exposure to antigen[5,8].

Many of the genes associated with the tolerance response might be predicted to have negative regulatory functions for maintaining the tolerant state (Fig. 4b). Egr-1 and Egr-2 are transcription factors implicated in B-cell mitogenesis[11,12], but their mitogenic activity is likely to be repressed in tolerant cells by relatively high expression of NAB2, an Egr family inhibitor[28]. Neurogranin and pcp-4 inhibit calmodulin[29] and may regulate the downstream effects of calcium oscillations in tolerant cells[7,21]. IgD represents the primary receptor isotype on tolerant B cells, through which repeated binding of self-antigen triggers low calcium oscillations. IgD signalling may be decreased relative to naive cells[6] by increased levels of CD72, which recruits the inhibitory tyrosine phosphatase SHP-1 (ref. 30) (Fig. 4b).

Inhibition of Egr-2 and CD72 by FK506 shows that this drug interferes with components of the active self-tolerance response, which may limit its efficacy in establishing or restoring tolerance in autoimmunity and transplantation. To develop more effective drugs, the unique transcript signature in anergic cells could be used in high-throughput assays as a surrogate marker for tolerance. A desired small molecule would suppress members of the subset of activation-only early response genes defined here, while leaving unaffected the subset of early response genes that also participate in tolerance, so that tolerance would be actively (re-)established. □

Methods

B-cell purification and stimulation

For each cell preparation, splenic B cells were purified and pooled from several mice at room temperature in 1% bovine calf serum in RPMI. The spleen cells were stained with CD4, CD8 and Mac-1 FITC-conjugated antibodies (Caltag) and depleted of T cells and macrophages with sheep anti-FITC magnetic beads (Perseptive Biosystems). The remaining cells were 85–95% B220 positive and were either lysed immediately (naive and tolerant cell preps) or stimulated in RPMI with 1% serum at 37 °C at (2–3) × 10^6 cells ml^{-1}. For stimulation experiments, HEL (Sigma) was used at 500 ng ml^{-1}, PD98059 (NEB) at 20 μM unless stated otherwise, ionomycin (a gift from G. Crabtree) at 1 μM and EGTA at 3 mM. The concentration of FK506 was chosen to be within the range maintained in the blood of kidney and liver transplant patients receiving FK506 (also called Tacrolimus and Prograf; information on dosing from http://www.fujisawa.com/info/medinfo/mnpginst. htm). Cells were pre-incubated for 45 min with PD98059, 15 min with FK506 and 2 min with EGTA before addition of HEL or anti-mu. Mock stimulations were carried out by addition of carrier alone for stimuli or inhibitors. At the end of the incubation, the cells were pelleted by centrifugation, resuspended in a minimal volume of medium (~50 μl) by pipetting and lysed in 0.5–1 ml Trizol (Gibco BRL). Naive and tolerant B cells were also purified by cell sorting for B220 positive, $CD21^{medium}$ cells. Marginal zone cells (CD21 high) were excluded from the gate. Between 10^7 and 5 × 10^7 magnetic-bead-selected B cells, or (1.5–3) × 10^6 cell-sorter-purified B cells were used for RNA preparation.

RNA preparation, array hybridization and data analysis

RNA labelling and array hybridization were done as described[10]. For detailed methods and statistical analysis, see Supplementary Information.

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Supplementary information is available on *Nature*'s World-Wide Web site (http://www.nature.com) or as paper copy from the London editorial offices of *Nature* and also at http://jcsmr/group_pages/mgc/MedGenCen.html and http://www.eosbiotech.com..

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Localization of apical epithelial determinants by the basolateral PDZ protein Scribble

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The generation of membrane domains with distinct protein constituents is a hallmark of cell polarization. In epithelia, segregation of membrane proteins into apical and basolateral compartments is critical for cell morphology, tissue physiology and cell signalling. *Drosophila* proteins that confer apical membrane identity have been found[1,2], but the mechanisms that restrict these determinants to the apical cell surface are unknown. Here we show that a laterally localized protein is required for the

apical confinement of polarity determinants. Mutations in *Drosophila scribble (scrib)*, which encodes a multi-PDZ (PSD-95, Discs-large and ZO-1) and leucine-rich-repeat protein, cause aberrant cell shapes and loss of the monolayer organization of embryonic epithelia. Scrib is localized to the epithelial septate junction, the analogue of the vertebrate tight junction[3], at the boundary of the apical and basolateral cell surfaces. Loss of *scrib* function results in the misdistribution of apical proteins and adherens junctions to the basolateral cell surface, but basolateral protein localization remains intact. These phenotypes can be accounted for by mislocalization of the apical determinant Crumbs. Our results show that the lateral domain of epithelia, particularly the septate junction, functions in restricting apical membrane identity and correctly placing adherens junctions.

We isolated *scrib* in a screen for maternal effect mutations that disrupt aspects of epithelial morphogenesis such as cell adhesion, shape and polarity. The structure of the embryonic cuticle was used to reflect the organization of the underlying epithelial epidermis that secretes it. The wild-type cuticle forms a smooth, continuous sheet (Fig. 1a), but embryos that are maternally and zygotically mutant for *scrib* (hereafter referred to as *scrib* embryos) produce a corrugated cuticular surface that is riddled with holes (Fig. 1b, hence the name *scribble*). Because *scrib* cuticles suggest a broad defect in epidermal organization, we examined the morphology of *scrib* embryos. *scrib* embryos proceed normally through precellular development, and the epithelial blastoderm forms as in wild type. After gastrulation, however, organization of the ectodermal epithelium is disrupted as cells lose their columnar shape and planar arrangement. These defects become progressively more severe as development proceeds. Instead of the wild-type tightly integrated columnar monolayer epithelium (Fig. 1c), the epidermis of late *scrib* embryos is frequently interrupted and consists of groups of irregular rounded cells that are separating from one another (Fig. 1d). Confocal microscope sections reveal that most of the epidermis is organized into multilayered strips or tubes of cells that have lost contact with the underlying tissue (Fig. 1f).

To understand the requirement of *scrib* for epithelial organization, we cloned the *scrib* gene (Fig. 2a; see also Methods). Plasmid rescue from two *scrib* P-element alleles included sequences identical to an expressed sequence tag (EST) in the genome database. The EST was used to screen an embryonic complementary DNA library and identify a clone (A2) containing an insert of 6.2 kilobases (kb), similar to the size seen on embryonic northern blots. This insert

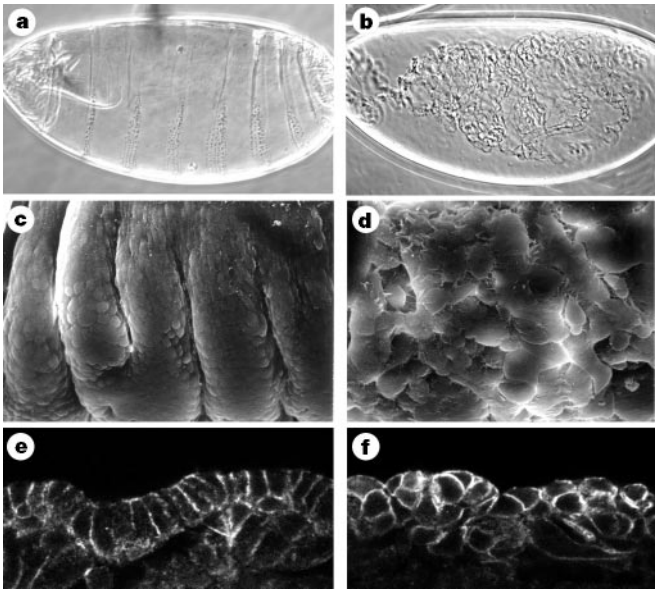

Figure 1 *scrib* mutations cause disorganization of epithelia. **a**, Wild-type cuticle. **b**, Cuticle from *scrib* GLC embryo. Only folded strips and whorls of disintegrating cuticle are produced. **c**, Scanning electron micrograph of a wild-type stage-14 embryo. **d**, Similar image of a *scrib* embryo. The highly integrated epithelium of wild-type embryos is falling apart; flattened and rounded cells are seen pulling away from their neighbours. **e**, Confocal microscope image of stage-15 wild-type embryonic epidermis stained for α-Spectrin. Note the regular columnar monolayer organization of the epithelium. **f**, Similar image of a *scrib* embryo. The epidermal cells are rounded, irregularly shaped, and pile up upon each other.

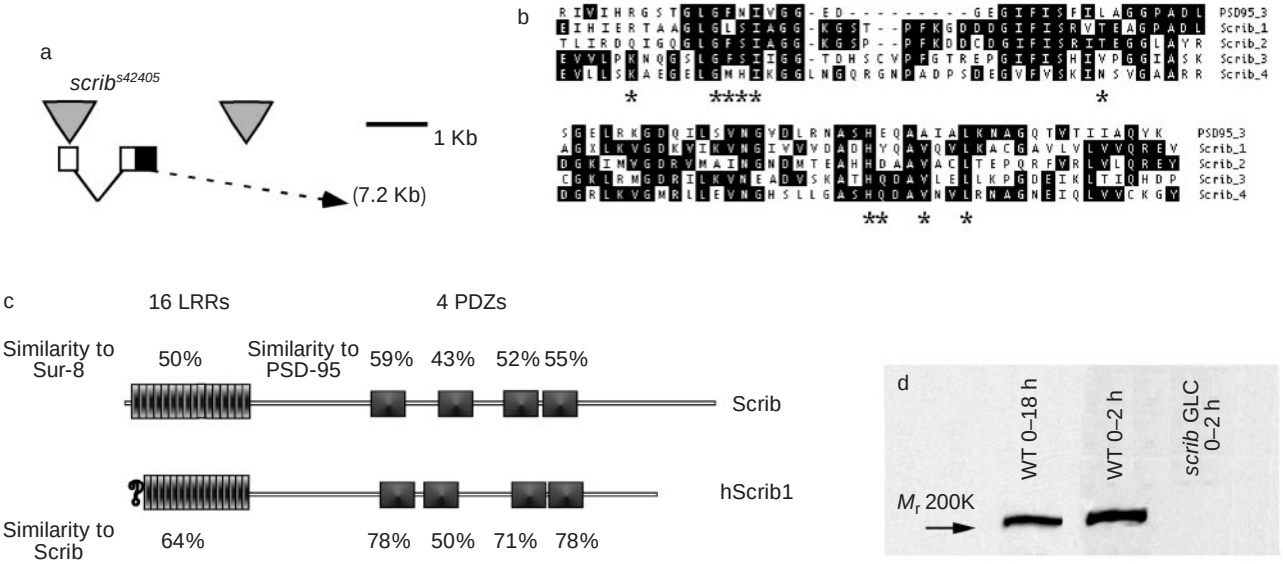

Figure 2 Cloning of *scrib*. **a**, Genomic structure of the *scrib* locus. Two P-element-induced hypomorphic *scrib* alleles map to 97B and insert in the 5′ untranslated region and the first intron of *scrib*. **b**, Diagram of the predicted Scrib protein. Similarity of Scrib regions to the LRRs of Sur-8 and to the third PDZ domain of PSD-95 is shown. Similarity of these Scrib regions to those in a partially sequenced human homologue hScrib1 is given below; other regions do not show significant conservation. The Genbank accession number for *scrib* sequence is AF190774. **c**, Alignment of the four Scrib PDZ domains with that of PSD-95 PDZ3; consensus identities are shaded in black. Asterisks indicate residues of PSD-95 that are predicted to determine its selectivity for binding an S/TXV consensus sequence at the C terminus of transmembrane proteins. **d**, Western blot of 0–18-hour and 0–2-hour wild-type (WT) embryos, and 0–2 hour *scrib* GLC embryos, probed with the anti-Scrib antibody. The antibody detects a large maternal contribution of Scrib that is absent in the mutant embryos.

includes a Kozak-like consensus sequence followed by a methionine codon at the start of a 5.8-kb open reading frame (ORF). Upon heat-shock induction, the A2 cDNA rescues the lethality of strong heteroallelic *scrib* combinations, showing that it encodes *scrib* function.

The *scrib* ORF is predicted to encode a protein of 1,731 amino acids with a relative molecular mass of 195,000 (M_r 195K). Sequence analysis reveals the presence of two known protein–protein interaction motifs (Fig. 2b). At the amino terminus of Scrib is a set of 16 leucine-rich repeats (LRRs). This region is very similar to the Ras-binding LRRs of the proteins Sur-8 from *Caenorhabditis elegans* and adenylate cyclase from yeast[4,5]. Distributed throughout the remainder of the protein are four PDZ domains. The PDZ domains of Scrib are highly similar to those of PDZ3 from PSD-95, a human homologue of fly Discs-large (Fig. 2c). Comparison of Scrib residues with those of PSD-95 indicates that all four Scrib PDZ domains are Type 1A, which are predicted to bind to the consensus S/TXV at the carboxy terminus of proteins[6]. Scrib has at least one human homologue, encoded by the KIAA0147 cDNA[7], which we call *hscrib1* (Fig. 2b). No obvious signal sequences or transmembrane domains are predicted in either Scrib or hScrib1 by standard algorithms, suggesting that Scrib is a cytosolic protein. We note, however, that Densin, a protein with 16 LRRs and one PDZ domain, has been suggested to have transmembrane toplogy[29]. We cannot exclude the possibility that Scrib is an atypical transmembrane protein.

To determine the subcellular localization of Scrib, we generated antibodies against Scrib peptides (see Methods). Two polyclonal sera recognize a band of ~210K on embryonic western blots. This band is absent in extracts from *scrib* embryos (Fig. 2d). In fixed tissue, Scrib is present at low levels in precellular embryos, where it is found in the actin caps that overlay the still-dividing blastoderm nuclei. As cellularization proceeds during cycle 14, Scrib staining becomes associated with the cell membranes as they invaginate basally towards the centre of the embryo (Fig. 3a). During gastrulation, however, Scrib relocates specifically to a relatively apical position

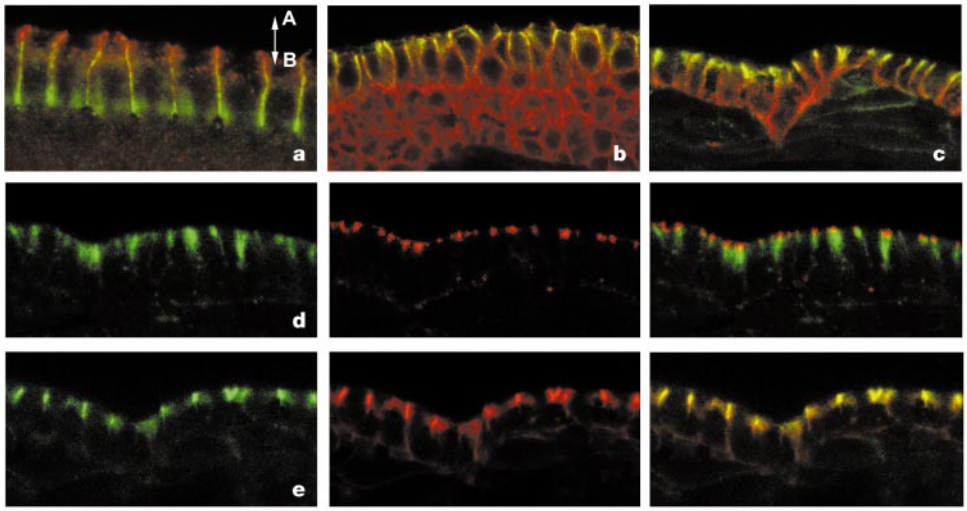

Figure 3 Expression and localization of Scrib. **a**, Confocal section of cellularizing embryo stained for Scrib (green) and the basolateral membrane marker Neurotactin (red). Scrib localizes at the basal tip of the ingrowing cell membranes. Apicobasal (A–B) axis is shown; apical orientation is up in all confocal images. **b**, Stage-8 embryo stained as in **a**. During gastrulation, Scrib relocalizes to the apicolateral region of the ectodermal epithelium. Note the much lower levels of Scrib in the non-epithelial mesoderm below the ectoderm. **c**, Stage-15 embryo stained for Scrib (green) and the basolateral protein FasIII (red). No Scrib is found in the apical cell membrane. **d**, Instead, Scrib (green) is in a small plaque that is basal to the adherens junction, revealed by Arm (red). it colocalizes with the septate junction marked by Cor (red) in **e**.

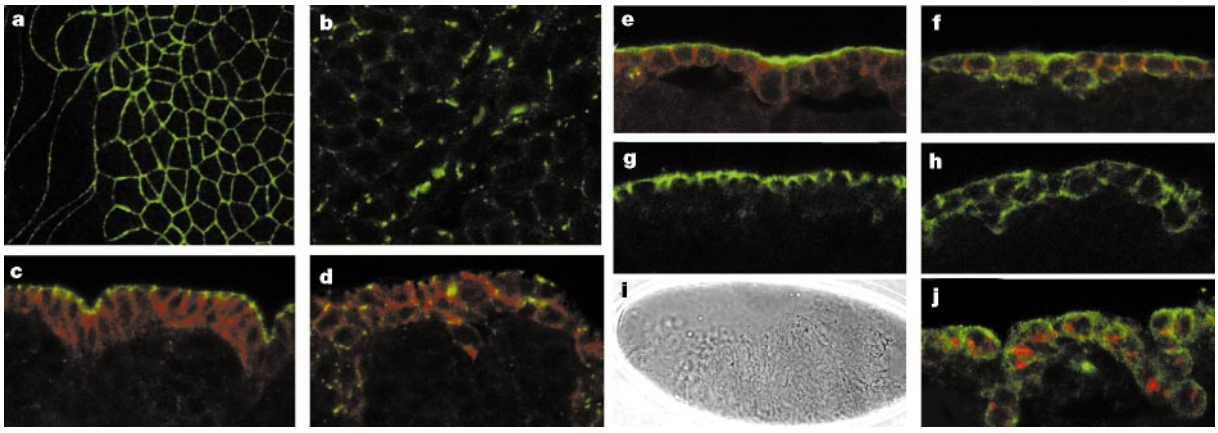

Figure 4 *scrib* embryos mislocalize apical proteins. Tangential confocal sections of wild-type (**a**) and *scrib* (**b**) stage-9 embryos stained for Arm. The regular apical band of Arm is severely disrupted in the mutant. Cross-sections of stage-13 wild-type (**c**) and *scrib* (**d**) epidermis stained for Arm (green) and Cor (red). In mutant tissues, Arm is found at ectopic sites around the surface of the cell instead of solely at the junction of contacting and non-contacting cell membranes. Note that Cor is not found at the apical surface of the *scrib* embryo. Cross-sections of wild-type (**e**) and *scrib* (**f**) stage-12 epidermis stained for Sas (green) and Neurexin Nrx (red). Sas is found basally and laterally as well as apically in the mutant, whereas Nrx remains basolateral. Cross-sections of stage 10 wild-type (**g**) and *scrib* (**h**) epidermis stained for Crb. Again, Crb is found around basal and lateral cell surfaces as well as apical. **i**, Cuticle produced by embryo expressing ectopic Crb protein shows a similar morphology to *scrib* cuticles. **j**, Ectopic Crb-producing embryos show basolateral mislocalization of Dlt (green) but not apical mislocalization of Cor (red), similar to *scrib* embryos.

along the lateral cell membrane of ectodermal cells (Fig. 3b). The apicolateral epithelial staining seen at gastrulation continues into late embryogenesis, where it has consolidated into a narrow subapical region (Fig. 3c).

Because the subapical epithelial membrane is the site of cell–cell junctions, we determined whether Scrib localizes specifically to a junction. In mature *Drosophila* epithelia, the adherens junction and the septate junction (analagous to the vertebrate tight junction[3]) are adjacent structures that are located at the margins of the apical and basolateral surfaces respectively[8]. Co-staining for Scrib and the adherens junction marker Armadillo (Arm), the homologue of vertebrate β-catenin, reveals that Scrib is located immediately basal to the adherens junction (Fig. 3d), whereas co-staining for Scrib and the septate junction marker Coracle (Cor), a protein 4.1 family member, reveals coincident protein distributions (Fig. 3e). These results show that Scrib localizes to septate junctions. Although the septate junction does not appear ultrastructurally until late embryogenesis at stage 14 (ref. 8), Scrib is enriched subjacent to the adherens junction after gastrulation at stage 8 (Fig. 3b). By contrast, the initial expression of Cor at stage 12 is spread throughout the basolateral membrane (Fig. 4c), with apicolateral enrichment visible only from stage 14. These data indicate that Scrib is an early marker for the site of the future septate junction, at the apical boundary of the basolateral compartment.

The septate junction localization of Scrib suggested that cell junctions might be defective in *scrib* mutants. We investigated the presence of cellular junctions using antibody probes specific for either the adherens or the septate junction. The initial assembly of adherens junctions in *scrib* cellular blastoderms is normal, but at stage 8, anti-Arm staining reveals a severe disruption of the forming circumferential adherens junction belts. The normally continuous 'honeycomb' pattern (Fig. 4a) seen in tangential apical sections of the epidermis is severely fragmented (Fig. 4b). Tissue cross-sections show that, rather than being lost, Arm is misdistributed. Instead of its wild-type localization exclusively at the apical tip of contacting cell membranes, much Arm is found around the cell periphery on the inside of the epidermis, in the midst of opposing cell membranes (Fig. 4d). At these sites, Arm colocalizes with E-cadherin (Ecad), the transmembrane component of the adherens junction (data not shown), indicating that adherens junctions form at ectopic basolateral membrane positions in *scrib* embryos.

The formation of adherens junctions at ectopic positions within *scrib* cells raised the issue of whether these cells have lost polarity. In the wild-type epidermis, apical proteins such as Stranded at Second (Sas) are localized to the non-contacting cell membrane at the surface of the embryo, whereas basolateral proteins such as Fasciclin III (Fas III) are found in a complementary domain along the contacting membranes of cells (Fig. 4e; Table 1)[1]. In *scrib* epidermal cells at stage 11, Sas is distributed throughout the plasma membrane, in both contacting and non-contacting cell surfaces (Fig. 4f). By contrast, Fas III localization is much less affected; in particular, it is not significantly mislocalized into apical regions. This preferential loss of apical restriction was seen with a panel of both cytoplasmic and transmembrane proteins through mid-embryogenesis (Table 1), after which late embryos repolarize some apical markers by a *scrib*-independent pathway (data not shown). Thus, the polarity defects in *scrib* epithelial cells arise not from a total loss of cell polarity but from a specific misdistribution of apical proteins.

Table 1 Localization of proteins in *scrib* embryos, stages 8–13

Protein	Topology	Localization in wild type	Localization in *scrib*
Crb	TM	A/J	A + BL
Ecad	TM	A/J	A + BL
Arm	Cyto	A	A + BL
Sas	TM	A	A + BL
Dlt	Cyto	A	A + BL
Nrt	TM	BL	BL
βSpec	Cyto	BL	BL
FasIII	TM	BL	BL (reduced)
Nrx	TM	BL	BL (reduced)
Cor	Cyto	BL	BL (reduced)

TM, transmembrane; Cyto, cytoplasmic; A, apical; J, junctional; BL, basolateral.

To place Scrib within the known pathway for *Drosophila* epithelial polarity determination, we examined the effect of *scrib* mutations on Crumbs (Crb). Crb is an apically localized transmembrane protein (Fig. 4g) that is necessary and sufficient to confer apical character on plasma membrane[1,9]. In *scrib* embryos, Crb shows unrestricted localization in both apical and basolateral regions (Fig. 4h). Discs-lost (Dlt), a second protein required for formation of the apical domain[2], is similarly mislocalized (Table 1). We considered whether *scrib* mutants are identical to a gain-of-function *crb* phenotype by comparing them with embryos in which GAL4-driven[10] Crb is present throughout the cell membrane. Ectopic Crb that is produced in this manner is sufficient to phenocopy several aspects of *scrib* embryos, including mislocalization of apical proteins and the cuticle pattern (Fig. 4i, j)[1,11]. These data indicate that a major function of Scrib in epithelial polarity is to exclude Crb from the basolateral domain. As ectopic Crb does not cause the epithelial morphology and multilayering defects seen in *scrib* embryos, Scrib may be required for the localization of additional epithelial determinants as well.

Analysis of the morphological and polarization phenotypes exhibited by *scrib* embryos shows that Scrib is a critical component of epithelial architecture in the *Drosophila* ectoderm, and suggests that its function is closely linked to that of Crb. Scrib, like Crb, is not required for the early localization of basal Dlt or apical Arm during blastoderm formation, and *scrib* mutants do not exhibit the defective cellularization or precipitous loss of cell adhesion seen when Dlt or Arm, respectively, are depleted in the embryo[2,12]. The increasingly severe cell shape, polarity and epithelial organization defects of *scrib* embryos are first manifested after gastrulation, coincident with the onset of defects in *crb* and *sdt* embryos. Loss of Crb results in loss of apical proteins from the plasma membrane and a failure to consolidate early adherens junction material into an apical band of ZAs[11,13], while in *scrib* embryos early adherens junctions become misdistributed basolaterally. Together with the similarities between *scrib* loss-of-function and *crb* gain-of-function phenotypes, these data place Scrib and Crb in a pathway required for the progression from the initially differentiated blastoderm membrane domains into a fully polarized epithelium with mature junctions.

Our results show that the junctional protein Scrib specifically restricts apical membrane determinants to the apical cell surface. This restriction allows the proper segregation of apical and basolateral domain components, and the appropriate placement of the adherens junction, resulting in full epithelial polarization. How does Scrib, a putative scaffolding[14] protein whose localizaton bounds the apical domain, dictate the proper confinement of apical proteins? Two models suggest themselves. Scrib could assemble a diffusion barrier that physically separates apical and basolateral compartments, similar to the 'fence' function proposed for the vertebrate tight junction[15]. To date, such a barrier has been shown to exist only for lipid diffusion in the outer leaflet of the plasma membrane[16,17]. If *scrib* mutations disrupt such a mechanical barrier, then secondary retention systems must serve to maintain basolateral protein restriction from the apical cell surface[18]. An alternative is that Scrib has a role in the polarized targeting of transport vesicles carrying apical proteins. The junctional complex, and in particular the tight junction, has been proposed to be a key sorting site for a subset of Golgi-derived vesicles[19]. In this model, Scrib might interact with the 'exocyst', a secretory targeting apparatus localized to the tight junction and involved in polarized segregation of transmembrane proteins[20]. PDZ domain proteins have been implicated at several different sites of the protein trafficking pathway[21,22], and occasional punctate intracellular staining of Scrib (data not shown)

is reminiscent of vesicles. Distinction between these models will rely on the identification of binding partners for Scrib. □

Methods

Fly stocks and cloning

scrib was mapped through assays of the ability of Y; autosome translocation chromosomes to provide paternal rescue of the *scrib* cuticle phenotype. About 15% of gametes from *T(Y;3)B158* males did not rescue the *scrib* germline clone (GLC) phenotype, whereas all gametes from *T(2;3)8r9* males did. This analysis limited the *scrib* region to 97BC, and a deficiency within this region (*Df(3R)Tl-x*) failed to complement *scrib*.

Non-complementing mutations were obtained from several collections; these new *scrib* alleles included two P-element-induced alleles. lethal phase analysis of the mutations and *Df(3R)Tl-x* indicates that the ethylmethane sulphonate–induced alleles *scrib*[1] (formerly *7c10;* D.B. and N.P., unpublished data) and *scrib*[2] (formerly *l(3)673;* K. Anderson, unpublished data) act as genetic nulls. *scrib j7B3* and *scrib s42405*[23,24] are induced by *P(lacW)* insertion. *scrib*[1] and *scrib*[2] were recombined onto *FRT82B* chromosomes for GLC production. The A2 cDNA was isolated from an embryonic cDNA library[25], and cloned into the vector pCasperhs for transgenic rescue experiments. For ectopic production of Crb, the strains *daGAL4* and *UAS-crb*[1] were crossed at 29 °C.

Antibody production and embryonic analysis

Peptides corresponding to N-terminal (RYSRTLEELFLDANHIRDLPKNF) and C-terminal (VDAEDMRNPLDEIEAVEFRS) portions of Scrib were used to immunize rabbits (QCB, Hopkinton, MA). An affinity-purified serum gave similar staining through mid-embryogenesis to that seen with the unpurified sera used here; this staining is absent in *scrib* embryos. Western blots were carried out using standard techniques with 100 embryos from 0–2-h collections of *y w* or *scrib* GLCs. Antibodies were obtained as follows: anti-α-Spectrin from D. Kiehart; anti-Sas from D. Cavener; anti-Dlt, anti-Neurexin and anti-Crb from M. Bhat; anti-Cor from R. Fehon; anti-Ecad from H. Oda; anti-Neurotactin, anti-FasIII and anti-Arm from the Developmental Studies Hybridoma Bank. Secondary antibodies were from Jackson Labs. Fixation and staining was done as described[26], except that 5′ fixation periods were used for anti-Scrib staining. Embryos were staged according to standard methods[27], and assayed for polarization prior to stage 14. Fluorescent images were collected on a Leica TCS confocal microscope. Scanning electron micrographs were performed as described[28].

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A tripeptide 'anticodon' deciphers stop codons in messenger RNA

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The two translational release factors of prokaryotes, RF1 and RF2, catalyse the termination of polypeptide synthesis at UAG/UAA and UGA/UAA stop codons, respectively[1–3]. However, how these polypeptide release factors read both non-identical and identical stop codons is puzzling[4]. Here we describe the basis of this recognition. Swaps of each of the conserved domains between RF1 and RF2 in an RF1–RF2 hybrid led to the identification of a domain that could switch recognition specificity. A genetic selection among clones encoding random variants of this domain showed that the tripeptides Pro-Ala-Thr and Ser-Pro-Phe determine release-factor specificity *in vivo* in RF1 and RF2, respectively. An *in vitro* release study of tripeptide variants indicated that the first and third amino acids independently discriminate the second and third purine bases, respectively. Analysis with stop codons containing base analogues indicated that the C2 amino group of purine may be the primary target of discrimination of G from A. These findings show that the discriminator tripeptide of bacterial release factors is functionally equivalent to that of the anticodon of transfer RNA, irrespective of the difference between protein and RNA.

A tRNA-like property has been speculated for release factors[5,6] in reading stop codons, and our aim was to identify a putative peptide anticodon equivalent in release factors. From sequence comparisons, we have previously proposed a seven-domain structure for release factors (domains A–G; Fig. 1a, top). We swapped these seven domains combinatorially between RF1 (UAG-specific) and RF2 (UGA-specific) to screen for active release-factor hybrids that would display altered codon specificity *in vivo*. For this purpose, common restriction sites were introduced into a clone at, or near, the sequences encoding the domain junctions so that these base changes would not affect activity (Fig. 1a, bottom). We examined a combinatory set of 128 release-factor hybrids for their ability