Merlin, the *Drosophila* homologue of neurofibromatosis-2, is specifically required in posterior follicle cells for axis formation in the oocyte

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SUMMARY

In *Drosophila*, the formation of the embryonic axes is initiated by Gurken, a transforming growth factor α signal from the oocyte to the posterior follicle cells, and an unknown polarising signal back to the oocyte. We report that *Drosophila* Merlin is specifically required only within the posterior follicle cells to initiate axis formation. *Merlin* mutants show defects in nuclear migration and mRNA localisation in the oocyte. Merlin is not required to specify posterior follicle cell identity in response to the Gurken signal from the oocyte, but is required for the unknown polarising signal back to the oocyte. Merlin is also required non-autonomously, only in follicle cells that have received the Gurken signal, to maintain cell polarity and limit proliferation, but is not required in embryos and larvae. These results are consistent with the fact that human Merlin is encoded by the gene for the tumour suppressor neurofibromatosis-2 and is a member of the Ezrin-Radixin-Moesin family of proteins that link actin to transmembrane proteins. We propose that Merlin acts in response to the Gurken signal by apically targeting the signal that initiates axis specification in the oocyte.

Key words: *Drosophila* oogenesis, *Merlin*, *gurken*, *bicoid*, *oskar*, TGFα, mRNA localisation, Oocyte microtubules, Embryonic axis formation, Tumour suppressor, ERM, Cell signalling

INTRODUCTION

The embryonic axes of *Drosophila* are established during oogenesis through the localisation of specific mRNAs to different regions of the oocyte cytoplasm. This process is initiated through bi-directional signalling between the oocyte and the overlying follicle cells (Schüpbach, 1987). While the localised mRNAs and some of the signalling components have been studied in detail, many of the genes involved in these processes are still unknown (Nilson and Schüpbach, 1999; van Eeden and St Johnston, 1999).

grk mRNA is localised in early oocytes in a posterior crescent between the nucleus and the follicle cells (Neuman-Silberberg and Schüpbach, 1993), thus targeting the Grk transforming growth factor α (TGFα) signal only to the adjacent follicle cells. The Grk signal is probably the ligand for Torpedo/DER, an epidermal growth factor receptor (EGFR) (Gonzalez-Reyes et al., 1995; Neuman-Silberberg and Schüpbach, 1993). Grk instructs 200 terminal follicle cells to adopt posterior instead of default anterior fates (Gonzalez-Reyes and St Johnston, 1998). Posterior, anterior and main body follicle cells originate from the same group of cells that divides five or six times before stage 6 and has equivalent columnar epithelial morphology up to stage 9 (Gonzalez-Reyes and St Johnston, 1998). However, anterior and posterior follicle cells express distinct cell fate markers (Deng and Bownes, 1998; Fasano and Kerridge, 1988; Micklem et al., 1997).

Once the Grk signal is received, an unknown signal is sent from the posterior follicle cells back to the oocyte, repolarising the oocyte microtubules (MTs). MT organisation and polarity have been visualised in fixed material with anti-Tubulin antibodies (Theurkauf et al., 1992) and β-galactosidase (βgal) fusions to MT-dependent motor domains (Clark et al., 1994; Clark et al., 1997) as well as a TauGFP fusion in living oocytes (Micklem et al., 1997). Before stage 7, a microtubule organising centre (MTOC) is located at the posterior of the oocyte, where the minus ends of MTs are localised. At stage 7, the posterior MTOC disassembles, a diffuse anterior MTOC forms and plus ends of MTs are found at the posterior. The polarity of MTs determines the site of localisation of different mRNAs in the oocyte. *bicoid* (*bcd*) mRNA is localised to the anterior of the oocyte, leading to a morphogenetic gradient of Bcd protein in the embryo (Driever and Nüsslein-Volhard, 1988). *osk* mRNA is localised at the posterior of the oocyte and embryo and specifies the future germ cells (Ephrussi et al., 1991).

The Grk signal also initiates formation of the dorsoventral (DV) axis when the oocyte nucleus moves from the posterior to the dorsoanterior corner. *grk* transcripts then become tightly

localised near the nucleus, so that Grk signalling instructs only the overlying follicle cells to adopt dorsal fates (Nilson and Schüpbach, 1999; van Eeden and St Johnston, 1999). The specification of appropriate populations of follicle cells along the DV axis leads to the secretion of egg shell structures such as the dorsal appendages. Later in development the embryonic DV axis is formed by signalling from ventral follicle cells, which leads to the formation of a graded nuclear-cytoplasmic distribution of Dorsal protein (Anderson, 1998).

Although the posterior polarising signal remains unidentified, a number of known genes are required for the process. Protein kinase A (PKA) is likely to be part of the machinery that receives the signal in the oocyte (Lane and Kalderon, 1994; Perrimon, 1994) but is not specific to this process. Mago nashi (Mago) is required for oocyte repolarisation and has an independent function in *osk* mRNA localisation. However, Mago is a novel nuclear protein that is ubiquitous in the egg chamber, and its role in signalling is unknown (Micklem et al., 1997; Newmark et al., 1997). Notch-Delta signalling is required among the posterior follicle cells to limit the number of polar posterior follicle cells that express Fasciclin III; it is also required earlier in oogenesis (Larkin et al., 1996). Notch is required for the specification of posterior follicle cell identity (Gonzalez-Reyes and St Johnston, 1998), and is therefore required indirectly for the generation of the polarising signal to the oocyte (Ruohola et al., 1991), rather than being directly involved in the signal itself. Two other neurogenic proteins, Brainiac (Brn) and Egghead (Egh) are required in the oocyte for follicle cell integrity, and it has been suggested that both proteins may interact with Notch and EGF-signalling (Goode et al., 1996a; Goode et al., 1996b). However, it is not known whether they are required for the polarising signal. Laminin A is a component of the extracellular matrix that is expressed and required in the posterior follicle cells for the polarising signal (Deng and Ruohola-Baker, 2000). A better understanding of the events associated with the polarising signal awaits the identification of the signal itself.

Here, we identify a new allele of *Mer* by screening a collection of temperature sensitive (ts) lethal alleles for defects in *grk* mRNA localisation and we show that Merlin functions in axis specification during oogenesis. *Drosophila Mer* was previously cloned by degenerate PCR (McCartney and Fehon, 1996) and mutations isolated by reverse genetic methods (Fehon et al., 1997). The human homologue is a tumour suppressor called neurofibromatosis-2 (NF2), which encodes Merlin (Moesin Ezrin Radixin Related Protein) (McCartney and Fehon, 1996). Merlin and Ezrin-Radixin-Moesin (ERM) proteins are members of the 4.1 family of proteins thought to link actin to transmembrane proteins (Mangeat et al., 1999; Tsukita et al., 1994) and *Drosophila* Merlin is apically localised in follicle cells (McCartney and Fehon, 1996). We show that Merlin is required only within the posterior follicle cells for mRNA localisation and axis specification in the oocyte. Merlin functions downstream of the Grk signal from the oocyte, but is only required if the posterior follicle cells receive the Grk signal. Merlin has no role in Notch-Delta signalling between the follicle cells, but is required upstream of the unknown polarising signal back to the oocyte. Merlin is also required non-autonomously in posterior follicle cells to limit their proliferation and maintain their polarity. We propose that Merlin functions by apically targeting the unknown polarising signal that initiates axis specification.

MATERIALS AND METHODS

Fly stocks

The collection of ts lethals was generated by EMS mutagenesis and subsequent selecting for male lethality at 29°C and viability at 21° (H. F.-L. and W. S., unpublished observations). $Merlin^{ts1}$ (Mer^{ts1}) stocks (yw^{67g}, Mer^{ts1}) were maintained at 18°C or 21°C, and mutant phenotypes analysed by shifting newly eclosed adult flies to 29°C on fresh food. For analysis of follicle cell and MT markers, Mer^{ts1} females were crossed to males from the following stocks: posterior follicle cell marker (*yw; 998/12/TM6b*: P. Deak, M. Bownes and D. Glover), border cell markers (*yw; 459/2/TM6b*: P. Deak, M. Bownes and D. Glover), anterior follicle cell marker (*L53b*: S. Kerridge), polar follicle cell *marker (w; P(w⁺)8523/CyO*: M. Heck, A. Spradling) and MT markers (*yw;Nod-lacZ* and *yw;Kin-lacZ, Tau-GFP*: D. St Johnston). The F₁ male progeny were then back crossed to Mer^{ts1} females.

Generation and detection of FRT/FLP follicle cell clones

Clones were induced by mitotic recombination at high frequency only in follicle cells using *en-Gal4,UAS-FLP* (Duffy et al., 1998) at 29°C in $Mer^3, FRT/nlsGFP, FRT$ (Davis et al., 1995) (FRT19A from S. Luschnig). Mer^3 Clones were identified by the lack of nlsGFP expression.

X-ray-induced germline clones

To create homozygous Mer^{ts1} germline clones, $ovoD^1$ males were mated to homozygous yw^{67g}, Mer^{ts1} females for two days and transferred to fresh food for 8 hours. The larvae (40-48 hours old) were exposed to 1000 rads of X-rays and allowed to recover at 21°C. 1000 surviving F1 females were crossed to yw^{67g} males in single pair matings, shifted to 29°C for three days, and their ovaries dissected, fixed and stored in methanol at –20°C. 10 females (1%) containing Mer^{ts1} germline clones were identified by the lack of male progeny, and their ovaries studied. 50 (5%) females with wild-type recombinant chromosomes were identified by the presence of male progeny and discarded.

In situ hybridisation

Ovaries were dissected and fixed in 3.7% formaldehyde in PBS with 0.1% Tween20 (PBT) and in situ hybridisation carried out using standard methods (Tautz and Pfeifle, 1989) with our previously described modifications (Wilkie and Davis, 1998; Wilkie et al., 1999). Mounting and imaging were performed as previously described (Davis, 2000).

X-Gal staining

For X-gal staining of the enhancer trap and other transgenic lines expressing βgal fusion proteins, ovaries were dissected and fixed in 0.05% glutaraldehyde in PBT for 15 minutes, and stained for 2 hours to overnight at 37°C using standard methodology.

Protein localisation

Actin

Ovaries were dissected and fixed without methanol and incubated in a 1:40 dilution of Texas-red-phalloidin (Molecular probes) in PBS overnight at 4°C and washed with PBT.

Centrosomes and spectrin

Ovaries were blocked in 2% BSA in PBS with 0.1% TritonX-100 (PBTX) for 2 hours at room temperature, washed several times in PBT and incubated with anti γ-tubulin monoclonal antibody (1:10,000

Sigma) or anti β$_H$-spectrin (1:200) in PBTX with 2% BSA overnight at 4°C, followed by AlexaFluor594- and AlexaFluor488-coupled secondary antibodies, respectively (Molecular Probes).

RESULTS

Identification of a temperature-sensitive mutation that disrupts mRNA localisation and oocyte nuclear migration

To identify new genes required for axis specification, we screened a collection of X-linked ts lethal mutations generated by selecting for male lethality at 29°C and viability at 21°C. We collected homozygous female progeny at 21°C from 73 viable ts lethal lines, shifted to 29°C for 3 days and performed *grk* RNA in situ hybridisation on ovaries. In wild type or *yw^{67g}* controls at 29°C or in all strains at 21°C, the oocyte nucleus migrates correctly to the antero-dorsal corner of the oocyte with *grk* mRNA localising between the nucleus and the overlying future dorsal follicle cells (Fig. 1A). In one line, *yw^{67g},l(1)ts594 {l(1)ts594}*, 55% (*n*=89) of oocyte nuclei fail to migrate and *grk* mRNA localises at the posterior after stage 8 (Fig. 1B). The remaining 45% of cases were similar to wild type, *l(1)ts594* at 21°C (Fig. 1A) and the same genetic background *yw^{67g} (yw)* chromosome at 29°C. In all subsequent
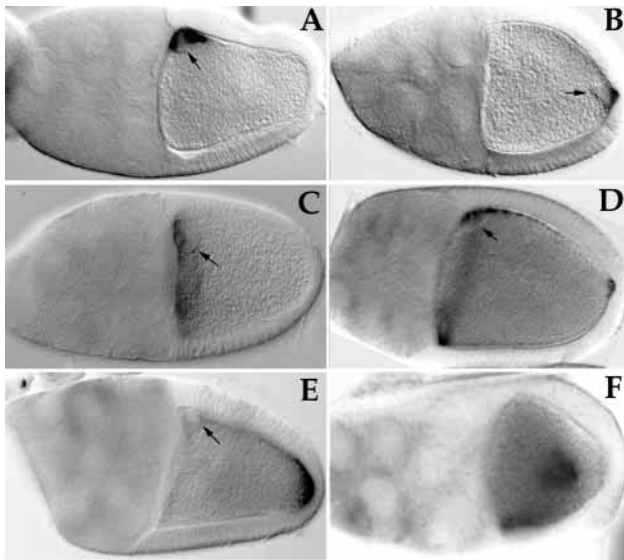

Fig. 1. Mutations in *Mer* disrupt mRNA localisation and nuclear migration in the oocyte. (A) Control showing the oocyte nucleus and *grk* mRNA at the dorso-anterior corner of the oocyte. (B) *Mer* mutant showing *grk* mRNA at the posterior near the oocyte nucleus (55% of cases). The other 45% of cases are the same as the control (not shown). (C) Control showing normal *bcd* mRNA localisation in an anterior ring. (D) *Mer* mutant showing some *bcd* mRNA abnormally localised at the posterior (83% of cases). (E) Control showing normal *osk* mRNA localisation at the posterior. (F) *Mer* mutant showing *osk* mRNA diffusely localised at the centre of the oocyte (89% of cases). The oocyte nucleus is in a different focal plane. (A,C,E) stage 10 *Mer^{ts1}* mutant 21°C, similar to wild type and *yw* strains at 29°C (not shown). (B,D) stage 10 and (F) stage 9 *Mer^{ts1}* mutants at 29°C. In situ hybridisation to detect *grk* (A,B), *bcd* (C,D) and *osk* (E,F) mRNA. Arrows indicate the oocyte nucleus.

experiments, similar controls were carried out, showing that the phenotype was not due to the temperature shift itself or the genetic background.

We also performed in situ hybridisation on *l(1)ts594* ovaries to detect *bicoid* (*bcd*) and *oskar* (*osk*) mRNA. We found that in 83% (*n*=47) of stage 9 or 10A mutants at 29°C, *bcd* mRNA is localised at the posterior as well as its normal accumulation in an anterior ring (Fig. 1C,D). In 89% (*n*=32) of stage 9 and 10A mutants, *osk* mRNA is mislocalised at the centre of the oocyte (Fig. 1F) instead of its normal posterior localisation (Fig. 1E).

To test whether the defects in the oocyte are primarily due to a defect in MT organisation, we examined MT polarity. We used Kin:βgal, a plus end-directed MT motor fusion that leads to βgal accumulation at the posterior of the oocyte (Clark et al., 1994). We also used Nod:βgal, a MT motor fusion that leads to βgal accumulation at the anterior, where the minus ends of MTs are thought to localise (Clark et al., 1997). The βgal motor fusions indicate that prior to stage 7, there is an MTOC at the posterior (data not shown). In wild-type oocytes after stage 7, the posterior MTOC disassembles, a diffuse MTOC appears at the anterior (Fig. 2A) with MT plus ends at the posterior (Fig. 2C). Prior to stage 7, *l(1)ts594* mutant oocytes show a similar MT organization to wild type (data not shown), but after stage 7, the MTOC fails to disassemble at the posterior and a second diffuse MTOC forms at the anterior (Fig. 2B). This leads to a symmetric organization of MTs, with their plus ends at the centre of the oocyte (Fig. 2D) and minus ends at the anterior and posterior (Fig. 2B). We also examined the overall distribution of MTs using a maternally expressed TauGFP line showing the highest concentration of MTs at the anterior cortex of wild-type oocytes (Micklem et al., 1997). We observed a similar Tau-GFP distribution in *l(1)ts594* oocytes at 21°C (Fig. 2E). In *l(1)ts594* at 29°C Tau-GFP showed an abnormally high level at the posterior, consistent with a failure to disassemble the posterior MTOC (Fig. 2F). We conclude that the mislocalisation of mRNA and failure of the oocyte nucleus to relocate in *l(1)ts594* oocytes are due primarily to defects in MT organisation.

l(1)ts594 is a strong loss-of-function allele of *Mer*

In order to determine the gene mutated in *l(1)ts594*, we mapped the mutation. Complementation analysis against deficiencies showed that the mutation lies in one of two gaps in the available deficiencies on the X chromosome, 18A2-A5 or 18D1-18E1-2 and recombination mapping showed that the lethality and oocyte phenotype both map to 18D-18E. Complementation analysis with all the available alleles in the region showed that three lethal alleles of *Merlin* (*Mer^1, Mer^2 and Mer^4*) (LaJeunesse et al., 1998) failed to complement the lethality of *l(1)ts594* at 29°C. A fusion of the *Mer* full-length cDNA with GFP and a cosmid containing a genomic DNA fragment including *Mer* are both able to fully complement the lethality, oocyte nuclear migration defects and mRNA mislocalisation of *l(1)ts594* (data not shown), suggesting that *l(1)ts594* is a ts allele of *Mer*.

Mer is the closest *Drosophila* homologue of human Merlin, a member of the ERM/4.1 family encoded by the NF2 tumour suppressor (Mangeat et al., 1999). ERM proteins are thought to link actin with transmembrane proteins at the cell membrane (Turunen et al., 1998) and may play a role in signalling (Mangeat et al., 1999). We sequenced the entire coding regions
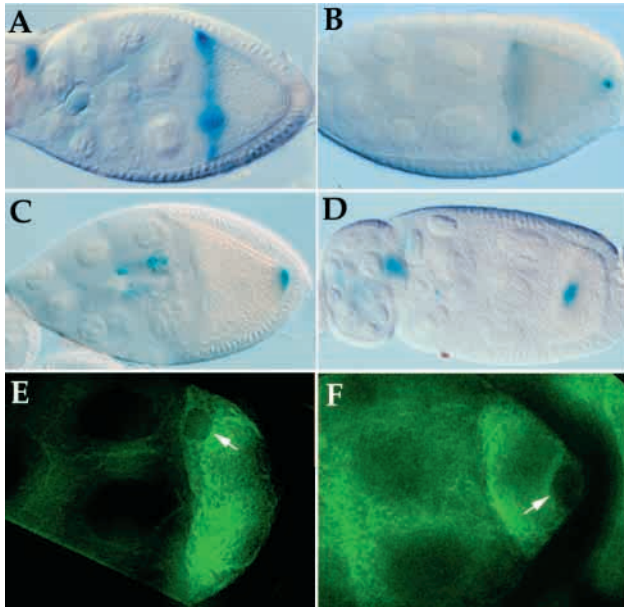
Fig. 2. Mutations in *Mer* disrupt microtubule (MT) organisation in the oocyte. (A,B) Minus ends of MTs visualised with Nod:βgal protein that marks the MT organising centre (MTOC). (A) Stage 9 control showing normal Nod:βgal localisation along the anterior cortex. (B) Stage 9 *Mer* mutant, showing Nod:βgal localisation at the posterior, owing to a failure of the posterior MTOC to disassemble. (C,D) Plus ends of MTs visualised with Kin:βgal expression. (C) Late stage 9 control showing Kin:βgal localised at the posterior of the oocyte. (D) Stage 9 *Mer* mutant showing abnormal Kin:βgal localisation in the centre of the oocyte. (E,F) MTs visualised in living egg chambers using Tau-GFP. Arrows indicate the nuclei. (E) Stage 9 control anterior-posterior gradient of MTs. (F) Stage 9 *Mer^{ts1}* mutant showing a symmetric array of MTs at the anterior and posterior cortex. (A,C,E) *Mer^{ts1}* 21°C. (B,D,F) *Mer^{ts1}* 29°C.
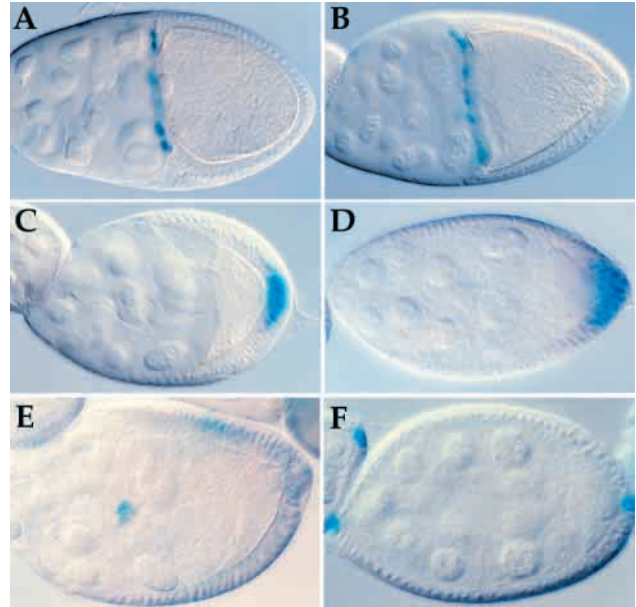

Fig. 3. Mutations in *Mer* do not disrupt follicle cell identity. (A-F) X-Gal staining of different *lacZ* lines crossed into control and *Mer^{ts1}* egg chambers. (A) Stage 10 control, expressing βgal protein in anterior follicle cells. (B) *Mer^{ts1}* egg chamber showing indistinguishable anterior follicle cells to A and no expression in posterior follicle cells. (C) Stage 9 control, expressing βgal in the posterior follicle cells. (D) *Mer^{ts1}* mutant showing normal posterior expression. Note the thicker layer of posterior follicle cells (also see Fig. 4) (E) *Mer^{ts1}* mutant showing normal expression of a border cell marker. (F) *Mer^{ts1}* mutant showing normal expression of polar posterior and polar anterior follicle cell marker.

of *Mer* in *l(1)ts594* and in the genetic background *yw^{67g}* chromosome. Two closely mapping non-conservative amino acid changes (F113L and I125F) were found in the conserved N-terminal domain involved in binding to transmembrane proteins (LaJeunesse et al., 1998). No modifications were detected in the C-terminal domain that has a putative regulatory role. We conclude that *l(1)ts594* is an allele of *Mer* and renamed it *Mer^{ts1}*.

To determine whether the phenotype we observed in *Mer^{ts1}* mutants was typical of existing loss-of-function *Mer* alleles, we studied the oogenesis phenotype of various other allelic combinations of *Mer*. We found similar defects in all the allelic combinations studied. Flies homozygous for *Mer^{ts1}*, and flies with *Mer^{ts1}* over a null allele (LaJeunesse et al., 1998) (*Mer^{ts1}/Mer^4*) showed almost identical phenotypes. *Mer^{ts1}* over a weak allele (Fehon et al., 1997) (*Mer^{ts1}/Mer^3*) showed a slightly reduced frequency of the oogenesis phenotype (data not shown). From these results, and the fact that a *Mer* transgene fully complements the *Mer^{ts1}* phenotype (data not shown), we conclude that *Mer^{ts1}* is a very strong loss-of-function allele, similar to a null.

Merlin is not required in the germline, or for Grk or Notch signalling

Merlin protein has previously been shown to be expressed in

the oocyte and in posterior follicle cells (McCartney and Fehon, 1996), but its function was only studied later in development (LaJeunesse et al., 1998; McCartney et al., 2000). To determine where Merlin functions in egg chambers, we generated homozygous *Mer^{ts1}* germline clones using X-rays in females raised at the restrictive temperature (29°C). We analysed 10 *Mer^{ts1}* oocytes surrounded by *Mer^{ts1}/+* follicle cells (see Materials and Methods), and they all showed normal mRNA localisation and lead to normal eggs (data not shown). We conclude that Merlin is not required in the germline derived nurse cells or oocyte.

To test whether Merlin is required within the somatically derived posterior follicle cells to receive the Grk signal from the oocyte, we studied the expression of different follicle cell markers in *Mer* egg chambers. The results show that *Mer^{ts1}* posterior follicle cells receive the Grk signal correctly, as they express posterior and not anterior markers (Fig. 3A-D). We conclude that Merlin is not required for any aspect of Grk signalling or its reception in the posterior follicle cells. Merlin is also not required for Notch signalling among the posterior follicle cells, which is required to specify the correct number of posterior cells (Gonzalez-Reyes and St Johnston, 1998).

We also tested whether Merlin is required for the formation or identity of other types of follicle cells by analysing markers for different follicle cell populations in *Mer^{ts1}*. These included a marker for border cells, stalk cells and polar follicle cells. Our results show that Merlin is not required for the correct
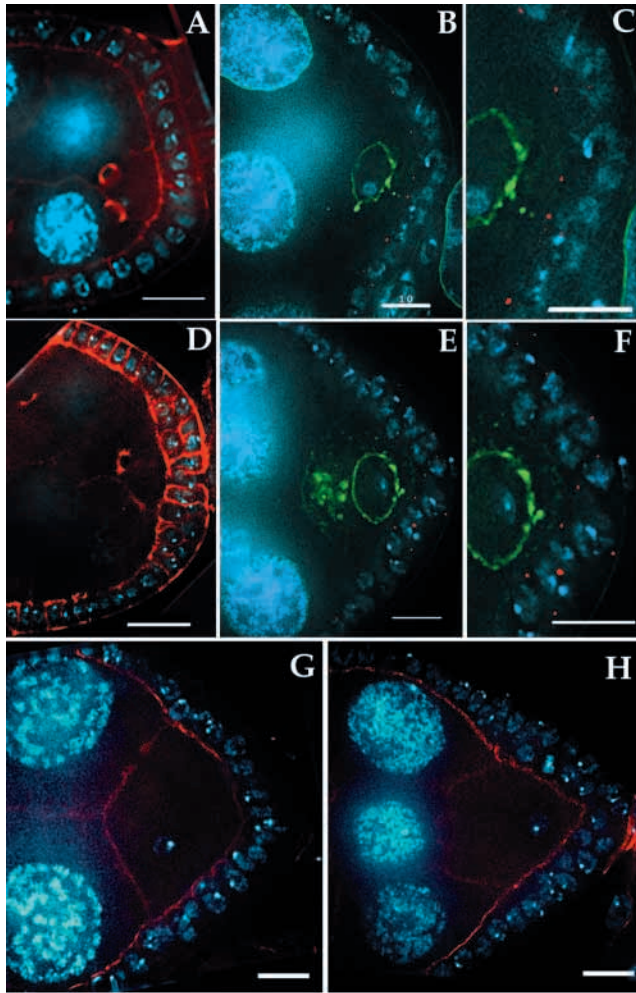
Fig. 4. *Mer* mutations disrupt posterior follicle cell organisation. (A,D) Follicle cell morphology visualised by phalloidin-rhodamine staining of actin (red) and DAPI staining of DNA (blue). (A) Control showing a columnar monolayer of follicle cells. (D) *Mer^{ts1}* mutant showing a double layer of follicle cells only at the posterior. (B,C,E,F) The polarity of follicle cells in control and *Mer^{ts1}* egg chambers showing centrosomes (γ-tubulin, red), the nuclear envelope (wheat germ agglutinin (WGA), green) and DNA (DAPI staining, blue). (B) Control showing that follicle cell centrosomes usually point to the apical surface, adjacent to the oocyte. (C) A higher magnification view of the posterior part of (B). (E) *Mer^{ts1}* egg chamber showing follicle cell centrosomes pointing both apically and basally. The oocyte nucleus has failed to migrate. WGA also stains the yolk particles, the boundary of the oocyte and outer edge of the follicle cells. (F) A higher magnification view of the posterior part of E. The centrosomes are not visible in some cells, as they are located in another focal plane. (G,H) Apical polarity visualised with anti-spectrin β heavy chain (β_H-spectrin) antibody (red) and DNA (blue). (G) Control showing apical localisation of β_H-spectrin in follicle cells. (H) *Mer^{ts1}* mutant showing normal apical β_H-spectrin in follicle cells adjacent to the oocyte, but no detectable β_H-spectrin in the outer layer of posterior follicle cells. Scale bars: 10 μm.
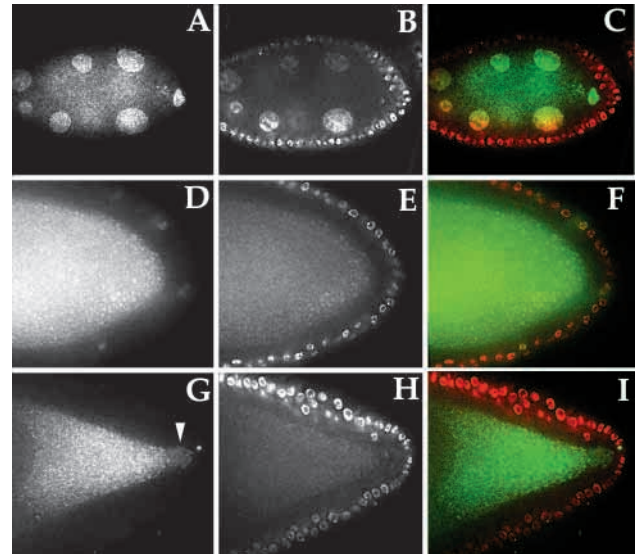

Fig. 5. Merlin is required non-autonomously in the posterior follicle cells to maintain their monolayer columnar morphology. (A-I) Examples of mosaic egg chambers with homozygous mutant *Mer* clones lacking nlsGFP expression. (A,D,G) nlsGFP expression (green in C,F,I). (B,E,H) DAPI staining showing all nuclei in the egg chambers (red in C,F,I). (A-C) Stage 8 in which all the follicle cells are *Mer^3*, but the germline is Mer^3/+, showing an identical phenotype to egg chambers from *Mer^3* mothers. The nucleus has failed to migrate. (D-F) Stage 10 with four very small patches of *Mer^3*/+ follicle cells at the posterior in a homozygous *Mer^3* mutant background. The few *Mer^3*/+ cells rescue the *Mer* mutant phenotype in all the neighbouring follicle cells and also rescue the nuclear migration phenotype. (G-I) Stage 10 with one *Mer^3*/+ follicle cell at the posterior in a *Mer^3* mutant background. The single *Mer/+* cell is able to rescue the mutant phenotype in the neighbouring follicle cells, but fails to rescue the nuclear migration phenotype. Arrowhead marks the oocyte nucleus.

specification or development of any subgroup of follicle cells, and is not required for Notch signalling among the follicle cells, which limits the number of polar follicle cells to two (Fig. 3E,F). Merlin is therefore likely to be required for cell

communication between the follicle cells and oocyte, downstream of Grk and upstream of the unknown polarising signal from the posterior follicle cells to the oocyte.

Merlin acts as a tumour suppressor in posterior follicle cells and is required for their polarity

We observed that the posterior follicle cells in fixed (Figs 3D-F, 4D-F,H) and living (data not shown) *Mer* egg chambers often have a slightly disrupted morphology. To study these defects in more detail, we covisualised actin and DNA to highlight each cell and its boundaries (Fig. 4A,D). Posterior follicle cells in controls have a uniform columnar appearance characteristic of epithelial sheets (Fig. 4A). However, after stage 6, *Mer^{ts1}* egg chambers have a double layer of follicle cells only at the posterior where follicle cells are in contact with the oocyte (Fig. 4D). To determine whether the double layer of posterior follicle cells is due to overproliferation, we counted the number of cells using three-dimensional microscopy and found a twofold increase in the number of posterior follicle cells, but no changes in other follicle cells (data not shown).

To determine whether the overproliferation of posterior follicle cells is accompanied by polarity defects, we studied MT polarity by covisualising DNA, the nuclear envelope and centrosomes (Fig. 4B,C,E,F). In control egg chambers, most

centrosomes lie on the apical side of each nucleus, where the minus ends of MTs are found (Fig. 4B,C). In contrast, Mer^{ts1} posterior follicle cells mostly loose the apical-basal polarity of their MTs (Fig. 4E,F).

To investigate whether other aspects of the apical-basal polarity of the posterior follicle cells are also disrupted, we studied the distribution of β-spectrin heavy chain (βH-spectrin) in Mer mutants (Fig. 4G,H). βH-Spectrin is normally restricted to the apical side of follicle cells within a Spectrin-based membrane skeleton (Fig. 4G) (Zarnescu and Thomas, 1999). In Mer^{ts1} mutants βH-spectrin is apically localised in the cells adjacent to the oocyte, but not detected in the second layer of follicle cells (Fig. 4H). These results suggest that in Mer mutants, the apical surface of posterior follicle cells contacts the oocyte correctly, and is probably competent to send and receive signals to the oocyte.

To determine whether the defects in cell proliferation and polarity in Mer egg chambers are dependent on receiving the Grk signal, we examined the follicle cells of Mer, grk double mutants. We found that even a hypomorphic allele of grk (grk^{2E12}) suppresses the Mer posterior follicle cell phenotype entirely (data not shown). We conclude that Merlin is required only in cells that receive the Grk signal and is not a constitutive factor required for cell polarity and proliferation.

Merlin is required non-autonomously in posterior follicle cells

To test directly whether Merlin is required only in posterior follicle cells, we used genetic mosaic analysis with the FRT/FLP system to make clones of homozygous Mer follicle cells located at posterior, anterior or central positions (Fig. 5A-I). We found that Merlin is required only in the posterior follicle cells for their correct morphology and migration of the oocyte nucleus. We produced Mer clones using Mer^3,FRT/nlsGFP,FRT; en-Gal4,UASFLP females. Mer^3 is a homozygous viable but sterile allele (Fehon et al., 1997) and en-Gal4,UASFLP expresses FLP recombinase at very high levels only in the follicle cells (Duffy et al., 1998). We examined a total of 43 egg chambers with Mer^3 clones, of which 29 were particularly revealing and analysed in detail. Of these, one egg chamber had follicle cells that were entirely Mer^3 (Fig. 5A-C), and three egg chambers had large mutant clones covering all the posterior (data not shown). These egg chambers showed a strong Mer phenotype indistinguishable from non-mosaic homozygous Mer^3 mutants. 21 egg chambers had large anterior or main body follicle cell clones without a Mer phenotype (data not shown). We conclude that Merlin is required only in posterior follicle cells.

To test whether Merlin is required cell autonomously to limit the proliferation and polarity of posterior follicle cells, we studied four egg chambers in which the follicle cells were Mer^3, except for one or more very small Mer^3/+ clones in the posterior follicle cells. Such egg chambers showed complete rescue of the Mer^3 phenotype when sufficient Mer^3/+ cells were present (Fig. 5D-F), indicating that Merlin acts cell non-autonomously among the posterior follicle cells. One of these clones had a single Mer^3/+ cell at the posterior tip surrounded by Mer^3 cells, showing that a single Mer^3/+ cell is able to rescue the overproliferation phenotype up to a distance of about six cell diameters (Fig. 5G-I). While the single Mer^3/+ cell was not able to rescue the oocyte nuclear migration defect, several
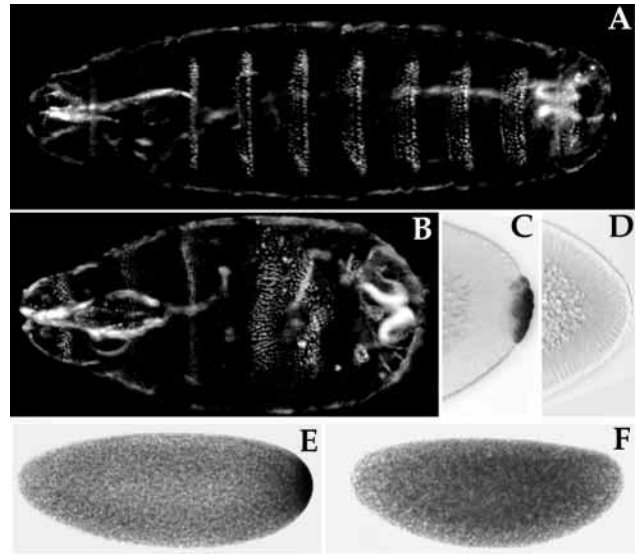

Fig. 6. Mer mutations disrupt osk mRNA localisation in some embryos, leading to abdominal defects and a lack of pole cells. (A,B) Cuticle preparations from Mer^{ts1} mutant eggs. (A) Normal cuticle of hatched eggs (79%). (B) Abdominal defects in unhatched eggs (21%). (C,D) Vasa antibody staining of pole cells in Mer^{ts1} embryos. (C) Normal numbers of pole cells (52% of cases). (D) Pole cells are absent (48% of cases). (E,F) osk mRNA localisation. (E) Control pre-blastoderm embryo showing normal posterior osk mRNA localisation. (F) Mer^{ts1} embryo showing unlocalised osk mRNA (50% of cases).

small Mer^3/+ clones were sufficient to do so. We conclude that Merlin is required non-autonomously in the posterior follicle cells to limit their proliferation.

Merlin is not required during embryogenesis

To test whether Merlin is required for embryogenesis we analysed the hatch rate of eggs laid by Mer^{ts1} mothers. At 29°C, 74% of eggs (n=100) hatch and develop normally until third instar larvae, compared with a hatch rate of 94% for Mer^{ts1} at 21°C and yw^{67g} at 29°C (a difference of 21%). All the unhatched embryos have abdominal cuticle defects similar to osk alleles (Fig. 6B), while the eggs that hatch have normal cuticles (Fig. 6A). We found that osk mRNA is mislocalised in 51% of embryos (n=104) (Fig. 6F) and 48% of embryos (n=103) show missing pole cells (Fig. 6D), explaining why many of the resulting flies have few germ cells (data not shown). These results are consistent with the fact that osk alleles are known to disrupt pole cell formation more readily than abdominal patterning (Lehmann and Nüsslein-Volhard, 1986).

Initially, it was surprising that most Mer eggs hatch, since mislocalised bcd mRNA would be expected to disrupt AP axis specification and cause embryonic lethality. However, we found that bcd mRNA, which was mislocalised at the posterior, partially or completely relocalised in older egg chambers (Fig. 7A-D). Consequently, Mer^{ts1} embryos have completely normal bcd localisation (Fig. 7E,F). The near normal hatch rate of Mer eggs was also initially surprising because 55% (n=89) of Mer mutants have misplaced oocyte nuclei and mislocalised grk mRNA, which would lead to embryonic lethality. However, we
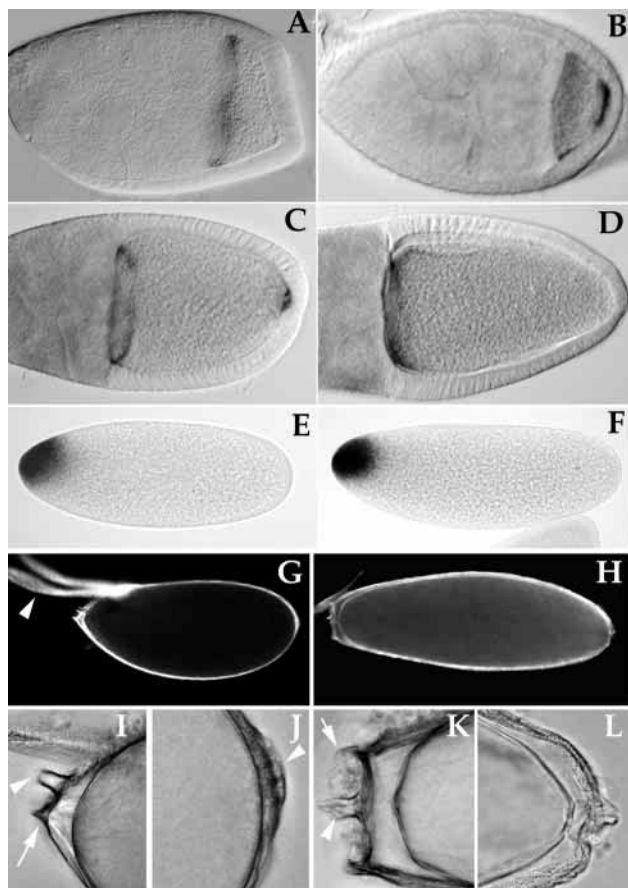
frequency than controls because of abdominal and dorsoventral defects that originate during oogenesis, rather than a direct requirement for Merlin in embryos. Therefore, Merlin is not required for embryogenesis and much of larval development.

DISCUSSION

We have shown that Merlin is required for the signal that initiates axis specification. Merlin is also required non-autonomously for signalling among the posterior follicle cells that limits their proliferation and maintains their polarity. Merlin is not required for other signals within the posterior follicle cells or in other parts of egg chambers and embryos.

Taking our data in the context of previous work, we propose that Merlin is involved in apical targeting of the unknown signal that initiates axis specification in the oocyte. Merlin is a member of the ERM/4.1 family of proteins and, in *Drosophila*, it is localised to the apical membrane of follicle cells and in the germline (McCartney and Fehon, 1996). ERM family members are thought to function as linkers between the cytoskeleton and the apical membrane, and they are probably required for apical targeting of signals, maintenance of epithelial adhesion, apical-basal polarity and to limit cell proliferation (Vaheri et al., 1997).

The overproliferation of the posterior follicle cells is consistent with overproliferation of mutant *Mer* cells seen in imaginal discs and with the function of human Merlin as a tumour suppressor causing neurofibromatosis-2. The changes in cell polarity we observe are also common in many other types of tumours. Interestingly, as in other *Drosophila* tissues (LaJeunesse et al., 1998), the *Mer* phenotypes we have studied are more similar in character to benign tumours seen in individuals with neurofibromatosis-2 than to the aggressive tumours produced in the mouse model (McClatchey et al., 1998). However, it is not known whether Merlin is required during mammalian oogenesis. We speculate that human Merlin may function in a similar non-autonomous manner in response to particular signals such as TGFα, which is known to be expressed in mammalian oocytes (Vaughan et al., 1992). Indeed, many parallels may exist between mammalian and fly oogenesis in respect of communication between the oocyte and follicle cells (Deng et al., 1997).

Is Merlin directly involved in signals that initiate axis specification?

We have shown that Merlin has a more specific and restricted function than previously thought, as it is required only in cells that receive the posterior Grk signal, despite being expressed more widely in egg chambers (McCartney and Fehon, 1996). Interestingly, the dorso-anterior follicle cells do not require Merlin, despite receiving the Grk signal.

Our data show that Merlin is required downstream of Grk but upstream of the unknown polarising signal. We propose that the effect of Merlin on the polarising signal is not indirectly due to the overproliferation and subtle changes in the polarity of the posterior follicle cells. βH-Spectrin is correctly distributed in *Mer* follicle cells adjacent to the oocyte, despite the centrosomes being disorganised and the second layer of follicle cells showing mislocalised βH-spectrin. Therefore, the inner layer of posterior follicle cells are probably competent to

Fig. 7. Mutant *Mer* mothers lay eggs with normal *bcd* mRNA localisation, but some dorsoventral defects. (A-F) *bcd* mRNA in *Mer* and control egg chambers and embryos. (A) Stage 9 control showing normal *bcd* mRNA localisation in an anterior ring. (B) Stage 9 *Mer^{ts1}* mutant showing *bcd* mislocalisation at the posterior. (C) Stage 10B *Mer^{ts1}* mutant showing a reduced level of *bcd* mRNA at the posterior. (D) Stage 11 *Mer^{ts1}* mutant showing no *bcd* mRNA localisation at the posterior. (E) Control pre-blastoderm embryo showing *bcd* mRNA localisation at the anterior. (F) Pre-blastoderm *Mer^{ts1}* embryo showing indistinguishable anterior *bcd* mRNA localisation to controls. (G,I,J) Control *Mer^{ts1}* at 21°C showing the same structures as wild type eggs or *yw* eggs at 29°C (not shown). (G) Egg with dorsal appendages (arrowhead). (I) Higher power anterior view of dorsally positioned micropyle (arrowhead) and centrally placed operculum (arrow). (J) High power posterior view of dorsally placed aeropyle (arrowhead). (H,K,L) Egg from *Mer^{ts1}* female at 29°C. (H) Egg showing a lack of dorsal appendages and a torpedo-like shape (11% of cases). The other 89% are normal (not shown). Females retain many of the defective eggs (not shown). (K) High power anterior view showing a centrally located micropyle (arrowhead) and a symmetric ringed operculum (arrow). (L) High power posterior view showing an enlarged aeropyle.

found that only 11% (*n*=158) of eggs laid by *Mer^{ts1}* mothers have strong dorsoventral defects (Fig. 7H,K,L), and the other defective egg chambers degenerate in females after stage 10A (data not shown). It is likely that egg chambers with mislocalised *osk* mRNA also degenerate in the mothers, explaining why there is a lower percentage of *osk* mRNA localisation defects in embryos compared with oocytes.

We conclude that *Mer* eggs hatch at a slightly lower

send the polarising signal in *Mer* mutants. Furthermore, some *Mer* egg chambers were found in which the polarising signal was not received, despite the posterior follicle cells showing no apparent defects in their proliferation or polarity (data not shown). We also found that *brn* mutant egg chambers show a similar specific morphological disruption of posterior follicle cells to that seen in *Mer* mutants (Goode et al., 1996a; Goode et al., 1996b) but *brn* mutations do not lead to any defects in oocyte axis specification (data not shown). Therefore, the morphological disruption of the posterior follicle cells in itself is not likely to be responsible for perturbing the unknown signal to the oocyte. Instead, we propose that Merlin may have a more direct role in targeting the polarising signal to the apical surface of posterior follicle cells.

What signals are disrupted by *Mer* mutations?

Since many genes involved in signalling among the follicle cells and between the oocyte and follicle cells are unknown, it is difficult to be certain which signals might be disrupted by *Mer* mutations. Nevertheless, our data conclusively rule out a role for Merlin in a number of known signalling processes. Merlin is not required for receiving the Grk signal via Torpedo, an EGF-like receptor, by the posterior or dorso-anterior follicle cells. Merlin is also not required for lateral inhibition via Notch-Delta signalling among the posterior follicle cells, that determines the correct number of posterior, polar posterior follicle cells and stalk cells between egg chambers. Nor is Merlin required for many kinds of essential signalling pathways throughout embryogenesis. Merlin is unlikely to play a direct role in the presumptive Egh/Brn signal from the oocyte, as these proteins are required in the oocyte and not in the follicle cells. Nevertheless, it is intriguing that the posterior follicle cells of *N*, *brn* or *egh* mutant egg chambers all show a similar overproliferation phenotype to *Mer* egg chambers. While it is possible that N, Egh or Brn are in some way related in function to Merlin, further experiments will have to be performed to explore these issues.

Our results show that Merlin is required for two distinct processes involving signalling, but we cannot distinguish whether the two processes depend on a single signal or two distinct signals. For example, the restriction of posterior follicle cell proliferation could require the same unknown signal that initiates MT repolarisation in the oocyte. Both processes could depend on the same signal secreted into the space between the follicle cells and oocyte. Indeed, it is intriguing that *Merlin* egg chambers have MT polarity defects in both the oocyte and the posterior follicle cells. However, further progress awaits the identification of the signal or signals involved.

The identity of the polarising signal is unknown, but some genes are known to be required for the signal, including PKA (Lane and Kalderon, 1995), Mago (Micklem et al., 1997) and Laminin A. Merlin is unlikely to be required for PKA and Mago functions as they are required in the oocyte. In contrast, Laminin A is expressed and required in posterior follicle cells as a component of the extracellular matrix (Deng and Ruohola-Baker, 2000). It is tempting to speculate that Merlin and Laminin A could be functionally linked as specialised structural components required specifically in the posterior follicle cells for the transduction of the polarising signal.

It is interesting to ask how many additional components are required for axis specification in the oocyte but it is not possible to estimate this number from our results. However, the fact that we identified even one mutation required for this process out from only 73 ts alleles, strongly argues that there are many more unrecognised genes required for axis formation.

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