kette and *blown fuse* interact genetically during the second fusion step of myogenesis in *Drosophila*

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Summary

***Drosophila* myoblast fusion proceeds in two steps. The first one gives rise to small syncytia, the muscle precursor cells, which then recruit further fusion competent myoblasts to reach the final muscle size. We have identified Kette as an essential component for myoblast fusion. In *kette* mutants, founder cells and fusion-competent myoblasts are determined correctly and overcome the very first fusion. But then, at the precursor cell stage, fusion is interrupted. At the ultrastructural level, fusion is characterised by cell-cell recognition, alignment, formation of prefusion complexes, electron dense plaques and membrane breakdown. In *kette* mutants, electron dense plaques of aberrant length accumulate and fusion is interrupted owing to a complete failure of membrane breakdown. Furthermore, we show that *kette* interacts genetically with *blown fuse* (*blow*) which is known to be required to proceed from prefusion complexes to the formation of the electron dense plaques. Interestingly, a surplus of Kette can replace Blow function during myogenesis. We propose a model in which Dumbfounded/Sticks and stones-dependent cell adhesion is mediated over Rolling Pebbles, Myoblast city, Crk, Blown fuse and Kette, and thus induces membrane fusion.**

Key words: Myoblast fusion, Attachment, Prefusion complex, Electron dense plaque *kette, blow, rols, sns, mbc, crk*

Introduction

In higher animals, skeletal muscles consist of bundles of syncytial myotubes, whereas in *Drosophila* larvae and in adult flies, a single syncytial myotube makes up one muscle. The multi-nucleated larval myotubes arise by myoblast fusion during embryogenesis. In adult birds and mammals, this process can be reactivated for repair following injuries or diseases because of the presence of myogenic stem cells – the so-called satellite cells – which can be induced to divide and subsequently fuse to the existing myotubes for regeneration. Similarly, myoblast fusion is reactivated in holometabolic insects during metamorphosis. The evolutionary conservation of the molecular players that control myoblast fusion in *Drosophila* may thus also provide insights into mammalian myogenesis.

Drosophila is an appropriate model system with which to study the basic mechanism of myoblast fusion, because myogenesis is completed within a few hours during embryogenesis (Bate, 1993) and can be analysed genetically (Dworak and Sink, 2002). In wild-type embryos, myoblast fusion starts at late stage 11 by the formation of the founder cells, which first fuse with two to three fusion-competent myoblasts (FCMs) to generate the precursor cells (Baylies et al., 1998; Frasch and Leptin, 2000; Paululat et al., 1999a). Until stage 14-15, these precursor cells then recruit additional FCMs until the desired muscle size is reached. At stage 16, muscles are correctly inserted into their epidermal attachment sites (Fig. 1A,D). Ultrastructural analyses revealed a number of characteristic steps during later myoblast fusion (Doberstein et al., 1997). Following cell-cell adhesion and alignment, vesicles are transported to the opposing membranes of precursor and fusion competent myoblasts. Here, they form the so-called prefusion complex, which is followed by electron-dense plaques and subsequent membrane breakdown (Doberstein et al., 1997).

Mutants with defects in myogenesis give an entry point into the functional analysis of fusion (Dworak and Sink, 2002; Paululat et al., 1999b; Taylor, 2002). Founder cells prefigure the muscle pattern. The individual founder cells express distinct transcription factors, e.g. Even skipped (Eve) or Krüppel (Kr) (Baylies et al., 1998; Ruiz-Gomez et al., 1997). After myoblast fusion, all nuclei of the resulting syncytia express these factors. In founder cells, the Ig-domain protein Dumbfounded/Kin of Irre (Duf/Kirre) acts in functional redundancy with Roughest (Rst) as a chemo-attractant for the fusion-competent myoblasts. Binding to the Ig-domain protein Sticks and stones (Sns), which is expressed by the fusion-competent myoblasts, then leads to cell adhesion (Bour et al., 2000; Ruiz-Gomez et al., 2000;

Strünkelnberg et al., 2001). Subsequently, the second fusion series requires the activity of Rolling Pebbles/Antisocial (Rols/Ants) in the precursor cell (Rau et al., 2001). Rols/Ants accumulates at the cell membranes during fusion (Menon and Chia, 2001). Moreover, there is biochemical evidence that Rols/Ants interacts directly with the intracellular domain of Duf/Kirre and thereby mediates a signal to the precursor to proceed in fusion (Chen and Olson, 2001). Additional cytoplasmic proteins such as Myoblast City (Mbc), the *Drosophila* homologue of Dock180 (Erickson et al., 1997; Nolan et al., 1998) and Blown Fuse (Blow) (Doberstein et al., 1997) are required in both founder/precursor cells as well as in fusion-competent myoblasts.

In order to identify further components for myoblast fusion, we screened EMS-induced mutations and such identified *kette* mutants displaying a strong muscle fusion defect. Kette [also called Hem (FlyBase), Nap1 and GEX-3] is well conserved during evolution (Baumgartner et al., 1995; Soto et al., 2002; Yamamoto et al., 2001). Mutations in *kette* were first identified based on their embryonic CNS phenotype, which is based on defects in neurite outgrowth (Hummel et al., 1999; Hummel et al., 2000). Subsequently, it was shown that *kette* affects the formation of the F-actin cytoskeleton, presumably by regulating the activity of two main regulators of F-actin nucleation Wasp and Wave (Scar – FlyBase) (Bogdan and Klämbt, 2003; Kunda et al., 2003; Rogers et al., 2003). Both Wasp and Wave are potent activators of the Arp2/3 complex, and their activity must thus be tightly controlled (Miki and Takenawa, 2003). Whereas Wasp is autoinhibited, inhibition of Wave function requires transacting factors. In vivo, Kette is found in a large cytosolic protein complex also comprising Sra-1 (also called PIR121, CYFIP), Abi, HSPC300 and Wave (Eden et al., 2002). Upon dissociation of this complex by binding to SH3 domains or to activated Rac1, Wave is released from the complex and is rendered active (Eden et al., 2002). Thus, in the cytosol Kette keeps Wave in an inactive state. By contrast, genetic data suggest that at the membrane Kette can activate Wasp function (Bogdan and Klämbt, 2003). In addition to these relatively ubiquitous acting proteins, regulation of F-actin dynamics is expected to involve cell type specific factors.

We have characterised the muscle phenotype of *kette* mutants and show that Kette is required for myoblast fusion. Further genetic and phenotypic analyses show that *kette* interacts with the mesoderm specific expressed gene *blow*. This interaction is essential for the correct formation of electron-dense plaques and the initiation of membrane breakdown.

Materials and methods

Drosophila stocks

The following strains were used for the analysis of the *kette*-phenotype: $kette^{C3\text{-}20}/TM3$, $kette^{J4\text{-}48}/TM3$, $kette^{G1\text{-}37}/TM3$, $kette^{J1\text{-}70}/TM3$ and $kette^{P168}/TM3$ (all Hummel et al., 2000). The enhancer trap insertion *rP298* (Nose et al., 1998) into the *duf* gene was used to label muscle founder cells. Kette was overexpressed in the mesoderm using the SG24 and TGX *twi*-Gal4 driver lines (gift of A. Michelson) and UAS-Hem transgenic flies (Hummel et al., 2000). As a wild-type control, we used $white^{1118}$ flies. $blow^2/CyO$ flies (Doberstein et al., 1997) were rebalanced with If/CyO_{hg} (provided by Markus Affolter) to $blow^2/CyO_{hg}$. From this strain, *blow* mutant fly lines carrying the *rP298* insertion were established. mbc^{C1} mutant flies (Rushton et al., 1995) were obtained from the Bloomington Stock Center.

Immunohistological staining and whole-mount in situ hybridisation

For immunohistological staining, the following antibodies were used: anti-β3-Tubulin (polyclonal from rabbit) (Leiss et al., 1988; Buttgereit et al., 1996), anti-β3-Tubulin (polyclonal from guinea-pig) (D. Buttgereit and R.R.-P., unpublished) anti-Kette (97/82) (Bogdan and Klämbt, 2003), anti-β-galactosidase (polyclonal, Biotrend), anti-Eve (from Developmental Studies Hybridoma Bank), anti-Kr (Kosman et al., 1998) and anti-Alien (Goubeaud et al., 1996). Embryos were fixed and stained as described previously (Stute et al., 2004). As detection system we used Vectastain ABC Elite-kit (Vector Laboratories) and the TSA-kit from Perkin Elmer. The application of the TSA-kit is essential to visualise Kette. Fluorescent secondary antibodies were obtained from Dianova.

Whole-mount in situ hybridisation was carried out essentially as described by Tautz and Pfeifle (Tautz and Pfeifle, 1989). DIG-labelled RNA antisense probes were synthesised by in vitro transcription using a RNA-DIG-labelling-Kit (Roche) and a *sns* cDNA clone in pBSKII kindly provided by Susan Abmayr (Bour et al., 2000). Analysis was performed with a Leica confocal laserscan microscope.

Electron microscopy

Embryos at different developmental stages were dechorionised by bleaching, prefixed in 18% glutaraldehyde/heptan 1:1 solution and mechanically peeled. Mutant embryos were selected using an immunohistochemical β-galactosidase staining which is restricted to the balancer carrying embryos while homozygous *kette* mutants lack *lacZ* activity (Stollewerk et al., 1996). The embryos were fixed by simultaneous glutaraldehyde/osmiumtetroxide-fixation with postosmication (Franke et al., 1969). Afterwards, embryos were stained en bloc with uranylacetate (Lin et al., 1994), dehydrated and embedded in Spurr's resin. After polymerisation semi- and ultra-thin sections were cut. For analyses in the TEM (Hitachi) ultra-thin sections were contrasted with lead citrate.

Results

Searching for further components that are involved in myoblast fusion, we screened a collection of EMS mutants from Hummel et al. (Hummel et al., 1999) using the anti-β3-Tubulin antibody which detects β3-Tubulin in myoblasts as well as in mature myotubes (Buttgereit et al., 1996; Leiss et al., 1988). The screen of the third chromosome revealed five alleles of *kette* that display strong fusion defects: $kette^{C3\text{-}20}$, $kette^{J4\text{-}48}$, $kette^{G1\text{-}37}$, $kette^{P168}$ and $kette^{J1\text{-}70}$. Four of these alleles were characterised at the molecular level: $kette^{C3\text{-}20}$ and $kette^{J4\text{-}48}$ are null mutants, while $kette^{J1\text{-}70}$ and $kette^{G1\text{-}37}$ are hypomorphic alleles (Hummel et al., 2000).

We first analysed the myoblast fusion phenotypes with regard to cell determination and ultrastructure, and then placed Kette within the described fusion cascade in relation to the other known fusion relevant proteins.

kette mutants are characterised by severe distortions in myoblast fusion

In *kette* mutants, we observe strong disturbances of myogenesis. Null mutants show many unfused myoblasts until stage 16, which cluster close to stretched minimuscles, presumably representing founder or precursor cells (Fig. 1B,E compare with wild type in 1A,D). In addition, the hypomorphic alleles show unfused myoblasts until stage 15, while at stage 16 hardly any unfused myoblasts are detectable (Fig. 1C,F). Apparently, in hypomorphic *kette* mutants fusion proceeds slower than in the wild type but muscles are eventually formed.

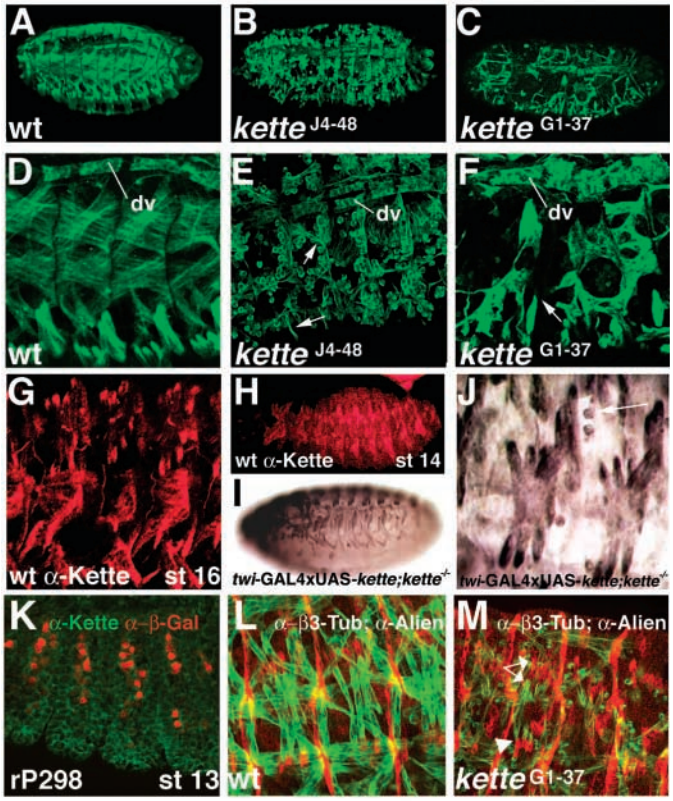

Fig. 1. Kette has a mesoderm intrinsic essential function for myoblast fusion. *kette* mutants show a strong defect in muscle fusion that is due to an intrinsic mesodermal function of Kette. (A-F) Anti-β3-Tubulin fluorescent staining of stage 16 embryos shows the myogenic defects of *kette* mutants. (A) Wild-type muscle pattern; (B) *kette*$^{J4-48}$ (null allele), which shows many unfused myoblasts, even at stage 16. (C) Hypomorphic allele *kette*$^{G1-37}$. (D-F) Detailed magnifications of A-C. The dorsal vessel (dv) is formed correctly in all *kette* mutants. (E) *kette*$^{J4-48}$ null mutant: mini-muscles are indicated by arrows. (F) *kette*$^{G1-37}$ stage 16 embryo: the unfused myoblasts have vanished. Large gaps in the muscle pattern and attachment defects (arrow) are visible. (G,H) Anti-Kette antibody staining on wild-type embryos shows the mesodermal expression of Kette. (G) In stage 16 embryos, the protein concentrates towards the muscle tips. (H) In stage 14 embryos, when muscle fusion takes place, Kette can be found in the whole somatic mesoderm. (I,J) Overexpression of Kette in the mesoderm in a *kette* mutant background with the help of a *twi*-GAL4 driver line rescues the *kette* phenotype. (I) Ventrolateral view of a rescued stage 16 embryo. (J) Higher magnification and lateral view of a rescued stage 16 embryo; only a few unfused myoblasts can be detected (arrow). (K) Anti-Kette (green) anti-β-galactosidase (red) double labelling of rP298-expressing stage 13 wild-type embryo. (L,M) Anti-β3-Tubulin and anti-Alien double labelling, monitoring the muscle attachment to the epidermis. (L) Wild-type stage 16 embryo with properly attached muscles. (M) Stage 16 *kette*$^{G1-37}$ mutant showing partly attached (arrowhead) and partly unattached (arrows) muscles. Unless otherwise stated, embryos in all figures are orientated with anterior towards the left.

The epidermal attachment, however, is often missing or incorrect (arrow in Fig. 1F). In order to visualise muscle attachments, we performed anti-Alien and anti-β3-Tubulin double labellings, as Alien allows the visualisation of attachment sites (Goubeaud et al., 1996). We could therefore observe that some muscles find their attachment sites, whereas others do not (Fig. 1L,M). This suggests that Kette plays a role in both myoblast fusion and muscle attachment.

We then asked at what level myogenesis was disturbed in *kette* mutants. As a first point, we excluded migration disturbances, which might affect the mesodermal cells: all *kette* mutant embryos showed correct dorsal closure that resulted in correct formation of the cardioblasts in the dorsal vessel (Fig. 1B,C,E,F). This indicates that after gastrulation, the mesodermal cells migrate correctly and that signalling from the epidermal cells to the underlying mesodermal cells can take place.

Second, we clarified whether Kette is expressed in the somatic mesoderm during myoblast fusion. We used the anti-Kette antibody (Bogdan and Klämbt, 2003) to determine whether the Kette protein is found in the mesoderm. As shown in Fig. 1H, Kette is detectable throughout the entire somatic mesoderm during the myogenic relevant stages. Double staining for Kette and β-galactosidase, visualising the founder cell marker rP298, in wild-type embryos clearly reveals that Kette is expressed in fusion-competent myoblasts as well as in founder/precursor cells (Fig. 1K). Thus, we propose that Kette fulfils an intrinsic function in the somatic mesoderm during myoblast fusion. Furthermore, in late stage 15 to stage 16, Kette accumulates at the tips of the mature myotubes that anchor them to the epidermis (Fig. 1G). This corresponds well to the incorrect attachment of muscles we observed in the hypomorphic alleles (Fig. 1M).

To support the idea that Kette fulfils an intrinsic function in the mesoderm, we employed the UAS-GAL4 system (Brand and Perrimon, 1993) by using a twi-GAL4 driver for expression of Kette in the mesoderm of *kette*$^{J4-48}$ mutant embryos. The strong fusion phenotype observed in *kette*$^{J4-48}$ mutants is almost completely rescued by mesodermal expression of Kette (Fig. 1I,J compare with Fig. 1B,E), which clearly demonstrates the intrinsic function of Kette in the somatic mesoderm. Overexpression of Kette in the wild-type background does not disturb muscle development (data not shown).

In summary, Kette is required for both myoblast fusion and muscle fibre insertion into the epidermis. Expression data and mesoderm-specific rescue experiments both suggest that Kette acts in the mesoderm.

Fusion-competent myoblasts and founder cells are correctly determined in *kette* mutants

In order to investigate whether the *kette* mutant phenotype is caused by abnormal myoblast fate determination or whether it is based on a specific defect in myoblast fusion, we first tested whether the two myogenic cell types, the founder cells and the fusion-competent myoblasts, are determined correctly in *kette*-null mutants.

Founder cell formation can be traced by analysis of *rP298-lacZ*, which is an enhancer trap that expresses β-galactosidase under the control of the Duf/Kirre regulatory region and thus allows to visualise all founder cells (Nose et al., 1998; Ruiz-Gomez et al., 2000). After fusion, each nucleus contains β-galactosidase and thus allows to monitor successful fusion. The comparison of *rP298* directed β-galactosidase expression in wild type (Fig. 2A,C) and *kette*$^{J4-48}$ mutant embryos clearly shows that at stage 13, founder cells are determined properly in time, in space and in number in *kette* loss-of-function

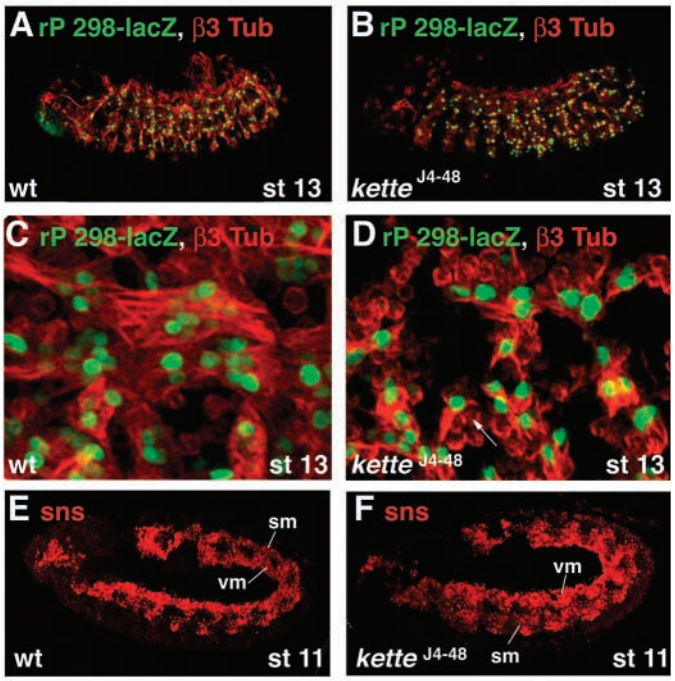

Fig. 2. Founders and fusion-competent myoblasts are determined in *kette*-null mutants. *kette* mutants were analysed for expression of the enhancer trap rP298-*lacZ* (A-D) and localisation of the sns-transcript (E,F) to examine the determination of founders and fusion-competent myoblasts. (A-D) Expression pattern of the rP298-*lacZ* enhancer trap (green), which resembles the founder cell marker *duf/kirre* expression pattern, shown by anti β-galactosidase fluorescent staining. β3-Tubulin fluorescent staining is red. (A) Stage 13 heterozygous wild-type embryo with TDLZ blue-balancer. (B) Stage 13 *kette*$^{J4-48}$-null mutant. (C) Late stage 13 wild-type embryos. After fusion, all nuclei of the syncytia express rP298. The forming dorsal muscles contain more than four nuclei and muscle structures is visible. (D) In stage 13 *kette*$^{J4-48}$-null mutants, founder cells are determined but no muscle structure are visible. Compared with wild type, less rP298-positive cells are detected that are surrounded by many fusion-competent β3-Tubulin-positive myoblasts (arrow). (E,F) Fluorescence in situ hybridisation with *sns*-antisense probe shows the correct determination of fusion-competent myoblasts in the somatic (sm) and visceral mesoderm (vm) of *kette* mutants. (E) Stage 11 wild-type embryo; (F) stage 11 *kette*$^{J4-48}$-null mutant embryo.

mutants (Fig. 2B,D). To monitor the *rP298*-positive nuclei relative to the forming muscles we used the anti β3-Tubulin antibody for counterstaining. In wild-type embryos the nuclei of the former FCMs become *rP298* positive after fusion with a founder/precursor cell. Therefore all nuclei of a forming muscle are *rP298* positive (Fig. 2C). *kette* mutants, however, show less *rP298*-positive nuclei, which are surrounded by many FCMs (Fig. 2D). This is indicative for fusion defects. The second essential cell population in the myogenic mesoderm is the pool of FCMs. We analysed *sns* expression in *kette*-null mutants and found that *sns* mRNA is expressed as in the wild type (Fig. 2E,F), suggesting that FCMs are correctly determined in *kette* mutants.

Altogether, these results show that Kette is required following determination of founder cells and fusion-competent myoblasts, indicating that it is directly involved in fusion.

Muscle precursors are established in *kette* and *blow* mutants

We previously proposed a two-step fusion model with a first step leading to precursor cells containing three or four nuclei, followed by a second step establishing the mature myotubes (Rau et al., 2001). Therefore, we aimed to clarify whether *kette* mutants arrest at the first or second fusion step. The expression of Eve and Krüppel indicates whether *kette* mutant embryos are able to perform the first fusion to precursor cells or are left with mononucleated founder cells.

Eve is expressed in the nuclei of dorsal muscle 1 (DA1) and in some pericardial cells. In stage 15-16 wild-type embryos, DA1 contains up to 14 nuclei (Ruiz-Gomez et al., 1997), while in *kette*$^{J4-48}$ mutant embryos, only minimuscles with three or four nuclei are formed (Fig. 3B).

The same proved to be true when we used Krüppel as a second founder cell marker that marks dorsal muscles DA1 and DO1. As we already observed for DA1 with anti Eve, we found Krüppel in three or four nuclei of minimuscles DA1 and DO1 in *kette*$^{J4-48}$ stage 13-14 embryos, but we could not observe any increase in Krüppel-positive nuclei at later stages (Fig. 3F). This corresponds to the wild type in stage 13 embryos, when the first fusion series is completed and precursors are formed (Fig. 3D). As *mbc* mutants stop at the first fusion step (Erickson et al., 1997) we used them as a control. As expected, single Krüppel-positive nuclei are observed in *mbc* mutants at stage 13, showing that myogenesis arrests at the founder cell stage (Fig. 3E). Taken together we could show that *kette* mutants undergo the first fusions to form precursor cells with three or four nuclei but stop afterwards.

Another gene that is known to regulate myoblast fusion is *blown fuse* (*blow*); *blow* mutants arrest fusion following the prefusion complex (Doberstein et al., 1997). To determine whether *blow* affects myogenesis at the first or the second fusion step, we analysed Eve and Krüppel distribution in *blow*-null mutants (*blow*^{2}). As in *kette* mutants, *blow*^{2} mutants reach the precursor cell stage and then fail to form mature myotubes and stop myogenesis during the second fusion step (Fig. 3C,G). The formation of precursor cells in *kette* mutants also proves true at the ultrastructural level. Fig. 4A shows a cluster of two or three nucleated precursor cells underlying the epidermis. Likewise in *blow*^{2} mutants we could confirm the formation of precursor cells (Fig. 4B), while in the *mbc* mutant embryos, only unfused mononucleated myoblasts are visible (Fig. 4C).

Ultrastructural analyses reveal that *kette* loss-of-function mutants progress fusion up to the plaque stage of the second fusion step

At the ultrastructural level, wild-type embryos clearly show that precursor cells with three or four nuclei and fusion-competent myoblasts have established contacts (Fig. 4D), electron dense vesicles start to form the perfusion complex (Fig. 4D, arrowhead, and higher magnification in E) (Doberstein et al., 1997). We observed that in the neighbourhood of this prefusion complex, electron-dense plaques start to be established within a cloud of electron-dense material that seems to result from the electron-dense vesicles of the prefusion complex (Fig. 4D, arrow, and magnification in F). Then, the membranes start to break down in the direct vicinity of the plaque, leading to the fusion of the precursor with the fusion-competent myoblast (Doberstein et al., 1997).

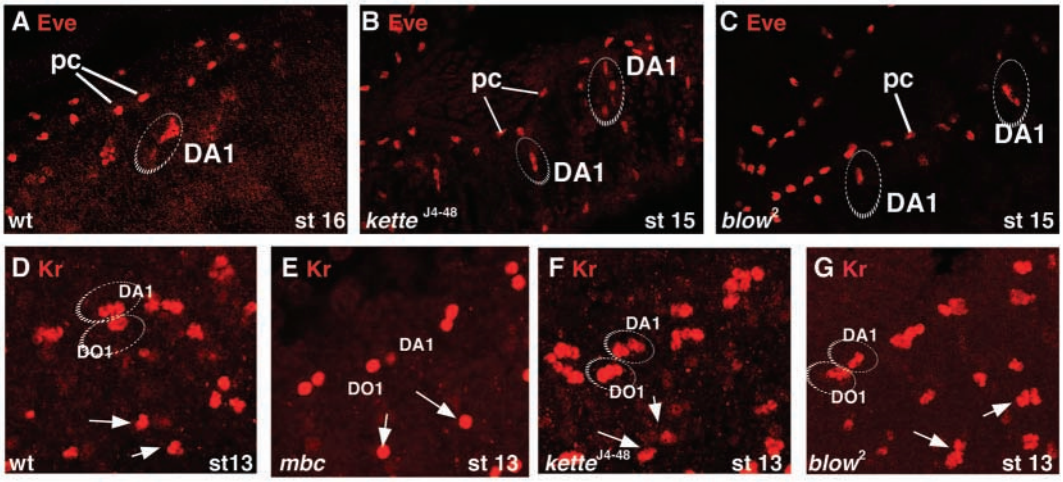

Fig. 3. *kette* and *blow* mutants form precursor cells with three or four nuclei. *kette* and *blow* mutants were analysed for founder/precursor cell status (using Eve and Kr) at (A-C) stage 15-16 and (D-G) stage 13. As a control for the Kr pattern of expression, we used *mbc* mutant embryos, which do not undergo any fusion step and arrest at the founder cell stage. It can be clearly seen that both *kette* and *blow* mutants build precursors that contain three or four nuclei, corresponding to what is seen in the wild type at stage 13. (A-C) Anti-Eve fluorescent staining in red (pc, pericardial cells). (A) Stage 16 wild-type embryo containing up to 14 Eve- and rP298-positive nuclei in DA1 muscle after fusion is completed. (B) Stage 15 *kette*$^{J4\text{-}48}$ null mutant embryo containing three or four Eve-positive nuclei in muscle DA1, corresponding to precursor cells. (C) Stage 15 *blow*^{2} mutant with two or three nuclei in DA1 muscle, which also indicates the presence of precursor cells. (D-G) Anti-Kr fluorescent staining. (D) Stage 13 wild-type embryo displays precursor cells of muscles DA1 and DO1 with three or four nuclei after the first fusion step occurs. (E) Stage 13 *mbc* mutant embryo does not undergo the first fusion step, indicated by the single *kr*-expressing cells that correspond to the founder cells of the muscles. (F) Stage 13 *kette*$^{J4\text{-}48}$-null mutant embryo with three or four nuclei in precursors of muscles DA1 and DO1. (G) Stage 13 *blow*^{2} mutant embryo with two or three nuclei in precursors of DA1 and DO1. Arrows in D-G indicate precursors of lateral muscles that are developing.

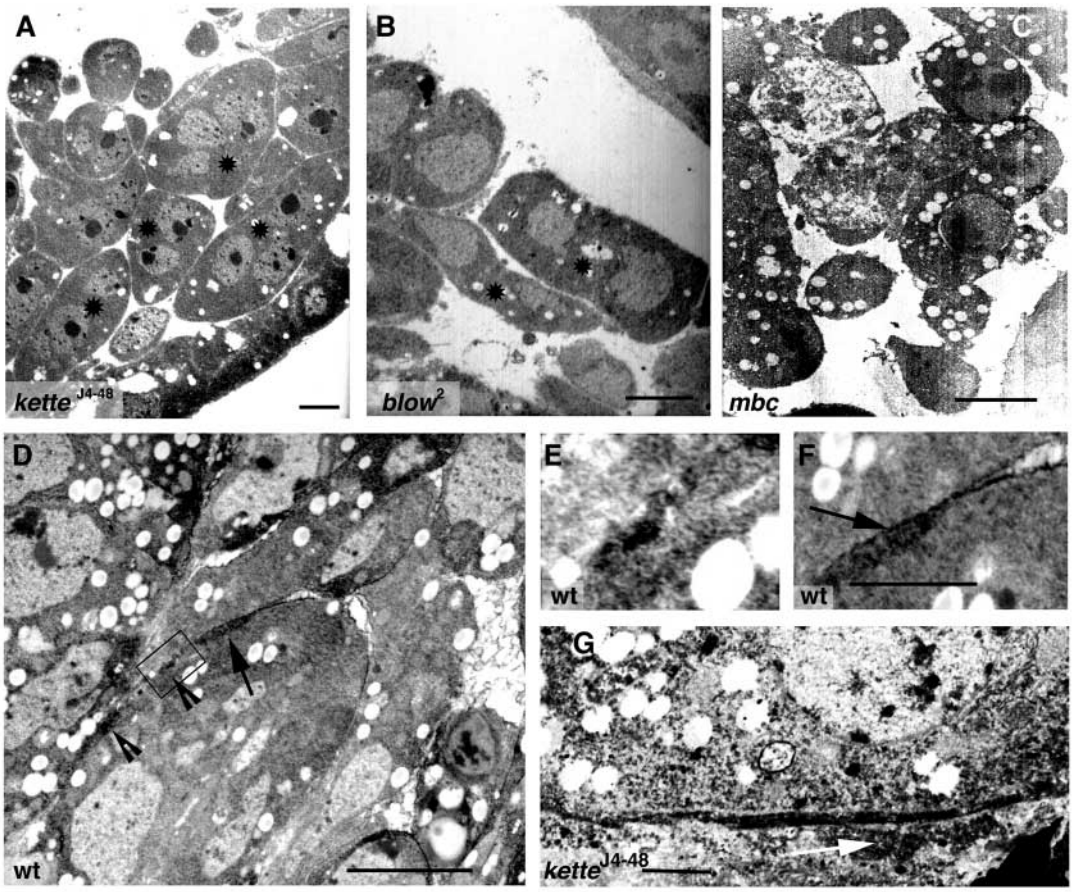

Fig. 4. *kette* mutants stop fusion during formation of electron-dense plaques. (A-C) Transmission electron microscope analyses of *kette*$^{J4\text{-}48}$, *blow*^{2} and *mbc* mutants confirm that *kette*$^{J4\text{-}48}$ and *blow*^{2} mutants do form muscle precursor cells, while *mbc* mutants do not. Scale bars: 2 μm. (A) Stage 14 *kette*$^{J4\text{-}48}$ mutant embryo; asterisks indicate precursors with two or three nuclei. (B) Stage 12-13 *blow*^{2} mutant embryo; developing precursors with two nuclei are clearly visible. (C) Stage 13-14 *mbc* mutant embryo. (D-F) Stage 13 wild-type embryo. (D) A muscle precursor has established contact with fusion-competent myoblasts, while groups of electron dense vesicles start to build the prefusion complex of paired vesicles (arrowheads). Nearby, a prefusion complex has already started to dissolve and will form a electron-dense plaque (arrow). Scale bar: 1.5 μm. (E,F) Detailed view of a group of electron dense vesicles in D. (F) Dissolving prefusion complex and developing electron dense-plaque (arrow) forming within a cloud of vesicles. Scale bar: 500 nm. (G) Developing electron-dense plaque in a stage 15 *kette*$^{J4\text{-}48}$ embryo. Remains of the dissolving prefusion complex are still visible (arrow); the length of the plaque is nearly twice that of the wild type plaque described by Doberstein et al. (Doberstein et al., 1997). Scale bar: 150 nm.

In *kette* mutants, fusion-competent myoblasts also attach to the precursors as in the wild type, and electron-dense material accumulates into plaques (Fig. 4G). In the wild type, the plaques are 500 nm in length. In *kette* mutants, however, the plaques are two to three times longer (Fig. 4G). There is no sign of membrane breakdown in the vicinity of the plaques. Instead, residues of the dissolving prefusion complex remain frequently visible until stage 15 (arrow in Fig. 4G).

Because, in contrast to *blow*, *kette* mutants still form electron-dense plaques, we postulate that Kette acts downstream of Blow.

kette interacts genetically with *blow* during myoblast fusion and is able to rescue the *blow* mutant phenotype

To test the relationship of *kette* and *blow* further, we performed epistasis experiments to detect possible genetic interactions. The hypomorphic *kette*$^{G1\text{-}37}$ allele (Fig. 5A) and *blow*^{2} loss-of-function mutants (Fig. 5B) can be distinguished in the light microscope by β3-Tubulin expression. We established balanced *Drosophila* strains carrying the hypomorphic *kette*$^{G1\text{-}37}$ mutation on the third chromosome and *blow*^{2} on the second chromosome. Loss of one copy of *blow* in a *kette*$^{G1\text{-}37}$ mutant background results in an enhanced fusion phenotype of *kette*$^{G1\text{-}37}$ mutants (Fig. 5C). This is even more prominent in homozygous double mutants (Fig. 5D). As the *blow* phenotype appears dominant, we conclude that Kette is acting later then Blow during myoblast fusion. This is confirmed by the phenotype of homozygous *blow*^{2} embryos lacking one copy of *kette*, which cannot be distinguished from homozygous *blow*^{2} mutants (data not shown). Owing to the fact that removal of one copy of the *blow* gene influences the phenotype of *kette*$^{G1\text{-}37}$ mutants, we propose that Kette and Blow interact during myoblast fusion.

We then asked whether Kette overexpression can rescue Blow function. When combining UAS-*kette* in a *blow*^{2} mutant background, we could observed that these embryos exhibit a partially rescued muscle pattern, although some muscles are missing (Fig. 5F, compare with *blow*^{2} phenotype in Fig. 5E). Although muscle orientation or attachment is sometimes disturbed in these rescued embryos, they are no longer embryonic lethal but survive until the first instar larval stage. This result confirms our idea that *kette* and *blow* interact genetically during myoblast fusion.

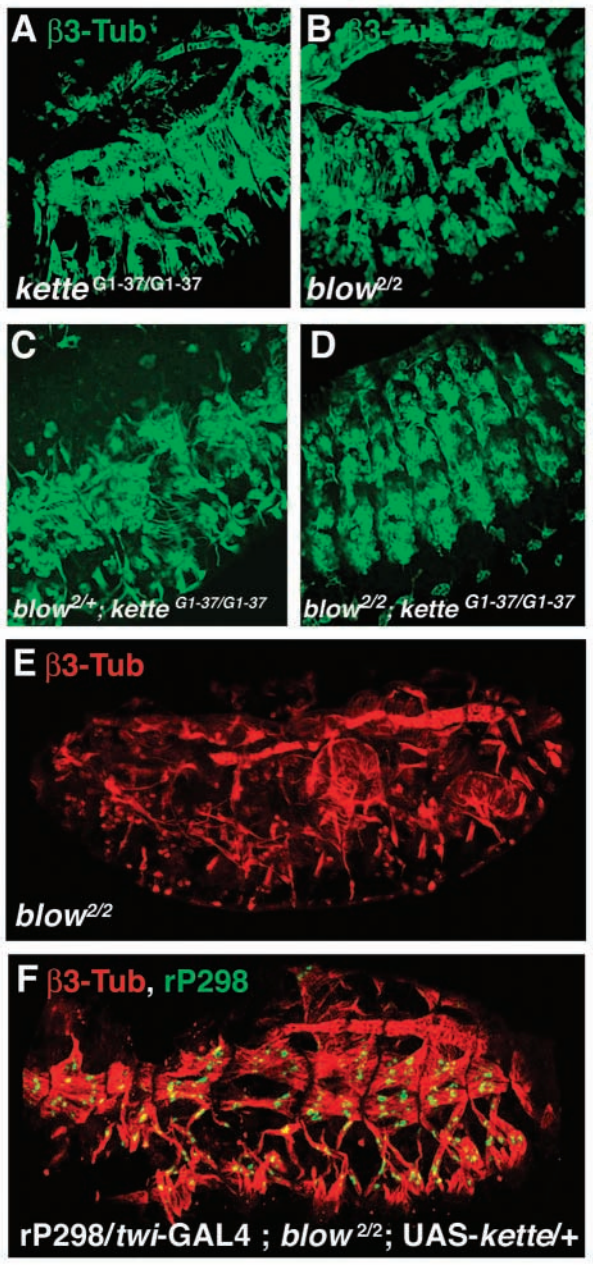

Fig. 5. Kette and Blown fuse interact genetically. (A-D) Anti-β3-Tubulin fluorescent staining of stage 15 embryos. (A) Homozygous *kette*$^{G1\text{-}37}$ hypomorphic mutant. (B) Homozygous *blow*^{2} null mutant. (C) Homozygous *kette*$^{G1\text{-}37}$ mutant with only one intact copy of *blow* (an enhancement of fusion defect takes place). (D) Double homozygous *blow*^{2} and *kette*$^{G1\text{-}37}$ mutant (further enhancement of fusion defects leads to a phenotype that resembles the original *blow*^{2} phenotype more than the *kette*$^{G1\text{-}37}$ phenotype). (E,F) Anti-β3-Tubulin fluorescent staining (red); anti-β-galactosidase staining (green) of rP298-*lacZ* enhancer trap. (E) Stage 16 *blow*^{2} mutant embryo (only minimuscles, presumably representing precursors, are formed, lareg gaps in the somatic mesoderm allow a direct view of the gut); β3-Tubulin-positive cardioblasts are properly arranged. At this stage, unfused myoblasts have been engulfed by macrophages. (F) Mesodermal overexpression of UAS-*hem* with a twist-GAL4 driver line in *blow*^{2} mutant background rescues the *blow* phenotype, at least partially, at stage 16. The embryo forms clearly fused and attached muscles, although defects in muscle number, size and attachment occur.

Discussion

Kette is essential in the second step of myoblast fusion

We describe a strong myoblast fusion phenotype in *kette* mutants. Null alleles of *kette* show numerous unfused myoblasts, while in hypomorphic alleles the fusion phenotype is less severe but defects in muscle attachment become obvious. These fusion defects are due to the intrinsic function of Kette in the myogenic mesoderm.

Furthermore, we show that founder cells and fusion-competent myoblasts are correctly determined in *kette* mutants and muscle precursor cells are properly formed during the first myoblast fusion step. Electron microscopic analysis of *kette* mutants revealed that the second myoblast fusion step is interrupted during formation of the electron-dense plaques and

thus *kette* mutants stop development shortly after *blow* but before *sns*^{15} mutants (Fig. 6).

Myoblast fusion requires intensive membrane rearrangements and thus an active modulation of the F-actin cytoskeleton, e.g. as seen during endocytosis (Engqvist-Goldstein and Drubin, 2003). In support of the idea that muscle development depends on a F-actin dynamics, myofibre atrophy is observed in the *Wave1* knockout mice (Dahl et al., 2003). Here, we show that the Wave regulator Kette is required for myoblast fusion in the fly embryo. Previous biochemical analyses showed that Kette fulfils a dual role in the regulation of the actin cytoskeleton. On the one hand, Kette promotes F-actin formation at the cell-membranes via Wasp (Bogdan and Klämbt, 2003); on the other hand, Kette inhibits Scar/Wave function in the cytosol (Bogdan and Klämbt, 2003; Kunda et al., 2003; Rogers et al., 2003).

In addition to a role of Kette during myoblast fusion, we noted high expression of Kette at the growing tips of mature myotubes. These structures are rich in F-actin and, like growth cones, migrate towards the muscle-attachment sites (Volk, 1999).

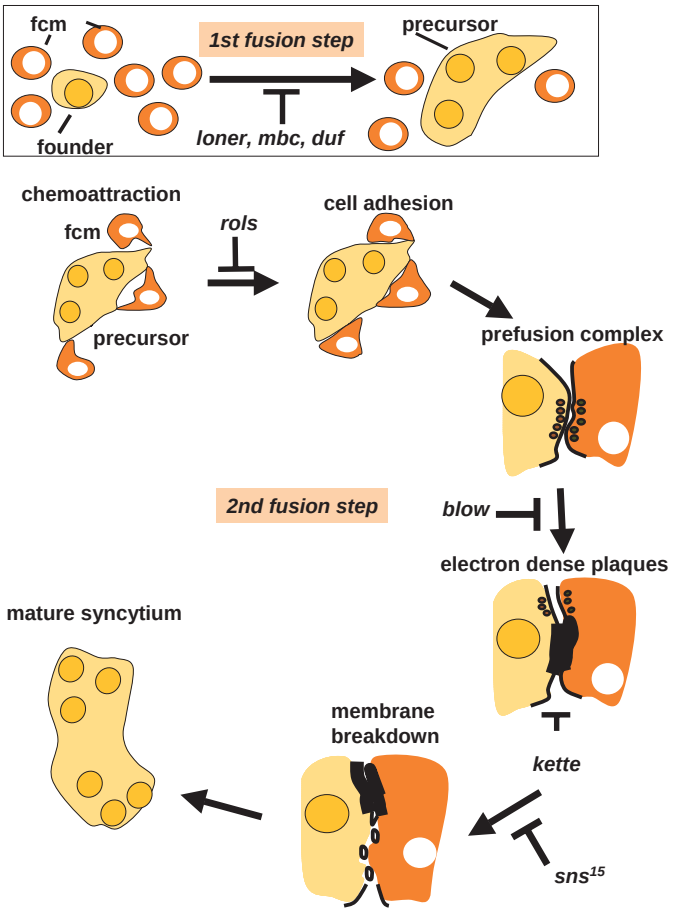

Fig. 6. Model of the two-step myoblast fusion process. The cell-cell recognition is mediated by Duf in the founder/precursor cells (yellow) and Sns in the fusion-competent myoblasts (orange). The first fusion step crucially depends on Mbc and Loner (Chen and Olson, 2003; Erickson et al., 1997). The second fusion step is characterised by formation of prefusion complexes, electron-dense plaques and membrane breakdown (Doberstein et al., 1997). This requires proper function of Rols, Blow and Kette. In this process, *blow*, *kette* and *sns*^{15} mutants are involved at different times. Kette acts during the formation of the electron-dense plaques shortly after Blow.

Integration of Kette in a model for myoblast fusion

Within the two-step model of myoblast fusion, we placed *kette* relative to other components of the fusion process (Fig. 6). The initial recognition between founder cells and fusion-competent myoblasts is mediated by the Ig-domain proteins Duf/Kirre and Rst in the founder cell. The extracellular domain of Duf/Kirre interacts with Sns, another member of the immunoglobulin superfamily, which is expressed in fusion competent myoblasts (Bour et al., 2000; Dworak and Sink, 2002). This interaction may signal into both cell types and thus initiate the first fusion step that leads to the formation of precursor cells.

It is possible that Duf/Kirre and Rst, as well as Sns, are also active in the second series of fusion events leading from the precursor cells to the mature myotubes. In the precursor cells, the Rols/Ants protein concentrates at the membrane (Chen et al., 2003; Menon and Chia, 2001) and we propose that Rols/Ants is needed to start the second series of fusion (Rau et al., 2001). Chen and Olson (Chen and Olson, 2001) have shown that in vitro Rols/Ants binds to the intracellular domain of Duf/Kirre, and we suggest that this might be the signal in the precursor cell that recruits further FCMs for fusion (Fig. 7). In the precursor cell, this interaction might initiate the formation of the prefusion complex and subsequently the formation of the electron-dense plaques and finally to membrane breakdown (Fig. 6).

We assume that Blow and Kette mediate the Duf/Kirre-Rols/Ants interaction signal in the precursor cell. Blow and Kette are also present in the fusion-competent cells, where we propose that Rols/Ants function is taken over by an, as yet, unidentified protein that interacts with Sns. In the precursor cell, Rols/Ants is proposed to mediate rearrangement of the cytoskeleton via Mbc/Dock180 (Chen et al., 2003; Erickson et al., 1997; Galleta et al., 1999). The electron-dense plaques and their connection to microfilaments are

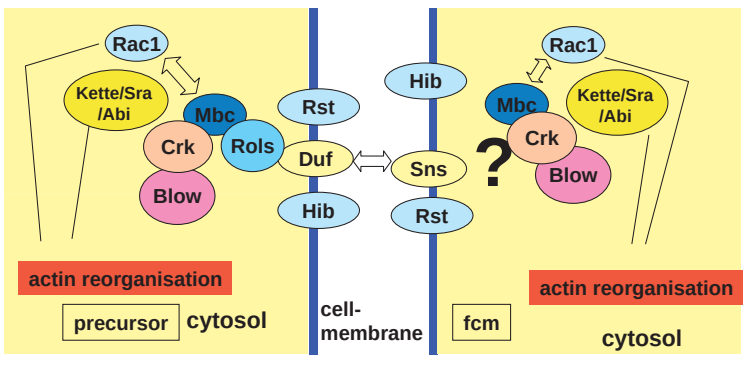

Fig. 7. Hypothesis for the function of Kette during the second myoblast fusion step compared with the known function of Kette during axonal outgrowth. We propose that the function of Dock/NCK, which mediates Kette function during neurogenesis (Bogdan and Klämbt, 2003), is taken over by Crk, which has also been shown to interact with Blow and Mbc, and to be involved in Rac1 activation and the formation of lamellipodia (see text for details).

symmetrical structures at opposing plasma membranes between precursor and fusion-competent myoblasts. We propose that the rearrangement of the actin filaments and their connection to electron-dense plaques is dependent on Kette and its interaction with Blow, as discussed in detail below.

Kette acts together with Blow in the second fusion step

The Blow protein is characterised by a pleckstrin homology (PH) domain that is often involved in mediation of membrane binding and in regulation of the cytoskeleton (Lemmon et al., 2002). As membrane association is important for Kette function (Bogdan and Klämbt, 2003), the observed genetic interaction may reflect a contribution of Blow in activating Kette. Interestingly, it has been recently reported that Blow binds to Crk (Giot et al., 2003), which in turn is able to associate with the Dock180 homolog Mbc (Galetta et al., 1999; Nolan et al., 1998). This adapter protein is proposed to link to the Duf/Kirre protein via Rols/Ants in precursor cells (Chen and Olson, 2001). We propose that a similar link between Sns and Mbc in the fusion-competent cells is mediated by a yet unidentified protein. This scenario would link the activation of membrane-bound receptors to the regulation of F-actin dynamics (Fig. 7).

The SH2-SH3 adaptor protein Crk has not yet been studied at the functional level in *Drosophila* but it is known from vertebrates that its orthologue CrkII and Dock180 form a complex after external stimulation (Hamasaki et al., 1996; Hasegawa et al., 1996; Klinghoffer et al., 1999; Lamorte et al., 2003; Li et al., 2002; Li et al., 2003; Ruest at al., 2001; Tachibana et al., 1997; Thomas et al., 1995; Vuori et al., 1996) and are able to promote Rac1 activation (Brugnera et al., 2002; Côté and Vuori, 2002; Guimienny et al., 2001; Kiyokawa et al., 1998). Rac1, in turn, is acting on the activation of Wave (Eden et al., 2002).

The interaction between Blow and Crk is supported by our finding of several potential binding motives in Blow that are described as potential recognition sites by both Crk-SH3-domains (Feller, 2001).

Therefore, we postulate that the function of Blow in myoblast fusion is dependent on its binding to Crk for which no mutants exist. We propose that this interaction leads to the activation of *kette*.

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