The *Drosophila* FHOD1-like formin Knittrig acts through Rok to promote stress fiber formation and directed macrophage migration during the cellular immune response

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**ABSTRACT**

A tight spatiotemporal control of actin polymerization is important for many cellular processes that shape cells into a multicellular organism. The formation of unbranched F-actin is induced by several members of the formin family. *Drosophila* encodes six formin genes, representing six of the seven known mammalian subclasses. Knittrig, the *Drosophila* homolog of mammalian FHOD1, is specifically expressed in the developing central nervous system midline glia, the trachea, the wing and in macrophages. *knittrig* mutants exhibit mild tracheal defects but survive until late pupal stages and mainly die as pharate adult flies. *knittrig* mutant macrophages are smaller and show reduced cell spreading and cell migration in *in vivo* wound experiments. Rescue experiments further demonstrate a cell-autonomous function of Knittrig in regulating actin dynamics and cell migration. Knittrig localizes at the rear of migrating macrophages in *in vivo*, suggesting a cellular requirement of Knittrig in the retraction of the trailing edge. Supporting this notion, we found that Knittrig is a target of the Rho-dependent kinase Rok. Co-expression with Rok or expression of an activated form of Knittrig induces actin stress fibers in macrophages and in epithelial tissues. Thus, we propose a model in which Rok-induced phosphorylation of residues within the basic region mediates the activation of Knittrig in controlling macrophage migration.

**KEY WORDS:** F-actin, *Drosophila*, Formin, Stress fiber, Tracheal development, Macrophages, Cellular immune response

**INTRODUCTION**

The development of multicellular organisms crucially depends on the ability of cells to respond to extracellular cues and to reshape and migrate in the context of the forming tissue (Pollard and Borisy, 2003; Pollard and Cooper, 2009). These processes require the tightly controlled polymerization of actin filaments. Eukaryotic cells have evolved different actin-nucleating proteins promoting the rate-limiting nucleation reaction, i.e. the formation of stable multimers of actin monomers (Firat-Karalar and Welch, 2011). The ubiquitous Arp2/3 complex is one of the best-studied actin-nucleating proteins promoting the actin filament branches on sides of pre-existing filaments (Pollard and Borisy, 2003; Pollard and Cooper, 2009). Unlike Arp2/3, all other known actin nucleators produce unbranched filaments, including the formins or tandem-monomer-

binding nucleators such as spire or JMY (Campellone and Welch, 2010; Carlier et al., 2011).

Formins assemble diverse cellular structures by nucleating unbranched actin filaments such as the cytokinetic contractile ring, stress fibers and filopodia (Goode and Eck, 2007). Defining features of all formins are formin homology domains 1 and 2 (FH1 and FH2). The homodimeric FH2 domain is thought to induce actin nucleation by stabilizing actin dimers, whereas the proline-rich FH1 domain promotes elongation by delivering profilin-bound actin monomers to the growing barbed filament end (Kovar, 2006). Most formins also contain additional conserved domains that regulate the activity, localization and interaction with regulatory binding partners. Mammals possess fifteen formins clustered into seven families based on domain similarities (Schönichen and Geyer, 2010). These families include the Diaphanous-related proteins (Dia), the Disheveled-associated activators of morphogenesis (Daam), the Formin-like proteins (FMNL), the Inverted formins (INF), the FH1/FH2 domain-containing proteins (FHOD/FHOS), the Delphin proteins and the group of FMN formins. Among all formins the Diaphanous-related formins (DRF) are best understood (Schönichen and Geyer, 2010). This group includes autoinhibited formins such as Dia, Daam and FMNL, which are direct effectors of Rho GTPases (Goode and Eck, 2007). The autoinhibition of DRF is mediated by an intramolecular interaction between the C-terminal Diaphanous autoregulatory domain (DAD) and the N-terminal Diaphanous inhibitory domain (DID/FH3) (Otomo et al., 2005). Binding of Rho GTPases to the GTPase-binding domain (GBD) adjacent to the DID/FH3 domain results in the activation of the formin protein.

Members of the FHOD formin family share the characteristic FH1, FH2 and DAD domains with other DRFs, but their mode of activation is less well understood. Unlike DRF family members, mammalian FHOD1 binds Rac and Rho but not Cdc42 (Gasteier et al., 2003; Westendorf and Koka, 2004). Although activated Rac1 recruits FHOD1 to the membrane, Rac binding is not sufficient for its activation (Gasteier et al., 2003; Gasteier et al., 2005). Structural analyses further confirmed significant differences of the N-terminal GBD of FHOD1, suggesting a different role beyond autoregulation (Schulte et al., 2008). Previous studies revealed that phosphorylation of the C-terminal DAD might be an important regulatory modification for FHOD activation. Co-expression of FHOD1 and the Rho-associated kinase Rok results in a strong induction of stress fibers. A similar phenotype is observed upon expression of a constitutively active FHOD1 protein lacking the C-terminal DAD (Takeya and Sumimoto, 2003; Takeya et al., 2008). Remarkably, recent *in vitro* actin polymerization assays with recombinant FHOD1-ADAD protein revealed that FHOD1 may act as a combined actin capping and bundling factor rather than an actin...
nucleator promoting actin polymerization (Schönichen et al., 2013). Indeed, constitutively active forms of FHOD1 inhibit actin assembly by capping growing actin filaments (Schönichen et al., 2013). Thus, the strong induction of stress fibers observed in cell transfection experiments might be the result of bundling pre-existing actin filaments.

The in vivo functions of the FHOD-related proteins in a multicellular context are less well studied. The muscle-specific FHOD3 has been implicated in myogenesis. FHod3 mutant mice display strong defects in myofibril maturation resulting in embryonic lethality (Iskratsch et al., 2010; Kan-O et al., 2012a; Kan-O et al., 2012b; Taniguchi et al., 2009). This muscle-specific function of FHOD isoforms seems to be evolutionarily conserved and C. elegans lacking the Fhod3-related gene shows prominent cytoskeletal organization defects in muscle cells (Mi-Mi et al., 2012). The developmental functions as well as the formin activity of Drosophila FHOD have not been addressed. However, recent microarray analyses revealed that the only member of the FHOD family in Drosophila becomes highly upregulated upon bacterial infection and in autophagic programmed cell death during pupal metamorphosis (Anhezini et al., 2012; Johansson et al., 2005).

Here, we present the functional analysis of knittrig, which encodes the Drosophila FHOD protein. During embryonic development knittrig is expressed in midline glia, subsets of tracheal cells and in macrophages. In larval stages, knittrig is expressed in the developing imaginal discs, including eye and wing imaginal discs. Mutant embryos show mild tracheal defects but can survive until adulthood and they show a characteristic wrinkled wing discs. Mutant embryos show mild tracheal defects but can survive until adulthood and they show a characteristic wrinkled wing phenotype. Rescue experiments indicate a cell-autonomous function of knittrig in macrophage spreading and migration. Live cell imaging and additional gain-of-function experiments further suggests a model in which the Rho-dependent kinase Rok activates knittrig to promote actin stress fiber formation mediating the trailing edge retraction of migrating macrophages.

RESULTS

Knittrig represents the Drosophila FHOD form3 subclass

The Drosophila genome encodes six of the seven known mammalian formin families (Liu et al., 2010; Schönichen and Geyer, 2010). Loss-of-function analyses of diaphanous (dia), cappuccino (capu), dishevelled activated formorogenesis (daam) and formin 3 (form3, CG33556) have previously been reported, representing the Dia, FMN, DAAM and INF family of formins, respectively (Castrillon and Wasserman, 1994; Emmons et al., 1995; Matussek et al., 2006; Tanaka et al., 2004). CG32030 encodes a member of the FHOD protein family (Phos). Based on the wing phenotype of mutant adult flies (see below) the gene CG32030 was termed knittrig (German for wrinkled).

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Identification of knittrig mutants

We first generated knittrig mutants by imprecise excision utilizing the AA142 enhancer trap insertion P(lwB)AA142 (Klämbt et al., 1991). The breakpoints of two deletions Δ140 and Δ1 were determined (Fig. 1A). Additionally, we generated one EMS-induced mutant allele (knittrigΔ140). The deletion Δ1 completely removes sequences encoding the knittrig isoforms PA, PB, PD, PF and PG (deletion spans from 8,762,007 to 8,818,577) and a cluster of small genes mostly encoding predicted chitin-binding proteins. The Δ140 deletion lacks the 5’ exons of isoforms PI, PJ and PH as well as the first exon of all mRNA encoding isoforms and parts of the Paramyosin (Prm) (Liu et al., 2003) gene (deletion spans from 8,736,953 to 8,762,911).

The knittrigΔ1 deletion results in reduced viability and only rare escaper flies are found in homozygosity or in trans to large deficiencies. Likewise, rare escaper flies are found in trans to knittrigΔ140, demonstrating that this phenotype is due to a specific loss of knittrig (Fig. 2A). The vast majority of mutants die during pupal stages or as pharate adult flies incapable of enclosing from the pupal case. Most of the escaper flies are unable to undergo complete wing inflation, causing characteristic wrinkled wings (Fig. 2B,C). Based on this phenotype the gene locus was termed knittrig. Some of the escaper flies show partially inflated and abnormally opaque wings, suggesting that the resorption of the wing epithelium or tracheal air-filling is incomplete (Fig. 2B). In agreement with this, mutant pupal wings appear normal (supplementary material Movie 1).

The knittrig gene is not uncovered by the deficiencies Df(3L)BSC816 and Df(3L)ED4416 (Fig. 1A, Fig. 2A). By contrast, knittrigΔ1 and knittrigΔ140 fail to complement the deficiencies Df(3L)hi22, Df(3L)ScfR6, Df(3L)ED4421 or Df(3L)ED4413. Prm1 complements knittrigΔ1 but not knittrigΔ140. Importantly, re-expression of Knittrig (PA isoform, single copy) using the ubiquitous arm-Gal4 driver completely rescues both lethality and the wing phenotype of homozygous knittrigΔ1 flies. Thus, both lethality and the wing phenotype result from a loss of Knittrig function and are not due to a loss of predicted chitin-binding proteins, which are also removed in knittrigΔ1 mutants.

Expression of knittrig during development

To gain insight into the developmental functions of Knittrig we examined the temporal and spatial expression of knittrig by in situ hybridization (Fig. 3). Knittrig expression can first be detected in early stage 12 embryos in the salivary gland placodes and midline glial progenitor cells (Fig. 3A,B). To study knittrig expression we also followed β-galactosidase expression directed by the AA142 enhancer trap element, which closely resembles the transcription pattern of the knittrig gene (Fig. 3). Note that expression of the AA142 enhancer trap marker is confined to nuclei due to expression of β-galactosidase with a nuclear import signal. Expression in the CNS midline glia persists throughout embryonic and larval development (Fig. 3E,F). In stage 16 embryos, knittrig RNA expression is also found in a ring of cells around the anal plates (Fig. 3G,H) and in hemocytes (Fig. 3F,G, arrows). In addition, knittrig is expressed in the tracheal system and the dorsal vessel (Fig. 3E,F; data not shown). Double-labeling experiments indicate that knittrig is expressed in the so-called tip cells that advance the tracheal tree towards the CNS (Fig. 3L, arrows). Later on in larval development, knittrig is expressed in the CNS midline glia and in several imaginal discs. In third instar wing imaginal discs knittrig expression is found in cells of the presumptive wing margin (Fig. 3I). In the eye imaginal discs, knittrig is expressed at the morphogenetic furrow (Fig. 3J).
Loss of *knittrig* function affects tracheal morphogenesis

Since *knittrig* shows prominent expression in the tracheal system, we examined *knittrig* mutant embryos for possible tracheal defects during development. In wild type, trachea development starts at embryonic stage 11 when epidermal cells invaginate. The resulting tracheal sacs extend branches in a stereotypic manner and finally
fuse to form an interconnected network of tracheal tubes by the end of embryogenesis (supplementary material Fig. S1A,C). In all knittrig mutant combinations, we observed a penetrant phenotype with reduced formation of ganglionic tracheal branches (supplementary material Fig. S1B,D). In wild type, these tracheae segmentally follow the peripheral nerves into the ventral nerve cord towards the ventral midline (Englund et al., 1999). In knittrig mutants, the majority of the embryos show disruption in at least one ganglionic branch (supplementary material Fig. S1B,D, arrows). Since the ganglionic tracheal branches supply oxygen to the ventral nerve cord, this might explain the ‘leaky’ lethality and the occurrence of a few eclosing homozygous knittrig flies. No abnormal phenotype was detected for the midline glial cells (data not shown).

knittrig function is required for macrophage cell spreading

The expression of knittrig in hemocytes suggests a possible function during the cellular immune response. Supporting this notion, knittrig expression is strongly upregulated in immune-challenged Drosophila hemocytes (Johansson et al., 2005; Valanne et al., 2007). To further analyze knittrig function in hemocytes we examined macrophages, which constitute the most abundant cell type in the hemolymph (Wood and Jacinto, 2007). We isolated macrophages from wild-type and homozygous knittrigΔ140 mutant larvae and prepupae. To visualize the actin cytoskeleton at high spatial resolution we applied structured-illumination microscopy to fixed cells stained with fluorescently labeled phalloidin (Fig. 4). Wild-type macrophages show a broad lamellipodial actin filament network at the cell front and actin stress fiber-like structures at the...
back around the nucleus (Fig. 4A) (Sander et al., 2013). Cell spreading is increased on cover glasses coated with the lectin concavalin A (ConA). We further differentiate between lectin-mediated cell spreading and integrin-mediated cell spreading on uncoated surfaces, as shown recently (Jani and Schöck, 2007; Kadandale et al., 2010). Loss of knittrig function does not affect lamellipodia formation, in contrast to what has been reported previously for downregulation of the WA VE-Arp2/3 pathway in hemocyte-like S2R+ cells or macrophages (Bogdan and Klämbt, 2003; Kunda et al., 2003; Zobel and Bogdan, 2013; Sander et al., 2013). However, we found that knittrig mutant macrophages display strong defects in cell flattening and spreading on both ConA-coated and uncoated surfaces (Fig. 4B,E). Consistently, hemocyte-specific RNAi-mediated knockdown of knittrig using the hmlΔ-Gal4 driver results in similar cell spreading defects, indicating a cell-autonomous requirement of Knittrig in cell spreading (Fig. 4E) (Sinenko and Mathey-Prevot, 2004). Supporting this notion, hemocyte-specific re-expression of a full-length Knittrig protein in knittrigΔ1 mutant cells substantially rescues the cell spreading defects (Fig. 4C,E).

Knittrig localizes at the rear of migrating macrophages in vivo
To further investigate the cellular requirement of Knittrig we analyzed its subcellular localization in macrophages. Since our antibodies failed to detect the endogenous protein, we generated an EGFP-tagged Knittrig transgene using the ΦC31 integrase system (Bischof et al., 2007). We expressed the EGFP-Knittrig-FL (isoform PA) transgene in larval and pupal macrophages using the hmlΔ-Gal4 driver. Spinning disc confocal imaging confirmed highly dynamic membrane protrusions of isolated transgenic macrophages ex vivo, as shown recently (Sander et al., 2013). EGFP-Knittrig localizes throughout the cytoplasm with nonspecific cytoplasmic filling extending into membrane ruffles (Fig. 4F; supplementary material Movie 2). Moreover, dynamic vesicular structures in the perinuclear region were often marked by EGFP-Knittrig (Fig. 4F; supplementary material Movie 2).

Since isolated macrophages become adherent shortly after cell plating, resulting in a non-motile cell behavior similar to S2 culture cells (Sander et al., 2013), we next analyzed the localization of
Knittrig in migrating wild-type and \textit{knittrig}^{\Delta1} mutant macrophages \textit{in vivo}. The EGFP-Knittrig-FL transgene was expressed only weakly to allow live cell imaging on dorsal subepidermal macrophages of 2- to 4-hour pupae (Moreira et al., 2013). At this stage, wild-type macrophages are morphologically highly polarized with broad lamellipodial protrusions and migrate randomly beneath the epidermis or along tracheae (Moreira et al., 2013) (Fig. 4G). Knittrig accumulates at the rear of these highly polarized macrophages in a dynamic fashion (Fig. 4G; supplementary material Movie 3). Next, we examined the localization of the EGFP transgene in homozygous \textit{knittrig}^{\Delta1} mutant macrophages lacking the endogenous protein (Fig. 1). This expression regime efficiently rescued the macrophage spreading and cell migration phenotype (see below). In addition, Knittrig localizes strongly to the retracting uropod-like structures of migrating mutant macrophages (Fig. 4G; supplementary material Movies 4a,b). These cells often show an extensive blebbing activity at the cell rear, which is thought to be controlled by the coordinated action of Rho/Rok-mediated

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\caption{\textit{knittrig} regulates macrophage cell spreading. (A-C) Structured illumination microscopy (SIM) images of \textit{Drosophila} larval (A) wild-type, (B) homozygous \textit{knittrig}^{\Delta1} and (C) homozygous \textit{knittrig}^{\Delta1} macrophages re-expressing full-length Knittrig driven by the \textit{hml}-\textit{Gal4} driver, stained with phalloidin (white, F-actin) and DAPI (blue, nuclei). (D) Quantification of cell spread area of wild-type, mutant and rescued macrophages plated on ConA-coated coverslips. (E) Macrophage-specific knockdown of \textit{knittrig} using the \textit{hml}-\textit{Gal4} driver results in similar cell spreading defects. Error bars represent s.e.m. ***P<0.0001. (F) Series of time-lapse images of an isolated macrophage expressing EGFP-Knittrig-FL. See also supplementary material Movie 2. (G,H) Series of time-lapse images of migrating macrophages expressing EGFP-Knittrig-FL taken from (G) wild-type and (H) homozygous \textit{knittrig}^{\Delta1} mutant prepupae (2-4 hours APF). See also supplementary material Movies 3, 4a,b.}
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actomyosin contractions (Fig. 4G; supplementary material Movie 4a) (Sánchez-Madrid and Serrador, 2009).

**knittrig function is required for macrophage migration**

Cell spreading defects related to the phenotypes mentioned above were recently observed in macrophages deficient for *Drosophila* Rho1 (Kadandale et al., 2010). Additionally, Rho1 mutant macrophages are impaired in their recruitment to epidermal wounds (Kadandale et al., 2010). Given the distinct localization of Knittrig to the cell rear we examined a possible role in directed cell migration and analyzed the migratory behavior of macrophages in *vivo* upon laser-induced wounding in pupal wings. Wild-type macrophages immediately migrate towards a laser-induced wound or single ablated cell (Fig. 5A; supplementary material Movie 5). To quantify this migratory behavior, a local histogram-based image analysis technique was developed to calculate an overall migration score (Fig. 5C-E). This score, called the histogram-based macrophage migration score (HMMS), measures the number of foreground pixels (i.e. macrophages) around the stimulus (wound) over time. High HMMS values indicate that most detected cells (relative to the overall cell density) are located around the wound, whereas small values (close to 0) indicate a sparse macrophage density around the wound. Furthermore, the slope of the score serves as an indirect measure of the migration speed, since a strong gradient is caused by a rapid increase of foreground pixels between consecutive frames (Fig. 5C,D). The median HMMS, plotted for wild-type, mutant and RNAi measurements over time clearly show that cells deficient for *knittrig* are still able to respond and migrate to wound sites but the speed as well as the direction of migration are clearly impaired (Fig. 5F; supplementary material Movies 5, 6, 8-10). The differences in directed migration between wild-type and mutant or RNAi depleted macrophages are most obvious between 200 and 800 seconds after wounding, when a rapid increase can be measured in wild-type cells compared with mutant and RNAi deficient macrophages.

**Fig. 5. Knittrig regulates macrophage cell migration during the immune response.** (A-B″) Frames of spinning disc microscopy videos of migrating wild-type (A-A″) and *knittrig* mutant (B-B″) macrophages at the indicated time points, visualized by the expression of cytoplasmic EGFP. (C) Sketch of an exemplary unprocessed image stack I₁, ..., Iₜ as well as the wound position p=(x, y). (D) Segmented image after applying thresholding procedure. White pixels are accounted as macrophage pixels. (E) Illustration of the local histogram regions. Concentric regions R₀, ..., R₄ around the stimulus and R₅ are highlighted. (F) Median HMMS values for wild-type (blue, n=10), *knittrig* RNAi (green, n=14), *knittrig* △1 mutant (red, n=11) and rescued *knittrig* △1 mutant (black, n=15) macrophage movies plotted for 1300 seconds after wounding. An overall higher median HMMS value is observed for the wild-type cells compared with *knittrig* RNAi and *knittrig* mutant cells, indicating more and faster movement towards the wound over time. See also supplementary material Movies 5-11.
depleted macrophages (Fig. 5F). Re-expression of Knittrig in homozygous knittrigΔ1 mutant macrophages restores the migration defects (Fig. 5F; supplementary material Movie 7). Remarkably, rescued cells even show an increased migration speed, resulting in higher HMMS values compared with wild-type macrophages (Fig. 5F; supplementary material Movie 11). Thus, we conclude that knittrig is important for directed macrophage migration during the cellular wound response.

**Knittrig promotes stress fiber formation in Drosophila S2 cells**

As shown previously for mammalian FHOD1, we could demonstrate an interaction of Knittrig with Rho GTPases such as Rac1 and RhoA (but not Cdc42), but in a guanine-nucleotide independent manner (Fig. 6A) (Westendorf, 2001). Thus, binding to Rho GTPases does not seem to be a sufficient regulatory mechanism for the activation of Knittrig.

Deletion of the C-terminal inhibitory DAD results in activation of mammalian FHOD1 (Takeya and Sumimoto, 2003). The induction of actin stress fibers is the most prominent phenotype evoked by constitutively active FHOD1-ΔDAD in mammalian cell culture (Takeya and Sumimoto, 2003). To test the role of the DAD of Knittrig we performed gain-of-function analyses to examine the ability of Knittrig in regulating actin cytoskeleton organization. We generated N-terminally EGFP-tagged fusions of mutant Knittrig proteins containing distinct deletions in functional domains (Fig. 6B). Expression of a full-length Knittrig protein or the FH2 domain alone did not affect the overall actin cytoskeleton architecture in transfected Drosophila S2R+ cells (Fig. 6C; supplementary material Movie 12). Both proteins mainly distribute throughout the cytoplasm or accumulate in large cytoplasmic clumps (Fig. 6C). However, truncations of the conserved C-terminal basic cluster (ΔB) or of the complete DAD (ΔDAD) result in a strong reorganization of the actin cytoskeleton of transfected cells (Fig. 6C). The expression of either truncated protein induces the formation of thick actin bundles (Fig. 6C). Such unbranched actin bundles are never observed in wild-type S2R+ cells.

The highly conserved C-terminal cluster of positively charged residues in the Knittrig protein appears sufficient to mediate the autoinhibition, although cells expressing the ΔDAD truncation exhibit stress fiber-like structures more frequently. Since it is known that rigid surfaces further increase the mechanical forces promoting stress fiber formation (Burridge and Wittchen, 2013), we plated transfected cells on uncoated glass coverslips. Under these conditions, S2R+ cells expressing Knittrig-ΔDAD strongly induce large stress fibers reminiscent of Rho-induced stress fibers in mammalian fibroblasts (Fig. 6C). Live imaging analysis revealed a dynamic turnover of activated Knittrig in stress fibers (supplementary material Movies 13a,b).

To further examine Knittrig protein dynamics we performed FRAP experiments with cells expressing EGFP-Knittrig-ΔDAD plated on glass coverslips (Fig. 6D). We observed a rapid homogeneous recovery of EGFP-Knittrig-ΔDAD along stress fibers with half-life times (t1/2) between 6 and 9 seconds and ~60-70% of the fluorescence recovered by 90 seconds (Fig. 6D,E). The turnover rates at the tips of stress fibers (t1/2=6.74 seconds), where actin filaments are anchored to focal adhesions, seem to be substantially faster than at central regions within stress fibers (t1/2=9.02 seconds), as shown recently for dorsal stress fiber formation in mammalian cell culture (Fig. 6D,E; supplementary material Movie 14) (Hotulainen and Lappalainen, 2006). These data indicate that active Knittrig constantly associates and dissociates with stress fibers.

**Knittrig can be activated by Rok to promote stress fiber formation**

Previous studies have demonstrated that phosphorylation of the C-terminal DAD by the Rho-dependent kinase Rok/Rock is an important regulatory modification for mammalian FHOD1 activation. The phosphorylated serine/threonine residues within the DAD are conserved between FHOD1 and Knittrig, suggesting that Knittrig might also be activated by Rok. We found that Knittrig can indeed be phosphorylated (Fig. 7A). Co-expression of EGFP-Knittrig-FL and an HA-tagged Rok in S2R+ cells results in increased serine phosphorylation of Knittrig (Fig. 7A). We next analyzed whether Rok is able to promote Knittrig-dependent actin stress fiber formation in S2R+ cells. Co-expression of full-length Rok (Rok-WT) and EGFP-Knittrig-FL indeed strongly induces stress fiber formation, whereas expression of Rok-WT or EGFP-Knittrig-FL alone has no effect on actin cytoskeleton organization (Fig. 7B, Fig. 6C). In contrast to wild-type Rok, a constitutively active variant (Rok-CAT 1-553aa) (Simões et al., 2010) already slightly induces actin stress fibers (Fig. 7C). Upon co-transfection of Rok-CAT and EGFP-Knittrig-FL stress fiber formation is strongly enhanced, even in cells plated on ConA (Fig. 7D,E). More importantly, these cells show dramatic cell contractions and dynamic membrane blebbing (supplementary material Movie 15). Remarkably, in those cells co-expressing Rok-CAT and EGFP-Knittrig-FL, Knittrig becomes entirely recruited to a ring-like structure in the cell center near the centrosome (supplementary material Movie 15). Additional live imaging analysis revealed a relocalization of Knittrig induced by Rok to regions of the microtubule organizing center (MTOC), where activated Rok has been found previously (supplementary material Movie 16) (Chevrier et al., 2002). In agreement with this observation, we frequently found cytokinesis defects upon Rok and EGFP-Knittrig-FL co-expression resulting in multinucleated cells (data not shown; Fig. 7H).

The functional interaction between Knittrig and Rho kinase in regulating stress fiber formation could also be observed in vivo in macrophages. Since early larval expression of active Knittrig or Rok gain-of-function inhibits the release of mature macrophages from the hematopoietic lymph gland into the hemolymph (data not shown), we used the Gal4/Gal80ts system to restrict expression to late third instar larvae (McGuire et al., 2003). Constitutively active Knittrig protein (ΔDAD) accumulates in actin stress fiber-like structures at the rear of isolated macrophages (Fig. 7F). Moreover, cells expressing elevated Knittrig-ΔDAD levels show an increased number of F-actin bundles, suggesting that Knittrig promotes the formation of these stress fiber-like structures in macrophages (Fig. 7F). Similar to observations in S2R+ cells, the co-expression of inactive full-length Knittrig with full-length Rok (Rok-WT) strongly induces stress fibers in macrophages (Fig. 7H). Again, expression of constitutively active Rok alone already induces stress fibers (Fig. 7I). Similarly, co-expression of Rok-CAT and Knittrig completely reorganizes actin filament bundles into a star-like pattern emerging from the cell center – a typical structure induced by Rok activation (Fig. 7J) (Leung et al., 1996).

**Knittrig is able to remodel epithelial architecture in vivo**

Finally, we examined whether active Knittrig also affects the organization of actin filaments in a multicellular context. Since ubiquitous expression of Knittrig-ΔDAD and Knittrig-ΔB results in early embryonic lethality we used the patched (ptc)-Gal4 driver to restrict expression. Overexpression of full-length Knittrig in wing imaginal discs did not affect the overall epithelial integrity or the level of F-actin. By contrast, expression of Knittrig-ΔB or Knittrig-
ΔDAD results in a significant elevation in the level of F-actin (Fig. 8A). Similar to S2R+ cell transfection, the effect of Knittrig-ΔDAD expression on F-actin induction is more striking than that of Knittrig-ΔB (Fig. 8A). Interestingly, both truncated proteins also affect epithelial morphology. Epithelial cells overexpressing Knittrig-ΔDAD or Knittrig-ΔB in the ptc stripe constrict, resulting in apical invaginations.

We extended these gain-of-function studies to larval salivary glands. Gland cells are large polyploid cells that form a monolayered epithelial tube. In wild-type salivary gland cells, F-actin is mainly concentrated along the lateral cell boundaries, in the periluminal region and at the basal surface of gland cells (Fig. 8B). Expression of Knittrig-ΔDAD or Knittrig-ΔB dramatically reorganizes this irregularly shaped basal actin cytoskeleton to long...
and thick actin bundles (Fig. 8B). This dramatic reorganization of the actin cytoskeleton also strongly affects overall gland morphology and multiple cyst-like lumina are found instead of the single lumen characteristic of wild-type glands (Fig. 8B).

Interestingly, this multiple cyst phenotype has been observed recently upon expression of constitutively active Rho, again indicating that Knittrig may act downstream of Rho signaling (Xu et al., 2008).

These data clearly show that deletions of the autoinhibitory C-terminus result in activation of the Knittrig protein. Most importantly, our data demonstrate that constitutively active formins are able to induce actin stress fibers in Drosophila. We suggest that the function of Knittrig in trachea and macrophages depends on its ability to remodel the cell architecture by controlling the formation of unbranched F-actin bundles.

DISCUSSION

Formin proteins have been shown to regulate a variety of morphogenetic processes by controlling the structure and dynamics of the actin cytoskeleton both in individual cells and in tissue. Drosophila knittrig encodes a well-conserved FHOD formin protein. Here we focused on cell spreading and cell migration defects displayed by knittrig mutant macrophages, which relate to a disrupted actin cytoskeleton.

Fig. 7. Knittrig can be activated by Rok to induce stress fiber formation in S2 cells and macrophages. (A) Co-immunoprecipitation of full-length Knittrig from S2R+ cell lysate co-expressing EGFP-Knittrig and wild-type Rho kinase (Rok). Cell lysates were immunoprecipitated with a control preimmune serum or anti-GFP antibody. The immunoprecipitates were probed for the presence of EGFP and phosphoserine as indicated. (B-E) SIM images of S2R+ cells expressing (B) EGFP-Knittrig-FL and full-length HA-Rok-WT, (C) constitutively active HA-Rok-CAT (1-553aa) alone or (C) HA-Rok-CAT together with EGFP-Knittrig-FL, stained with phalloidin (white, F-actin), anti-HA (red, Rok) and EGFP (green, Knittrig). (F) SIM images of isolated macrophages expressing EGFP-Knittrig-ΔDAD, stained with phalloidin (white, F-actin), anti-Knittrig (red) and DAPI (blue, nuclei). Cells with elevated Knittrig-ΔDAD expression are marked by an asterisk. (G,H) SIM images of isolated macrophages expressing (G) full-length HA-Rok-WT, (H) EGFP-Knittrig-FL and HA-Rok-WT, (I) constitutively active HA-Rok-CA and (J) HA-Rok-CAT together with EGFP-Knittrig-FL. Cells are stained with phalloidin (white, F-actin) and DAPI (blue, nuclei). For expression in macrophages the combined Gal4/Gal80ts system was used. Heat shock was carried out in late third instar larvae at 29°C. Scale bars: 10 µm.
The molecular activities of formins on actin have been analyzed extensively. Formins are able to efficiently assemble linear actin filaments from profilin-bound actin monomers (Goode and Eck, 2007). The FH2 dimer nucleates filament assembly by stabilizing actin intermediates, whereas an additional processive capping activity of formins is thought to promote actin filament elongation by protecting growing barbed ends from capping proteins. Nucleation and processive elongation seem to be the major conserved activities of formins, although a number of studies also revealed additional effects on filament bundling, severing or even depolymerization (Harris et al., 2004; Harris et al., 2006; Schönichen et al., 2013). The broad range of biochemical activities suggest that formins regulate different aspects of the actin cytoskeleton architecture and actin dynamics during morphogenesis.

The phenotypic analyses of the formin mutants demonstrated distinct cellular functions in Drosophila development. The founding member of formins, Dia, is the key regulator of mitotic and meiotic cytokinesis in both the germline and all somatic cells, whereas the only member of the FMN formin subclass Capu is essential for anterior-posterior and dorsal-ventral patterning during oogenesis and early embryogenesis (Castrillon and Wasserman, 1994; Emmons et al., 1995; Manseau and Schüpbach, 1989). form3 and daam mutants are embryonic/larval lethal owing to severe defects in tracheal development (Matusek et al., 2006; Tanaka et al., 2004). Knittrig is also required for proper tracheal development. Based on the distinct mutant phenotypes, all three formins seem to differentially regulate different aspects of tracheal morphogenesis. Tracheal tubes of daam mutant larvae fail to secrete the cuticle, whereas form3 is required for tracheal cell fusion (Matusek et al., 2006; Tanaka et al., 2004).
2006; Tanaka et al., 2004). The knittrig phenotype is much less dramatic and only affects the formation of distinct tracheal branches. Knittrig is not only expressed at tracheal tip cells but also strongly marks midline glial cells. However, we did not find significant defects in the morphology of knittrig-deficient glial cells (data not shown). This might suggest that Knittrig does not have a pronounced role in glia cell architecture. Alternatively, functional redundancy might exist between Knittrig and CG32138, which encodes a member of the FRL subclass of formins. CG32138 has not been characterized but it is also expressed in CNS midline glia (Sánchez-Soriano et al., 2007).

Unlike other Drosophila formins, Knittrig is required for macrophage dynamics during the cellular wound response. Given its asymmetric enrichment at the rear of migrating macrophages we propose that Knittrig acts downstream of Rho/Rok-dependent cell protrusion. Like mammalian FHOD1, Knittrig binds GTPases such as Rho1 in a non-specific, nucleotide-independent manner. Thus, Knittrig seems not to be directly regulated by Rho but rather by the Rho effector kinase (Rok). In mammals, Rok in turn directly phosphorylates FHOD1 at three specific sites within the polybasic region of the DAD (Takeya et al., 2008). The phosphorylated serine/threonine residues are conserved in the Knittrig protein, which implies that its activity might also be regulated by phosphorylation in Drosophila. Serine phosphorylation is indeed enhanced upon Rok co-expression, indicating that Knittrig might be a direct substrate of Rok. Our gain-of-function experiments provide further evidence that Rok mediates the activation of Knittrig in vivo. Co-expression of Knittrig and Rok results in a strong induction of stress fibers. Consistently, we found that deletion of the polybasic region in Knittrig-ΔAB promotes similar actin stress fiber formation. Thus, Rok-catalyzed phosphorylation is likely to disrupt an autoinhibitory interaction in Knittrig, as proposed recently for FHOD1 activation (Takeya et al., 2008).

Since the truncation of the complete DAD further increases the activity of Knittrig, additional regulatory mechanisms might be required for its full activation. Given the dramatic gain-of-function phenotype in flies, the cooperative functions of such regulatory signals might be essential to tightly restrict Knittrig activity during development. A possible regulatory scenario is simultaneous Rho1 binding and Rok-mediated phosphorylation, or even a phosphorylation-dependent increase in Rho binding. Alternative splicing would provide an additional regulatory mechanism to control formin function. The shortest isoform, KnittrigPB, lacks the C-terminal DAD and hence constitutes a constitutively active protein. The expression of this active isoform seems to be upregulated in pupal salivary glands during edcsylone-induced programmed cell death (Anhezini et al., 2012). Thus, uncontrolled, massive induction of F-actin might be an efficient way to induce apoptosis or to extrude single cells from the epithelium, as proposed previously (Farhadifar et al., 2007).

It still remains unclear how formins, such as the FHOD proteins, induce actin stress fiber formation (Burridge and Wittchen, 2013). Based on sequence similarity, FHOD proteins have been grouped into the DRF class of formins likely to promote actin nucleation. Interestingly, the opposite has been observed recently in vitro (Schönichen and Geyer, 2010). In pyrene actin polymerization assays a recombinant FHOD1-ΔDAD behaves like an actin filament capping protein, inhibiting actin polymerization in vitro. High-speed sedimentation assays further showed that an N-terminal fragment lacking the FH2 domain is sufficient to bundle actin filaments. Based on these observations, the authors propose a model in which the dimeric FH2 domain protects barbed actin filament depolymerization, whereas the N-terminal region of FHOD bundles actin filaments. Thus, stress fiber formation might depend on the combination of actin capping and bundling activity rather than on actin nucleation and elongation (Schönichen and Geyer, 2010). However, recent studies on mouse Dia1 (Dia1p) revealed that the DAD contains conserved residues essential for its nucleation activity (Gould et al., 2011). Thus, the nucleation activity of the truncated FHOD1-ΔDAD might have been lost and the remaining FH2 domain only stabilizes actin filaments. Interestingly, the critical residues for nucleation activity were mapped to a lysine followed by an arginine within the polybasic region, which is conserved in Knittrig as well as in mammalian FHOD1. In vivo, we found that constitutively active Knittrig is able to induce actin assembly. Expression of Knittrig-ΔDAD protein in the wing results in a significant increase of F-actin. However, it remains unclear whether increased F-actin is due to de novo actin nucleation or to stabilization of pre-existing actin filaments. Thus, in the future the activity of truncated proteins with an intact KR motif (the conserved lysine-arginine motif within the DAD) should be measured in vitro. Unfortunately, all attempts to purify soluble full-length or truncated Knittrig proteins have proven unsuccessful so far.

In conclusion, the biological functions of Knittrig seem to be well conserved. Knittrig promotes stress fiber formation and macrophage migration. Re-expression of Knittrig not only rescues the migration defects of mutant cells but also increases the cellular immune response of macrophages. A similar positive activity of Knittrig in cell migration has recently been observed for human FHOD1, the expression of which is upregulated during epithelial-mesenchymal transition promoting cancer cell migration (Gardberg et al., 2013). Overexpression of human FHOD1 in melanoma and breast cancer cells promotes cell migration and cell invasion (Koka et al., 2003; Jurmeister et al., 2012). Likewise, recent in vitro data confirmed that FHOD1 is needed for directed forces and adhesion during cell spreading and migration (Iskratsch et al., 2013). Our data provide first insights into how FHOD proteins might promote cell migration in vivo. Given the asymmetric subcellular localization of Knittrig in vivo we propose that Knittrig promotes the formation of actin bundles at the rear of the cell in a Rok-dependent manner. Thus, Rok is in a central position to simultaneously coordinate actin assembly by Knittrig phosphorylation and the activation of actomyosin-based retraction by myosin light chain kinase (MLCK) phosphorylation in the trailing edge of migrating macrophages.

**MATERIALS AND METHODS**

**Fly genomics**

All crosses were performed at 25°C unless indicated otherwise. Excision mutagenesis was performed with the P(lwB)AA12 insertion as described (Stephan et al., 2011). The following deficiency chromosomes were used for mapping: Df(3L)Scf-R6, Df(3L)hi22, Df(3L)BSC612, Df(3L)ED4421, Df(3L)ED4413 (obtained from the Bloomington Stock Center). To identify EMS-induced knittrig alleles we genotyped isogenic st e marked chromosomes and screened in trans to knittrig excision alleles. Transcriptic flies were generated using FCo31-mediated transgenesis targeted to the M{3xP3-RFP.attP}ZRH-86F landing site (Bischof et al., 2007). RNAi lines were obtained from the Vienna Drosophila RNAi Center (VDRC, ID 34034 and 34035). Flies bearing the pUASP-HA-Rok-WT and pUASP-HA-Rok-CAT (1-553aa) were obtained from Jennifer Zallen (Simões et al., 2010).

**Cell culture, cell transfection and cloning**

Drosophila S2R+ cells were propagated in 1X Schneider’s Drosophila medium as described previously (Fricke et al., 2009). S2R+ cells were transfected as described previously (Fricke et al., 2009). A full-length Knittrig-PA cDNA construct was assembled using 5′ sequences of LD24110.
to the first XhoI site and the remaining 3’ sequences from SD08909. cDNAs were amplified by PCR and subcloned into Gateway entry vectors (pENTR D-TOPO, Invitrogen) according to the manufacturer’s instructions (Escherichia coli Expression System with Gateway Technology, Invitrogen). The QuikChange XL Site-Directed Mutagenesis Kit (Stratagene, Agilent Technologies) was used to generate distinct deletions. For primer details see supplementary material Table S1. The inserts were sequenced and cloned by LR in vitro recombination into corresponding destination vectors (Drosophila Genomics Resource Center) containing UAS promoters and N-terminal eGFP. The pQE-His-tag system (Qiagen) was used to express fusion proteins used for immunization of rabbits. pUASp-HA-Rok-WT and pUASp-HA-Rok-CAT (1-553aa) plasmids were obtained from Jennifer Zallen (Simões et al., 2010).

Fluorescence recovery after photobleaching (FRAP) analysis
FRAP analysis of EGFP-Knirp-G-ΔAD was performed with an UltraVIEW Vox 3D live cell imaging system (PerkinElmer) coupled to a Nikon Eclipse Ti inverted microscope. The system incorporated a CSU-X1 spinning disk scanner (Yokogawa), a C9100-50 EM-CCD camera (1000×1000 pixels; Hamamatsu) and Volocity software (PerkinElmer). Cells were imaged via a Nikon 60× (NA 1.49) oil-immersion objective lens. The fluorescence recovery was monitored by time-lapse imaging at low-intensity illumination and quantified with Volocity for FRAP (PerkinElmer).

Structured illumination microscopy (SIM) imaging
SIM images were taken with an ELYRA S.1 Microscope (CellObserver SD, 63×/1.4 oil-immersion objective; Zeiss) using software Zen 2010 D (Zeiss). Image acquisition was performed as described previously (Zobel and Bogdan, 2013). For isolation of macrophages, five white pupae or ten third instar larvae were bled into 400 μl Shields and Sang M3 medium containing 10% FBS and 1× penicillin/streptomycin. Cell suspensions were transferred to a chambered cover glass and cells were incubated for 1 hour at 25°C. Cell fixation and phalloidin staining were performed as previously described (Zobel and Bogdan, 2013).

In vivo migration assay of pupae
Live imaging of macrophages in prepupae and in the pupal wing was performed with a CellObserver SD spinning disk microscope (Zeiss) as reported (Sander et al., 2013). The cuticle of staged pupae (16 hours after puparium formation (APF)) were completely removed and pupae were placed on the lateral side, glued on glass-bottom culture plates. Cells were imaged through a 40× Plan-Apochrmat (NA 1.3) oil-immersion objective using an inverted Zeiss Axio Observer.Z1 microscope with an Axioimager MRm CCD camera (4.45 μm × 6.45 μm) and 488 nm laser line. For wounding assays we used the UV laser ablation system DL-355/14 direct and quantified with Volocity for FRAP (PerkinElmer).

Quantification of in vivo macrophage cell migration
To quantify the migration behavior we introduce the histogram-based macrophage migration score (HMMS). The score utilizes local histograms around the laser-induced wound to measure the macrophage concentration over time.

\[ \psi(R_m,t) = \sum_{j=0}^{255} h^j(t), \]

where \( h^j(t) \) is the normalized histogram value for intensity \( j \) in region \( R_m \) at time \( t \). Again, if macrophages migrate towards the stimulus, \( \psi(\sigma, t) \) decreases over time. Given all macrophage occurrences for all regions over time, the HMMS is calculated by:

\[ \text{HMMS}(\sigma) = \sigma_0 R_0(0, t) + \sigma_1 R_1(0, t) + \sigma_2 R_2(0, t) + \sigma_3 R_3(0, t) + \sigma_4 R_4(0, t), \]

with weights \( \sigma_0, \sigma_1, \sigma_2, \sigma_3 > 0 \) and \( \sigma_4 < 0 \). The weights \( \sigma_0, \sigma_1, \sigma_2, \sigma_3 \) are selected based on the two-dimensional Gaussian distribution \( N(\mu, \Sigma) \) with mean value \( \mu \) and covariance matrix:

\[ \Sigma = \begin{pmatrix} \sigma^2 & 0 \\ 0 & \sigma^2 \end{pmatrix}, \]

where \( \sigma \) is the volume (integral) covered by ± one standard deviation for each direction around the mean. Similarly, \( \mu \) is the volume within \((–\sigma, +\sigma)\) without the volume within \((–\sigma, +\sigma)\). In the same manner, \( \sigma_1, \sigma_2, \sigma_3 \) are defined by the intervals \((–3\sigma, +3\sigma)\) and \((–4\sigma, +4\sigma)\), respectively. To punish macrophages remaining in region \( R_0 \), \( \sigma_4 \) is set to −0.25. The HMMS will therefore increase over time, assuming that the macrophages migrate from region \( R_0 \) towards region \( R_0 \). To compare the migration behavior, HMMS values for several wild-type (n=11), knitrig RNAi (n=11) and knitrig mutant (n=15) movies were calculated. Finally, these values are normalized by HMMS\((\sigma) = U_{\alpha0}(R_m, t)\), where the denominator is the percentage of foreground pixels relative to the total number of pixels. In this way, the score is adaptively restricted to cell migration only and independent of varying cell densities in different movies. Subsequently, median densities for all types were plotted (Fig. 5F).

GST pulldown and co-immunoprecipitation
An excess of recombinant GST-Rac1, GST-Cdc42 and GST-Rho1 (Grosshans et al., 2005) proteins was immobilized on glutathione resin according to the manufacturer’s instructions (GE Healthcare). About \( \times 10^6 \) S2R+ cells were harvested and lysed in 2 ml ice-cold lysis buffer (1× PBS, 1% NP40, 1 mM DTT and 1 mM PMSF). Lysates were centrifuged for 10 minutes at 10,000 g (4°C) and 500 μl of the cytoplasmic supernatant were added to 30 μl loaded glutathione resin. Following incubation for 1 hour at room temperature, unbound proteins were removed by washing four times with 1 ml lysis buffer at room temperature. 10% of the preparation was used per lane on standard SDS-PAGE and analyzed by western blot. The immobilized GST proteins were pre-treated with either 1 mM GDP or 1 mM GTP-γS plus 25 mM MgCl₂ supplement for 10 minutes at 30°C. Co-immunoprecipitation experiments were performed as previously described (Fricke et al., 2009). Phosphorylated serine/threonine residues were detected using an anti-phosphoserine antibody (PSR-45, Abcam).

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Competing interests
The authors declare no competing financial interests.

Author contributions
S.B. oversaw the biochemical, genetic and cell biological work. U.L. generated the knitrig mutant flies, performed the complementation analysis and in situ
hybridization experiments. M.B. characterized the kni1tig mutant macrophages, performed the rescue and live-imaging experiments. B.R. and D.B. established the histogram-based image analysis. A.F. performed the S2 cell transfection and western blot analysis. I.B. sequenced the mutant alleles. J.S.O. oversaw the bioinformatic analyses. C.K. oversaw the initial fly genetics experiments. S.B. wrote the manuscript with assistance from all authors.

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