Clathrin heavy chain plays multiple roles in polarizing the Drosophila oocyte downstream of Bic-D

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ABSTRACT
Bicaudal-D (Bic-D), Egalitarian (Egl), microtubules and their motors form a transport machinery that localizes a remarkable diversity of mRNAs to specific cellular regions during oogenesis and embryogenesis. Bic-D family proteins also promote dynein-dependent transport of Golgi vesicles, lipid droplets, synaptic vesicles and nuclei. However, the transport of these different cargoes is still poorly understood. We searched for novel proteins that either mediate Bic-D-dependent transport processes or are transported by them. Clathrin heavy chain (Chc) co-immunopurifies with Bic-D in embryos and ovaries, and a fraction of Chc colocalizes with Bic-D. Both proteins control posterior patterning of the Drosophila oocyte and endocytosis. Although the role of Chc in endocytosis is well established, our results show that Bic-D is also needed for the elevated endocytic activity at the posterior of the oocyte. Apart from affecting endocytosis indirectly by its role in osk mRNA localization, Bic-D is also required to transport Chc mRNA into the oocyte and for transport and proper localization of Chc protein to the oocyte cortex, pointing to an additional, more direct role of Bic-D in the endocytic pathway. Furthermore, similar to Bic-D, Chc also contributes to proper localization of osk mRNA and to oocyte growth. However, in contrast to other endocytic components and factors of the endocytic recycling pathway, such as Rabenosyn-5 (Rbsn-5) and Rab11, Chc is needed during early stages of oogenesis (from stage 6 onwards) to localize osk mRNA correctly. Moreover, we also uncovered a novel, presumably endocytosis-independent, role of Chc in the establishment of microtubule polarity in stage 6 oocytes.

KEY WORDS: Clathrin heavy chain, Drosophila oogenesis, RNA localization, Germ line, Microtubules, Polarity formation

INTRODUCTION
Studies in different organisms portray Bic-D homologs as factors that link the dynein-dynactin minus end-directed microtubule (MT) motor complex to different cargoes, moving them to particular cellular regions (Claussen and Suter, 2005; Vazquez-Pianzola and Suter, 2012). Drosophila Bic-D (also known as BicD) directly binds Egalitarian (Egl), which engages with Dynein light chain (Dlc; also known as Ctp) and specific mRNA localization signals (Navarro et al., 2004; Dienstbier et al., 2009). This complex is thought to associate with further proteins that regulate translation and stability of transported mRNAs, and the resulting larger complex translocates with the help of motors along the MT cytoskeleton.

The Bic-D-dependent transport machinery is used repeatedly during Drosophila development. In oogenesis, Bic-D is needed for the transport of osk, bcd and grk mRNAs from the nurse cells into the oocyte and then to specific compartments within the oocyte (Suter and Steward, 1991; Ran et al., 1994; Clark et al., 2007). Correct localization of these factors within the oocyte is crucial for specifying anteroposterior and dorsoventral axes of oocytes and embryos. Later in the life cycle, the mRNA localization machinery delivers specific mRNAs to the apical side of the syncytial embryo (Bullock and Ish-Horowicz, 2001) and promotes localization of inscuteable mRNAs in neuroblasts (Hughes et al., 2004).

Drosophila Bic-D binds mRNA cargoes not only through the cargo-binding adaptor Egl, but also through the Fragile-X Mental Retardation Protein (Fmr1) (Bianco et al., 2010). Mammalian BicD2 regulates centrosome and nuclear positioning during mitotic entry (Splinter et al., 2010) and mammalian Bic-D isoforms bind Rab6 to control COPI-independent Golgi-endoplasmic reticulum transport (Matanis et al., 2002). Furthermore, Rab6B-BicD1 interaction regulates retrograde membrane transport in human neurites (Wanschers et al., 2007). Fly and worm Bic-D genes are also involved in nuclear migration in photoreceptors, oocytes and hypodermal precursor cells (Swan et al., 1999; Swan and Suter, 1996; Fradillofson et al., 2010), and Bic-D also dynamically regulates transport of lipid droplets (Larsen et al., 2008). Given the involvement of Bic-D in the localization of surprisingly diverse cargos, we searched for adaptor proteins that mediate cargo binding as well as for novel cargo molecules.

RESULTS
Chc associates with Bic-D in ovaries and embryos
Using two different monoclonal anti-Bic-D antibodies that recognize different epitopes, we immunopurified Bic-D complexes and identified Chc as a complex component in both approaches through mass spectrometry (Fig. 1A). Beads alone and beads coupled to control antibodies did not precipitate Chc. Myc-tagged Chc expressed under UAS control and a Flag-Tetracysteine (4C)-tagged Chc expressed from its endogenous promoter (Kasprowicz et al., 2008) were also immunoprecipitated with both anti-Bic-D antibodies from embryonic (Fig. 1B) and ovarian (Fig. 1C) extracts. Li et al. showed that Chc interacts directly with Bic-D and is its major interactor in the nervous system (Li et al., 2010). In contrast to this, Bic-D interacts with Egl, Chc, Pabp and others in embryos (Fig. 1A) (Vazquez-Pianzola et al., 2011; P.V.P. and B.S. unpublished data). Thus, in non-neuronal tissues, such as young embryos and ovaries, Chc appears to be one of many partners of Bic-D.

A fraction of Chc dynamically colocalizes with Bic-D in cortical regions of the oocyte
Anti-Bic-D antibody staining of ovaries that were fixed in a healthy state does not reveal aggregated structures, suggesting that native complexes are small particles that cannot be resolved with normal
Fig. 1. Chc forms complexes with Bic-D in ovaries and embryos and is enriched in the oocyte. (A) Coomassie-stained SDS-PAGE showing anti-Bic-D IPs of total embryo extracts. Anti-Cdk7 antibodies were used as controls for nonspecific binding. Gel areas in which Chc, Bic-D and Egl were identified by mass spectrometry are indicated. Chc was identified in IPs performed with both anti-Bic-D antibodies, but was not found in the corresponding gel position after IPs with control antibodies. (B) IP of total embryo extracts expressing a Myc-tagged Chc expressed with the Nullo-Gal 4 driver. Antibodies used for IPs are indicated on top. Bic-D antibodies (4C2 and 1B11), a negative control mouse monoclonal antibody (HTm4) and beads alone were used. Western blots of the precipitated material were tested for the presence of Bic-D, Myc::Chc and Egl. (C) IPs with the antibodies indicated on top and input controls. Extracts were from ovaries of wild-type (wt) flies and from ovaries expressing a Flag-tagged Chc from the 4C-CHC genomic construct. Anti-Bic-D (1B11) and anti-GFP antibodies were used. IP material was analyzed by western blot to check for the presence of Bic-D, Myc::Chc and Egl. Chc was detected with anti-Flag and with rabbit anti-mammalian Chc antibodies. (D) Immunostaining of wild-type ovaries with Drosophila anti-Chc antibody (red). (E) Enlarged view of the posterior part of stage 6, 8 and 9 egg chambers. Endogenous Chc is enriched in the oocyte in early stage egg chambers. Around stage 8, Chc localizes to the oocyte cortex and gets specifically enriched at the posterior pole. It becomes also concentrated in the center of the oocyte by stage 8 (arrowhead). At stage 9-10 it is enriched in the oocyte cortex and also at the posterior cortex (arrowhead). (F) Immunostaining of egg chambers expressing a Flag-tagged genomic Chc construct (4C-Chc transgene) with anti-Flag antibody (red) showing a similar germline staining as shown in D. Although 4C-CHC signal is reduced in follicle cells, the apparent Chc distribution pattern is very similar in the germ line, showing clear oocyte enrichment and a similar subcellular localization in the oocyte. Intensity differences appear to be due to different expression levels, nonspecific staining of the Chc antibody and penetration differences between antibodies. (G) Enlarged views of stage 6, 9 and 10 oocytes from F. Arrowhead indicates Chc enrichment at the posterior cortex. (H) Partial colocalization of Flag-tagged Chc (green) and Bic-D (red) is seen as yellow signal at the posterior cortex in stage 6-7 oocytes (arrowheads) and also at the anterior cortex at stage 8 (arrowhead). (I) Immunostaining of wild-type ovaries with anti-Cic antibody (green) and anti-Bic-D antibody (red). Cic is also enriched in the oocyte. Partial Cic/Bic-D colocalization is also observed in stage 6-7 oocytes at the posterior cortex (arrowheads). However, Cic is more cortical and more uniformly distributed throughout the cortex in stage 8 egg chambers and no clear posterior enrichment is observed from stage 8 onwards. In D-I, ovarioles were also stained with Hoechst (blue) to visualize the DNA. Scale bars: 10 µm.
confocal microscopy. Colocalization studies can therefore only reveal whether Bic-D and Chc colocalized in the same cellular compartment. Indeed, a fraction of differently tagged (V5, Myc, Flag and GFP) or un-tagged Chc colocalizes with Bic-D in specific regions of the oocyte (Fig. 1D-H; data not shown). The germ line Chc signal became enriched in the oocyte from early stages onwards (Fig. 1D,F) and further signal enrichment became apparent at the posterior cortex of the oocyte from stage 6 onwards (Fig. 1E-G). During stage 6, Chc shows a partial colocalization with Bic-D and Egl at the posterior cortex (Fig. 1H; supplementary material Fig. S1). From stage 8-9 onwards, Chc signals localized to the oocyte cortex with higher enrichment at the posterior pole (Fig. 1E,G). Another partial colocalization between Bic-D/Egl and Chc is evident at the anterior cortex in stage 8 oocytes (Fig. 1H; supplementary material Fig. S1B).

As Clathrin activity in endocytosis involves also Clathrin light chain (Clc), we also studied Clc localization (Fig. 1I; supplementary material Fig. S1). Anti-Clc antibody staining also revealed Clc enrichment in the oocyte from early stages onwards. Clc concentrates at the posterior cortex, partially colocalizing with Bic-D in stage 6-7 oocytes. However, at stage 8, Clc is more uniformly distributed throughout the oocyte cortex and no clear posterior enrichment is observed from stage 8 onwards (Fig. 1I; supplementary material Fig. S1). Co-staining for Chc and Clc showed that, although both proteins are enriched in the oocyte and specifically at the oocyte cortex, they do not completely colocalize during development (supplementary material Fig. S1). This finding could suggest that Chc may play roles in oocyte development independently of Clc.

**Bic-D localizes Chc mRNA to the oocyte and Chc protein to the posterior oocyte cortex**

Bic-D and Egl seem to localize normally in Chc<sup>4</sup> mutant ovarioles.

The converse experiment, testing the function of Bic-D in later processes of oogenesis, is complicated by the fact that loss of Bic-D prevents oocyte formation (Ran et al., 1994). To deplete the germ line of Bic-D protein after oocyte determination, we used Bic-D<sup>dom</sup> flies in which Bic-D is provided solely from an inducible promoter that can be turned off once oocyte fate is established (Swan and Suter, 1996). Around 4 days after shutting down Bic-D, these ovaries contain mid-oogenesis germ line devoid of Bic-D. Once Bic-D protein became undetectable, Chc protein accumulation in the oocyte and at the posterior cortex of the oocyte became significantly reduced (Fig. 2). This finding could suggest that Bic-D may play roles in oocyte development independently of Chc.
oocyte cortex became reduced as well (Fig. 2A), even though Chc levels were not affected (Fig. 2B). Therefore, in the female germ line Bic-D acts upstream in localizing Chc.

The dominant mutant Bic-D alleles affect the localization of the cargo osk mRNA (Ephrussi et al., 1991; Kim-Ha et al., 1991). We therefore tested whether they also affect Chc localization. In stage 10 oocytes heterozygous for the dominant Bic-D alleles Bic-DIIE48 or Bic-D71.34 or Bic-Dnull, Chc accumulated strongly at the posterior in most cases (79%, n=44 in Bic-DIIE48/+ or Bic-Dnull/+; 89%, n=38 in Bic-D71.34/+ or Bic-Dnull/+). Thus, only 10-20% of oocytes with one normal copy of Bic-D showed reduced accumulation of Chc at the posterior. By contrast, posterior accumulation of Chc was reduced in 49% (Bic-DIIE48/Bic-Dnull; n=49) and 44% (Bic-D71.34/Bic-Dnull; n=99) of the mutant stage 9-10 oocytes (Fig. 2C). Interestingly, the observed effects are not due to an abolished interaction between Chc and the dominant mutant Bic-D protein because Chc is efficiently precipitated with Bic-D from Bic-DIIE48/Bic-Dnull and Bic-D71.34/Bic-Dnull mutant extracts (not shown). Furthermore, Chc protein levels are also normal in these ovaries (Fig. 2D,E). Because a significant portion of dominant Bic-D mutant protein accumulates strongly at the anterior cortex, it appears that there may not be sufficient functional Bic-D available to transport Chc cargo to the posterior cortex.

Because Bic-D transports mRNAs into the oocyte, we analyzed the distribution of Chc mRNA in wild-type and Bic-Dnull ovaries (Fig. 3A,B). Once the female germ line was depleted of Bic-D, Chc mRNA accumulation in the oocyte also became reduced, even though total levels of Chc mRNA remained normal (Fig. 3C). Bic-D thus functions to localize Chc mRNA into the oocyte, rather than in stabilizing the transcript. Similar to the mRNA levels, ovarian Chc protein levels were also normal in Bic-Dnull mutants (Fig. 2B).

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**Fig. 3. Chc mRNA is transported into the oocyte by the Bic-D/Egl machinery.** (A) In situ hybridization to yw controls (upper panels), to egg chambers 4 days after turning off Bic-D expression (Bic-Dnull, middle panels) and to Bic-D71.34/Bic-Dnull egg chambers (lower panels) showing Bic-D mRNA (red) and Chc mRNA (green). Chc mRNA fails to efficiently accumulate in Bic-Dnull oocytes lacking Bic-D mRNA. Oocytes are on the right side of each egg chamber. Chc mRNA still accumulates in Bic-D71.34/Bic-Dnull oocytes. (B) Quantification of the number of egg chambers showing oocyte enrichment of Chc from the experiment described in A. (C) Levels of Bic-D, Chc, osk, grk and Tub67C mRNA in ovaries as measured by qRT-PCR 1 day (when Bic-D levels are still normal) and 4 days after shutting down Bic-D expression. A 1:1 mixture of heterozygous dominant mutants (+/Bic-D71.34) and heterozygous null mutants (+/Bic-Dnull) was used as control for Bic-Dnull oocytes. Values were normalized to Tub67C levels and fold change calculated from two independent biological samples using the Pfaffl method (Pfaffl, 2001). Bars show the average fold change and the lines show the maximum and minimum values. Results with two other samples and analysis by semi-quantitative PCR showed similar results (not shown). Chc transcripts are stable in egg chambers 4 days after shutting down Bic-D and at a time when Bic-D mRNAs are clearly reduced. (D) Wild-type embryo extracts (control IP) and extracts expressing an Egl::GFP fusion protein were subject to immunoprecipitation using anti-GFP antibodies. qPCR was used to assay the presence of Chc mRNA, known localized mRNAs (h, ftz, osk) and negative controls (Kr, rp49, Tub67C). The values represent the average of two IPs for each treatment. qPCR for all samples were performed in triplicate. Scale bars: 30 µm.
Reduced oocyte accumulation of Chc protein in Bic-D-depleted egg chambers therefore seems to be due to the lack of Chc mRNA transport. To test whether the Bic-D/Egl localization machinery associates with Chc mRNA, we immunopurified Bic-D/Egl::GFP complexes. This procedure resulted in enrichment of Chc mRNAs to a similar level as the enrichment of the known Bic-D/Egl targets ftz, hairy and osk (Fig. 3D). By contrast, Kr, a non-localizing mRNA, and the housekeeping mRNAs rp49 (also known as RpL32) and Tub67C were much less enriched in these immunoprecipitates.

Because Bic-D is involved in localizing Chc mRNA and protein, it became important to assess the dynamics of Chc::GFP transport in the presence and absence of Bic-D in the female germ line (Fig. 4). As seen for Chc in fixed samples, Chc::GFP accumulates at the oocyte cortex in the wild type, but much less so in Bic-D\textsuperscript{mono} oocytes (Fig. 4A). Chc::GFP spots move in strictly linear directions in mutant and wild-type oocytes, suggesting that Chc::GFP is transported along MT tracks. Clusters of similar size and shape to the moving spots are located in the vicinity of the cortex in Bic-D\textsuperscript{mono} oocytes (Fig. 4B) but not in wild-type oocytes, in which the strong accumulation of cortical Chc::GFP leads to a more homogenous and brighter signal (Fig. 4B). Particles moved frequently and with similar directionality along the oocyte cortex in wild type and mutants (Fig. 4C,D), but average particle speed was significantly higher in mutants (Fig. 4E). It therefore seems that lack of Bic-D affects movement of Chc on MTs and that this defect prevents cortical accumulation of Chc protein. The increased particle speed observed upon loss of Bic-D may point towards an inhibitory function of Bic-D and dynein-mediated transport towards kinesin-based transport. Interestingly, the particle speed for Rab6, another Bic-D cargo, increases also after inhibition of the dynein-dynactin interaction by expressing p50-dynamitin (Januschke et al., 2007). Furthermore, overexpression of Chc::GFP in the hypomorphic and the dominant Bic-D mutant background enhanced the Bic-D-dependent osk mislocalization phenotypes. Different cargoes competing for limited Bic-D could lead to such an enhancement of the hypomorphic phenotype. Alternatively, Chc overexpression could modify the cortex in a way that improves anchoring of ectopic osk mRNA (supplementary material Fig. S2).

Like Bic-D, Chc is needed for proper localization of osk mRNA and for oocyte growth

Although Chc\textsuperscript{1}, Chc\textsuperscript{2} and Chc\textsuperscript{3} are lethal alleles, Chc\textsuperscript{4} is a non-lethal mutant allele that causes a remarkable reduction in endocytic capacity and a wide range of phenotypes, including sterility (Bazinet et al., 1993; Peralta et al., 2009). In homozygous, hemizygous and transheterozygous (Chc\textsuperscript{1}/Chc\textsuperscript{4}) Chc\textsuperscript{4} females, oogenesis arrests mostly at stage 8, but escaper follicles develop into eggs. This Chc\textsuperscript{4} oogenesis arrest phenotype is rescued by different Chc\textsuperscript{+} constructs, revealing also that differently tagged transgenes had Chc\textsuperscript{+} rescuing activity. A single genomic copy of Chc\textsuperscript{+} with a Flag-tag (4C-Chc) (Kasprowicz et al., 2008) rescued sterility, poor viability and locomotive defects of Chc\textsuperscript{1}/Chc\textsuperscript{4} females, and two copies of this transgene also rescued Chc\textsuperscript{1} lethality. pUASP-Chc\textsuperscript{+}, pUASP-Chc::eGFP (Li et al., 2010) and pUASP-nyc-Chc, driven by an Actin-Gal4 driver, were able to rescue the sub-lethality and sterility of Chc\textsuperscript{1}/Chc\textsuperscript{4} females, but not the Chc\textsuperscript{1} lethality. The reduced rescue efficiency could be due to low or non-ubiquitous expression of the Gal4 driver or the pUASP vector. Chc\textsuperscript{+} mutant egg chambers showed an overall reduced size, smaller oocytes and smaller, but normally localized, oocyte nuclei (Fig. 5A). Receptor-mediated endocytic yolk uptake into the oocyte depends on Clathrin and is initiated normally around stage 8 (Tanaka et al., 2011). Chc\textsuperscript{+} mutant oocytes and mutant larval Garland cells showed reduced endocytosis (Peralta et al., 2009) (data not shown).

In Chc\textsuperscript{4} mutants, maternal mRNAs seemed to be imported normally from the nurse cells into the oocyte and grk, orb, Bic-D and Chc mRNAs showed mostly normal localization (Fig. 5B). By contrast, osk mRNA accumulated in oocytes, but it was found uniformly distributed in Chc\textsuperscript{4} mutant oocytes or ectopically localized in the middle of stage 9-10 oocytes unless a rescue transgene was provided (Fig. 5C). osk mRNA localization at the anterior and posterior cortex of stage 8 oocytes was mostly not observed in mutant egg chambers. Osk protein levels remain low in Chc\textsuperscript{4} stage 9 egg chambers, and 12 out of 20 egg chambers did not show Osk expression at the posterior cortex compared with only three out of 20 in the wild type (data not shown). Germ line clones of the lethal allele Chc\textsuperscript{4} (Yan et al., 2009) showed morphological defects, but, nevertheless, grk mRNA localization was mostly normal (Fig. 6A). By contrast, osk mRNA failed to enrich at the oocyte posterior from stage 6 onwards (Fig. 6B), further supporting the view that Chc is needed more specifically for proper localization of osk mRNA.

Chc organizes oocyte MT polarity already before bulk endocytosis starts

Mutations in Rab11 and Rabenosyn-5 (Rbsn-5), two genes involved in endocytosis, cause defects in osk mRNA localization.
(Jankovics et al., 2001; Dollar et al., 2002; Tanaka and Nakamura, 2008). However, as opposed to Rab11 and Rbsn-5, Chc<sup>4</sup> affects osk mRNA localization earlier than stage 9. We therefore tested whether problems in MT organization in Chc<sup>4</sup> egg chambers could lead to osk mRNA mislocalization (Fig. 7). Indeed, Kinesin heavy chain (Khc), a marker for stable MT plus ends, is enriched at the posterior cortex only in wild-type oocytes and in rescued mutants, but not in Chc<sup>4</sup> mutants (Fig. 7A). Posterior enrichment of EB1, a marker for growing MT plus ends, was also affected in stage 6-9 Chc<sup>4</sup> mutant egg chambers (Fig. 7B). Furthermore, α-Tubulin staining showed that MTs are enriched at the posterior oocyte cortex at stage 6, before they re-focus at the anterior and anterodorsal cortex by stage 8 (Fig. 7C). In Chc<sup>4</sup> mutant egg chambers, α-Tubulin shows either a dotted pattern or no staining at the posterior cortex in stage 6. Also, stage 8 oocytes do not show anterior α-Tubulin accumulation, indicating again problems in focusing the MT network. In agreement, focusing of MTs was also not observed in germ line clones of the Chc<sup>GF23</sup> allele (supplementary material Fig. S3). Such early functions in MT organization were not described for other genes involved in the endocytic pathway (Jankovics et al., 2001; Dollar et al., 2002; Tanaka and Nakamura, 2008). Revisiting Rbsn-5 and Rab5 mutant phenotypes in our laboratory, we confirmed normal localization of Khc in early oogenesis and that Khc localization was only affected after stage 9 (supplementary material Fig. S4). Therefore, Rbsn-5 and Rab5 (involved in the formation of early endosomes) mutations that interfere with endocytosis, affect MT organization only after stage 9 of oogenesis. By contrast, Chc is involved already earlier, around stage 6, in the organization of MT polarity and the MT network. Because bulk endocytosis starts only after MT reorganization at stage 8 and because the other endocytic genes tested are not important for MT organization prior to stage 9, it appears that Chc may play an endocytosis-independent role in MT organization around stage 6. Interestingly, in germ lines lacking Bic-D, Khc is also delocalized and detached from the posterior cortex (Fig. 7D) and α-Tubulin staining revealed abnormal MT organization (data not shown). Even though the defects observed in Chc mutants are clearly stronger than the ones observed in the hypomorphic Bic-D<sup>Dmmom</sup> situation, this result indicates that, like Chc, Bic-D is also involved in controlling MT organization.

**Chc links Bic-D to the regulation of the endocytic pathway and posterior patterning**

Clathrin plays a major role in the formation of coated vesicles during endocytosis. As Bic-D interacts with Chc and is needed for proper localization of Chc in oocytes (Figs 1, 2), we analyzed the levels of endocytosis in Bic-D<sup>Dmmom</sup> egg chambers by visualizing FM4-64 uptake and autofluorescence of yolk granules (Fig. 8A,B). Wild-type stage 8-9 egg chambers show an accumulation of FM4-64 in the follicle cells and at the oocyte cortex with a higher accumulation at the posterior (Fig. 8A). Similarly, yolk granules accumulate at high amounts and are clearly enriched at the posterior in wild-type oocytes (Fig. 8A). However, in Bic-D<sup>Dmmom</sup> egg chambers in which Bic-D has become undetectable (labeled ‘day 4’), FM4-64 dye uptake and yolk granule autofluorescence are reduced and mostly lack enrichment at the posterior cortex (Fig. 8A,B). Therefore, Bic-D is needed to support oocyte endocytosis. Elevated levels of posterior endocytosis have been linked to the establishment of posterior identity (Vanzo et al., 2007; Tanaka and Nakamura, 2008). To find out whether the Bic-D-mediated elevated accumulation of Chc at the posterior cortex can be linked to the elevated endocytosis at the posterior pole, we took
Discussion

Chc and Bic-D are present in the same complexes in embryos and ovaries, where their functions show considerable overlap. As opposed to the nervous system, in which Li et al. describe Chc as the major Bic-D-associated protein in Drosophila heads (Li et al., 2010), we found that Chc is one of many Bic-D partners in ovaries and embryos. The magnitude of these differences suggests that they are not solely caused by the different purification protocols employed. Instead, they seem to reflect differences in tissue-specific complex formation and additional activities of Bic-D during oogenesis and embryogenesis that are mediated by different adaptor and cargo proteins.

Dual role of Bic-D in stimulating endocytosis at the posterior pole of the oocyte

Chc is the main structural component of clathrin-coated pits and vesicles, and a central player in clathrin-mediated endocytosis (Brodersky, 2012; Faini et al., 2013). The association of Bic-D with Chc led us to discover that Bic-D is needed for endocytosis in the oocyte through two different mechanisms. On one hand, Bic-D seems to localize Chc mRNA into the oocyte. But in addition, Bic-D also appears to transport Chc protein to the posterior oocyte cortex. In Bic-D dominant mutants Chc mRNA localization is normal, but Chc protein is reduced at the posterior and much Bic-D protein remains anteriorly, pointing to insufficient protein transport towards posterior. Direct observation of moving particles also show that lack of Bic-D alters the dynamics of Chc::GFP transport in oocytes.

Li and co-workers proposed that Bic-D is needed for localization of Chc to periactive zones through a role in synaptic vesicle recycling (Li et al., 2010). A Chc recycling mechanism may be at work at the oocyte cortex, but our data are also consistent with a function of Bic-D in transporting Chc to the oocyte and then to its cortex. It seems possible that both mechanisms are at work in the female germ line, but further studies are needed to clarify their individual contribution and to find out whether they occur simultaneously or successively.

Endocytosis-independent function of Chc in establishing MT polarity

From oogenesis stage 8 on, Chc also functions in posterior localization of osk mRNA. However, as opposed to Bic-D and Egl, Chc does not get recruited to localizing mRNAs injected into syncytial embryos (data not shown), suggesting a more indirect role in mRNA transport. At stage 6, slightly before osk mRNA localization defects become visible, Chc is involved in organizing the MTs that are needed for mRNA transport. Therefore, although many endocytosis-related genes play roles later in oogenesis for MT maintenance, Chc is needed for the establishment of MT organization already around oogenesis stage 6. Furthermore, establishment of MT polarity by Chc appears to be endocytosis independent because Rbsn-5null mutant egg chambers (showing strong endocytosis defects) and Rab-5null mutants show MT polarity defects also only after stage 9 (Tanaka and Nakamura, 2008) (supplementary material Fig. S4). Therefore, although many endocytosis-related genes play roles later in oogenesis for MT maintenance, Chc is needed for the establishment of MT organization already around oogenesis stage 6. Furthermore, establishment of MT polarity by Chc appears to be endocytosis independent because Rbsn-5null mutant egg chambers (showing strong endocytosis defects) and Rab-5null mutants (also required for vesicle fusion in early endocytosis) do not reveal a requirement for this process (Tanaka and Nakamura, 2008; Morrison et al., 2008) (supplementary material Fig. S4).

Interestingly, mammalian Chc (CHC17; also known as CLTC) associates with mitotic spindles and forms a complex with the advantage of a dominant Bic-D allele that prevents this elevated accumulation of Chc at the posterior pole. In Bic-D71.34/Bic-Dnull oocytes, FM4-64 dye uptake is also reduced (Fig. 8A). In addition, these oocytes show reduced yolk granule autofluorescence and, although they still show more yolk granules towards the posterior, these granules are smaller and not clearly enriched at the very posterior cortex where they concentrate in wild-type oocytes (Fig. 8A,B). Interestingly, in synapses lacking functional Chc small vesicles fail to be formed and these synapses contain larger vesicles (Kasprowicz et al., 2008) This is consistent with Bic-D-dependent posterior localization of its interaction partner Chc being an essential step in upregulating posterior endocytosis and patterning. Similarly, the absence of Bic-D and the presence of the Bic-D71.34 mutation interfere with posterior oocyte localization of Rab11 and Rbsn-5 (Fig. 8C,D).
MT-associated proteins TACC3 and ch-TOG (also known as CKAP5) (Royle et al., 2005; Booth et al., 2011). This complex stabilizes kinetochore fibers by mediating short-distance interactions between adjacent MTs (Booth et al., 2011). Similar to Chc, Drosophila ch-TOG, encoded by mini spindles (msps), is also required for organization and structure of oocyte MTs and for the mRNA localization of bicoid (bcd) mRNA (Moon and Hazelrigg, 2004). However, the hypomorphic msp mutants did not show defects in osk mRNA localization. Stronger reduction of msp activity may help in determining whether the hypomorphic msp mutants still provide sufficient msp activity to localize osk mRNA or whether the roles of Chc in MT organization and osk mRNA localization can be separated. Chc-coated vesicles also form at the trans-Golgi network (TGN). Interestingly, non-centrosomal MTs nucleating at the Golgi have been found to be indispensable for establishing proper cell polarity (Ori-McKenney et al., 2012; Zhu and Kaverina, 2013). Further studies should therefore address whether Chc organizes oocyte MT polarity by nucleating MT at the TGN.

Model for Bic-D and Chc functions in establishing pole plasm and anterior-posterior (A-P) axis
Incorporating our results into a model, we show how endocytosis, pole plasm assembly and anchoring of the pole plasm are tightly linked processes involving feedback loop regulation (Fig. 9). Bic-D contributes to osk mRNA localization and pole plasm assembly in two ways. First, from earlier studies we learned that the dynein adaptor Bic-D acts directly by transporting osk mRNA from the nurse cells into the oocyte (Suter and Steward, 1991; Swan and Suter, 1996; Clark et al., 2007). Here, we found that Bic-D controls the endocytic pathway also by transporting Chc mRNA into the oocyte and by localizing Chc protein to the oocyte cortex and enriching it at the posterior cortex. Thus, through these mechanisms, Bic-D promotes endocytosis at the posterior, which then stimulates pole plasm anchoring and osk mRNA localization.
localization at the posterior. Chc is also needed for establishing the MT cytoskeleton during the middle stages of oogenesis and it may be needed for the maintenance of this A-P polarity. In summary, we have found a novel and central role for Chc and Bic-D proteins in controlling pole plasm assembly by affecting MT polarity, endocytosis and osk mRNA localization.

MATERIALS AND METHODS

**Drosophila stocks**

Gal4 lines, Chc and Chc stocks were obtained from the Bloomington Drosophila Stock Center. The transgenic genomic rescue constructs pCHC3w and 4C-CHC [FLAG-TetraCysteine tag (4C)] were from C. Bazinet and P. Verstreken (Bazinet et al., 1993; Kasprowicz et al., 2008), and UAS-Chc::eGFP. Tub-Egl::eGFP flies were from S. Bullock (Dienstbier et al., 2009; Li et al., 2010).

Bic-D71.34 and Bic-DIIIE48, the two dominant alleles, the null allele Bic-Dr5 and Bic-Dmom were described previously (Suter et al., 1989; Ran et al., 1994; Swan and Suter, 1996) except that Df(2L)Exel7068 (Exelixis) was used as Bic-D deficiency. ChcGF23 (from T. Schüpbach) (Yan et al., 2009), Rab52 (Lu and Bilder, 2005) and Rbsn-5C241 (from A. Nakamura) (Tanaka and Nakamura, 2008) were used to generate mutant germ line clones with hsFLP; Ubi-GFP (S65T)nls FRT40A, His2Av-GFP, hsFLP, FRT19A/FM7a or Ubi-GFP, hsFLP, FRT19A/FM7. Clones were induced twice per day at 37°C for 2 h for 2 consecutive days. Flies were dissected 2-4 days after hatching. The Chc4 chromosome from the Bloomington Drosophila Stock Center was cleaned up by recombination with the multiply marked X chromosome cv m w y sd os, resulting in a lethal-free Chc4 chromosome marked with sd. Production of homozygous Chc4 females was described previously (Bazinet et al., 1993).

pUASP-Chc-V5-K10-attB and pUASP-myc-chc-K10-attB transgenic flies were generated with the phiC31 integrase transgenesis system and the yw;+; attP-64A line as host (Bischof et al., 2007; Koch et al., 2009).

To express UAS-Chc::eGFP in the Bic-Dmom mutant background, the P[mata4-GAL-VP16] driver was recombined with the Bic-D deficiency Df(2L)Exel7068. Flies of the genotype Df(2L)Exel7068, P[mata4-GAL-VP16]/SM1; hs-Bic/D/TM3Sb were crossed to BicDr5/SM1; UAS-Chc::eGFP/TM3Sb flies and the progeny of this cross was heat shocked twice per day (37°C, 2 h) from L3 till at least day two of adulthood, before shutting off Bic-D expression. Otherwise, flies were kept at 25°C.

**DNA constructs**

To construct pUASP-myc-chc-K10-attB and pUASP-Chc-V5-K10-attB, the Chc open reading frame was amplified from LD43101 (BDGP) using primers with XhoI sites for cloning in-frame into pUASP-myc-K10-attB and pUASP-V5-HisA (Life Technologies), respectively. From the
resulting pMT-Chc-V5 template, the Chc-V5 region was amplified with primers containing NotI and KpnI sites to facilitate cloning into the pUASP-K10-attB vector.

**Immunoprecipitations and western blots**

Immunoprecipitations (IPs) were performed according to Vazquez-Pianzola et al. (Vazquez-Pianzola et al., 2011) with embryonic extracts from 0- to 8-hour-old translin<sup>null</sup> mutant embryos and from same age embryos laid by Null-o-Gal4/SM1; pUASP-myc-Chc-K10 attB/TM3Sb females. For IPs, 70 pairs of ovaries were used per ovarian IP, whereas seven pairs were collected for one western blot lane. These were boiled for 2 min in 20 µl of SDS buffer, vortexed for 15 s, boiled for another 8 min and loaded on a SDS-PAGE gel. Western blots were performed using the mouse monoclonal anti-myc 9E10 [1:5, Developmental Studies Hybridoma Bank (DSHB)], SDS-PAGE gel. Western blots were performed using the mouse monoclonal anti-Flag (1:200, Sigma, F3165), mouse anti-α-Tubulin (1B11 plus 4C2, 1:10) (Suter and Steward, 1991), rat anti-Chc (1:35) (Wingen et al., 2009) or rabbit anti-Chc (1:200) (Kametaka et al., 2010), rabbit anti-Clc (1:500) (Heerssen et al., 2008). Both anti-Flag antibodies stained the oocyte nucleus in wild-type ovaries. To reduce this background cross-reactivity, antibodies were preadsorbed with 1 mg/ml bovine serum albumin and protase inhibitors (Roche). The following were added to the hypotonic buffer: 1 mM DTT, 0.5% Tween-20, protease and RNase inhibitors, 100 U/ml (Biolabs). The wash buffer contained 200 mM KCl. For each IP, 40 µl of protein G sepharose beads (Roche) and 1 ml of monoclonal anti-GFP were used (a gift from A. Marcil). Coated beads were washed with non-hypotonic buffer (hypotonic buffer adjusted to 150 mM KCl and 20% glycerol). For extract preparation, 1 g of 0- to 8-hour-old embryos were homogenized in 1 ml hypotonic buffer. The extract was cleared by 20 min with 2.5 µg/ml Hoechst 33258 and 1 unit/ml rhodamine-conjugated phalloidin (Molecular Probes), respectively, during the final washing steps. Imaging was with a Leica TCS-SP2 confocal microscope. Mouse anti-α-tubulin DM1A staining of ovaries is described by Sato et al. (Sato et al., 2011) and EB1 staining by Sanghavi et al. (Sanghavi et al., 2012).

**In situ hybridization to whole-mount ovaries**

Digoxigenin-labeled RNA probes were synthesized from linearized pBS-oskar, ESTs LD32255 and LD43101 (BDGP), pBS-OrbE4 cDNA (Lantz et al., 1992) and pBS-Bic-D. In situ hybridizations experiments (Vazquez-Pianzola et al., 2011) utilized 5% milk for blocking and as antibody dilution buffer. For dual detection of mRNA and GFP signal, the proteinase K step was omitted. Rabbit anti-GFP (1:300, AMS Biotechnology, 210-PS-1GF) was incubated overnight together with sheep anti-digoxigenin antibodies (Roche, 1 333 089). Donkey anti-sheep A647 (1:200, Jackson ImmunoResearch, 713-606-147) and donkey anti-rabbit A568 (1:1500, Invitrogen, A10042) secondary antibodies were incubated overnight.

**Immunostaining**

Whole-mount immunostainings were performed with mouse anti-Bic-D (1B11 plus 4C2, 1:10) (Suter and Steward, 1991), rat anti-α-Chc (1:35) (Wingen et al., 2009) or rabbit anti-α-Chc (1:200) (Kametaka et al., 2010), rabbit anti-α-Osk (1:500) (Markussen et al., 1995), rabbit anti-α-Egl (1:500) (Mach and Lehmann, 1997), rabbit anti-Chc (1:200, Sigma, F3165), rabbit anti-Flag (1:200, Sigma, F7425), rabbit anti-Rab11 (1:8000) (Tanaka and Nakamura, 2008), rabbit anti-Rbsn-5 (1:5000) (Tanaka and Nakamura, 2008), rabbit anti-Ark (1:150, Cytokeleton), mouse anti-α-tubulin DM1A (1:500, Sigma, T9026), rabbit anti-EB1 (1:500) (Rogers et al., 2002) and rabbit anti-α-Cdc (1:500) (Heerssen et al., 2008). Both anti-Flag antibodies stained the oocyte nucleus in wild-type ovaries. To reduce this background cross-reactivity, antibodies were preadsorbed on wild-type embryos. Secondary antibodies were Cy3-conjugated anti-mouse (712-165-150), Cy5-conjugated anti-rabbit (111-175-144), Cy5-conjugated anti-mouse (115-175-146) (Jackson ImmunoResearch) and A488-conjugated goat anti-rabbit (A11008, Molecular Probes). Nuclei and F-actin were stained with 20 min with 2.5 µg/ml Hoechst 33258 and 1 unit/ml rhodamine-conjugated phalloidin (Molecular Probes), respectively, during the final washing steps. Imaging was with a Leica TCS-SP2 confocal microscope. Mouse anti-α-tubulin DM1A staining of ovaries is described by Sato et al. (Sato et al., 2011) and EB1 staining by Sanghavi et al. (Sanghavi et al., 2012).

**RT-qPCR assays and RNA immunoprecipitation**

RNA from five to ten ovaries was extracted using the RNeasy Kit (Qiagen), DNasel digestion was performed according to the manual. RNA IPs were performed essentially as described (Easow et al., 2007) with the following modifications. Blocking buffer was supplemented with 1 mg/ml bovine serum albumin and protease inhibitors (Roche). The following were added to the hypotonic buffer: 1 mM DTT, 0.5% Tween-20, protease and RNase inhibitors, 100 U/ml (Biolabs). The wash buffer contained 200 mM KCl. For each IP, 40 µl of protein G sepharose beads (Roche) and 1 ml of monoclonal anti-GFP were used (a gift from A. Marcil). Coated beads were washed with non-hypotonic buffer (hypotonic buffer adjusted to 150 mM KCl and 20% glycerol). For extract preparation, 1 g of 0- to 8-hour-old embryos were homogenized in 1 ml hypotonic buffer. The extract was cleared at 10,000× g for 20 min. For each IP, 600 µl of cleared supernatant was adjusted to 20% glycerol and 150 mM KCl, and supplemented with 2 µl of RNase-free DNase.
I (20 U/ml Roche). IP was performed overnight. The immunoprecipitate was washed eight times. Beads were Protease K treated, RNA extracted using Trizol (Invitrogen) and resuspended in 25 μl DEPC-treated water, ready for RT-qPCR analysis.

cDNAs were synthesized with the SuperScript II Reverse Transcriptase and oligo dT primers. Primers used for the RT-qPCR analysis amplified fragments between 100 and 150 bp and one primer of a pair spanned an exon junction. The Quant iTect SYBR Green PCR Kit (Qiagen) and a Rotor Gene (Qiagen) were used, and the Pfaff method was applied to normalize to Tubulin67C mRNA levels and to calculate fold changes (Pfaffl, 2001).

Endocytosis assay and live imaging

For FM4-64 (Molecular Probes) incorporation assays, see Sommer et al. (Sommer et al., 2005). For live imaging of egg chambers, five to seven females were raised with five to seven males on freshly prepared fly food supplemented with fresh yeast paste. For BicD

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