Dynein-mediated apical localization of crumbs transcripts is required for Crumbs activity in epithelial polarity

Zhouhua Li,1 Liwei Wang,1 Thomas S. Hays,3 and Yu Cai1,2
1Temasek Lifesciences Laboratory and 2Department of Biological Science, National University of Singapore, Singapore 117604
3Department of Genetics, Cell Biology, and Development, University of Minnesota, Minneapolis, MN 55455

Introduction

Metazoan epithelial cells are polarized along the apicobasal (A/B) axis and this polarization is important for the formation and function of the epithelial structures they comprise (Tepass et al., 2001; Knust and Bossinger, 2002; Nelson, 2003; Macara, 2004; Shin et al., 2006). Along with this polarization, their plasma membranes are compartmentalized into several distinct domains with different protein complexes differentially localized along the A/B axis, including the Crumbs (Crb) complex (Crb, Stardust [Sdt], and Patj/Dlt), the PAR complex (PAR-3, PAR-6, and aPKC), and the Scribble complex (Discs large, Lethal giant larvae, and Scribble). Genetic studies in Drosophila melanogaster have revealed that these protein complexes function in a sequential yet interdependent manner to regulate the establishment, elaboration, and maintenance of cellular polarity (Tepass et al., 2001; Bilder et al., 2003; Tanentzapf and Tepass, 2003).

The transmembrane protein Crb localizes on the apical domain and acts as an apical domain determinant by organizing a protein network that regulates A/B polarity (Tepass et al., 1990; Wodarz et al., 1995). However, it remains unclear how Crb is targeted onto the apical domain. Here, we show that the cytoplasmic dynein complex transports both Crb protein and transcripts to the apical domain of Drosophila melanogaster follicular cells (FCs). The crb 3′ untranslated region (UTR) is necessary and sufficient for the apical localization of its transcript and this apical transcript localization is crucial for crb function. In crb mutant FCs, Crb protein derived from transgenes lacking the 3′ UTR does not effectively localize to the apical domain and does not effectively restore normal epithelial polarity. We propose that dynein-mediated messenger RNA transport coupled with a localized translation mechanism is involved in localizing Crb to the apical domain to mediate epithelial apicobasal polarity and that this mechanism might be widely used to regulate cellular polarity.

Results and discussion

To identify genes that regulate epithelial polarity, we performed a mosaic screen in D. melanogaster follicle cells (FCs) and identified mutants in D. melanogaster dynein heavy chain 64C

Abbreviations used in this paper: A/B, apicobasal; Co-IP, coimmunoprecipitation; Crb, Crumbs; Dhc64C, Drosophila melanogaster dynein heavy chain 64C; EMS, ethyl methanesulfonate; FC, follicle cell; MT, microtubule; Sdt, Stardust; UTR, untranslated region; wt, wild type.

The online version of this paper contains supplemental material.

Correspondence to Y. Cai: caiyu@ill.org.sg

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showed cytoplasmic localization (100%, A–F). The adherens junction (Armadillo; 100%, FCs are polarized along the A/B axis and form a single layer for screen and mapping details). In the wild type (wt), the loss of A/B polarity (Fig. 1, B, C, E, and F). Examination of the apical region in both Dhc64C mutants (100%, n > 1,000; Fig. 1, A and D). However, Dhc64C and Glued mutants often became multiple layered when located at the posterior end of the follicle after stage six (98%, n = 125), which indicates a loss of A/B polarity (Fig. 1, B, C, E, and F). Examination of known polarized membrane markers showed that Dhc64C and Glued mutants primarily affect apical localization of the Crb complex without markedly affecting the localization of other polarity complexes regardless of the position and developmental stage of the mutant clones induced. Crb localizes to the apical region in wt FCs (100%, n > 1,000) but is absent from the apical region in both Dhc64C and Glued mutant FCs (100%, n = 245; Fig. 2, A–C), whereas Sdt and Patj/Dlt showed cytoplasmic localization (100%, n = 156; see Fig. 4, A–F). The adherens junction (Armadillo; 100%, n = 175), as well as the PAR complex (aPKC; 100%, n = 212) largely retained their normal localization, although their levels were somewhat reduced (Fig. 2, D–I); the lateral Scribble complex was slightly expanded into the apical domain (100%, n = 260; Fig. 2, J–L). Identical phenotypes were also observed in previously identified alleles (Dhc64C and Glued) or when p25 (another subunit of dynactin) function was compromised using double-stranded RNA–mediated knockdown (termed p25RNAi; Fig. S2 and not depicted). Although recent data showed that dynein activity is required for Bazooka (Baz, the fly homologue of Par3) localization in embryonic epithelia (Harris and Peifer, 2005), our data favor the model that dynein functions primarily through the Crb complex to establish FC polarity. This may reflect the different requirement of these two systems. A wt Dhc64C transgene fully rescued the polarity defects in Dhc64C mutant FCs (100%, n > 500; Fig. S1). Dynein and dynactin form a complex that mediates microtubule (MT)-based transport and both Dhc64C and Glued mutants showed virtually identical polarity defects. For simplicity, we show data mainly for Dhc64C.

The dynein complex transports cargos toward MT minus ends. To examine the effects of disrupting the MT cytoskeleton, we used colchicine to depolymerize MTs. Similar polarity defects were observed in these FCs with specific loss of Crb from their apical domains without markedly affecting other cell polarity complexes (100%, n > 200 for each marker; Fig. 2, M–P). Furthermore, the MT cytoskeleton is largely unaffected in dynein mutant FCs (unpublished data), which is consistent with the notion that the observed polarity defects seen in dynein mutant and MT-depolymerized FCs are caused by defective dynein-mediated transport.

We next investigated how dynein mediates the apical localization of Crb. Restricted mRNA localization coupled with local translation is widely used to generate cellular asymmetry. It has been reported that crb mRNA localizes apically in embryonic epithelia (Tepass et al., 1990). This prompted us to investigate whether dynein functions via the localization of crb mRNA to localize Crb protein.

In the wt, crb mRNA is highly enriched on the apical domain. However, in the dynein mutant, crb mRNA is no longer apically enriched (100%, n = 241; Fig. 3, A and B; and Fig. S3, available at http://www.jcb.org/cgi/content/full/jcb.200707007/DC1). Consistent with the notion that dynein-mediated transport requires an intact MT cytoskeleton, crb mRNA is mislocalized in colchicine-treated FCs (100%, n = 127; Fig. S3). Together, these data show that crb mRNA localizes to the apical domain via dynein-mediated transport.

Because of difficulties in studying how dynein transports crb mRNA in FCs, we examined crb mRNA localization in blastoderm embryos, as it has been found that dynein-mediated transport is conserved between embryogenesis and oogenesis (Karlins-Mcginness et al., 1996; Bullock and Ish-Horowicz, 2001). We took advantage of a functional crb minigene, crb intra-myc (this minigene contains the crb 3′ untranslated region [UTR]; see Fig. S2 for a schematic presentation of the transcripts used in this study; Wodarz et al., 1995). Fluorescently labeled crb intra-myc transcripts rapidly localized to the apical domain after injection into the basal cytoplasm of embryos (100%, n = 13; Fig. 3 C). However, preinjection with the anti-Dhc64C antibody P1H4 (100%, n = 12) or colchicine (100%, n = 9) but not anti-Myc antibody (100%, n = 8) completely blocked apical localization of crb intra-myc.
transcripts (Fig. 3, D–F). These data demonstrate that dynein activity is required for the apical localization of in vitro labeled crb transcripts in embryos.

To test whether the crb 3’ UTR mediates its localization, we injected crb intra-myc-wt into the crb 3’ UTR was replaced by the 3’ UTR of the SV40 large T antigen, into blastoderm embryos (Simmonds et al., 2001). These transcripts did not localize apically, which suggests that the crb 3’ UTR is required for its apical localization (100%, n = 9; Fig. 3 G). In a complementary experiment, we fused the crb 3’ UTR to an exogenous β-galactosidase gene and found that this chimeric transcript localized apically (100%, n = 10; Fig. 3 H) when injected into the embryo, whereas control β-galactosidase transcripts (lacZ-SV40 3’ UTR) did not (100%, n = 8; Fig. 3 I). We conclude that the crb 3’ UTR is necessary and sufficient for its apical localization in embryos. We next investigated whether the crb 3’ UTR also mediates its apical localization in FCs. We again used crb intra-myc and crb intra-myc-wt minigenes and the subcellular localization of these transcripts was determined by RNA in situ. When ectopically expressed in wt FCs, crb intra-myc transcripts were predominantly found on the apical domain, whereas crb intra-myc-wt transcripts were unlocalized (Fig. 3, J and K; and Fig. S3, compare with endogenous crb transcripts in Fig. 3 A). Together, these results demonstrate that the crb 3’ UTR mediates apical localization of crb transcripts in FCs.

Next, we investigated whether apical localization of crb transcripts is a prerequisite for Crb protein localization. Both transgenes are expressed at an equivalent level when driven by the same driver in both FCs and embryos, which suggests that the different 3’ UTRs do not affect protein expression levels (Fig. S2 F). In wt FCs, both crb intra-myc transcripts and Crb intra-myc protein localized to the apical domain (100%, n > 200; Fig. 3, J and L). Interestingly, in wt FCs expressing crb intra-myc-wt, crb intra-myc-wt transcripts did not apically localize (Fig. 3 K), yet Crb intra-myc-wt protein remained localized to the apical domain in the great majority of FCs (95%, n = 73) in several independent transgenic lines (Fig. 3 M and not depicted). Only in ~5% (n = 73) of the FCs examined did Crb intra-myc-wt protein show cytoplasmic localization (Fig. 3 N). These results suggest that apical localization of Crb protein can be independent of apical transcript localization and that dynein also transports Crb protein to the apical domain. It was found that endogenous oskar mRNA can direct the proper localization of exogenous transcripts derived from a transgene bearing the oskar 3’ UTR during D. melanogaster oogenesis (Hachet and Ephrussi, 2004). Thus, it is possible that endogenous crb transcripts/protein may have an impact on the behavior of these transgene products. Therefore, we examined the behavior of the transgene products in a crb mutant background where endogenous Crb protein is absent. Interestingly,
The localization of the Crb transcripts is required for effective Crb function in epithelial polarity. Furthermore, these results suggest that a localized translational machinery near the apical domain may be involved in the generation of full Crb activity on the apical domain.

It has been found that, in embryonic epithelial cells, Crb binds Sdt and the two are mutually dependent for their localization and function (Tepass and Knust, 1993; Bachmann et al., 2001; Hong et al., 2001). Crb protein levels are markedly reduced in the sdt mutant and vice versa. Our data show that in dynein mutant FCs, Crb is undetectable by immunofluorescence, whereas Sdt is mainly cytoplasmic (Fig. 4, A–C). There are several possibilities for the inability to detect Crb in the dynein mutant. First, the apical localization and stability of Crb requires dynein activity. In the absence of dynein function, unlocalized Crb is not stable and is degraded. Second, the apical localization but not stability of Crb requires dynein activity. In the dynein mutant, Crb may be uniformly distributed in the cytoplasm and hence fall below the threshold of detection by immunofluorescence. To address these possibilities, we took advantage of MT-depolymerized although Crb intra-myc efficiently localized to the apical domain in the crb mutant (100%, n = 66), Crb intra-myc-wo showed largely cytoplasmic localization in 64.3% (n = 45) of the crb mutants (Fig. 3, O and P). Thus, endogenous wt crb product is required, directly or indirectly, for the apical localization of exogenous Crb intra-myc-wo, although the reasons for this remain unclear. These data suggest that, in crb mutant FCs, Crb intra-myc-wo protein, derived from transcripts that do not apically localize, is not effectively localized to the apical domain.

Thus far, we have shown that apical localization of crb mRNA contributes to the apical localization of Crb protein. Does this have any functional relevance? Consistent with previous reports that Crb intra-myc can rescue crb mutant defects in embryonic epithelial cells (Wodarz et al., 1995), apically localized Crb intra-myc recruits Sdt to the apical region and fully rescues the polarity defects in crb mutant FCs (100%, n = 55; Fig. 3 Q). However, in 60% (n = 20) of crb mutant FCs expressing crb intra-myc-wo Sdt remains in the cytoplasm and polarity defects are not rescued, which is consistent with the Crb intra-myc-wo localization in crb mutant FCs (Fig. 3 R). These data strongly indicate that apical localization of the crb transcripts is required for effective crb function in epithelial polarity. Furthermore, these results suggest that a localized translational machinery near the apical domain may be involved in the generation of full Crb activity on the apical domain.

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FCs that mimic the effects of dynein mutants (Fig. 2, M–P). Interestingly, although no protein can be detected in these FCs by immunostaining, normal levels of Crb protein are detectable by Western blotting (Fig. 4 L), which is consistent with the notion that the apical localization but not stability of Crb requires dynein activity. Furthermore, in the crb mutant, Sdt also displays cytosolic localization (Fig. 4, G–I), suggesting that apical localization but not stability of Sdt protein requires Crb activity.

We next examined whether Crb and Sdt form a complex when in the cytosol. When expressed in wt FCs, Crb intra-myc largely colocalizes with Sdt (Fig. 4 J). In addition, an anti-Myc antibody can bring down Sdt in coimmunoprecipitation FCs that mimic the effects of dynein mutants (Fig. 2, M–P). Immunosblotting with an anti-Crb antibody (lane 1) wt (crb intra-myc expressed in wt background) input (10%). (lane 2) Anti-Myc immunoprecipitation from wt sample. (lane 3) Anti-Flag immunoprecipitation (negative control) from wt sample. (lane 4) Anti-Myc immunoprecipitation from a colchicine-treated sample. (lane 5) Anti-Flag immunoprecipitation (control) from a colchicine-treated sample. Bars, 5 μm.
(Co-IP) experiments (Fig. 4 M, lane 2). These suggest that in the wt, Cbr\textsuperscript{intra-myc} forms a complex with Sdt. In the \textit{crb} mutant, whenever Cbr\textsuperscript{intra-myc-wo} apically localized, it colocalized with Sdt. However, when Cbr\textsuperscript{intra-myc-wo} was found in the cytosol, it did not colocalize well with Sdt (unpublished data), which suggests that these two molecules may not form a complex when not localized. To test this possibility, we again took advantage of colchinic-treated FCs. When ectopically expressed in colchinic-treated FCs, Cbr\textsuperscript{intra-myc} does not bring down Sdt in Co-IP experiments, which suggests that these proteins do not form a complex when both are cytoplasmic (Fig. 4 M). Similarly, an anti-Crb antibody could bring down Sdt in wt FCs but not p25\textsuperscript{RNAi} FCs, confirming that colchinic-treated FCs actually reflect loss of dynein activity and not other MT-based activity (Fig. S2). Collectively, these data suggest that Crb and Sdt form a complex on the apical cortex and that this complex is stabilized, directly or indirectly, by dynein activity.

When expressed in wt FCs (in the presence of wt \textit{crb}), Cbr\textsuperscript{intra-myc} localizes apically and forms a complex with Sdt. However, in dynein mutant FCs (in the presence of wt \textit{crb}), Cbr\textsuperscript{intra-myc} is localized to the cytoplasm and does not form a complex with Sdt (Figs. 4 and S2). Our data suggest that in the wt, an endogenous Cbr product is required, directly or indirectly, for the apical localization of exogenous Cbr\textsuperscript{intra-myc} protein. It is possible that apically localized endogenous Cbr could be involved in this process via its requirement for A/B polarity, which in turn directs exogenous Cbr\textsuperscript{intra-myc} apical localization and subsequently allows the formation of the Crb–Sdt complex. In the dynein mutant, although endogenous Cbr is still present (as confirmed by Western blotting), it is not localized apically. As a result, A/B polarity is not properly established. Consequently, exogenous Cbr does not form a complex with cytoplasmic Sdt when in the cytosol.

Our data suggest that dynein transports both Cbr protein and mRNA to the apical domain, where Cbr protein interacts with Sdt to form an apical complex. The \textit{crb} 3' UTR is necessary and sufficient for the apical localization of \textit{crb} mRNA. The apical localization of \textit{crb} mRNA appears to be crucial for \textit{crb} function. \textit{crb}\textsuperscript{intra-myc} produces transcripts and protein that apically localize and can fully rescue the polarity defects associated with \textit{crb} mutant FCs. In contrast, \textit{crb}\textsuperscript{intra-myc-wo} produces transcripts and proteins that do not fully localize apically and show reduced ability to rescue the polarity defects of \textit{crb} mutant FCs. Based on these observations, we propose that localized transcripts coupled with a local translation mechanism contribute to the apical localization of Cbr and its ability to mediate epithelial polarity.

In general, transmembrane proteins are cotranslationally produced and inserted into the ER and trafficked via the exocytic pathway to generate epithelial polarity. As the \textit{crb} 3' UTR is highly conserved through evolution (unpublished data), together with the conserved function of MT-based dynein activity, our results suggest that this directional transport of mRNA plus local translation may be a widely used mechanism to generate epithelial polarity.
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anti-c-myc (9E10, 1:500), mouse anti-Dhc64C (P1H4), and rabbit and mouse anti-

Center for Developmental Biology, Kobe, Japan), guinea pig anti-Scrib

anti-aPKC (1:1,000), mouse anti-Flag (1:2,000; Santa Cruz Biotechnology,

To depolymerize the MT cytoskeleton, 100 μg/ml colchicine (Sigma-Aldrich) was injected

In situ hybridization

crb template was amplified using Crb-S' (ATACGGCCAAGGAGGACG)

and Crb-S' (CTAAATGTCGGCCTCTCCGGC) primers. Probes were digoxi-

Capped fluorescent RNA synthesis and injection

crb or crbmycmyc were amplified from transgenic flies (Wodarz et al., 1995) using crb-S' (GGGAATGGAAAGTTTCCCGCACCAAACTGACGT) and crb-S' (GGCTCAGGTACAAAATTGGTTATTAGTG) and crbmycmyc (AGATCCTC-

Embryo injections were performed with 200 ng/μl of labeled RNA. To depolymer-

Immunocytochemistry

Ovaries were fixed according to standard protocols. Anti-Crb (C44, 1:50), anti-Arm (N2 71A1, 1:50), Developmental Studies Hybridoma Bank), rabbit anti-pJUPC (1:1,000), mouse anti-Flag (1:2,000; Santa Cruz Biotechnology, Inc.), rabbit anti-pShit/Dim (1:1,000; Bhat et al., 1999), rabbit anti-Rabz (1:1,000; a gift of F. Matsuzaki, Institute of Physical and Chemical Research Center for Developmental Biology, Kobe, Japan), guinea pig anti-Scrub (1:1,000; a gift of D. Bilder, University of California, Berkeley, Berkeley, CA), rabbit anti-Sdt (1:1,000; Bachmann et al., 2001), rabbit and mouse anti-c-myc (PE10, 1:500), mouse anti-a-tubulin (1:1,000; Sigma-Aldrich), mouse anti-Dhc64C (P1H4), and rabbit and mouse anti-galectosidase (Invitrogen). Fluorescently conjugated goat anti-mouse and rabbit secondary antibodies and rhodamine phalloidin were used (Invitrogen).

Microscopy

Samples were mounted in Vectashield mounting medium (Vector Laboratories). Images were collected using a microscope (Axioplan 2) with an upright confocal system (LSM510 META; both from Carl Zeiss, Inc.) at room temperature. The objective lens used was a Plan NEOFUAR 40× 1.3 oil and the imaging software used was Zeiss LSM510 (both from Carl Zeiss, Inc.). The confocal images were extracted with LSM510 browser software (Carl Zeiss, Inc.) and then processed in Photoshop 7.0.1 (Adobe). Scale bars are indicated in each individual image.

Immunoprecipitation

Ovaries with corresponding genotype were dissected in S2 culture medium (Invitrogen) and collected in PBS buffer. Protein extract and CoIP experiments were performed according to standard protocols and probed with corresponding antibody (Fig. 4, L and M) and detected with an ECL kit (Thermo Fisher Scientific).

Online supplemental material

Fig. S1 shows molecular lesions of Dhc64C202 and Glued1 and the rescue of apical Crb localization in the Dhc64C202 mutant by a Dhc64C transgene. Fig. S2 shows phenotypic analyses of p25TM4 FCS, schematic presentation of the transcripts used in this study, and that Crb forms a complex with Sdt on the apical domain. Fig. S3 shows that apical localization of crb transcripts depends on dynein function in FCS. Online supplemental material is available at http://www.jcb.org/cgi/content/full/jcb.200707007/DC1.

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Note added in proof. A complementary study examining the contributions of Dynein and Sdt mRNA localization to A/B polarity regulation has been performed (Home-Badonvica, S., and D. Bilder. 2007. PLoS Genet. doi:10.1371/ journal.pgen.0040208).

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(Drosophila), and gel was recovered using standard kits (GE Healthcare). The fragments were sequenced using a set of sequencing primers. Sequencing of the Dhc64C202 mutant identified a premature stop codon at Trp 173 causing a truncated product before the DHC_N2 domain and deleting all four ATPase domains. Furthermore, no signaling could be detected using an anti-Dhc64C antibody that recognizes the N-terminal region of Dhc64C polypeptide in immunofluorescent staining, which suggests that this is a protein null allele. There is also a premature stop codon identified in Glued1 mutant (Gln121 stop), which lies in the second coiled-coil domain implicated for protein–protein interactions (Fig. S1).

MT drug treatment

To depolymerize the MT cytoskeleton, flies were starved at 25°C for 2.5 h and fed with 200 μg/ml colchicines for 24 h before dissection.

Generation of crbmycmyc transgene stocks

crbmycmyc was amplified from a crbmycmyc transgene fly and cloned into a pUAST vector. This construct was microinjected into FRT182-crb114D2/Tmd6b embryos after standard transgene protocol to generate crbmycmyc transgene stock. Three independent lines were selected for further analysis. Hs-eGFP; Ayal-Gal4-uar-lacZ was used to drive the expression of transgenes in FCS.

In situ hybridization

crb template was amplified using Crb-S' (ATTACGCCCAAGGAGGACG) and Crb-S' (CTAAATGTCGGCCTCTCCGGC) primers. Probes were digoxi-

Microscopy

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