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Abi, Sra1, and Kette Control the Stability and Localization of SCAR/WAVE to Regulate the Formation of Actin-Based Protrusions

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Summary

Background: In animal cells, GTPase signaling pathways are thought to generate cellular protrusions by modulating the activity of downstream actin-regulatory proteins. Although the molecular events linking activation of a GTPase to the formation of an actin-based process with a characteristic morphology are incompletely understood, Rac-GTP is thought to promote the activation of SCAR/WAVE, whereas Cdc42 is thought to initiate the formation of filopodia through WASP. SCAR and WASP then activate the Arp2/3 complex to nucleate the formation of new actin filaments, which through polymerization exert a protrusive force on the membrane.
Results: Using RNAi to screen for genes regulating cell form in an adherent *Drosophila* cell line, we identified a set of genes, including Abi/E3B1, that are absolutely required for the formation of dynamic protrusions. These genes delineate a pathway from Cdc42 and Rac to SCAR and the Arp2/3 complex. Efforts to place Abi in this signaling hierarchy revealed that Abi and two components of a recently identified SCAR complex, Sra1 (p140/PIR121/CYFIP) and Kette (Nap1/Hem), protect SCAR from proteasome-mediated degradation and are critical for SCAR localization and for the generation of Arp2/3-dependent protrusions.
Conclusions: In *Drosophila* cells, SCAR is regulated by Abi, Kette, and Sra1, components of a conserved regulatory SCAR complex. By controlling the stability, localization, and function of SCAR, these proteins may help to ensure that Arp2/3 activation and the generation of actin-based protrusions remain strictly dependant on local GTPase signaling.

Introduction

In animal cells, actin polymerization at the cell cortex generates the force required for the generation of membrane protrusions [1]. The precise actin-based structure formed is thought to be determined, in part, by the relative contributions of different members of the Rho family of small GTPases [2]. Thus, active Cdc42 promotes the formation of parallel bundles of actin filaments and fine actin-based processes, or filopodia [2], whereas Rac loaded with GTP induces the formation of branched networks of actin filaments and therefore broad protrusions, termed lamellipodia [2]. Cdc42 is thought to induce new actin filament formation by binding to the WASP family proteins WASP/N-WASP [3, 4]. This induces a conformational change that releases the WASP VCA domain from auto-inhibition [5], enabling it to bind G-actin and to activate the Arp2/3 complex. Although Rac signals to the Arp2/3 complex in an analogous fashion [6, 7] via a WASP-related protein, SCAR/WAVE [8, 9], Rac does not directly interact with SCAR/WAVE [1]. Moreover, unlike WASP/N-WASP, purified SCAR is constitutively active in vitro [7, 10, 11]. In an effort to elucidate the molecular events linking Rac and SCAR, a recent study isolated an inhibitory SCAR complex from mammalian brain extracts that was responsive to Rac signaling [12]. In its inactive state this complex included Abi/E3B1 [13, 14], homologs of *Drosophila* Kette and Sra1 [15–18] (Nap1/NCKAP1/Hem2/GEX3 and p140/PIR121/CYFIP/GEX2, respectively), proteins previously found in association with Rac-GTP [19–21] and genetically linked to Rac [18, 22], together with a small, highly conserved actin regulator, HSPC300 [12]. Upon addition of Rac-GTP, the complex dissociated, freeing SCAR and HSPC300 to induce actin polymerization through activation of the Arp2/3 complex [12]. Although the complex provides the first clear mechanistic link between Rac and SCAR activation, these findings have yet to be verified within the context of a cell. To identify the genes involved in the generation of cell form, we recently collaborated in the development of a complementary approach [23], in which double-stranded RNA-mediated interference (RNAi) is used to systematically silence genes in an adherent *Drosophila* cell line. In this paper, we have used this RNAi technology to delineate a pathway, from Cdc42 and Rac to SCAR and the Arp2/3 complex, that controls the nucleation of actin filaments in *Drosophila* [16, 17]. This analysis also confirms that proteins identified as part of the mammalian SCAR complex perform a conserved function in the regulation of SCAR in *Drosophila*. Strikingly, although the complex inhibits SCAR activity in vitro, in the context of a *Drosophila* cell, individual complex components appear to play a positive role in the generation of actin-rich protrusions.

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Results

With the advent of RNAi it is possible to use *Drosophila* cells in culture as a model system to test the cell-biological function of genes identified by genomic sequencing; such genes include those involved in the generation of actin-based protrusions [23, 24]. S2R+ cells [25] are particularly amenable to this type of loss-of-function analysis because a large number of distinct actin-related phenotypes can be readily distinguished in this cell type [23]. Using such an approach (see Experimental Procedures), we identified several genes from a set of putative actin regulators that are absolutely required for the maintenance of S2R+ cell shape and for the formation of lamellipodia. In each case, the gene-specific dsRNA identified caused cells to assume a starfish-like mor-

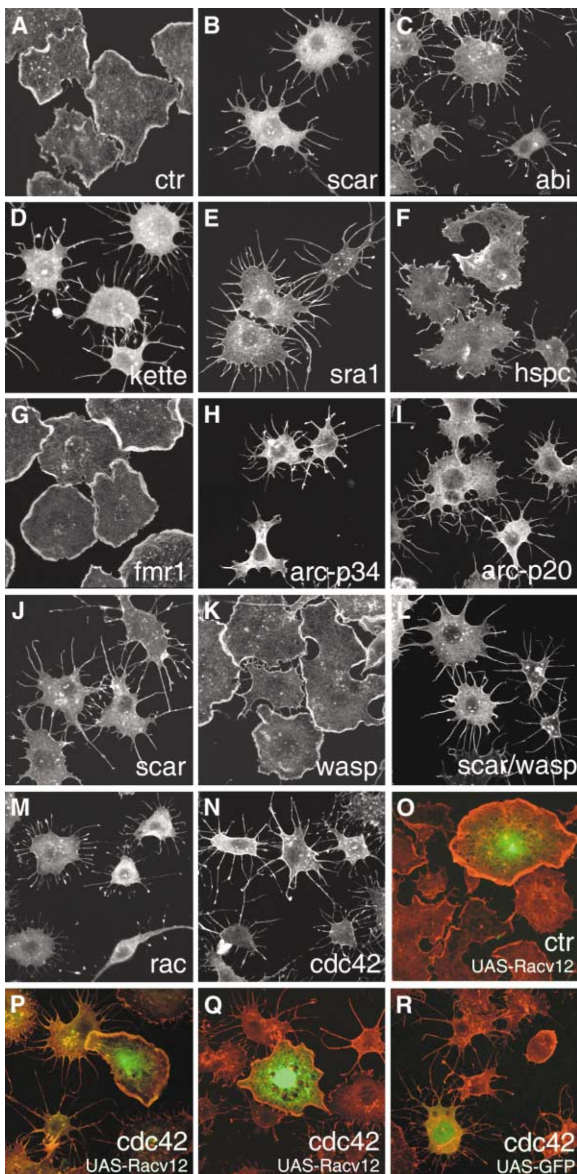

Figure 1. Identification of Abi, Kette, and Sra1 as Positive Effectors in the SCAR Pathway

F-actin was visualized with TRITC-labeled phalloidin approximately 6 days after the addition of specific dsRNA in (A) control S2R+ cells and in (B and J) SCAR, (C) Abi, (D) Kette, (E) Sra1, (F) HSPC300, (G) FMR1, (H) Arc-p34, (I) Arc-p20, (K) WASP, (L) WASP and SCAR, (M) Rac1, Rac2, and Mtl, and (N) Cdc42 RNAi cells. F-actin (red) and GFP (green) were imaged in (O) control or (P–R) Cdc42^{RNAi} cells after cotransfection of either (O–Q) UAS-Racv12, Actin5C-Gal4, and UAS-GFP or (R) Actin5C-Gal4 and UAS-GFP. Images were captured at different settings to enable residual actin filaments to be visualized. There was a marked reduction in the level of cortical actin in SCAR, Abi, Kette, Sra1, Arc-p34, Arc-p20, Rac (Rac1, Rac2, and Mtl), and Cdc42 RNAi cells.

phology with multiple slender cell extensions (Figure 1). This change in form was accompanied by the loss of actin filaments from the cell periphery, resulting in a more diffuse, non-cortical F-actin distribution (Figure 1). Genes with this characteristic RNAi phenotype included a known activator of the Arp2/3 complex, the sole *Drosophila* SCAR/WAVE homolog ([16]; Figures 1B and 1J), and *Drosophila* Abi [26], an SH3 domain-containing Abl substrate (Figure 1C). In contrast, *Drosophila* WASP [16, 27], another Arp2/3 complex activator, had no discernable RNAi phenotype in this assay (Figure 1K) and did not visibly accentuate the SCAR^{RNAi} phenotype (Figure 1L). RNAi targeting of Arc-p34 and Arc-p20, two components of the *Drosophila* Arp2/3 complex [17], led to a similar change in S2R+ cell shape (Figures 1H and 1I), implying that this spiky phenotype reflects the inability to nucleate new cortical actin filaments [1].

In order to follow the development of this characteristic morphological phenotype, we imaged SCAR^{RNAi} and Abi^{RNAi} cells every 60 s as they spread on a serum-coated glass substrate (Figures 2A–2D; Movies 1A–1D in the Supplemental Data available with this article online). Both control cells and cells treated with SCAR or Abi dsRNA were able to flatten on the substrate (Figures 2A–2D), probably as a result of integrin-mediated adhesion [23]. Whereas control cells rapidly extended broad lamellipodia together with fine filopodia as they spread (Figure 2A), SCAR^{RNAi} or Abi^{RNAi} cells failed to develop significant numbers of new protrusions (see arrows in Figures 2B–2D). Instead, existing, stable protrusions flattened, whereas the cortex of SCAR^{RNAi} and Abi^{RNAi} cells remained relatively smooth. From these data, it can be concluded that SCAR and Abi are specifically required for the formation of dynamic actin-based protrusions in *Drosophila* cells. We used immunofluorescence to test whether this function of SCAR is reflected in its subcellular localization. SCAR was found at the tips of both broad and fine protrusions in control S2R+ cells, distal to F-actin (Figures 2E–2G and arrowhead in Figures 2H and 2I), but it was often absent from thicker F-actin bundles, likely to represent retraction fibers (arrow in Figures 2H and 2I). SCAR is therefore concentrated at the tips of elongating processes in *Drosophila* cells (as are SCAR and Abi in mammalian cells [28–30]), where it is required, together with Abi, for new actin filament formation.

Because SCAR is thought to function downstream of activated Rac in several systems [1, 16], we tested the morphological effects of using RNAi to target Rac. In *Drosophila*, three Rac homologs that function in a partially redundant fashion have been identified [31]. We targeted all three 3 genes, Rac1, Rac2, and Mtl, singly and in combination to assess the loss-of-function phenotype. Although adding dsRNA specific for any one of these Rac homologs had a mild effect on cell form, dsRNAs targeting all three *Drosophila* Racs induced a phenotype very similar to that seen after loss of SCAR or Abi (Figure 1M). The effects of constitutively active Rac were also tested in S2R+ cells. Rac1V12 caused a dramatic increase in the level of cortical F-actin (Figure 1O), showing that the activation of Rac is a critical event in the generation of a protrusion in S2R+ cells, as it is in many other systems [2]. Cdc42 was also found to have a profound SCAR-like RNAi phenotype (Figure 1N), even though WASP, its putative downstream regulator [5], had no discernable RNAi phenotype (Figures 1K and 1L). Strikingly, however, the Cdc42^{RNAi} phenotype differed from that observed in SCAR^{RNAi}, Abi^{RNAi}, or Arc-p20^{RNAi} cells in that it could be completely suppressed

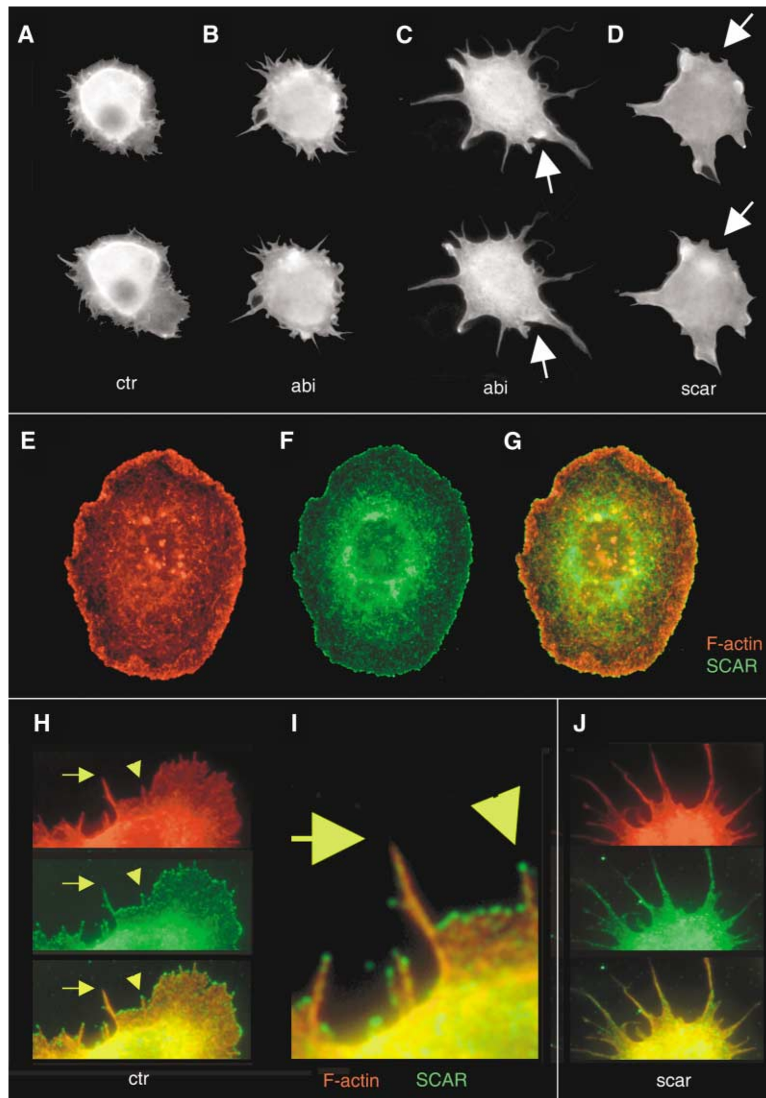

Figure 2. SCAR and Abi Are Required for the Formation of Actin-Based Protrusions in S2R+ Cells

In (A–D), F-actin was imaged with moesin-GFP every 60 s as control (A), Abi^{RNAi} (B and C) or SCAR^{RNAi} (D) cells were spread onto serum-coated cover slips. Stills, separated by 60 s, show cells in the first few minutes after their attachment to the substrate. Complete image sequences are shown in Movies 1A–1D available with this article online. Most cellular extensions visible in Abi^{RNAi} and SCAR^{RNAi} cells were present as cells first touched the substrate. The slender processes that characterize these cells contained microtubules (data not shown) and resemble those formed in S2R+ cells after treatment with actin cytoskeletal inhibitors [23]. In Abi^{RNAi} and SCAR^{RNAi} cells, F-actin accumulated at cortical sites that underwent cycles of swelling and contraction (arrows in [C] and [D]). Although present in actin-rich puncta, Arp2/3 complex components were absent from SCAR-like processes and from smooth actin-rich cortical structures in fixed Abi^{RNAi} cells (data not shown). (E–J) SCAR protein (green) was localized together with F-actin (red). In control S2R+ cells, SCAR was found at the tips of both broad and fine cellular protrusions, distal to F-actin (E–I), but was specifically lost from SCAR^{RNAi} cells (J). A fine, actin-based protrusion is indicated with a small arrowhead, and a retraction fiber-like structure is indicated with an arrow (H and I).

by the expression of activated Rac1V12 (Figures 1O-1Q and data not shown). Taken together, these data show that Cdc42, Rac, SCAR, and the Arp2/3 complex constitute elements of a pathway or functional module that controls the formation of actin filaments in *Drosophila* cells. In these cells, Cdc42 appears to facilitate the activation of Rac [2]. Cdc42 may therefore exert its effect on the *Drosophila* actin cytoskeleton organization primarily through modulation of Rac-SCAR pathway activity.

Although a role for Abi in the SCAR pathway was initially unexpected, a mammalian homolog of Abi was recently found to bind SCAR as part of an inhibitory complex, together with homologs of Kette, Sra1, and HSPC300 [12, 32, 33]. If Abi were to negatively regulate SCAR, loss of Abi would be expected to lead to ectopic actin filament formation. The fact that Abi^{RNAi} S2R+ cells had a SCAR-like phenotype, in apparent contradiction with this model of SCAR regulation [12], prompted us to test the function of the other putative complex components. Strikingly, dsRNA-mediated silencing of Kette or Sra1 phenocopied the effects of SCAR^{RNAi} or Abi^{RNAi} (Figures 1D and 1E), whereas the fifth complex component, HSPC300, had a somewhat variable, although related, RNAi phenotype (Figure 1F). In contrast, dsRNAs targeting several other putative pathway regulators, Dock (a *Drosophila* Nck homolog), Sos, and FMR1 [18, 22] (Figure 1G and data not shown), had no visible effect on actin organization or S2R+ morphology. Therefore, in the context of a *Drosophila* cell, Abi, Kette, and Sra1 appear to act together with SCAR to promote the formation of actin-based protrusions.

To further explore the role of Abi, Kette, and Sra1 in the regulation of SCAR, SCAR protein levels were analyzed in dsRNA-treated cells (Figure 3). As expected, SCAR protein was almost undetectable in cells grown in the presence of SCAR dsRNA (Figure 3A). More surprisingly, however, SCAR protein levels were also significantly reduced (routinely >90%) in Abi, Kette, and Sra1 dsRNA-treated cells and were reproducibly lower (<50% of the control) in cells in which HSPC300 was targeted (Figures 3A and 3B). These effects were not specific to S2R+ cells because they were seen in another *Drosoph-*

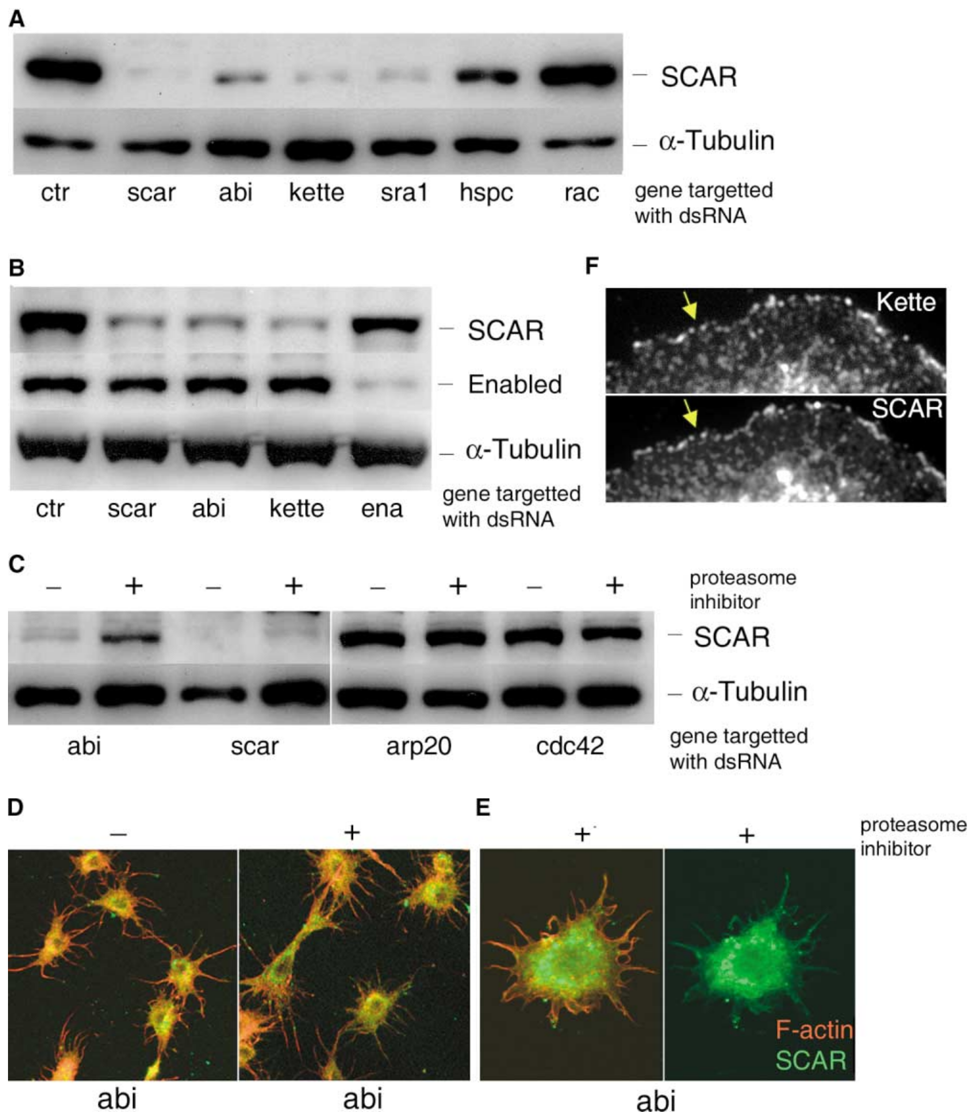

Figure 3. Loss of Abi, Kette, or Sra1 Leads to the Degradation of SCAR by the Proteasome

(A–C) SCAR protein levels were assessed by Western blotting approximately 6 days after the addition of specific dsRNAs targeting SCAR, Abi, Kette, Sra1, HSPC300, Rac (Rac1, Rac2, and Mtl), Ena, or Arc-p20. Similar results were seen in 3–5 experiments and in another *Drosphila* cell line, UC88 cells (B). A control protein, α-Tubulin, was unaffected by treatment with dsRNA, and depletion of Ena, Sra1, Kette, and WASP proteins was confirmed by Western analysis after addition of the corresponding dsRNA ([B] and data not shown). (C) Loss of SCAR protein from Abi^{RNAi} S2R+ cells is partially rescued by the addition of proteasome inhibitors for 4 hr (+), a treatment that has little effect on the level of SCAR in other dsRNA-treated cells or in SCAR^{RNAi} cells.

(D and E) The addition of proteasome inhibitors to Abi^{RNAi} cells did not alter actin organization (TRITC-labeled phalloidin, in red), SCAR localization (green), or cellular morphology. (F) SCAR and Kette were found to colocalize in S2R+ cells by immunofluorescence.

ila cell line, UC88 (Figure 3B). DsRNAs targeting Cdc42, Rac homologs, or components of the Arp2/3 complex had little, if any, effect on SCAR protein levels (Figures 3A and C), confirming that loss of SCAR was not an indirect effect of the change in cell morphology or due to a perturbation in pathway signaling. To test whether the reduction in SCAR protein levels in Abi^{RNAi} and Kette^{RNAi} cells was due to increased SCAR degradation, we added a cocktail of proteasome inhibitors (Lactacystin and VELCADE) to control and dsRNA-treated cells (see Experimental Procedures). Inhibition of the proteasome had little effect on the level of SCAR in control cells or in SCAR^{RNAi} cells but dramatically increased the amount of SCAR protein in Abi^{RNAi} cells (Figure 3C) and did so to a somewhat lesser extent in Kette^{RNAi} and Sra1^{RNAi} cells (data not shown). These data demonstrate that SCAR is subject to proteasome-mediated degradation in the absence of other putative components of the complex. The interactions between SCAR, Abi, Kette, Sra1, and HSPC300 suggest the possibility that *Drosophila* SCAR is regulated by a complex analogous to that found in mammalian cells [12]. In support of this conclusion, SCAR and Kette proteins were found to colocalize in S2R+ cells (Figure 3F).

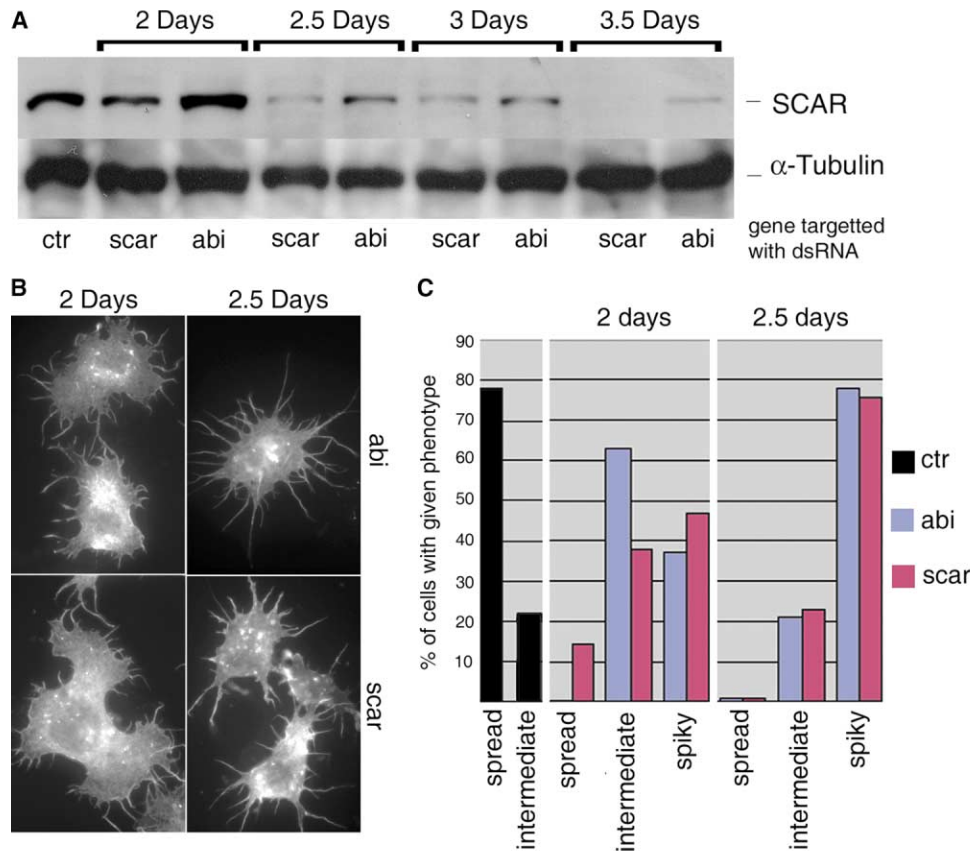

Figure 4. Abi^{RNAi} Cells Develop Their Characteristic Morphological Phenotype prior to the Loss of SCAR Protein

(A) SCAR protein levels were assayed in Abi^{RNAi} and SCAR^{RNAi} cells 2–3.5 days after the addition of dsRNA. α-Tubulin was used as a loading control.

(B) At 2 and 2.5 days, as SCAR levels were in decline, cells were replated on serum-coated cover slips, and actin filaments were visualized with TRITC-phalloidin.

(C) For monitoring development of the morphological phenotype, the percentage of Abi^{RNAi} and SCAR^{RNAi} cells with spread, intermediate, or spiky morphologies was calculated. At no point in time was a substantial increase in F-actin levels observed in Abi^{RNAi} cells.

Although the reduction in SCAR levels seen in SCAR^{RNAi}, Abi^{RNAi}, Kette^{RNAi}, and Sra1^{RNAi} cells explains their superficial phenotypic similarities, it is conceivable that Abi, Kette, and Sra1 serve additional functions that are masked by their effect on SCAR protein levels. To investigate this possibility, we examined Abi^{RNAi} cells in which SCAR levels had been partially restored by inhibition of the proteasome. The SCAR accumulating in these cells failed to become properly localized at the tips of protrusions (Figure 3E) or to rescue the defects in cell morphology (Figures 3D and 3E). To gain further support for this finding, we correlated loss of SCAR in Abi^{RNAi} and SCAR^{RNAi} cells with the development of the distinctive morphological defect. A decline in the level of SCAR protein was seen 2 days after the addition of SCAR dsRNA and several hours later, at 2.5 days, in Abi^{RNAi} cells (Figure 4A). In contrast, changes in cell shape and in cortical F-actin organization appeared concurrently in the two cultures (Figures 4B and 4C). As a result, Abi^{RNAi} cells take on their characteristic spiky morphology while still expressing normal levels of SCAR (compare Figures 4A, 4B, and 4C), proving that the morphological defect first observed in Abi^{RNAi} cells is not a simple consequence of a reduction in the level of SCAR protein. Finally overexpression of full-length SCAR failed to induce the formation of actin-rich lamellipodia in Abi^{RNAi} cells (Figures 5A–5C).

Data from these three experiments are consistent in showing that Abi has an additional function, independent of its role in the control of SCAR stability, in the localization of SCAR and in the generation of actin-based protrusions.

Purified SCAR is sufficient to activate the Arp2/3 complex [7, 10, 11]. Within the context of the cell, however, it is possible that components of the SCAR complex facilitate the interaction between SCAR and the Arp2/3 complex. To test this hypothesis, we expressed a GFP-tagged truncated form of SCAR, which we refer to as SCAR-PVCA (which includes the portion of the protein known to bind both G-actin and the Arp2/3 complex [7, 10, 11]), in dsRNA-treated S2R+ cells. SCAR-PVCA accumulated in the perinuclear region of control cells (Figure 5D), where it induced the local accumulation of F-actin (yellow in Figures 5D–5K). In Arc-p34^{RNAi} or Arc-p20^{RNAi} cells, however, SCAR-PVCA failed to elicit this response (Figure 5F), confirming that these aggregates of actin filaments result from activation of the Arp2/3 complex. Furthermore, components of the Arp2/3 com-

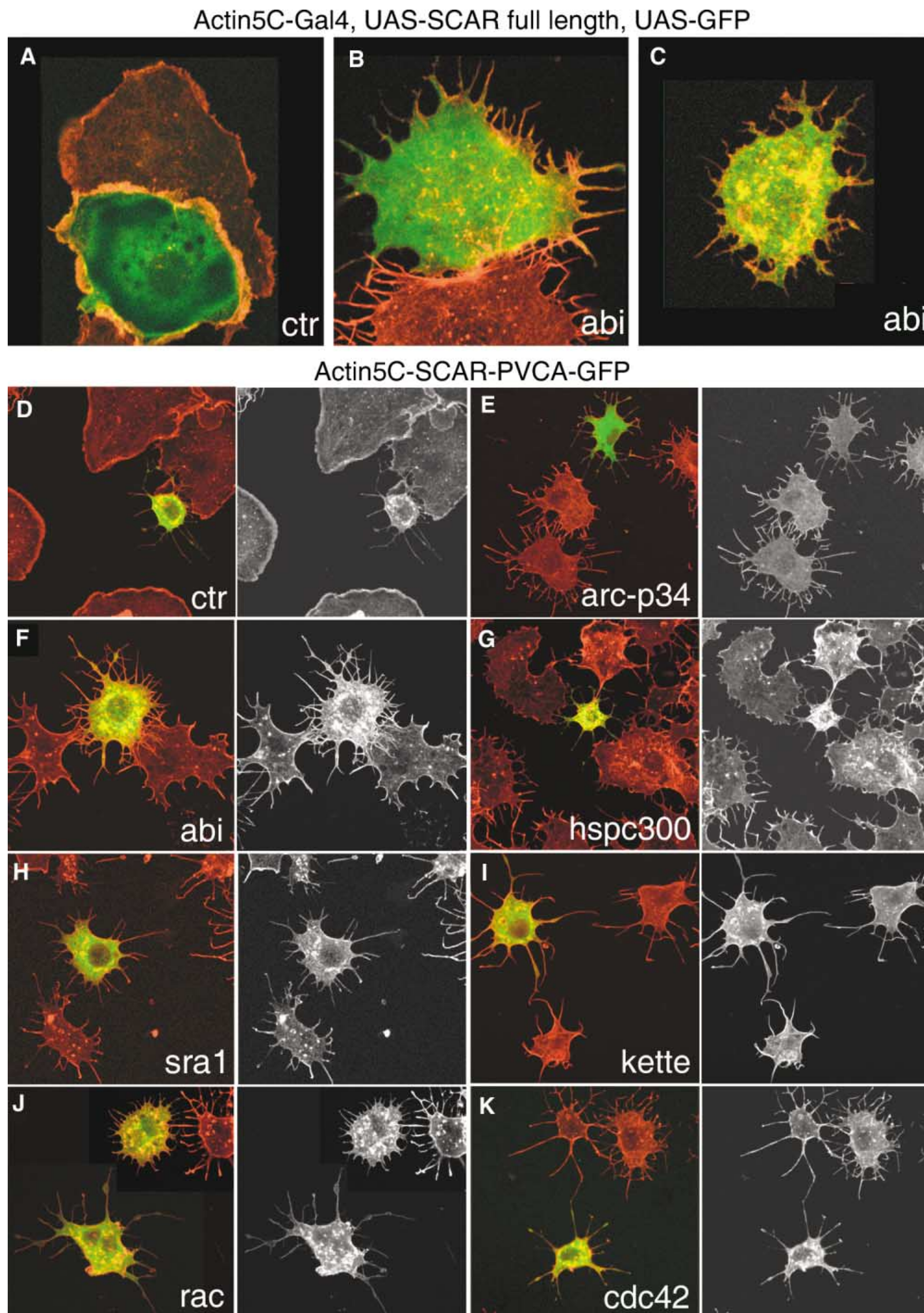

Figure 5. The C-Terminal SCAR PVCA Domain Is Able to Activate the Arp2/3 Complex in the Absence of Complex Components

DsRNA-treated cells were transfected with full-length SCAR (Actin5C-Gal4, UAS-SCAR, UAS-GFP) (A–C) or SCAR-PVCA-GFP (D–K). Actin filaments were visualized (red and white) in (A and D) control, (B, C, and F) Abi, (E) Arc-p34, (G) HSPC300, (H) Sra1, (I) Kette, (J) Rac1, Rac2 and Mtl, and (K) Cdc42 RNAi cells. Full-length SCAR was unable to rescue the Abi^{RNAi} phenotype (B) and induced actin filament formation in the center of control or Abi^{RNAi} cells if highly expressed (C). Perinuclear aggregates of F-actin were also seen in cells transfected with SCAR-PVCA-GFP (D, F–K). In this case, SCAR-PVCA-GFP (green) was seen to colocalize with accumulation of F-actin (in red; colocalization is in yellow) and with components of the Arp2/3 complex (data not shown). Note that SCAR-PVCA (or full-length SCAR) was unable to induce ectopic actin filament formation in Arc-$p34^{RNAi}$ cells. Expression of SCAR or SCAR-PVCA was frequently accompanied by a loss of F-actin from the cell periphery. It may therefore compete with endogenous SCAR for components of the actin polymerisation machinery.

plex were found to colocalize with clumps of GFP and F-actin in these cells (data not shown). Having established this gain-of-function assay, we tested whether additional components of the signaling cascade are required for Arp2/3 activation. SCAR-PVCA was still able to generate ectopic actin filaments in cells treated with

Abi, Kette, HSPC300, Rac, or Cdc42-specific dsRNA (Figures 5D–5K). Thus, although Abi, Kette, Sra1, and HSPC facilitate the generation of actin-based protrusions, they are not required for efficient activation of the Arp2/3 complex by SCAR.

Discussion

In this study we used RNAi to identify a set of genes, including SCAR and Abi, that are essential for the generation of actin-based cellular protrusions in an adherent *Drosophila* cell line (Figures 1A–1I). Although this analysis delineated a putative pathway (Cdc42>Rac>SCAR> Arp2/3 complex) that promotes the nucleation of actin filaments [1], it was not clear where to place Abi within this signaling hierarchy. A recent biochemical study, however, noted that Abi copurifies with mammalian homologs of Kette, Sra1, and HSPC300 as part of a regulatory SCAR complex in extracts from mammalian brains [12, 33]. By using RNAi to test the functions of the equivalent *Drosophila* proteins, we found that Abi, Sra1, and Kette are essential for the generation of protrusions and for the stability of SCAR protein. Similarly, in parallel studies in *Drosophila* [24] and in *Dictyostelium* [34], reduced levels of SCAR protein were observed in cells lacking individual components of the complex. These data suggest that the presence of SCAR in a regulatory complex and its sensitivity to degradation have been highly conserved during evolution. Furthermore, the idea that these proteins form a physical complex in *Drosophila* is supported by the colocalization of Kette and SCAR at the tips of protrusions (Figure 3F). Our data concerning the function of the fifth component of the putative SCAR complex, HSPC300 [12, 33], were more equivocal. Although treatment of S2R+ cells with HSPC300 dsRNA compromised their ability to form lamellipodia and caused a reproducible, if partial, reduction in SCAR protein levels, we were not able to measure the extent of RNAi-mediated HSPC300 silencing. Therefore, although our data support a role for HSPC300 in the regulation of SCAR, we cannot yet determine whether it is absolutely required for the generation of SCAR-dependent protrusions, as are Abi, Kette, and Sra1.

Given the apparent sensitivity of SCAR to proteolysis, changes in local or global SCAR stability could modulate the rate of actin filament formation. Furthermore, if SCAR is released from the complex after the binding of Rac-GTP, as predicted [12], SCAR degradation could also act as a brake to limit SCAR-dependent actin filament nucleation [34]. In either case, one would expect SCAR protein to exhibit a relatively short half-life in vivo. In actively ruffling S2R+ cells, however, SCAR appears to be relatively stable because proteasome inhibitors or inhibitors of transcription or translation have little effect on SCAR protein levels (Figure 3C and data not shown). These findings lead us to conclude that most SCAR is present in stable complexes in wild-type cells. For this reason, the conserved instability of SCAR protein may simply provide cells with a mechanism to rapidly eliminate free, nascent, or mislocalized SCAR, protecting them from the potentially adverse effects of this potent, constitutively active protein. Nevertheless, under special circumstances or in other cell types [34], proteasome-mediated degradation of SCAR may help to limit the extent of actin filament nucleation induced after a burst of Rac-GTP (see below).

Although Abi, Kette, and Sra1 are required for preventing SCAR degradation, our data clearly point to their having additional functions. Most importantly, the morphological changes observed in Abi^{RNAi} cells precede the loss of SCAR protein (Figure 4). In addition, increasing the SCAR protein levels in Abi^{RNAi} cells fails to rescue their morphological defects (Figures 3D and 5B); it also fails to do so in $Kette^{RNAi}$ and $Sra1^{RNAi}$ cells (data not shown). This might seem unexpected given that SCAR is able to activate the Arp2/3 complex on its own, both in vitro [7, 10, 11] and in *Drosophila* cells (this study). In the absence of complex components, however, SCAR fails to become properly localized at the cell cortex (Figure 3E). So, by localizing SCAR at the cortex, the complex may play a critical role in harnessing its activity for the generation of protrusive force.

Three recent genetic studies reported observations that conflict with data presented here and by Rogers and Vale [24]. In particular, data presented in these studies show that Sra1/Kette and SCAR display an antagonistic relationship in the *Drosophila* nervous system [18, 22] and in *Dictyostelium* [34]. Although more work will have to be done to unravel such apparently contradictory data, some of these discrepancies may reflect differences in the relative levels of SCAR and components of the inhibitory complex in the model system under investigation. If the total cellular pool of Abi, Sra1, and Kette is bound up in stable, Rac-responsive SCAR complexes, a reduction in the level of any one component will lead to a reduction in Arp2/3-dependent actin nucleation (as observed in this study and in [24]). On the other hand, if Abi, Kette, and Sra1 are present in excess of SCAR, they will limit the ability of free, active SCAR to nucleate actin filaments (as in [18, 22]).

Conclusions

Below, we outline a speculative model for the regulation of SCAR. This model attempts to reconcile our findings with data from recent in vitro [12] and genetic studies [18, 22, 34]. We propose that nascent SCAR is rapidly incorporated into an inhibitory complex that contains Abi, Sra1, and Kette [12, 33] and protects the protein from proteolysis. The complex localizes at the cell cortex ([28, 22] and this study), where it is responsive to Rac signaling. The binding of Sra1 to Rac-GTP [19, 20] may induce a transient change in the makeup [12] or conformation of the complex [12], which may free the SCAR VCA domain to interact with the Arp2/3 complex, whose activation triggers a burst of new actin filament formation. Finally, the extent of actin filament formation in response to a pulse of Rac-GTP may be limited by the presence of the inhibitory complex and to a lesser extent by proteasome-mediated degradation [34]. In summary, we propose that cells regulate SCAR stability, localization, and activity to ensure that actin nucleation and the formation of cellular protrusions are precisely regulated in time and space.

Experimental Procedures

Drosophila S2R+ Cell Culture Methods

Drosophila S2R+ cells were propagated and treated with dsRNA as previously described [23, 35]. For dsRNA synthesis, primer sequences flanked with T7 sites were chosen for amplification of 300–600 bp of exonic sequence with <21 bp stretches of identity with any other gene. PCR products were then used as a template for the MEGAscript T7 reaction. In the case of SCAR, Abi, WASP, and HSPC300, different portions of the genes were targeted via RNAi with identical results. Control experiments were carried out in parallel without dsRNA. Cells were grown for 4–7 days before being harvested for microscopic analysis or Western blotting. After approximately 2 weeks of growth, cells were able to recover from RNAi. In ongoing screens in the laboratory, Abi was found to share the SCAR/Cdc42 RNAi phenotype, identified in an earlier study [23]. The *Drosophila* SCAR-PVCA fragment, representing amino acids 238–611, and full-length SCAR were PCR amplified from a SCAR cDNA. SCAR-PVCA was GFP tagged (Clonetech pEGFP-N1) and inserted into a modified pFastBac vector (Life Technologies) that includes the *Drosophila* actin5C promoter (gift from Aaron Straight). Fugene 6 was used for transfecting constructs into cells after 3–4 days of RNAi, and F-actin and GFP were visualized 15–24 hr later. Actin5C-Gal4 was used to drive expression of UAS-GFP or UAS-Rac1V12 (the latter was a gift from Liqun Luo). UAS-GFP was included at one-tenth the concentration. For F-actin visualization in live cells, the modified baculovirus was used to express a fragment of moesin fused to GFP (gift from Dan Kiehart). Virus particles were added to media, and infected S2R+ cells were replated 15–24 hr later. Cells were imaged on a Nikon 2000E microscope with a 40× lens at 60 s intervals as they spread on a serum-coated cover slip. Two consecutive representative images of cells are shown in Figures 2A–2D in the first minutes after adhesion. (See Movies 1A–1D in the Supplemental Data).

Immunofluorescence and Western Blotting

To visualize F-actin, we replated cells on serum-coated slides for 1–2 hr before fixation in 4% formaldehyde in PBS for 10 min and stained them with TRITC-labeled phalloidin as previously described [23]. For SCAR and Kette localization, S2R+ cells were fixed as above or washed in cytoskeletal buffer (10 mM PIPES [pH 6.1], 138 mM KCl, 3 mM $MgCl_2$, 2 mM EGTA, and 0.32 M sucrose) and fixed for 20 min in buffer with 4% formaldehyde. Cell lysates were processed for Western blotting as previously described [23]. SCAR antibody (a gift from Jen Zallen) was used at 1:100; Ena antibody (Iowa Hybridoma Bank) was used at 1:100; and anti-α tubulin antibody (GM1, Sigma) was used at 1:500. Kette anitibody (a gift from Christian Klambt) was used at 1:2000 or 1:300 for immunofluorescence; Sra1/CYFIP antibody (a gift from Angela Giangrande) was used at 1:1000. To inhibit the proteasome, we added 5 μM lactacystin (Sigma) and 1 μM VELCADE (a gift from Millenium) in DMSO to cells for 4 or 8 hr. DMSO was added to the control. Similarly, we added cyclohexamide or actinomycin to cells for 4–8 hr to inhibit translation and transcription, respectively. Over this period, inhibitors had no discernable affect on the form of S2R+ cells or on F-actin distribution.

Supplemental Data

Four movies are available with this article online. These four movies show control (S1A), Abi^{RNAi} (S1B-C), and SCARRNAi (S1D) S2R+ cells expressing GFP-moesin as they adhere to a serum-coated cover slip. Images were taken every 60 s, and stills from these movies are shown in Figures 2A–2D.

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