Differential regulation of adherens junction dynamics during apical–basal polarization

Juan Huang1,*, Lynn Huang1, Yi-Jiu Chen1, Erin Austin1, Caitlin E. Devor1,2, Fabrice Roegiers3 and Yang Hong1,∗

1Department of Cell Biology and Physiology, University of Pittsburgh School of Medicine, 3500 Terrace Street, Pittsburgh, PA 15261, USA
2Department of Biology, Allegheny College, 520 North Main Street, Meadville, PA 16335
3Institute for Cancer Research, Cancer Biology Program, Fox Chase Cancer Center, 333 Cottman Ave. R356, Philadelphia, PA 19111, USA

*Present Address: National Institute of Biological Sciences, 7 Kexueyuan Road, ZGC Life Sciences Park, Beijing 102206, China

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Summary
Adherens junctions (AJs) in epithelial cells are constantly turning over to modulate adhesion properties under various physiological and developmental contexts, but how such AJ dynamics are regulated during the apical–basal polarization of primary epithelia remains unclear. Here, we used new and genetically validated GFP markers of Drosophila E-cadherin (DE-cadherin, hereafter referred to as DE-Cad) and β-catenin (Armadillo, Arm) to quantitatively assay the in vivo dynamics of biosynthetic turnover and membrane redistribution by fluorescence recovery after photobleaching (FRAP) assays. Our data showed that membrane DE-Cad and Arm in AJs of polarizing epithelial cells had much faster biosynthetic turnover than in polarized cells. Fast biosynthetic turnover of membrane DE-Cad is independent of actin- and dynamin-based trafficking, but is microtubule-dependent. Furthermore, Arm in AJs of polarizing cells showed a faster and diffusion-based membrane redistribution that was both quantitatively and qualitatively different from the slower and exchange-based DE-Cad membrane distribution, indicating that the association of Arm with DE-Cad is more dynamic in polarizing cells, and only becomes stable in polarized epithelial cells. Consistently, biochemical assays showed that the binding of Arm to DE-Cad is weaker in polarizing cells than in polarized cells. Our data revealed that the molecular interaction between DE-Cad and Arm is modulated during apical–basal polarization, suggesting a new mechanism that might be crucial for establishing apical–basal polarity through regulating the AJ dynamics.

Key words: Adherens junction, Apical–basal polarity, DE-cadherin, Armadillo, Drosophila

Introduction
Establishing and maintaining apical–basal polarity is essential for epithelial cell morphology, function and tissue integrity. One hallmark of apical–basal polarity in epithelial cells is the demarcation of their membrane domains by polarized formation of cell junctions such as adherens junctions (AJs). The cell adhesion molecule E-cadherin and its cytosolic partner β-catenin (Armadillo, Arm) are the major components of the AJ complex. As a transmembrane protein, E-cadherin in AJs is constantly under turnover through vesicle trafficking, and such a dynamic nature of the AJ complex is required for modulating the adhesion properties during tissue morphogenesis (Clasen et al., 2005; Gumbiner, 2005; Ulrich et al., 2005). Formation of AJs is a crucial process in apical–basal polarization in epithelial cells and it is conceivable that establishing and maintaining the polarity requires proper regulation of AJ dynamics. However, systematic and quantitative assays of in vivo AJ dynamics during apical–basal polarization have yet to be done. It is known that Drosophila E-cadherin (DE-Cad) is regulated by actin-based endocytosis in polarized pupal epithelial cells (Georgiou et al., 2008; Leibfried et al., 2008) and in morphogenetically active neuroectoderm (Duncan and Peifer, 2008; Harris and Tepass, 2008), but quantitative data measuring such turnover are not available.

AJ dynamics can be regulated by distinct mechanisms involving dramatically different spatial-temporal scales. The biosynthesis-based turnover of the AJ complex often is a rather slow process, for instance the membrane E-cadherin in AJs of polarized MDCK cells showed a long half-life of approximately 5 hours (Shore and Nelson, 1991). Similarly, ectopically expressed DE-Cad::mEosFP fusion protein in AJs of polarizing Drosophila embryonic epithelia also showed very little turnover within a 1 hour period (Cavey et al., 2008). These results seem to suggest that the biosynthetic turnover of membrane E-cadherin remains slow in AJs of either polarized or polarizing cells, and that its dynamics are not subjected to stage-specific regulations during polarization. However, such interpretations are complicated by the fact that the DE-Cad::mEosFP was ectopically expressed and assayed in a heterogeneous background, which might obscure the dynamics of endogenous DE-Cad. Furthermore, MDCK cells polarize through mesenchymal–epithelial transition that is based on contact-initiated cell adhesion, whereas Drosophila embryonic epithelial cells polarize through pre-existing polarity cues (Cavey and Lecuit, 2009), making their results not directly comparable. In contrast to the slow biosynthetic turnover of E-cadherin, its local turnover at the membrane (i.e. membrane redistribution) is a much faster process, with half-times often measured in seconds (Cavey et al., 2008; de Beco et al., 2008), and has long been assumed to be based on the adhesion-dependent lateral diffusion of E-cadherins (Cavey et al., 2008). Recently, de Beco et al. showed that membrane redistribution of E-cadherin in matured AJs of polarized MDCK cells contained no lateral diffusion, but was an exchange-based process requiring dynamin-mediated endocytosis (de Beco et al., 2009). Again, little is known about how membrane redistribution dynamics of E-cadherin and Arm are regulated...
during apical–basal polarization and how such regulations might be implicated in establishing the cell polarity.

For the purpose of assaying the in vivo AJ dynamics during apical–basal polarization, *Drosophila* embryonic epithelia can serve as an excellent genetically tractable system. The apical–basal polarity in *Drosophila* embryonic primary epithelia is established in a stepwise fashion that correlates well with embryogenesis (Tepass, 1997) (Fig. 1A), making it possible to precisely and reproducibly identify cells at distinct stages of polarization simply by staging the embryos. In addition,
we have recently made two new and genetically validated AJ markers in *Drosophila*: a *DE-Cad::GFP* knock-in allele generated by our genomic engineering approach (Huang et al., 2009) and an *arm::GFP* allele obtained through a gene-trap screen (Clyne et al., 2003). These markers enabled us to quantitatively assay the in vivo dynamics of biosynthetic turnover and membrane redistribution of DE-Cad and Arm using fluorescence recovery after photobleaching (FRAP) assays in a clean homogenous background. Our data revealed that during apical–basal polarization the biosynthetic and local membrane turnovers of AJ complex are dramatically and differentially regulated, and that modulated DE-Cad–Arm interactions might be a mechanism that is crucial for regulating AJ dynamics and establishing apical–basal polarity.

**Results**

**Membrane DE-Cad and Arm show fast biosynthetic turnover, specifically in AJs of polarizing epithelial cells**

To investigate whether the turnover of the AJ complex is differentially regulated during apical–basal polarization, we first carried out whole-cell FRAP assays in which DE-Cad::GFP in a patch of approximately 20–50 cells of the sample epithelium was bleached (supplementary material Fig. S1A; see Materials and Methods). Because the photobleaching by laser was not limited to the focus plane but penetrates deep inside cells, both surface and intracellular pools of DE-Cad::GFP were erased in the bleached cells. The whole-cell FRAP assay effectively measures the biosynthetic turnover of membrane DE-Cad::GFP in AJs, because only newly synthesized DE-Cad::GFP contributes to the fluorescence recovery. In addition to the DE-Cad::GFP knock-in allele, which is fully homozygous viable and expresses at the identical level as the endogenous DE-Cad gene shotgun (*shg*) (Huang et al., 2009), we used an arm::GFP allele in which a GFP-coding artificial exon was fused in frame into the endogenous arm locus through Wee-P transposon-based gene trapping (Clyne et al., 2003) (Fig. 2A). Flies homozygous for the arm::GFP allele are also viable, fertile and healthy. Western blot results confirmed that arm::GFP embryos expressed exclusively Arm::GFP fusion protein with virtually no detectable wild-type Arm (Fig. 2B), demonstrating that the Arm::GFP fusion protein fully substitutes the wild-type Arm for the normal development. We used embryos between stages 9 and early 11 and embryos between stages 15 and 16 to specifically assay the AJ dynamics in polarizing and polarized cells, respectively (Tepass, 1997; Tepass and Hartenstein, 1994) (Fig. 1A). We consider epithelial cells in stage 9 to early 11 as polarizing cells based on two criteria: they are sensitive to the loss of function of polarity proteins Crumbs (Crb) and Stardust (Sdt), which are required for cell polarization; and they lack basolateral junctions such as septate junctions (SJs). By contrast, epithelial cells in stage 15–16 embryos are considered to be fully polarized because of their fully elaborated apical AJs and basolateral SJs (Tepass, 1997). Earlier embryos at stages such as cellularization were excluded for assaying polarizing cells due to their weak GFP signal and rapid morphogenetic activity.

Because not all of the whole-cell FRAP data could be fit to the exponential rise to maximum curve (see Materials and Methods), to make results more comparable among different sample pools we first calculated the initial recovery rates in all assays by linear regression (see Materials and Methods). As shown in Fig. 1B–D, initial recovery rate of membrane DE-Cad::GFP averaged 1.6%/minute in AJs of polarizing epithelial cells (*n*=14), but only 0.26%/minute in polarized late embryonic epithelial cells (*n*=9). Thus, the DE-Cad biosynthetic turnover is dramatically and differentially regulated during apical–basal polarization. Furthermore, the initial recovery rates of membrane Arm::GFP in AJs of polarizing cells and polarized cells (1.73 vs 0.34%/minute; Fig. 1D and Fig. 2C,D) were very close to the DE-Cad::GFP results, consistent with β-catenin functioning as a core component of AJ complex by binding to E-cadherin in the early phase of AJ complex biosynthesis (Chen et al., 1999). By further fitting the whole-cell FRAP data in polarizing cells (Fig. 1C and Fig. 2D) to the exponential rise equation, we confirmed that DE-Cad and Arm in polarizing cells showed very similar mobile fractions (73.1 and 76.4%, respectively; Fig. 1E), recovery half-time (*t*1/2) (28.5 and 25.0 minutes, respectively; Fig. 1F), and initial recovery rates at *t*=0 (1.98 and 2.25%/minute, respectively). Because slow recoveries of DE-Cad::GFP and Arm::GFP in polarized cells make similar curve-fitting impossible, we can only estimate that a theoretical 50% recovery would require more than 3 hours, which is comparable to the 5-hour half-time of membrane E-cadherin in AJs of polarized MDCK cells (Shore and Nelson, 1991). We also carried out whole-cell FRAP assays using the ectopically expressed DE-Cad::GFP (ubi-DE-Cad::GFP) used in previous studies of DE-Cad dynamics (Cavey et al., 2008; McGill et al., 2009; Oda and Tsukita, 2001; Shindo et al., 2008). In the presence of untagged endogenous proteins, the biosynthetic turnover of ectopically expressed DE-Cad::GFP also showed differential regulation during polarization (supplementary material Fig. S3A,B). Its FRAP recovery rate in polarizing cells was similar to DE-Cad::GFP knock-in samples (1.4 vs 1.6%/minute), although

![Fig. 1.](url)
in polarized cells it was much more reduced than for DE-Cad::GFP knock-in samples (0.11 vs 0.26%/minute).

Overall, our results showed that membrane AJ complexes of polarizing cells are highly dynamic at the biosynthetic level, as over 70% of membrane DE-Cad and Arm proteins are subjected to turnover with a half-time of less than 30 minutes. To exclude the possibility that the fast biosynthetic turnover of DE-Cad and Arm is regulated by the stage of development rather than by the stage of cell polarization, we carried out the same whole-cell FRAP assays in the epithelia of early pupae at 20% pupal development (20% pd). Early pupal epithelial cells are well studied for their apical–basal polarity and are considered fully polarized (Langevin et al., 2005). We measured an average DE-Cad::GFP recovery rate in pupal epithelium of 0.54%/minute.
DE-Cad biosynthetic turnover in polarizing epithelial cells requires intact microtubules, but not actin cytoskeleton or dynamin

We also investigated the potential trafficking regulators that might be specifically required in the fast biosynthetic turnover of AJs in polarizing cells. In polarized cells, actin and microtubule cytoskeletons often play pivotal roles in vesicle trafficking (Apodaca, 2001; Kizhatil et al., 2007; Lock and Stow, 2005). We treated early DE-Cad::GFP embryos with the F-actin-disrupting drug latrunculin B (LatB) and several different microtubule-disrupting drugs (Fig. 2E and supplementary material Fig. S2A). Consistent with previous reports (Cavey et al., 2008; Harris and Peifer, 2007), the initial recovery rate of DE-Cad::GFP was reduced in all embryos treated with three different drugs (Fig. 2E). Of these, Colcemid caused the most severe disruption of microtubules and the most significant reduction of DE-Cad::GFP recovery (Fig. 2E and supplementary material Fig. S2A). On the basis of these pharmacological results, we concluded that the fast biosynthetic turnover of DE-Cad::GFP in polarizing epithelial cells does not require an intact actin network, but does require an intact microtubule network. As a control, we also carried out whole-cell FRAP assays in embryos homozygous of Crb::GFP-A knock-in allele (Huang et al., 2009). Crb::GFP-A showed a medium rate of biosynthetic turnover (0.64%/minute) in early embryos and, interestingly, its recovery was independent of microtubules but sensitive to the disruption of F-actin (supplementary material Fig. S2C). Such results suggest that disruption of microtubule or F-actin does not necessary repress or enhance translation in general, and, although both DE-Cad and Crb are transmembrane proteins and share similar subcellular localization, their biosynthetic trafficking appears to rely on distinct mechanisms.

Finally, because dynamin-mediated vesicle trafficking appears to be important for remodeling the AJ during pupal epithelial morphogenesis (Classen et al., 2005), we tested whether the fast recovery of DE-Cad::GFP in polarizing cells requires dynamin-dependent vesicle trafficking. We combined the DE-Cad::GFP knock-in allele with shi<sup>D</sup>, a well-characterized temperature-sensitive mutant of shibire, which encodes the Drosophila dynamin. Interestingly, we found the rates of DE-Cad::GFP recovery were virtually identical in wild-type and in shi<sup>D</sup> embryos at either the permissive (25˚C) or restrictive (31˚C) temperature (Fig. 2F and supplementary material Fig. S2D). Thus, the biosynthetic turnover of AJs in polarizing epithelial cells is independent of dynamin.

AJs in polarizing and polarized cells show different membrane redistribution dynamics of DE-Cad and Arm

Why is the biosynthetic turnover of the AJ complex faster in polarizing epithelial cells? Previous studies indicated that the total amount of AJs increases during early embryonic stages (Tepass, 1997; Tepass and Hartenstein, 1994), therefore a higher rate of DE-Cad and Arm biosynthesis might be required for building more AJs in polarizing cells. Because the differences in AJ abundance between distinct stages of apical–basal polarization have not been quantitatively measured, we used live DE-Cad::GFP embryos to specifically quantify the amount of membrane DE-Cad in AJs in primary epithelia (see Materials and Methods). Our data suggest that during approximately 10 hours of embryogenesis from early embryonic stage 9 to late embryonic stage 15 there is a threefold increase in membrane DE-Cad in AJs (Fig. 2G,H). Once the cells are polarized, the
steady-state amount of membrane DE-Cad is also stabilized, as pupal epithelial cells showed only mild increase of AJ compared with late embryonic cells (Fig. 2G,H). Our data provided the first quantitative evidence that polarizing cells build up more AJ s in the process of establishing their apical–basal polarity. Such an increase in AJ s, however, did not contribute to the measured rates of DE-Cad::GFP or Arm::GFP recovery in whole-cell FRAP experiments based on our formula (see Materials and Methods).

More importantly, we speculated that the fast biosynthetic turnover of membrane DE-Cad and Arm in polarizing cells also suggests that AJ complexes in polarizing cells are qualitatively different from those in polarized cells. To test this hypothesis, we measured the dynamics of membrane redistribution of DE-Cad and Arm within the AJ s by carrying out diffusion or exchange-based FRAP assays in which a spot of ~0.84 μm diameter on an AJ segment was bleached by a 488 nm laser and its recovery recorded every 2 seconds (supplementary material Fig. S1B). To avoid confusion with the whole-cell FRAP assays, we refer to such FRAP assays as ‘spot-FRAP’, which share a similar setup and protocol to the FRAP assays previously described (Cavey et al., 2008). Because the majority of the spot-FRAP recoveries appear to be non-diffusion based (see below), we measured the mobile fraction and half-time of recovery ($t_{1/2}$) using the same exponential rise equation used in whole-cell FRAP assays (see Materials and Methods) (Figs 3, 5). To make our spot-FRAP data more comparable with previous studies based on the assumption of lateral membrane diffusion, we also processed the data with the diffusion-based formula (Klonis et al., 2002; Tsuji and Ohnishi, 1986) (supplementary material Fig. S5). Because the recovery rates of DE-Cad::GFP and Arm::GFP in spot-FRAP assays were at least an order of magnitude faster than their biosynthetic recovery rates in whole-cell FRAP assays, the recovery of spot-FRAP can be assumed to be exclusively based on the diffusion or exchange of the unbleached GFP proteins, and the whole-cell FRAP recovery to be based exclusively on the de novo synthesis of the GFP protein.

A recent study showed that AJ s in Drosophila epithelia contain persistently immobile microdomains called spot-AJs that are crucial for a two-tier regulatory mechanisms controlling the maturation and stability of AJ s (Cavey et al., 2008). Consistently, we found that at the submicron level the fluorescence intensity of DE-Cad::GFP and Arm::GFP along AJ segments varied, although discreet microdomains resembling spot-AJs were much less pronounced than shown by Cavey and colleagues (Cavey et al., 2008) (supplementary material Fig. S1B). The relative fluorescence intensity of the spots (spot intensity) that we randomly selected for spot-FRAP ranged roughly from 0.6 to 1.6 before bleaching, with a standard deviation of ~0.6 to 1.6 ($t_{1/2}$) using the same exponential rise equation used in whole-cell FRAP assays (see Materials and Methods). We also carried out spot-FRAP assays in early embryos of ubi-DE-Cad::GFP and again found that its membrane redistribution dynamics matched well with DE-Cad::GFP knock-in samples (supplementary material Fig. S3C,D), making our spot-FRAP data comparable with previous studies (Cavey et al., 2008). Our results also agree with previous studies on the local dynamics of overexpressed DE-Cad::GFP based on FRAP assays similar to our spot-FRAP (McGill et al., 2009; Shindo et al., 2008).

Molecular interactions between DE-Cad and Arm are regulated during apical–basal polarization

In spot-FRAP assays, although the $t_{1/2}$ of DE-Cad::GFP recovery remained similar at different cell polarization stages, we noticed that the $t_{1/2}$ of Arm::GFP recovery was significantly reduced in polarizing cells (Fig. 3C). In polarized epithelial cells, however, the $t_{1/2}$ of Arm::GFP recovery increased to a level similar to that of DE-Cad::GFP (Fig. 3C), consistent with previous studies that E-cadherin and β-catenin showed similar local turnover dynamics in mature mammalian AJ s (Yamada et al., 2005). Nonetheless, the difference between the membrane redistribution dynamics of Arm and DE-Cad in polarizing cells suggests that the association of Arm with DE-Cad is probably more dynamic in polarizing cells, and only becomes stable in polarized epithelial cells.

To test this hypothesis, we further analyzed the spot-FRAP data of DE-Cad::GFP and Arm::GFP to see whether their membrane-redistribution mechanisms are qualitatively different. It was recently discovered (de Beco et al., 2009) that in MDCK cells the membrane-redistribution of E-cadherin in stable AJ s requires endocytic trafficking and is a strictly exchange-based process (e.g. endocytic recycling). According to de Beco and colleagues, diffusion-based and exchange-based membrane redistributions show distinctly different temporal profiles of the Gaussian width ($σ$) of the fluorescence intensity profile after photobleaching: the $σ$ increases over time in diffusion-based recovery but remains constant in exchange-based recovery. Because the individual spot-FRAP kymographs of DE-Cad::GFP were too noisy to reliably calculate $σ$, we averaged the spot-FRAP kymographs of DE-Cad::GFP in wild-type and shi$^{A}$ assays (supplementary material Fig. S4) for measuring the temporal profiles of $σ$. As shown in Fig. 4A,B, in each of eight different spot-FRAP sample pools the $σ$ of the DE-Cad::GFP intensity profile after photobleaching remained strictly constant during the recovery, regardless of the genotype (wild-type or shi$^{A}$), assay temperature (25 or 31°C) or polarization stage.

Nonetheless, given the heterogeneous nature of the DE-Cad distribution along the AJ s (Fig. 3A) (Cavey et al., 2008), it is possible that a specific category of DE-Cad spots and/or microdomains might contain diffusion-based recovery that was obscured when all spot-FRAP kymographs in a given sample pool were averaged together. From DE-Cad spot-FRAP data in early wild-type samples at 25°C, we selected ten kymographs for each of three categories: those of highest spot-intensity before bleaching (“bright”), lowest spot-intensity before bleaching (“dim”), and shortest $t_{1/2}$ of recovery (“fast”) (Fig. 4C; also see Materials and Methods). In each category, the top ten spot-FRAP kymographs were averaged for $σ$ calculation, and the data showed no significant increase of $σ$ during the recovery in any of these categories (Fig. 4D). Thus, our data confirmed that membrane redistribution of DE-Cad::GFP in AJ s of either polarizing or polarized cells is a strictly exchange-based process.
By contrast, Arm::GFP intensity profiles after photobleaching showed a dramatic increase of σ in spot-FRAP assays in early embryos (Fig. 4E,F), indicating that lateral diffusion of membrane Arm contributes significantly to its membrane redistribution in polarizing epithelial cells. Spot-FRAP recovery of Arm::GFP in polarized cells showed a constant σ over the entire recovery (Fig. 4E,F), suggesting that the Arm::GFP recovery becomes strictly exchange-based just like that of DE-Cad::GFP. Such imaging data are consistent with a model in which DE-Cad remains relatively stable on the junctional membrane, but Arm binds rather dynamically to DE-Cad in polarizing cells and only becomes tightly associated with DE-Cad in polarized cells. To further test this hypothesis, we immunoprecipitated DE-Cad::GFP/Arm complex from early and late embryos (Fig. 4G). Although Arm was recovered equally well in both staged samples, in early but not late samples its binding to DE-Cad::GFP was sensitive to mild acidic wash (pH 5.5) (Fig. 4G). Such biochemical evidence strongly suggests that the molecular interaction between Arm and DE-Cad is indeed regulated during apical–basal polarization.

Efficient membrane redistribution of DE-Cad::GFP in polarizing epithelial cells requires dynamin

Because the membrane redistribution of DE-Cad::GFP was strictly exchange-based throughout apical–basal polarization, we wondered whether it requires dynamin-mediated endocytosis which in MDCK cells is essential for the membrane redistribution of E-cadherin in matured AJs (de Beco et al., 2009). We measured the $t_{1/2}$ of DE-Cad::GFP recovery in early and late embryos of both wild-type and shi<sup>es</sup>, at either permissive or restrictive temperatures. As summarized in Fig. 5, we found that in early wild-type embryos DE-Cad::GFP recovery became significantly faster at higher (restrictive) temperatures (i.e. shorter $t_{1/2}$; Fig. 5A,C). Such temperature-dependent reduction of recovery $t_{1/2}$ was completely eliminated in shi<sup>es</sup> early embryos (Fig. 5B,D), suggesting a partial requirement of dynamin in DE-Cad membrane redistribution. By contrast, both wild-type and shi<sup>es</sup> late embryos showed slightly reduced $t_{1/2}$ at a higher temperature, although the differences were not statistically significant (Fig. 5). We conclude that dynamin-mediated endocytosis is required for efficient membrane redistribution of DE-Cad in polarizing cells, but is not essential for the membrane redistribution of DE-Cad. Such dynamin-independent membrane redistribution of DE-Cad is reminiscent of that observed in MCF7 mammalian cells (de Beco et al., 2009).

Discussion

Differential regulation of biosynthetic turnover of AJ complexes during apical–basal polarization

We have reported the use of two distinct FRAP assays to quantitatively measure the in vivo dynamics of biosynthetic turnover and membrane redistribution of membrane DE-Cad and Arm. Our studies revealed that membrane DE-Cad::GFP in AJs of polarizing cells showed much faster biosynthetic recovery than in polarized cells. Specifically, more than 70% of membrane DE-Cad::GFP in AJs of polarizing cells showed significantly faster recovery (i.e. shorter $t_{1/2}$) than DE-Cad::GFP. Error bars indicate s.d.
Fig. 4. See next page for legend.
membrane redistribution of DE-Cad::GFP in AJs of both polarizing and polarized cells showed a strictly exchange-based recovery identical to that of E-Cad::GFP in matured AJs of polarized MDCK cells, but distinct from the diffusion-based recovery in disassembling or immature mammalian AJs (de Beco et al., 2009). In addition, despite the finding that polarizing cells showed higher mobile fractions in DE-Cad::GFP membrane redistribution, the $t_{1/2}$ of DE-Cad::GFP spot-FRAP recovery remained nearly the same in both polarizing and polarized cells. Together, the data suggest that mobile membrane DE-Cad engages in similar trans-interactions regardless of the polarization stage, and that mechanisms other than DE-Cad trans-interaction regulation probably contribute to the differential regulation of its biosynthetic kinetics during apical–basal polarization.

**Molecular interaction between DE-Cad and Arm is modulated in vivo during apical–basal polarization**

In contrast to the model that Arm and DE-Cad form a strong and static complex on the membrane and show similar or identical membrane redistribution dynamics, Arm::GFP in polarizing cells showed a faster and lateral diffusion-based membrane redistribution that was both quantitatively and mechanistically different from that of DE-Cad::GFP. We postulate that in polarizing cells the association between Arm and DE-Cad must be dynamic, allowing Arm proteins to exchange their DE-Cad binding partners to yield a diffusion-based recovery in spot-FRAP assays. Consistently, biochemical assays showed that the binding of Arm to DE-Cad is weaker in polarizing cells but stronger in polarized cells. Our results revealed for the first time that the interaction between DE-Cad and Arm is regulated during apical–basal polarization. Regulating the Arm–DE-Cad interaction could be a potential mechanism for controlling the AJ biosynthetic dynamics, because the dynamic and less stringent interactions between DE-Cad and Arm make AJ complexes in polarizing cells more susceptible to degradation, requiring increased biosynthesis to maintain the AJs. In fully polarized cells, DE-Cad and Arm are tightly coupled with each other to form stable and mature AJs that require much slower biosynthesis to maintain. Such dynamic regulation of Arm–DE-Cad interaction can be crucial for establishing apical–basal polarity. For instance, it has been shown that DE-Cad::Arm fusion protein fails to complement the function of DE-Cad in cell polarity and formation of AJs, and in fact behaves like a dominant negative (Pacquet et al., 2005).

Several molecular mechanisms can be responsible for regulating the DE-Cad–Arm interactions during apical–basal polarization. First, the phosphorylation of the DE-Cad intracellular domain (Huber and Weis, 2001) directly enhances the binding of Arm and is an obvious candidate mechanism for modulating the Arm binding to DE-Cad during polarization. To test this hypothesis, we are at present using a genomically engineered approach (Huang et al., 2009) to generate and characterize an array of DE-Cad phosphorylation knock-in mutants. Second, it is also possible that certain cofactors might be involved in regulating the formation of DE-Cad–Arm complex. For instance the Echinod–Canoe complex, the *Drosophila* homolog of the nectin–afadin complex, has been shown to regulate AJ formations (Lin et al., 2007; Sawyer et al., 2009; Wei et al., 2005) and could be a candidate for modulating the DE-Cad–Arm interactions. Finally, fast biosyntheses of DE-Cad and Arm measured at AJs of polarizing cells are microtubule-dependent, and it will be of interest to investigate whether any microtubule-dependent motors such as dynein or kinesin are involved in the fast delivery of AJ complexes to junctions.

**Potential roles of regulating the AJ dynamics in vivo in establishing apical–basal polarity**

One implication from our results is that polarizing cells might require specific mechanisms to sustain the fast AJ complex turnover at the biosynthetic and endocytic levels in order to build up their AJs and to maintain polarity. It is well characterized that establishing apical–basal polarity requires several groups of polarity proteins (Wang and Margolis, 2007), but much remains to be learned about the molecular and cellular mechanisms by which these proteins control the apical–basal polarity. In particular, the polarity protein Crumbs and its partner Stardust are dispensable for maintaining the polarity in fully polarized epithelial cells, but are specifically required for maintaining AJs in polarizing cells and in morphogenetically active cells undergoing AJ remodeling (Bachmann et al., 2001; Campbell et al., 2009; Hong et al., 2001). On the basis of the differential regulation of AJ dynamics through DE-Cad–Arm interaction during polarization, we speculate that a major function of the Crumbs–Stardust complex is to support the weaker and dynamic AJs, by possibly enhancing DE-Cad phosphorylation, promoting additional partners to stabilize the AJ complex, or regulating the trafficking of the AJ complex. In fact, it has been shown that Pals1, the mammalian homolog of Stardust, is required for
surface expression of E-cadherin in MDCK cells (Wang et al., 2007). Testing how polarity proteins might regulate AJ dynamics to establish apical–basal polarity in vivo is technically challenging because manipulating AJ dynamics through a general vesicle trafficking pathway might cause pleiotropic phenotypes. For instance, formation of AJs requires Rab11-mediated vesicle trafficking of DE-Cad (Roeth et al., 2009), but disruption of Rab11 function also disrupts at least the trafficking and localization of the transmembrane polarity protein Crumbs (Roeth et al., 2009), making it difficult to clarify the role of regulated AJ dynamics in establishing apical–basal polarity. To solve this problem, we are again using a genomic engineering approach to generate several DE-Cad gene knock-in mutants that carry mutations specifically affecting AJ complex formation and trafficking.

The fast biosynthetic turnover of DE-Cad and Arm should also facilitate the AJ remodeling that requires constant disassembling and reassembling of AJs in active-morphogenesis. Embryos from stage 6 to 13 show active morphogenesis through so-called germband extension and retraction, in which the epithelial layer extends and intercalates. Previous characterization of the requirements of DE-Cad in embryogenesis suggested that DE-Cad was subjected to active turnover, preferentially in more morphogenetically active epithelia such as neuroectoderm (Tepass et al., 1996). We initially carried out whole-cell FRAP assays in both the ventral neuroectoderm and the less morphogenetically active medial-lateral epithelia, and observed similarly fast biosynthetic recovery of DE-Cad::GFP and Arm::GFP in both. However, active neuroblast delamination makes it exceedingly difficult to consistently quantify the FRAP data using our established methods (supplementary material Fig. S1). We used medial-lateral epithelia exclusively for whole-cell FRAP assays in this study and our data showed that less morphogenetically active epithelia feature fast AJ biosynthetic turnover in early embryos, so the biosynthetic turnover of AJ is probably determined by the stage of epithelial polarization rather than by pure morphogenetic activity.

**Spot-AJs in polarizing cells are biosynthetically active**

In our DE-Cad::GFP and Arm::GFP knock-in samples, there were much fewer conspicuously bright and discreet spots than shown in other studies (Cavey et al., 2008), but the spot-intensity of both markers varied along the junctional membrane in each sample pool. There was also a clear correlation between spot intensity and mobile fraction (Fig. 3A), consistent with the idea that junctional membrane contains microdomains that can be classified according to mobile fractions in AJ complex local turnover. Nevertheless, our DE-Cad::GFP whole-cell FRAP data does not reveal any discrete microdomains that are of significantly slower recovery in polarizing cells, even in the...
absence of intact actin cytoskeleton (Fig. 2E and supplementary material Fig. S2A,B). This is in contrast to the similar FRAP assays of ectopically expressed DE-Cad::mEosFP, showing that spot-AJs or those discreet microdomains of high fluorescence intensity had virtually no biosynthetic turnover even in morphogenetically active early embryonic epithelia. We suspect that the discrepancy is due to the difference in chromophore maturation time between EGFP and mEosFP. The precise maturation time of mEosFP chromophore is unknown in Drosophila embryos, but in Xenopus embryos this process could take hours (Jorg et al., 2009), whereas the maturation of EGFP chromophore can be less than 30 minutes (Jorg et al., 2009). It is possible that the detectable fluorescence of mEosFP is severely biased to the old DE-Cad::mEosFP molecules retained largely in the immobile fraction of biosynthetic turnover, thus skewing the FRAP assays towards apparently stagnant recoveries. Our data suggest that the biosynthetic stability of spot-AJs or discreet microdomains of higher DE-Cad intensity in polarizing cells must be distinct from those in polarized cells. The fast biosynthetic turnover might require extra mechanisms to help maintain the stability and immobility of these spot-AJs or discrete microdomains.

Requirement of dynamin-mediated endocytosis in E-cadherin membrane redistribution is partially conserved

Dynamin-mediated endocytosis is required for AJ turnover during Drosophila pupal epithelial morphogenesis (Classen et al., 2005) and in the membrane redistribution of E-cadherin in mature AJs of MDCK cells (de Beco et al., 2009). Although dynamin is well characterized for its role in vesicle trafficking in Drosophila, quantitative in vivo analyses of dynamin-mediated vesicle trafficking have not been carried out in Drosophila prior to our studies. Surprisingly, our experiments using the well-established shibashi mutants showed that dynamin-mediated vesicle trafficking is not required in either the fast DE-Cad biosynthetic turnover in polarizing cells or the membrane redistribution of DE-Cad, suggesting that dynamin-independent vesicle trafficking must be essential for such processes. Although recent literature showed that endocytosis-independent diffusion of E-cadherin contributed to its membrane redistribution in AJs of mammalian A431 cells (Hong et al., 2010), in our studies the membrane redistribution of DE-Cad is strictly an exchange-based process that is consistent with the results of membrane E-cadherin in MDCK cells. Probably, the requirement for dynamin-mediated endocytosis in E-cadherin membrane redistribution is specific to epithelial cell types, and in Drosophila such requirement is only partially conserved. It will be of great interest to identify which dynamin-independent endocytosis mechanisms (Damke et al., 1995; Doherty and McMahon, 2009) are responsible for the membrane redistribution of DE-Cad, or whether the observed membrane redistribution behavior of DE-Cad is based on endocytosis-independent mechanisms such as lateral exchange of DE-Cad within the plane of the membrane (de Beco et al., 2009).

Materials and Methods

Fly stocks

w; DE-Cad::GFP and w; Crb::GFP-A were previously described (Huang et al., 2009). y w shiba10; DE-Cad::GFP was made by recombining the DE-Cad::GFP with shiba10 (Poodry et al., 1973; Roegiers et al., 2005). w arm::GFP was obtained from a GFP-trap screen similar to Clyne et al. (Clyne et al., 2003). w; ubi-DE-Cad::GFP was obtained from Kyoto Stock center (Kyoto, Japan).

Molecular characterization of arm::GFP

Inverse PCRs were carried out as described (Clyne et al., 2003). A ~1.5 kb product was obtained by the second round of PCR using MspI-digested arm::GFP genomic DNA as template. Sequences of this PCR product were used to search the Drosophila genome to identify the exact insertion of GFP transposon. Western blots of arm::GFP and wild-type embryos were carried out as described (Huang et al., 2009). Primary antibodies used in western blots were rabbit anti-GST::GFP (Huang et al., 2009), 1:20,000; mouse anti-Arm (N2 7A1, DSHB, Iowa City, Iowa), 1:500; mouse anti-α-tubulin (AA4.3, DSHB), 1:5000.

Live imaging assays of AJ in DE-Cad::GFP and arm::GFP embryos and pupae

Staged DE-Cad::GFP or arm::GFP embryos were collected and their eggshells manually removed. Dechorionated embryos were placed in air-permeable chambers filled with halocarbon oil (#95) on custom-made slides, to ensure their normal development throughout the imaging session (Lu et al., 2001). DE-Cad::GFP or arm::GFP pupae were staged and dissected for live imaging on their notum epithelia according to the published protocol (Roegiers et al., 2001). To image embryos at 31°C, we used a modified temperature-controlled stage (PDMI-2 microscope; Zeiss, Germany) at 2°C above the ambient temperature (Air Chamber, Holst Apparatus, Holliston, MA) so that the temperature of the embryo being imaged could be directly monitored in real time by a microthermensor (Flex Microprobe Implantable, 0.02 mm diameter, Kent Scientific, Torrington) positioned within 1 mm distance.

Drug treatments in embryos

Embryos were dechorionated in bleach, washed and then placed in 1:1 mixture of Schneider’s medium and n-octane (Tedodo and O’Farrell, 2003). Drugs or an equal amount of DMSO (dimethyl sulfoxide) was added to the Schneider’s medium and embryos were then shaken at 400 rpm for 30 minutes. Embryos were then quickly rinsed in n-octane, briefly dried in air and immediately mounted in halocarbon oil (#95) in an air-permeable chamber for imaging.

Whole-cell FRAP assays

Whole-cell FRAP assays (supplementary material Fig. S1A) were carried out on an Olympus FX1000 confocal microscope (Center for Bioimaging, University of Pittsburgh, PA) under 2–15% 488 nm laser, 40× objective (NA 1.4), 3× zoom, and a 512×512 pixel or 512×384 pixel window at the optical resolution of 0.28 μm/pixel. GFP in an approximately 200×200 pixel region of interest (ROI) window was bleached to approximately 5–15% level by a 405 nm UV laser at 100% power for 10–20 seconds. GFP recovery was then recorded by time-lapse Z-sections (2–3 μm depth) over 20–40 minutes at 1- or 2-minute intervals. Photobleaching by 488 nm laser at 100% power for 60–90 seconds yielded similar results in FRAP. We did not observe any arrests of dividing cells after photobleaching (Fig. 2C), and embryos went through morphogenesis during the recording period without obvious abnormality.

To maintain the consistency of optimal Z-depth throughout different time points, maximum intensity projection of Z-stacks was manually carried out in Photoshop. Two custom Photoshop javascripts, namely TIMEJET and FRAPJET, were then used to standardize the processing of FRAP data as depicted in supplementary material Fig. S1A. TIMEJET converts FRAP time-lapse images into a montage by automatically aligning the user-specified ROI from each time point (supplementary material Fig. S1A) with necessary compensations for sample drifting. The second script, FRAPJET, allows auto-measurement of GFP signal at each time point in the montage. For a user-specified ROI, FRAPJET first calculates its mean pixel value, mean(P), which is used as a threshold to split all pixels in the ROI into two groups: PFGF [pixels with values equal or above mean(P)] and PBackground [pixels with values below mean(P)]. Final GFP value is calculated as GFP = mean(PFGF) − mean(PBackground). This threshold calculation is very effective in distinguishing the junctional GFP signals from the cytosolic background, and thus specifically recognizes and measures the membrane-enriched (i.e. junctional) GFP signal (supplementary material Fig. S1A).

We only measured cells at the center of a bleached epithelial patch whose neighboring cells were also completely bleached. By excluding the GFP recovery in partially bleached cells, only newly synthesized GFP contribute to the GFP recovery in our whole-cell FRAP assays. The GFP recovery at a given time point t is calculated and normalized as the percentage of the difference between normalized GFP levels before bleaching (b.b.) and right after bleaching (t = 0) as:

\[
\text{GFP recovery} = \frac{[B_C/(C_1 - B_C)]/[B_C/(C_0 - B_C)]}{\text{GFP value before bleaching in to-be-bleached or unbleached control areas, respectively.}}
\]

For early stage samples, mobile fractions and recovery half-times (t1/2) of DE-Cad::GFP and Arm::GFP were calculated in SigmaPlot by fitting the averaged FRAP data to the exponential rise to maximum curve y = α (1 − e−t/t1/2). For late stage samples, linear regression was applied to the entire 20 or 40 minute FRAP data.
For each stage, samples were first calculated the \( t_{1/2} \) and \( t_{1/4} \) based on the average recovery 0.5%, then data up to \( t_{1/2}\) in each individual recovery curve were used for linear regression. Time point \( t_{1/2}\) was empirically determined to maximize the number of data points for linear regression while maintaining a good and consistent approximation to the linearity.

**Spot-FRAP assays**

For spot-FRAP assays (supplementary material Fig. S1B), embryos were imaged under Olympus FV-1000 confocal microscope at the optical resolution of 0.042 \( \mu \text{m/pixel} \) using 100 x objective (NA 1.4) with \( \times 8 \) digital zoom. A spot of AJ of 21 pixels (i.e. 0.84 \( \mu \text{m} \) in diameter) was randomly chosen along an AJ segment of a sample embryonic cell. Photobleaching of the spot was done by a 1 - second exposure to a 488 nm laser. Images (512 x 256 pixels, line averaged twice) were captured at 2-second intervals, from 4 seconds before photobleaching to 120 seconds after bleaching. No more than 80% of GFP intensity was bleached at the spot to ensure a proper Gaussian distribution profile of fluorescence intensity after photobleaching (Cavey et al., 2008). FRAP recording was first aligned in ImageJ (NIH, Bethesda, MD) using the StackReg plug-in (http://bigwww.epfl.ch/thevenaz/stackreg/) to compensate for potential sample drifting. Aligned recordings were then processed into kymographs using custom macros in ImageJ. The FRAP data (supplementary material Fig. S1B) were measured using custom macros in ImageJ according to the procedures described previously (Cavey et al., 2008). The data were processed by curve-fitting in SigmaPlot. For an assumption-free processing, we used the exponential rise to maximum equation \( y = y_{0} + (a - y_{0}) \exp(-x/b) \) to calculate the mobile fraction \( a \) and half-time of recovery \( t_{1/2} \) of each spot-FRAP sample. In addition, a diffusion-based formula previously described (Klomis et al., 2002; Tsuji and Ohnishi, 1986) was used to calculate the characteristic time of recovery \( t_{0} \), diffusion coefficient \( D \), and mobile fraction \( a \).

We processed all samples through curve-fitting and then excluded samples that either failed to converge or showed >200% mobile fraction. Here, the 200% mobile fraction was arbitrarily chosen to exclude samples that contained significantly insufficient FRAP recording for reliably calculating the FRAP parameters. Overall, less than 20% of our samples were excluded on the basis of the above criteria. For statistic analysis, unpaired Student’s \( t \)-test was used.

**Quantification of AJ in epithelial cells by DE-Cad::GFP**

Standard brightness curves of laser power and gain factor of FV-1000 confocal microscope were generated first. Live DE-Cad::GFP epithelial images were then captured under identical imaging conditions with only adjustments on laser power and gain factor. After GFP brightness was normalized by standard curves, the amount of DE-Cad::GFP in a given ROI in the epithelium was quantified in two ways: the average intensity of GFP at AJs, and the total amount of junctional DE-Cad::GFP normalized to values per square micrometer.

**Gaussian curve fitting of fluorescence intensity profiles**

Kymographs of a given sample pool were first stacked and aligned manually in Photoshop. Stacked images were then average-projected into a single layer in ImageJ using a custom macro. Artificial pixels introduced by Photoshop during stacking and alignment were excluded from averaging process. The projected image was then normalized using a custom ImageJ macro to compensate for photobleaching during time-lapse recording and to smooth the GFP intensity profiles across the AJ. Normalized pixel values were then exported into SigmaPlot and Gaussian-curve fitting was applied at specified time points. For presentation purposes, gray-scale kymographs were pseudo-colored by Fire-LUT (Lookup Table) in ImageJ, and FRAP images were contrast-enhanced by level adjustments in Photoshop. However, all quantifications were made on unmodified raw images. Custom Photoshop scripts and ImageJ macros used in this report are available upon request.

**Immunoprecipitation of DE-Cad/Arm complex from embryos**

Stage early and late embryos were collected for 2 hours at 25°C and aged for 4 hours at 25°C for collecting early embryos, and for 10 hours at 25°C for late embryos. Embryos were then extracted with lysis buffer [25 mM Tris pH 8.0, 27.5 mM NaCl, 20 mM KCl, 25 mM sucrose, 10 mM EDTA, 10 mM EGTA, 10% glycerol, 0.5% Nonidet P-40, 1% Triton X-100, with 1 mM DTT, 1 mM PMSF, 1 mM protease inhibitor (Roche, Indianapolis, IN) and 1 mM phenylmethylsulfonyl fluoride added immediately before homogenization] for 1 hour on ice. Embryo lysates were immunoprecipitated with home-made rabbit anti-GFP antibody crosslinked to Protein G magnetic beads (Millipore, Billerica, MA), separated by SDS-PAGE, blotted onto nitrocellulose membranes, and probed with chicken anti-GFP (1:5000) and mouse anti-armsH (1:200) antibodies. The pH 5.5 wash buffer for immunoprecipitates was PBST (1 × PBS plus 0.1% Triton X-100) adjusted with 2 M HCl and used immediately.

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**References**


