centrosomin’s beautiful sister (cbs) encodes a GRIP-domain protein that marks Golgi inheritance and functions in the centrosome cycle in Drosophila

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Summary
The mechanism of inheritance of the Golgi complex is an important problem in cell biology. In this study, we examine the localization and function of a Golgi protein encoded by centrosomin’s beautiful sister (cbs) during cleavage in Drosophila melanogaster. Cbs contains a GRIP domain that is 57% identical to vertebrate Golgin-97. Cbs undergoes a dramatic relocalization during mitosis from the cytoplasm to an association with chromosomes from late prometaphase to early telophase, by a transport mechanism that requires the GRIP domain and Arl1, the product of the Arf72A locus. Additionally, Cbs remains independent of the endoplasmic reticulum throughout cleavage. The use of RNAi, Arf72A mutant analysis and ectopic expression of the GRIP domain, shows that cycling of Cbs during mitosis is required for the centrosome cycle.

Introduction
The eukaryotic Golgi complex is an essential organelle involved in many cellular processes, including lipid biosynthesis, protein modification and intracellular membrane trafficking. A major question and area of debate in cell biology is how the Golgi complex is inherited by daughter cells during mitosis. This has led to two different mechanistic models. One model proposes that the Golgi is a derivative of the endoplasmic reticulum (ER) and arises de novo from the ER during late telophase, whereas the other model proposes the Golgi complex is a unique organelle that arises by a template-based mechanism, requiring existing Golgi subunits for reassembly after mitosis (Barr, 2002; Barr, 2004; Glick and Malhotra, 1998; Rossanese and Glick, 2001; Roth, 1999; Shorter and Warren, 2002).

In vertebrate cells, it is generally agreed that, during mitosis the Golgi breaks down into individual stacks at prophase (Lucocq and Warren, 1987), which then fragment into many mitotic Golgi vesicle clusters by prometaphase (Shima et al., 1997). Do clusters merge with the ER at mitosis and re-emerge at telophase to self-assemble the Golgi, or do they remain intact and independent of the ER during mitosis, acting as a template to reform the Golgi after mitosis? Persuasive evidence exists that mitotic clusters remain independent of the ER throughout mitosis (Jesch and Linstedt, 1998; Jesch et al., 2001; Jokitalo et al., 2001; Seemann et al., 2002; Shima et al., 1998; Shima et al., 1997), and that clusters reorganize during metaphase to associate with the mitotic spindle, centrosomes and metaphase plate during mitosis (Jesch et al., 2001; Jokitalo et al., 2001; Shima et al., 1998). It has also been shown that, if the Golgi is removed from cytoplasts, the Golgi complex does not form de novo from the ER (Pelletier et al., 2000). Additionally, the initiation of Golgi fragmentation and dispersal, prior to cluster formation, is required for cells to enter mitosis and may act as a Golgi-damage sensor regulating the cell cycle, which would serve to link the Golgi cycle to the centrosome cycle (Sutterlin et al., 2002).

In Drosophila melanogaster the morphology and mitotic behavior of the Golgi is variable, based on cis-Golgi proteins and depending on the tissue type investigated. During syncytial embryogenesis maternally supplied Golgi membranes are concentrated at the embryonic cortex as several thousand punctate structures that remain unchanged during the syncytial nuclear divisions (Frescas et al., 2006; Ripoche et al., 1994; Stanley et al., 1997). The ER is present at the embryonic cortex and as an interconnected membrane network throughout the cytoplasm of the oocyte at fertilization (Bobinnec et al., 2003). Additionally, the cortical ER and Golgi are present as a continuous network in syncytial preblastoderm embryos, which fragments into individual units associated with nuclei following nuclear migration to the cortex in syncytial blastoderm embryos (Frescas et al., 2006). In cellular embryos in Drosophila tissue culture cells the interphase Golgi exists as discrete units throughout the cytoplasm that break...
down and disperse during mitosis (Stanley et al., 1997). Finally, in imaginal disc cells during pupariation, the Golgi is present as multiple stacked cisternae (Rabouille et al., 1999), which form from pre-existing Golgi units (Kondyli et al., 2001); and these cisternae have polarity, suggesting that the Drosophila Golgi has distinct functional compartments (Yano et al., 2005).

Several peripheral membrane proteins called golgins have been identified that are involved in formation of the Golgi matrix and that link the Golgi to the cytoskeleton. These proteins function within specific domains of the Golgi, have a coiled-coil structure, and are regulated by small GTPases (Barr and Short, 2003). Several cis-Golgi matrix golgins can reform a structural Golgi skeleton, in the absence of ER-derived Golgi membranes, suggesting that the matrix represents a template that gives the Golgi an autonomous identity (Seemann et al., 2000). Mitotic Golgi-vesicle clusters are enriched for matrix golgins and these vesicles maintain a cis to trans polarity throughout mitosis (Shima et al., 1997). This has led to the proposal that these vesicles are required for inheritance of the Golgi during mitosis (Seemann et al., 2002). In vertebrates, the golgins are clearly important for normal Golgi structure and regulation of the cell cycle. In Drosophila several cis-Golgi golgins have been investigated, but very little is known about the trans Golgi and the proteins that are specific to this compartment.

A subset of peripheral membrane golgins contain a 42-amino-acid, functionally conserved Golgi-localization GRIP domain, which acts to localize vesicles to the trans Golgi (Barr, 1999; Kjer-Nielsen et al., 1999). Subsequent work has shown that this functional domain is highly conserved across the eukaryotes (Gilson et al., 2004; Luke et al., 2003; McConville et al., 2002). The function of GRIP proteins is still poorly understood, but ectopic expression of either the human Golgin-245 or Golgin-97 GRIP domains in HeLa cells leads to the displacement of endogenous GRIP proteins and mislocalization of other trans-Golgi proteins. This expression was also associated with a disruption of the trans-Golgi structure and interference with vesicle trafficking (Yoshino et al., 2003). The expression of either the Golgin-97 or Golgin-245 GRIP domains causes similar defects in Golgi structure, suggesting that the GRIP domains from both proteins share a common binding partner. However, both endogenous proteins form homodimers and thus potentially have independent functions (Luke et al., 2005). These findings make the GRIP domain a reliable marker of the trans Golgi in eukaryotes.

Most GRIP proteins are regulated by the ADP-ribosylation-factor-like (ARL) GTPases (Barr and Short, 2003). The GTP-bound form of Arl1 GTPase is required for recruitment of the GRIP domain to the trans Golgi in yeast (Behnia et al., 2004; Panic et al., 2003b) and vertebrates (Lu and Hong, 2003). The Arfl2A gene, which encodes Arl1 in Drosophila has been identified and an embryonic recessive lethal allele has been recovered (Tamkun et al., 1991). Unfortunately, the mutant phenotype has not been investigated. Although Arl1 interacts with other, non-GRIP-containing proteins (Panic et al., 2003b), its interaction with most eukaryotic GRIP domains appears to be highly conserved.

The Golgin-97 GRIP data discussed above suggests a function in maintaining Golgi structure, possibly as a component of the trans-Golgi matrix. Injection of anti-Golgin-97 antibody into cells causes the fragmentation of the entire Golgi complex, supporting a matrix function required for the global organization of this organelle (Lu et al., 2004). Interestingly, Golgin-97 also localizes to the centrosome throughout the cell cycle and it may act as a Golgi-nucleating protein at the centrosome, analogous to γ-tubulin nucleation of microtubules (Takatsuki et al., 2002). Golgin-97 is emerging as an important component of the trans Golgi in vertebrates, but has yet to be characterized in any other organism.

Here, we report the initial functional analysis of the maternal products of the Drosophila gene centrosomin’s beautiful sister (cbs), during early embryogenesis. cbs encodes three isoforms, including a functional homolog of vertebrate Golgin-97. During mitosis Cbs protein undergoes a dramatic subcellular relocation, similar to the relocation described for the vertebrate Golgi matrix during mitosis. We show that Cbs and the GRIP domain are required for maintenance of the Drosophila trans-Golgi complex during embryogenesis, and for normal centrosome maturation during the cell cycle. Our results suggest that several characteristics of the Drosophila trans Golgi during the cell cycle are similar to vertebrates, making this a useful system for investigating the compartmentalization and inheritance of this complex organelle.

Results

**cbs encodes two golgin isoforms**

We identified cbs (CG4840) in our studies of centrosomin (cnn) (Heuer et al., 1995). The cbs locus lies adjacent to cnn in the 50A region of the right arm of the second chromosome in Drosophila. Our initial characterization included screening of cDNA libraries and expressed-sequence-tag (EST) collections to produce transcript profiles for both genes, and saturation mutagenesis screens to recover recessive lethal and sterile mutations. We recovered multiple mutations in cnn and other genes in this region (our unpublished data), but failed to recover any mutations in cbs.

The cbs gene is approximately 3 kb long and contains two transcription start sites located 134 and 278 base pairs distal to the most distal cnn transcription start site. The gene encodes at least five transcripts, which translate into a total of three different acidic protein isoforms (Fig. 1A). The two larger isoforms differ by the presence or absence of a functional GRIP domain and are similar to human Golgin-97. The longest isoform of Cbs is 611 amino acids long and has a predicted molecular mass of 70.8 kDa. Although the overall sequence conservation between Cbs and Golgin-97 is moderate this isoform contains a 42-amino-acid GRIP domain located near the C-terminus (aa 551-593) that is 57% identical to the GRIP domain in human Golgin-97 (Fig. 1B). The alternatively spliced long Cbs isoform is missing amino acids 558 to 567, which eliminates the GRIP motif. The third short Cbs isoform is 367 amino acids long, has a predicted molecular mass of 42.7 kDa and is also similar to several golgins.

The predicted secondary structure of Cbs is predominately coiled-coil, with short disordered regions that interrupt the coils (Fig. 1C), similar to the structure of all known golgins (Barr and Short, 2003). There are also nine putative phosphorylation sites, consistent with the finding that several golgins undergo post-translational regulation (Dirac-Svejstrup et al., 2000; Preisinger and Barr, 2001) and three putative myristylation
Cbs undergoes a dramatic reorganization and associates with DNA during mitosis

To characterize the wild-type pattern of Cbs accumulation during early embryogenesis, we coinmunistained for Cbs with various combinations of antibodies against: Cnn, a core component of the centrosome (Megraw et al., 1999), Lava Lamp (Lva), a cis-Golgi protein required during cellularization (Sisson et al., 2000) and microtubules (α-tubulin), as well as for DNA and PDI::GFP, a fusion protein that marks the ER (Bobinnec et al., 2003). We followed Cbs throughout mitosis during four stages of embryogenesis: preblastoderm (cycles 1-9), syncytial blastoderm (cycles 10-13), cellularization, and early gastrulation (Foe et al., 1993). In addition to the pattern of Cbs described below, there is a pool of Cbs particles at the embryonic cortex that becomes gradually depleted during syncytial development.

During the early preblastoderm divisions (cycles 1-7), Cbs is difficult to detect in the cytoplasm at prophase. However, by prometaphase Cbs is localized to chromatin, where it remains until early telophase. At this point Cbs appears to fragment into small particles and is undetectable by the completion of mitosis (data not shown). The association of Cbs with chromosomes from metaphase throughout anaphase is characteristic of all four stages of development studied.

During late preblastoderm and syncytial blastoderm stages, the prophase and telophase pattern of Cbs becomes more complex. As chromosomes begin to condense during early prophase Cbs particles begin to form in a haze around nuclei (Fig. 2A-D). During late prophase, cytoplasmic Cbs staining becomes more particulate and Cbs begins to accumulate within the nuclei during early chromosome congression (Fig. 2E-H and supplementary material Fig. S1). As spindles elongate during prometaphase Cbs is localized to the spindles and chromosomes (Fig. 2I-L), and is present only at chromosomes sites, necessary for post-translational modifications associated with protein-membrane interactions (Fig. 1C).

On a western blot of 0-hours- to 2-hours-old embryos, we detect a strong Cbs band that runs just below the 77 kDa marker and a relatively weaker band that runs below the 50 kDa band of /H9251-tubulin 84B, but well above the 34 kDa marker. The apparent molecular mass of both bands is in close agreement with the predicted sizes for the three Cbs isoforms, although both bands run slightly higher than expected. On the basis of our western blot data and the isolation of both long isoforms from embryonic cDNA libraries, all three isoforms are present in early Drosophila embryos (Fig. 1D). Taken together these data suggest that Cbs produces three, potentially functionally distinct, golgin-like peripheral membrane proteins of the Drosophila trans Golgi.

Cbs in Golgi function and inheritance

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by late metaphase (Fig. 2M-P), maintaining this association until late anaphase B (Fig. 2Q-T). By late telophase, as mid-bodies begin to break down, Cbs staining appears to fragment into smaller particles (Fig. 2U-X). These Cbs particles appear to completely break down by the completion of telophase, again forming a dense cytoplasmic haze around nuclei.

To our knowledge, the association of Golgi proteins with chromosomes during mitosis has not been reported in *Drosophila*, suggesting that preparative conditions may be important for their detection. Our primary fixation method has been optimized for the preservation of syncytial microtubule structures, so we tested two different formaldehyde preparations with and without a Taxol pre-treatment to preserve microtubules (see Materials and Methods). Under these latter conditions, in the absence of Taxol, the early prophase pattern of Cbs is the same (Fig. 2A-H), but the association of Cbs with chromosomes is barely detectable or even absent. However, when embryos were treated with Taxol prior to formaldehyde fixation Cbs association with chromosomes is readily detectable from metaphase to early telophase, although immunostaining intensity is lower than what we observe with our initial preparative method. Thus, it appears that microtubule stability is required for the detection of Cbs on chromosomes, whereas the early prophase changes

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**Fig. 2.** Cbs cycles from the cytoplasm to DNA during mitosis. Mitotic series (PDI::GFP stock) immunostained for GFP to mark the ER lumen (green), Cbs (red), and stained for either Cnn, microtubules or DNA TOTO-3 (blue) during mitosis in syncytial embryos. From early prophase (A-D) to late prophase (E-H), Cbs rapidly changes from a diffuse cytoplasmic haze, to small particles that accumulate near centrosomes and begin to migrate into the nucleus. During prometaphase Cbs is present in elongating spindles and at chromatin (I-L), but is present only at chromatin by late metaphase (M-P). The ER forms an envelope around the spindle during these stages, but is absent from the spindle proper. Throughout anaphase (Q-T) Cbs remains associated with DNA, although, as the ER begins to disperse, small cytoplasmic Cbs particles are present. At late telophase (U-X) Cbs moves towards centrosomes and astral microtubules, and rapidly fragments into small particles that disperse into the cytoplasm. The ER is present throughout the cytoplasm and at the spindle mid-bodies during telophase, but does not colocalize with Cbs. Bar, 20 μM.
in the localization of Cbs from the cytoplasm to the nucleus is less sensitive to the preservation of microtubules. The differential detection of Cbs during these two stages of Cbs reorganization during mitosis suggests that there are different mechanisms required for these processes.

To quantify Cbs and Cnn immunostaining during these stages of development we analyzed the relative fluorescence intensity of protein around nuclei at different stages of mitosis. Fluorescent intensity (FI) of Cbs is highest during prophase, with considerable variation between individual nuclei. The FI and variation decreases approximately 50% during metaphase and begins to increase during anaphase B and telophase (Fig. 3 and supplementary material Table S1). The only significant differences in FI values during cycles 8-13 are 30% reductions in Cbs intensity during cycle 13 prophase and during cycle 12 and 13 metaphase. The FI values for Cnn remain relatively constant in wild-type embryos, with minimal variation among nuclei, throughout mitosis during syncytial development.

During the cellularization stage, as nuclei enter the first prolonged interphase in Drosophila development, centrosomes replicate and move 2-4 μm apart along the apical surface of nuclei, where they remain throughout cellularization of the blastoderm (Callaini and Anselmi, 1988; Foe et al., 1993). The accumulation pattern of Cbs displays two separate components during this stage of development. Immediately after centrosome replication, Cbs particles form a concentrated region of staining that fills the gap between the centrosomes and extends partway into the region between the centrosomes and nucleus (Fig. 4A-D). During formation of this region of Cbs concentration, we detect weak colocalization with Cnn within replicated centrosomes (arrowheads, Fig. 4B-D). As cellularization continues, Cbs staining is also present as a cytoplasmic haze surrounding the entire lateral surface of each columnar nucleus (Fig. 5F,G), and there is a fourfold increase in the Cbs FI level. As is the case for Cbs association with the mitotic chromosomes, the outer portion of the Cbs pericentriolar concentrated region and the lateral domain appears to be microtubule-dependent because it is best preserved with the same preparative methods. The concentrated pericentriolar region and cytoplasmic haze of Cbs staining persists throughout most of cellularization, but recedes to just the pericentriolar region prior to cytokinesis.

During gastrulation, the pattern of Cbs in mitotic domains is the same as that described for late syncytial nuclear cycles. In interphase cells during very early gastrulation, and in cells along segments and embryonic furrows, Cbs forms a dense perinuclear structure and a cytoplasmic haze that is closely apposed to the nuclei (Fig. 4E-H). However, by late gastrulation and throughout the rest of embryogenesis, most interphase cells have multiple Cbs foci of variable size that are surrounded by a weak cytoplasmic haze (Fig. 4I-L).

In general, the amount of detectable cytoplasmic Cbs during late telophase and early prophase increases between the preblastoderm and syncytial blastoderm nuclear cycles. During all stages of development, Cbs FI cycles from high levels at prophase to reduced levels during metaphase and anaphase when Cbs is associated with chromosomes. The association of Cbs with chromosomes during mitosis and the later expansion of Cbs during cellularization appear to be microtubule-dependent. Throughout cellularization and early gastrulation, Cbs remains concentrated between centrosomes on the apical surface of nuclei. This region of Cbs fragments and disperses throughout the cytoplasm during late gastrulation. We also observe a persistent cytoplasmic pool of Cbs throughout mitosis during syncytial blastoderm cycles, which is undetectable at mitosis during the preblastoderm divisions.

Cbs partially colocalizes with Lva during syncytial mitosis and at cellularization

All known GRIP domain proteins are found at the trans Golgi, and are implicated in vesicle tethering and transport, strongly supporting a role for Cbs at the trans Golgi in Drosophila. Of the known Drosophila Golgi proteins, Lava Lamp (Lva) seemed a likely candidate to interact with Cbs. Lva is a golgin-like Golgi protein that interacts with actin, microtubules, CLIP190, Spectrin complexes and the cis-Golgi protein p120. It is also required for dynein-dependent membrane vesicle transport from the Golgi to the growing furrow front during cellularization (Papoulas et al., 2005; Sisson et al., 2000). Although the localization of Lva is complex during cellularization, a fraction of Lva maintains a pericentriolar location throughout the process, similar to the pericentriolar Cbs staining described above.

During the preblastoderm and syncytial blastoderm stages, in addition to the cytoplasmic pool of Lva, we find that Lva colocalizes with
chromosome-associated Cbs transiently during early chromosome congression (Fig. 5A-D and supplementary material Fig. S2). This association is no longer detectable by the time chromosomes are tightly aligned along the metaphase plate. Additionally, we only observe this colocalization when microtubules are rapidly fixed (Fig. 5A,B) or stabilized with Taxol prior to formaldehyde fixation (Fig. 5C,D), and the FI is strongest in rapidly fixed embryos.

At the start of cellularization we detect strong colocalization between Cbs and Lva at the pericentriolar region of spherical nuclei (Fig. 5E,F), and this association persists throughout most of cellularization. As cellularization continues, a second population of Lva vesicles that is basal to the nuclei moves to the pericentriolar region by a microtubule-dependent mechanism (Sisson et al., 2000), concomitant with the formation of the cytoplasmic haze of Cbs along the lateral surface of nuclei described above (Fig. 5G,H). Throughout this process Lva FI levels increase in a similar manner to Cbs,
although Lva intensity is significantly less than Cbs. Small puncta of Cbs and Lva colocalization are present throughout this lateral domain but we did not observe the larger, basal and apical Lva particles in this region (Fig. 5G). Cbs and Lva do not colocalize at growing membrane furrows above nuclei, and the only Cbs present above the pericentriolar region are scattered cortical particles.

On the basis of the previously described dynamic behavior of Lva during cellularization, the known interactions between Lva and other cis-Golgi proteins, and the strong colocalization with Cbs at the pericentriolar region, we propose that Lva moves from the cis Golgi to the trans Golgi where it associates with Cbs before it is transported to the growing-furrow fronts. Additionally, Cbs and Lva colocalize transiently during chromosome congression prior to formation of a compact metaphase plate, which may be important for Golgi maintenance.

Cbs does not colocalize with the ER during syncytial divisions and cellular mitosis
The relationship between the ER and the Golgi complex during mitosis is a key issue in the debate over the mechanism of Golgi inheritance in animal cells. We used a transgenic fly line that expresses PDI::GFP under control of the endogenous PDI promoter to mark the ER lumen throughout the cell cycle (Bobinnec et al., 2003) and to determine whether Cbs is present within the ER lumen during mitosis. It is important to note that the immunostaining pattern for PDI::GFP in embryos fixed within the ER lumen during mitosis. It is important to note that the immunostaining pattern for PDI::GFP in embryos fixed with our initial preparative method is similar to the published live pattern for this ER marker.

During synaptotagmin blastoderm and cellular stages, the ER and Cbs are present together throughout the cytoplasm during early prophase, but there is no evidence for colocalization of the two proteins. The ER begins to condense around nuclei during late prophase, concomitant with the movement of Cbs from the cytoplasm to the interior of the nuclei (Fig. 2E,F,H). Throughout metaphase and anaphase, the ER forms an envelope around the mitotic spindle (Fig. 2I,J,L), but is excluded from the spindle proper and the regions of Cbs localization. ER dispersal to the cytoplasm begins during early telophase, and the ER is prophase-like by the time Cbs fragmentation begins at late telophase (Fig. 2U,V,X).

During cellularization and early gastrulation, the ER accumulates above the centrosomes, and we detect weak colocalization between ER projections and the closely apposed pericentriolar Cbs region (Fig. 4D,H). This is consistent with EM data from rat kidney cells, where it has been proposed that the direct transfer of lipids from the trans ER to the trans Golgi, rather than the typical transport through the Golgi, provides a rapid mechanism to generate new plasma membrane (Ladinsky et al., 1999).

From these data and the original report on the PDI::GFP marker (Bobinnec et al., 2003) we conclude that, Cbs is not present in the lumen of the ER throughout mitosis in Drosophila and is actively inherited by daughter cells via a mechanism independent of the ER.

Reduced levels of Cbs cause defects in centrosome maturation and replication
We wanted to know what effect the loss of Cbs has on early embryogenesis to ascertain the cellular function of Cbs. Because of the lack of any known mutations in Cbs, we used the Gal4/UAS-inducible expression system to target all possible cbs transcripts for RNA interference (RNAi)-induced degradation by using the Symp-UAST RNAi vector (Giordano et al., 2002). Additionally, we raised flies at different temperatures to regulate GAL4-induced transcription (see Materials and Methods). The majority of embryos produced by females raised at 18°C develop normally and there is no apparent sterility in the nanos--cbs females. Approximately 25% of the embryos produced by females that were kept at 25°C and 83% of the embryos produced by females that were kept at 29°C had severe defects (Tables 1 and 2). Despite these differences, we see a similar range of protein depletion that varies from a moderate depletion to an apparent complete loss of Cbs in embryos, as evidenced by significantly reduced FI

| Table 1. Metaphase phenotype classification for cbs RNAi |
|---------------------------------|----------------|----------------|----------------|---------------- |
| Phenotype class                   | Asymmetric centrosomes | Single centrosome | Collapsed spindles | Acentrosomal spindles |
| Moderate, 146 nuclei              | <=50%                  | <=10%              | <=15%             | <=5%         |
| Severe, 170 nuclei                | >25%                   | >30%                | >15%              | >10%         |

Characterization of nuclei from five embryos with moderate defects and five embryos with severe defects.

| Table 2. Percent of embryos with developmental defects under different conditions |
|---------------------------------|----------------|----------------|----------------|---------------- |
| Genotype                        | Moderate defects in syncytial embryos | Severe defects in syncytial embryos | Severe defects in cellular embryos | Wild-type embryos |
| cbs RNAi at 18°C, n=53          | 5.7%                  | 0%                | 0%              | 94.3%         |
| cbs RNAi at 25°C, n=98          | 40.8%                 | 17.3%             | 7.1%             | 34.7%         |
| cbs RNAi at 29°C, n=127         | 13.4%                 | 68.5%             | 14.2%            | 3.9%          |
| Arf72A^1, n=78                  | (0)%                  | 37.2%             | 62.8%            | 0%           |

Cbs RNAi embryos were scored based on Table 1. Arf72A mutant embryos were scored based on Table 1 and overall morphology. GFP::GRIP embryos were scored based on centrosome number per nucleus and morphology, as well as overall morphology of the embryo.
values at 25°C and 29°C (supplementary material Table S1). A fraction of embryos fail during syncytial blastoderm and cellularization stages at both temperatures, but the frequency of failure is much higher in embryos produced by females raised at 29°C (Table 2). None of the temperatures investigated caused any obvious defects during the preblastoderm stage of embryogenesis. The phenotypes described below were not observed in control embryos.

During syncytial blastoderm stages, the severity of the defects in embryos correlates with the amount of reduction in FI levels for Cbs. Moderate phenotypes are present in embryos with at least half the wild-type FI levels of Cbs, and severe defects are associated with significant reductions in FI levels (Fig. 3). During prophase, moderate reductions in Cbs results in centrosomes that vary in size, and Cnn staining is less compact than that observed in wild-type centrosomes. Additionally, there frequently are acentrosomal nuclei present (Fig. 6A,B). In more severely depleted embryos, cytoplasmic Cbs-particle formation is barely detectable during prophase, Cnn staining at centrosomes is reduced, the frequency of acentrosomal nuclei increases and the nuclei appear to be degraded (Fig. 6C,D). During metaphase moderate depletion of Cbs results in aberrant spindle formation, which varies from short, broad spindles, to completely collapsed spindles (Fig. 6E). Aberrant spindles usually have asymmetric centrosomes based on Cnn staining, even when the detectable amount of Cbs at metaphase chromosomes appears to be normal (Fig. 6F). In syncytial blastoderm embryos that have no detectable Cbs staining, metaphase spindles typically have a single centrosome with Cnn staining, most likely due to defective spindle attachment (Fig. 6G,H), and these embryos probably die. In all the embryos with a significant reduction in the FI levels of Cbs, the FI levels of Cnn are also significantly reduced (supplementary material Table S1) as compared with wild-type embryos.

If Cbs is significantly depleted at the start of cellularization (IJ), centrosome maturation defects become more prevalent (arrowheads), and Cbs fails to form a dense pericentriolar structure, remaining as an amorphous haze above nuclei. As cellularization continues (KL) Cbs fails to move down over nuclei and many nuclei fall into the syncytium, creating large gaps in the embryonic cortex. Bars, 20 μM (A-D,IJ); 40 μM (E-H,K,L).

When Cbs is significantly depleted during metaphase (G,H) centrosome replication is blocked, resulting in monopolar spindles, although in some examples (arrows) the loss of centrosomes may be due to poor attachment to spindles. At the start of cellularization (IJ, centrosome replication defects become more prevalent (arrowheads), and Cbs fails to form a dense pericentriolar structure, remaining as an amorphous haze above nuclei. As cellularization continues (KL) Cbs fails to move down over nuclei and many nuclei fall into the syncytium, creating large gaps in the embryonic cortex. Bars, 20 μM (A-D,IJ); 40 μM (E-H,K,L).
this stage of development and zygotic gene expression could allow cells to recover.

On the basis of these RNAi results, we conclude that a significant pool of maternally loaded cbs transcript is necessary to perpetuate the normal cycling of Cbs during mitosis. The Cbs cycle is necessary for maintaining centrosome number and morphology, and for maintaining sufficient levels of Lva. Although significantly lower than wild-type levels of Cbs appear to be sufficient for development to proceed, when Cbs levels fall below a minimum value development fails. During syncytial development failure is due to centrosome defects, whereas failure at cellularization is minimally due to the combined loss of Cbs, Lva and defective centrosomes. We conclude that normal cycling and sufficient protein levels of Cbs are required during all stages of development following cortical migration in Drosophila.

Arf1 is required for Cbs localization to DNA during mitosis. Because the RNAi experiments potentially eliminate all isoforms of Cbs and our antibody does not distinguish between the three, we next wished to know the effects of the loss of GRIP-domain function on embryogenesis and Cbs behavior. It is known that the GTPase Arf1 interacts directly with the GRIP domains in the yeast Imh1p, human Golgin-245 and human Golgin-97 proteins, and is required for GRIP function (Hickson et al., 2003; Lu and Hong, 2003; Lu et al., 2004; Panic et al., 2003a; Panic et al., 2003b; Setty et al., 2003; Van Valkenburgh et al., 2001; Wu et al., 2004). To gain insight into the function of the Cbs GRIP isoform, we looked at Cbs and Cnn in an Arf72A1-mutant background during early embryogenesis, using the Arf72A1-mutant allele of the Drosophila Arf72A1-encoding ortholog (Tamkun et al., 1991). Arf72A1 is a recessive lethal allele, and mutant embryos fail at cellularization and early gastrulation (Table 2).

The mitotic localization pattern of Cbs is dramatically changed in an Arf72A1-mutant background. During early prophase, Cbs changes from a haze to larger particles, similar to wild type (Fig. 7A, compare with Fig. 2D), but particles do not concentrate around nuclei and the density of Cbs shows considerable variation across the embryo. Cbs particles persist in the cytoplasm throughout mitosis, occasionally forming large aggregates, but fail to localize with DNA from metaphase to telophase (Fig. 7B,C). These particles fail to completely fragment during telophase and appear to be degraded as development proceeds. Centrosome maturation proceeds normally in most embryos through the early syncytial blastoderm stage, but a low percentage of centrosomes are either small or fused (Fig. 7B arrowheads). During the late syncytial blastoderm stage, the number of centrosome and nuclear fusions increases, and the total amount of Cbs present decreases significantly (Fig. 7D).

Throughout late preblastoderm and syncytial blastoderm cycles the FI levels of Cbs are relatively constant and variation between nuclei remains high during all stages of mitosis (Fig. 3 and supplementary material Table S1). Although Cbs particles appear to be uniformly scattered throughout the embryo during these stages, Cnn FI values vary from approximately double normal levels to nearly zero (supplementary material Table S1) and Cnn immunostaining is highly variable among nuclei, in contrast to wild-type embryos.

During cellularization Cbs intensity is significantly less than wild type in Arf72A1 embryos and Cbs fails to concentrate in the pericentriolar region, remaining as a haze above the apical surface of nuclei (Fig. 7E). Additionally, Cbs is absent at the lateral surface of nuclei, and centrosomes vary in size between members of a pair and from cell to cell; however, they are larger than those found in RNAi embryos. In addition to loss of Cbs, Lva FI values are 80-85% lower in these embryos, and many nuclei drop away from the cortex (Fig. 7E), similar to the RNAi cellularization phenotype. Unlike the RNAi embryos, the alignment of the remaining nuclei at the cortex of Arf72A1 mutant embryos is almost normal, suggesting that centrosomes are partially functional, and that the loss of Lva and failure to cellularize is directly due to the loss of Arf1 function.

Approximately 63% of the embryos complete cellularization and initiate gastrulation prior to failure (Table 2), but furrow-and segment-formation is weak or incomplete, giving the cortex a smooth, flat appearance. Mitotic domains have fewer cells than in wild type and many spindles have a single centrosome (Fig. 7F arrowheads). In general, the density of nuclei is low in these embryos, many nuclei appear to be
aneuploid, and both Cbs and Lva are significantly reduced or absent in most cells.

The Arf72A mutant data shows that Arl1 is necessary for the transport of Cbs from the cytoplasm to the chromosomes, and that this process is required to maintain Cbs and Lva protein levels during syncytial development. The initial formation of Cbs particles during early prophase is normal, suggesting that this process involves one or both of the non-GRIP isoforms of Cbs. Additionally, because the transport of Cbs to the chromosomes appears to be microtubule-dependent, this suggests that Arl1 and the GRIP domain are required for localizing Cbs to microtubules. Although the loss of Arl1 function most probably affects other proteins, the fact that localization of Cnn to centrosomes is aberrant, and Lva is lost in both RNAi depletion and Arf72A-mutant embryos, suggests that the interaction between Arl1 and the GRIP domain is conserved in Drosophila, and Cbs is a target of Arf72A.

The GRIP domain is transported to DNA and causes centrosome hypertrophy

The GRIP domain has been shown to be a trans-Golgi localization signal in eukaryotes, and overexpression of this domain interferes with the function of endogenous GRIP proteins in a concentration-dependent manner. Based on the Arf72A-mutant data, ectopic expression of the GRIP domain during embryogenesis should interfere with Cbs transport during mitosis, causing cellularization and centrosome defects. To test the Cbs GRIP function in Drosophila during embryogenesis, we expressed a 45-amino-acid fragment that aligned with the Golgin-97 GRIP sequence at different temperatures (see Materials and Methods) to obtain a range of phenotypic responses to low and high levels of GFP::GRIP expression (Table 2).

The localization of the GFP::GRIP fusion protein during syncytial cycles is very similar to endogenous Cbs, although the early prophase cytoplasmic haze for the fusion peptide is weaker. During the early stages of centrosome replication, GFP::GRIP localizes to both centrosomes, but is significantly more concentrated at one centrosome (Fig. 8B). In 37% of the embryos assayed at 25°C, we observe excess Cnn at prophase centrosomes, based on immunostaining and FI values, leading to centrosome hypertrophy and asymmetric centrosome pairs (Fig. 8A,D and Table 2).

In an additional 15-28% of the embryos assayed, centrosome hypertrophy is associated with re-replication of individual centrosomes (Fig. 8E-H arrowheads and Table 2). We have observed up to seven centrosomes associated with a single nucleus, and frequently find enlarged centrosomes that appear to be in the process of separating again (Fig. 8E-H arrow). Whenever centrosome hypertrophy is severe, there are free cytoplasmic centrosomes and an increase in the number of nuclei that are acentrosomal or contain a single centrosome, suggesting that the fusion peptide also interferes with centrosome attachment to the early spindle during prophase.

During metaphase and anaphase the GRIP domain localizes strongly to centrosomes and spindle microtubules, but is nearly

Fig. 8. Ectopic expression of the GRIP domain causes centrosome hypertrophy. To investigate the function of the Cbs GRIP domain we expressed a GFP::GRIP fusion protein during embryogenesis under control of nanos::Gal4 and immunostained embryos for Cnn (green, A,E,I) or microtubules (green, M), GFP (red, B,F,J,N), and Cbs (blue, O) or DNA stained with TOTO-3 (blue, C,G,K). (A-D) During early prophase the GRIP domain initially localizes to one centrosome, resulting in an asymmetric accumulation of Cnn at replicated centrosomes (arrowheads). A cytoplasmic pool of the GRIP domain is also present around nuclei during prophase. (E-H) When the GRIP domain remains concentrated at centrosomes during prophase, individual centrosomes (arrowhead) and pairs of centrosomes (arrow), undergo additional rounds of replication and frequently become dissociated from nuclei. (I-P) During metaphase the GRIP domain accumulates as cytoplasmic particles and localizes strongly at centrosomes and the microtubule spindle, but does not cause further accumulation of Cnn at centrosomes or centrosome hypertrophy. Low levels of the GRIP domain are present at DNA (J,N), but the transport efficiency and DNA-binding affinity of endogenous Cbs appears higher (O). However, the GRIP domain does cause excessive accumulation of cytoplasmic Cbs particles that are not seen in wild-type embryos during mitosis. Bars, 20 μM (A-D, I-P); 5 μM (E-H).
absent from chromatin (Fig. 8I–L). Interestingly, high concentrations of the GRIP domain at metaphase and anaphase centrosomes do not lead to precocious replication, because we never see replicating centrosomes during these stages of mitosis.

The GRIP domain also interferes with the transport of native Cbs protein, because there is a significant increase in the number of cytoplasmic Cbs particles during metaphase, which are not present at this stage in wild-type embryos (Fig. 8M–P). Additionally, the FI values for Cbs at nuclei and spindles are lower during prophase and metaphase, suggesting that the GFP::GRIP domain interferes with proper localization of Cbs.

If centrosome hypertrophy is severe, embryos fail because of mitotic defects – including multipolar spindles and severe aneuploidy – prior to cellularization. However, presumably due to the inherent variability of the GAL4 system, we also see less severely affected animals, and these embryos could potentially complete cellularization and embryogenesis. Consistent with this possibility is the fact that even at 29°C nanos–GFP::GRIP mothers can produce viable progeny. Interestingly, in the defect-free animals the fusion protein does not persist in cellularized embryos, implying that the maternal supply of GFP::GRIP is degraded at this point in development.

From these findings and the Arf72A mutant data, we conclude that the GRIP domain is required for the transport of Cbs from the cytoplasm to the chromosomes, but that it is not the primary motif for chromosome or microtubule attachment. Additionally, the GFP::GRIP fusion peptide colocalizes with Cnn at the center of the centrosomes throughout mitosis, and interferes with the normal localization and function of Cnn. This association may be an exaggeration of the weak colocalization between Cnn and Cbs at centrosomes during early cellularization. Although the mechanism that links Cbs and Cnn transport to the centrosome remains unclear, it appears that, by blocking the efficient transit of Cbs to the chromosomes due to excess GFP::GRIP protein at centrosomes during prophase, the centrosome cycle can be uncoupled from the cell cycle.

**Discussion**

The complex structure of the vertebrate Golgi, its pericentriolar location in cells and the mechanism of inheritance for this organelle appears to be a unique characteristic of vertebrates. The *Drosophila* Golgi complex exists as many discrete stacks *Drosophila* during embryogenesis. The first potential interphase function of Cbs occurs during cellularization, when the approximately 6000 cortical nuclei cellularize simultaneously to form the cellular blastoderm (Foe et al., 1993). During cellularization, Cbs and Lva colocalize in the pericentriolar region, which we propose corresponds to the site of the *trans* Golgi in *Drosophila*. In addition to Lva, projections from the ER colocalize with the pericentriolar Cbs region during cellularization and gastrulation, possibly for the direct transfer of new membrane from the ER to the *trans* Golgi. Taken together these data suggest that, at developmental
stages that require large amounts of membrane production and transport, at least the trans Golgi may form a single, large continuous structure that maintains a pericentriolar location in cells and a close association with the ER, much like the vertebrate Golgi complex.

Our RNAi depletion, Arf72A1-mutant analysis and GFP::GRIP ectopic expression data provide insight into the function of Cbs and the GRIP domain during embryogenesis. The RNAi depletion of the maternal pool of cbs transcript causes a significant reduction in the protein levels of Cbs, Cnn and Lva during the syncytial cleavage divisions. Additionally, Cnn fails to properly localize to mitotic centrosomes. If the loss of these proteins is excessive development fails, but if some minimal level of Cbs is present development appears to proceed normally, consistent with a study by Warren and colleagues, showing that after removing the Golgi in cytoplasts, the addition of 2-5% of the normal amount of Golgi restored 35% of the transport function assayd (Pelletier et al., 2000).

Unlike RNAi-depleted embryos, in Arf72A1-mutant embryos, Cbs localization during syncytial prophase is similar to wild type, but transport of Cbs to the chromosomes is completely abrogated. When Cbs is not inherited by a chromosome-based mechanism during the syncytial blastoderm stage, Cbs and Lva protein accumulation is decreased similar to RNAi depletion of Cbs, and cellularization fails, suggesting that GRIP function is required for the inheritance and maintenance of the Golgi. In Arf72A1-mutant embryos, the effect on the centrosome cycle during early syncytial blastoderm stages is less severe than RNAi but, by cellularization and early gastrulation, Cbs is nearly absent and the incidence of centrosomal defects increases, including centrosome fusions and loss of centrosomes. These data suggest that, GRIP domain function is required to maintain high levels of Cbs during late cleavage, so that normal prophase cycling of Cbs can occur. When Cbs levels are significantly reduced (similar to Cbs depletion by RNAi) Cbs fails to form aggregates during prophase and Cnn fails to localize to the centrosomes. The finding that this early relocation of Cbs might not require microtubules is similar to the finding that Cnn localization to the centrosome is by a microtubule-independent process (Megraw et al., 1999).

The ectopic expression of a GFP::GRIP fusion protein supports a role for this domain in the inheritance of Cbs via the chromosomes, and links Cbs to the centrosome cycle. In GFP::GRIP-expressing embryos, both the fusion protein and endogenous Cbs are transported to chromosomes but Cbs appears to bind to chromosomes more efficiently than the fusion protein. Nevertheless, large particles of Cbs accumulate in the cytoplasm during mitosis, similar to the Arf72A1 mutant phenotype. This block in Cbs transport also leads to an excessive accumulation of Cnn at centrosomes, causing centrosome hypertrophy and re-replication. These data suggest that, the transport of Cbs and Cnn from the cytoplasm to chromosomes and centrosomes, respectively, is linked in a time-dependent manner. The GRIP protein could interfere with Cbs transport by competing with Cbs for Arl1 binding, by affecting transport-machinery binding or a combination of the two. Alternatively, accumulation of the GRIP peptide at centrosomes might physically block transport of Cbs by directly interfering with Cnn function. Discerning between these possibilities will, of course, require further investigation. Nonetheless, it is clear that the normal cycling of Cbs is important for normal centrosome maturation, replication and spindle attachment during mitosis, and the linkage of this process to the normal nuclear cycle.

Taken together, our results support a model that requires Cbs function for inheritance and maintenance of a subset of the Golgi complex in Drosophila, and links Golgi inheritance to the cell cycle via the centrosome cycle. During syncytial cleavage divisions, we propose that the initial prophase changes in Cbs are required for centrosome replication and maturation by an Arl1-independent mechanism, because both processes are normal in an Arf72A1-mutant background, provided sufficient levels of cytoplasmic Cbs particles form during prophase. In RNAi-depleted animals the maternal load of cbs transcripts is reduced, resulting in an early loss of Cbs and an increase in centrosome defects. Because Cbs particles do not need to associate with centrosomes for normal replication and maturation, we propose an indirect link between Cbs and Cnn cycles, requiring a set period of time of Cbs particle formation for sufficient Cnn localization to centrosomes. The combined data suggest that, when Cbs levels are reduced there is not sufficient time for localization of Cnn to centrosomes and, conversely, when the traffic time is extended by the ectopic expression of the GRIP domain, Cnn localization continues, resulting in centrosome hypertrophy. This mechanism functions only during prophase, because reduced centrosomes fail to mature during late mitosis in RNAi mutants and centrosome replication does not occur after prophase in embryos that express the GRIP fusion peptide.

Our characterization of Cbs suggests that many aspects of Golgi inheritance in animal cells are more similar between flies and vertebrates than previously perceived. Although the Drosophila Golgi complex is much less complex than that of the vertebrate, flies offer a powerful genetic and molecular system in vivo animal system that represents an evolutionary intermediate, useful for addressing several questions regarding the animal Golgi that are difficult to address using tissue culture and in vitro systems alone. Even though many Golgi components in Drosophila remain uncharacterized, Cbs, Lva and several other cis-Golgi proteins offer a starting point for a more complete understanding of the role and regulation of golgins in Golgi structure and inheritance, how Golgi-vesicle transport is linked to the microtubule cytoskeleton and how the inheritance of this organelle is linked to the cell cycle in animals. Future work on Cbs and the Drosophila Golgi promises to be interesting and useful for reaching a more complete understanding of this fascinating organelle.

Materials and Methods

Antibody production
To produce Cbs protein in E. coli we amplified the first 600 bases of coding sequence with the following primers: ChsAb5'-X: 5'-AAC ATG CAA TGG TAC ATC CAC TGA AA AA CA A G-3' and ChsAb5'-H: 5'-AAA AGC TTC TAC GCC AGT GTT GTG GTC AG-3' (MWG, Inc.). PCR products were cloned into a Topo-TA pCR2.1 vector (Invitrogen) and their sequence was verified by ABI Big Dye 3 reagents on an ABI 3700 sequencer. The PCR product was digested with EcoRI, filled with Klenow polymerase, digested with XbaI, and ligated into a pW930-1 lacZ fusion cassette that was digested with HindIII, filled with Klenow polymerase and digested with Xhel. Ligation and protein production were done as previously described (Matthews et al., 1989). Antibodies were produced in guinea pigs by Coicalico Biologicals, Inc.
Western blotting
To test the specificity of whole sera, we collected 0-hours-old to 2-hours-old embryos (in 1:1 and mounted on glass slides at 20% glycerol and 10% PBS, with 0.2 M n-propyl gallate (Sigma). The following antibodies were used: guinea pig whole serum anti-Cbs at 1:50, guinea pig (whole serum) anti-Cnn at 1:500 (Megraw et al., 1999) or rabbit anti-Cnn at 1:300 (Heuer et al., 1995), rabbit anti-actin-1:400 (Arf72A; a gift from Bill Sullivan (University of California, Santa Cruz, CA). All fluorescent secondary antibodies were used at 1:200 (Jackson ImmunoResearch Labs). Proteins were detected using Pierce SuperSignal West Pico Chemiluminescence Substrate and developed on Kodak film.

Drosophila stocks
All flies used in this study were grown on standard corn-meal agar medium at 25°C except where noted. Wild-type flies were Oregon R. To follow the ER during embryogenesis we used the promoter-trap transgenic line 74-1, which expresses a PEn-GFP chimera under its endogenous promoter (Bogin et al., 2003), a gift from Alain Debec (Univ. Pierre et Marie Curie, Villefranche-sur-mer, France). To express the GRIP fusion and the RNAi transgenes during embryogenesis the GRIP line ChBr2R15-H (w; P[w+6-2-2+1-50-1400USP-GFP-GRIP]) and the RNAi line ChB30-III (w; P[w+6-2-2+1-50-1400UAST-cbs-RNAi-UAST]) were crossed with a nanos:Gal4 line (P[GAL4::VP16-ox-UTR]) (Van Doren et al., 1998). These crosses were performed at 18°C and 29°C to regulate Gal4-induced transcription and obtain a range of phenotypic responses to the presence of the fusion protein or dsRNA. Embryos collected from P[GAL4::VP16-ox-UTR] MVD; P[w+6-2-2+1-50-1400UAST-cbs-RNAi-UAST] or P[GAL4::VP16-ox-UTR] MVD; P[w+6-2-2+1-50-1400UAST-GFP-GRIP] mothers that had been kept at either of the three temperatures were collected and stained for the presence of Cbs or GFP to assess the efficacy of RNAi depletion or to determine the pattern of accumulation of the chimERIC protein, respectively. To analyze the effect the loss of Ar11 protein function has on Cbs we used Arf72A, an EMS-induced mutant allele (Tamura et al., 1991).

Characterization of the cbs locus
To characterize the cbs locus, we screened ovarian, embryonic and testes cDNA libraries, as previously described (Heuer et al., 1995). Additionally, the following BDGP ESTs were completely sequenced: GH06072, LD35238 and LP08185. EST clones were acquired from Research Genetics (Huntsville, AL). Sequences were aligned with the genomic sequence and maps were determined with MacVector software (Accelrys). Predictions of the secondary protein structure were completed using PredictProtein (http://www.predictprotein.org/).

Cloning and transformation
To construct the GRIP fusion protein we PCR-amplified GFP with the following primers: SpeGfp-5′/H11032 2000 3′, ACG CTG CTC CGA GCC GAC GAC-3′ and GfpMlu-3′: 5′-ACG CGT CTT GTA CCA CTC CAT G-3′. We amplified the GRIP domain with the following primers: MluChBr-5′/H11032 2000 3′, 5′-GGGA CGG GCT TTC AAT ATT TTA AAC ACC-3′ and ChBrGatX-3′: 5′-CCT CAT GAC TAG TCG TGT AGC ACC TTC CCC-3′ using standard PCR techniques and added restriction sites within the primers. PCR products were cloned and sequenced as described above. All other cloning was done using in-gel ligation techniques as previously described (Kalvakolanu and Livingston, 3rd, 1991). PCR products were digested with appropriate enzymes and subcloned into a pBluecript vector, digested with NolI and shuttled into a pUASP vector (Rorth, 1998). The RNAi construct was made with the antibody clone (described above) and a modified UAST vector that has sites within the primers. 2-3 P-element helper plasmid, a gift from Joseph Duffy (Indiana University, Bloomington, IN), and we transformed a w1108 Drosophila stock. We recovered homozygous transgenic lines for both constructs on chromosomes I, II and III (w; P[w+6-2-2+1-50-1400USP-GFP-GRIP] and w; P[w+6-2-2+1-50-1400UAST-cbs-RNAi-UAST]).

Fixation and immunostaining
Embryos were collected every 2-4 hours, dechorionated in 1:1 bleach and NaCl-Triton (0.2% NaCl, 0.02% Triton X-100) followed by two NaCl-Triton and one H2O wash. Embryos were fixed for 12 minutes in 50% heptane and 50% MeOH-EGTA wash. Embryos were fixed for 10 minutes in 50% heptane for 10 minutes, followed by MeOH removal of the vitelline membrane. Embryos were then treated with 10 μm Taxol prior to formaldehyde or paraformaldehyde fixation to stabilize microtubules (Foe et al., 2000). Embryos were immunostained as previously described (Gorman and Kaufman, 1995; Miller et al., 1995) or stained on glass slides at 90% glycerol and 10% PBS, with 0.2 M n-propyl gallate (Sigma). The following antibodies were used: guinea pig (whole serum) anti-Cbs at 1:50, guinea pig (whole serum) anti-Cnn at 1:500 (Megraw et al., 1999) or rabbit anti-Cnn at 1:300 (Heuer et al., 1995), rabbit anti-actin-1:400 (Arf72A; a gift from Bill Sullivan (University of California, Santa Cruz, CA). All fluorescent secondary antibodies were used at 1:200 (Jackson ImmunoResearch Labs). Proteins were detected using Pierce SuperSignal West Pico Chemiluminescence Substrate and developed on Kodak film.

Microscopy and imaging
All images were captured on a Leica TCS confocal microscope, with a 63× HX Plan Apo oil-immersion objective, using TCSNT software. Images are all Z-series of 2.5-6.0 μm thickness, and are composed of 0.5-0.9 μm thick sections. Projected images were processed and assembled into figures with Adobe Photoshop version 7.0 software.

Fluorescence and phenotype quantification
To quantify the effects of different treatments we used the Leica TCSNT quantification software and analyzed fluorescent intensity (Fl) of eight to 14 individual nuclei or spindles in projected images of equal thickness at different stages of synvical development and mitosis. The mean and standard deviation for each image was determined and student’s t-tests of equivalent wild-type and mutant images were performed (supplementary material Table S1).

To quantify the cbs RNAi phenotypes, we chose five images with moderate defects and five images with severe defects. Each nucleus was counted once and put into one of the categories in Table 1. This standard was used to characterize cbs RNAi images used in Table 2 that were coimmunostained for Cbs, DNA and either Cnn or microtubules. The Arf72A mutant images were coimmunostained for Cbs, DNA and either Cnn or microtubules, and were characterized using the cbs RNAi standard or based on morphology. The GFP::GRIP images used in Table 2 were coimmunostained for GFP, DNA and Cnn.

The authors thank Alain Debec, Joseph Duffy, Maria Furia, William Sullivan, John Sisson and the Bloomington Stock Center for reagents and Drosophila stocks, Jeff Cecil for characterizing the transcript map for cbs, Lei Gong for assistance with the western blotting and Kevin Cook, Claire Walzak, Willian Sullivan and John Sisson for invaluable comments on the manuscript. An NSF Integrative Graduate Education and Research Traineeship (DGE-9972830) supported R.E. The work reported here was supported in part by equipment and facilities supplied by INGEN.

References
Barr, F. A. (1999). A novel Rab6-interacting domain defines a family of Golgi-targeted coiled-coil proteins. Curr. Biol. 9, 381-384.
Barr, F. A. (2002). Inheritance of the endoplasmic reticulum and Golgi apparatus. Curr. Opin. Cell Biol. 14, 496-499.
Barr, F. A. (2004). Golgi inheritance: shaken but not stirred. J. Cell Biol. 164, 955-958.
Barr, F. A. and Short, B. (2003). Golgins in the structure and dynamics of the Golgi apparatus. Curr. Opin. Cell Biol. 15, 405-413.
Behnia, R., Panic, B., Whyte, J. R. and Munro, S. (2004). Targeting of the Arflike GTase Arflp to the Golgi requires N-terminal acetylation and the membrane protein Syp1p. Natl. Cell Biol. 6, 405-413.
Bocchini, Y., Marcaillou, C., Morin, X. and Debec, A. (2003). Dynamics of the endoplasmic reticulum during early development of Drosophila melanogaster. Cell Motil. Cytoskeleton 54, 217-225.
Callaini, G. and Anselmi, F. (1988). Centrosome splitting during nuclear elongation in the Drosophila embryo. Exp. Cell Res. 178, 415-421.
Dirac-Svejstrup, A. B., Shorter, J., Waters, M. G. and Warren, G. (2000). Phosphorylation of the vesicle-tethering protein p151 by a caspin kinase II-like enzyme is required for Golgi reassembly from isolated mitotic fragments. J. Cell Biol. 150, 475-488.
Foe, V. A., Oddell, G. M. and Edgar, B. A. (1993). Mitosis and morphogenesis in the Drosophila embryo: point and counterpoint. In The Development of Drosophila melanogaster (ed. A. Martinez-Arias and M. Bate), pp. 499-300. Cold Spring Harbor, NY: Cold Spring Harbor Laboratory Press.
Foe, V. E., Field, C. M. and Odell, G. M. (2000). Microtubules and mitose cyclic phase modulate spatiotemporal distributions of F-actin and myosin II in Drosophila syncytial blastoderm embryos. Development 127, 1767-1778.
Frescas, D., Navrakis, M., Lorenz, H., Delotto, R. and Lippincott-Schwartz, J. (2006). The secretory membrane system in the Drosophila syncytial blastoderm embryo exists as functionally compartmentalized units around individual nuclei. J. Cell Biol. 173, 219-230.
Gilson, P. R., Vergara, C. E., Kjer-Nielsen, L., Teasdale, R. D., Bacic, A. and Gleeson, J.

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