Par-1 kinase establishes cell polarity and functions in Notch signaling in the Drosophila embryo

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Summary
The Drosophila protein kinase Par-1 is expressed throughout Drosophila development, but its function has not been extensively characterized because of oocyte lethality of null mutants. In this report, we have characterized the function of Par-1 in embryonic and post-embryonic epithelia. Par-1 protein is dynamically localized during embryonic cell polarization, transiently restricted to the lateral membrane domain, followed by apicolateral localization. We depleted maternal and zygotic par-1 by RNAi and revealed a requirement for Par-1 in establishing cell polarity. Par-1 restricts the coalescing adherens junction to an apicolateral position and prevents its widespread formation along the lateral domain. Par-1 also promotes the localization of lateral membrane proteins such as Delta. These activities are important for the further development of cell polarity during gastrulation. By contrast, Par-1 is not essential to maintain epithelial polarity once it has been established. However, it still has a maintenance role since overexpression causes severe polarity disruption. Additionally, we find a novel role for Par-1 in Notch signal transduction during embryonic neurogenesis and retina determination. Epistasis analysis indicates that Par-1 functions upstream of Notch and is critical for proper localization of the Notch ligand Delta.

Key words: Par-1, Drosophila, Notch, Polarity, Development

Introduction
The distribution of cellular components in asymmetric patterns produces polarized cells, which are critical for the building and maintenance of multicellular organisms. Par-1 is a serine-threonine protein kinase that acts in this process. Par-1 was first identified in Caenorhabditis elegans where it is required in the one-cell zygote for asymmetries that produce distinct daughter cells, subsequently yielding distinct cell lineages (Kemphues et al., 1988). Par-1 is essential for asymmetric inheritance of P granules to the P1 cell as well as the eccentric positioning of the mitotic spindle (Cheeks et al., 2004; Guo and Kemphues, 1995). The Drosophila Par-1 kinase acts within the developing fly oocyte to establish the anteroposterior axis of the resulting embryo through localization and stabilization of polarized mRNAs and proteins that ultimately pattern the embryo (Riechmann and Ephrussi, 2004; Riechmann et al., 2002; Shulman et al., 2000; Tomancak et al., 2000). Reduction of Par-1 activity in hypomorphic mutant oocytes results in embryos with abnormal posterior patterning and missing germ cells (Shulman et al., 2000; Tomancak et al., 2000). Thus, Par-1 is critical for establishing a polarized anterior-posterior axis in early embryonic development. Analysis of null par-1 alleles has shown that Par-1 is also critical for earlier events during oogenesis such as the maintenance of oocyte fate (Huynh et al., 2001).

Par-1 functions in the somatic follicle epithelium surrounding the Drosophila oocyte. As it does in mammalian epithelia, Drosophila Par-1 localizes to the basolateral domain of follicle cell membranes (Bohm et al., 1997; Doerflinger et al., 2003; Vaccari et al., 2005). It acts to restrict the localization of the conserved Par-3/Par-6/atypical protein kinase C (aPKC) complex away from the basolateral domain by phosphorylating the Drosophila Par-3 homolog, Bazooka (Baz), thereby generating binding sites on Baz for the 14-3-3 protein Par-5 (Benton and St Johnston, 2003a; Benton and St Johnston, 2003b). Binding of Baz to Par-5 prevents its association with aPKC. Interestingly, a conserved residue in the spacer domain of Par-1 protein is phosphorylated by aPKC (Vaccari et al., 2005). This residue is required for restricting Par-1 from the subapical region (marginal zone) in the follicle epithelium where Baz/Par-6/aPKC is localized. Human Par-1 is also phosphorylated by aPKC in its homologous spacer domain, and phosphorylation triggers redistribution of Par-1 from the cell cortex to intracellular compartments (Hurov et al., 2004). These observations indicate that Par-1 and the Par-3/Par-6/aPKC complex occupy distinct membrane domains in epithelia due to mutual negative regulation.

Par-1 not only affects localization of the Baz complex in follicle cells, but also the localization of the apical membrane protein Crumbs (Crb), and the adherens junction (AJ) (Doerflinger et al., 2003). Another function of Par-1 in follicle cells is the regulation of the cytoskeleton. Par-1 limits microtubule accumulation but stabilizes the microtubules that are made. However, the mechanism of microtubule regulation in Drosophila remains unknown. Although mammalian Par-1 phosphorylates microtubule-associated proteins such as Tau, Map2, and Map4 (Drewes et al., 1997; Illenberger et al., 1996), Drosophila Tau does not play a role in microtubule organization within the follicular epithelium or the oocyte (Doerflinger et al., 2003).
Aside from a role in regulating cell polarity, Par-1 also functions in signal transduction. *Drosophila* Par-1 phosphorylates Dishevelled and allows canonical Wnt signaling through stabilization of β-catenin (Sun et al., 2001). Mammalian Par-1 phosphorylates the cell-cycle regulator Cdc25 to create a 14-3-3 binding site, which results in a complex of Cdc25 and 14-3-3, blocking progression through the cell cycle (Peng et al., 1998). Complex dissociation in response to growth factor signaling is achieved by reduction of Par-1 activity by Pim-1 (Bachmann et al., 2004). In a similar manner, Par-1 regulates Ras signaling by phosphorylating Ksr to generate a 14-3-3 binding site on Ksr (Kao et al., 2004; Muller et al., 2001). When non-phosphorylated, Ksr is localized at the plasma membrane where it acts as a scaffold that localizes proteins important for Ras/Raf signaling. Thus, Par-1 negatively regulates Ras signaling.

The wealth of well-characterized epithelia in *Drosophila*, makes it an attractive organism in which to study the role of proteins in establishing and maintaining cell polarity. We sought to understand the function of Par-1 in development of the embryonic blastoderm and ectoderm, and in development of the eye epithelium. We present evidence that Par-1 is essential for restriction of the AJ within the blastoderm. Interestingly, we identified a novel role for Par-1 in promoting Notch-mediated lateral inhibition during embryonic neurogenesis.

### Results

Par-1 is expressed throughout embryonic development and in imaginal discs

The first embryonic epithelium in *Drosophila* to develop is the blastoderm, which forms by cellularization. Nuclear division without cytokinesis continues for 13 rounds until nearly 5000 nuclei are present in a syncitium. The nuclei migrate peripherally beneath the plasma membrane, divide once more, and become enveloped by invaginating membrane. Membrane growth occurs by insertion of new membrane at distinct sites, first apically and then apicolaterally (Lecuit and Wieschaus, 2000). The leading edge of each membrane invagination forms a furrow canal, and is associated with basal spot adherens junctions (SAJs) composed of E-cadherin and β-catenin (Hunter and Wieschaus, 2000; Tepass and Hartenstein, 1994). Apical SAJs also arise during lateral membrane growth and eventually coalesce to form the apicolateral AJ at mid-gastrulation, while the basal SAJs disappear (Muller and Wieschaus, 1996). The blastoderm epithelium is the direct progenitor of the ectoderm, from which such diverse epithelia as the epidermis and imaginal discs are derived (Tepass, 1997).

To explore the role Par-1 plays in *Drosophila* zygotic development, we examined localization of Par-1 protein in embryos and detected anti-Par-1 staining throughout cellularization. At early stages of cellularization, staining was detected along the original plasma membrane and in the vicinity of the furrow canal (Fig. 1A,A’). As cellularization progressed, staining was detected in the vicinity of the furrow canal and along the lateral membrane, while the original plasma membrane staining was attenuated (Fig. 1B,B’). When cellularization was complete, Par-1 staining was associated with the lateral domain of blastoderm cells, as well as punctate staining in the cytoplasm (Fig. 1C,C’). Apical staining of the original plasma membrane was not apparent, and Par-1 did not overlap with E-cadherin in the AJ (Fig. 2C). At mid-gastrulation, the apical and lateral membrane regions of ectodermal cells were stained, as was the cytoplasm at a lower level (Fig. 1D,D’). Low levels of cytoplasmic staining were also detected in neuroblasts and mesodermal cells (Fig. 1D,D’). Late stage embryos exhibited punctate Par-1 staining, with a conspicuously higher level in the central nervous system relative to other tissues (Fig. 1E,E’). To determine the specificity of anti-Par-1 embryo staining, we performed RNAi on *par-1* by injecting embryos with double-stranded RNA (dsRNA). Depletion of *par-1* transcripts by RNAi resulted in

![Fig. 1. Localization of Par-1 in the *Drosophila* embryo and eye imaginal disc. (A-E) Embryos stained for PAR-1 protein (green) and nuclei (red). All views except B are mid-sagittal; B is of the blastoderm at the apicolateral plane. (A,B,C,D,E) Low magnification views of embryos at different stages. (A’,B’,C’,D’,E’) High magnification views of the developing blastoderm and ectoderm, with the apical region oriented towards the top. (A,A’) Early cellularization. (B,B’) Mid-cellularization; PAR-1 is reduced apically and localized laterally, including the furrow canal (arrow). (C,C’) Late cellularization. (D,D’) Stage 8 gastrulating embryo showing apical and apicolateral PAR-1 in the ectoderm. Weak staining is also present in neuroblasts. (E,E’) Stage 15 embryo, with higher levels present in the nervous system and midgut. (F-H) Localization of PAR-1 (green) in eye imaginal discs. (F) A low magnification view of the eye disc. The band of strong staining is the morphogenetic furrow. (G) A transverse view of three ommatidia with the apical region oriented toward the top. PAR-1 is concentrated in apical and apicolateral regions. E-cadherin (red) marks the AJ in projections of photoreceptor neurons. (H) A planar view at the apicolateral plane of disc cells. Par-1 is detected around cell membranes and in the photoreceptor AJs containing E-cadherin (red).](image-url)
almost no immunostaining, demonstrating that the staining pattern we observed is specific for the Par-1 protein (Fig. 2A,B).

Anti-Par-1 staining was maintained in epithelia that were derived from the blastoderm. The eye imaginal disc is a fully polarized epithelium that is directly derived from mid-embryonic stage ectoderm epithelium without a mesenchymal intermediate (Tepass, 1997). We observed specific anti-Par-1 staining in the eye imaginal disc of third-instar larvae in the apical domain, marginal zone, and AJ (Fig. 1F-H). This pattern was reminiscent of the pattern observed in the gastrulating embryonic ectoderm, from which the eye disc is derived. At this stage, Par-1 overlapped with E-cadherin in the disc cell AJ.

\textbf{par-1(RNAi) mislocalizes blastoderm and ectoderm polarity markers}

Genetic analysis of an embryo completely devoid of maternal and zygotic Par-1 was not possible. \textit{par-1} is a maternal-effect gene and germline clones fail to develop beyond stage 5 of oogenesis (Shulman et al., 2000). We were interested, however, in the role \textit{par-1} might play in early stages of embryogenesis. Homozygous zygotic mutants do not exhibit any detectable phenotype in early embryonic epithelia although mutants are embryonic lethal (Doerflinger et al., 2003; Shulman et al., 2000). This suggested that a maternal contribution precludes blastoderm analysis of \textit{par-1} in zygotic mutants. RNAi permitted us to remove both maternal and zygotic \textit{par-1} mRNA in pre-blastoderm embryos, thus circumventing the genetic quandary. Injection of dsRNA into embryos prior to cellularization allows for all cells to take in dsRNA and destroy the target mRNA (Kennerdell and Carthew, 1998). The RNAi effect is sustained through subsequent embryonic development, rendering it possible to analyze loss of gene activity in early and late stage embryos. Effectiveness of \textit{par-1}(RNAi) was confirmed by injection of dsRNA into syncytial embryos, which were then fixed and stained for Par-1 protein (Fig. 2A,B). To determine if the \textit{par-1}(RNAi) phenotypes we observed were specific for the \textit{par-1} gene, we compared phenotypes in embryos that were injected with three distinct dsRNAs generated from non-overlapping regions of the \textit{par-1} gene. All three dsRNA triggers resulted in identical embryonic phenotypes, confirming that the RNAi phenotypes we observed are attributable to the \textit{par-1} gene.

We examined an early marker of blastoderm cell polarity. Towards the end of cellularization, SAJs coalesce near the apical surface (Lecuit and Wieschaus, 2000). Concurrent with this coalescence, basal SAJs associated with the furrow canal dissipate (Muller and Wieschaus, 1996). \textit{Drosophila} E-cadherin is a component of the cadherin-catenin complex, which is present in SAJs during cellularization (Hunter and Wieschaus, 2000). Indeed, towards the end of cellularization, anti-E-cadherin staining was concentrated near the apical domain of a control blastoderm (Fig. 2C). Double-labeling of E-cadherin and Par-1 indicated that the two proteins are localized in non-overlapping domains, with Par-1 extending along the lateral domain up to a position immediately basal to the E-cadherin domain (Fig. 2C). When we examined E-cadherin localization in the blastoderm of \textit{par-1}(RNAi) embryos, we observed that E-cadherin was dramatically expanded along a large span of the lateral membrane domain (Fig. 2D). The expansion in E-cadherin distribution suggested that Par-1 is required for proper SAJ localization in the cellularized blastoderm.

\textbf{Fig. 2.} Par-1 is required to establish blastoderm polarity. (A,B) Stage 15 embryos stained for Par-1 protein (red). Embryos were injected with (A) buffer or (B) with \textit{par-1} dsRNA. (C-P) Embryos were injected prior to cellularization with buffer (C,E,G,I,K,M,O) or \textit{par-1} dsRNA (D,F,H,J,L,N,P) and stained with antibodies near the end of cellularization. Blastoderm nuclei are shown in blue in some panels and all panels are oriented apical side towards the top. (C,D) Blastoderm stained with anti-Par-1 (red) and anti-E-cadherin (green). (E,F) Anti-phosphotyrosine (green) staining. (G,H) Anti-Patj staining (green) is expanded along the lateral domains of \textit{par-1}(RNAi) blastoderm. (I,J) Anti-Crb staining (green) in the apical domain. (K,L) Anti-Dlg staining (green) is present along the lateral membranes. (M,N) Anti-Delta staining (green) is reduced basolaterally by \textit{par-1}(RNAi). (O,P) Anti-Tau (green) stains the apical cytoplasm capping the nuclei and extends down past the nuclei.
To further test this hypothesis, we examined the localization of another SAJ marker in par-1(RNAi) embryos. Many proteins with phosphorylated tyrosine residues strongly localize in cellularized epithelia at SAJs and the AJ (Müller and Wieschaus, 1996). Control blastoderm cells nearing completion of cellularization were stained at apical and basal SAJs with anti-phosphotyrosine (Fig. 2E). By contrast, par-1(RNAi) blastoderm cells showed an expansion of staining along the lateral membrane in addition to the apical and basal SAJ staining (Fig. 2F). Thus, Par-1 is required to restrict SAJ proteins to the apicolateral and basal regions only.

We next determined whether Par-1 is required for proper localization of the PDZ protein Patj (also called Dlt), which is localized at gastrulation in the basal domain and in the marginal zone (Bhat et al., 1999) (Fig. 2G). Patj was also detected in the basal domain and marginal zone of par-1(RNAi) cells, indicating that these structures do not require Par-1 (Fig. 2H). There was a weak expansion of Patj localization and increased cytoplasmic staining in depleted embryos. To test whether the expansion of Patj expression was due to an expansion of the marginal zone, we stained par-1(RNAi) embryos for Crb. In early gastrulating embryos, Patj and Crb interact in the marginal zone (Bhat et al., 1999). However, Par-1 was not required to restrict Crb since Crb protein did not expand into the lateral domain of par-1(RNAi) embryos (Fig. 2H,J). Thus, the restriction of the marginal zone adjacent to the apical surface does not require Par-1, even though Par-1 restricts SAJs near the apical region.

It was possible that the AJ proteins were mislocalized because of a global retargeting of transport vesicles to the lateral membrane. Alternatively, it was the result of a more specific alteration in basolateral membrane properties. To test this possibility, we examined a membrane protein that is normally localized to the basolateral membrane during cellularization. Delta is the ligand for the Notch receptor and is localized at gastrulation in the basal domain and contains Scribbled (Scrib) and discs large (Dlg). Normally, Dlg is present along the lateral domain in blastoderm cells and was more concentrated near the apical region. However, Par-1 restricts Crb since Crb protein did not expand into the lateral domain of par-1(RNAi) embryos (Fig. 2H,J). Thus, the restriction of the marginal zone adjacent to the apical surface does not require Par-1, even though Par-1 restricts SAJs near the apical region.

Apical polarity is affected by a protein complex that is localized to the lateral domain and contains Scribbled (Scrib) and Discs large (Dlg). Normally, Dlg is present along the lateral domain in blastoderm cells, enriched just below the site of AJ formation (Fig. 2M) (Tanentzapf and Tepass, 2003). In dlg mutants apical AJ proteins such as E-cadherin are localized more basally than normal. Thus, it was possible that Par-1 was acting through Dlg to restrict AJ proteins. When we examined the relationship between Par-1 and Dlg, we found that anti-Dlg staining was not altered in par-1(RNAi) embryos (Fig. 2N). Thus, Par-1 functions independently of Dlg expression to block lateral accumulation of SAJs. This is consistent with observations that dlg mutants affect AJ protein localization considerably later in embryogenesis (Bilder and Perrimon, 2000).

Since membrane proteins are delivered to specific membrane regions by the microtubule network during cellularization, we investigated whether Par-1 organizes the network in the cellularizing embryos. During cellularization, microtubules grow out from apically positioned centrosomes down over the surface of nuclei and project their plus ends into the interior, forming ‘inverted baskets’ around each nucleus (Foe and Alberts, 1983). Throughout cellularization, proteins important for furrow canal movement move along the microtubules towards the furrow, and transport of new membrane to specific domains depends on microtubules (Lecuit and Wieschaus, 2000). We visualized microtubule organization in blastodermers by staining the microtubule-binding protein Tau. Anti-Tau staining was localized to cytoplasm on the apical side of nuclei (Fig. 2O). This localization is consistent with Tau associating with microtubules. In par-1(RNAi) embryos, the cap of Tau staining was denser but was nevertheless normally located apical to nuclei (Fig. 2P). The increased Tau staining suggests a higher microtubule density in par-1(RNAi) blastoderm cells, a phenomenon also observed in par-1 mutant follicle cells (Doerflinger et al., 2003). However, the apical cap staining of par-1(RNAi) cells suggests that microtubule organization is not dramatically affected by Par-1. Consistent with this, localization of a plus-ended kinesin fusion protein was not expanded in blastoderm cells by RNAi depletion of Par-1 (data not shown).

We determined whether Par-1 plays a role in establishing polarity in the blastoderm to ectoderm transition that occurs during gastrulation. At this stage, E-cadherin normally localizes to the developing AJ of ectoderm and mesoderm cells (Fig. 3A). par-1(RNAi) embryos, however, exhibited abnormally high levels of E-cadherin, with expansion of E-cadherin over entire cell cortices of ectoderm, mesoderm and neuroblast cells (Fig. 3B). Thus, restriction of the developed AJ in ectoderm and derived tissues also requires Par-1. Patj

![Fig. 3](Image)

**Fig. 3.** Par-1 is required for gastrula ectoderm cell polarity. Embryos injected at the syncitial stage with buffer (A,C,E) or par-1 dsRNA (B,D,F) are shown at stage 9. (A,B) Anti-E-cadherin (red) shows expanded localization within ectoderm, mesoderm and neuroblasts in par-1(RNAi) embryos. (C-F) Embryos triple-labeled for Patj (cyan), Dlg (green) and E-cadherin (red). (C,D) Patj staining is not significantly expanded along lateral membranes in par-1(RNAi). (E,F) Dlg staining is similar in control and par-1(RNAi) embryos.
localization at the same stage is normally in the marginal zone (Fig. 3C). par-1(RNAi) had little effect on Patj localization in ectoderm cells (Fig. 3D), and Dlg localization was normal in ectoderm and neuroblasts of par-1(RNAi) gastrulas (Fig. 3E,F). Thus, the Crb/Patj and Dlg/Scrib complexes are not significantly affected in ectoderm. Moreover, epithelial architecture was relatively normal in depleted embryos, in contrast to mid-gastrula embryos devoid of other polarity factors such as Par-3 and aPKC (Wodarz et al., 2000). These results suggest that the pre-polarity pattern established by Par-1 during cellularization is retained in the ectoderm of the gastrula. However, generation of epithelial architecture of the ectoderm does not require Par-1.

Par-1 is not essential for maintenance of epithelial polarity in the eye imaginal disc

Par-1 is important for establishing the primary polarity of early blastoderm cells. Many epithelia are derived from the blastoderm without a transition to a non-epithelial state and temporary loss of polarity (Tepass, 1997). One such epithelium is that of the eye imaginal disc. We investigated whether Par-1 is required for maintenance of polarity in the eye disc. This was done by establishing normal blastoderm and ectoderm polarity in the presence of Par-1, and then removing Par-1 activity by eliminating the wild-type par-1 gene. Eye disc cells were induced to undergo mitotic recombination using the FLP-FRT system, changing the cells’ heterozygous genetic state to a homozygous null mutant state. Mutant cells showed a normal staining pattern with the neuron-specific marker Elav, indicating photoreceptor fate specification was unaffected (data not shown). Mutant cell polarity appeared to be normal as determined by staining with antibodies to E-cadherin, Patj, phosphotyrosine and Crb (Fig. 4A-E, data not shown). Since the loss of par-1 in these cells occurred from 6 to 70 hours before analysis, it is unlikely that much residual Par-1 protein was present. Thus, Par-1 is not necessary to maintain cell polarity in the eye imaginal disc.

Although we found no apical-basal polarity defects, a subtle morphological phenotype revealed itself in null clones. The eye disc epithelium appeared to locally deform basally in regions...
containing a clone. Confocal z-sections showed apical domains of mutant cells and basal domains of surrounding wild-type cells (Fig. 4B-E). This deformation was not due to a change in cell shape or size. Comparative analysis of cell dimensions by tracing wild-type and mutant cells and counting the number of z-sections for each, as well as comparing size in the xy plane, failed to yield distinguishable differences (data not shown).

The polarized localization of Par-1 in the eye disc suggests that it plays a role in eye disc polarity but it is not essential because of genetic redundancy. To test whether Par-1 regulates polarity, we overexpressed Par-1 in eye disc cells. We used GMR-Gal4 to drive expression of UAS-Par-1 in cells posterior to the disc morphogenetic furrow. Par-1 protein was overexpressed, as determined by anti-Par-1 staining, although the localization of the protein was normal (Fig. 4F,F'). Disruption of eye disc morphology was apparent (Fig. 4I). Differentiation was also affected. Neurons, marked by anti-Elav, were present but reduced in number and were in a disorganized pattern (Fig. 4K,K',L,L'). Expanded BrdU labeling and Acridine Orange staining indicated an excess of cell proliferation and apoptosis, respectively (Fig. 4M,N,P,Q). Markers of the AJ such as E-cadherin and phosphotyrosine were irregularly localized (Fig. 4I',J', and data not shown). In addition, organization of the apical membrane domain and septate junction was disrupted (Fig. 4O,R).

To demonstrate that the phenotype caused by overexpressed Par-1 was due to increased Par-1 kinase activity, we also overexpressed two mutant forms of Par-1 that were predicted to be kinase inactive. Par-1(AA) has the residues S640 and T636 in the kinase P loop replaced with alanines. The homologous serine and threonine residues in human Par-1 were shown to activate kinase activity when phosphorylated (Drewes et al., 1997). Par-1(KR) has an arginine in place of the critical lysine residue in the ATP-binding site. Overexpression of Par-1(AA) and Par-1(KR) with GMR-Gal4 had no detectable effect upon eye disc morphology, polarity and development (Fig. 4J,J',L,L',N,Q,R). Antibody labeling of these discs showed similar Par-1 levels to discs overexpressing wild-type Par-1, and localization was normal (Fig. 4F-H'). Thus, the effect of Par-1 overexpression is completely dependent on its kinase activity.

Par-1 functions in neural lateral inhibition
To understand the role of Drosophila Par-1 in cell-cell signaling, we examined the effect of par-1(RNAi) on embryonic patterning that is controlled by signal transduction. Drosophila embryonic systems that are derived from ventrolateral ectoderm begin at stage nine (Artavanis-Tsakonas et al., 1995). Clusters of ectoderm acquire the potential for a neural fate by expressing a set of proneural genes. However, most cells in each cluster do not adopt a neural fate because of lateral inhibition. Signaling between cells in the form of interactions between the Notch receptor and its ligand Delta represses the neural fate decision, ensuring that only a small number of ectoderm cells acquire neural identity (Fig. 5A,B). When we stained par-1(RNAi) embryos to visualize neurons, we saw severe hyper-neuralization in 78% (n=86) of embryos (Fig. 5C,D). The remaining embryos displayed milder defects in neural development. The hyper-neural phenotype was highly similar to the neuralization seen in the absence of Notch (Fig. 5E,F). We investigated whether the neuralization caused by par-1(RNAi) was due to a general loss of polarity in the ectoderm. We immunostained maternal mutants of other polarity genes with a neural-specific antibody. Flies mutant in the genes shg (encodes E-cadherin), baz and crb displayed disorganized nervous systems but were not hyper-neuralized (Fig. 5I-N). We also examined embryos that were depleted of maternal and
zygotic *scrib* by RNAi. Although abnormalities were evident in the neural patterning of *scrib*(RNAi), hyper-neuralization was not evident to the extent seen with *par-1*(RNAi) (Fig. 5G,H). A comparison of the number of neuro-positive cells in peripheral chordotonal sensory organs for each mutant confirmed that only *par-1*(RNAi) embryos had an enhanced number of neural cells comparable to those mutant for Notch (Table 1). We conclude that the neurogenic phenotype caused by loss of Par-1 is not a general consequence of disrupting ectoderm cell polarity.

One of the proneural genes initially expressed in the clusters is *achaete*. Its expression progressively increases in one cell per cluster and decreases in all other cells due to Notch signaling (Fig. 6A). To ascertain if Par-1 regulates *achaete* expression like Notch, we stained *par-1*(RNAi) embryos with anti-Achaete. Embryos had normal numbers of proneural clusters, with each cluster containing many extra Achaete-positive cells (Fig. 6B). This result indicates that Par-1 is required for lateral inhibition.

Par-1 acts in Notch signaling

We investigated whether Par-1 acts in the Notch signaling pathway by performing epistasis analysis. We expressed a membrane-tethered form of Notch that is able to signal independently of ligand binding (Rebay et al., 1993), resulting in significant loss of embryonic nervous tissue (Fig. 6C). When Par-1 was depleted by RNAi in embryos expressing constitutively activated Notch, there was loss of nervous tissue that was indistinguishable from the Notch phenotype when Par-1 was present (Fig. 6D). This result indicates that Par-1 acts upstream of Notch, and is not required for subsequent steps in the signaling pathway.

It was possible that loss of Par-1 affected other signaling events occurring in the ectoderm at the time of lateral inhibition. We examined this issue by analyzing the expression pattern of Engrailed in *par-1*(RNAi) embryos. Engrailed is expressed in the ectoderm in a manner dependent upon both the Wingless and Hedgehog signaling pathways. During early neurogenesis (stage nine) and later neurogenesis (stage 11), the expression pattern of Engrailed was normal in *par-1*(RNAi) embryos (Fig. 6E,F and data not shown). Our data is thus consistent with a specific requirement for Par-1 in Notch signaling.

We had observed an effect of Par-1 on Delta localization at an earlier stage. We therefore tested if Delta and Notch localization were Par-1-dependent during neurogenesis. At this stage, Notch is distributed along the lateral surfaces of ectoderm cells below the AJ, and along the basal surfaces of ectoderm cells and neuroblasts (Fig. 7A). Notch membrane localization was unaffected by *par-1*(RNAi) although there was enhanced cytoplasmic staining (Fig. 7B-D). Delta is normally localized along the lateral membrane of ectoderm cells up to and including the AJ (Fig. 7E). In *par-1*(RNAi) embryos, Delta was strongly reduced in the basolateral domain, with concomitant enhanced cytoplasmic localization (Fig. 7F-H).

Par-1 synergistically acts with Notch to stimulate cell growth

Notch signaling affects developmental decisions throughout the *Drosophila* life cycle. We investigated whether Par-1 functions in the Notch pathway at other stages of development. Notch is required for several aspects of eye disc development. One role is to promote determination of the disc towards an eye identity and promote growth (Kumar and Moses, 2001; Kurata et al., 2000). Loss of Par-1 in early disc cells had no

### Table 1. Numbers of neurons per embryonic chordotonal organ in different polarity backgrounds

| Genotype    | Mean number neurons/organ | Standard deviation |
|-------------|----------------------------|--------------------|
| Wild type   | 5.0                        | 0                  |
| *par-1*(RNAi) | 52.4                       | 32.8               |
| *N*~65ac~/ | 65.4                       | 38.5               |
| *Scrib*(RNAi) | 10.3                       | 4.0                |
| *bac*~2006~ | 13.5                       | 2.4                |
| *shg*~1A2~ | 6.7                        | 1.4                |
| *crb*~1A2~ | 8.7                        | 3.2                |

Fig. 6. Par-1 acts specifically in the Notch pathway. Embryos were injected with buffer (A,C,E) or *par-1* dsRNA (B,D,F) before analysis. (A,B) Anti-Achaete staining of four segments at stage 9 shows one positive cell per proneural cluster in control, whereas many proneural cells are positive after *par-1* RNAi treatment. (C,D) Stage 15 *arm-GAL4 UAS-N*~E~ embryos stained for Neuroglian show severe reduction in neural tissue. (E,F) Stage 10 embryos stained for Engrailed.
detectable impact on their specification and growth (Fig. 4A-E). To determine if Par-1 nevertheless influences Notch signaling, we overexpressed Par-1 in early eye disc cells using the ey-GAL4 driver. This resulted in a small or non-existent eye disc, or transformation of the disc to an antennal identity (Fig. 8A-C). This phenotype is identical to a lack of Notch signaling in the eye disc (Kumar and Moses, 2001). The kinase-dead versions of Par-1 had no effect on eye disc growth and specification (Fig. 8D and data not shown), indicating that Par-1 kinase activity is required for the phenotype. If the effect of overactive Par-1 is to disrupt Notch signaling in a manner similar to the embryonic ectoderm, then Notch should be epistatic or downstream of Par-1 in the eye disc as it is in the embryo. Expression of constitutively active Notch in the early eye disc results in eye disc hyperplasia, as had been previously observed (Fig. 8E) (Kurata et al., 2000). To determine the epistatic relationship of Par-1 and Notch, we analyzed the phenotype when both Par-1 and activated Notch were co-expressed in the early eye disc. The eye discs were present and properly specified, indicating that Notch is downstream of Par-1 (Fig. 8F). Surprisingly, we observed a synergistic interaction between Par-1 and Notch, in that there was a profound hyperplasia of the eye disc. This indicates that the relationship between Par-1 and Notch is not simply linear. Probably, Par-1 and Notch signaling also act in a parallel manner to stimulate cell growth within the eye disc.

**Discussion**

Par-1 plays a role in establishing proper cell polarity in the blastoderm and ectoderm. The data presented here indicate that Par-1 is specifically involved in restricting SAJs to a region adjacent to the future apical domain during cellularization. Par-
Par-1 also restricts the AJ to the same region in older cells as they undergo gastrulation. Depletion of Par-1 results in expansion of the AJ into the lateral domain. This effect is not unique to par-1 (RNAi) embryos. The Dlg/Scrib complex is required to restrict the AJ from the lateral domain. However, three observations indicate that Par-1 is not simply acting through this complex to localize the AJ. First, Par-1 does not regulate the localization of Dlg in blastoderm or ectoderm. Second, the Dlg complex does not begin to restrict the AJ until gastrulation (Bilder and Perrimon, 2000), a time considerably later than Par-1 initially acts. Third, loss of Scrib results in lateral expansion of apical Crb (Bilder and Perrimon, 2000), a phenomenon not observed when Par-1 is depleted. Altogether, these data indicate that Par-1 restricts the AJ by a mechanism independent of the Dlg/Scrib complex, perhaps preceding it. Interestingly, the presence of the Dlg complex in gastrulating ectoderm is not sufficient to re-establish an apical AJ when Par-1 is depleted, suggesting that the Par-1 and Dlg/Scrib pathways are obligatory.

It might not be that Par-1 simply defines the limits of the AJ. Rather, it might also act positively to specify the basolateral domain. Par-1 is required for localization of Delta to the basolateral region. This positive effect of Par-1 on Delta is not due to preventing the AJ from inhibiting Delta. Delta colocalizes with the AJ both normally and when Par-1 is depleted. So how does Par-1 guide membrane regionalization during cellularization? Par-1 protein is distributed along the lateral membrane immediately basal to the incipient AJ. Based on this, three models suggest themselves. Par-1 could assemble a diffusion barrier that physically blocks movement of SAJs into the lateral region and limits movement of Delta into the apicolateral region. If par-1 (RNAi) disrupts such a barrier, then other mechanisms must maintain apical Crb restriction from the lateral region. An alternative is that Par-1 has a role in the polarized targeting of transport vesicles carrying SAJ and Delta proteins. In this model, Par-1 might interact with the ‘exocyst’, a secretory targeting apparatus involved in polarized segregation of transmembrane proteins. Data from yeast Par-1 indicate that it directly associates with a t-SNARE, a membrane-bound component of the exocyst (Elbert et al., 2005). Moreover, Par-1 phosphorylation of the t-SNARE protein triggers its release from the cell membrane. If Drosophila Par-1 also interacts with the exocyst, then it might selectively block the fusion of SAJ exocytic vesicles to the basolateral membrane. In support of this model, punctate intracellular staining of Par-1 can be seen during cellularization and is reminiscent of vesicles. Par-1 might also stimulate targeting of other cargo, such as Delta-loaded vesicles, to the basolateral membrane. Consistent with this notion, Par-1-depleted ectoderm cells accumulate Delta-positive cytoplasmic vesicles. Finally, Par-1 could differentially affect the stability of proteins in the lateral domain, by de-stabilizing some and stabilizing others. This could occur through degradation or rapid recycling via endocytosis.

How directly would Par-1 participate in these mechanisms? This is unclear at present. None of the known substrates for Drosophila Par-1 kinase include Delta or AJ components. In ovarian follicle cells, Par-1 phosphorylation of Baz prevents Baz association with Par-6 – aPKC (Benton and St Johnston, 2003a; Benton and St Johnston, 2003b). Baz and Par-6 are among the earliest acting proteins in polarization of blastoderm. During cellularization, Baz associates with the apicolateral membrane, whereas Par-6 is localized to the apical cortex (Harris and Peifer, 2004; Hutterer et al., 2004). If either Baz or Par-6 is mutated, the apical AJ proteins do not coalesce but disperse along the lateral membrane. Thus, Baz could be a candidate for mediating the effects of Par-1 in the blastoderm. However, several observations indicate that Par-1 phosphorylation of Baz is not necessarily essential to establish AJ localization. First, Par-1 does not have a polarized distribution during early cellularization and is detected in the apicolateral regions where Baz and Par-6 are already localized (Harris and Peifer, 2004; Hutterer et al., 2004). A similar co-distribution is seen by the time the ectoderm has reached mid-gastrula stage. Second, although Baz is required for apical localization of Crb and Patj (Bilder et al., 2003; Muller and Wieschaus, 1996; Wodarz et al., 2000), Par-1 has no significant effect on their apical localization. Third, establishment of the AJ in follicle cells is not dependent upon Par-1 phosphorylation of Baz (Benton and St Johnston, 2003b).

Par-1 plays a curious role in maintenance of polarity of imaginal disc epithelia that derive directly from ectoderm. Par-1 is localized to the apical and marginal zones of imaginal disc cells but is not essential for their polarity. Possibly, redundant mechanisms operate in the absence of Par-1. This idea is supported by overexpression experiments. When Par-1 is overexpressed, the AJ and apical domain are disorganized, and cells are compromised for differentiation, growth and death. This result argues that Par-1 normally plays a role in maintaining cell polarity that is sensitive to its activity level. By contrast, Par-1 is essential to maintain polarity in follicle cell epithelia surrounding adult egg chambers (Benton and St Johnston, 2003a; Benton and St Johnston, 2003b), suggesting that redundancy is restricted to imaginal discs.

Par-1 also regulates Notch signaling and it acts upstream of Notch as determined by epistasis analysis. We detected two different Notch signaling decisions regulated by Par-1. The first was in the embryonic ectoderm where Par-1 depletion disabled Notch-mediated lateral inhibition. The second was in the eye imaginal disc where Par-1 overexpression disabled Notch-mediated eye cell determination. Since Notch was disabled when Par-1 was missing or overactive, it suggests that Par-1 is not playing an instructive role in Notch signaling. Rather, it is probably a permissive effect that is related to cell polarity regulation. Indeed, localization of Delta is dramatically reduced along the basolateral domains of blastoderm and ectoderm cells of par-1 (RNAi) embryos. It is reasonable to think that Par-1 acts in Notch signaling by localizing Delta to a region of the membrane where it can make a productive interaction with Notch. This permissive model of Notch signaling is nevertheless specific; other regulators of ectoderm polarity do not affect Notch signaling. Moreover, we found that other signaling pathways active in the ectoderm are unaffected by Par-1. However, our observations are at odds with those of Sun (Sun et al., 2001) who examined the effect of par-1 (RNAi) on Wingless/Dishevelled signaling. They did not look directly at Wingless signaling but at a late embryonic readout of signalling – cuticle patterning – and observed a weak dishevelled-like phenotype. Since Notch has been linked to Dishevelled function in Wingless signaling in Drosophila (Axelrod et al., 1996), it is possible that a weak disruption of Dishevelled by dysfunctional Notch in par-1 (RNAi) embryos led to the mild cuticle phenotype.
Interestingly, a synergetic interaction between Par-1 and Notch was found in the eye imaginal disc. Disc growth significantly increased when Par-1 was overexpressed with ligand-independent Notch. The extra eye tissue developed photoreceptors, indicating the ectopic cells are properly specified. Since loss of cell polarity is associated with hyperplasia in the eye disc (Bilder, 2004; Brumby and Richardson, 2003), this supports the notion that Par-1 exerts this effect through perturbation of eye disc cell polarity. The synergetic interaction with Notch may be useful in the future for screening of genes involved in tumor formation or progression to a cancerous state.

Materials and Methods

dsRNA synthesis and RNAi injections

dsRNA was synthesized as described previously (Kennerdell and Carthew, 1998). Primers used for Par-1: 5′-ACTGATCCATCITAGACACATGA-3′, 5′-GTTCACATCTAGAGGGTTG-3′, 5′-AGCCAGAGCTCAGTACTGATCA-3′, 5′-GCCACCTCAGGAAGCTGT-3′, 5′-CTTCACTGGTCTGGCTTCATTGCA-3′. Primers used for Scribble: 5′-TTGGTTGTAAGCCACATGTC-3′, 5′-CAAGGACACACAAGGACATC-3′. Par-1 dsRNAs were 685, 266 and 201 base pairs, respectively. RNAi injections were performed as described previously (Kennerdell and Carthew, 1998).

Immunohistochemistry

Primary antibodies used were 1:5,000 rabbit anti-Par-1 (Shulman et al., 2000), 1:20 rat anti-DE-cadherin (Oda et al., 1994), 1:1,000 mouse anti-C17.9C6 (Notch) (Heidary and Fortini, 2001), 1:3 mouse 4F3 anti-Dlg (Parnas et al., 2001), 1:3 rat anti-C17.9C6, 1:20 rat anti-DE-cadherin (Oda et al., 1994), 1:1,000 mouse anti-C17.9C6 (Notch) (Heidary and Fortini, 2001), 1:3 mouse 4F3 anti-Dlg (Parnas et al., 2001), 1:3 rat anti-Elav 7EBA10, 1:3 mouse AbD4 (Engrailed), and 1:3 mouse GS4 anti-brdu. For immunofluorescent staining, Alexa-Fluor-conjugated IgG secondary antibodies were used (Molecular Probes). Nuclear dyes used were either TOPRO (Molecular Probes) or propidium iodide (Sigma Aldrich). Most fixation and staining experiments followed published methods (Kaufmann et al., 1996; Kennerdell and Carthew, 1998). Notch and Delta staining were done following published methods (Kauffmann et al., 1996; Bennet, R., and St Johnston, D., 2003a). A conserved oligomerization domain in drosophila Bazooka/Par-3 is important for apical localization and epithelial polarity. Curr. Biol. 13, 1330-1334. Bennet, R. and St Johnston, D. (2003b). Drosophila Par-1 and 14-3-3 inhibit Bazooka/Par-3 to establish complementary cortical domains in polarized cells. Cell 115, 691-704.

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