Inscuteable-dependent apical localization of the microtubule-binding protein Cornetto suggests a role in asymmetric cell division

Silvia Bulgheresi, Elke Kleiner and Juergen A. Knoblich*

Research Institute of Molecular Pathology (I.M.P.), Dr Bohr Gasse 7, A-1030 Vienna, Austria

*Author for correspondence (e-mail: knoblich@nt.imp.univie.ac.at)

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SUMMARY

Drosophila **neuroblasts divide asymmetrically along the apical-basal axis. The Inscuteable protein localizes to the apical cell cortex in neuroblasts from interphase to metaphase, but disappears in anaphase. Inscuteable is required for correct spindle orientation and for asymmetric localization of cell fate determinants to the opposite (basal) cell cortex. Here, we show that Inscuteable also directs asymmetric protein localization to the apical cell cortex during later stages of mitosis. In a two-hybrid screen for Inscuteable-binding proteins, we have identified the coiled-coil protein Cornetto, which shows a highly unusual subcellular distribution in neuroblasts. Although the protein is uniformly distributed in the cytoplasm during metaphase, it concentrates apically in anaphase and forms an apical crescent during telophase in an *inscuteable*-dependent manner. Upon overexpression, Cornetto localizes to astral microtubules and microtubule spin-down experiments demonstrate that Cornetto is a microtubule-binding protein. After disruption of the actin cytoskeleton, Cornetto localizes with microtubules throughout the cell cycle and decorates the mitotic spindle during metaphase. Our results reveal a novel pattern of asymmetric protein localization in *Drosophila* neuroblasts and are consistent with a function of Cornetto in anchoring the mitotic spindle during late phases of mitosis, even though our *cornetto* mutant analysis suggests that this function might be obscured by genetic redundancy.**

Key words: Cell division, Cell polarity, Mitotic spindle

INTRODUCTION

Some cells can divide asymmetrically into two different daughter cells with distinct cell fates (Horvitz and Herskowitz, 1992). Such asymmetric cell divisions involve the segregation of a determinant into one of the two daughter cells, which then establishes a particular developmental pathway in this cell but not in its sister cell (Doe and Bowerman, 2001; Knoblich, 2001; Lu et al., 2000). *Drosophila* neuroblasts delaminate from an epithelium in the ventral neuroectoderm and divide asymmetrically along the apical-basal axis. During this asymmetric division, the transcription factor Prospero (Hirata et al., 1995; Knoblich et al., 1995; Spana and Doe, 1995) is transported by the coiled-coil protein Miranda (Ikeshima-Kataoka et al., 1997; Schuldt et al., 1998; Shen et al., 1997) into the smaller basal daughter cell, in which Prospero is required for correct cell fate specification (Doe et al., 1991; Vaessin et al., 1991). Prospero and Miranda colocalize in a crescent that forms during late prophase in the area of the cell cortex overlying the basal centrosome and is maintained until telophase, when cytokinesis segregates both proteins into the basal daughter cell. Several other proteins, including Numb (Rhyu et al., 1994), Partner of Numb (Pon) (Lu et al., 1998) and Staufen (Broadus et al., 1998; Li et al., 1997; Schuldt et al., 1998), colocalize with Prospero and Miranda at the basal cortex and segregate into the same daughter cell. Even though their function in neuroblasts is less clear, they presumably use the same machinery for basal protein localization during mitosis.

Another set of proteins colocalizes at the opposite (apical) cell cortex in mitotic neuroblasts. This set includes the PDZ domain proteins Bazooka (Kuchinke et al., 1998; Schober et al., 1999; Wodarz et al., 1999) and DmPar-6 (Petronczki and Knoblich, 2000), the kinase DaPKC (Wodarz et al., 2000), the TPR repeat protein Pins (Schaefer et al., 2000; Yu et al., 2000) and Inscuteable (Kraut et al., 1996), a protein with no homology to other known proteins (Kraut and Campos-Ortega, 1996). In contrast to the basal proteins, asymmetric localization of these proteins begins in late interphase. Apical localization is maintained upon entry into mitosis but, in anaphase, all apical proteins disappear until they reappear at the apical cell cortex during late interphase of the next cell cycle. In the absence of any of the apical proteins, either the basal localization of Prospero and Miranda during mitosis does not occur or crescents form at incorrect positions and are no longer correlated with one of the two spindle poles. In addition, mitotic spindles in neuroblasts fail to rotate into an apical-basal orientation (Kaltschmidt et al., 2000) and neuroblast divisions are misoriented. Although all these proteins are required for correct orientation of neuroblast divisions, Inscuteable stands out because, unlike the other apical proteins, it is not only required but also sufficient for spindle reorientation (Kraut et al., 1996). When the *inscuteable* gene is expressed ectopically in epithelial cells, which normally divide parallel to the epithelial plane, this

can cause an apical-basal reorientation of the mitotic spindle. Thus, Inscuteable is both required and sufficient for orienting mitotic spindles along the apical-basal axis.

The molecular mechanisms by which Inscuteable orients mitotic spindles in *Drosophila* are not very well understood. Laser ablation experiments in *Caenorhabditis elegans* and genetic experiments in *Saccharomyces cerevisiae* have suggested that mitotic spindles in these organisms are oriented by astral microtubules that attach to the cell cortex and exert a pulling force on one of the two centrosomes (Carminati and Stearns, 1997; Hyman, 1989; Hyman and White, 1987). While the molecular nature of the microtubule/cell-cortex interaction is unclear in *C. elegans*, cortical attachment of microtubules in yeast is mediated by the protein Bim1 (Korinek et al., 2000; Lee et al., 2000; Miller et al., 2000). Bim1 is a microtubule-associated protein that can bind directly to Kar9, a protein that is localized to the region of the cell cortex towards which the mitotic spindle is drawn, thus providing a direct molecular link between astral microtubules and the cell cortex. Even though this link is clearly involved in positioning the mitotic spindle, neither *bim1* nor *kar9* are essential genes, suggesting that spindle orientation in yeast is mediated by functionally redundant pathways.

To obtain insights into Inscuteable function during asymmetric cell division, we searched for Inscuteable binding partners. In a two-hybrid screen for Inscuteable-binding proteins, we have identified the novel coiled-coil protein Cornetto. Like Inscuteable, Cornetto localizes apically in neuroblasts, and its apical localization is Inscuteable dependent. In contrast to Inscuteable, Cornetto localization occurs during anaphase and telophase, and the protein is cytoplasmic during earlier stages of mitosis. Upon disruption of the actin cytoskeleton by treatment with latrunculin A, Cornetto colocalizes with microtubules during metaphase, and microtubule spin-down experiments suggest that Cornetto is indeed a microtubule-binding protein. Thus, Cornetto provides the first molecular link between the apically localized Inscuteable protein and the mitotic spindle. In addition, the unusual apical localization of Cornetto not only shows that Inscuteable is involved in basal protein localization during metaphase but also suggests another function in apical protein targeting during late stages of asymmetric cell division.

MATERIALS AND METHODS

Identification of Cornetto

The two-hybrid bait vector pBHAK was generated from pBHA (Shen et al., 1997) by replacing the fragment encoding the ampicillin resistance with a kanamycin resistance fragment from the plasmid pBSL86. A DNA fragment corresponding to amino acids 252-615 of the Inscuteable protein was cloned into pBHAK and used to screen a 3-12 hour embryonic two-hybrid library (Shen et al., 1997), essentially as described (Shen et al., 1997). Among 10 million clones, 100 positives were identified, 11 of which contained gene fragments that interact specifically with Inscuteable but not with Lamin. Sequence analysis revealed that four of these fragments correspond to the same gene, which was named *cornetto* based on the subcellular localization of the protein.

Database searches identified a sequenced EST (CK00492) that corresponds to *cornetto* but this does not encode the whole open reading frame. One of the DNA fragments obtained in the two-hybrid screen was therefore used to screen a *Drosophila* embryonic cDNA library (Brown and Kafatos, 1988). The sequence of the longest cDNA clone was determined and encoded a 2883 bp open reading frame preceded by stop codons in all frames. The full length *cornetto* cDNA sequence has been submitted to GenBank (accession number AY032851). The *cornetto* coding region corresponds to the open reading frame CG8621 predicted by the *Drosophila* genome project. Partial sequence and restriction analysis of the other identified clones did not reveal any splice variants of *cornetto* and so this cDNA was used in all experiments.

In vitro binding assay

A PCR fragment corresponding to amino acids 252-615 (GST-Insc-cen) of Inscuteable (Knoblich et al., 1999) was cloned into pGEX4T-1 (Pharmacia). The GST-Insc-cen fusion protein and GST alone (empty vector) were expressed in the bacterial strain HB101 and were bound to glutathione agarose beads (Pharmacia). Full-length *cornetto* was translated in the presence of ^{35}S-methionine using the TNT kit (Promega), bound to the beads for 30 minutes and washed six times with PBS containing 0.1% NP40. The beads were boiled in SDS sample buffer and proteins were separated by SDS-PAGE. Gels were dried and exposed to X-ray film overnight.

Antibodies and immunofluorescence

Polyclonal antibodies were raised in rabbits following standard procedures against a peptide corresponding to amino acids 941-960 of Cornetto. The anti-Cornetto antibody specifically recognized a single band of ~116 kDa in immunoblots of extracts from wild-type embryos. This band corresponds to Cornetto because it is absent after preincubation of the antibody with the peptide used for immunization (Fig. 2E) and from extracts prepared from *cornetto* mutant embryos (data not shown).

For immunofluorescence, embryos were dechorionated using 50% household bleach, fixed for 20 minutes in 5% or 8% paraformaldehyde and devitellinized with methanol following standard protocols. Immunofluorescence experiments were performed essentially as described (Rhyu et al., 1994). Primary antibodies were rabbit anti-Cornetto (1:500), mouse anti-Inscuteable (1:200; M. Schaefer and J.A.K., unpublished) and mouse monoclonal antibody 22C10 (1:300). Drug treatment of embryos was performed by incubating dechorionated *Drosophila* embryos for 30 minutes in a 1:1 mixture of n-heptane and 200 μM latrunculin A in PBS (essentially as described by Knoblich et al. (Knoblich et al., 1997)) before fixation as usual. Secondary antibodies made in donkey and coupled to Alexa 488 or Alexa 568 (Molecular Probes) were used. DNA was visualized by staining embryos for 5 minutes in 1 μg ml^{-1} Hoechst 33258 (Fig. 3G,H) or for 10 minutes with 1 μg ml^{-1} propidium iodide following a 1 hour treatment with 2 mg ml^{-1} RNAse A (all other figures). Images were recorded on a Leica TCS-NT confocal microscope equipped with a pulsed near-infrared laser to visualize Hoechst 33258 by two-photon microscopy.

Microtubule binding assays

Microtubule binding assays were performed essentially as described (Saunders et al., 1997). 0-8-hour-old *Drosophila* embryos were dechorionated for 3 minutes in 50% household bleach and homogenized in 2 volumes homogenization buffer (0.1 M PIPES / NaOH, pH 6.6, 5 mM EGTA, 1 mM $MgSO_4$, 0.9 M glycerol, 1 mM DTT, 1 mM PMSF, 1 mg ml^{-1} chymostatin, 1 mg ml^{-1} leupeptin, and 1 mg ml^{-1} pepstatin). Microtubules were depolymerized by 15 minutes of incubation on ice and high-speed supernatants were prepared by centrifugation first for 30 minutes at 20,000 ***g***, then for 30 minutes at 140,000 ***g***. Extracts were incubated for 30 minutes at room temperature with or without 1 mM GTP and 20 μM Taxol. After centrifugation through a 30% sucrose cushion for 30 minutes at 80,000 ***g***, pellets and supernatants were analysed by SDS-PAGE and western blotting.

Generation of Cornetto overexpression lines and mutants

To overexpress *cornetto*, a *Dra*I/*Not*I fragment of the *cornetto* cDNA containing 926 bp 5′ untranslated region, the complete coding region and 219 bp 3′ untranslated region was cloned into pUAST (Brand and Perrimon, 1993). Transgenic flies were generated using standard methods and the protein was expressed in embryos using *hairy-Gal4* (Brand and Perrimon, 1993).

The cytological position of the *cornetto* gene is 65E. Database searches revealed that the sequenced, *rosy*$^{+}$-marked P-element insertion P[PZ]04202, which had previously been described as male sterile (*ms(3)65E*) (Castrillon et al., 1993), was inserted into the *cornetto* 5′ untranslated region. The male sterility could be recombined away from the P-element and the cleaned chromosome was homozygous viable even though the levels of Cornetto protein were reduced. To generate Cornetto null mutants, 500 *rosy*$^{-}$ revertants were generated by mobilization of the P-element using a constitutively active transposase source. From these, 18 partial excisions were selected in which the P-element end proximal to the *cornetto* coding region had been lost and the other end remained intact. One large directional deletion was found to remove all but the last 384 nucleotides of the *cornetto* coding region. Even though no Cornetto protein was detectable in flies homozygous for this deletion, these cornetto null mutants were viable and fertile, and did not show any visible defects in asymmetric cell division.

RESULTS

Cornetto is an Inscuteable-binding protein

In neuroblasts and epithelial cells of the procephalic neurogenic region, Inscuteable localizes to the apical cell cortex and directs apical-basal orientation of the mitotic spindle (Kraut et al., 1996). We used the yeast two-hybrid system to find proteins that interact with the Inscuteable asymmetry domain, a 364 amino acid region of Inscuteable that is required and sufficient for the known functions of the protein (Knoblich et al., 1999; Tio et al., 1999). Among 10 million clones from a library generated from 3-12-hour embryonic cDNA (Shen et al., 1997), 11 clones showed specific interaction with Inscuteable. Four clones corresponded to the same gene and were chosen for further analysis based on the expression of this gene in neuroblasts (see below). The gene was named Cornetto because of the characteristic subcellular localization of the corresponding protein (see below). Inserts from the two-hybrid clones were used to isolate a *cornetto* full-length cDNA from a 4-8-hour embryonic cDNA library (Brown and Kafatos, 1988). The longest identified cDNA was 4260 nucleotides in length (GenBank accession number AY032851) and contained a single long open reading frame preceded by stop codons in all reading frames, suggesting that it represents the complete *cornetto* coding region. The predicted Cornetto protein (Fig. 1A) has a molecular weight of 105 kDa. Computer searches did not identify any known protein domains, but both the central region of the protein (amino acids 370-420 and 470-590) and the C terminus (amino acids 890-960) are predicted by the COILS program to form a coiled coil at very high probability (Fig. 1B). All two-hybrid clones contained the coiled coil domains, suggesting that they are the Inscuteable-binding part of the protein. The coiled-coil regions of Cornetto show high homology to other coiled-coil proteins, but sequence similarity searches failed to identify clear orthologues of *cornetto* in other species.

To verify the molecular interaction between Cornetto and Inscuteable, we performed in vitro binding assays (Fig. 1C). At least 20% of in vitro translated full-length Cornetto protein could be bound to a GST fusion of the Inscuteable asymmetry domain, but no interaction could be detected with GST alone. We conclude that Cornetto and Inscuteable can bind to each other, even though the fact that we were not able to coimmunoprecipitate the two proteins from embryonic extracts suggests that the interaction in vivo is very transient.

Cornetto is expressed in epithelial cells and neuroblasts

To test whether Cornetto is present in the cells in which Inscuteable is functional, we analysed *cornetto* expression by in situ hybridization. Inscuteable is specifically expressed in cells of the nervous system (Kraut and Campos-Ortega, 1996) but, when ectopically expressed in epithelial cells of the prospective epidermis, it can cause a reorientation of the mitotic spindle (Kraut et al., 1996). Other genes needed for Inscuteable function should therefore be expressed both in neural precursors and in these epithelial cells. Unlike Inscuteable, *cornetto* is strongly expressed in early embryos before cellularization, suggesting a strong maternal contribution (Fig. 2A). After cellularization, the gene is ubiquitously expressed until neurogenesis, when expression becomes stronger in developing neuroblasts (Fig. 2B). In these neuroblasts, the *cornetto* RNA was found to localize to the apical part of the cell (Fig. 2D), similarly to Inscuteable RNA and the RNA of other genes involved in asymmetric cell division. In germ-band-extended embryos, the expression in the epidermis is progressively downregulated until germ-band retraction, when *cornetto* is only expressed in the nervous system. In germ-band-retracted embryos, expression remains strong in the nervous system but can also be detected in the gut (Fig. 2C). Thus, the expression of *cornetto* is consistent with a role in mediating Inscuteable function.

To analyse Cornetto protein expression and subcellular location, we generated an antibody against a peptide corresponding to the Cornetto C terminus. The antibody detects a single band on immunoblots of whole *Drosophila* extracts (Fig. 2E). Both this protein band and all immunofluorescence stainings described can be completely blocked by preincubation of the Cornetto antibody with peptide (Fig. 2E) and are absent from Cornetto-deficient embryos (see below). Unlike *cornetto* RNA, very little Cornetto protein was detected in pre-blastoderm embryos by either immunofluorescence (Fig. 2F) or immunoblotting (data not shown). During gastrulation, the protein was present in all cells (Fig. 2G) but it became restricted to the nervous system during germ-band retraction and was specifically detected in cells of the central and peripheral nervous systems, and the gut in germ-band-retracted embryos (Fig. 2H). In the central nervous system of these germ-band-retracted embryos, Cornetto was mostly present on axons (data not shown), whereas, in the peripheral nervous system, the protein showed a distinct and characteristic localization to the cap cell of lateral chordotonal organs (Fig. 2I). Thus, Cornetto protein expression is similar, but not identical, to expression of *cornetto* RNA.

Cornetto is asymmetrically localized in neuroblasts

To analyse the subcellular localization of Cornetto protein,

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  1 MSATLFEMDS QTEEQQQTVC LEEQQLVVGM TSLELSESVV TPGNCDGSDS  GLDVASCCHS RITLQRGLSS TSGGYTSSNG LKEIYDSCEL KVTTVAQTPS 100
101 ETGSEKTSPP RRTGSVKKKV AMFEPEPTGT PAKDQLRGRV ANLKRAESLP  RNSAAPGTPG TPSTHRDKAR LSTSSFRVPS TTGTPSSSRL ARPQKPDTLP 200
201 NALNRAQSVQ RLTQVQRTPS LSRARTPGTP SDDGRWPANR GAARRGMSVT  PEVTTNRMRG SPAPGGTLPR RRKQQSVEDL TGGRLSRSNS ISRAAIVDAR 300
301 MTSSVMVVPT SRRSLAPAAA KVNSLRRPQP ATPRTRIYHE TAVQTALTSE  DLEQVLGGGL LQTRAVDAVE QRNQSTQAEP DQREFELEQL RHEVRQLSGK 400
401 LQREREEKLA MQQELHLNTE RVMGMLELAR VVSASAGTPT SEDSGDGSGH  DSLLMLESQI QISGHELFER QQEIGQLRSM CRALQLEMRR SLATQQLLLQ 500
501 EKAAIEQESS ELQDFLQHEK AAQCDALRDL EADYQAVKLQ LANREEEAKV  LRDECRHLVR LNEQRRQENR LLQTKFTALE NKSRELIHQQ NASVAGASTA 600
601 LSGLHSRLDG LVEQLVYSYS ISEQDLEDIR FQAETEHVQN GQRPNGLDLP  ICPAASGDAI HTLVLASDGS LSPQRNQSFI AAVIGAIRQA TTHSGKRLSL 700
701 RQAGKKNGMG LGSAQAVSAG TQGQEPQGNG DDSDSTEMLD SETEPCLLMM  DNVLEDVVHH DSHSHNMVSS CTGMISQIEL PTELISQCSH QNNINGGAGG 800
801 DDSLQQLSQA ITNRQQMELH MHKMNGMPMN PRDQCNTEEL SCHDSLAELT  ELPSLMEYST AQAVVDQVIE VDTLVTKLLK VLRLVQLDND NCIQQLIVDK 900
901 NKLQQHKEDM LEKLKDLEDV NLKLQDELMD ATQELMIKGS DLSGAKAEMQ  RHRNEIDVRF                                            961
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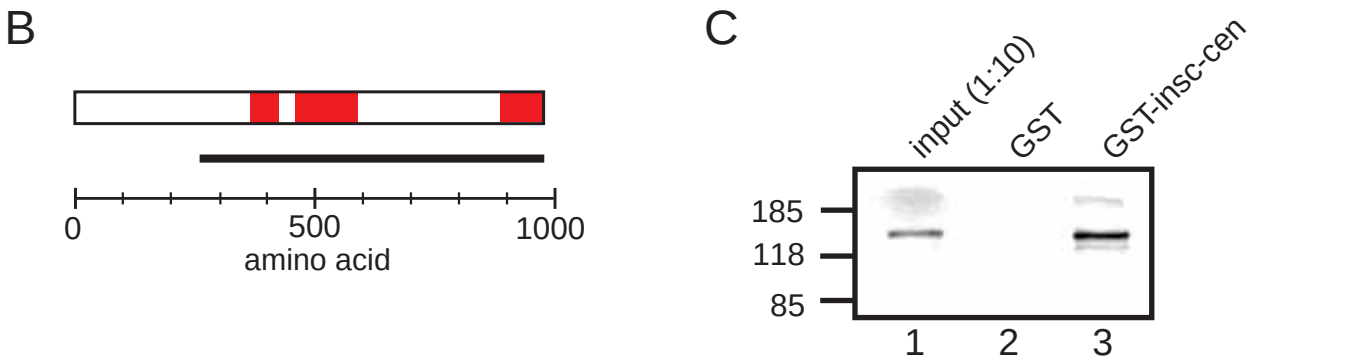

Fig. 1. The Cornetto protein. (A) Predicted amino acid sequence of the Cornetto protein. The part of Cornetto that was found to interact with Inscuteable is underlined. (B) Domain structure of Cornetto. Three predicted coiled-coil domains are indicated by red boxes; the black bar shows the extent of the two-hybrid clone. (C) In vitro translated Cornetto protein binds to a GST fusion of the Inscuteable asymmetry domain (GST-insc-cen; lane 3), but not to GST alone (GST; lane 2). One-tenth of the input used in the in vitro binding assays is shown in lane 1.

stage 11 embryos were double stained for Cornetto and DNA (Fig. 3). In epithelial cells, Cornetto protein is found in the cytoplasm with a slightly higher concentration in the apical half of cells (Fig. 3A,B). In neuroblasts, the protein was found to be evenly distributed in the cytoplasm during interphase (data not shown). This distribution is not changed when cells enter mitosis and, until metaphase (Fig. 3C), no signs of asymmetric protein distribution can be seen. Starting in early anaphase (Fig. 3D), however, Cornetto starts to concentrate apically in the area of the neuroblast at which the apical aster forms. During telophase (Fig. 3E), this apical concentration becomes more prominent and Cornetto localizes into an apical crescent overlying the newly forming larger daughter nucleus. This asymmetric localization is initially maintained after exit from mitosis (Fig. 3F) but, during interphase, Cornetto becomes progressively delocalized. Thus, Cornetto is the first protein that localizes asymmetrically during late stages of mitosis in *Drosophila* neuroblasts.

Like Cornetto, Inscuteable localizes apically in dividing neuroblasts, but its asymmetric localization starts during neuroblast delamination (Kraut et al., 1996). Inscuteable remains localized until metaphase of mitosis but, starting in anaphase, the protein becomes delocalized (Kraut et al., 1996) and degraded (J.A.K., unpublished). To test whether the unique subcellular localization of Cornetto requires proteins that localize apically during earlier phases of the cell cycle, we stained embryos homozygous for an Inscuteable deletion for Cornetto and DNA. Most *inscuteable* mutant telophase (Fig. 3I) and early interphase (Fig. 3J) neuroblasts did not show any asymmetric or cortical localization of Cornetto, but weak crescents could be detected in a subset of cells. Whereas 18±2.7 crescents per embryo (10 embryos analysed) could be detected in controls, this was reduced to 4±2.9 in *inscuteable* mutants (10 embryos analysed), even though the total number of anaphase and telophase neuroblasts was unchanged. We conclude that Inscuteable, which localizes apically in metaphase, is required for the apical localization of Cornetto during anaphase and telophase.

Double staining of neuroblasts both for Inscuteable and Cornetto, however, showed that the two proteins do not colocalize in neuroblasts (Fig. 3G,H). In metaphase, when Inscuteable localized into an apical crescent, Cornetto was in the cytoplasm (Fig. 3G). In telophase, when Inscuteable was progressively degraded and lost from the apical cell cortex, both Cornetto and residual Inscuteable were apically localized (Fig. 3H). Whereas Inscuteable localized to the cell cortex, Cornetto localized to an apical structure that might be the apical microtubule aster of the mitotic spindle (Fig. 3H). This difference became even more prominent when Cornetto was overexpressed using the UAS/Gal4 system (Brand and Perrimon, 1993). Both apical concentration in epithelial cells and cytoplasmic localization during early phases of mitosis in

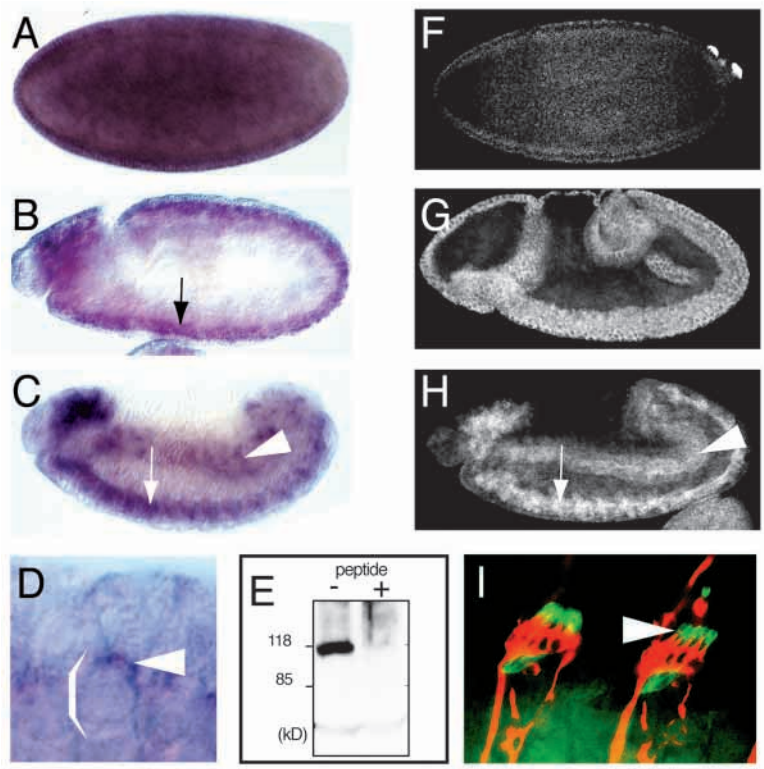

Fig. 2. RNA and protein expression from the *cornetto* gene. (A-D). In situ hybridizations to wild-type *Drosophila* embryos using the *cornetto* cDNA fragment identified in the two-hybrid screen as a digoxigenin-labelled probe (Tautz and Pfeifle, 1989). (A) RNA from *cornetto* is uniformly expressed in embryos during the syncytial blastoderm stage, indicating a strong maternal contribution. (B) Expression becomes progressively restricted to the central nervous system (arrow) in germ-band-extended embryos. (C) After germ-band retraction, *cornetto* RNA is specifically detected in the central nervous system (arrow) and the developing gut (arrowhead). (D) A high-magnification view of a neuroblast (indicated by bracket) shows the apical asymmetric localization of the *cornetto* RNA (arrowhead). (E) Antibodies directed against a Cornetto C-terminal peptide detect a single band on immunoblots of *Drosophila* whole embryonic extracts (–), which can be completely blocked by preincubation of antibodies with excess peptide (+). (F-H) *Drosophila* embryos at various stages were stained by immunofluorescence using anti-Cornetto-peptide antibodies. (F) Low levels of Cornetto are detected during syncytial blastoderm stages. (G) Cornetto protein is uniformly expressed during germ-band extension. (H) During germ-band retraction, Cornetto protein is detected in the central nervous system (arrow) and the gut (arrowhead). (I) Stage 13 embryos were double stained using anti-Cornetto antibodies (green) and the monoclonal antibody 22C10 (red) to visualize neurons. Cornetto is strongly expressed on axons of the central nervous system (data not shown) and in cap cells of chordotonal organs (arrowhead).

neuroblasts were maintained and no obvious phenotype was observed in *cornetto* overexpressing embryos (data not shown). In telophase neuroblasts, Cornetto was still concentrated apically but was localized into filaments (Fig. 3E), which are more clearly visible in optical cross sections through the apical part of *cornetto* overexpressing neuroblasts (Fig. 3L). Their astral organization and the fact that they converge on the apical centrosome (data not shown) suggest that these filaments are microtubules and that overexpressed Cornetto localizes to the apical aster of the mitotic spindle. Even though the quality of

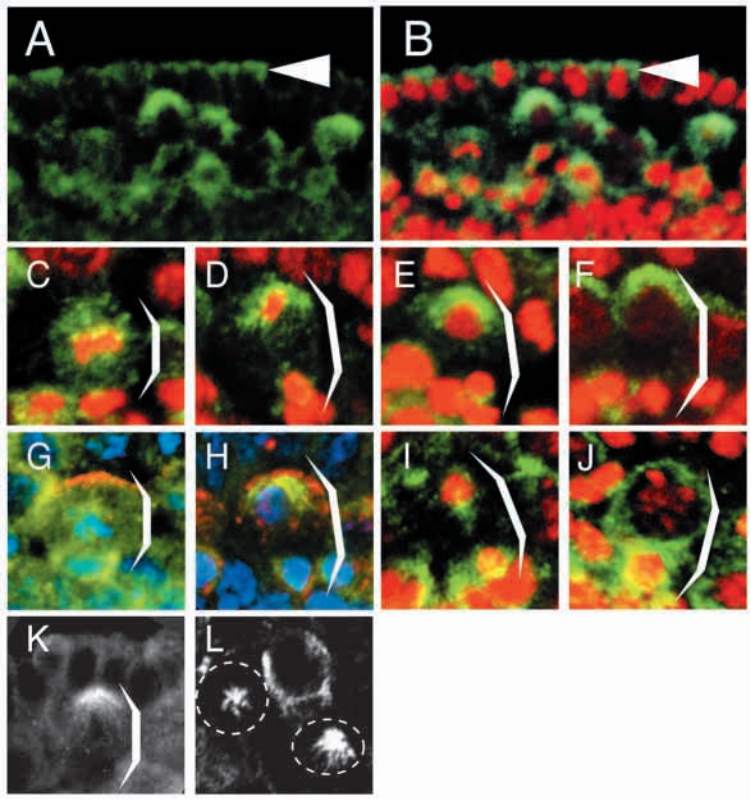

Fig. 3. Inscuteable-dependent asymmetric localization of Cornetto protein. Stage-10 wild-type *Drosophila* embryos (A-H), embryos homozygous for the *inscuteable*^{P72} null allele (I,J) or embryos overexpressing full-length *cornetto* using the UAS-Gal4 system (K,L) were double stained for Cornetto protein (green in A-J) and DNA (propidium iodide; red in A-F, blue in G,H, not shown in I,J), and for Inscuteable in G and H (red). Optical cross sections through the prospective epidermis and developing nervous system (A,B) or high-magnification views of neuroblasts (C-K, neuroblasts indicated by brackets) are shown. (A,B) In epithelial cells of the developing epidermis, Cornetto protein is concentrated in the apical cytoplasm (arrowhead). (C-F) In wild-type neuroblasts, Cornetto is uniformly distributed in the cytoplasm during metaphase (C). During anaphase (D), the protein starts to accumulate apically. In telophase (E), it localizes into an apical crescent that is maintained until interphase of the next cell cycle (F). (G) In metaphase, Inscuteable (red) localizes into an apical crescent but Cornetto (green) is in the cytoplasm. (H) In telophase, when Inscuteable becomes delocalized, Cornetto localizes apically. The Cornetto crescent forms in the apical cytoplasm in the area of the apical microtubule aster, whereas Inscuteable localizes to the cell cortex. (I,J) No asymmetric localization of Cornetto is detected in most *inscuteable*^{P72} (Kraut et al., 1996) mutant neuroblasts during telophase (I) or early interphase (J). (K,L) Upon overexpression of Cornetto, filaments become visible; an optical cross section (K) and a top view onto the apical cortex (L) of telophase neuroblasts are shown. The dashed circles in L indicate the outlines of cells.

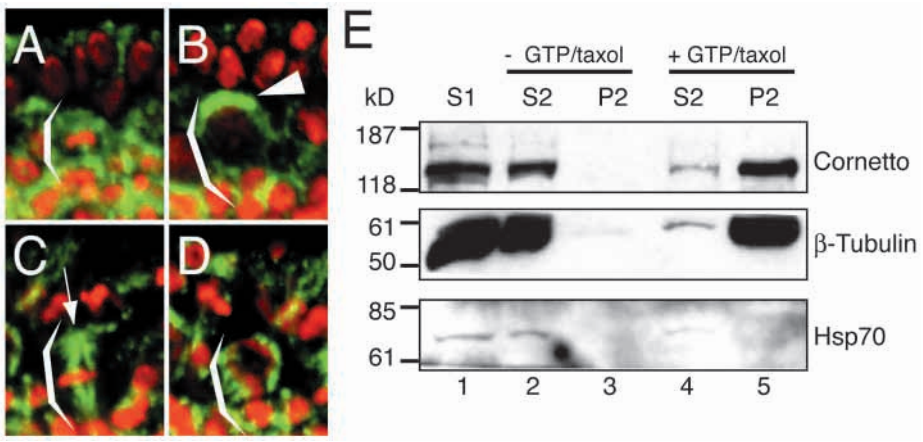

Fig. 4. Cornetto is a microtubule-binding protein and colocalizes with microtubules after disruption of the actin cytoskeleton. (A-D) Permeabilized control embryos (A,B) or embryos treated for 30 minutes with 200 μM latrunculin A (C,D) were stained for Cornetto (green) and DNA (propidium iodide, red). Brackets indicate neuroblasts. In control embryos, Cornetto is uniformly distributed in the cytoplasm of neuroblasts in metaphase (A) and forms an apical crescent in telophase (B, arrowhead). In latrunculin-A-treated embryos, the protein colocalizes with microtubules of the mitotic spindle in metaphase (C, arrow) and fails to localize asymmetrically in telophase (D). (E) High-speed supernatants of *Drosophila* embryo extracts were prepared under microtubule-depolymerizing conditions (see Materials and Methods). Microtubules were repolymerized by incubation with GTP and Taxol and microtubules and microtubule-associated proteins were sedimented by ultracentrifugation. β-Tubulin and Cornetto are found in the pellet under microtubule-polymerizing conditions (lanes 4 and 5), whereas both proteins remain soluble in a control experiment in the absence of GTP and Taxol (lanes 2 and 3). By contrast, a cytoplasmic protein (Hsp70) remains soluble under both conditions. An amount of extract equivalent to that used in the experiment was loaded in lane 1.

our Cornetto antibody did not allow us to analyse the microtubule localization of endogenous Cornetto protein, these observations suggest a functional connection between Cornetto and the mitotic spindle.

Cornetto is a microtubule-binding protein

To test the actin dependence of Cornetto localization, the actin cytoskeleton was disrupted using latrunculin A (Fig. 4). Stage 10 *Drosophila* embryos were stained for Cornetto and DNA after permeabilization and mock treatment or treatment with 200 μM latrunculin A for 30 minutes. Even though permeabilization did not disrupt Cornetto localization in control embryos (Fig. 4B), asymmetric localization of Cornetto during telophase was abolished in drug-treated embryos (Fig. 4D). During prophase and metaphase, Cornetto was evenly distributed in the cytoplasm of control embryos (Fig. 4A) but relocalized to the mitotic spindle in drug-treated embryos (Fig. 4C). This surprising colocalization with microtubules was not restricted to neuroblasts but was also observed in mitotic epithelial cells (data not shown). Relocalization to the mitotic spindle is microtubule dependent because it was not observed in embryos in which both the actin and microtubule cytoskeletons were disrupted by double treatment with latrunculin A and colcemid (data not shown). We conclude that asymmetric localization of Cornetto during telophase is an actin-dependent process and that Cornetto has the capacity to colocalize with microtubules after disruption of the actin cytoskeleton.

Because the colocalization of Cornetto with the mitotic spindle suggests a potential interaction with microtubules, we performed microtubule-binding assays (Fig. 4E). Protein extracts were generated from *Drosophila* embryos under microtubule-depolymerizing conditions. Microtubules were repolymerized by addition of GTP and Taxol, sedimented by centrifugation and both soluble and co-sedimenting proteins were analysed by immunoblotting. Both β-tubulin and Cornetto were found in the microtubule fraction and remained soluble in a control experiment without Taxol, whereas Hsp70 remained in the soluble fraction in both experiments. Taken together, these experiments suggest that Cornetto is a microtubule-binding protein whose colocalization with microtubules during mitosis is prevented by an actin-dependent process.

cornetto mutants have no defects in asymmetric cell division

To assess Cornetto function genetically, we generated mutants in the *cornetto* locus. We found a P-element insertion in the *cornetto* 5′ untranslated region that had been identified in a screen for mutations causing male sterility. However, the sterility of this mutant and the P-element insertion could be separated by recombination and the clean P-element insertion was homozygous viable and used as a starting strain for imprecise excision. P-element revertants were screened for directional deletions that remove the P-element end proximal to the *cornetto* coding region but leave the other end intact. Even though the P-insertion is separated from the *cornetto* coding region by a large 8 kb intron, one imprecise excision could be recovered that removes all but the last 385 nucleotides of the *cornetto* coding region. Even though no Cornetto protein could be detected in homozygous mutant embryos and a dilution series indicated that protein levels were reduced by at least 30 times, *cornetto* mutants were homozygous viable and fertile, and no defects in asymmetric cell division could be detected (data not shown).

DISCUSSION

The *Drosophila* Inscuteable protein is required in neuroblasts for apical-basal orientation of the mitotic spindle and for the basal localization of cell fate determinants during mitosis (Kraut et al., 1996). Our experiments show that Inscuteable is also required for the apical localization of the Cornetto protein in neuroblasts during anaphase and telophase. Cornetto is a microtubule-binding protein and interacts with the functional domain of Inscuteable, suggesting that it could provide a direct physical link between apically localized Inscuteable protein and the mitotic spindle during late stages of mitosis.

Inscuteable-dependent apical localization of Cornetto

Two distinct patterns of asymmetric protein localization can be distinguished in *Drosophila* neuroblasts. The proteins Inscuteable (Kraut et al., 1996), Pins (Parmentier et al., 2000; Schaefer et al., 2000; Yu et al., 2000), Bazooka (Kuchinke et al., 1998; Schober et al., 1999; Wodarz et al., 1999), DmPAR-6 (Petronczki and Knoblich, 2000) and DaPKC (Wodarz et al., 2000) colocalize at the apical cell cortex during interphase, prophase and metaphase, but become delocalized in anaphase and telophase. They are required during metaphase for correct orientation of the mitotic spindle and for the correct segregation of a second set of proteins into the basal daughter cell. The second set includes the cell fate determinants Numb (Rhyu et al., 1994) and Prospero (Knoblich et al., 1995; Spana and Doe, 1995), the RNA-binding protein Staufen (Broadus et al., 1998; Li et al., 1997; Schuldt et al., 1998), and the adapter proteins Pon (Lu et al., 1998) and Miranda (Shen et al., 1997). Starting in late prophase, these proteins move to the basal cell cortex, where they colocalize until the exit from mitosis. The asymmetric localization of Cornetto does not fit into any of these categories. Cornetto is homogeneously distributed in the cytoplasm during prophase and metaphase but starts to localize apically in anaphase and telophase. Thus, a third mechanism for asymmetric localization of proteins with distinct spatial and temporal regulation must exist in neuroblasts to transport Cornetto and possibly other proteins apically during anaphase. The Inscuteable dependence of Cornetto localization suggests a novel function for Inscuteable in providing directional information for this late localization machinery.

Inscuteable and all other known apically localized proteins become delocalized or degraded during anaphase, suggesting that the apical positional cue is resolved. At the onset of the second mitosis, however, these proteins reappear on the apical cell cortex and they are again required for spindle orientation during the second mitotic division (Kraut et al., 1996) (S.B. and J.A.K., unpublished). The directional cue for apical protein localization during the second cell cycle is not known. Our experiments show that Cornetto is the first protein that remains apically localized even after Inscuteable has disappeared, suggesting that a cortical mark exists at the apical cell cortex even after completion of the first division. We could not detect abnormalities in the orientation of the second neuroblast division in *cornetto* mutants but other proteins transported by the same localization machinery could function in repolarization of the neuroblast after the first division.

Actin dependence of Cornetto localization

Cornetto is found in the cytoplasm during prophase and metaphase but concentrates apically in anaphase. Upon disruption of the actin cytoskeleton, the protein colocalizes with microtubules throughout mitosis in every cell type analysed and fails to concentrate apically during late stages of mitosis. Although the failure to concentrate apically might indirectly reflect the actin dependence of Inscuteable localization (Kraut et al., 1996), the remarkable microtubule colocalization demonstrates a novel type of cross-talk between the actin and microtubule cytoskeletons. Either actin-dependent binding of Cornetto to another protein prevents microtubule association or this association is prevented by actin-dependent modification of microtubules or the Cornetto protein itself. The identification of Cornetto-binding proteins or post-translational modifications of Cornetto will help to distinguish between these possibilities.

The cytoplasmic localization of Cornetto in unperturbed cells suggests that the protein does not bind microtubules during metaphase, but the asymmetric localization during anaphase could reflect the association with astral microtubules. This would explain why Cornetto crescents are wider than crescents observed for proteins like Inscuteable, which are thought to be restricted to the apical cell cortex in neuroblasts. Furthermore, when Cornetto is overexpressed, the protein localizes to the apical microtubule aster in telophase. Thus, the subcellular localization pattern of Cornetto is consistent with a cell-cycle-regulated binding to a subset of microtubules.

What is the function of Cornetto?

Inscuteable is required for correct orientation of the mitotic spindle along an apical-basal axis and for the accumulation of cell fate determinants at the basal cell cortex in metaphase neuroblasts. Spindle orientation involves the attachment of astral microtubules to particular sites at the cell cortex (Hyman, 1989; Hyman and White, 1987) and Inscuteable must be involved in generating such a site in neuroblasts. Inscuteable could directly or indirectly interact with a microtubule-binding protein and localize this protein to the apical cell cortex, where it would bind astral microtubules and attract one of the two spindle poles. Alternatively, such a microtubule-binding protein could be part of the basal Numb-Miranda complex and attract one spindle pole to the basal cell cortex. The identification of Cornetto as a protein that can bind both microtubules and Inscuteable argues for the first possibility. Even though spindle orientation in neuroblasts during metaphase is normal in *cornetto* mutants, the protein could stabilize the orientation of the mitotic spindle during later stages of mitosis to ensure precise orientation of cell division.

A function of Cornetto in anchoring the mitotic spindle to the cell cortex could be masked by genetic redundancy, which has been observed in other systems for proteins involved in spindle orientation. In yeast, the proteins Bim1 and Kar9 provide a molecular link between astral microtubules and the cell cortex (Korinek et al., 2000; Lee et al., 2000; Miller et al., 2000) but neither *kar9* nor *bim1* are essential for viability and only mild defects in spindle orientation are observed in these mutants. Both genes are synthetic lethal with mutations in the *dynein* gene suggesting that the Kar9/Bim1 pathway and a dynein-based mechanism provide functionally redundant means to orient the mitotic spindle correctly. All the genes known to be involved in spindle orientation in *Drosophila* neuroblasts are functionally connected to Inscuteable and we fail to detect any synthetic lethality between Cornetto and Bazooka or Inscuteable. Mutations in Drosophila *dynein* exist but no allelic combinations are available that would allow us to test a function in neuroblasts. Synthetic lethal screens for genes that are essential in the absence of *cornetto* should reveal whether Cornetto functions redundantly with another microtubule-binding protein to orient mitotic spindles in *Drosophila* neuroblasts.

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