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WAVE/SCAR, a multifunctional complex coordinating different aspects of neuronal connectivity

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Abstract

Although it is well established that the WAVE/SCAR complex transduces Rac1 signaling to trigger Arp2/3-dependent actin nucleation, regulatory mechanisms of this complex and its versatile function in the nervous system are poorly understood. Here we show that the *Drosophila* proteins SCAR, CYFIP and Kette, orthologs of WAVE/SCAR complex components, all show strong accumulation in axons of the central nervous system and indeed form a complex in vivo. Neuronal defects of SCAR, CYFIP and Kette mutants are, despite the initially proposed function of CYFIP and Kette as SCAR silencers, indistinguishable and are as diverse as ectopic midline crossing and nerve branching as well as synapse undergrowth at the larval neuromuscular junction. The common phenotypes of the single mutants are readily explained by the finding that loss of any one of the three proteins leads to degradation of its partners. As a consequence, each mutant is unambiguously to be judged as defective in multiple components of the complex even though each component affects different signaling pathways. Indeed, SCAR-Arp2/3 signaling is known to control axonogenesis whereas CYFIP signaling to the Fragile X Mental Retardation Protein fly ortholog contributes to synapse morphology. Thus, our results identify the *Drosophila* WAVE/SCAR complex as a multifunctional unit orchestrating different pathways and aspects of neuronal connectivity.

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Keywords: WAVE/SCAR; CYFIP/Sra-1/PIR121; Kette/Hem-2/NAP-1/NAP125; Rac1 GTPase pathway; Axon guidance; Midline crossing; Synapse morphology; Neuromuscular junction; Actin cytoskeleton remodeling

Introduction

The heteropentameric WAVE/SCAR complex, composed of WAVE/SCAR, PIR121/Sra-1/CYFIP, Hem-2/NAP1/Kette, Abi and HSPC300 proteins, relays Rac1

signaling to the Arp2/3 complex (Eden et al., 2002; Innocenti et al., 2004), which directs actin cytoskeleton remodeling (Pollard and Beltzner, 2002). Mechanisms of WAVE/SCAR complex signal transduction and consequences of its manipulation in different cellular systems have been recently under debate (Blagg et al., 2003; Bogdan and Klambt, 2003; Eden et al., 2002; Gautreau et al., 2004; Innocenti et al., 2004; Kunda et al., 2003; Rogers et al., 2003; Steffen et al., 2004) (for an overview, see Blagg and Insall, 2004). Whereas it is unquestionable that the WAVE/SCAR protein can bind and activate the Arp2/3 complex, dissenting views exist about the mechanisms

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triggering activation of the WAVE/SCAR protein. Initially, the four WAVE/SCAR-associated proteins have been thought to inhibit WAVE/SCAR activity until they dissociate from the protein upon Rac1 signaling (Eden et al., 2002). Since these pioneer in vitro data, some cellular studies have presented data supporting such a negative regulation (Blagg et al., 2003; Bogdan and Klambt, 2003), whereas others have shown that WAVE/SCAR protein activity is positively regulated by its associated proteins (Innocenti et al., 2004). Furthermore, the four proteins have been implicated in control of localisation and stability of the WAVE/SCAR protein (Blagg et al., 2003; Innocenti et al., 2004; Kunda et al., 2003; Rogers et al., 2003; Steffen et al., 2004). Despite these mechanistic discrepancies, such studies clearly highlight the importance of the associated proteins as WAVE/SCAR regulators.

In the present study, we have addressed the role of the WAVE/SCAR complex in development of the nervous system, an issue that is of major importance, given the instructive role of Rac1 signaling pathways in neuronal actin remodeling underlying axon as well as synapse development (reviewed in Luo, 2002). Furthermore, mutations of several genes directly affecting Rho/Rac regulatory or effector proteins cause hereditary cases of mental retardation (reviewed in Chelly and Mandel, 2001; Ramakers, 2002), strongly emphasizing the importance of associated signaling cascades in establishment of a functional neuronal network. Although direct proof for requirement of the human WAVE/SCAR complex in establishment of a properly wired nervous system is missing at present, functional studies on single subunits of the complex in *Drosophila* have provided compelling evidence for their importance in development of the nervous system (Bogdan and Klambt, 2003; Hummel et al., 2000; Schenck et al., 2003; Zallen et al., 2002). Finally, it was recently shown that one of the SCAR-associated proteins, CYFIP, links dRac1 signaling to dFMR1, the fly ortholog of the Fragile X Mental Retardation Protein (FMRP) (Schenck et al., 2001, 2003). FMRP is thought to act as a regulator of translation required in synapse formation, learning and memory (Bardoni and Mandel, 2002; Willemsen et al., 2004). This suggested the possibility that the WAVE/SCAR complex coordinates several molecular and functional pathways.

By using the fly animal model, we here show that the WAVE/SCAR complex is an evolutionarily conserved multi-functional unit that controls and thereby coordinates specific aspects of axonal development and synapse morphology. Mutation in any one of three examined WAVE/SCAR complex components—SCAR, CYFIP or Kette—leads to instability of its partners and must be therefore considered as a multiple mutation. Thus, the common axonal and synaptic phenotypes observed in the single *SCAR*, *CYFIP* and *Kette* mutations are due to disruption of the signaling cascades associated with the three proteins. Furthermore, our data on a developing organism support the view that WAVE/SCAR complex function is controlled at the level of protein

stability, subcellular localisation and possibly post-translational modification.

Materials and methods

Genetics

The wild-type strain was *Sevelen*. The utilised mutant strains were *SCAR*^{Δ37}, *SCAR*^{K13811}, *CYFIP*^{Δ85.1}, and Kette alleles *Kette*⁰³³³⁵, *Kette*^{G1-37}, *Kette*^{J1-70}, and *Kette*^{Δ2-6}. *Elav-gal4* and *Kette*⁰³³³⁵ strains were obtained from the Berkeley stock centre; other *Kette* mutants and a *UAS-Kette* transgenic strain were kindly provided by C. Klambt. For the rescue, we recombined *UAS-Kette* onto the *Kette*^{J1-70} chromosome. The used *UAS-SCAR* and *UAS-CYFIP* transgenic strains were *UAS-SCAR#3* and *UAS-CYFIP13.2*, the latter one kindly provided by N. Harden. Blue balancers were used to identify homozygous mutant embryos. *CyO GFP* and *TM6 Tb* balancers allowed identification of homozygous mutant larvae.

In situ hybridisation and immunolabeling

In situ hybridisation using digoxigenin-labeled riboprobes and immunolabeling on whole-mount embryos were performed according to standard procedures. *CYFIP*, *Kette* and *SCAR* riboprobes were generated from full-length cDNAs. Polyclonal Kette antibody #2081 was raised in rabbit against peptide RHNDNPPLLKNKGC. Anti-Kette was affinity purified using the same peptide on Sulfolink Coupling Gel columns (Pierce) and was used at 1:100. Other antibodies used in immunolabeling were anti-Fas II (1:50) (gift of C. Goodman), anti-β-gal (1:500) (Sigma, Cappel), anti-SCAR (gift of J. Zallen) and anti-CYFIP #1719 at 1:100. Secondary antibodies coupled with Cy3 or FITC (Jackson) were used at 1:400. For evaluation of NMJs, larvae open-book preparations were performed as described in Bellen and Budnik (2000), and immunolabeled with anti-DLG (1:20) (DSHB). At least 10 animals were analysed per genotype. Pictures of synapses were imported in the in-house developed TCS/timt software that quantified synaptic length by automatic measurement of redrawn synaptic terminals. Statistical significance was calculated using ANOVA and the Newman–Keuls Method for post hoc pairwise analyses. Larvae of examined genotypes were all of normal body size.

Immunoprecipitations

S2 cells were cultured in Schneider cell medium (Gibco BRL) + 10% fetal calf serum. Cytoplasmic extracts were prepared by lysing S2 cells in buffer [300 mM NaCl, 20 mM Tris–HCl pH 7.5, 5 mM MgCl₂, 0.4% Triton X-100, protease inhibitor cocktail (PIC)], kept on ice for 10 min. The supernatant of a 2000 × g centrifugation was recovered. Aliquots were incubated for 6 h with 4 μg of

either anti-CYFIP #1719 or rabbit IgG and protein A Sepharose. Beads were extensively washed in lysis buffer, directly boiled in SDS-PAGE loading buffer and part of the reaction was subjected to SDS-PAGE analysis.

Embryonic extracts

For extracts of homozygous mutant embryos, overnight (18 h) cages of the heterozygous mutant strains carrying blue balancer chromosomes were harvested and embryos were subjected to X-gal staining without fixation step. Wild-type embryos were subjected to the same procedure. Late-stage embryos (stage 12–17; according to Campos-Ortega and Hartenstein, 1985) lacking X-gal staining were hand-selected and counted, transferred into an Eppendorf tube and mashed with a pestle in (150 mM NaCl, 20 mM Tris-HCl pH 7.5, 1 mM EDTA, 0.1 mM MgCl₂, 1% Triton X-100, PIC), followed by an incubation on ice for 10 min. The supernatant of a 12,000 × *g* centrifugation was briefly sonicated, and the amount of total protein was determined by Bradford assay.

Western blot analysis

Proteins were separated in 7% polyacrylamide gels. SDS-PAGE and blotting were performed according to standard procedures. Primary antibodies used in Western blot analysis were anti-CYFIP #1719 (1:200), anti-SCAR (1:1500), anti-Kette #2081 and anti- β -tubulin (1:4000) (Chemicon). HRP-conjugated secondary antibodies (Jackson) were used at 1:5000.

Microscopy

The confocal microscope was a Leica TCS-SP1. Confocal images were assembled using an in-house developed software. Synapse images were obtained using a Zeiss Axiophot2 microscope.

Supplemental data

A supplemental table shows quantitative analysis of central axon defects observed in *SCAR* ^{Δ 37}, *CYFIP* ^{Δ 85.1}, and *Kette*⁰³³³⁵ homozygous mutant embryos, as depicted in Fig. 2. A supplemental figure allows direct comparison between endogenous and overexpressed SCAR protein in an immunolabeling experiment.

Results

The *Drosophila* proteins CYFIP, SCAR and Kette form a complex

To provide formal evidence that the *Drosophila* SCAR, CYFIP and Kette proteins form a complex, we performed co-immunoprecipitation experiments from cytoplasmic

extracts of *Drosophila* Schneider (S2) cells using antibodies raised against CYFIP (Schenck et al., 2003). Extract and co-immunoprecipitated material were subjected to Western blot analysis using antibodies against SCAR (Zallen et al., 2002) and Kette (see Materials and methods), the fly orthologs of WAVE and Hem-2/NAP125, respectively, which both associate with the human CYFIP2 protein (Eden et al., 2002). Anti-Kette antibody reveals a band of 112 kDa (Fig. 1A, right lane), whereas anti-SCAR reveals a doublet of about 66 and 70 kDa (Fig. 1A, right lane), one band of which may represent a post-translationally modified SCAR protein. Further evidence for antibody specificity is presented below (see Fig. 3B). The two *Drosophila* proteins are found to specifically co-immunoprecipitate with CYFIP (Fig. 1A, left lane). Neither Kette nor SCAR proteins are detected in a control experiment using rabbit IgG for precipitation (Fig. 1A, middle lane).

Using the same antibodies, we found that, in wild-type embryos, Kette is present in longitudinal connectives as well as commissures during establishment of the axonal network (Fig. 1B), like SCAR and CYFIP (Fig. 1B and Schenck et al., 2003; Zallen et al., 2002). By late stages, all three proteins accumulate in longitudinal connectives (see Fig. 3, wt panels), strongly suggesting that they act as a physical and functional unit during embryogenesis. In summary, the WAVE/SCAR complex is conserved in *Drosophila* and its members accumulate in axons of the nervous system.

The WAVE/SCAR complex regulates axon pathfinding and motor nerve branching

We recently demonstrated that CYFIP loss results in axon pathfinding defects at the ventral midline and in

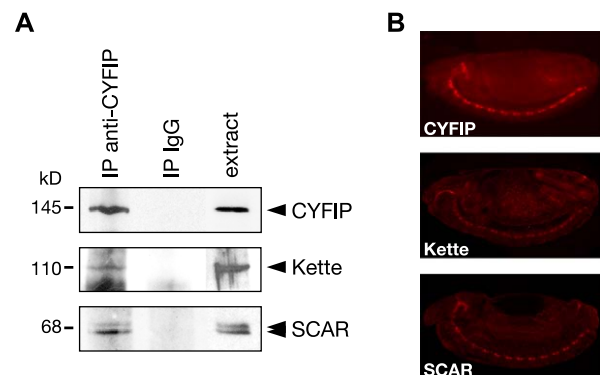


Fig. 1. *Drosophila* proteins CYFIP, Kette and SCAR co-localise and form a complex in vivo. (A) Co-immunoprecipitation experiments of CYFIP, Kette and SCAR proteins from *Drosophila* S2 cytoplasmic cell extract demonstrate physical association of the endogenous proteins. Lanes, from left to right: anti-CYFIP immunoprecipitation, IgG control immunoprecipitation, input (cytoplasmic extract). Proteins are indicated on the right; their molecular weights are indicated on the left. Note the doublet revealed by anti-SCAR antibody. (B) Localisation of endogenous CYFIP, Kette and SCAR proteins in embryogenesis. Antibodies used are indicated on the left. Embryos at stage 14–16, lateral views. Images are projections of few confocal sections near the midline. Commissural labeling is observed. Scale bar: 50 μ m.

ectopic motor nerve branching (Schenck et al., 2003). The pleiotropic defects within and outside the nervous system previously reported in zygotic *Kette* null embryos (Hummel et al., 2000) and in embryos devoid of maternal and zygotic SCAR (Zallen et al., 2002) make it difficult to pinpoint the cause of their axonal phenotypes, which prompted us to score for defects in milder mutant conditions. Immunolabeling with anti-FasII, which specifically recognises three longitudinal fascicles and motor axons (Figs. 2A,H), reveals

prominent axon defects in hypomorphic *Kette* embryos (P-insertion *Kette*⁰³³³⁵) with overall wild-type morphology and intact somatic musculature (data not shown). These defects include ectopic midline crossing (Figs. 2D,G) and motor nerve branching (Fig. 2K) of variable severity, which strongly resemble those displayed by *CYFIP*^{Δ85.1} embryos (Figs. 2B,E,I; Schenck et al., 2003). Albeit at lower frequencies, three weak hypomorphic *Kette* alleles, *Kette*^{GI-37}, *Kette*^{JL-70} and *Kette*^{Δ2-6} (Hummel et al., 2000) show

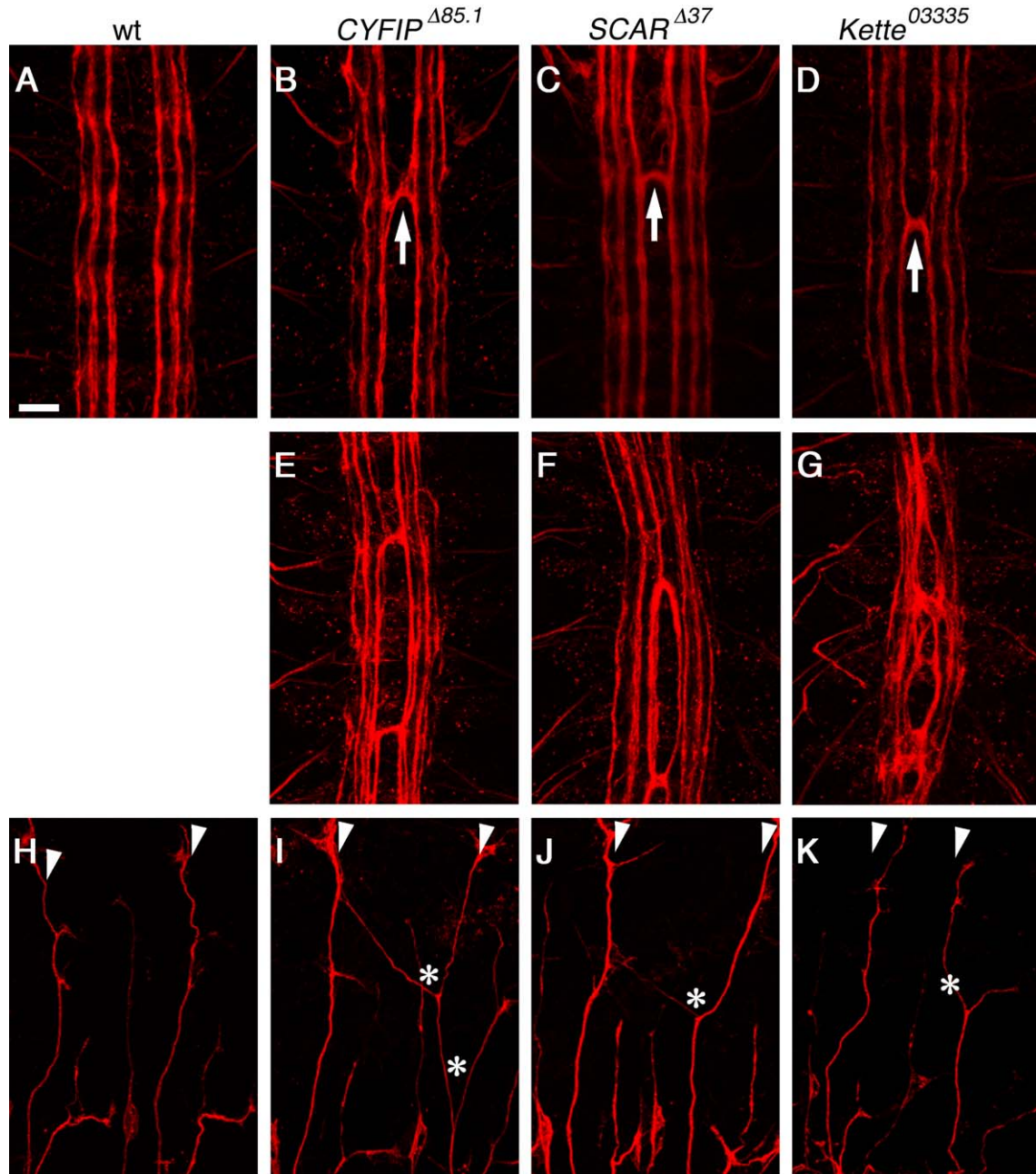


Fig. 2. Axonal phenotypes of *CYFIP*, *SCAR* and *Kette* mutant embryos. All panels show homozygous embryos of stage 16–17, labeled by neuronal marker anti-FasII. Genotypes are indicated to the top. (A–G) Central axon tracts, anterior on top. (H–K) Peripheral motor nerves, dorsal to the top, anterior to the left. Arrowheads label intersegmental nerves (ISN) of two abdominal segments. Arrows in panels B–D indicate axon guidance defects at the ventral midline in *CYFIP*, *SCAR* and *Kette* mutants, respectively. Panels E–G showing severe phenotypes observed in the same mutant lines, which illustrates defect variability. Asterisks indicate ectopic ISN branching (panels I–K). Scale bar: 20 μ m.

the same phenotypes (data not shown), indicating that they are specifically due to loss of Kette activity.

The same type and variability of defects are also observed in *SCAR*^{Δ37} (zygotic) null embryos (Figs. 2C,F,J) that appear rather normal using the more general central axon marker BP102 (Bogdan and Klambt, 2003; Zallen et al., 2002 and data not shown). *SCAR*^{k13811}, a weak hypomorph, only shows occasional defects (data not shown). A quantitative analysis of central axon defects in *CYFIP*^{Δ85.1}, *Kette*⁰³³³⁵ and *SCAR*^{Δ37} mutants is available as Supplemental Data.

In conclusion, biochemical and genetic data show that Kette and SCAR are implicated in establishment of the same axon pathways in the nervous system.

Mutations in any of the three genes (SCAR, Kette and CYFIP) result in loss of the remaining complex components

In *Hela*, *Dictyostelium* and *Drosophila* cellular systems, it was recently reported that knockdown of either CYFIP (alias PirA, Sra1) or Kette causes SCAR protein instability (Blagg et al., 2003; Kunda et al., 2003; Rogers et al., 2003; Steffen et al., 2004). In agreement, SCAR axonal labeling is dramatically reduced in both *CYFIP*^{Δ85.1} and *Kette*⁰³³³⁵

embryos compared to its wild-type pattern. Strikingly, we observed that the converse is also true; that is, CYFIP axonal labeling is completely missing in both *SCAR*^{Δ37} and *Kette*⁰³³³⁵ embryos and Kette axonal labeling is absent in *SCAR*^{Δ37} and *CYFIP*^{Δ85.1} embryos (Fig. 3A). In all cases, axonal protein patterns are disrupted throughout embryogenesis (data not shown).

To corroborate that defective axonal labeling in mutants is a consequence of protein loss, not mislocalisation, we performed a quantitative Western blot analysis using extracts of wild-type and homozygous mutant late-stage embryos (see Materials and methods). CYFIP and Kette proteins are indeed undetectable in *SCAR*, *CYFIP* and *Kette* embryonic mutant extracts. In agreement with immunolabeling results, extracts contain residual levels (3–9%) of the SCAR protein (Fig. 3B). These data demonstrate for the first time that levels of any of the three proteins, SCAR, CYFIP and Kette, depend on the presence of its complex partners.

To determine the cause of such SCAR, CYFIP and Kette protein loss, we performed whole mount in situ hybridisation using probes specific for each of the three genes on wild-type and mutant embryos. These experiments unequivocally demonstrate that the mRNA levels of any one of the

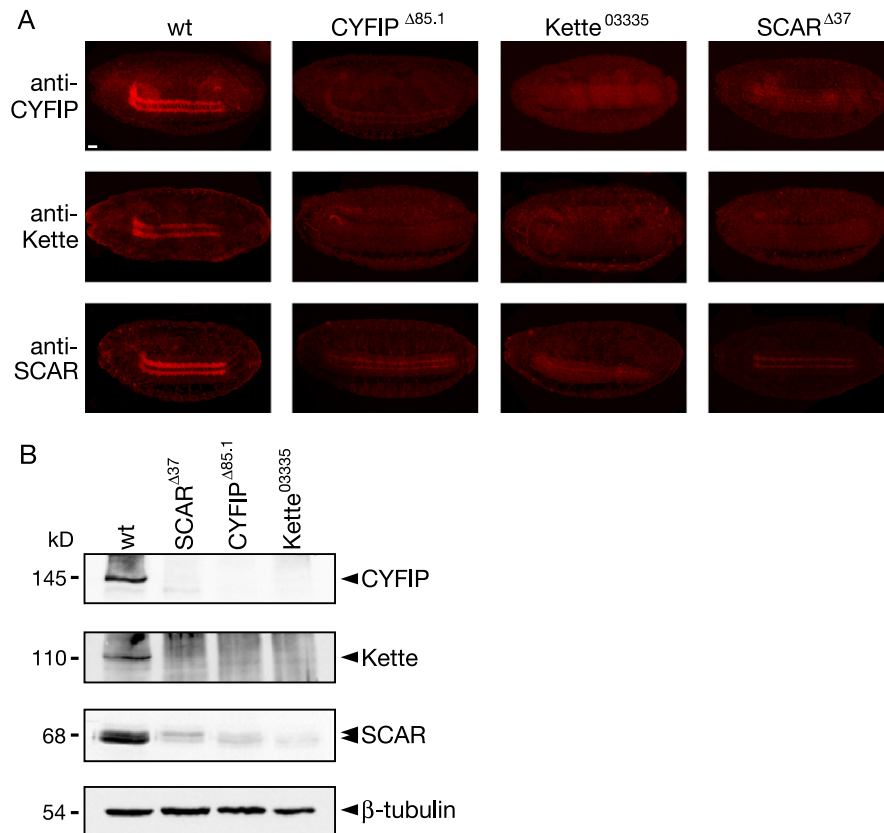


Fig. 3. CYFIP, Kette and SCAR protein pattern in *CYFIP*, *Kette*, *SCAR* mutant embryos. (A) Whole-mount immunohistochemistry on embryos of indicated mutant genotypes (top). Antibodies are indicated to the left. All panels show ventral views of stage 16/17 embryos, anterior to the left. Wild-type expression patterns (Fig. 1) have been added for convenience. Whereas residual labeling in *CYFIP*^{Δ85.1} and *SCAR*^{Δ37} mutants highlights the maternal contribution of the two proteins, Kette amounts in *Kette*⁰³³³⁵ embryos are below the detection level. (B) Quantitative analysis of CYFIP, Kette and SCAR protein levels by Western blotting. Extracts are from wt and homozygous mutant embryos stage 12–17, genotypes as above. (A, B) Note that mutations in any gene also result in dramatic loss of the remaining complex components. Scale bar: 50 μm.

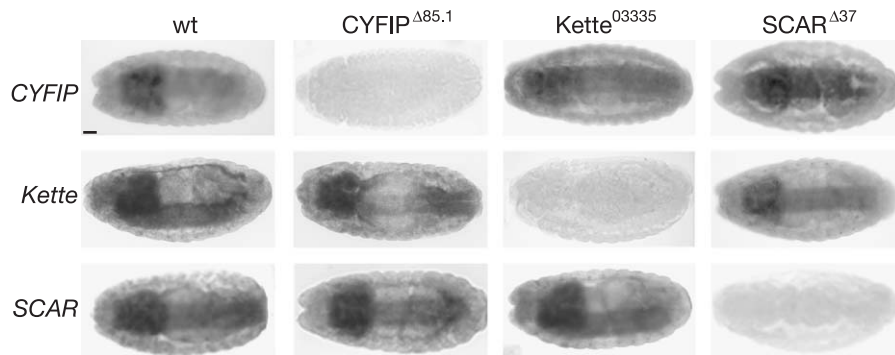


Fig. 4. *CYFIP*, *Kette* and *SCAR* mRNA pattern in wild-type and in *CYFIP*, *Kette*, *SCAR* mutant embryos. In situ hybridisation using *CYFIP*, *Kette* or *SCAR* specific probes on wild-type and mutant embryos (genotypes). Mutations in *CYFIP*, *Kette* or *SCAR* have no effect on mRNA levels of the remaining complex components. Scale bar: 50 μ m.

three genes remain unaltered in embryos carrying mutations in any other component of the complex (Fig. 4). Hence, the observed loss of *CYFIP*, *Kette* and *SCAR* proteins (Fig. 3) is not due to transcriptional defects. The fact that, in mutant conditions, all three complex components are targeted to degradation pinpoints an additional level of control, which may regulate signaling in addition to the proposed regulation by complex dissociation (Eden et al., 2002) or localisation (Bogdan and Klambt, 2003; Innocenti et al., 2004; Kunda et al., 2003; Steffen et al., 2004).

Multiple levels of *WAVE/SCAR* complex regulation in vivo

Because it appears not possible to target single components of the *WAVE/SCAR* complex by loss of function conditions for the above-mentioned reasons, we wondered whether gain of function studies could help to allocate specific roles. For this purpose, we separately overexpressed *SCAR*, *CYFIP* or *Kette* using the *elav-gal4* panneuronal driver. Surprisingly, while recombinant *WAVE/SCAR* protein is constitutively active in vitro (Eden et al., 2002; Innocenti et al., 2004), in vivo *SCAR*, as well as *CYFIP* or *Kette* overexpression does not cause any gain of function phenotype and flies are perfectly viable (Fig. 5, bottom

panels, data not shown, previously described for *Kette*; Bogdan and Klambt, 2003). Lack of overexpression phenotypes is not due to protein degradation, because protein levels are strongly increased compared to those observed in wild-type embryos, as measured by Western blot analysis (data not shown) and immunolabeling experiments (Fig. 5 and Supplemental Data).

Interestingly, in addition to central axon labeling also detected in wild-type embryos, very strong labeling of overexpressed *CYFIP* and *Kette* is detected in neuronal cell bodies of the ventral cord (Fig. 5). The fact that excess *CYFIP* and *Kette* proteins fail to localize at central axons suggests a need for active transport. Cell body accumulation of *CYFIP* and *Kette* is likely not due to titration of the transport machinery because overexpressed *SCAR* protein does succeed in accumulating at central axons (Fig. 5, see arrows and Supplemental Data). It is then intriguing to note that overexpressed *SCAR*, which does reach its normal destination and is thought to be in the active state on its own (Blagg and Insall, 2004; Eden et al., 2002; Innocenti et al., 2004), still does not disturb wiring of the nervous system. Thus, while in vitro studies reveal the potential mode of action of the *SCAR* protein, additional mechanisms must exist, which control activity of the *SCAR* protein in vivo.

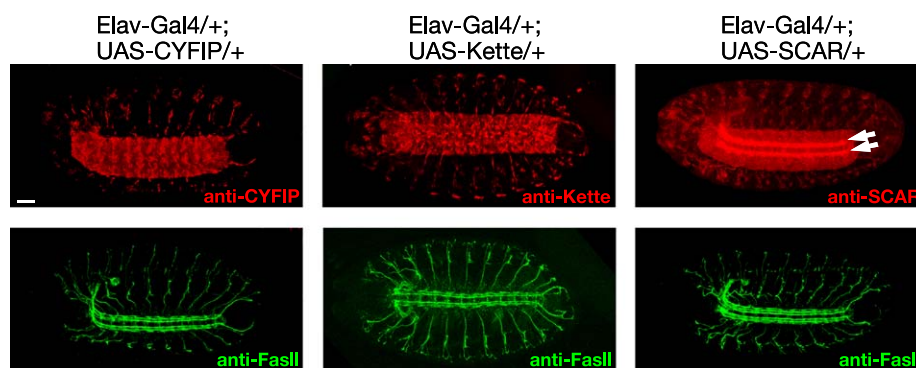


Fig. 5. *CYFIP*, *Kette* and *SCAR* gain of function phenotypes. In vivo overexpression of *CYFIP*, *Kette* or *SCAR* using the panneuronal driver *elav-gal4*. Genotypes on top. Overexpressed protein is detected by the respective antibody, as indicated in top panels. Bottom panels show the same embryos labeled by marker *FasII*, which reveals wild-type morphology. Note that overexpressed *CYFIP* and *Kette* proteins accumulate in neuronal cell bodies, whereas excess *SCAR* protein is also detected at central axons (arrows). For a direct comparison between endogenous and overexpressed *SCAR* protein, see Supplemental Data. Scale bar: 50 μ m.

Kette and *SCAR*, like *CYFIP*, regulate synaptic morphology at the neuromuscular junction

One of the WAVE/SCAR complex components, *CYFIP*, genetically and biochemically interacts with the FMRP fly ortholog *dFMR1*. Interestingly, *dFMR1* is dispensable for

development of embryonic central axons (data not shown), but affects synapse morphology (Zhang et al., 2001), where its function is counteracted by *CYFIP* (Schenck et al., 2003). Indeed, loss of *CYFIP* or *dFMR1* leads to undergrown vs. overgrown synapses at the larval neuromuscular junction (NMJ), the fly model currently used to study

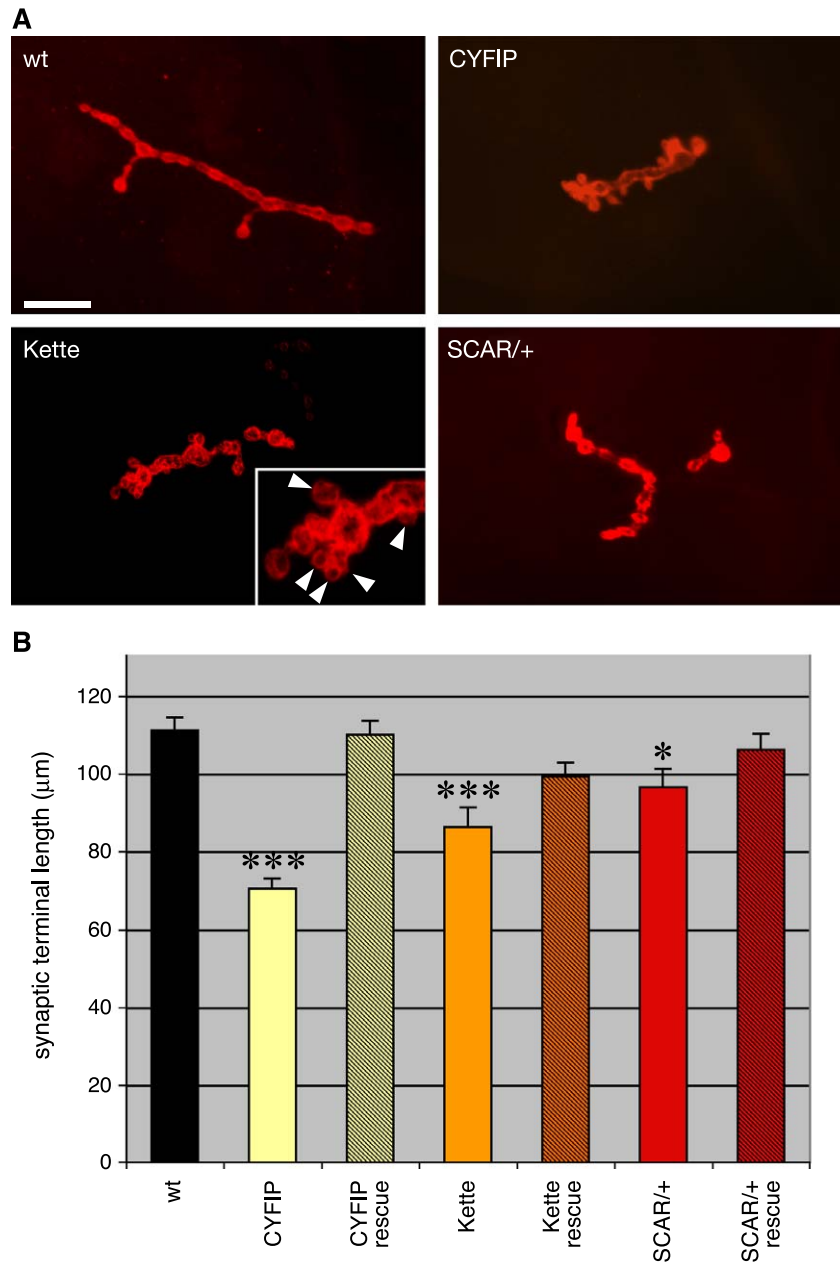


Fig. 6. Synaptic phenotypes of *CYFIP*, *Kette* or *SCAR* mutants. (A) DLG immunolabeling of muscle 4 synaptic terminals. Representative synapses of third instar larvae of the following genotypes are shown: *wt*, *CYFIP^{Δ85.1}*, *Kette^{J1-70/Kette^{Δ2-6}}* and *SCAR^{Δ37/+}*. Compared to *wt*, *CYFIP* and *Kette* synapses display undergrowth and supernumerary budding. Inset in the *Kette* panel shows a high magnification of the same synapse, arrowheads labeling supernumerary buds. Synapses of heterozygous *SCAR* mutants also show a growth defect. Scale bar: 20 or 7.5 μm (inset). (B) Statistic evaluation of the NMJ growth phenotypes displayed by *CYFIP*, *Kette* or *SCAR* mutants, and their respective rescue experiments (shaded bars). Genotypes: *wt*, *CYFIP^{Δ85.1}* (*CYFIP*), *elav-gal4/+; UAS-CYFIP*, *CYFIP^{Δ85.1}* (*CYFIP* rescue), *Kette^{J1-70/Kette^{Δ2-6}}* (*Kette*), *elav-gal4/+; UAS-Kette*, *Kette^{J1-70/Kette^{Δ2-6}}* (*Kette* rescue), *SCAR^{Δ37/+}* (*SCAR/+*), *elav-gal4/+; SCAR^{Δ37}/UAS-SCAR* (*SCAR* rescue). Sample size (number of muscle 4 junctions scored) was 32 per genotype. Error bars indicate SEM, statistical significance was calculated using ANOVA and the Newman–Keuls method for post hoc pairwise analyses. Significant differences vs. *wt* are indicated on top of bars (* $P \leq 0.025$, *** $P \leq 0.001$). No asterisk on top of a bar indicates $P > 0.05$. Length of synaptic terminals in μm, as measured using TCS/timt software (see Materials and methods).

synaptic plasticity. Because mutations in any gene, *SCAR*, *CYFIP* or *Kette*, lead to very similar axonal abnormalities, we attempted to address whether the *CYFIP* synapse phenotype (70.4 μm vs. 111.1 μm wild-type length, $P < 0.001$; Fig. 6; Schenck et al., 2003) is also shared.

SCAR and most *Kette* mutations are embryonic lethal (Zallen et al., 2002), (Hummel et al., 2000), preventing us from analyzing homozygous null larvae. However, the hypomorphic *Kette*^{J1-70} mutation gives rise to larval escapers that indeed show undergrown synapses compared to wt. Because synapse morphology is disturbed in *CYFIP* (Schenck et al., 2003) and *Kette* mutants (this study), it is not possible to count boutons, the parameter currently used to evaluate synapses. We therefore used a computer-assisted program that allows us to measure the overall length of the synapse, a parameter that we have previously shown to correlate well with bouton number (Schenck et al., 2003). This quantitative evaluation revealed a highly significant difference between *Kette*^{J1-70} and wild-type synapses (85.8 μm vs. 111.1 μm wild-type length, $P < 0.001$). To exclude an impact of genetic background on this phenotype, we also scored synapses of larvae carrying the *Kette*^{J1-70} chromosome in *trans* with the hypomorphic excision allele *Kette* ^{Δ 2-6} (Hummel et al., 2000). Synapses of these larvae are significantly shortened as well (86.2 vs. 111.1 μm wild-type length, $P < 0.001$, Fig. 6). Interestingly, synaptic terminals of *Kette* mutants share the abnormally high number of synaptic buds (Fig. 6A, inset) (5.1 vs. 1.6 buds per synapse, $P < 0.001$) shown by homozygous *CYFIP* mutants (7.8 buds, $P < 0.001$) (Schenck et al., 2003). Because buds arising from existing boutons have been described as an intermediate structure toward establishment of a new bouton (Zito et al., 1999), this phenotype likely reflects a defect in synapse maturation. Most importantly, as previously shown for *CYFIP*, *Kette* synaptic defects (length and bud number) are rescued by transgenic expression of *Kette* (*UAS-Kette*) in neurons (Fig. 6B; rescued animals: 2.3 buds). This provides evidence for the specificity of the defects observed in the hypomorph and indicates that such defects are of presynaptic origin.

To determine whether *SCAR* mutations affect synapse morphology, we took advantage of the observation that not only homozygous but also heterozygous *CYFIP* mutants exhibit a reduction in synaptic length (to 94.0 vs. 111.1 μm wild-type length), suggestive of a dose-dependent effect (Schenck et al., 2003). We thus analysed synapses of larvae heterozygous for the *SCAR* ^{Δ 37} null allele and found that they are indeed significantly shorter than their wt counterparts (96.5 vs. 111.1 μm wild-type length, $P \leq 0.05$) (Figs. 6A,B), to an extent comparable to those of heterozygous *CYFIP* animals. Like *CYFIP*⁺ synapses (Schenck et al., 2003), *SCAR* ^{Δ 37/+} synapses do not show abnormal budding (Fig. 6A) (2.3 buds per synapse). Finally, the length of *SCAR* ^{Δ 37/+} synaptic terminals is rescued by neuronal *SCAR* expression (Fig. 6B). In summary, our data identify the WAVE/SCAR complex as a crucial component required

for normal development of synapse morphology at the neuromuscular junction.

Genetic interactions between the WAVE/SCAR complex components and the fly Fragile X Mental Retardation ortholog

To characterise the impact of WAVE/SCAR complex signaling to the dFMR1 pathway, we performed genetic interaction experiments at the NMJ using both loss and gain of function conditions. As a prerequisite for these experiments, we completed the evaluation of heterozygous mutant phenotypes for the three WAVE/SCAR complex components and dFMR1. Results are summarised in Table 1. In brief, *CYFIP*, *SCAR* and *Kette* heterozygous synapses are significantly undergrown, whereas *dFMR1* heterozygous synapses are significantly extended compared to wild-type structures. Heterozygous combinations between *dFMR1* and any one of *CYFIP*, *SCAR* or *Kette* genes suppresses the *dFMR1* overgrowth phenotype, which suggests an antagonistic relationship between the WAVE/SCAR complex and dFMR1. In clear contrast, synapse undergrowth defect caused by overexpression of dFMR1 (Schenck et al., 2003; Zhang et al., 2001) is suppressed by co-overexpression of *CYFIP* but, notably, not by co-overexpression of *Kette* or *SCAR* (Table 1 and Schenck et al., 2003).

Thus, while *CYFIP* and dFMR1 show genetic interaction in both loss and gain of function experiments, *Kette* or *SCAR* interact with dFMR1 only in loss but not in gain of function experiments. This demonstrates that *Kette* and *SCAR* cannot directly antagonise dFMR1 function. Genetic conditions that reduce their levels will, however, necessarily affect levels of their partner *CYFIP*, which, as previously demonstrated, can directly antagonise dFMR1-dependent

Table 1
Genetic interactions at the NMJ between proteins of the WAVE1/SCAR complex and the Fragile X protein

Genotype	Synaptic length \pm SEM (μm)	
Wild type (+)	111.1 \pm 3.3 ^a	
<i>CYFIP</i> ⁺	93.4 \pm 2.3 ^b	$P^+ < 0.05$
<i>Kette</i> ⁺	91.9 \pm 4.1	$P^+ < 0.05$
<i>SCAR</i> ⁺	96.5 \pm 4.6 ^a	$P^+ < 0.05$
<i>SCAR</i> ^{+/+} ; <i>Kette</i> ⁺	90.1 \pm 4.1	$P^+ < 0.05$
dFMR1 ^{+/+}	119.8 \pm 5.1	
dFMR1/ <i>CYFIP</i>	100.2 \pm 4.9	$P^{\text{dFMR1}/+} < 0.05$
dFMR1/ <i>Kette</i>	103.8 \pm 3.9	$P^{\text{dFMR1}/+} < 0.05$
<i>SCAR</i> ^{+/+} ; dFMR1 ^{+/+}	104.4 \pm 4.4	$P^{\text{dFMR1}/+} < 0.05$
UAS-dFMR1	70.0 \pm 3.6 ^b	
UAS-dFMR1/UAS- <i>CYFIP</i>	88.7 \pm 4.1 ^b	$P^{\text{UAS-dFMR1}} < 0.05$
UAS-dFMR1/UAS- <i>Kette</i>	72.5 \pm 2.9	n.s.
UAS-dFMR1/UAS- <i>SCAR</i>	73.7 \pm 4.9	n.s.

CYFIP, *Kette*, and *SCAR* alleles as in Fig. 4. Overexpression of UAS constructs was driven by *elav-Gal4*. Sample size per genotype was 28–32. P values vs. indicated genotypes (superscript) are determined by ANOVA and post hoc Newman–Keuls Test. n.s.: not significant ($P > 0.05$).

^a See Fig. 6.

^b See Schenck et al. (2003).

regulation of synapse morphology (Schenck et al., 2003; Zhang et al., 2001).

Finally, we found that *Kette* synapse undergrowth is not modified by reduced levels of SCAR (Table 1), even though heterozygous *SCAR* mutants already show reduced synaptic length. This is in line with our view that, in conditions of reduced levels of two or more complex components, the most limiting of them determines the amount of its partners and thus the degree of synapse phenotype.

Discussion

The mammalian WAVE/SCAR complex is recently shown to be an integral part of Rac1 GTPase signaling pathways that coordinate actin cytoskeleton remodeling. Although mutations in single components call for a role of these proteins in construction of the nervous system (Bogdan and Klambt, 2003; Hummel et al., 2000; Schenck et al., 2003; Zallen et al., 2002), little is known about regulation and function of the WAVE/SCAR complex in this tissue. In this study, we provide evidence that the three *Drosophila* proteins, SCAR, CYFIP and *Kette*, co-localise during embryogenesis, form a complex in vivo and that they are submitted to interdependent, posttranscriptional, control. Moreover, we show that the WAVE/SCAR complex acts as a functional unit coordinating different aspects of axonal and synapse development, revealing its role in core signaling pathways underlying neuronal connectivity.

The WAVE/SCAR complex—a multifunctional unit

The analysis of *CYFIP*, *SCAR* and *Kette* mutant phenotypes and their genetic interaction with *dFMR1* call for distinct pathways being triggered by the WAVE/SCAR unit. Better understanding of specific contribution requires a more complete knowledge on these signaling pathways. First conclusions, however, can be drawn. Guidance of embryonic central axons is, for example, affected in *WAVE/SCAR* complex but not in *dFMR1* mutants and is hence controlled by *dFMR1*-independent pathways downstream of the WAVE/SCAR complex. In fact, central axons may be under control of the SCAR-Arp2/3 pathway, because mutations in different subunits of the Arp2/3 complex result in disruption of these axon tracts (Zallen et al., 2002).

In contrast, *dFMR1* as well as *WAVE/SCAR* complex mutants affect NMJ morphology (Zhang et al., 2001), suggesting a role of one or more complex components in this process. Indeed, overexpressed CYFIP rescues the *dFMR1* gain of function phenotype, while overexpressed *Kette* and *SCAR* do not. This indicates that only CYFIP can signal to *dFMR1* and suggests that the *Kette* and *SCAR* synaptic phenotypes are indirect consequences of CYFIP protein degradation. The fact that nevertheless, *CYFIP*, *Kette* or *SCAR* mutations compensate for the *dFMR1* loss of function phenotype further supports the view that the

WAVE/SCAR complex acts as an integral unit. While these studies do not exclude a direct role of WAVE/SCAR-mediated Arp2/3-dependent actin nucleation in synapse morphology, they clearly highlight the importance of CYFIP signaling to *dFMR1*. Interestingly, it has been recently shown that WASP, the second actin nucleation promoting factor, as well as its interacting protein Nervous wreck, control NMJ morphology (Coyle et al., 2004). WASP is also directly linked to the WAVE/SCAR complex by its interaction with the *Abi* protein (Bogdan and Klambt, 2003), indicating that proper synapse morphology requires integration of several related signaling pathways. Understanding the molecular bases of neuronal connectivity clearly implies evaluation of the specific contribution and integration of Arp2/3 and Fragile X Mental Retardation Protein mediated pathways at the synapse.

CYFIP, SCAR and Kette are interdependent members of the fly WAVE/SCAR complex

Two recent studies on fly and vertebrate cell cultures have shown that overexpressed SCAR or WAVE2 in cells that were knocked down for other components of the complex fail to be recruited to the cell periphery and do not rescue cytoskeletal defects (Kunda et al., 2003; Steffen et al., 2004). Our loss and gain of function data show that WAVE/SCAR complex function relies on the integrity of all its components and that not only SCAR, but also its partners require proper control of protein stability and localisation. Surprisingly, the overexpressed SCAR protein can still accumulate, at least in part, at central axons, whereas excess CYFIP and *Kette* proteins cannot, suggesting the possibility that SCAR is directly connected to the translocation machinery responsible for axonal recruitment of the WAVE/SCAR complex.

Our observation, that even upon simultaneous overexpression in pairwise or triple combinations SCAR is found in axons whereas excess CYFIP and *Kette* are not (data not shown), is explained by the recent finding that CYFIP and *Kette* do not bind SCAR directly (Gautreau et al., 2004; Innocenti et al., 2004) and must hence fail to travel piggybaggy with SCAR.

Even properly localised excess of SCAR, however, is not capable of inducing an aberrant phenotype. Localisation of SCAR is hence a prerequisite but not sufficient to activate Arp2/3-dependent changes in the actin cytoskeleton, calling for an additional level of SCAR activity control. Whether this control occurs through phosphorylation, as in the case of the WAVE/SCAR related protein WASP (Torres and Rosen, 2003) (Cory et al., 2003) and as suggested by the doublet revealed by anti-SCAR in immunoblotting (Fig. 1), remains to be determined.

Direct comparison of axonal and synaptic phenotypes displayed by *CYFIP*, *Kette* and *SCAR* mutant alleles has revealed that they are undistinguishable, a finding that suggested a common pathogenic mechanism. Indeed, we

determined that, in the developing nervous system, not only SCAR is subjected to protein turnover if either CYFIP or Kette are missing, as predictable from studies in cellular systems (Blagg et al., 2003; Kunda et al., 2003; Rogers et al., 2003; Steffen et al., 2004), but also CYFIP and Kette are lost if one of their partners is absent. These results demonstrate for the first time that not only SCAR levels are regulated by CYFIP and Kette dose, but also CYFIP and Kette levels depend on the dose of their protein partners. Thus, instead of being considered as single mutants, *CYFIP*, *Kette* and *SCAR* mutants have unambiguously to be judged as defective in multiple components of the WAVE/SCAR complex. This common biochemical basis (i.e., lack of all three proteins) clearly accounts for the identical observed phenotypes in the loss of function conditions, regardless of any effect these proteins may exert on each other in this tissue.

An important question that has remained so far unanswered by studies on the WAVE/SCAR complex is why WAVE/SCAR requires four associated proteins to transduce Rac1 signaling to the Arp2/3 complex, whereas the WAVE/SCAR-related protein WASP is capable of doing this job on its own (see Blagg and Insall, 2004 for an overview). We speculate that the hetropentameric WAVE/SCAR complex constitutes a checkpoint for a multitude of signaling pathways, which ensures their simultaneous activation. Several hints exist now in the literature indicating additional functions of Kette, Abi and CYFIP proteins. Whereas the functional significance of Kette interaction with signaling proteins like dynamin and Eps8 (Tsuboi et al., 2002) and Abi interaction with the Abl nonreceptor tyrosin kinase (Dai and Pendergast, 1995; Shi et al., 1995) remain to be validated, our work has delineated a first pathway specific to one of the WAVE/SCAR-associated proteins, CYFIP signaling to dFMR1.

In the midst of mental retardation genes—the WAVE/SCAR complex and the molecular basis of cognition

Our data show that integrity of the WAVE/SCAR complex plays a pivotal function in nervous system development and that CYFIP and Kette do not simply function as SCAR silencers or proteins merely stabilising/localizing SCAR. This is of particular interest if one considers that a series of genes connected to the WAVE/SCAR complex and its associated signaling pathways are implicated in human mental retardation. First, several mutations directly affecting Rho/Rac regulatory or effector proteins cause X-linked mental retardation (reviewed in Chelly and Mandel, 2001; Ramakers, 2002). Moreover, the most frequent cause of hereditary mental retardation is due to mutations in the Fragile X Mental Retardation gene, which is connected to Rac1 via CYFIP (Billuart and Chelly, 2003; Schenck et al., 2003) and thereby to the WAVE/SCAR complex (present study). Finally, *MEGAP* (mental disorder-associated GAP protein), also known as *WRP* or *srGAP3*, encoded by one of the few so far identified

autosomal mental retardation genes (Endris et al., 2002), is directly linked to the WAVE/SCAR complex. Indeed, *MEGAP/WRP/srGAP3* is a negative regulator of the Rac1 GTPase and binds directly to WAVE1, suggesting that the protein terminates Rac1 signaling to the complex (Soderling et al., 2002). The WAVE/SCAR complex is thus central to signaling pathways mutated in impaired conditions of neuronal functioning.

In light of the data obtained in fly, one can speculate that also (some of) the different human genetic conditions mentioned above may have a common biochemical basis. If it can be formally proven that, analogous to flies, also the recently reported WAVE1 knockout mouse, notably characterised by cognitive deficits (Soderling et al., 2003), is devoid of CYFIP and Kette proteins, this would provide the first direct evidence for the implication of this complex not only in neuronal connectivity but also in cognitive function.

Dissecting the WAVE/SCAR complex-dependent pathways and understanding the role of such pathways in local actin cytoskeleton remodeling constitute challenging questions in the field of neuronal plasticity and cognitive functions.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at [doi:10.1016/j.ydbio.2004.07.009](https://doi.org/10.1016/j.ydbio.2004.07.009).

References

- Bardoni, B., Mandel, J.L., 2002. Advances in understanding of fragile X pathogenesis and FMRP function, and in identification of X linked mental retardation genes. *Curr. Opin. Genet. Dev.* 12, 284–293.
- Bellen, H.J., Budnik, V., 2000. The neuromuscular junction. In: Sullivan, W., Ashburner, M., Hawley, R.S. (Eds.), *Drosophila* Protocols. Cold Spring Harbor Laboratory Press, New York, pp. 175–200.

- Billuart, P., Chelly, J., 2003. From Fragile X Mental Retardation Protein to Rac1 GTPase. New insights from Fly CYFIP. *Neuron* 38, 843–845.
- Blagg, S.L., Insall, R.H., 2004. Solving the WAVE function. *Nat. Cell Biol.* 6, 279–281.
- Blagg, S.L., Stewart, M., Sambles, C., Insall, R.H., 2003. PIR121 regulates pseudopod dynamics and SCAR activity in *Dictyostelium*. *Curr. Biol.* 13, 1480–1487.
- Bogdan, S., Klambt, C., 2003. Kette regulates actin dynamics and genetically interacts with Wave and Wasp. *Development* 130, 4427–4437.
- Campos-Ortega, J.A., Hartenstein, V., 1985. *The Embryonic Development of Drosophila melanogaster*. Springer-Verlag, Berlin.
- Chelly, J., Mandel, J.L., 2001. Monogenic causes of X-linked mental retardation. *Nat. Rev.* 2, 669–679.
- Cory, G.O., Cramer, R., Blanchoin, L., Ridley, A.J., 2003. Phosphorylation of the WASP-VCA domain increases its affinity for the Arp2/3 complex and enhances actin polymerization by WASP. *Mol. Cell* 11, 1229–1239.
- Coyle, I.P., Koh, Y.H., Lee, W.C., Slind, J., Fergestad, T., Littleton, J.T., Ganetzky, B., 2004. Nervous wreck, an SH3 adaptor protein that interacts with Wsp, regulates synaptic growth in *Drosophila*. *Neuron* 41, 521–534.
- Dai, Z., Pendergast, A.M., 1995. Abi-2, a novel SH3-containing protein interacts with the c-Abl tyrosine kinase and modulates c-Abl transforming activity. *Genes Dev.* 9, 2569–2582.
- Eden, S., Rohatgi, R., Podtelejnikov, A.V., Mann, M., Kirschner, M.W., 2002. Mechanism of regulation of WAVE1-induced actin nucleation by Rac1 and Nck. *Nature* 418, 790–793.
- Endris, V., Wogatzky, B., Leimer, U., Bartsch, D., Zatyka, M., Latif, F., Maher, E.R., Tariverdian, G., Kirsch, S., Karch, D., Rappold, G.A., 2002. The novel Rho-GTPase activating gene MEGAP/srGAP3 has a putative role in severe mental retardation. *Proc. Natl. Acad. Sci. U. S. A.* 99, 11754–11759.
- Gautreau, A., Ho, H.Y., Li, J., Steen, H., Gygi, S.P., Kirschner, M.W., 2004. Purification and architecture of the ubiquitous Wave complex. *Proc. Natl. Acad. Sci. U. S. A.* 101, 4379–4383.
- Hummel, T., Leifker, K., Klambt, C., 2000. The *Drosophila* HEM-2/NAP1 homolog KETTE controls axonal pathfinding and cytoskeletal organization. *Genes Dev.* 14, 863–873.
- Innocenti, M., Zucconi, A., Disanza, A., Frittoli, E., Areces, L.B., Steffen, A., Stradal, T.E., Di Fiore, P.P., Carlier, M.F., Scita, G., 2004. Abi1 is essential for the formation and activation of a WAVE2 signalling complex. *Nat. Cell Biol.* 6, 319–327.
- Kunda, P., Craig, G., Dominguez, V., Baum, B., 2003. Abi, Sra1, and Kette control the stability and localization of SCAR/WAVE to regulate the formation of actin-based protrusions. *Curr. Biol.* 13, 1867–1875.
- Luo, L., 2002. Actin cytoskeleton regulation in neuronal morphogenesis and structural plasticity. *Annu. Rev. Cell Dev. Biol.* 18, 601–635.
- Pollard, T.D., Beltzner, C.C., 2002. Structure and function of the Arp2/3 complex. *Curr. Opin. Struct. Biol.* 12, 768–774.
- Ramakers, G.J., 2002. Rho proteins, mental retardation and the cellular basis of cognition. *Trends Neurosci.* 25, 191–199.
- Rogers, S.L., Wiedemann, U., Stuurman, N., Vale, R.D., 2003. Molecular requirements for actin-based lamella formation in *Drosophila* S2 cells. *J. Cell Biol.* 162, 1079–1088.
- Schenck, A., Bardoni, B., Moro, A., Bagni, C., Mandel, J.L., 2001. A highly conserved protein family interacting with the fragile X Mental Retardation Protein and displaying selective interactions with the FMRP related proteins FXR1P and FXR2P. *Proc. Natl. Acad. Sci. U. S. A.* 98, 8844–8849.
- Schenck, A., Bardoni, B., Langmann, C., Harden, N., Mandel, J.L., Giangrande, A., 2003. CYFIP/Sra-1 controls neuronal connectivity in *Drosophila* and links the Rac1 GTPase pathway to the Fragile X Protein. *Neuron* 38, 887–898.
- Shi, Y., Alin, K., Goff, S.P., 1995. Abl-interactor-1, a novel SH3 protein binding to the carboxy-terminal portion of the Abl protein, suppresses v-abl transforming activity. *Genes Dev.* 9, 2583–2597.
- Soderling, S.H., Binns, K.L., Wayman, G.A., Davee, S.M., Ong, S.H., Pawson, T., Scott, J.D., 2002. The WRP component of the WAVE-1 complex attenuates Rac-mediated signalling. *Nat. Cell Biol.* 4, 970–975.
- Soderling, S.H., Langeberg, L.K., Soderling, J.A., Davee, S.M., Simerly, R., Raber, J., Scott, J.D., 2003. Loss of WAVE-1 causes sensorimotor retardation and reduced learning and memory in mice. *Proc. Natl. Acad. Sci. U. S. A.* 100, 1723–1728.
- Steffen, A., Rottner, K., Ehinger, J., Innocenti, M., Scita, G., Wehland, J., Stradal, T.E., 2004. Sra-1 and Nap1 link Rac to actin assembly driving lamellipodia formation. *EMBO J.* 23, 749–759.
- Torres, E., Rosen, M.K., 2003. Contingent phosphorylation/dephosphorylation provides a mechanism of molecular memory in WASP. *Mol. Cell* 11, 1215–1227.
- Tsuboi, D., Qadota, H., Kasuya, K., Amano, M., Kaibuchi, K., 2002. Isolation of the interacting molecules with GEX-3 by a novel functional screening. *Biochem. Biophys. Res. Commun.* 292, 697–701.
- Willemsen, R., Oostra, B.A., Bassell, G.J., Dichtenberg, J., 2004. The fragile X syndrome: from molecular genetics to neurobiology. *Ment. Retard. Dev. Disabil. Res. Rev.* 10, 60–67.
- Zallen, J.A., Cohen, Y., Hudson, A.M., Cooley, L., Wieschaus, E., Schejter, E.D., 2002. SCAR is a primary regulator of Arp2/3-dependent morphological events in *Drosophila*. *J. Cell Biol.* 156, 689–701.
- Zhang, Y.Q., Bailey, A.M., Matthiensi, H.J.G., Renden, R.B., Smith, M.A., Speese, S.D., Rubin, G.M., Broadie, K., 2001. *Drosophila* Fragile X-related gene regulates MAP1B homolog Futsch to control synaptic structure and function. *Cell* 107, 591–603.
- Zito, K., Parnas, D., Fetter, R.D., Isacoff, E.Y., Goodman, C.S., 1999. Watching a synapse grow: noninvasive confocal imaging of synaptic growth in *Drosophila*. *Neuron* 22, 719–729.