin.

Although we showed that Fascin is a downstream target of PG signaling during nurse cell dumping, Villin (Quail), another actin-bundling protein required for nurse cell dumping, does not interact with PGs. Previous work indicates that Villin mediates initial bundle formation during nurse cell dumping (Mahajan-Miklos and Cooley, 1994b; Cant *et al.*, 1998; Matova *et al.*, 1999). Fascin then bundles the filaments more tightly, increasing bundle strength. We find that heterozygosity for *villin* fails to enhance the dumping defects due to reduced PG synthesis. These results may seem surprising, given that overexpression of Villin partially rescues *fascin*-mutant phenotypes (Cant *et al.*, 1998). However, such bundles are structurally different, and overexpression may not completely reflect endogenous function. Therefore we interpret our findings to mean that Villin is not likely to be regulated by PGs. Supporting this model, Villin expression and localization are grossly normal in *pxt* mutants. An alternative interpretation is that Villin levels may not have been reduced enough to detect an interaction by our assays.

Prostaglandins could regulate Fascin activity in a number of ways. In human cells, protein kinase C (PKC) phosphorylates Fascin-1, blocking filamentous actin (F-actin) binding (Ono *et al.*, 1997; Adams *et al.*, 1999). In addition, human Fascin-1 competes with caldesmon and tropomyosin for F-actin (Ishikawa *et al.*, 1998). Calmodulin, and thus Ca^{2+}/cAMP signaling, negatively regulates these two proteins, promoting Fascin-1's bundling activity. Rac, a Rho-type GTPase, also positively regulates human Fascin-1 (Adams and Schwartz, 2000; Parsons and Adams, 2008; Hashimoto *et al.*, 2007). A recent study revealed that Fascin-1 is also regulated by Rho via LIM kinase 1 (Jayo *et al.*, 2012). Of note, PGs are known to signal through all of these mechanisms. Given that *Drosophila* Fascin PKC-site phosphomutants (S52A/E) restore nurse cell dumping in *fascin* mutants (Zanet *et al.*, 2009), it is unlikely that PGs regulate Fascin in this manner during this process. However, an additional phosphorylation site (S289), associated with a bundling-independent function, has recently been identified in *Drosophila* (Zanet *et al.*, 2012); perhaps this role of Fascin contributes to cortical actin integrity. Because both cAMP (Lane and Kalderon, 1993; Schneider and Spradling, 1997; Lannutti and Schneider, 2001; Fan and Schneider, 2003) and Rho GTPase (Murphy and Montell, 1996; Genova *et al.*, 2000) regulate nurse cell dumping, it will be important to determine whether PGs signal via these pathways to regulate Fascin. It remains possible that PGs regulate Fascin by a previously unidentified means.

One alternative mechanism by which PGs could regulate Fascin is through direct modification. 15-Deoxy-prostaglandin J_2, produced by nonenzymatic processing of $PGF_{2\alpha}$, modifies (prostanylates) cysteine residues on proteins in mammalian cells. It is intriguing that actin and cytoskeletal regulatory proteins, including tropomyosin, have been shown to be prostanylated (Stamatakis *et al.*, 2006; Yamamoto *et al.*, 2011). Therefore PGs could directly modify Fascin or a protein that regulates Fascin.

This work is the first evidence linking PGs to Fascin. Of interest, high levels of both PGs (Rolland *et al.*, 1980; Chen *et al.*, 2001; Khuri *et al.*, 2001; Gallo *et al.*, 2002; Denkert *et al.*, 2003) and Fascin-1 (Hashimoto *et al.*, 2004; Yoder *et al.*, 2005; Lee *et al.*, 2007; Okada *et al.*, 2007; Li *et al.*, 2008; Chan *et al.*, 2010) independently correlate with highly aggressive cancers in patients. In addition, both are critical for cancer cell invasion in human cell culture and mouse models (PGs; Tsujii *et al.*, 1997; Chen *et al.*, 2001; Lyons *et al.*, 2011). Specifically, Fascin-1 bundles actin filaments within filopodia (Vignjevic *et al.*, 2006; Hashimoto *et al.*, 2007) and invadopodia (Li *et al.*, 2010; Schoumacher *et al.*, 2010), structures required for cancer metastasis. These parallel actin bundles are nearly structurally identical to those found in the nurse cells during dumping (Guild *et al.*, 1997). This leads us to speculate that PGs control Fascin-1 within human cancer cells to tightly regulate the formation of invasive cytoskeletal structures and thus cancer metastasis. It will be critical to determine the detailed mechanisms and signaling cascade by which PGs regulate Fascin and whether this regulation is conserved from *Drosophila* follicle development to human cancer progression.

MATERIALS AND METHODS

Fly strains

Fly stocks were maintained at 21, 25, or 27.5°C, as indicated, on standard cornmeal-agar-yeast food. Flies were fed with wet yeast paste daily for ovary analysis, including in vitro follicle maturation, immunofluorescence, and Western blotting. *yw* was used as the wild-type control in experiments except where indicated otherwise. $pxt^{EY03052}$ (referred to as pxt^{EY} in all the figures), sn^{34e}, sn^{2}, sn^{36a}, matαGal4 (second and third chromosomes), and $qua^{6\text{-}396}$, $qua^{8\text{-}1062}$, and $qua^{EY03072}$ fly stocks were obtained from the Bloomington *Drosophila* Stock Center (Bloomington, IN); pxt^{f01000} (referred to as pxt^{f} in all the figures) was obtained from Harvard Exelixis (Cambridge, MA); and the UASp Fascin transgenic fly line was a generous gift from François Payre (Zanet *et al.*, 2009).

In vitro follicle maturation

This was performed as previously described (Tootle and Spradling, 2008). Aspirin, NS-398, and fluprostenol ($PGF_{2\alpha}$ analogue) were dissolved in either dimethyl sulfoxide or ethanol (Cayman Chemical Company, Ann Arbor, MI). Experiments were performed a minimum of three times, and standard errors are shown. Paired *t* tests were performed using Excel (Microsoft, Redmond, WA).

Immunofluorescence

Whole-mount samples were fixed for 10 min at room temperature in 4% paraformaldehyde in Grace's insect media (Lonza, Walkersville, MD). Samples were processed using standard procedures (Cox and Spradling, 2003) with the following exceptions: samples were blocked by washing in antibody wash (1× phosphate-buffered saline [PBS; 135 mM NaCl, 3.2 mM Na_2HPO_4, 1.3 mM KCl, 0.5 mM KH_2PO_4, pH 7.4], 0.1% bovine serum albumin, 0.1% Triton X-100) six times for 10 min each and mounted in 1 mg/ml phenylenediamine in 50% glycerol, pH 9 (Platt and Michael, 1983).

In Figure 1, samples were subjected to a fixation protocol designed to better preserve actin structures (modified from that described by Frydman and Spradling, 2001). In short, samples are fixed for 10 min at room temperature in 4% paraformaldehyde, 2% Triton X-100, and 1 U/ml fluorophore-conjugated phalloidin in Grace's insect media. This was followed by two rinses in Triton-antibody wash (PBS, 0.2% TX-100). Blocking was performed by washing samples three times for 10 min each in antibody wash supplemented with 1 U/ml fluorophore-conjugated phalloidin, followed by washing three times for 10 min each in antibody wash alone. Staining and mounting were carried out as indicated.

Mouse α-quail (6B9) (Cooley, L.) was obtained from the Developmental Studies Hybridoma Bank (DSHB; developed under the auspices of the National Institute of Child Health and Human Development and maintained by the University of Iowa, Department of Biology, Iowa City, IA). The mouse anti-phosphotyrosine antibody (4G10), used 1:500, was from EMD Millipore (Billerica, MA). Additional stains used and their concentrations are as follows: rhodamine::phalloidin, 1:500; Alexa Fluor (AF) 488::phalloidin, 1:500; 4′,6-diamidino-2-phenylindole (DAPI; 5 mg/ml), 1:10,000 (all from Invitrogen, Grand Island, NY). The following secondary antibodies were used at 1:1000 to 1:2000 as appropriate: AF488::goat anti-mouse, AF568::goat anti-mouse, and AF633::goat anti-mouse (Invitrogen).

Quantification of actin defects by confocal microscopy

The S10B–S11 follicles were examined by confocal microscopy to score actin bundle defects and cortical actin breakdown. Either the top and bottom of each S10B follicle was marked and a single 20× image at the center of the follicle was acquired for both phalloidin and DAPI or complete *Z*-stacks were acquired. The DAPI staining was used to assess how far the centripetal follicle cells had migrated over the oocyte; this was used to define the point during S10B that each follicle was at, allowing its bundle formation to be compared with similarly staged wild-type or control follicles. Phalloidin staining was used to assess the bundle and cortical actin defects. Follicles were scored as having either: 1) normal bundles, of the expected length for the developmental point in S10B, straight, and uniformly distributed along the nurse cell membranes; 2) defective bundles, of variable lengths or the wrong length for the developmental point, bent/aggregated, and/or not uniformly distributed along the membranes; or 3) no bundles, the absence of elongated bundles. Follicles were also scored for cortical actin breakdown, as observed by incomplete cortical actin around the nurse cell and two or more nuclei enclosed in one cortical actin loop.

Microscope image acquisition and processing

All microscopy images were acquired via Zen software on a Zeiss 700 LSM mounted on an Axio Observer.Z1 using a Plan-Apochromat 20×/0.8 M27 or EC Plan-Neofluar 40×/1.30 DIC M27 objectives (Carl Zeiss Microscopy, Thornwood, NY). Complete S14 follicles (Figure 1) were imaged at 20× and acquired by tiling (10% overlap) and stitching using the Zen software. Maximum projections, image rotation, and cropping were performed in ImageJ (Abramoff *et al.*, 2004). To aid in visualization, all panels for Figure 1 were brightened by 30% in all channels using Photoshop (Adobe, San Jose, CA).

Western blot

Standard Western blotting techniques were used. The following primary antibodies were obtained from the DSHB and were used at the following concentrations: Fascin (mouse anti–sn 7C) 1:20 (Cooley, L.); Villin (mouse anti-6B9, concentrated) 1:50 (Cooley, L.); and Vasa (rat anti-Vasa) 1:100 (Spradling, A. C. and Williams, D.). Fascin and Villin blots had 0.2% Tween 20, whereas all others had 0.1% Tween 20 added to the primary antibody. The following secondary antibodies were used: Peroxidase-AffiniPure Goat Anti-Rat IgG (H+L) (1:5000) or Peroxidase-AffiniPure Goat Anti-Mouse IgG (H+L) (1:5000; Jackson ImmunoResearch Laboratories, West Grove, PA). Blots were developed with SuperSignal West Pico Chemiluminescent Substrate (Thermo Scientific, Waltham, MA) and imaged using

ChemiDoc-It Imaging System and VisionWorksLS software (UVP, Upland, CA). Bands were quantified using the gel analyzer function of ImageJ (Abramoff *et al.*, 2004).

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REFERENCES

Abramoff MD, Magalhaes PJ, Ram SJ (2004). Image processing with ImageJ. Biophotonics Int 11, 36–42.

Adams JC (1997). Characterization of cell-matrix adhesion requirements for the formation of fascin microspikes. Mol Biol Cell 8, 2345–2363.

Adams JC, Clelland JD, Collett GD, Matsumura F, Yamashiro S, Zhang L (1999). Cell-matrix adhesions differentially regulate fascin phosphorylation. Mol Biol Cell 10, 4177–4190.

Adams JC, Schwartz MA (2000). Stimulation of fascin spikes by thrombospondin-1 is mediated by the GTPases Rac and Cdc42. J Cell Biol 150, 807–822.

Birukova AA, Zagranichnaya T, Fu P, Alekseeva E, Chen W, Jacobson JR, Birukov KG (2007). Prostaglandins PGE(2) and PGI(2) promote endothelial barrier enhancement via PKA- and Epac1/Rap1-dependent Rac activation. Exp Cell Res 313, 2504–2520.

Bos CL, Richel DJ, Ritsema T, Peppelenbosch MP, Versteeg HH (2004). Prostanoids and prostanoid receptors in signal transduction. Int J Biochem Cell Biol 36, 1187–1205.

Brand AH, Perrimon N (1993). Targeted gene expression as a means of altering cell fates and generating dominant phenotypes. Development 118, 401–415.

Bulin C, Albrecht U, Bode JG, Weber AA, Schror K, Levkau B, Fischer JW (2005). Differential effects of vasodilatory prostaglandins on focal adhesions, cytoskeletal architecture, and migration in human aortic smooth muscle cells. Arterioscler Thromb Vasc Biol 25, 84–89.

Cant K, Knowles BA, Mahajan-Miklos S, Heintzelman M, Cooley L (1998). *Drosophila* fascin mutants are rescued by overexpression of the villin-like protein, quail. J Cell Sci 111, 213–221.

Cant K, Knowles BA, Mooseker MS, Cooley L (1994). *Drosophila* Singed, a Fascin homolog, is required for actin bundle formation during oogenesis and bristle extension. J Cell Biol 125, 369–380.

Chan C, Jankova L, Fung CL, Clarke C, Robertson G, Chapuis PH, Bokey L, Lin BP, Dent OF, Clarke S (2010). Fascin expression predicts survival after potentially curative resection of node-positive colon cancer. Am J Surg Pathol 34, 656–666.

Chen L, Yang S, Jakoncic J, Zhang JJ, Huang X-Y (2010). Migrastatin analogues target fascin to block tumour metastasis. Nature 464, 1062–1066.

Chen WS, Wei SJ, Liu JM, Hsiao M, Kou-Lin J, Yang WK (2001). Tumor invasiveness and liver metastasis of colon cancer cells correlated with cyclooxygenase-2 (COX-2) expression and inhibited by a COX-2-selective inhibitor, etodolac. Int J Cancer 91, 894–899.

Cooley L, Verheyen E, Ayers K (1992). chickadee encodes a profilin required for intercellular cytoplasm transport during *Drosophila* oogenesis. Cell 69, 173–184.

Cox RT, Spradling AC (2003). A Balbiani body and the fusome mediate mitochondrial inheritance during *Drosophila* oogenesis. Development 130, 1579–1590.

Denkert C *et al.* (2003). Elevated expression of cyclooxygenase-2 is a negative prognostic factor for disease free survival and overall survival in patients with breast carcinoma. Cancer 97, 2978–2987.

Dormond O, Bezzi M, Mariotti A, Ruegg C (2002). Prostaglandin E2 promotes integrin alpha Vbeta 3-dependent endothelial cell adhesion, rac-activation, and spreading through cAMP/PKA-dependent signaling. J Biol Chem 277, 45838–45846.

Edwards RA, Bryan J (1995). Fascins, a family of actin bundling proteins. Cell Motil Cytoskeleton 32, 1–9.

Fan S, Schneider LE (2003). The role of maternal and zygotic Gprk2 expression in *Drosophila* development. Biochem Biophys Res Commun 301, 127–135.

Frydman HM, Spradling AC (2001). The receptor-like tyrosine phosphatase lar is required for epithelial planar polarity and for axis determination within *Drosophila* ovarian follicles. Development 128, 3209–3220.

Funk CD (2001). Prostaglandins and leukotrienes: advances in eicosanoid biology. Science 294, 1871–1875.

Gallo O, Masini E, Bianchi B, Bruschini L, Paglierani M, Franchi A (2002). Prognostic significance of cyclooxygenase-2 pathway and angiogenesis in head and neck squamous cell carcinoma. Hum Pathol 33, 708–714.

Gates J, Nowotarski SH, Yin H, Mahaffey JP, Bridges T, Herrera C, Homem CC, Janody F, Montell DJ, Peifer M (2009). Enabled and Capping protein play important roles in shaping cell behavior during *Drosophila* oogenesis. Dev Biol 333, 90–107.

Genova JL, Jong S, Camp JT, Fehon RG (2000). Functional analysis of Cdc42 in actin filament assembly, epithelial morphogenesis, and cell signaling during *Drosophila* development. Dev Biol 221, 181–194.

Guild GM, Connelly PS, Shaw MK, Tilney LG (1997). Actin filament cables in *Drosophila* nurse cells are composed of modules that slide passively past one another during dumping. J Cell Biol 138, 783–797.

Hashimoto Y, Parsons M, Adams JC (2007). Dual actin-bundling and protein kinase c-binding activities of Fascin regulate carcinoma cell migration downstream of rac and contribute to metastasis. Mol Biol Cell 18, 4591–4602.

Hashimoto Y, Shimada Y, Kawamura J, Yamasaki S, Imamura M (2004). The prognostic relevance of fascin expression in human gastric carcinoma. Oncology 67, 262–270.

Hashimoto Y, Skacel M, Adams JC (2005). Roles of fascin in human carcinoma motility and signaling: prospects for a novel biomarker? Int J Biochem Cell Biol 37, 1787–1804.

Hudson AM, Cooley L (2002a). A subset of dynamic actin rearrangements in *Drosophila* requires the Arp2/3 complex. J Cell Biol 156, 677–687.

Hudson AM, Cooley L (2002b). Understanding the function of actin-binding proteins through genetic analysis of *Drosophila* oogenesis. Annu Rev Genet 36, 455–488.

Hwang JH, Smith CA, Salhia B, Rutka JT (2008). The role of fascin in the migration and invasiveness of malignant glioma cells. Neoplasia 10, 149–159.

Ideses Y, Brill-Karniely Y, Haviv L, Ben-Shaul A, Bernheim-Groswasser A (2008). Arp2/3 branched actin network mediates filopodia-like bundles formation in vitro. PLoS One 3, e3297.

Ishikawa R, Yamashiro S, Kohama K, Matsumura F (1998). Regulation of actin binding and actin bundling activities of fascin by caldesmon coupled with tropomyosin. J Biol Chem 273, 26991–26997.

Jayo A, Parsons M, Adams JC (2012). A novel Rho-dependent pathway that drives interaction of fascin-1 with LIM kinase1/2 to promote fascin-1/actin binding and filopodia stability. BMC Biol 10, 72.

Khuri FR, Wu H, Lee JJ, Kemp BL, Lotan R, Lippman SM, Feng L, Hong WK, Xu XC (2001). Cyclooxygenase-2 overexpression is a marker of poor prognosis in stage I non-small cell lung cancer. Clin Cancer Res 7, 861–867.

Lane ME, Kalderon D (1993). Genetic investigation of cAMP-dependent protein kinase function in *Drosophila* development. Genes Dev 7, 1229–1243.

Lannutti BJ, Schneider LE (2001). Gprk2 controls cAMP levels in *Drosophila* development. Dev Biol 233, 174–185.

Lee TK, Poon RT, Man K, Guan XY, Ma S, Liu XB, Myers JN, Yuen AP (2007). Fascin over-expression is associated with aggressiveness of oral squamous cell carcinoma. Cancer Lett 254, 308–315.

Li A, Dawson JC, Forero-Vargas M, Spence HJ, Yu X, Konig I, Anderson K, Machesky LM (2010). The actin bundling protein fascin stabilizes actin in invadopodia and potentiates protrusive invasion. Curr Biol 20, 339–345.

Li X, Zheng H, Hara T, Takahashi H, Masuda S, Wang Z, Yang X, Guan Y, Takano Y (2008). Aberrant expression of cortactin and fascin are effective markers for pathogenesis, invasion, metastasis and prognosis of gastric carcinomas. Int J Oncol 33, 69–79.

Liu R, Abreu-Blanco MT, Barry KC, Linardopoulou EV, Osborn GE, Parkhurst SM (2009). Wash functions downstream of Rho and links linear and branched actin nucleation factors. Development 136, 2849–2860.
Lyons TR, O'Brien J, Borges VF, Conklin MW, Keely PJ, Eliceiri KW, Marusyk A, Tan AC, Schedin P (2011). Postpartum mammary gland involution drives progression of ductal carcinoma in situ through collagen and COX-2. Nat Med 17, 1109–1115.
Mahajan-Miklos S, Cooley L (1994a). Intercellular cytoplasm transport during *Drosophila* oogenesis. Dev Biol 165, 336–351.
Mahajan-Miklos S, Cooley L (1994b). The villin-like protein encoded by the *Drosophila* quail gene is required for actin bundle assembly during oogenesis. Cell 78, 291–301.
Matova N, Mahajan-Miklos S, Mooseker MS, Cooley L (1999). *Drosophila* quail, a villin-related protein, bundles actin filaments in apoptotic nurse cells. Development 126, 5645–5657.
Minn AJ, Gupta GP, Siegel PM, Bos PD, Shu W, Giri DD, Viale A, Olshen AB, Gerald WL, Massague J (2005). Genes that mediate breast cancer metastasis to lung. Nature 436, 518–524.
Murphy AM, Montell DJ (1996). Cell type-specific roles for Cdc42, Rac, and RhoL in *Drosophila* oogenesis. J Cell Biol 133, 617–630.
Nemethova M, Auinger S, Small JV (2008). Building the actin cytoskeleton: filopodia contribute to the construction of contractile bundles in the lamella. J Cell Biol 180, 1233–1244.
Okada K, Shimura T, Asakawa K, Hashimoto S, Mochida Y, Suehiro T, Kuwano H (2007). Fascin expression is correlated with tumor progression of extrahepatic bile duct cancer. Hepatogastroenterology 54, 17–21.
Ono S, Yamakita Y, Yamashiro S, Matsudaira PT, Gnarra JR, Obinata T, Matsumura F (1997). Identification of an actin binding region and a protein kinase c phosphorylation site on human Fascin. J Biol Chem 272, 2527–2533.
Parsons M, Adams JC (2008). Rac regulates the interaction of fascin with protein kinase C in cell migration. J Cell Sci 121, 2805–2813.
Peppelenbosch MP, Tertoolen LGJ, Hage WJ, de Laat SW (1993). Epidermal growth factor induced actin remodeling is regulated by 5-lipoxygenase and cyclooxygenase products. Cell 74, 565–575.
Pierce KL, Fujino H, Srinivasan D, Regan JW (1999). Activation of FP prostanoid receptor isoforms leads to Rho-mediated changes in cell morphology and in the cell cytoskeleton. J Biol Chem 274, 35944–35949.
Platt JL, Michael AF (1983). Retardation of fading and enhancement of intensity of immunofluorescence by p-phenylenediamine. J Histochem Cytochem 31, 840–842.
Rolland PH, Martin PM, Jacquemier J, Rolland AM, Toga M (1980). Prostaglandin in human breast cancer: evidence suggesting that an elevated prostaglandin production is a marker of high metastatic potential for neoplastic cells. J Natl Cancer Inst 64, 1061–1070.
Schneider LE, Spradling AC (1997). The *Drosophila* G-protein-coupled receptor kinase homologue Gprk2 is required for egg morphogenesis. Development 124, 2591–2602.
Schoumacher M, Goldman RD, Louvard D, Vignjevic DM (2010). Actin, microtubules, and vimentin intermediate filaments cooperate for elongation of invadopodia. J Cell Biol 189, 541–556.
Spradling AC (1993). Developmental genetics of oogenesis. In: The Development of *Drosophila melanogaster,* ed. B. Martinez-Arias, Cold Spring Harbor, NY: Cold Spring Harbor Laboratory Press, 1–70.
Stamatakis K, Sanchez-Gomez FJ, Perez-Sala D (2006). Identification of novel protein targets for modification by 15-deoxy-delta12,14-prostaglandin J2 in mesangial cells reveals multiple interactions with the cytoskeleton. J Am Soc Nephrol 17, 89–98.
Tamma G, Wiesner B, Furkert J, Hahm D, Oksche A, Schaefer M, Valenti G, Rosenthal W, Klussmann E (2003). The prostaglandin E2 analogue sulprostone antagonizes vasopressin-induced antidiuresis through activation of Rho. J Cell Sci 116, 3285–3294.
Tootle TL, Spradling AC (2008). *Drosophila* Pxt: a cyclooxygenase-like facilitator of follicle maturation. Development 135, 839–847.
Tootle TL, Williams D, Hubb A, Frederick R, Spradling A (2011). *Drosophila* eggshell production: identification of new genes and coordination by Pxt. PLoS One 6, e19943.
Tsujii M, Kawano S, DuBois RN (1997). Cyclooxygenase-2 expression in human colon cancer cells increases metastatic potential. Proc Natl Acad Sci USA 94, 3336–3340.
Vignjevic D, Kojima S, Aratyn Y, Danciu O, Svitkina T, Borisy GG (2006). Role of fascin in filopodial protrusion. J Cell Biol 174, 863–875.
Vignjevic D, Yarar D, Welch MD, Peloquin J, Svitkina T, Borisy GG (2003). Formation of filopodia-like bundles in vitro from a dendritic network. J Cell Biol 160, 951–962.
Wheatley S, Kulkarni S, Karess R (1995). *Drosophila* nonmuscle myosin II is required for rapid cytoplasmic transport during oogenesis and for axial nuclear migration in early embryos. Development 121, 1937–1946.
Xing P, Li JG, Jin F, Zhao TT, Liu Q, Dong HT, Wei XL (2011). Fascin, an actin-bundling protein, promotes breast cancer progression in vitro. Cell Biochem Funct 29, 303–310.
Yamamoto Y, Takase K, Kishino J, Fujita M, Okamura N, Sakaeda T, Fujimoto M, Yagami T (2011). Proteomic identification of protein targets for 15-deoxy-delta(12,14)-prostaglandin J2 in neuronal plasma membrane. PLoS One 6, e17552.
Yoder BJ, Tso E, Skacel M, Pettay J, Tarr S, Budd T, Tubbs RR, Adams JC, Hicks DG (2005). The expression of fascin, an actin-bundling motility protein, correlates with hormone receptor-negative breast cancer and a more aggressive clinical course. Clin Cancer Res 11, 186–192.
Zanet J, Jayo A, Plaza S, Millard T, Parsons M, Stramer B (2012). Fascin promotes filopodia formation independent of its role in actin bundling. J Cell Biol 197, 477–486.
Zanet J, Stramer B, Millard T, Martin P, Payre F, Plaza S (2009). Fascin is required for blood cell migration during *Drosophila* embryogenesis. Development 136, 2557–2565.