**RNAi Screen in Tribolium Reveals Involvement of F-BAR Proteins in Myoblast Fusion and Visceral Muscle Morphogenesis in Insects**

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**ABSTRACT** In a large-scale RNAi screen in *Tribolium castaneum* for genes with knock-down phenotypes in the larval somatic musculature, one recurring phenotype was the appearance of larval muscle fibers that were significantly thinner than those in control animals. Several of the genes producing this knock-down phenotype corresponded to orthologs of *Drosophila* genes that are known to participate in myoblast fusion, particularly via their effects on actin polymerization. A new gene previously not implicated in myoblast fusion but displaying a similar thin-muscle knock-down phenotype was the *Tribolium* ortholog of *Nostrin*, which encodes an F-BAR and SH3 domain protein. Our genetic studies of *Nostrin* and *Cip4*, a gene encoding a structurally related protein, in *Drosophila* show that the encoded F-BAR proteins jointly contribute to efficient myoblast fusion during larval muscle development. Together with the F-Bar protein Syndapin they are also required for normal embryonic midgut morphogenesis. In addition, *Cip4* is required together with *Nostrin* during the profound remodeling of the midgut visceral musculature during metamorphosis. We propose that these F-Bar proteins help govern proper morphogenesis particularly of the longitudinal midgut muscles during metamorphosis.

**KEYWORDS** muscle development, *Tribolium*, *Drosophila*, RNAi screen, F-Bar domain, myoblast fusion, visceral musculature, metamorphosis

As described in the accompanying paper (Schultheis et al. 2019), we participated in large-scale screens with systemic RNAi in the flour beetle *Tribolium castaneum* aiming to identify new genes that regulate the development of the somatic musculature. One screen was for knock-down phenotypes in muscles of late stage embryos and first instar larvae, which involved injecting double stranded RNAs into pupae of a tester strain that expressed EGFP in all somatic (and visceral) muscles. A second screen was for knock-down phenotypes in the adult indirect flight muscles of the thorax of late stage pupae, which involved injections into larvae of a strain expressing EGFP in these muscles. A broad overview over these screens, which included screening for various other phenotypes, has been presented in Schmitt-Engel et al. (2015). After identifying new genes associated with knock-down phenotypes in the somatic musculature in *Tribolium* our main strategy was to utilize the superior genetic tools and accrued body of information in *Drosophila* to study the functions of their fly orthologs in detail and place them into the known regulatory framework of muscle development in the fly.

Herein we focus on genes that we selected based on their larval muscle phenotypes in the pupal injection screen. Specifically, this is a group of genes that produced a phenotype of somatic muscles in embryos that were significantly thinner as compared to controls, which led to anomalous gaps between parallel muscle fibers. *Drosophila* myoblast fusion is an increasingly well-characterized process, during which a set number of fusion-competent myoblasts
fuses with a single muscle founder cell and with the nascent myotube formed by this process. The asymmetry of this process relies on the cell type-specific expression of several of the key components of the recognition and fusion machinery (Kim et al. 2015; Deng et al. 2017). In particular, the recognition and adhesion of the two types of myoblast involves the engagement of the immunoglobulin domain proteins Sticks-and-stones (Sns) and Hibris (Hbs) on the surface of the fusion-competent myoblasts with the structurally related proteins Kin of irre (Kirre) (aka, Dumbfounded, Duf) and Roughest (Rst, aka, IrreC) on the surface of the muscle founder cells. This interaction then triggers downstream events in both cell types, which culminate in the differential assembly of polymerized actin structures at the prospective fusion site in fusion-competent vs. founder myoblasts. Membrane breakdown and fusion pores occur upon the extension of actin-propelled protrusions from the fusion-competent myoblasts that invade the founder cells, and of F-actin sheaths thought to act as counter-bearers underneath the opposing membranes of the founder cells. The concomitant assembly of ring-shaped multiprotein complexes and the removal of cell adhesion proteins such as N-Cadherin at these sites additionally promote and orchestrate the formation and extension of fusion pores at these sites (Önel and Renkawitz-Pohl 2009; Önel et al. 2014). Whether any fusogens, as known to be active in other contexts of cell fusion (Seger et al. 2018), are involved in membrane fusions in Drosophila myoblast fusion is currently not known. Consecutive rounds of myoblast fusions generate the multinucleated muscle precursors in this manner.

A new gene identified based on its thin-muscle phenotype in Tribolium castaneum (Tc) was TCO13784 (Tc-Nostrin), homologs of which previously have not been implicated in Drosophila myoblast fusion. This gene encodes a protein with an F-BAR domain within its N-terminal half and an SH3 domain at its C-terminus. F-BAR proteins associate as curved homo-dimers with the inner face of the plasma membrane via binding to phospholipids and regulate membrane curvature as well as actin polymerization in various contexts (Roberts-Galbraith and Gould 2010; Liu et al. 2015; Salzer et al. 2017). Here we focus on the analysis of Drosophila Nostrin and two related genes, Cip4 and Syndapin (Synd), within this superfamily that encode F-BAR plus SH3 domain proteins, during muscle development. Previously, the functions of these Drosophila genes have been characterized within other developmental contexts, including germ line cell encapsulation (Nostrin; Zobel et al. 2015), the formation of proper numbers of wing hairs (Cip4; Fricke et al. 2009), and postsynaptic membrane organization (Synd; Kumar et al. 2009b). As described herein, our genetic analysis of these F-BAR genes in Drosophila muscle development shows that their encoded proteins, particularly Nostrin and Cip4, make joint contributions to myoblast fusion during embryogenesis. We also show that these F-BAR proteins, with a predominant role of Cip4, are critical for normal morphogenesis of the adult visceral muscles, which undergo major remodeling processes during metamorphosis.

**MATERIALS AND METHODS**

**Tribolium iBeetle database**
RNAi phenotypes from the screen and additional gene information can be looked up in the iBeetle database (http://ibeetle-base.uni-goettingen.de).

**Drosophila strains**
All Drosophila melanogaster stocks were kept on standard medium at 25°C. The following Drosophila strains were used in this study: Cip4^{4,32} (Fricke et al. 2009); Cip4^{4,32}, Synd^{4,32}/TM3, twi>>GFP (this work); Cip4^{4,32}, Synd^{4,32}/TM3, twi>>GFP (this work); Cip4::YFP (PBac[754.P.FSVS-0]Cip4^{4,32}.331, Kyoto Stock Center); hsFLP/TM6 (Bloomington Drosophila Stock Center, #279); imd/TM3, Kr->lacZ (Duan et al. 2001); UAS-Nostrin-EGFP (Zobel et al. 2015): Nostrin^{4,32} (this work): Nostrin^{4,32} (this work); Nostrin^{4,32}, Cip4^{4,32}, Synd^{4,32}, Synd^{4,32}/TM3, twi>>GFP (this work); Nostrin^{4,32}, Cip4^{4,32}/TM3, twi>>GFP (this work); Nostrin^{4,32}, Cip4^{4,32}/TM3, twi>>GFP (this work); Nostrin^{4,32}, Synd^{4,32}/TM3, twi>>GFP (this work); Nostrin^{4,32}, Synd^{4,32}/TM3, twi>>GFP (this work); Nostrin^{4,32}, Synd^{4,32}/TM3, twi>>GFP (this work); Nostrin^{4,32}, Synd^{4,32}/TM3, twi>>GFP (this work); P[XP]CG10962.108142 (Harvard Medical School); PBac[WH]/CG10962.102373 (Harvard Medical School); PBac[WH]/06363 (Harvard Medical School); r2p89-lacZ (Nose et al. 1998); Synd^{4,32}/TM3, twi>>GFP (Kumar et al. 2009b); Synd^{4,32}/TM3, twi>>GFP (Kumar et al. 2009a); Mef2-GAL4 (Ranganayakulu et al. 1998).

**Generation of Nostrin mutants**
Nostrin mutants were generated via the flp/FRT-system as described in Parks et al. (2004). For the generation of Nostrin deletions either the strain P[XP]CG10962.108142 or the strain PBac[WH]/CG10962.102373 in combination with PBac[WH]/06363 were used (Figure S2). The deletions were identified via PCR. The resulting Nostrin deletion mutant strains Nostrin^{4,32} and Nostrin^{4,32} are fully viable and fertile.

**Generation of homozygous Nostrin and Cip4 and of Cip4 double mutants**
To obtain adult flies homozygous mutant for both Nostrin and Cip4 (lacking the zygotic and maternal expression of both genes) the strain Nostrin^{4,32}/Cip4^{4,32}/TM3,Js-hid3 see also Zobel et al. 2015) was used. Heat shocking the progeny for 1.5 hr at 37°C during 3–4 days resulted in the survival of only homozygous double mutant escapers flies and the death of all animals carrying the balancer chromosome. These escapers were mated with each other for obtaining Nostrin^{4,32}, Cip4^{4,32} (m+z) embryos and (rare) adults for analyzing the embryonic somatic and adult midgut musculatures.

Meiotic recombinants carrying both Cip4 and Synd on chromosome 3 were identified by checking for lethality (due to Synd) along with the presence of wing hair duplications (not present in Synd single mutants).

**Staining procedures**
Drosophila embryo fixations, immunostainings for proteins and RNA in situ hybridization were performed as described previously (Azpiazu and Frasch 1993; Knirr et al. 1999). The Elite ABC-HRP kit (Vector Laboratories) and TSA Cyanine 3 System and TSA Fluorescein System (PerkinElmer Inc.) were used for fluorescent detection of RNA and One-Step NBT/BCIP (Thermo Scientific) for the non-fluorescent detection of RNA. The following antibodies were used: sheep anti-Digoxigenin (1:2000; Roche), sheep anti-Digoxigenin conjugated with alkaline phosphatase (1:2000; Roche), mouse anti-Even-Skipped (1:100; DSHB, Iowa), mouse anti-GFP (1:100; Invitrogen), rabbit anti-GFP (1:2000; Invitrogen), rabbit anti-Mef2 (1:750) (Bour et al. 1995), rat anti-Organ (1:100) (Schaub et al. 2012), rabbit anti-Timpan (1:750) (Yin et al. 1997), rat anti-Tropomyosin (1:200; Babraham Institute), rabbit anti-Beta Tubulin (1:3000; gift from R. Renkawitz-Pohl), rabbit anti-β-Galactosidase (1:1500; Promega), mouse anti-β-Galactosidase (40-1a) (1:20; DSHB, Iowa), mouse anti-lamin (T40) (1:25; Frasch et al. 1988) and digoxigenin labeled nostrin, cip4 and syndapin antisense RNA probes (all 1:200). Secondary antibodies used were conjugated with DyLight 488, DyLight 647 or DyLight 549 (1:200; Jackson Immuno Research) or with biotin (1:500; Dianova). The following digoxigenin-labeled RNA antisense probes were used: Nostrin, Cip4 and Syndapin. T7 promoter-tagged templates were generated by PCR (for primers see supplement) from cDNA clones.
obtained from the Drosophila Genomics Resource Center (Nostro: clone #IP202041; Cip4: clone #FI02049; Syndapin: clone #LD46328). Tribolium fixation and in situ hybridization were performed as described previously (Tautz and Pfeifle 1989; Patel et al. 1994). For the generation of RNA antisense probes the same primers as for the dsRNA fragments were used (see http://ibeetle-base.uni-goettingen.de/gb2/gbrowse/tribolium/). Images were acquired on a Leica SP5II confocal laser scanning microscope using a HC PL APO20x/0.70 and HCX PL APO 63x/1.3 objectives with glycerol and the LAS AF (Leica) software, on an Axioscope (Zeiss) equipped with an ApoTome (Zeiss) and Plan Apochromat 20x/0.8, 40x/1.3, 63x/1.4 objectives using the Axiosvision 4.8 software or on an Axios Scope A1 (Zeiss) using the ProgRes CapturePro (jenopik) software. The final figures were obtained using Photoshop CS5 (Adobe).

Analysis of adult gut phenotypes and wing bristle phenotypes

Adult flies were narcotized with CO2. After cutting off the head, the flies were pinned through the thorax with their ventral side facing up onto a wax dish. After covering the flies with PBT the abdomen was opened along the ventral side. Next the gut was removed from the abdomen using forceps, transferred into a staining dish, and fixed for 20 – 40 min in PBS containing 3.7% formaldehyde. Guts were stained over night at 4°C with a Leica SP5II confocal laser scanning microscope using a HC PL APO20x/0.70 and HCX PL APO 63x/1.3 objectives (with glycerol) and the LAS AF (Leica) software, on an Axioscope (Zeiss) equipped with an ApoTome (Zeiss) and Plan Apochromat 20x/0.8, 40x/1.3, 63x/1.4 objectives using the Axiosvision 4.8 software or on an Axios Scope A1 (Zeiss) using the ProgRes CapturePro (jenopik) software. The final figures were obtained using Photoshop CS5 (Adobe).

Materials produced in this study are available upon request. The authors affirm that all data necessary for confirming the conclusions of this article are represented fully within the article and its tables and figures with the exception of sequence information (e.g., for amplification primers) that is available at http://ibeetle-base.uni-goettingen.de/gb2/gbrowse/tribolium/. Supplemental material available at Figshare: https://doi.org/10.25387/g3.7680020.

RESULTS

Knock-downs of orthologs of Drosophila genes involved in myoblast fusion cause ‘thin-muscle’ phenotypes

When we inspected the muscle phenotypes of genes for which their Drosophila orthologs have been implicated in myoblast fusion in the iBeetle database, we noticed that in many (albeit not all) cases these displayed significantly thinner muscles after their knock-down (Figure 1A - F). This phenotype is particularly obvious for the dorsal and ventral longitudinal muscles, which normally are broad and touch their neighbors aligned in parallel (Figure 1A). By contrast, upon knock-down of the Tribolium orthologs of Drosophila Cdc-12 (aka Elmo), Crk oncogene (Crk), schizo (siz) (aka loner) and Verprolin1 (Vrp1) (aka solitary, stl), all of which are known to participate in myoblast fusion, these muscles are thinner and therefore clear gaps are present between them (Figure 1B - E) (Chen et al. 2003; Balagopalan et al. 2006; Kim et al. 2007; Massarwa et al. 2007; Geisbrecht et al. 2008; Jin et al. 2011). Similar effects are seen upon knock-down of the Tribolium ortholog of laminin (lmnl), which in Drosophila is needed for specifying fusion-competent myoblasts (Figure 1F) (Duan et al. 2001). Of note, this phenotype differs from the prototypical myoblast fusion phenotype in Drosophila, which is characterized by the presence of large numbers of unfused myoblasts. However, also in Drosophila fusion mutants the unfused myoblasts tend to disappear at late embryonic stages, presumably because of cell death. In Drosophila mutants for genes with less prominent functions, with weak alleles, or with partial functional rescue by maternal products, the muscles are thinner as well due to the reduced uptake of fusion competent cells (e.g., Hamp et al. 2016). We propose that the ‘thin muscle’ phenotypes in Tribolium knock-downs of most myoblast fusion genes (including some weak phenotypes with Tcas kirrel/rst; Schultheis et al. 2019) result from similar effects of incomplete functional knock-down and rapid disappearance of the unfused myoblasts. The absence of the GFP marker at earlier stages unfortunately prevented the detection of unfused myoblasts in control and RNAi treated embryos to confirm this explanation.

Knock-downs of the F-BAR domain encoding gene Tc-Nost cause similar muscle phenotypes as those of myoblast fusion genes in Tribolium

A new gene with a ‘thin muscle’ phenotype upon RNAi not previously implicated in myoblast fusion was TC013784, the ortholog of Drosophila CG42388, which subsequent to our screen was named after its mammalian ortholog, Norstr (Nost) (Zimmermann et al. 2002; Zobel et al. 2015). The encoded Tc-Nost is a member of the family of F-BAR proteins that are known to regulate membrane curvature and actin turnover in a variety of contexts (Fricke et al. 2010; Liu et al. 2015; Salzer et al. 2017). The phenotype was present with similar strength upon injections of different amounts of the TC013784 iB dsRNA and of a non-overlapping Tc-Nost dsRNA into pig-19, as well as upon iB dsRNA injection into the SB strain of Tribolium castaneum (Figure 1G - J; c.f. Figure 1A). In all cases, the penetrance of the phenotype was high (80 - 100% in pig-19, 43 - 62% in SB).

Tc-Nost is the only F-Bar domain encoding gene that is annotated in the iBeetle database with a highly penetrant or specific muscle phenotype (http://ibeetle-base.uni-goettingen.de). In the Tribolium genome there exists only one representative for Nost and, likewise, for Syndapin (Synd; TC007515), which encodes a protein with a related F-Bar domain. However, there are two representatives for the F-Bar domain encoding gene Cip4, TC014985 and TC034900. In this case, we cannot exclude that the lack of a muscle phenotype is due to functional redundancy of the paralogs. Other Bar domain genes are more distantly related such as TC005182 (Tc-CG8176) (A/H Bar domain class).

Because the observed muscle phenotype with Tc-Nost RNAi and its similarity to those of the knocked-down orthologs of the myoblast fusion genes described above were indicative of a role of Tribolium Nost in myoblast fusion, we tested whether Tc-Nost (TC013784) is expressed in the somatic mesoderm at embryonic stages when myoblast fusion is expected to occur. In situ hybridizations showed that TC013784 mRNA is present at highest levels in the forming somatic muscles as well as in the CNS and posterior gut rudiment of embryos at the fully retracted germ band stage, whereas lower levels are present in epidermal cells (Figure 2A). Hence, TC013784 expression is compatible with a role in myoblast fusion and/or other functions in Tribolium muscle development. Lateral views show TC013784 mRNA expression in subepidermal cells of the body wall and the legs, which include muscles and potentially also cells of the peripheral nervous system, as well as in specific epidermal cells (Figure 2B).

Drosophila Nostroin and related F-BAR domain encoding genes are expressed in the somatic and visceral mesoderm

Because of the much wider availability of immuno-histological and genetic tools in Drosophila we performed in-depth analyses of Nost and related F-BAR domain encoding genes in this insect species. As shown
in Figure 3A, Drosophila Nost (CG42388) mRNA is deposited maternally. The maternal transcripts vanish during stage 4 in the cellular blastoderm (except for the germ plasm and germ cells; data not shown) and zygotic expression is first seen at stage 10 in the entire mesoderm (Figure 3B). At early stage 12, Nost mRNA expression becomes more restricted to segmental subsets and an anterior-posterior band of mesodermal cells, which appear to correspond to somatic and visceral mesodermal cells, respectively (Figure 3C). To define the Nost mRNA expression pattern more carefully we performed fluorescent in situ hybridizations in conjunction with other markers for known mesodermal cell types. Double labeling for Tinman protein showed that, at stage 11, Nost is expressed specifically in the fusion-competent myoblasts of the trunk visceral mesoderm and the hindgut visceral mesoderm, but not in the visceral muscle founder cells and cardiogenic progenitors marked by Tinman (Figure 3D) (Azpiazu and Frasch 1993). Double-labeling for Org-1, which marks the founder cells of visceral muscles and of a small subset of somatic muscles, confirmed the specific expression of Nost in the visceral mesodermal fusion-competent cells at stage 12, as well as in somatic mesodermal cells adjacent to the Org-1 expressing somatic muscle founder cells (Figure 3E) (Schaub et al. 2012). At mid stage 12, there is a wide overlap between Nost mRNA and Mef2 protein expression in the somatic (and visceral) mesoderm, but not in the cardiac mesoderm (Figure 3F) (Lilly et al. 1994; Nguyen et al. 1994). Co-stainings for Nost and the founder cell marker rP298-LacZ (aka duf-LacZ) indicated mutually exclusive patterns, further suggesting that Nost is expressed specifically in the fusion competent myoblasts of the somatic mesoderm as well (Figure 3G, G') (Ruiz-Gómez et al. 2000). This interpretation was fully confirmed by the results of Nost in situ hybridizations in lameduck (lmd) mutants, which lack fusion-competent myoblasts and do not show any Nost expression (Figure 3H) (Duan et al. 2001). As expected for F-BAR domain proteins and shown for Nost in follicle epithelium cells (Zobel et al. 2015), a Nost-EGFP fusion protein localizes to the cell membranes in the somatic mesoderm (Fig. S1).

In addition to Nost, we included two other F-BAR domain encoding genes in our analysis that were characterized previously in other contexts, namely Cip4 and Syndapin (Synd) (Leibfried et al. 2008; Fricke et al. 2009; Kumar et al. 2009b). At stages 12-13, Cip4 mRNA is expressed prominently in the trunk visceral mesoderm, but low levels are also detected in Mef2-marked somatic mesodermal cells (in addition to ectodermal expression; Figure 31 - 1'). The same result was obtained with GFP stainings (co-stained for Tropomyosin I) of embryos from a line in which Cip4 was tagged endogenously with GFP. Similar to Nost-EGFP, Cip4-YFP fusion protein is also located at the membranes of the cells, which is most obvious for the strongly expressing visceral mesodermal, CNS, ectodermal cells, and salivary

Figure 1 Examples of muscle phenotypes of Tribolium orthologs of known Drosophila genes required for myoblast fusion as indicated. Thinner muscles and concomitantly larger distances of adjacent muscles are evident. (G) Embryo from primary screen (using injections of 1 mg dsRNA) with RNAi knock-down of ortholog of TCO13784 (Tc-Nost), also exhibiting narrower muscles that are spaced apart. (H) Embryo from verification screen upon pupal injections of 3 mg dsRNA for Tribolium Nost into pig-19. The phenotype is similar and not stronger as compared to (G). (J) Embryo from verification screen upon pupal injection of 1 mg dsRNA for Tribolium Nost (non-overlapping fragment (NOF) relative to original fragment from primary screen) into pig-19. (J) Embryo from verification screen upon pupal injection of 1 mg dsRNA for Tribolium Nost (original iBeetle fragment (iB) as in primary screen) into SB strain. Scale bar in A, also applicable for B - J: 100 mm.

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Embryos from primary screen with RNAi knock-down of orthologs of for EGFP. (A) Control embryo from uninjected female pupa. (B) to (F) Embryos from primary screen with RNAi knock-down of orthologs of known Drosophila genes [brackets] required for myoblast fusion and of TCO13784 (Tc-Nost). Shown are lateral (A-E, G), ventral-lateral (F, I, J), or dorsal-lateral (H) views of fully developed pig-19 embryos live imaged for EGFP. (A) Control embryo from uninjectected female pupa. (B) to (F) Embryos from primary screen with RNAi knock-down of orthologs of
mRNA expression in stage 12 embryo homozygous for gland (Figure 3J, J). Low levels of Cip4-YFP are present in the somatic mesoderm. The expression of Synd mRNA during embryogenesis is quite broad, but co-staining with Mef2 shows that it includes the early mesodermal layer (Figure 3K - K') as well as the somatic mesoderm during subsequent stages (Figure 3L, L'). Similar levels of Synd mRNA are present in the ectodermal and endodermal germ layers (Figure 3K - L').

**Functionally redundant contributions of F-BAR domain genes to somatic muscle development**

The specific expression of Nost in fusion-competent myoblasts prompted us to generate Nost null mutations to examine its potential functions during somatic and visceral muscle development (see Materials & Methods). In the two alleles obtained, all (Nost<sup>d16</sup>) or almost all (Nost<sup>d25</sup>) protein-coding exons of each isoform were deleted (Fig. S2). Homozygous flies for both alleles were fully viable, fertile, and lacked any obvious defects, including in locomotion, as was also shown with a Nost allele presumably identical to Nost<sup>d25</sup> that was made in parallel (Zobel et al. 2015). Furthermore, embryos collected from homozygous Nost mutant strains, which therefore lacked both the maternal and the zygotic activity of Nost, did not exhibit any defects in their somatic muscle patterns (Figure 4D, cf. Figure 4A, B). Likewise, embryos from crosses of homozygous null mutant flies for Cip4, which completely lack Cip4 activity (Fricke et al. 2009), also did not show any somatic muscle phenotype (Figure 4E), and neither did homozygous Synd null mutant embryos (which do contain maternal products, as}

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**Figure 2** mRNA expression of TCO13784 (Tc-Nost). Shown are in situ hybridizations of embryos at the fully retracted germ band stage (A, ventral view, B, lateral view; optical sections of dissected embryos). (A) Higher levels of TCO13784 mRNA expression are seen in the developing somatic muscles (sm), central brain (cbr), posterior gut rudiment (pg), as well as epidermal (ep) and subepidermal cells. (B) TCO13784 expression is seen in somatic muscles (sm) and epidermal (ep) cells of the body wall and limbs. Scale bars: 100 μm.

**Figure 3** Embryonic expression of Drosophila F-BAR domain genes and of Cip4-YFP fusion protein. (A) Ubiquitous distribution of maternal Nost mRNA in stage 1 embryo. (B) Uniform zygotic mesodermal expression of Nost mRNA in stage 10 embryo. (C) Segmented mesodermal expression of Nost mRNA in stage 11-12 embryo. In (D) to (L) mRNAs or fusion proteins of F-BAR domain genes are labeled in green and various tissue markers in red, as indicated. (D) Nost mRNA expression in visceral mesodermal fusion-competent myoblasts (vm-fcm) and hindgut visceral mesoderm in stage 11 embryo (dorsal view of posterior germ band). Visceral muscle founder cells (vm-fc) and cardiac mesoderm (cm) are marked by anti-Tinman (Tin) staining and lack Nost mRNA. (E) Nost mRNA expression in fusion-competent myoblasts of the visceral (vm-fcm) and somatic (sm-fcm) muscles (lateral high magnification view, stage 12). Visceral (vm-fc) and somatic (sm-fc) muscle founder cells are marked by anti-Org-1 staining. (F) Nost mRNA expression in fusion-competent myoblasts of the somatic mesoderm (sm; lateral view, stage 12). All somatic mesodermal cells and cardioblasts are marked by anti-Mef2 staining. (G, G') Nost mRNA expression in fusion-competent myoblasts of the somatic muscles (sm-fcm); lateral view, stage 12 (p298-lacZ line). Somatic muscle founder cells (sm-fc) are marked by p298-LacZ enhancer trap staining (anti-βGalactosidase) and lack Nost mRNA. (H) Absent Nost mRNA expression in stage 12 embryo homozygous for Imd, which lacks sm-fcm’s. (I) Cip4 mRNA expression in stage 13 embryo co-stained for Mef2 protein. (I', I") High magnification view of embryo in (I), showing Cip4 mRNA expression in the visceral mesoderm (vm), epidermis (bottom), and more weakly in the somatic mesoderm (sm, arrow heads). (J, J') High magnification view of stage 14 embryo from line tagged with YFP at native Cip4 locus, showing Cip4-YFP expression in the visceral mesoderm (vm), central nervous system (CNS), and more weakly in the somatic mesoderm (sm, arrow heads). (K - K’) Synd mRNA expression in stage 11 embryo in mesoderm (ms), neuroectoderm (ne), and anterior as well as posterior midgut primordia (amg, pmg), with mesoderm counterstained for Mef2. (L, L') Synd mRNA expression in stage 14 embryo (high magnification of ventral-lateral area) in somatic but not visceral mesoderm (sm, vm, counterstained against Mef2) and in endoderm (en). Scale Bars: D, F, G, H, I, K, K’, 50 μm; E, G, I’, J, L, 25 μm.
Synd is homozygously larval lethal (Kumar et al. 2009a) and fertile homozygous Synd\textsuperscript{Dex22} mutant females could not be obtained (Figure 4F).

Because of the possibility of functional redundancies among these different F-BAR proteins we examined Nost Cip4 double mutants and Nost Cip4 Synd triple mutants at stage 16 for embryonic muscle phenotypes. Synergistic activities of Nost and Cip4 have already been demonstrated by the appearance of egg chamber defects in Nost Cip4 double mutants (Zobel et al. 2015). In addition, simultaneous knockdowns of Nost and Cip4 led to increased duplicated and frequent multiple wing hair phenotypes as compared to Nost mutants that lack any wing hair phenotype and Cip4 mutants that show duplicated wing hairs at lower frequency (Zobel et al. 2015). As shown in Figure 4H - I (c.f. Figure 4C, G - G''), mutant embryos completely lacking both Nost and Cip4 products (see Materials & Methods) indeed displayed frequent muscle defects. In several segments, certain muscle fibers were missing or strongly reduced in size, and instead, mononucleated myoblasts were present at the corresponding positions. These can be detected as

Figure 4 Embryonic somatic muscle phenotypes in mutants for F-BAR domain genes. Shown are stage 16 embryos derived from inter se crosses of homozygous single mutant parents for Nost (D) or Cip4 (E), homozygous mutant embryos from heterozygous Synd mutant parents (F) and from homozygous Nost Cip4 double mutant escaper parents (H - I'') and embryos homozygous for mutations in all three genes from homozygous Nost mutant parents that were heterozygous for Cip4 and Synd (J - K'). (A) Somatic muscle pattern in control embryo (yw) stained for \(\beta\)-3-tubulin. (B) High magnification view of lateral muscles in two abdominal segments from control embryo stained for tropomyosin I (TM1). (C) High magnification view of two abdominal segments from embryo in A (boxed). (D, E, F) Views of normal ventral and lateral muscles of four abdominal segments from maternally + zygotically mutant (m+z) Nost\textsuperscript{df004}, Cip4\textsuperscript{D32}, and of zygotic Synd\textsuperscript{id} mutant, respectively, all stained for \(\beta\)-3-tubulin. (G - G'') Somatic muscle pattern in control embryo (yw) stained for tropomyosin I (TM1, red) and Mef2 (green) (G', high magnification view of boxed area in G; G'' single channel for TM1). (H - I'') Somatic muscle pattern in maternally + zygotically mutant (m+z) Nost\textsuperscript{id};Cip4\textsuperscript{D32} double mutants stained and depicted as in (G - G''). Arrows indicate mononucleated myoblasts. (J - K'') Somatic muscle pattern in maternal + zygotic (m+z) Nost\textsuperscript{id};zygotic (2) Cip4\textsuperscript{D32} Synd\textsuperscript{Dex22} triple mutants stained for TM1 and depicted as in (G - G''). Arrows indicate mononucleated myoblasts (highlighted in J', K''), asterisks an example of area with missing muscles, and arrow heads a dorsal bulge due to expanded midgut. Abbreviations: m+z, zygotically homozygous and lack of maternal contribution; z, zygotically homozygous with presence of maternal contribution. Scale Bars: A, D, E, F, G, H, I, J, K, 50 \(\mu\)m; B, C, G', H', I', J', K', 25 \(\mu\)m.
Tropomyosin I-positive cells that contain a single Mef2-positive nucleus each (Figure 4H’, H”, G’, G”; c.f. Figure 4C, G’, G”). Whereas control embryos do not contain any unfused myoblasts at stage 16, unfused myoblasts were present inappropriately in all Nost (m+z) Cip4 (m+z) double mutant embryos at this stage, and about half of these had muscles missing in one to four segments (Table S1A). We did not detect any preferential distribution of these muscle defects with respect to specific segmental or muscle identities. No defects were visible in the dorsal vessel (Figure 4I).

We found that, similar to Cip4 and Nost, Cip4 and Synd also have synergistic activities during epithelial planar polarity and wing hair formation (Fig. S3). Therefore we examined the embryonic musculature in Cip4 Synd double mutant embryos lacking the synergistic activities of both Cip4 and Synd. Although no muscle defects were detected in these embryos (Fig. S3), such defects were present in embryos in which the synergistic activities of Synd and Cip4 were missing together with both the maternal and synergistic activities of Nost (Figure 4) - K”; c.f. Figure 4C, G”). Like in Nost (m+z) Cip4 (m+z) double mutant embryos, muscle fibers were variably missing and, unlike in the controls, unfused myoblasts were seen in late stage embryos. In the triple mutants (Nost (m+z) Cip4 (z) Synd (z)) this phenotype appeared to be slightly more severe, even though Cip4 activity is only removed synergistically in these embryos. This indicates that Synd also contributes to normal somatic muscle development, in cooperation with Nost and Cip4. Altogether, the observed phenotypes suggested a role of these F-BAR proteins in the process of myoblast fusion. To describe the muscle phenotypes more quantitatively in the different genetic backgrounds, we counted syncytial nuclei at consecutive developmental stages of double and triple mutant embryos. For this analysis we used the well-characterized dorsal muscle DA1 (aka, M1) that expresses Even-skipped, which was used in development, in cooperation with Nost previously by Bataillé et al. 2012. The same pattern as in control embryos and in embryos of the genotypes, particularly the longitudinal muscle fibers, show strongly disrupted morphologies. As shown in Figure 7C - C”", in the absence of Cip4 the longitudinal muscle fibers display numerous branches, particularly near their ends, which often contact neighboring longitudinal fibers. In addition, the longitudinal fibers appear shorter, as unlike in the wild type they do not span large extents of the length of the midgut, and they are also not arranged strictly in parallel. In Nost (m+z) Cip4 (m+z) double mutant flies (see Materials & Methods), analogous but even more severe disruptions of midgut muscle morphologies are observed. In these flies, the ends of the longitudinal muscle fibers are even more frayed and some

As the difference between the double and triple mutants were not statistically significant, it is not clear whether Synd indeed contributes to the functions of Nost and Cip4 during this process.

F-BAR domain encoding genes are required for normal embryonic midguts and for midgut muscle morphogenesis during metamorphosis

In embryos lacking the zygotic activities of both Synd and Cip4, and likewise in embryos lacking zygotic Synd together with zygotic Cip4 and maternal + zygotic Nost, a subtle but consistent phenotype was seen in the midgut. In embryos with these genetic backgrounds, the anterior chamber was slightly expanded, thereby causing a bulge at the dorsal side of the embryos (Figure 6 B, c.f. Figure 6A; Fig. S3E; c.f. Fig. S3D; Figure 4J, K; c.f. Figure 4G). This phenotype was not present in embryos that were only homozygous for Synd, thus indicating that Cip4 and potentially Nost cooperate with Synd during normal midgut morphogenesis. Because disruptions in midgut morphology are often due to developmental defects in the visceral musculature (Lee et al. 2005) and because particularly Nost and Cip4 showed prominent expression in the visceral mesoderm, we examined whether loss of these F-BAR domain proteins or of Synd caused any gut muscle phenotypes.

In embryos (and, likewise, in larvae), none of the mutant backgrounds described above displayed any overt gut muscle phenotypes, which is in line with the relatively mild alterations in their midgut morphologies. By contrast, strong defects were seen in midguts of adults with some of these mutant backgrounds. Normal adult midguts are ensheathed by an orthogonal network of binucleated circular and multinucleated longitudinal visceral muscles, which run in parallel and are arranged equidistantly to their neighboring fibers (Strasburger 1932; Klapper 2000). The same pattern as in control flies is observed in midguts of Nost (m+z) mutant flies (Figure 7B, c.f. Figure 7A). However, in Cip4 (m+z) mutant flies, particularly the longitudinal muscle fibers display numerous branches, particularly near their ends, which often contact neighboring longitudinal fibers. In addition, the longitudinal fibers appear shorter, as unlike in the wild type they do not span large extents of the length of the midgut, and they are also not arranged strictly in parallel. In Nost (m+z) Cip4 (m+z) double mutant flies (see Materials & Methods), analogous but even more severe disruptions of midgut muscle morphologies are observed. In these flies, the ends of the longitudinal muscle fibers are even more frayed and some

![Figure 5 Quantification of nuclei within muscle DA1 syncytia of control, Nost Cip4 double, and Nost Cip4 Synd triple mutant embryos.](image)

*Figure 5* Quantification of nuclei within muscle DA1 syncytia of control, Nost Cip4 double, and Nost Cip4 Synd triple mutant embryos. Even-skipped + Lamin stained nuclei of muscle DA1 syncytia, counter stained for tropomyosin I, were counted at stages 14, early and late stage 15, and stage 16 in yw control embryos and in embryos of the genotypes Nost(w004)(m+z);Cip4A32 (m+z) and Nost(w004)(m+z); Cip4A32(m+z), SyndA22(z) (***, P < 0.0005; *, P < 0.05; ns, differences not significant; m, maternal; z, zygotic).
fibers are split toward their middle portions (Figure 7D - D*). Furthermore, the arrangement and thickness of the longitudinal fibers is irregular. In Cip4 (m+z) mutants and, even more often, in Nost (m+z) Cip4 (m+z) double mutants the normally parallel arrangement of the circular midgut muscles is also disrupted (Figure 7C - C*, Fig. 7D - D*). Their distances vary widely and often fibers cross over each other. Closer inspection showed that the abnormal feathered extensions of the longitudinal fibers tend to contact the circular fibers at their ends, and it appears that the circular fibers are being pulled in anterior-posterior directions by the contractions of the attached longitudinal fibers (Figure 6C - D*). Hence, the circular muscle phenotype may be largely secondary to the observed longitudinal muscle phenotype. These data show that Cip4 and Nost cooperate during the process of longitudinal midgut muscle metamorphosis. A contribution of Synd cannot readily be tested because homozygous Synd mutant flies are not viable.

**DISCUSSION**

**F-BAR proteins promote myoblast fusion**

Prompted by the observed thin-muscle phenotype upon Tc-Nostrin (TC013784) knock-down and the highly specific expression of Dm-Nostrin in fusion-competent myoblasts of embryonic somatic and visceral muscles, we focused on the characterization of the potential roles of Dm-Nostrin (Nost) and two structurally related proteins, Cip4 and Syndapin (Synd), in Drosophila muscle development. All three proteins contain F-BAR domains within their N-terminal half and an SH3 domain at their C-terminus (Fricke et al. 2009; Kumar et al. 2009a; Zobel et al. 2015). These F-BAR proteins belong to the NOSTRIN, CIP4, and PACSIN subfamilies of the F-BAR protein superfamily and are the only representatives of this subfamily in Drosophila, and likewise, in Tribolium (although there are two Tc-Cip4 paralogs, see above). Members of this F-Bar protein subfamily are known to provide a molecular link between the plasma membrane or nascent vesicular membranes and actin dynamics (Liu et al. 2015; Salzer et al. 2017). Chiefly, the interaction of the homodimeric crescent-shaped F-BAR domains of these proteins with membrane phospholipids creates membrane curvatures, and their SH3 domains interact with the Wiskott-Aldrich syndrome protein (WASP), neural (N)-WASP, WASP family verproline-homologous protein (WAVE), and dynamin. The ensuing activation of these proteins leads to the binding of actin-related protein 2/3 (Arp2/3) which, in turn, promotes actin nucleation and polymerization. Actin polymerization then can further propel membrane curvatures, which in the case of the NOSTRIN, CIP4, and PACSIN family proteins has been reported to promote the formation of filopodia, lamellipodia, podosomes, invadopodia, and to stimulate endocytosis (Chen et al. 2012; Liu et al. 2015; Salzer et al. 2017). Cellular events with these characteristics are also known to be hallmarks during Drosophila myoblast fusion, particularly in fusion-competent myoblasts, in which Dm-Nostrin is expressed (Onel and Renkawitz-Pohl 2009; Kim et al. 2015; Deng et al. 2017). Thus, during the earliest steps of myoblast fusion, fusion-competent myoblasts extend filopodia to the muscle founder cells before attaching to them
The actual fusion process is driven to a large part by the formation of a dense F-actin focus surrounded by a fusion-restricted myogenic-adhesive structure (FuRAMAS) in fusion-competent myoblasts, which propels an invadopodia-like membrane protrusion into the attached founder myoblast or nascent myotube (Önel and Renkawitz-Pohl 2009; Kim et al. 2015; Deng et al. 2017). This process is thought to provide the key force for membrane rupture and cell fusion (Sens et al. 2010). F-actin polymerization is activated downstream of the activated Ig domain receptors Sticks-and-stones (Sns) and Hibriss (Hbs) upon their engagement with the extracellular domains of the related receptors Kirre (aka Dumbfounded, Duf) and Roughest (Rst) on the surface of the founder myoblast. Links between the intracellular domains of active Sns/Hbs are provided by the adaptor proteins Dock and Crk, which bind to activated Sns and Hbs and through their SH3 domains interact with WASp and the WASp regulator Verprolin 1 (Vpr1; aka Solitary, Sltr). In turn, these nucleate linear and branched actin polymerization via activation of Arp2/3, which is additionally regulated by activated WAVE (aka SCAR), the WASp family member WHAMY, and by the formin Diaphanos (Dia) (Önel and Renkawitz-Pohl 2009; Kim et al. 2015; Brinkmann et al. 2016; Deng et al. 2017). As our data demonstrate a contribution of F-BAR proteins, particularly Nostrin and Cip4, to the process of myoblast fusion, it is conceivable that these proteins provide an additional, perhaps receptor-independent link between the plasma membrane and actin polymerization at the fusion site within fusion-competent myoblasts. In addition to their activation of actin polymerization, they could influence membrane bending at the fusion site directly by binding to the plasma membrane, and help coordinating membrane bending and the formation of the F-actin focus. However, attenuation of fusion efficiency even after the complete elimination of both Nost and Cip4 (and additionally of zygotic Synd) is relatively mild as compared to the generally stronger block of myoblast fusion in mutants for the various actin nucleators and their upstream activators (unless there are strong maternal contributions) (e.g., Richardson et al. 2007; Gildor et al. 2009; Kaipa et al. 2013). This indicates that these F-BAR proteins play a supporting rather than an essential role during this process. In addition, as shown herein, there is functional redundancy among different F-BAR proteins during this process. Overall, this and published information support the view that the system has a significant amount of back-up pathways built in. In Tribolium, we have not attempted any double or triple knock-downs of the different subfamily members to determine whether this would cause more severe muscle phenotypes.

As F-BAR proteins including Nostrin, Cip4, and Syndapin in both mammals and Drosophila have been shown to regulate receptor-mediated endocytosis (Kessels and Quaumann 2002; Icking et al. 2005; Itoh et al. 2005; Leibfried et al. 2008; Fricke et al. 2009; Feng et al. 2010; Zobel et al. 2015; Sherlekar and Rikhy 2016), this mode of action could also be involved in promoting myoblast fusion. In Drosophila myoblast fusion, there is evidence that local clearance of N-cadherin at the fusion site by endocytosis in fusion-competent cells and nascent myotubes is needed prior to fusion to allow progression of the fusion process (Dottermusch-Heidel et al. 2012). Perhaps related to this observation, Nost and Cip4 were shown to cooperate in sequential steps of endocytic E-Cadherin membrane turnover in the Drosophila thoracic epithelium and in developing egg chambers, which in the latter case is important for proper germline cell adhesion, egg chamber encapsulation by follicle cells, and normal fertility (Zobel et al. 2015). In growing myotubes, endocytosis appears to be involved also in the clearance of Sns (but not Duf) in addition to N-cadherin, which may be beneficial for efficient later rounds of myoblast fusion (Haralalka et al. 2014). In future experiments, these processes could be monitored in Nost Cip4 double or Nost Cip4 Synd triple mutant embryos to determine a possible role of these F-BAR proteins in endocytic events during myoblast fusion.

In mouse, several observations from in vitro models have pointed to the involvement of F-BAR and other BAR superfamily proteins in myoblast fusion. (George et al. 2014) reported that the CIP4 subfamily member Toca-1 is required for normal myoblast fusion and myotube formation in differentiating C2C12 cells, which appears to involve downstream activation of the actin regulator N-WASP. In an in vitro model for cell-to-cell fusion initiated by protein fusogens of influenza virus and baculovirus, curvature generating proteins, including the F-BAR domain protein FCHO2 as well as GRAF1 that contains a C-terminal SH3 domain in addition to the N-terminal Bar domain and central RhoGAP and pleckstrin homology (PH) domains, were shown to promote syncytium formation (Richard et al. 2011). GRAF1 is enriched in skeletal muscle and was reported to promote terminal differentiation and myoblast fusion of C2C12 cells, which involves its Rho-GTPase activating function for actin remodeling and BAR domain-dependent membrane sculpting (Doherty et al. 2011). Myoblasts isolated from GRAF1 knock-out mice and regenerating muscles in GRAFI−/− mice showed reduced myoblast fusion (Lenhart et al. 2014). In addition to its influence on the actin metabolism, this function may be mediated by regulating vesicular trafficking of the fusogenic ferlin proteins to promote membrane coalescence (Lenhart et al. 2014). Drosophila Graf is known to regulate hematopoiesis through endocytosis of EGFR, but its potential expression in the somatic mesoderm and any role in myoblast fusion have not been examined (Kim et al. 2017). Yet another Bar family member, the N-Bar domain protein Bin3, has also been implicated in mouse myoblast fusion, as myoblasts from Bin3−/− mice show a reduced fusion index and Bin KO mice show delayed regeneration upon injury (Simionescu-Bankston et al. 2013).

F-BAR proteins are important during adult visceral muscle morphogenesis

The strongest muscle phenotypes of Drosophila F-BAR gene mutants are present in the longitudinal midgut muscles of adult flies, which instead of being linear and arranged in parallel are highly branched at their ends, connected to their neighbors, and oriented irregularly. In this case, the phenotype is already seen in Cip4 single mutants but is enhanced in Nost Cip4 double mutants, similar to the situation in wing hair formation (Zobel et al. 2015). The phenotype is most likely explained by the drastic events of cellular remodeling of the longitudinal midgut muscles during metamorphosis. During pupariation, the larval longitudinal muscles dedifferentiate, first forming numerous cytoplasmic projections that are shed (Klapper 2000), then fragmenting into smaller syncytia and finally into mononucleated myoblasts (Aghajanian et al. 2016, D. Schultheis & M. Frasch, unpublished data). At this stage of maximal dedifferentiation the myoblasts are connected to each other by a network of fine filopodia. The reconstitution of the visceral muscle syncytia is accompanied by a progressive disappearance of the lateral extensions to neighboring cells and syncytia (D. Schultheis & M. Frasch, unpublished data). Ultimately parallel, multinucleated fibers are re-established that very much resemble the original larval longitudinal midgut muscles. Because the longitudinal midgut muscles in Cip4 and even more so in Nost Cip4 mutant flies are shorter, display frayed ends connected to neighboring muscles, and are not neatly arranged in parallel, we propose that these F-BAR domain proteins act especially during the steps of redifferentiation. Apart from myoblast fusion, we propose that shape changes and removal of the extensive filopodial connections during these extreme remodeling events involve...
coordinated interactions between membranes and actin turnover, as well as regulated endocytosis, in which these F-BAR proteins are likely involved. Future experiments with fluorescent plasma membrane reporters in longitudinal muscles in *Drosophila* mutant pupae can test these possibilities, although so far our attempts in this direction were hampered by the very low fertility of these double mutants, which has limited the number of developmental stages that could be examined.

**CONCLUSION**

Aiming to utilize the identified *Tribolium* genes from the iBeetle screen for gaining new insight into *Drosophila* muscle development, we demonstrated that F-BAR domain proteins, particularly Nostrin and Cip4, play roles in myoblast fusion during embryonic somatic muscle development and during visceral muscle remodeling at metamorphosis. The examination of orthologs of additional genes with muscle phenotypes identified in the iBeetle screen will likely further advance our understanding of muscle development in *Drosophila* and other species.

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