Characterisation of the role of Vrp1 in cell fusion during the development of visceral muscle of *Drosophila melanogaster*

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Abstract

**Background:** In *Drosophila* muscle cell fusion takes place both during the formation of the somatic mesoderm and the visceral mesoderm, giving rise to the skeletal muscles and the gut musculature respectively. The core process of myoblast fusion is believed to be similar for both organs. The actin cytoskeleton regulator Verprolin acts by binding to WASP, which in turn binds to the Arp2/3 complex and thus activates actin polymerization. While Verprolin has been shown to be important for somatic muscle cell fusion, the function of this protein in visceral muscle fusion has not been determined.

**Results:** Verprolin is specifically expressed in the fusion competent myoblasts of the visceral mesoderm, suggesting a role in visceral mesoderm fusion. We here describe a novel Verprolin mutant allele which displays subtle visceral mesoderm fusion defects in the form of mislocalization of the immunoglobulin superfamily molecule Duf/Kirre, which is required on the myoblast cell surface to facilitate attachment between cells that are about to fuse, indicating a function for Verprolin in visceral mesoderm fusion. We further show that Verprolin mutant cells are capable of both migrating and fusing and that the WASP-binding domain of Verprolin is required for rescue of the Verprolin mutant phenotype.

**Conclusions:** Verprolin is expressed in the visceral mesoderm and plays a role in visceral muscle fusion as shown by mislocalization of Duf/Kirre in the Verprolin mutant, however it is not absolutely required for myoblast fusion in either the visceral or the somatic mesoderm.

Background

In general there are three major muscle types in vertebrates as well as in insects; visceral muscle, cardiac muscle and skeletal muscle. *Drosophila* muscle progenitors, i.e. myoblasts, arise during embryogenesis and undergo the central process of myoblast fusion during the development of both the visceral and the somatic muscles. The mechanisms underlying cell fusion are actively studied in musculature of *Drosophila melanogaster*, with significant focus on the process of fusion within the somatic mesoderm (SM), although the phenomenon of myoblast fusion also occurs during the formation of the visceral muscle. The visceral mesoderm (VM) of the fruitfly consists of an inner layer of circular muscles, formed after one round of myoblast fusion, surrounded by an outer layer of longitudinal muscles [1-3]. Although the process of fusion in the VM is generally considered to be similar to SM fusion, VM fusion has not been as extensively studied and is not entirely understood [4-7]. To date, a number of molecules that are required for SM fusion have been identified, leading to the development of models describing the process of SM fusion [8]. Central to this, two different myoblast subtypes have been identified, founder cells (FCs) and fusion competent myoblasts (FCMs), which differentially express a number of transcription factors and adhesion molecules [9]. The FC is destined to become the first cell of each SM muscle, fusing with FCMs to generate the multinucleated muscle. FCMs continue to fuse with the growing myotube ultimately resulting in a muscle of the appropriate mass [10,11]. Attraction between the FC and the FCM is mediated, at least in part, by immunoglobulin-domain containing...
proteins such as protein Dumbfounded/Kin of Irre (Duf/Kirre) and Sticks and Stones (SNS) which are expressed on the cell membrane of the FCs and FCMs respectively [12-15]. The subsequent fusion of the myoblast plasma membrane is to a large extent dependent on signaling pathways regulating the actin cytoskeleton.

The significance of the actin machinery in SM fusion has become evident from studies of mutants of the Scar-Wasp signaling network. Scar (WAVE in mammals) and Wiskott-Aldrich syndrome protein (Wasp) are multidomain proteins which are structurally different at their NH2-terminal domains, but which both contain a common Verprolin-homology, cofilin-homology, and highly acidic (VCA) - region at the COOH-terminal region, through which they bind to and activate the Arp2/3 complex [16]. The Arp2/3 complex is a well characterized actin nucleator, and thus Scar and Wasp are important regulators of actin polymerization [16]. A number of additional proteins are necessary for the proper function of both Scar and Wasp: Scar acts in a complex with four other proteins, including Kette (NAP125 in mammals), while Wasp functions in a complex with Verprolin (Vrp) [17]. Vrp is also known as Wasp interacting protein (WIP) in mammals [18] and in Drosophila Vrp is known as Verprolin1 (Vrp1) [19]/D-WIP [20]/Solitary [21]/and Solas [22]. Both Scar and Wasp are activated by small GTPases such as Rac and Cdc42 [23]. Rac, in turn, is regulated by the guanine nucleotide exchange factor Myoblast city (Mbc) [24].

Drosophila mutants in Scar, Wasp, Vrp, Arp2, Kette, mbc, Rac1, Rac1-Rac2-mtl and Cdc42 all show SM fusion defects during embryonic stages, although the severity of fusion phenotypes varies extensively between the different mutants, probably due to redundancy as well as maternal contribution in certain cases [20,21,25-28]. The fusion defects in these mutants, characterized by unfused SM cells as well as abnormal actin accumulations at the cell-attachment sites (in the case of Scar, Wasp, Kette, Rac1-Rac2-mtl), confirm the importance of the actin machinery in SM cell fusion [25,29].

In this work we have investigated the process of myoblast fusion in the VM. VM cells in Drosophila melanogaster express the ALK (Anaplastic lymphoma kinase) receptor tyrosine kinase (RTK), which activates a signaling cascade resulting in the specification of VM FCs [4-6]. The immunoglobulin-domain containing molecules Duf/Kirre and Sns are expressed in the VM FCs and FCMs respectively, and play a role in VM fusion, mediating adhesion between the FCs and FCMs. We identified the actin regulatory protein Vrp1 as a molecule important in the process of muscle fusion the SM and VM development, based on a deficiency screen for VM fusion mutants carried out in our laboratory. A role for Vrp in the SM fusion process has previously been reported [20-22,26], however, Vrp is also strongly expressed in the FCMs of the VM suggesting a role in VM fusion. Here we show that Vrp1 mutants display defects in the development of the visceral muscle, although the defects observed in the VM are more subtle than those observed in the SM.

Results

Vrp1f06715 is an insertion in the Vrp locus which exhibits severe somatic muscle fusion defects

We initially identified the deficiency Df(2R)ED3943 as displaying a strong muscle fusion phenotype (Figure 1B). Examination of the genes contained within this region revealed the presence of CG13503 (originally named Vrp1 in Flybase), which had previously been identified as an FCM specific gene in the elegant microarray analysis of Estrada and Michelson 2008 [30]. Subsequent examination of the Harvard Exelixis stock collection revealed the presence of a potential Vrp1 mutant fly strain. This fly strain contained a piggyBac element inserted within the coding region of the Vrp1 gene (Figure 1C) and was therefore named vrp1f06715. This insertion completely disrupts Vrp1 gene function and causes lethality and a severe somatic muscle phenotype at the embryonic stage, both alone (Figure 1E) and in combination with Df(2R) ED3943 (Figure 1F). Vrp1f06715 embryos display a similar degree of somatic muscle fusion phenotype as the previously published Vrp1 mutant D-WIPD30 [21] [Additional file 1: Supplemental Figure 1], and Vrp1f06715/D-WIPD30 transheterozygotes [Additional file 1: Supplemental Figure 1], confirming Vrp1f06715 as a novel Vrp1 mutant allele. In addition, expression of the piggyBac transposase in Vrp1f06715 flies resulted in the mobilization and excision of Vrp1f06715 and reversion of the muscle fusion phenotype (Figure 1G), demonstrating that the Vrp1f06715 insertion indeed causes the fusion phenotype.

Vrp expression pattern

The Vrp protein domain structure has been conserved throughout evolution from yeast to Drosophila and further on to higher organisms such as mouse and human. Vrp is a probe rich protein with two WH2 domains in the N-terminal region of the protein and a WASP-binding domain in the C-terminal portion (Figure 2A). Vrp1 mRNA is expressed in both the developing visceral (Figure 2B, arrows) and somatic muscles (Figure 2B, arrowheads). Anti-Vrp1 antibodies were generated in order to analyze the expression of the Vrp1 protein. Vrp1 protein is strongly expressed in muscles (Figure 2C and 2E) and is not detectable in Vrp1f06715 (Figure 2F) or in Vrp1f06715/Df(2R)ED3943 embryos (Figure 2G). Analysis of rp298lacZ embryos, which express beta galactosidase (lacZ) in the founder
cell specific pattern of the Duf/Kirre gene[13,31], indicates that Vrp expression is specific for FCMs, since no overlap between Vrp and lacZ expression was detected (Figure 2D).

**Vrp** is specifically expressed in the FCMs of the VM

An essential role for Vrp1 in somatic muscle fusion has been elegantly described in previous work [20-22], however its role in other tissues has not been studied. We observed that both Vrp1 mRNA and protein are found not only in the somatic muscles but also in the visceral mesoderm (Figure 2B arrow, and Figure 3A, arrow) as well as at muscle attachment sites (data not shown). The VM forms the midgut in the fruitfly, and at early embryonic stages, prior to fusion, columnar shaped FCs and the rounder FCMs of the VM can be distinguished morphologically as described previously [1,3]. Analysis of the VM of control embryos revealed expression of

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**Figure 1** Vrp1<sup>106715</sup> is new Vrp1 allele which exhibits severe somatic muscle fusion defects. (A-B, D-G) Stage 16 embryos were stained with antibodies against β3-Tubulin to visualize somatic muscles. (A) Wild type embryo (WT). (B) Df(2R)ED3943 mutant embryo with severe muscle fusion defects. Arrow indicates unfused cells. (C) Schematic representation of the Vrp1<sup>106715</sup> allele. The genomic location of the Vrp1 locus on 2R is indicated. mRNA representing exons and introns are shown as yellow and grey boxes respectively, and correspond to the longest predicted mRNA splice variant (CG13503-RA). The Vrp1<sup>106715</sup> allele has a piggyBac insertion (WH06715) in the coding region of the 7<sup>th</sup> intron, which disrupts gene function. Other genes in close proximity of the Vrp1 locus are illustrated with white boxes and their transcriptional direction with arrows (FlyBase [20]). (D) Wild type embryo (WT). (E) Vrp1<sup>106715</sup> mutant embryo (arrow indicates unfused cells). (F) Vrp1<sup>106715/Df(2R)ED3943</sup> transheterozygous embryo displaying the same muscle fusion defects as Vrp1<sup>106715</sup> (arrow indicates unfused cells). (G) The Vrp1<sup>106715</sup> phenotype was reverted by precise excision of the WH06715 piggyBac element.
Vrp1 specifically in the FCMs (Figure 3B, arrow), while the columnar FCs lack Vrp1 expression (Figure 3B; arrowhead). Both FCMs and FCs express Alk, which outlines all VM cells (Figure 3A, B, D). Vrp1 protein localization was further examined in \textit{rp298lacZ} embryos, in which the FCs express lacZ [13,31], confirming the specificity of Vrp1 expression in FCMs (Figure 3C, arrow indicates FCMs, arrowhead indicates FCs). The specific expression of Vrp1 in FCMs can clearly be observed in \textit{sns} mutant embryos, in which the FCMs and FCs of the VM separate as a result of defective adhesion between the FCMs and the FCs and the VM cells as a result of defective adhesion between the FCMs and the FCs (Figure 3D, arrow indicates FCMs, arrowhead indicates FCs)[3,32]. Taken together, these results clearly demonstrate that Vrp1 is a specific protein in the developing visceral mesoderm.

**The VM of Vrp1 mutant embryos displays a subtle phenotype**

While the expression of Vrp1 in the VM is confined to the FCM subtype as in the developing somatic muscle, the role of Vrp1 in VM muscle fusion does not seem to be as profound as in the SM. In the VM of Vrp1\(^{f06715}\) embryos the FCMs and the FCMs appear to fuse despite the absence of Vrp1 protein (Figure 4A). The VM of the Vrp1\(^{f06715}\) mutants appears slightly disorganized at early stages (Figure 4A), however the development of the gut proceeds, and the Vrp1\(^{f06715}\) mutants develop a gut structure with midgut constrictions at later stages (Figure 4D). Examination of Duf/Kirre expression in the VM of stage 13 Vrp1\(^{f06715}\) embryos employing the \textit{rp298lacZ} reporter [13,31] indicates that all VM myoblasts have fused as all cells appear to express LacZ (Figure 4C).

We also investigated the development of the longitudinal visceral muscles in \textit{vrp1}^{f06715} mutants, employing UAS-LacZ expressed under the control of 5053-GAL4 as a readout. At stage 12 in both \textit{vrp1}^{f06715} and control embryos (Figure 4H and 4I) the longitudinal muscles surround the circular musculature, and at later stages, both in mutants and controls, the longitudinal muscles form a characteristic longitudinal pattern (Figure 4F and 4G). These results indicate that longitudinal muscle development is not obviously affected by Vrp1 mutation.
To further examine the VM development in Vrp1<sup>f06715</sup> mutant embryos we investigated a number of molecules which are known to play a role in myoblast fusion and muscle development. One such molecule; Duf/Kirre, is known to play a role in muscle cell fusion. Interestingly, we observed that Duf/Kirre protein is inappropriately expressed in muscles of Vrp1<sup>f06715</sup> mutant embryos. During the course of this work we have observed that Duf/Kirre protein is normally highly expressed in the VM during stage 11, but after muscle cell fusion has occurred Duf/Kirre is downregulated and protein expression is undetectable after fusion, in keeping with a previous report from Menon et al. [33] in the SM. In contrast, Duf/Kirre is observed in a punctuate pattern and appears not to be downregulated correctly in either the VM or SM of later stage Vrp1<sup>f06715</sup> mutant embryos (Figure 5A and 5B, arrows, compared to wild type embryo Figure 5C, arrow), a phenomenon also observed in several SM fusion mutants [33]. One explanation is that these Duf/Kirre rich accumulations arise between VM FCs and FCMs when fusion does not proceed as normal.

Since actin foci have been reported to be formed at the cell-cell attachment sites between fusing FCs and FCMs, and to contain fusion proteins such as Sns, Rol, Loner, Blow and Mbc [25], we investigated if the Duf/Kirre accumulations in the Vrp1<sup>f06715</sup> mutant could involve such actin structures. For this we employed the <i>twip-GFP-actin</i> fly strain in which a GFP-actin fusion protein is expressed under the control of the twist promoter [25], and examined actin localization in Vrp1<sup>f06715</sup> mutant animals. Analysis of these mutants revealed that the Duf/Kirre accumulations do not contain elevated levels of actin (Figure 5D arrowheads), suggesting that the Duf/Kirre containing structures we observe are different than the above described actin foci.

**Mutations in additional components of the Scar-Wasp signaling network display similar phenotypes as Vrp1<sup>f06715</sup>**

Because the development of the VM appears to be less sensitive to perturbations in the actin regulating machinery than the SM, we decided to investigate the consequence of manipulating additional actin regulating proteins.
proteins in the VM. We examined three mutants for components of the scar-wasp signaling network; *kette* J4-48, *wasp* 3D3-035, and *arp3-wasp*. *Kette* J4-48 is a null mutant for the *kette* gene [27,34], *wasp* 3D3-035 is a mutant allele that encodes a dominant negative form of Wasp and thereby also inhibits maternally contributed Wasp protein [35], and *arp3-wasp* is a double mutant of *wasp* 3D3-035 and *arp3* (which the latter encodes a component of the Arp2/3 complex [26]). We find that all three mutants; *kette* J4-48, *wasp* 3D3-035, and *arp3-wasp*, exhibit normal VM development, resulting in the formation of a gut (Figure 6B - D, arrows indicate gut development).
and Figure 6D”, arrow indicates VM) despite severe SM fusion phenotypes (Figure 6B ‘- D’, arrowheads indicate unfused SM cells). The Duf/Kirre rich accumulations observed in the Vrp1f06715 mutant were found to be present in all mutants examined (Figure 6E, and data not shown). Taken together, these data suggest that Arp2/3 mediated actin polymerization is not essential for the formation of the embryonic VM, in contrast to its indispensable role in the somatic muscle fusion process. It is possible that complete fusion is not an essential process in embryonic VM formation, and that therefore disrupting fusion mechanisms does not affect VM development significantly. However, the presence of Duf/Kirre accumulations indicates that some as yet uncharacterised defect in development of the VM exists.

*Vrp1* mutant VM cells are capable of migrating and fusing
In addition to having a role in muscle cell fusion, Vrp1 and other actin regulating proteins have, in other experimental systems, been suggested to have roles in cell motility [36-39]. In order to test the role of Vrp1 in both muscle fusion and cell motility experimentally we analyzed Alk mutant embryos. In Alk10 mutant embryos at stage 12 there is no fusion of the VM and a number of mutant Alk expressing VM myoblasts have migrated to the SM (Figure 7B, arrowhead). Vrp1 protein can be detected in the leading tip of cells stretching towards a SM cell (Figure 7B’, arrowhead), suggesting a possible role for Vrp1 in the
migration mechanism. However, we observe that loss of Vrp1 has no appreciable effect on the migration of Alk mutant myoblasts of the VM, since Alk-positive cells can be detected in the somatic muscle cell populations of Alk-Vrp1 double mutants with a similar efficiency to that of Alk mutants. (Figure 7C, arrowhead, and 7C', arrow).

To further investigate whether Alk-Vrp1 mutant myoblasts are capable of fusing with somatic FCs, we examined Alk-Vrp1 double mutant embryos carrying the rp298lacZ enhancer trap, which marks the FC population [13,31]. In these embryos, Alk positive lacZ expressing cells could readily be detected suggesting that Alk-Vrp1 mutant cells of the VM are able to both migrate and fuse with cells of the SM (Figure 7D and 7D', arrow). The fact

Figure 6 Several mutants for components of the Scar-Wasp signaling network develop a normal gut, but display Duf/Kirre accumulation phenotype. (A-D) Stage 17 embryos stained with FasIII to visualize VM and β3-Tubulin to visualize SM. All mutants in B-D display no obvious VM phenotypes, shown by the presence of a developed gut (arrows), this is despite severe SM mutant phenotypes (arrowheads indicate unfused SM cells in B'-D'). (A-A') Wild type. (B-B') kette14-48. (C-C') wasp3D3-035. (D-D') arp3-wasp. (E) arp3-wasp mutant embryo, stage 15, stained with Duf/Kirre (red) and Alk (green). Duf/Kirre accumulations are observed in both the VM (box) and SM (arrowhead). (E') Close up of box in D. Duf/Kirre is accumulated in foci indicated by arrowheads. (E'') Close up of box in D, Alk staining marks VM (arrow). Arrowhead indicates Duf/Kirre accumulations as in H'.
that Vrp1 mutant cells are capable of fusing suggests that the fusion defects observed in the somatic muscles of Vrp1 mutant embryos are not caused by a complete block in fusion, but may reflect an inability of Vrp1 mutant cells to either complete the fusion process or to go through multiple rounds of fusion.

Expression of Vrp1 in the FCM population rescues fusion
The Vrp1 protein contains a number of functional domains; two WH2 domains at the NH2-terminal region, which are predicted to be actin binding domains, a central proline rich region, which are often involved in multi-protein complex formation, and at the COOH-terminal a WASP-binding domain, which facilitates binding to WASP [18,40]. In order to investigate the importance of the various domains of Vrp1 functionally we generated a set of transgenic Drosophila carrying UAS-Vrp1 transgenes (shown schematically in Figure 8A). In embryos employing either the Twist-Gal4 or Sns-Gal4 driver lines to ectopically express the various Vrp1 proteins we were unable to observe any visible phenotypes in the VM, nor with overexpression of the same proteins in imaginal discs (data not shown). In rescue experiments we found that both the full length Vrp1 transgene and the Vrp1ΔWH2 proteins were able to fully rescue the Vrp1 mutant phenotype when overexpressed specifically in FCMs of Vrp1 mutants using the sns-Gal4 driver [41] (Figure 8A), as well as with the stronger muscle specific driver TwistGal4 (data not shown). In contrast, those transgenes which lacked the WASP-binding domains; Vrp1ΔProΔWASP and Vrp1ΔWASP, were both unable to rescue either lethality (Figure 8A) or the somatic muscle phenotype to any extent using either of the two drivers [Additional file 2: Supplemental Figure 2B-D].

In parallel, we examined the effect of the various Vrp1 proteins on the organization of the actin cytoskeleton in
Figure 8 Expression of Vrp1 in the FCM population rescues fusion and lethality in Vrp1<sup>f06715</sup> mutants. (A) Overview of the transgenic constructs generated for UAS-Gal4 fly experiments and for cell culture overexpression experiments. Dark grey boxes represent the WH2 domains, light grey box denotes the proline rich domain (Pro), black box is the WASP-binding domain (WBD). Myc-tag is indicated by an oval. Various domains of the Vrp1 protein were deleted as shown. Transgenes containing the WASP-binding domain were able to rescue the Vrp1<sup>f06715</sup> mutant lethality when specifically expressed in the FCMs using a sns-GAL4 driver, while those transgenes lacking the WASP-binding domains were unable to rescue the lethality of the Vrp1<sup>f06715</sup> mutant as indicated in the table. (B) Ectopic expression of the full length Vrp1 transgene, but not the truncated forms, induced a dramatic reorganization of the actin filament system in form of the assembly of thick bundles and the formation of actin dots, resulting in loss of stress fibers. Actin dots (accumulation of actin in foci, red arrowhead) and thick bundles (thick actin filaments, red arrow) are known to be formed upon ectopic expression of actin reorganizing proteins, such as mammalian Vrp1, at the expense of the stress fibers. A detailed description of the phenotypes are given in [Additional file 2: Supplemental Figure 2E]. Filamentous actin was visualized by TRITC-labeled phalloidin (red). Vrp1-expressing cells were detected by co-transfecting an EGFP- and Vrp1-expressing plasmids. Bar represents 20 μm. (C) Quantification of the effects on the actin organization caused by ectopic expression of the Vrp1 transgenes in PAE cells was performed; the percentage of cells displaying extensive stress fiber loss, thick bundles and actin dots were counted manually employing a 63x immersion oil objective. The values represent triplicates of analyzes of at least 100 transfected cells.
porcine aortic endothelial (PAE) cells, reasoning that in this system we would be able to analyze the effect of the various Vrp1 protein domains on the morphology of the actin cytoskeleton. We have previously found that ectopic expression of mammalian Verprolin results in a profound reorganization of filamentous actin [42]. We observe a shift in the balance between monomeric and filamentous actin, seen as the bundling of stress fibers into thick actin filaments and the formation of actin foci (Figure 8B). Here, the full length Vrp1 transgene, but not the truncated forms, induced thick bundles, actin dots and stress fiber loss (Figure 8B and quantification in C), indicating that ectopic expression of Vrp1 regulates the organization of the actin cytoskeleton in PAE cells, in a similar manner to the mammalian Verprolins WIRE and WIP [42][Additional file2: Supplemental Figure 2E].

Discussion and Conclusions

Df(2R)ED3943 was identified in a deficiency screen designed to identify novel genes with roles in VM development. Subsequent work led to the identification of the Vrp1<sup>WH2F06715</sup> mutant allele, present in the Exelixis mutant collection maintained at Harvard [43], which carries a piggyBac insertion in the Vrp1 gene. Closer examination of both Df(2R)ED3943 and the Vrp1<sup>WH2F06715</sup> mutant, lead to the identification of a subtle VM-phenotype as well as a severe somatic mesoderm (SM) fusion phenotype. At this time the SM fusion phenotype of independent mutants in the Vrp1 locus, which is characterized by a large number of unfused myoblasts, was unpublished. However, several elegant studies have subsequently described the role of Vrp1/D-WIP/Solitary/solas [20-22]. Therefore, we have focused upon investigation of the role of Vrp1 in the development of the visceral musculature.

The VM phenotype observed in Vrp1<sup>WH2F06715</sup> mutants is not as explicit as that in the SM. Both Df(2R)ED3943 and Vrp1<sup>WH2F06715</sup> exhibit defects in gut structure, however, we cannot definitively address how much of this is due to the lack of structural support of a surrounding somatic musculature. More detailed analysis of the developing VM of Vrp1<sup>WH2F06715</sup> mutant embryos was performed, leading to the discovery of a VM phenotype characterized by mislocalization of the adhesion molecule Duf/Kirre (see below for further discussion).

To date, there are few published mutants with strong VM fusion phenotypes, and even mutants with a complete block of fusion between myoblasts in both the SM and the VM, such as sns [1] and myoblast city [1,3] mutants, display subtle VM fusion phenotypes which can be difficult to identify. While mutants such as Alk and Jeb, which do not specify founder cells [4-6,44,45] display clear fusion phenotypes which are easily identified during embryonic development, many more muscle specific genes which are expressed both in the SM and the VM, have been reported to have weak VM phenotypes when mutated, although they give severe fusion phenotypes in the SM. Examples include mutants in rolling pebbles [46], antisocial [47] roughest [12], blown fuse [48,49], lame duck [50,51], loner [52] and kette [49]. Our work adds Vrp1 the list of mutants belonging to this category.

The Vrp1 protein contains several domains, which are conserved throughout evolution (Figure 2A), [18,19]. By asking which domains of Vrp1 are required to rescue the Vrp1<sup>WH2F06715</sup> mutant phenotype we have investigated the importance of the different domains of Vrp1 in Drosophila, and find that only the WASP-binding domain is required for muscle fusion, while the actin binding domains are dispensable. These findings are contradictory to results previously published by Kim et al. 2007, who reported that the WH2 domains were required for rescuing the solitary mutant phenotype [20]. Our results indicate that the Vrp1-WASP interaction is critical in muscle fusion. However, the effects on the organization of the actin cytoskeleton, caused by Vrp1 expression in PAE cells, indicate that all conserved domains have actin cytoskeleton modulating properties, suggesting that the WH2 domains may be of importance in other contexts than myoblast fusion. Two additional proteins - Wasp and Scar - are nucleation promoting factors that act in parallel to activate the Arp2/3 complex, and mutants for the genes that encode these proteins display similar SM fusion phenotypes as the Vrp1<sup>WH2F06715</sup> mutant [26], indicating that many members of the Scar-Wasp signaling network work together to regulate myoblast fusion. We have analyzed VM fusion in additional single and double mutants for some of the components in this pathway; kette, wasp, and arp3-wasp, and observed that these mutants also develop a gut, suggesting that either VM fusion takes place in these mutants as in Vrp1<sup>WH2F06715</sup>, or that the VM manages to develop normally despite fusion blockage. Interestingly accumulation of Duf/Kirre is observed in all examined mutants of the Scar-Wasp signaling network.

Taken together, we suggest that VM fusion is initiated in mutants of components in the Scar-Wasp signaling network, and that these molecules are involved in an increased efficiency of the fusion process.

In addition to the Arp2/3 complex, other molecular pathways are able to nucleate actin. These include proteins such as formins, Spire and Cordon-bleu. Molecules of these protein families are structurally different to the Arp2/3 complex and produce linear instead of branched actin filaments. (discussed in Campellone and Welch 2010 [53], and Aspenstöm 2010 [54]). Spire and several formins, including Diaphanous and Cappuccino, have
been identified in *Drosophila*, were they have been associated with cellular processes such as vesicle transport and actin-microtubule interactions [53], but not yet with muscle development. Thus, loss of Arp2/3 function does not inhibit all actin polymerization in the cell, although the strong SM phenotypes observed in different Scar-Wasp signaling pathway mutants suggests that the Arp2/3 complex is an important actin nucleator in muscles. Our data suggests that actin polymerization by the Arp2/3 complex pathway is not required for VM fusion. Whether additional modes of actin assembly contribute to VM fusion is an interesting prospect and remains to be further investigated.

Duf/Kirre, together with Sns, is important for myoblast fusion in both the VM and the SM, as these immunoglobulin receptors facilitate attachment between FCs and FCMs, and therefore a mislocalisation of this molecule suggests that the process of fusion does not proceed in the normal fashion. We observe that Duf/Kirre protein is not downregulated in the VM of *Vrp1* f06715 mutants, possibly reflecting a stalled or inefficient fusion process. However, a recognizable embryonic gut is developed despite this phenotype, and the longitudinal muscles of *Vrp1* f06715 mutants appear morphologically wild type, suggesting that fusion defects do not affect VM development. Interestingly, we also observed a significant accumulation of Duf/Kirre protein in the SM of the analyzed *Vrp1* f06715 mutants, strengthening the hypothesis that this particular phenotype is the result of an inability of myoblasts to fuse properly. Accumulation of Duf/Kirre in the SM has previously been reported and suggested to reflect an imbalance in Duf/Rols signaling during fusion [33], a conclusion that is supported by recent study investigating Duf/Kirre signaling in myoblast fusion efficiency [55]. Our findings in the VM of *Vrp1* mutants, together with our and others reports in the SM [20-22,26] indicate that Vrp1 and components of the Scar-Wasp signaling network are also important for fusion efficiency. Ultrastructural analysis with electron microscopy has shown that SM cell fusion is a process of many steps, including the adherence of the myoblasts to each other, the appearance of vesicles and elongated plaques on both sides of the plasma membranes, the formation of fusion pores which lead to mixing of cell content, and then an anticipated enlargement of the pores as the plasma membranes are broken down, which finally results in complete fusion of the two cells [48]. The Duf/Kirre accumulation in the mutants examined in this study may reflect an inability of fusing cells to proceed through all the above described fusion steps, resulting in an incomplete or stalled fusion event. This would still produce an obvious fusion defective phenotype in the SM, but appears to have little effect in the embryonic VM. Clearly, it remains to be investigated whether loss of Vrp1 results in later developmental defects.

As a result of our experiments investigating Vrp1 function in the VM we conclude that Vrp1 is not absolutely required for muscle cell fusion in *in vivo*. This is evidenced by the fact that *Alk10-Vrp1* double mutant FCMs originating from the VM are clearly capable of fusing with FCMs of the SM. Naturally, one major difference between the fusion process in the VM and the fusion process in the SM, is that in the VM one FC fuses with only one FCM, whereas in the SM one FC per myotube fuses with up to 25 FCMs to form much larger muscle syncytia. It is possible that the many fusion events that take place in the SM require significantly more efficient actin rearrangement machinery than the few fusion events in the VM, and this would then explain why the fusion phenotypes that are caused by Vrp1, scar, wasp and arp3 disruption are more visible in the SM than in the VM. It follows that evaluation of VM developmental defects will be difficult given current markers, and that study of the VM during larval stages will provide insight. Thus, although the VM of the *Vrp1* f06715 mutant displays only minor defects at embryonic stages, the gut may be non-functional as the animal develops further. Unfortunately, at present time we are unable to test the functionality of the mutant larval gut since the Vrp1 mutation causes an embryonic lethal phenotype precluding an investigation of the mutant larval gut. For this, a SM specific tissue rescue would be required, something which is currently not possible. Future development of tools to allow investigation of the function of Vrp1, and indeed other molecules, in the *Drosophila* visceral muscle at later stages must now be a priority for analyzing the gut muscle specific function of Vrp1 *in vivo*.

**Methods**

**Fly strains**

Standard *Drosophila* husbandry procedures were followed. The following stocks were used: w1118, referred to as WT in Figures and text (Bloomington, stock number 5905), *Df(2R)ED3943* (Bloomington, stock number 9158), *P(Tub-PBac \(\setminus T\))2/wg^{Sp-1}* (Bloomington, stock number 8285), *rp298lacZ* [31], *Vrp1 f06715* (Exelixis Collection at the Harvard Medical School [43]), *sns20* [33], referred to as *sns* in Figures and text [13], *twistp-GFP-actin* [25], *UAS-LacZ*, *5053-GAL4* [56], *kette* [34], *Arp3schwächling wasp^{3D3-035}* referred to as *Arp3-WASP* in text [26], *wasp^{3D3-035}* [35], *Alk10* [45], *Sns-GAL4* [41]. Transgenic fly strains: *UAS-Vrp1^{full length}* , *UAS-Vrp1^{ΔW1H2}* , *UAS-Vrp1^{ΔProΔWASP}* and *UAS-Vrp1^{ΔWASP}* were generated as described below.

**Crosses**

*P(Tub-PBac \(\setminus T\))2/wg^{Sp-1}* flies were crossed to *Vrp1 f06715* flies to induce expression of piggyBac transposase, in order to remodelize the WH^{106715} element. To drive
LacZ expression in the longitudinal muscles of Vrp1Δ86715 mutant as well as heterozygous controls, flies with the genotype Vrp1-UAS: lacZ/CyO\textit{Wg}LacZ were crossed to flies with the genotype Vrp1/CyO\textit{Wg}LacZ; S053-GAL4. For studies of migration and fusion of VM cells in the SM, fly strains with the genotype Alk10-Vrp1/CyO\textit{Wg}LacZ were generated as well as flies with the genotype rp298lacZ:Alk10-Vrp1/CyO\textit{Wg}LacZ. For rescue experiments flies of the genotype Vrp1Δ86715/ CyO\textit{Wg}LacZ; UAS-Vrp1 transgene (all four UAS-transgenes, Figure 8A) were crossed with flies of the genotype Vrp1Δ86715; snS-GAL4/CyO\textit{Wg}LacZ, and in the case of rescue of lethality straight winged flies were counted. For studies of actin expression in muscles a \textit{twistp-GFP-actin}-Vrp1Δ86715 fly strain was generated via recombination.

**Generation of Vrp1 transgenic constructs**

The Vrp1 cDNA clone GH25793 (Drosophila Genomics Resource Center) was used as a PCR template to generate four different myc tagged Vrp1 transgenic constructs; Vrp1 \textit{full length} (2250 bp), Vrp1 2X\textit{WH2} (1830 bp), Vrp1 Δ\textit{ProΔWBD} (450 bp) and Vrp1 Δ\textit{WBD} (2140 bp). The primers added a BamHI restriction site to the 5' end of the PCR product and a \\textit{Xhol} restriction site and a myc sequence to the 3' end. Primers for Vrp1 \textit{full length} were: 5’ primer: GGA TCC GCC ATG CCT ATT CCG CCA CCC CCG GGA, 3’ primer: CTC GAG CTA CAG ATC TTC AGA GAT GAG TTT CTG CTC CAT ACC ATT GGT GCC CTG TAA. Primers for Vrp1 Δ\textit{WH2} were; 5’ primer: GGA TCC GCC GCC ATG ACA ACG AAC TCA TCC GCT CAG, 3’ primer: CTC GAG CTA CAG ATC TTC AGA GAT GAG TTT CTG CTC CAT ACC ATT GGT GCC CTG TAA. Primers for Vrp1 Δ\textit{ProΔWBD} were; 5’ primer: GGA TCC GCC ATG CCT ATT CCG CCA CCC CCG GGA, 3’ primer: CTC GAG CTA CAG ATC TTC AGA GAT GAG TTT CTG CTC GTG CCG CTG CAA GTG. Primers for Vrp1 Δ\textit{WBD} were; 5’ primer: GGA TCC GCC ATG CCT ATT CCG CCA CCC CCG GGA, 3’ primer: CTC GAG CTA CAG ATC TTC AGA GAT GAG TTT CTG CTC GTG CCG CTG CAA GTG. Primers for Vrp1 Δ\textit{WBD} were; 5’ primer: GGA TCC GCC ATG CCT ATT CCG CCA CCC CCG GGA, 3’ primer: CTC GAG CTA CAG ATC TTC AGA GAT GAG TTT CTG CTC GTG CCG CTG CAA GTG. Standard PCR programs were used to amplify DNA fragments. PCR products were then digested with BamHI and \textit{Xhol} and subcloned into the p\textit{UAST} plasmid [57] and pc\textit{DNA3} (Invitrogen), and the resulting constructs were confirmed by DNA sequencing prior to injection and generation of transgenic fly strains (BestGene Inc).

**Embryo Immunostainings and in situ hybridization**

Unless otherwise stated, embryos were collected, fixed and immunostained as described previously [58], prior to dehydration and mounting in methylsalicylate on glass slides for analysis. The following primary antibodies were used: Rabbit anti-β3 Tubulin (1:5000) [59], guinea pig anti- β3 Tubulin (1:10 000) [59], rabbit anti-βGal (1:150, Cappel), mouse anti-βGal (1:1000, Promega), mouse anti-Mef2 (1:500, gift from B. Paterson), rabbit anti-Alk (1:1000)[45], guinea pig anti-Alk (1:1000)[5], mouse anti-FasIII (1:50, Developmental Studies Hybri dyoma Bank), rabbit anti-Duf/Kirre (1:300)[60]. Guinea pig anti-Vrp1 was generated by injection of guinea pigs with recombinant HIS-tagged protein corresponding to residues 837-936 of Vrp1 in p\textit{ETM11} [61]. The resulting guinea pig antiserum (Medprobe) was IgG-purified on a Protein A column (Pierce) prior to use at 1:1000 for immunostaining. Fluorescent secondary antibodies employed were: goat anti-rabbit Cy3 (1:1000, Amersham), goat anti-mouse Cy3 (1:1000, Jackson), donkey anti-guinea pig Cy3 (1:200, Jackson), goat anti-rabbit Cy2 (1:1000, Amersham), goat anti-mouse Cy2 (1:1000, Amersham), donkey anti-guinea pig Cy2 (1:1000, Jackson), donkey anti-rabbit Cy5 (1:200, Jackson), donkey anti-mouse Cy5 (1:200, Jackson), donkey anti-guinea pig Cy5 (1:400, Jackson). For \textit{in situ} hybridization a digoxigenin-labelled RNA probe was made using cDNA encoding Vrp1 and a DIG RNA labelling kit (Roche). \textit{In situ} hybridization of whole-mount wild type \textit{Drosophila} embryos was carried out as described [62].

**Cell line experiments**

Porcine aortic endothelial (PAE) cells were cultured in Ham’s F12 medium, Supplemented with 10% FBS and penicillin/streptomycin at 37°C in an atmosphere of 5% CO2. For immunostaining experiments, the cells were seeded on coverslips and transiently transfected by Lipofectamine (Invitrogen Life Technologies) employing the protocol provided by the manufacturer. Twenty hours post-transfection, the cells were fixed in 3% paraformaldehyde in phosphate buffered saline (PBS) for 20 minutes at 37°C and washed with PBS. The cells were thereafter permeabilized in 0.2% Triton X-100 in PBS for 5 minutes, washed again in PBS and incubated in 5% FBS in PBS for 30 minutes at room temperature. To visualize filamentous actin, cells were incubated with tetramethyl rhodamine isothiocyanate (TRITC)-conjugated phalloidin (Sigma) diluted in 5% PBS in PBS for 30 minutes at room temperature. The coverslips were washed in PBS and mounted on object slides by the use of Fluoromount-G (Southern Biological Associates). Cells were photographed by a Hamamatsu ORCA CCD digital camera employing the QED Imaging System software using a Zeiss Axioplan2 microscope. Thick bundles, actin dots and stress fibers were quantified manually in microscope by calculating the percentage of transfected PAE cells displaying these structures or cells displaying extensive loss of...
stress fibers (see legends to Figure 8). All samples were analyzed blind.

**Additional material**

Additional file 1: Supplemental Figure 1; Comparison of SM phenotypes between the Vrp1Δf06715 and WIPΔD30. Comparison of SM phenotypes between the Vrp1Δ f06715 and WIPΔ D30 mutants reveals a similar degree of myoblast fusion defects in both mutants. Somatic embryonic muscles are stained with β3-Tubulin antibodies. (A) Wild type embryo, (B) Vrp1Δ f06715, (C) D-WIPΔD30, (D) Vrp1Δ f06715/D-WIPΔD30 transheterozygotes.

Additional file 2: Supplemental Figure 2; Rescue experiments of Vrp1Δf06715 mutant embryos with different Vrp1 constructs, and description of mutant phenotypes observed in PAE cells upon expression of the different Vrp1 constructs. Rescue of the Vrp1Δ f06715 embryonic mutant phenotype performed with different Vrp1 constructs as described in Figure 2. UAS-Vrp1Δ f06715 and UAS-Vrp1ΔD30 are both able to fully rescue the SM fusion phenotype of the Vrp1Δ f06715 mutant when expressed with the Sns-G4H driver, while UAS-Vrp1ΔVBD and UAS-Vrp1ΔProABD are not. A representative embryo from each cross is shown. Unfused cells are indicated by arrows. (A) Vrp1Δ f06715; Sns > > Vrp1Δ f06715; (B) Vrp1Δ f06715; Sns > > Vrp1Δ f06715; (C) Vrp1Δ f06715; Sns > > UAS-Vrp1ΔProABD; (D) Vrp1Δ f06715; Sns > > UAS-Vrp1ΔVBD. (E) The white arrow indicates normal stress fibers (SF). Non transfected PAE cells contain numerous stress fibers in contrast to cells that ectopically express full length Vrp1. The Vrp1-expressing cells undergo a very characteristic reorganization of the actin filament system; the cells appear almost empty of the bulk filamentous actin, apart from few and thick bundles of actin filaments and a formation of focal points of actin, so called actin dots. Red arrows indicate the presence of thick bundles and actin dots, as well as stress fiber loss (SF loss).

**List of abbreviations**

Arp2/3: Actin-related protein 2 and 3; ALK: Anaplastic lymphoma kinase; lacZ: β-galactosidase; Duf/Nire: Dumbfloundered/Kin of irle; Fasll: Fasciclin III; Fcs: founder cells; FCMs: fusion competent myoblasts; PAE: paralogue of A4; SNS: Somatic mesoderm; Vrp1: Verprolin; VCA: Verprolin-homology, coiled-homology, and highly acidic; VM: visceral mesoderm; Wip: Wasp interacting protein; Wisp: Wiskott–Aldrich syndrome protein.

**Authors’ contributions**

TE and RHP designed the study, TE carried out the genetic and molecular characterization, PA carried out the PAE cell experiments and GV performed TE and RHP designed the study, TE carried out the genetic and molecular characterization, PA carried out the PAE cell experiments and GV performed in situ hybridization analysis TE, PA and RHP wrote the manuscript. All authors read and approved the final manuscript.

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