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A novel role for an APC2-Diaphanous complex in regulating actin organization in *Drosophila*

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The rearrangement of cytoskeletal elements is essential for many cellular processes. The tumor suppressor Adenomatous polyposis coli (APC) affects the function of microtubules and actin, but the mechanisms by which it does so are not well understood. Here we report that *Drosophila* syncytial embryos null for *Apc2* display defects in the formation and extension of pseudocleavage furrows, which are cortical actin structures important for mitotic fidelity in early embryos. Furthermore, we show that the formin Diaphanous (DIA) functions with APC2 in this process. Colocalization of APC2 and DIA peaks during furrow extension, and localization of APC2 to furrows is DIA-dependent. Furthermore, APC2 binds DIA directly through a region of APC2 not previously shown to interact with DIA-related formins. Consistent with these results, reduction of *dia* enhances actin defects in *Apc2* mutant embryos. Thus, an APC2-DIA complex appears crucial for actin furrow extension in the syncytial embryo. Interestingly, EB1, a microtubule +TIP and reported partner of vertebrate APC and DIA1, may not function with APC2 and DIA in furrow extension. Finally, whereas DIA-related formins are activated by Rho family GTPases, our data suggest that the APC2-DIA complex might be independent of RHOGEF2 and RHO1. Furthermore, although microtubules play a role in furrow extension, our analysis suggests that APC2 and DIA function in a novel complex that affects actin directly, rather than through an effect on microtubules.

KEY WORDS: Adenomatous polyposis coli (APC), Diaphanous, *Drosophila* syncytial development

INTRODUCTION

Orchestrated cytoskeletal rearrangements play fundamental roles in a diverse array of cellular and developmental processes, from cytokinesis and cell migration to cell shape changes that underlie morphogenesis. The Adenomatous polyposis coli (APC) family of tumor suppressors can influence microtubules, and has been suggested to regulate actin (reviewed by Nathke, 2004). In addition, APC proteins (see Fig. 1A,B) are essential negative regulators of Wnt signaling (reviewed by Logan and Nusse, 2004). Although it is clear that disruption of APC signaling plays a key role in the initiation of colon cancer, excessive Wnt signaling alone may not explain the role of APC in tumorigenesis (reviewed by Nathke, 2004).

APC proteins may affect both microtubules and actin in a variety of ways (reviewed by Nathke, 2004). The microtubule-associated functions of APC proteins have become clearer in recent years with the identification of a role for APC in kinetochore-microtubule interactions (Green and Kaplan, 2003), and as part of a 'cortical template' that directs microtubule network formation (Reilein and Nelson, 2005). Via its Armadillo repeats (Fig. 1A), APC can interact with Kinesin-associated protein 3 (KAP3) and appears to be transported along microtubules to the cortex via KIF3A/3B (Jimbo et al., 2002). The movement of APC along microtubules is enhanced in cell projections involved in migration (Mimori-Kiyosue et al., 2000; Nathke et al., 1996). In cultured cells, mammalian APC promotes microtubule stability (Kroboth et al., 2007), and in some contexts it promotes stability together with the formin DIA1 and the microtubule plus-end tracking protein (+TIP) EB1 (Wen et al., 2004). APC also affects microtubule dynamic instability independent of EB1 (Kita et al., 2006).

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APC2 localizes with actin in multiple contexts in *Drosophila* embryos and epithelial cells (McCartney et al., 1999; Townsley and Bienz, 2000; Yu and Bienz, 1999). Such APC-actin interactions may influence Rho family GTPases, as APC has been shown to interact with the RacGEF ASEF and with IQGAP, an effector of RAC1 and CDC42 in cultured cells (Kawasaki et al., 2003; Kawasaki et al., 2000; Watanabe et al., 2004). Despite many reports of APC-cytoskeletal interactions, the specific mechanisms by which APC proteins affect the cytoskeleton are still poorly understood, particularly in coordinated cytoskeletal rearrangements.

The *Drosophila* syncytial blastoderm is a superb system in which to study dynamic coordinated actin and microtubule rearrangements. Early *Drosophila* embryogenesis is syncytial, with nuclear division occurring without cytokinesis (reviewed by Sullivan and Theurkauf, 1995). By interphase of nuclear cycle 10, most nuclei have migrated to the cortex to form the syncytial blastoderm. These nuclei undergo four rounds of roughly synchronous mitoses (cycles 10-13) before cellularization. During interphase, actin is organized into caps above each nucleus (see Fig. 1C,D) (reviewed by Schejter and Wieschaus, 1993). These caps 'expand' into diffuse rings at the periphery of each nucleus during prophase. Cortical actin becomes focused into tight rings surrounding each nucleus during prophase of cycles 11-13, and the actin extends into the embryo to form pseudocleavage furrows that surround each spindle (Fig. 1E,F). Reaching their maximum depth during metaphase, and quickly retracting during anaphase and telophase, these furrows serve as physical barriers between adjacent nuclei that prevent collisions. Such collisions might otherwise result in abnormal nuclei and nuclear loss from the cortex (reviewed by Sullivan and Theurkauf, 1995). As daughter nuclei reform during telophase, actin redistributes into caps. These cytoskeletal rearrangements continue through each cortical syncytial nuclear cycle.

We previously showed that APC2 localizes with actin caps and pseudocleavage furrows during syncytial development, and that hypomorphic mutations in *Drosophila Apc2* result in nuclear loss without significant defects in actin or microtubule organization

(McCartney et al., 1999; McCartney et al., 2001). This led to a model whereby APC2 facilitates interactions between actin and microtubules that are important for nuclear tethering. Here we show that complete loss of APC2 results in defects in actin pseudocleavage furrow initiation and extension, with no apparent microtubule defects. Further, we demonstrate that APC2 works together with the formin Diaphanous to promote furrow extension, and we propose that this complex affects actin directly. Finally, the novel APC2-DIA complex we describe may function independently of the microtubule +TIP EB1 and RHO1 signaling.

MATERIALS AND METHODS

Fly stocks

The following were used: *Apc2*$^{\Delta S}$ (McCartney et al., 1999), *Apc2*^{d40} and *Apc2*^{g10} (McCartney et al., 2006), *dia*^{5} *FRT40A* (Afshar et al., 2000; Castrillon and Wasserman, 1994), *dia*^{k07135}*/CyO* [Bloomington Stock Center (BSC)], *dia*^{2}*/CyKrGFP* (Castrillon and Wasserman, 1994), *FRTG13 Rho1*^{L3} (Hacker et al., 2003), *FRTG13 RhoGEF2*^{04291} (Hacker and Perrimon, 1998) and wild type (WT; *Oregon-R-S*, BSC). The *Eb1*-null allele (*Eb1*^{B13}) is a deletion resulting from the imprecise excision of a local P-element (L. Lee, personal communication). *FRT Eb1*^{B13} was a gift from P. Kolodziej (Vanderbilt University, Nashville, TN, USA). Mutant embryos maternally *dia*^{5} *FRT40A*, *FRTG13 Eb1*^{B13}, *FRTG13 Rho1*^{L3} or *FRTG13 RhoGEF2*^{04291} were generated using FLP/FRT/DFS (Chou and Perrimon, 1996). During syncytial development, zygotic transcription is not significantly active. Genotypes referred to are maternal, and females were mated to WT males.

Immunolocalization and imaging

Embryo preparation

Embryos were collected for 2 hours (syncytial) or 6 hours (gastrulated) at 27°C, fixed and stained as described by McCartney et al. (McCartney et al., 1999), or were hand devitellinized. Antibodies and labels were as follows. Anti-β-tubulin (E7, 1:500, Developmental Studies Hybridoma Bank), anti-acetylated tubulin (1:350, Sigma), anti-APC2 [1:500 (McCartney et al., 1999)], anti-DIA [1:5000 (Afshar et al., 2000)] and anti-Anillin [1:1000 (Field and Alberts, 1995)]. Secondary antibodies were labeled with Alexa Fluor 488, 568 or 647 (1:1000, Invitrogen). Actin was detected using Alexa Fluor 488-phalloidin (1:500, Invitrogen). DNA was stained with DAPI (1:1000, Sigma) or propidium iodide (25 μg/ml, Invitrogen) for 30 minutes, following a 2-hour incubation with RNase A (10 mg/ml). Embryos were mounted in Aqua-Poly/Mount (Polysciences).

Image acquisition and analysis

Images were acquired with a spinning-disc confocal microscope (Solamere Technology Group) with a Yokogawa scanhead on a Zeiss Axiovert 200M using QED InVivo software. ImageJ and Adobe Photoshop were used for image analysis. *z*-stacks of 0.2 μm optical slices were taken from the apical surface to below the cortical nuclei/microtubules. To generate cross-sections from *z*-stacks, the *x*-*y* image stack was resliced in the *x*-*z* plane in ImageJ (Figs 2, 5, 7 and 8). Alternatively, cross-sectional single images of embryos were acquired (Fig. 4; see Fig. S4 in the supplementary material). Embryos were assigned to nuclear and cell cycle stage according to features of their DNA and/or microtubules.

Live imaging

Dechorionated embryos containing ZeusGFP, a microtubule marker (Morin et al., 2001), were mounted in halocarbon oil (700 series, Halocarbon Products) on PetriPERM dishes (Sigma). Images were acquired every 30 seconds.

Furrow depth analysis

The most apical actin section of the embryo was designated as the 0 μm position, and –0.8, –1.6, and –2.2 μm depths were also analyzed. This captured the entire furrow in the WT. Each slice was scored for complete and incomplete actin rings (i.e. those missing any portion of the ring) (see Fig. S1 in the supplementary material). Five embryos (~200 actin rings) for each genotype were assessed. Statistics employed the binomial approximation of the normal distribution.

Spindle dynamics

We measured the pole-to-pole distance of ten spindles in three embryos for each genotype, from metaphase through anaphase B. We measured the midbodies in telophase. Two iterations of adaptive deconvolution using Autodeblur Gold CF software version X2.1.1 (Media Cybernetics) were performed on the images in Fig. 3D-E′ and Fig. S2K-N (see Fig. S2 in the supplementary material).

Colocalization

We examined the localization patterns of APC2 and DIA in multiple nuclear cycle 12 WT embryos throughout the cell cycle at approximately –1.0 μm. Colocalization was defined as when pixel intensities 150 to 255 were detected in the same position in the APC2 image and the correlated DIA image using the ImageJ Colocalization plug-in (Fig. 4Ae-Fe).

Plasmid construction

N-terminal Glutathione S-transferase (GST) and Maltose-binding protein (MBP) fusions were generated by PCR, followed by subcloning of fragments encoding EB1 (amino acids 1-291), human APC^{BasicEB1bd} (2167-2843), human APC^{Basic} (2167-2674), human APC^{EB1bd} (2673-2843) and *Drosophila* APC^{Basic} (2135-2412) into pGEX-4T1 (GE Healthcare); APC2^{N} (1-490) into pGEX-4T3 (GE Healthcare); APC2^{C} (491-1067), DIA^{N} (1-506) and DIA^{C484} (484-1091) into pLM1 (Pai et al., 1996); EB1 (1-291) and Chickadee (CHIC, *Drosophila* profilin; amino acids 1-126) into pMAL-c2x (NEB). His-DIA^{C519} (pQE80, 519-1091) was provided by H. Muller (Grosshans et al., 2005).

Direct protein-protein interactions

Bound protein (10 μg) was incubated for 1 hour at 4°C with free protein (10 μg) in HKT buffer (Miles et al., 2005) or modified RIPA buffer (Wen et al., 2004) with 1 mM DTT. The amount of protein in the bead (B) lanes was four times that in the input and supernatant (S) lanes. Immunoblots probed with HRP-conjugated anti-MBP (1:10,000, NEB) or anti-DIA (1:10,000) were developed using the HRP SuperSignal West Pico Chemiluminescent Substrate Kit (Pierce).

Immunoprecipitation

WT 0-2 hour embryos (27°C) were lysed in 50 mM HEPES (pH 7.5), 115 mM KAc, 2.5 mM $Mg(Ac)_2$, 0.5% Nonidet P40 substitute (Sigma), 0.5 mM EDTA, 0.5 mM EGTA, 1× Complete protease inhibitor cocktail (Roche), 1× Phosphatase inhibitor cocktail 2 (Sigma). Anti-Myc (DSHB) immunoprecipitations were at 1:40 and anti-APC2 (McCartney et al., 1999) at 1:50. Complexes were precipitated with Protein G-agarose (Zymed). Western blots probed for APC2 (1:1000) or DIA (1:5000) were developed as described above.

RESULTS

Complete loss of APC2 results in furrow extension defects

In our original study of APC2 function in the early embryo (McCartney et al., 2001), the alleles of *Apc2* were not null; both *Apc2*$^{\Delta S}$ and *Apc2*^{d40} produce mutant proteins (McCartney et al., 1999; McCartney et al., 2006). *Apc2*^{g10} is a nonsense mutation that changes amino acid 383 to a stop (Fig. 1B). No truncated protein was detected and the allele acts as a genetic null (McCartney et al., 2006). We previously showed that embryos maternally *Apc2*^{g10} exhibit loss of cortical syncytial nuclei, and this loss was rescued by expression of *P[Apc2*$^{+}$*]*, an *Apc2* cDNA driven by the endogenous promoter (McCartney et al., 2006).

To assess cytoskeletal defects in *Drosophila Apc2* mutants, we examined actin and microtubules in fixed, stage-matched wild-type (WT) and mutant syncytial embryos. Metaphase *Apc2*^{g10} embryos exhibited incomplete actin rings that were occasionally associated with apparent spindle collisions (Fig. 2A, arrow and arrowhead). We also observed incomplete actin rings in *Apc2*^{d40} embryos (Fig. 2A, arrow). To determine whether these reflect defects in furrow extension, we examined stage-matched WT and mutant embryos in

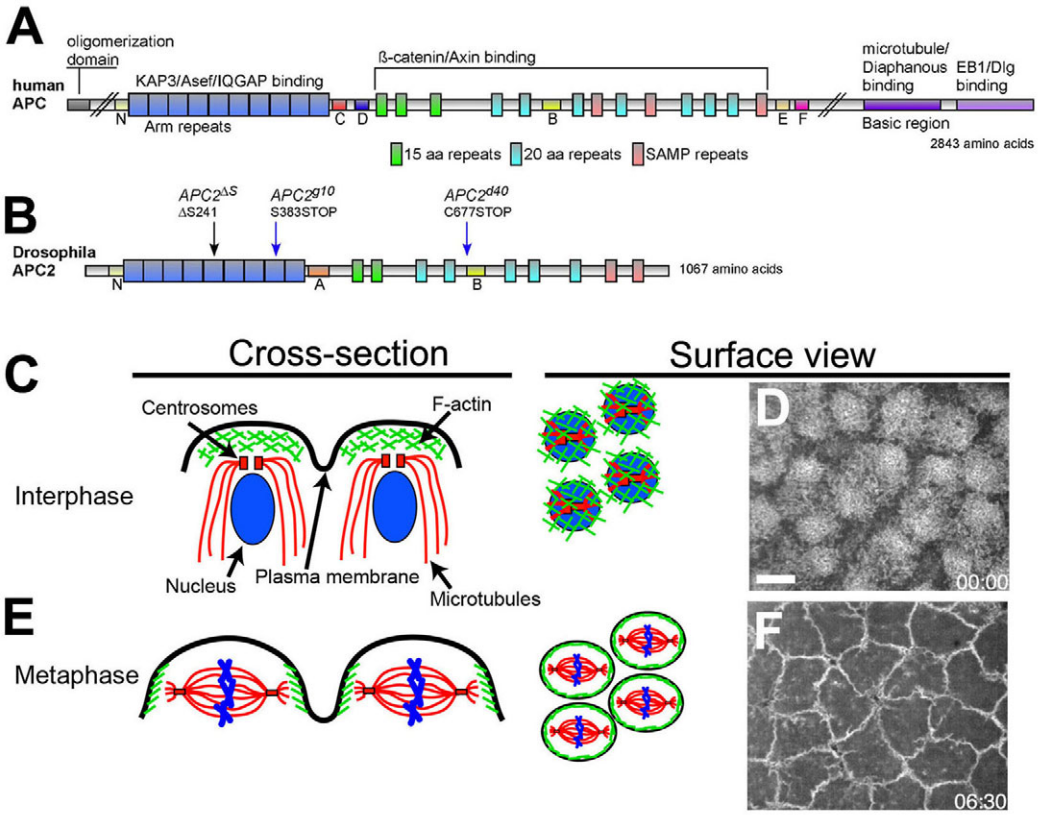

Fig. 1. Schematics of APC proteins and cytoskeletal rearrangements in the *Drosophila* syncytial embryo. (**A**) Multiple domains of mammalian APC interact with the cytoskeleton, either directly or indirectly. (**B**) *Drosophila* APC2 with relevant alleles indicated. Blue arrows, stop codons. A and B are modified with permission from McCartney et al. (McCartney et al., 2006). (**C**) At interphase, cortical actin is organized into apical caps above each nucleus. Centrosomes are located between each nucleus and its actin cap and nucleate microtubules. (**D**) Actin caps in a wild-type (WT) embryo expressing MoesinGFP, an actin marker. (**E**) At metaphase, actin has reorganized into rings (see surface view) that extend into actin pseudocleavage furrows (see cross-section). Centrosomes and microtubules have reorganized into the spindle. (**F**) Actin rings in the same embryo as in D, 6 minutes 30 seconds later. Scale bar: 10 μm.

cross-section. Although furrows initiated in some $Apc2^{d40}$ and $Apc2^{g10}$ embryos, they did not extend normally (Fig. 2B, arrows). By contrast, furrow extension in $Apc2^{\Delta S}$ embryos appeared largely as in WT (Fig. 2B). To quantify actin defects, we assessed actin rings as viewed from the surface at four depths, using the presence of incomplete actin rings as a measure of furrow depth (see Fig. S1 in the supplementary material). This analysis clearly demonstrated a statistically significant increase in the percentage of incomplete rings in the $Apc2^{g10}$ mutants at all depths as compared with WT (Fig. 2C) (P<0.001); this was partially rescued by one copy of $P[Apc2^{+}]$ (Fig. 2C). We observed an equally severe furrow extension defect in $Apc2^{d40}$ mutants (Fig. 2C). $Apc2^{\Delta S}$ mutants exhibited a weak furrow extension defect that was apparent only at –2.2 μm (Fig. 2C). These results suggest that APC2 plays a role in the organization of actin pseudocleavage furrows, and thus has functions beyond spindle tethering (McCartney et al., 2001).

APC2 may influence furrow formation directly through actin, or indirectly through microtubules. APC proteins function in microtubule stability (Kroboth et al., 2007), and although APC2 lacks the direct microtubule-binding domain and the EB1-binding domain (Fig. 1A,B), it could play a similar role in the syncytial embryo. We asked whether stabilized microtubules, characterized by post-translational modifications such as acetylation and detyrosination (reviewed by Hammond et al., 2008), are present in the syncytium. Syncytial blastoderm embryos did not contain acetylated microtubules (Fig. 3A-B″), consistent with previous observations that neither acetylated nor detyrosinated microtubules are found in the early embryo (Warn et al., 1990; Wolf et al., 1988). This suggests that if APC2 normally affects microtubules, it is not affecting their stability.

Anaphase microtubules are important in furrow formation in the subsequent cell cycle (Riggs et al., 2007). We examined *Apc2* mutant microtubule organization and dynamics during cycle 12 anaphase. Spindles in *Apc2* mutants did not exhibit morphological defects (see Fig. S2A-J in the supplementary material), and exhibited the same dynamic behavior as WT (Fig. 3C). Furthermore, non-kinetochore microtubules and astral microtubules in *Apc2* mutants appeared to have the same density and organization as in WT (Fig. 3D-F′; see Fig. S2K-L′ in the supplementary material), as did interphase microtubule arrays (see Fig. S2M,N in the supplementary material). Finally, we analyzed WT and $Apc2^{g10}$ astral microtubules in cycles 9 and 10 when asters are robust, and observed no significant differences in microtubule density, in the distribution of microtubules around the astral center, or in the average maximum length of microtubules (Fig. 3G-H′; see Fig. S3 in the supplementary material). The lack of stabilized microtubules in the syncytial embryo, coupled with the lack of any discernable defects in *Apc2* mutant microtubules, suggest that APC2 might affect furrow extension directly through actin.

diaphanous mutant embryos exhibit defects in furrow extension

To understand how loss of APC2 affects actin organization, we asked what other proteins function with APC2 in this process. Formins nucleate and elongate unbranched actin filaments (reviewed by Goode and Eck, 2007), and, consistent with this function, the *Drosophila* formin Diaphanous (DIA) influences actin furrow assembly in the syncytial embryo and during cytokinesis (Afshar et al., 2000; Castrillon and Wasserman, 1994). Interestingly, mouse DIA1 (also known as DIAP1) is reported to function with

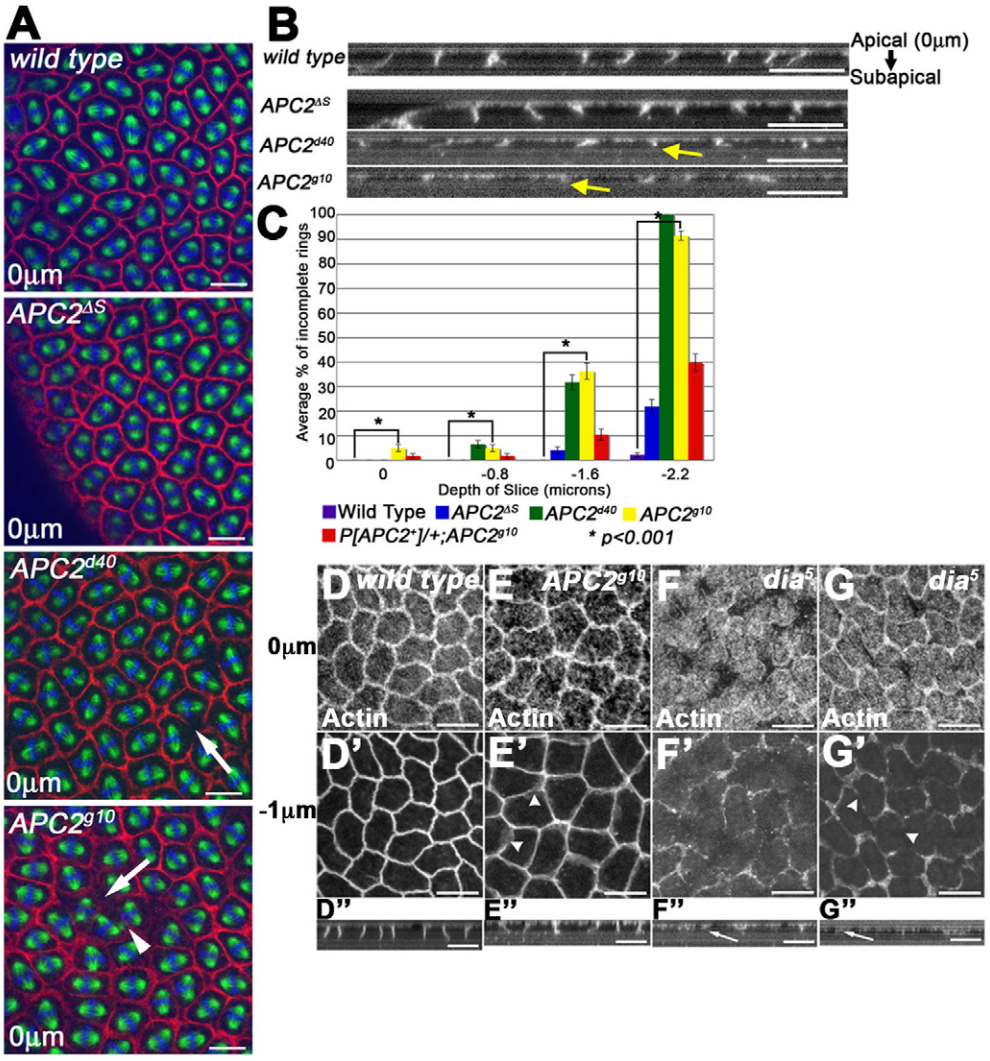

Fig. 2. *Apc2* mutants exhibit defects in actin rings and furrow extension; *dia*^{5} mutants exhibit actin defects similar to those of *Apc2* mutants. (**A**) Apical views of cycle 12 metaphase *Drosophila* embryos. *Apc2*^{d40} and *Apc2*^{g10} embryos display incomplete actin rings (arrows) that are occasionally associated with spindle collisions (arrowhead). A surface (0 μm) view is shown. (**B**) Cross-sectional views of metaphase actin show that *Apc2*$^{\Delta S}$ furrows appear largely WT, whereas furrow extension in *Apc2*^{d40} and *Apc2*^{g10} mutants is grossly defective (yellow arrows). (**C**) Quantification of furrow extension. The percentage of incomplete actin rings at four depths for five embryos of each genotype is shown. The increase in incomplete actin rings in *Apc2*^{g10} embryos as compared with WT is significant at all depths. (**D-G″**) Apical (D-G), subapical (D′-G′) and cross-sectional (D″-G″) views of actin in cycle 12 metaphase WT (D-D″), *Apc2*^{g10} (E-E″) and *dia*^{5} (F-G″) embryos. Defects in *dia*^{5} embryos range from actin primarily in caps during metaphase (F,F′) to embryos with incomplete actin rings (G,G′, arrowheads) that are similar to, but more severe than, those in *Apc2*^{g10} (E,E′, arrowheads). Arrows in F″,G″ indicate effective furrow extension Scale bars: 10 μm.

APC to stabilize microtubules in migrating cultured cells (Wen et al., 2004). To further explore the role of DIA in pseudocleavage furrow formation, and assess the potential similarities with *Apc2* mutants, we examined the *dia*-null (*dia*^{5}) phenotype in more detail. In some cases, most actin remained in caps during metaphase in *dia*^{5} mutants (Fig. 2F,F′), as previously reported (Afshar et al., 2000), but more frequently actin was located in weak rings (Fig. 2G,G′), similar to those of *Apc2* mutants (Fig. 2E,E′). Furrow extension was significantly impaired in all *dia*^{5} mutants (Fig. 2F″,G″).

APC2 and DIA colocalize during furrow extension, and the localization of APC2 to furrows is DIA-dependent

If APC2 and DIA function together during furrow extension, they should colocalize. At the time when furrows begin to extend late in prophase, both APC2 and DIA were detected in the furrow (see Fig. S4B1-4, arrow, in the supplementary material), but DIA appeared to temporally precede APC2 (compare Fig. 4Bc with 4Bb). As furrows reached their maximum depth in metaphase, APC2 and DIA displayed the most extensive colocalization (Fig. 4Cd,e,Dd,e). In some cases, DIA was enriched at the furrow tip, whereas actin and APC2 were uniformly distributed (Fig. 4G, arrow; see Fig. S4B4-E4 in the supplementary material) (Afshar et al., 2000), or DIA extended beyond the domains of actin and APC2 (see Fig. S4D4 in the supplementary material). By late anaphase and telophase when furrows have retracted, the colocalization of APC2 and DIA was reduced (Fig. 4Ed,e,Fd,e). At telophase, APC2 exited the furrows before DIA; APC2 was detected only at the poles (Fig. 4Fb), whereas DIA resided along the entire ring and in the remnant furrow (Fig. 4Fc; see Fig. S4F3 in the supplementary material). Taken together, these observations reveal that APC2 and DIA localize to actin structures throughout the cell cycle, but colocalize most extensively when furrows are extending and have extended. Furthermore, APC2 appears to arrive in the furrow after DIA and to leave the furrow before DIA.

The distinct temporal pattern of DIA and APC2 colocalization suggests that APC2 localization to the furrow could be DIA-dependent. In *dia*-null embryos, APC2 localized to actin caps during interphase and early prophase as in WT (Fig. 5A,B and data not shown). However, APC2 failed to localize with actin in the partial metaphase rings in *dia*^{5} mutants (Fig. 5Fa,b, arrows), and remained in the residual caps (data not shown). This trend continued through anaphase (Fig. 5Ha,b, arrows). By contrast, Anillin (Scraps – FlyBase) localized to all remaining cortical actin structures in *dia* mutants (Fig. 5Fc,Hc, arrows), suggesting that the loss of APC2 localization is a specific consequence of the loss of DIA. During telophase, APC2 was located at the poles of actin rings (Fig. 5Ja,b arrows) and in reforming

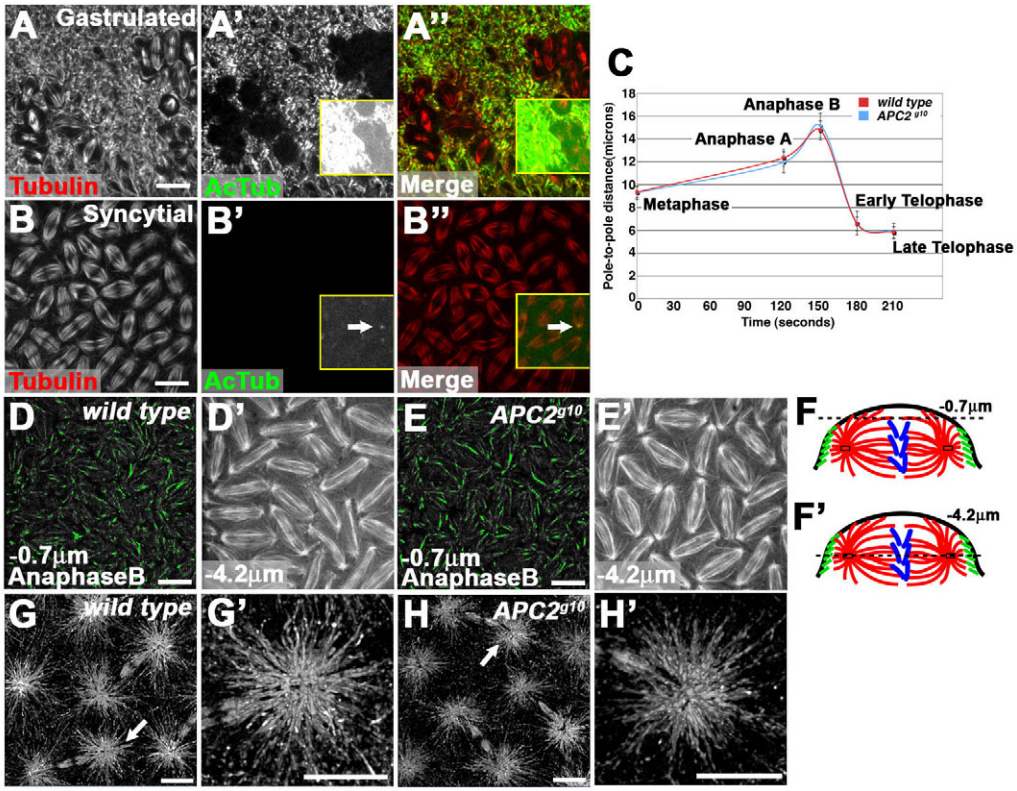

Fig. 3. Syncytial embryos do not contain acetylated microtubules and *Apc2* mutants have no visible microtubule defects. (**A-B″**) Staining for tubulin (A,B, red in merge) versus acetylated tubulin (A′,B′, green in merge) in gastrulated (A-A″) and syncytial (B-B″) *Drosophila* embryos. Unlike the acetylated microtubules in gastrulated embryos (A′), anaphase microtubules in syncytial embryos are not acetylated (B′). Overexposure (insets) reveals weak staining for acetylated microtubules at spindle poles (arrow) in syncytial embryos. (**C**) Pole-pole distance measurements in live cycle-12 embryos; *n*=30 for each. (**D-E′**) Anaphase microtubules in WT (D,D′) and $Apc2^{g10}$ (E,E′) cycle-12 embryos have similar densities and organization. (**F**,**F′**) Schematics indicating the plane of section in D-E′. (**G-H′**) Three-dimensional volume-rendered views of deconvolved cycle 9 anaphase asters reveals qualitatively similar astral microtubule organization in WT (G,G′) and $Apc2^{g10}$ (H,H′) embryos. Arrows in G and H indicate the asters that are shown at high magnification in G′ and H′. Scale bars: 10 µm.

actin caps (data not shown). The dependence of APC2 on DIA specifically when rings and furrows are prominent largely parallels the timing of colocalization between APC2 and DIA in WT embryos (Fig. 4), and supports the hypothesis that APC2 and DIA function together specifically during actin furrow extension.

We then asked whether the localization of DIA is dependent on APC2. Not only was DIA localized to $Apc2^{g10}$ actin rings as in WT (Fig. 5K,L), but DIA also was enriched at the tip of the furrow in defective $Apc2^{g10}$ furrows, also as in WT (Fig. 5K′,L′, arrows). This suggests that the basic association of DIA with actin and with the leading edge of the furrow occurs independently of APC2, and that the localization of DIA alone is insufficient for normal furrow extension.

Reduction of *dia* enhances the actin defects in *Apc2* mutant embryos

We predicted that if APC2 and DIA function together during furrow extension, a 50% reduction in the dose of *dia* in $Apc2^{g10}$ embryos would enhance the $Apc2^{g10}$ phenotype. We reduced the dose of *dia* using two different alleles: dia^{2}, a deletion and reported null allele (Afshar et al., 2000), and dia^{k07135}, a P-element insertion in the 5′UTR (Berkeley *Drosophila* Genome Project). Because both alleles similarly affected the *Apc2* phenotype, we report the findings for dia^{k07135} (dia^{k}) only. Reduction of *dia* enhanced the $Apc2^{g10}$ phenotype in two ways. The first conspicuous difference between metaphase $Apc2^{g10}$, dia^{k}/CyO and dia^{k}/CyO; $Apc2^{g10}$ (Fig. 6B-D) embryos was at the apical surface, where some actin remained cap-like in dia^{k}/CyO; $Apc2^{g10}$ embryos (Fig. 6D, arrow), reminiscent of dia^{5} mutants (Fig. 2F,G). This metaphase cap-like actin is a striking enhancement; cap-like actin was not observed in $Apc2^{g10}$ mutants or dia^{k} heterozygotes (Fig. 6B,C). Second, we evaluated how reduction of *dia* affects $Apc2^{g10}$ furrow extension. We did not score rings at 0 µm for dia^{k}/CyO; $Apc2^{g10}$ embryos because the cap-like actin obscured the actin rings. Surprisingly, dia^{k} heterozygotes exhibited furrow extension defects similar in magnitude to those of $Apc2^{g10}$ at –1.6 µm and at –2.2 µm (data not shown), suggesting that DIA is limiting for furrow extension. Therefore, we focused on –0.8 µm where both $Apc2^{g10}$ and dia^{k} heterozygotes exhibited only weak defects (Fig. 6E). A 50% reduction in the dose of maternal *dia* in $Apc2^{g10}$ resulted in a significant increase in incomplete rings (47%) (Fig. 6E), as compared with either $Apc2^{g10}$ or dia^{k}/CyO alone (5-10%; $P<0.001$) (Fig. 6E). Together, these data demonstrate a dose-dependent genetic interaction between *Apc2* and *dia*, suggesting that APC2 and DIA function in a common pathway to promote furrow extension and the dissolution of actin caps.

APC2 directly binds DIA

Previous work demonstrated that a complex including mouse DIA1, APC and EB1 influences microtubule stability in cultured cells (Wen et al., 2004). There, the C-terminus of mouse DIA1 (FH1 and

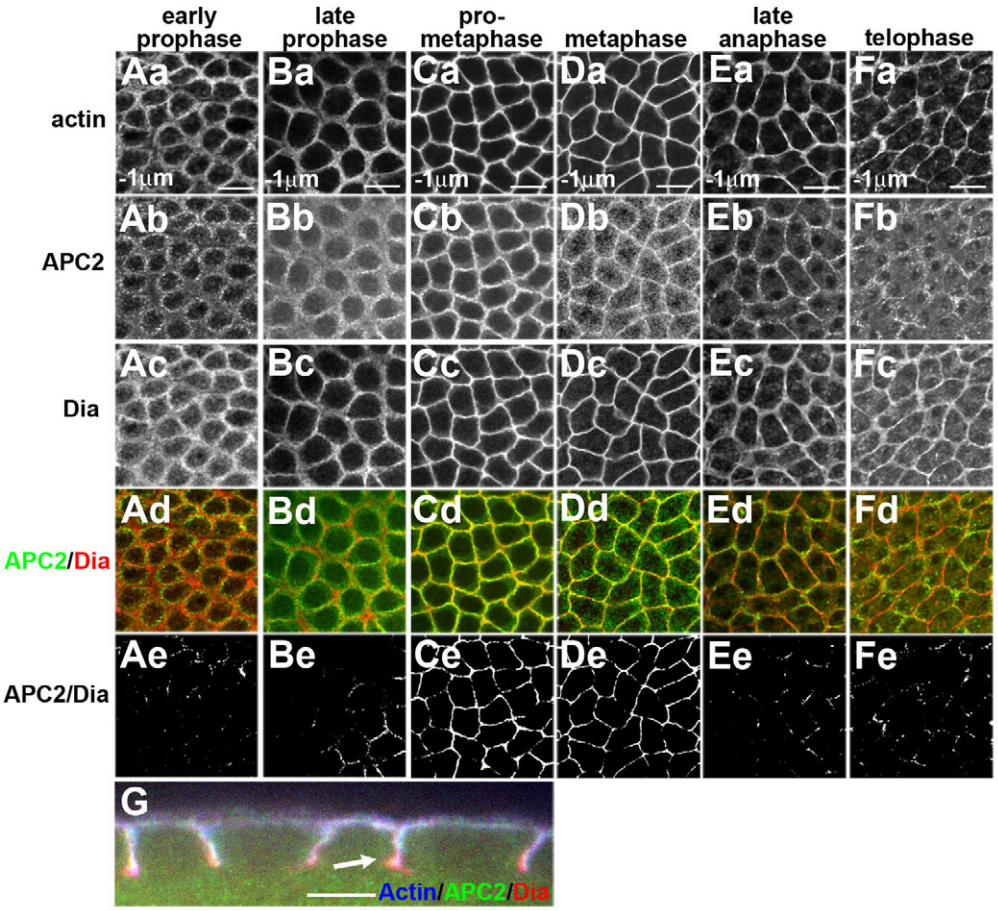

Fig. 4. APC2 and DIA proteins colocalize together and with actin in WT embryos. (**Aa-Fe**) APC2 (Ab-Fb) and DIA (Ac-Fc) colocalize with actin (Aa-Fa) throughout the cell cycle in WT cycle-12 *Drosophila* embryos. Colocalization of APC2 and DIA (yellow in Ad-Fd and white in Ae-Fe) is not uniform throughout the cell cycle, but peaks during prometaphase (Cd,Ce) and metaphase (Dd,De). (**G**) Metaphase; in cross-section APC2 (green) and DIA (red) colocalize with actin (blue) as the furrows extend. DIA is enriched at the furrow tip (arrow). Scale bars: 10 μm.

FH2 domains) binds directly to the basic domain of APC and to the N-terminal domain of EB1, while the EB1-binding domain ($EB1^{bd}$) of APC binds to the C-terminus of EB1. These pairwise interactions led Wen et al. (Wen et al., 2004) to propose the formation of a ternary complex. Unlike mouse APC, *Drosophila* APC2 contains neither the basic domain shown to interact with DIA, nor the $EB1^{bd}$ (Fig. 1B).

To determine whether APC2 interacts with DIA via other domains, we asked whether His or GST fusions of APC2 and DIA bind directly in vitro. The C-terminal half of DIA (His-DIA^{C519}) directly bound the C-terminal half of APC2 ($APC2^{C}$), but not GST alone, nor the N-terminal half of APC2 ($APC2^{N}$) (Fig. 6F). His-DIA^{C519} also bound directly to the predicted binding partner Chickadee (Chic, *Drosophila* Profilin), to *Drosophila* EB1, and to the basic domain of APC (Fig. 6F). These data indicate that although both mouse APC and *Drosophila* APC2 can bind DIA proteins directly, the underlying molecular interactions are distinct; mouse APC interacts with DIA1 through its basic domain (Wen et al., 2004), whereas *Drosophila* APC2 interacts with DIA through its central repeat region that includes the 15 and 20 amino acid repeats and the SAMP repeats (Fig. 6H).

To determine whether APC2 and DIA form a complex in vivo, we immunoprecipitated APC2 from 0-2 hour WT embryos and probed the blots with an anti-DIA antibody. DIA co-immunoprecipitated with APC2, but not with control antibody (Myc) or beads alone (Fig. 6G and data not shown). The results of these biochemical assays, together with our phenotypic, protein localization and genetic interaction data, strongly support a model in which APC2 and DIA function together to extend actin furrows during cortical syncytial mitoses.

An APC2-DIA complex may function independently of EB1 in furrow extension

In mouse, EB1 is reported to function with DIA1 and APC1 to promote microtubule stability (Wen et al., 2004). We examined whether *Drosophila* EB1 plays a role in furrow extension, first by asking whether *Eb1* mutants exhibit actin defects similar to those of *Apc2* and *dia* mutants. In contrast to $Apc2^{g10}$ or dia^{5} mutants, maternal $Eb1^{B13}$ (null) embryos did not have incomplete actin rings (Fig. 7A,B) and exhibited partially extended furrows (Fig. 7C, arrows); the average percentage of incomplete actin rings was similar to that of WT at 0, –0.8 and –1.6 μm (data not shown). At –2.2 μm, ~40% of the rings were incomplete (data not shown), compared with 92% for $Apc2^{g10}$ (Fig. 2C). Approximately 25% of $Eb1^{B13}$ embryos had actin caps during metaphase, similar to dia^{5} mutants; however, unlike dia^{5} mutants, these caps were associated with, and might be the result of, severe spindle disruptions (data not shown). *Eb1* mutants also exhibited a wide array of spindle morphologies (Fig. 7A′,B′), as predicted from studies in cultured *Drosophila* S2 cells and in syncytial embryos injected with anti-EB1 antibodies (Rogers et al., 2002). This might account for the higher frequency of nuclear loss in *Eb1* mutants as compared with *Apc2* or *dia* mutants. Furthermore, ~75% of $Eb1^{B13}$ embryos exhibited disordered ‘mats’ of actin associated with regions of significant nuclear loss (Fig. 7A,B, arrows). These actin organization defects were not observed in $Apc2^{g10}$ or dia^{5} mutants.

To further test the model that EB1 plays a role with the APC2-DIA complex during furrow extension, we reduced the dose of *Eb1* in $Apc2^{g10}$ embryos. We predicted that if EB1 functions with

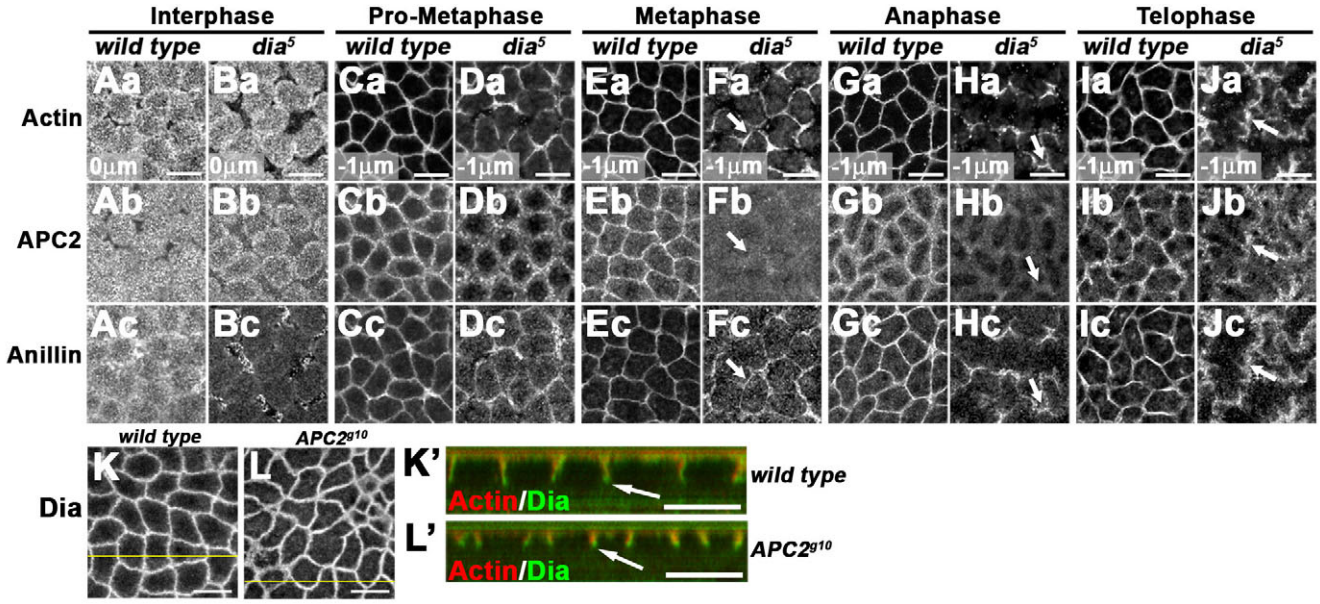

Fig. 5. DIA is required for normal APC2 localization, but APC2 is not required for DIA localization. (**Aa-Jc**) Stage-matched cycle-12 WT (A,C,E,G,I) and *dia*^{5} (B,D,F,H,J) *Drosophila* embryos showing the localization of actin (Aa-Ja), APC2 (Ab-Jb) and Anillin (Ac-Jc). APC2 (Fb,Hb, arrow) fails to localize to actin rings (Fa,Ha, arrow) during metaphase and anaphase in *dia*^{5} mutant embryos, whereas Anillin continues to colocalize with actin (Fc,Hc, arrow). During telophase, APC2 localizes to remnant actin rings and reforming caps in *dia*^{5} embryos (Ja,Jb, arrow). (**K**,**L**) In WT (K) and *Apc2*^{g10} (L) cycle-12 metaphase embryos, DIA localizes to actin rings. (**K′**,**L′**) In cross-section (at the yellow lines in K,L), DIA (green) is enriched at the furrow tips in *Apc2*^{g10} embryos (L′, arrow) as in WT (K′, arrow). Scale bars: 10 µm.

APC2-DIA, reduction of *Eb1*, like the reduction of *dia* (Fig. 6A-E), would result in the presence of cap-like actin during metaphase, and in a more severe furrow extension defect. Unlike *dia*^{k}*/CyO; Apc2*^{g10} embryos (Fig. 6D), apical actin appeared WT in *Eb1*^{B13}*/CyO; Apc2*^{g10} (Fig. 7G) embryos. Furthermore, there was no significant increase in the percentage of incomplete actin rings (Fig. 7H).

Finally, we asked whether *Drosophila* EB1 forms a complex with APC2 and DIA in vitro. Although APC2 lacks the defined EB1^{bd} (Fig. 1B), it might bind EB1 through a novel domain. As predicted, EB1 bound directly to DIA^{C484} and human APC^{EB1bd} (Fig. 7I). However, neither APC2^{N} nor APC2^{C} interacted with EB1 (Fig. 7I). APC2^{N} and APC2^{C} did bind to the known partners KAP3 and Armadillo, respectively (data not shown). Thus, differences in syncytial actin phenotypes and the lack of a genetic interaction with *Apc2*, coupled with the lack of direct binding to APC2, suggest that an APC2-DIA complex might function independently of EB1 in furrow extension.

Rho1 and *RhoGEF2* mutant phenotypes are distinct from those of *Apc2* and *dia*

Our data support a model in which an APC2-DIA complex functions in the development of actin furrows during cortical syncytial mitoses. Because RHO activates DIA-related formins (DRFs) (reviewed by Goode and Eck, 2007), we predicted that APC2-DIA functions downstream of RHO. To test this hypothesis, we examined the actin phenotypes of embryos maternally mutant for a hypomorphic allele of *Rho1* [*Rho1*^{L3} (Padash Barmchi et al., 2005)] and a null allele of *RhoGEF2* [*RhoGEF2*^{04291} (Hacker and Perrimon, 1998)]. The syncytial actin defects associated with these two mutants were similar (Fig. 8D,E), but those of *RhoGEF2* were more severe, consistent with the fact that *RhoGEF2*^{04291} is a null. If DIA is a direct downstream effector of RHO1 signaling during syncytial actin rearrangements, we predicted that the *RhoGEF2* and *Rho1* mutant phenotypes would include the *Apc2* and *dia* mutant phenotypes. Contrary to expectation, the actin defects in *RhoGEF2* and *Rho1* mutants were distinct from those of *Apc2* and *dia*. *Rho1* (Fig. 8D) and *RhoGEF2* (Fig. 8E) mutant rings exhibited areas of decreased actin (arrows) and areas of excessive actin accumulation (arrowheads) that appeared gauzy (Fig. 8D, inset) and sometimes included protruding actin ‘bulbs’ (Fig. 8E, inset). We did not observe such actin defects in WT, *Apc2* or *dia* mutants (Fig. 8A-C, insets). In addition, we never observed actin remaining in apical caps during metaphase in *Rho1* or *RhoGEF2* mutants (data not shown) as we saw in *dia*^{5} and *dia*^{k}*/CyO; Apc2*^{g10} mutants (Fig. 2F,G; Fig. 6D). Finally, although the actin furrows in *RhoGEF2* and *Rho1* mutants are not WT, they do not have the extension defects exhibited by *Apc2* and *dia* mutants (Fig. 8F). Similar to the surface views, *RhoGEF2* and *Rho1* mutant furrows in cross-section often appeared thickened (Fig. 8F, arrows), consistent with areas of excessive actin accumulation. Furthermore, both DIA and APC2 localized to actin in *RhoGEF2* mutants (Fig. 8G-G″), suggesting that the actin associations of DIA and APC2 are not disrupted when RHO1 signaling is disrupted. The distinct mutant phenotypes of *RhoGEF2* and *Rho1* mutants suggest that APC2 and DIA are not in a simple linear pathway downstream of RHOGEF2 and RHO1.

DISCUSSION

Cytoskeletal functions of APC2

APC family proteins have many well-documented effects on the microtubule cytoskeleton, whereas APC functions with actin are much less well understood. The *Drosophila* syncytial embryo is an excellent in vivo system in which to study the role of APC2 and its partners in organizing the cytoskeleton. We previously demonstrated

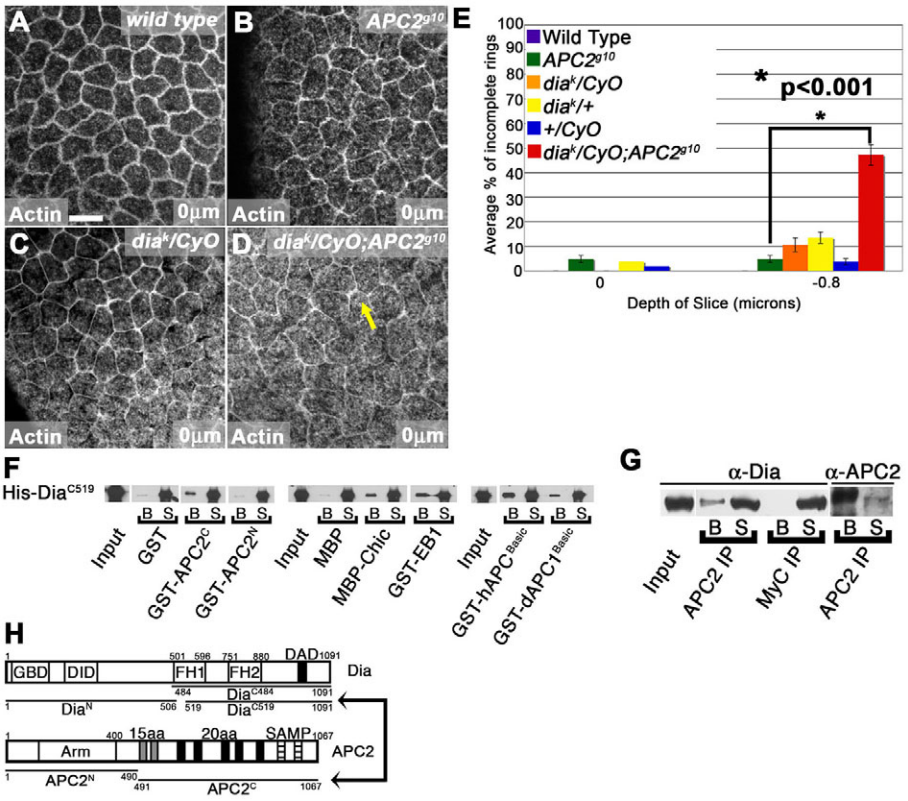

Fig. 6. *Apc2* and *dia* genetically interact, and APC2 and DIA bind directly. (**A-D**) Actin in cycle-12 metaphase *Drosophila* embryos at 0 μm. In comparison to WT (A), $Apc2^{g10}$ (B) and dia^{k}/CyO (C) embryos, $dia^{k}/CyO;\ Apc2^{g10}$ (D) embryos have increased cap-like actin (arrow). Scale bar: 10 μm. (**E**) Quantification of furrow extension at –0.8 μm reveals enhancement of the $Apc2^{g10}$ phenotype with reduction of *dia*. dia^{k} is dia^{k07135}. (**F**) His-DIA^{C519} binds directly to $APC2^{C}$ and to Chickadee, EB1, human $APC1^{Basic}$ and *Drosophila* $APC1^{Basic}$ (positive controls) in RIPA buffer. (**G**) DIA co-immunoprecipitated with APC2, but not with Myc (negative control) from 0-2 hour embryo lysates. (**H**) Schematic summary of APC2 and DIA constructs and interactions. B, bead; S, supernatant.

that *Drosophila* APC2 localizes to actin in syncytial embryos, and suggested a role for APC2 in tethering cortical microtubules to actin (McCartney et al., 2001).

Here we report the cytoskeletal consequences of eliminating all APC2 in the syncytial embryo. *Apc2*-null mutants exhibit incomplete actin rings and a failure of actin furrow extension (Fig. 2). $Apc2^{\Delta S}$ mutants exhibit more nuclear loss than do null mutants (McCartney et al., 2006), but have weaker actin defects (Fig. 2), suggesting that the $APC2^{\Delta S}$ protein might interfere with a tethering process for which APC2 is not essential. The presence of actin furrow defects in embryos that are mutant for multiple alleles of *Apc2*, including a null (Fig. 2), strongly suggests that APC2 functions in the normal organization of actin furrows.

An APC2-Diaphanous complex

We demonstrate a novel role for an APC2-DIA complex in the organization of the actin cytoskeleton. Formins such as DIA are best known for their ability to nucleate unbranched actin filaments and accelerate filament elongation (reviewed by Goode and Eck, 2007). *Drosophila* DIA functions in actin-based furrow assembly during cellularization and conventional cytokinesis (Afshar et al., 2000; Castrillon and Wasserman, 1994; Padash Barmchi et al., 2005). *dia* mutant syncytial embryos have defects in the initiation and elongation of actin furrows, consistent with DIA subcellular localization and known roles for formins (Figs 2 and 4) (Afshar et al., 2000). We show that APC2 and DIA colocalize together and with actin specifically at times when furrows are elongating (Fig. 4). The fact that APC2 and DIA bind directly in vitro (Fig. 6), but their colocalization is cell cycle-dependent, suggests that the interaction is regulated in vivo.

The simplest model for the function of an APC2-DIA complex in actin furrow formation is that DIA-dependent nucleation and elongation of unbranched actin filaments is essential for furrow extension, and that APC2 promotes DIA activity (Fig. 9A). The fact that the *dia*-null phenotype is more severe than that of *Apc2* (Fig. 2), coupled with the enhancement of the *Apc2*-null phenotype by a reduction of *dia* (Fig. 6), support the model that APC2 is not essential for, but might enhance, DIA activity. The dependence of APC2 on DIA for localization (Fig. 5) indicates that DIA may directly affect the regulation of its own activity.

One mechanism regulating the activity of formins has been extensively studied. DIA-related formins (DRFs) are autoinhibited through the binding of the N-terminal DIA inhibitory domain (DID) to the C-terminal DIA autoregulatory domain (DAD) (Fig. 6H). Binding of the GTPase-binding domain (GBD) by RHO-GTP relieves the autoinhibition and activates DRFs, which function as dimers (reviewed by Goode and Eck, 2007). Although RHO1 and RHOGEF2 have been reported to act upstream of DIA during *Drosophila* cellularization and embryonic morphogenesis (Grosshans et al., 2005; Homem and Peifer, 2008), other reports suggest that they are in a parallel pathway during these times (Mulinari et al., 2008; Padash Barmchi et al., 2005). The distinct actin defects in *Rho1* and *RhoGEF2* mutants as compared with *Apc2* and *dia* mutants (Fig. 8) suggest that RHO1 is not the GTPase that directly activates DIA during furrow formation in the syncytial embryo. However, because *Rho1*-null embryos cannot be generated genetically owing to requirements during oogenesis, it is possible that there is a role for RHO1 in activating DIA independent of RHOGEF2. In addition, although we cannot rule out the activity of other GEFs and GTPases, these observations, along with those of formins in other systems (reviewed by Higgs, 2005), suggest the existence of alternative mechanisms for DRF activation.

Here we show that APC2 and DIA can bind directly to each other. Thus, APC2 could directly affect the function of DIA, perhaps by stabilizing the open conformation required for optimal DIA activity.

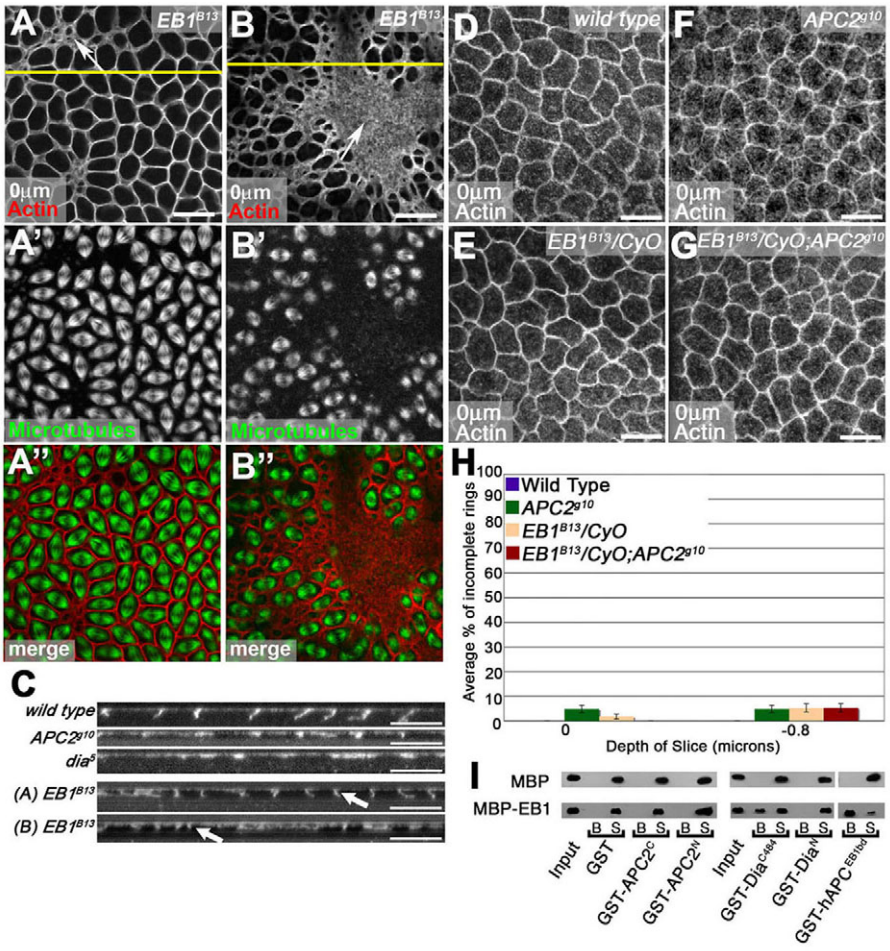

Fig. 7. EB1 may function independently of APC2-DIA. (**A-B″**) Apical views of actin (A,A″,B,B″) and microtubules (A′,A″,B′,B″) in cycle-12 metaphase $Eb1^{B13}$ embryos. Arrows in A and B indicate actin mats; yellow lines indicate plane of sections in C. (**C**) Partial furrow extension (arrows) in $Eb1^{B13}$ embryos in cross-section. $Eb1^{B13}$ mutants exhibit a wide array of spindle morphologies, including apparently normal spindles (A′) and severely disrupted spindles (B′). (**D-G**) Actin in cycle-12 metaphase embryos of the indicated genotypes. (**H**) Quantification of furrow extension shows that there is no significant effect of reduction of *Eb1* on the $Apc2^{g10}$ phenotype. (**I**) Free MBP-EB1 binds directly to DIA^{C484} and human APC^{EB1bd} (positive control), but to neither *Drosophila* APC2 fragment in HKT buffer. B, bead; S, supernatant.

Alternatively, APC2 could enhance the activity of DIA by binding to and recruiting other DIA-activating factors to the complex (Fig. 9A). Once DIA is activated it might dissociate from this complex, resulting in two pools of DIA. This notion is supported by our observation that DIA, but not APC2, is enriched at the furrow tip (Fig. 4G; see Fig. S4 in the supplementary material). We propose that in the absence of APC2, the efficiency of DIA activation is reduced, resulting in a decrease in the amount of unbranched actin filaments and a consequent production of shallow furrows (Fig. 9A). Consistent with this model for APC2 function, APC proteins are thought to play a scaffolding role in the Wnt regulatory 'destruction complex' (reviewed by Kennell and Cadigan, 2008). Furthermore, vertebrate APC binds ASEF and IQGAP, activators of RAC and RAC/CDC42, respectively, through its N-terminal Armadillo repeats (Kawasaki et al., 2000; Watanabe et al., 2004). Thus, APC2 may promote the association of DIA with GEFs and GTPases, or with other proteins that promote the open conformation or otherwise enhance DIA activity.

Role of EB1 in an APC2-DIA complex

EB1 regulates microtubule function in many organisms including *Drosophila*, in which its disruption affects spindle positioning and dynamics in the early embryo (Rogers et al., 2002). Mouse EB1 can bind both DIA1 and APC1 and the binary interactions identified have suggested a ternary complex (Wen et al., 2004). We tested the possibility that EB1 plays a role in an APC2-DIA complex. Although *Eb1*-null syncytial embryos exhibit weak defects in furrow extension (Fig. 7C), the lack of a genetic interaction with *Apc2* (Fig. 7D-H), and the lack of direct binding to APC2 (Fig. 7I), argue that EB1 function in the syncytial embryo is independent of the APC2-DIA complex.

Taken together, the lack of post-translationally modified, stabilized microtubules in the syncytial embryo (Fig. 3A-B″), the lack of discernable microtubule defects in the *Apc2* mutant (Fig. 3C-F′), and the lack of APC2 interactions with EB1 (Fig. 7), strongly suggest that the *Drosophila* APC2-DIA complex has a distinct cytoskeletal function from that of the mouse DIA1-APC-EB1 complex that stabilizes microtubules in cultured cells (Wen et al., 2004). In addition, the colocalization of APC2 and DIA specifically when furrows are extending during prophase and metaphase (Fig. 4), rather than during the critical period of microtubule function in anaphase, supports the model that the APC2-DIA complex primarily affects actin. It is intriguing that interactions between APC proteins and formins have been conserved, and that such complexes can affect both the actin and microtubule networks.

Building a furrow

Unlike conventional cytokinesis that uses actomyosin-based contraction to drive membrane invagination, Myosin II function is dispensable for pseudocleavage furrow formation (Royou et al., 2004). Many proteins are known to affect the dynamic organization of syncytial actin (Fig. 9B). Centrosomin, a core centrosome component, Sponge, a putative unconventional RacGEF, and Scrambled, a novel protein, all have roles in normal cap formation (Postner et al., 1992; Stevenson et al., 2001; Vaizel-Ohayon and Schejter, 1999). Cap formation and expansion require the Arp2/3

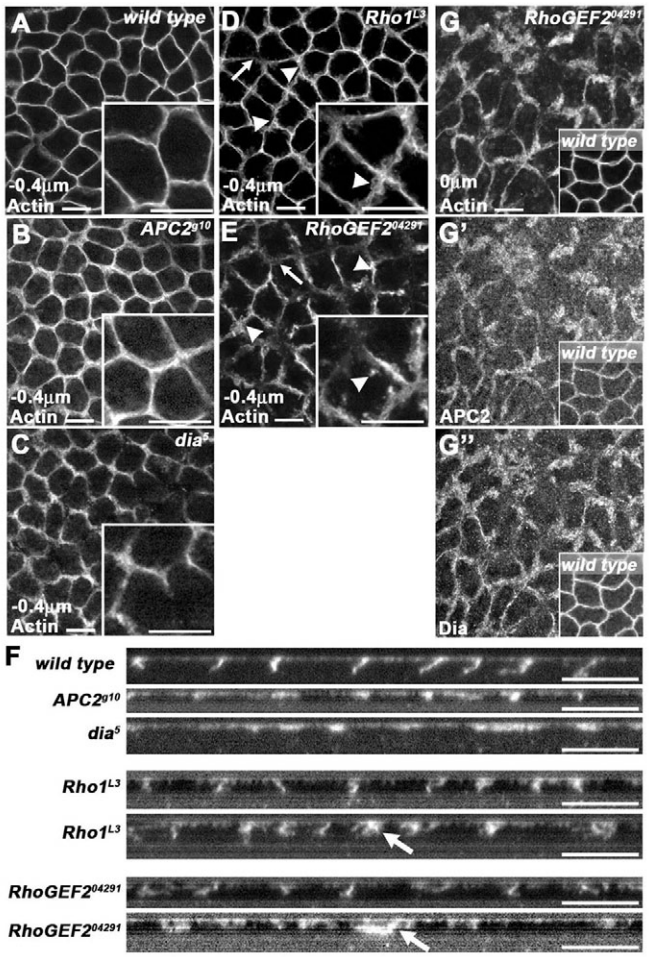

Fig. 8. *Rho1* and *RhoGEF2* mutants. (**A-F**) Cycle-12 metaphase *Drosophila* embryos. *Rho1* (D) and *RhoGEF2* (E) mutants display areas of decreased actin (D,E, arrows), as well as areas of excessive accumulation of actin associated with rings (D,E, insets, arrowheads), which are not observed in WT (A), *Apc2*^{g10} (B) or *dia*^{5} (C) embryos. (F) Cross-sections stained for actin show defective thickened furrows in *Rho1* and *RhoGEF2* embryos (arrows). (**G-G″**) APC2 (G′) and DIA (G″) localize to cortical actin (G) in a *RhoGEF2*^{04291} mutant cycle-12 anaphase embryo. Scale bars: 10 μm.

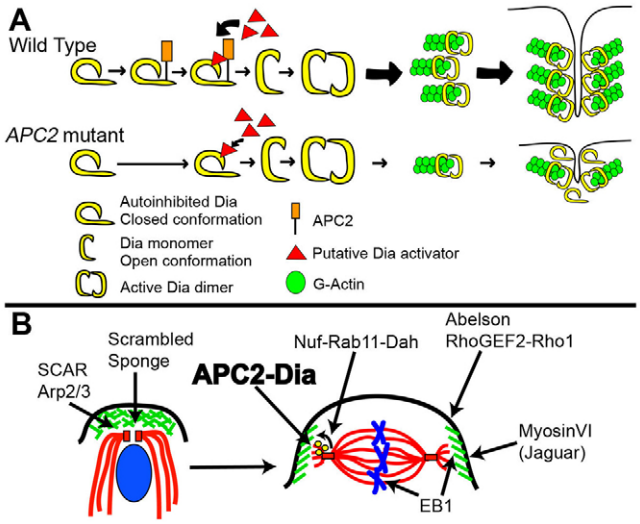

Fig. 9. Model for *Drosophila* APC2-DIA function. (**A**) We propose that APC2 promotes the activity of DIA by facilitating the interaction between DIA and an activator. This interaction enhances the efficiency of actin nucleation in the pseudocleavage furrow. In the absence of APC2, DIA and its activator interact less efficiently, resulting in defective furrow extension. (**B**) Many proteins affect syncytial actin rearrangments (see Discussion).

complex and its activator SCAR (Stevenson et al., 2002; Zallen et al., 2002). Well-known actin regulators such as RHO1, RHOGEF2 and Abelson (Abl tyrosine kinase) (Grevengoed et al., 2003; Grosshans et al., 2005; Padash Barmchi et al., 2005; Postner et al., 1992; Sullivan et al., 1993) may not affect furrow extension directly (Fig. 9B), but rather regulate the overall balance of actin activity. The Myosin VI protein Jaguar appears to play a specific role in furrow extension (Mermall and Miller, 1995), where it might stabilize actin filaments, as it does in the *Drosophila* testis (Noguchi et al., 2006).

The best-understood mechanism for pseudocleavage furrow extension is the NUF (Arfophilin)-RAB11-DAH (Dystrophin) pathway, which utilizes microtubule-based transport from the recycling endosome to move membrane and actin to the site of furrow extension (Fig. 9B) (Riggs et al., 2007). Once actin has been delivered to this site, APC2-DIA might promote the nucleation and elongation of unbranched filaments necessary for furrow extension. Interestingly, we have shown that in the absence of *dia*, and in *Apc2* mutant embryos reduced for *dia*, some actin remains cap-like (Figs 2 and 6). This intriguing observation suggests that there is a relationship between the dissolution of caps and the formation of furrows, and that these distinct pools of branched and unbranched actin might be in a balance. The APC2-DIA complex has emerged as a key factor affecting actin organization in the early embryo, and further study will reveal how the many regulatory pathways converge to influence dynamic changes in actin organization.

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Supplementary material

Supplementary material for this article is available at http://dev.biologists.org/cgi/content/full/136/8/1283/DC1

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