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Article

Centrioles Regulate Centrosome Size by Controlling the Rate of Cnn Incorporation into the PCM

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Summary

Background: Centrosomes are major microtubule organizing centers in animal cells, and they comprise a pair of centrioles surrounded by an amorphous pericentriolar material (PCM). Centrosome size is tightly regulated during the cell cycle, and it has recently been shown that the two centrosomes in certain stem cells are often asymmetric in size. There is compelling evidence that centrioles influence centrosome size, but how centrosome size is set remains mysterious.
Results: We show that the conserved *Drosophila* PCM protein Cnn exhibits an unusual dynamic behavior, because Cnn molecules only incorporate into the PCM closest to the centrioles and then spread outward through the rest of the PCM. Cnn incorporation into the PCM is driven by an interaction with the conserved centriolar proteins Asl (Cep152 in humans) and DSpd-2 (Cep192 in humans). The rate of Cnn incorporation into the PCM is tightly regulated during the cell cycle, and this rate influences the amount of Cnn in the PCM, which in turn is an important determinant of overall centrosome size. Intriguingly, daughter centrioles in syncytial embryos only start to incorporate Cnn as they disengage from their mothers; this generates a centrosome size asymmetry, with mother centrioles always initially organizing more Cnn than their daughters.
Conclusions: Centrioles can control the amount of PCM they organize by regulating the rate of Cnn incorporation into the PCM. This mechanism can explain how centrosome size is regulated during the cell cycle and also allows mother and daughter centrioles to set centrosome size independently of one another.

Introduction

Centrioles are complex, microtubule (MT)-based structures that organize two important cellular organelles: cilia and centrosomes. Cilia regulate many cell and developmental processes, whereas centrosomes function as major MT organizing centers (MTOCs), which play an important part in many aspects of cell organization. There is increasing evidence linking cilia and centrosome dysfunction to various human diseases [1].

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Centrosomes generally comprise a pair of centrioles surrounded by an amorphous pericentriolar material (PCM). The centrioles are required to organize the PCM [2, 3], which is highly enriched in proteins that nucleate and organize MTs. Centrosome size (defined here as the total amount of PCM organized by the centrioles) is tightly regulated during the cell cycle: centrosomes are typically small during interphase but increase in size (mature) as cells prepare to enter mitosis [4]. Centrosome size can be asymmetrically regulated in certain stem cells, where the two centrosomes within the same cell differ in size; this size asymmetry appears to be important for the efficient asymmetric division of these cells [5, 6]. Despite the importance of centrosome size regulation, the molecular mechanisms involved in setting centrosome size are largely unknown.

Two pieces of evidence indicate that centrioles play an important part in setting centrosome size. First, in *C. elegans* embryos, artificially varying centriole size leads to corresponding variations in PCM size [7]. Second, in *Drosophila* male germline stem cells (GSCs), the older, mother, centriole seems to always organize more PCM than its daughter, ensuring the specific retention of the mother centriole in the stem cell through repeated rounds of asymmetric division [8]. This observation strongly suggests that mother and daughter centrioles can independently set centrosome size. It remains unclear, however, how centrioles influence centrosome size.

Mutational and/or RNA interference analysis in various organisms has identified several proteins that are important for the proper recruitment of the PCM [4, 9]. In a genome-wide RNA interference (RNAi) screen in *Drosophila* S2 cells, Polo kinase and centrosomin (Cnn) were found to have the largest influence on PCM recruitment [10]. These proteins are codependent for their centrosomal localization, and Cnn is phosphorylated during mitosis in a Polo-dependent manner. Cnn is a large coiled-coil protein required for the efficient recruitment of most, if not all, PCM components [11–13]. Proteins related to Cnn are found in species from yeast to man and have been implicated in centrosome and MTOC function [14, 15]. In humans, there are two Cnn-related proteins, CDK5RAP2 and Myomegalin, and, as in flies, vertebrate CDK5RAP2 homologs are required to establish and/or maintain proper PCM organization [14, 16, 17]. Moreover, mutations in human CDK5RAP2 have been linked to autosomal-recessive primary microcephaly [18], a condition closely associated with defects in centrosome function [19].

Although Cnn is clearly required for proper PCM organization in flies, it is not clear how it is recruited to the PCM. Here we investigate the dynamics of Cnn recruitment and explore how this relates to the regulation of centrosome size in *Drosophila* embryos.

Results

GFP-Cnn Is Only Incorporated into the PCM Closest to the Centrioles

To study PCM recruitment in vivo, we analyzed various PCM components fused to GFP in *Drosophila* embryos coexpressing the centriolar marker RFP-PACT. We photobleached the GFP-PCM marker and monitored fluorescence recovery.

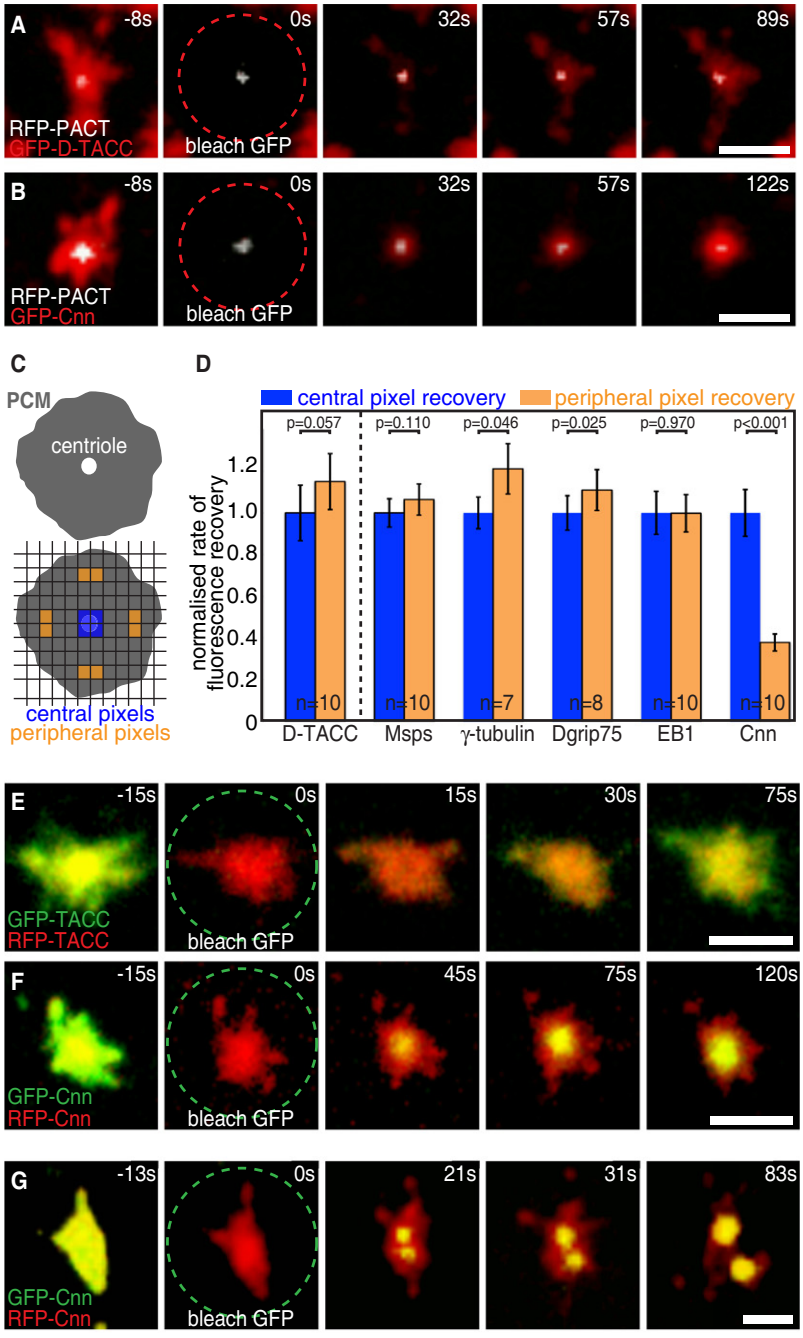

Figure 1. Cnn Is Initially Incorporated into the PCM that Is Closest to the Centrioles

(A and B) Images show the dynamic behavior of GFP-D-TACC (A) or GFP-Cnn (B) (pseudocolored red) at centrosomes in embryos coexpressing the centriolar marker RFP-PACT (pseudocolored white). Time before and after photobleaching (t = 0 s) is indicated.

(C) Schematic diagram indicating the positions of central (blue) and peripheral (orange) pixels used to analyze fluorescence recovery in different regions of the PCM.

(D) Graphs show the rate of initial fluorescence recovery in the peripheral pixels (orange) relative to the central pixels (blue) for several PCM components.

(E–G) Images show the dynamic behavior of GFP-D-TACC (E) or GFP-Cnn (F and G; green) in embryos coexpressing RFP-D-TACC (E) or RFP-Cnn (F and G; red). Error bars denote standard error. Scale bars represent 3 μm. See also Figures S1 and S2 and Movie S1, Movie S2, Movie S3, and Movie S4.

We expected PCM components to have binding sites throughout the PCM so that fluorescence recovery would occur throughout the entire PCM volume (see Figure S1A available online for schematic). This appeared to be true for the conserved PCM components D-TACC (Figure 1A; Movie S1), Msps, γ-tubulin, DGrip75, and EB1 (data not shown). Strikingly, however, GFP-Cnn fluorescence appeared to initially recover in the PCM closest to the centrioles and then spread outward throughout the rest of the PCM (Figure 1B; Movie S2).

We measured rates of initial fluorescence recovery relative to prebleach intensities in PCM closest to the centrioles (central pixels) and farther away from the centrioles (peripheral pixels) (see Figure 1C for schematic). The rates of recovery for D-TACC, Msps, γ-tubulin, DGrip75, and EB1 were similar in the different PCM regions (Figure 1D), suggesting that their binding sites were distributed throughout the PCM. In contrast, GFP-Cnn fluorescence recovered much faster in the central pixels (Figure 1D). This behavior was most dramatically illustrated when we photobleached GFP-Cnn (or, as a control, GFP-D-TACC) in embryos coexpressing RFP-Cnn (or RFP-D-TACC), thus allowing us to monitor the recovery of GFP fluorescence within a pool of unbleached (RFP) protein (Figures 1E and 1F; Figures S1D and S1E; Movie S3; Movie S4). Moreover, when we bleached centrosomes as their centrioles started to separate, GFP-Cnn incorporated around each separating centriole within the single centrosome (Figure 1G), suggesting that each centriole incorporates its own independent pool of GFP-Cnn.

Centrosomes display a behavior known as flaring, in which large particles of PCM that do not contain centrioles detach from the PCM and move away along centrosomal MTs. This behavior has been observed for several PCM proteins, including Cnn [20] and D-TACC [21], and many flares contain both Cnn and D-TACC [20]. When we bleached well-detached GFP-Cnn-containing flares in GFP/RFP-Cnn embryos, no fluorescence recovered within the flares (Figures S2A and S2C). In contrast, when we bleached well-detached GFP-D-TACC-containing flares in GFP/RFP-D-TACC embryos, the fluorescence recovered at similar rates within the flares and the centrosomes (Figures S2B and S2D). Taken together, these results indicate that centrioles are required to incorporate Cnn into the PCM; once incorporated, the Cnn molecules appear to move away from the centrioles and spread throughout the rest of the PCM (see Figures S1B and S1C for schematic).

The Rate of Cnn Incorporation into the PCM Is an Important Determinant of Centrosome Size

These observations immediately suggested a simple model for how centrioles might influence centrosome size (defined here as the total amount of PCM organized by the centrioles).

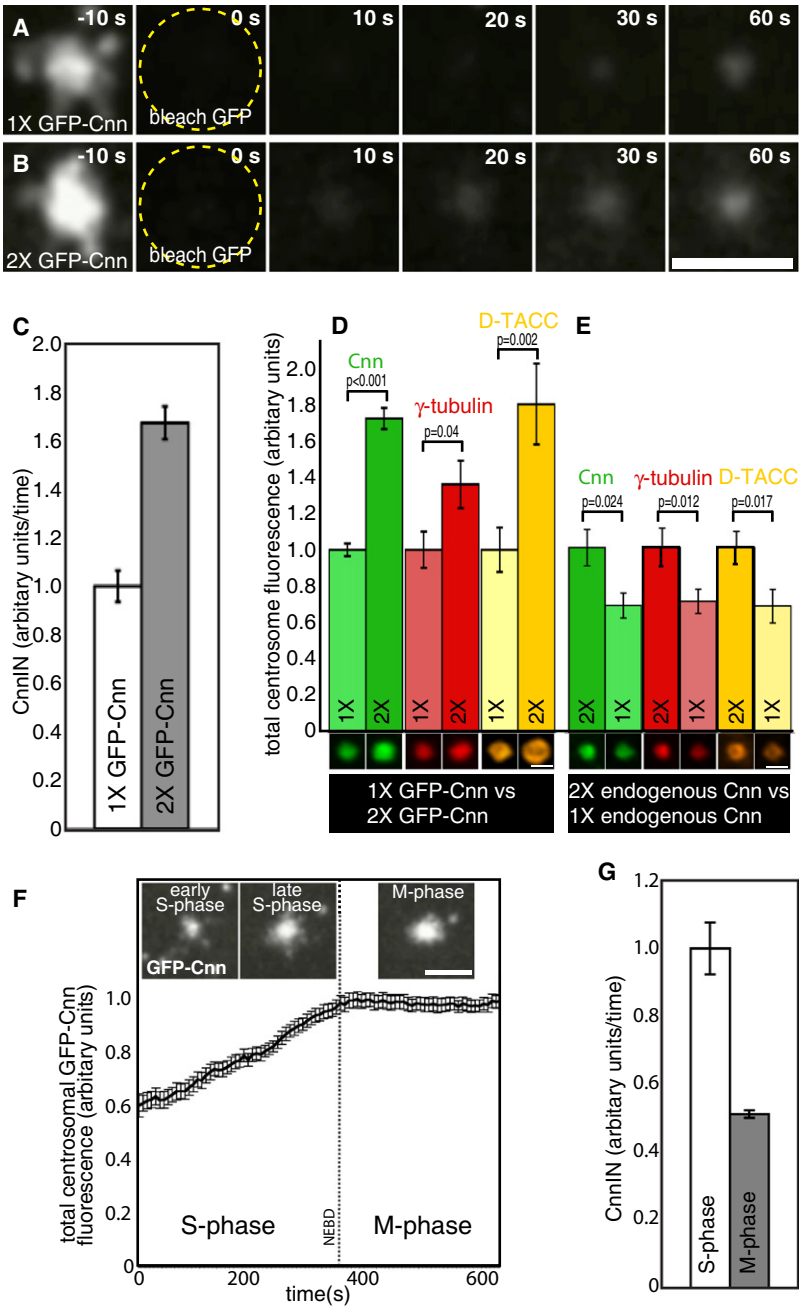

Figure 2. Changes to CnnIN Lead to Changes in Centrosome Size

(A–C) Images (A and B) and graph (C) show the amount of Cnn incorporating into the PCM per unit time (CnnIN) in *cnn* null mutant embryos expressing either one copy (A) (white bar in C, n = 10 centrosomes) or two copies (B) (gray bar in C, n = 10 centrosomes) of GFP-Cnn. Time before and after photobleaching (t = 0 s) is indicated.

(D and E) Graphs show the total centrosomal fluorescence intensity of Cnn (green bars), γ-tubulin (red bars), and D-TACC (yellow bars) in fixed embryos laid by mothers expressing either one or two copies of GFP-Cnn (D; n = 21 embryos for each genotype), or two or one copies of the endogenous *cnn* gene (E; n = 20 embryos for each genotype).

(F) Images and graph show how the amount of GFP-Cnn in the PCM increases during S phase and then remains constant after the entry into mitosis (n = 20 centrosomes).

(G) CnnIN is ~2-fold higher in S phase (white bar, n = 8 centrosomes) than in M phase (gray bar, n = 4 centrosomes). Error bars denote standard error. Scale bars represent 2 μm. See also Figures S3, S4, and S7.

Because Cnn is required for the proper centrosomal localization of many PCM components, it seemed plausible that the amount of Cnn in the PCM might play an important part in determining the amount of other proteins in the PCM. The amount of Cnn in the PCM depends on the balance between the amount of Cnn incorporating into the PCM per unit time (hereafter, CnnIN) and the amount of Cnn dissociating from the PCM per unit time (hereafter, CnnOUT; see Experimental Procedures for a full explanation of these terms). Therefore, if the centrioles could influence CnnIN, they could modulate centrosome size by modulating the amount of Cnn in the PCM (see Figure S3 for schematic summary). To directly test this possibility, we examined whether artificially varying CnnIN could change centrosome size.

We first analyzed the effects of artificially increasing CnnIN. We examined GFP-Cnn dynamics in *cnn* null mutant embryos expressing either one or two copies of GFP-Cnn, reasoning that increasing the cytoplasmic concentration of Cnn might increase CnnIN. Increasing the cytoplasmic concentration of GFP-Cnn (Figure S4A) did increase CnnIN (Figures 2A–2C), and this led to a significant increase in the amount of GFP-Cnn in the PCM, as well as to an increase in the amount of γ-tubulin and D-TACC (Figure 2D). In contrast, increasing the cytoplasmic concentration of GFP-D-TACC (Figure S4B) significantly increased the amount of GFP-D-TACC in the PCM, but not the amount of Cnn or γ-tubulin (Figure S4E).

When we decreased CnnIN by decreasing the cytoplasmic concentration of Cnn (by comparing wild-type embryos to embryos expressing just one endogenous copy of *cnn*; Figure S4C), the amounts of Cnn, γ-tubulin, and D-TACC in the PCM were all significantly reduced (Figure 2E). In contrast, decreasing the cytoplasmic concentration of D-TACC (Figure S4D) significantly decreased the amount of D-TACC in the PCM, but not the amounts of Cnn or γ-tubulin (Figure S4F). Somewhat surprisingly, when the cytoplasmic concentration of Cnn was decreased, the cytoplasmic concentration of γ-tubulin reproducibly increased (Figure S4C). Importantly, however, the amount of γ-tubulin, Cnn, and D-TACC in the PCM all decreased under these conditions, indicating that the amount of Cnn in the PCM has a stronger influence on centrosome size than the cytoplasmic concentration of γ-tubulin. We conclude that increasing or decreasing CnnIN leads to a corresponding increase or decrease in the amount of Cnn in the PCM, and thus to an increase or decrease in overall centrosome size.

CnnIN Is Regulated during the Cell Cycle in Embryos

Although these experiments demonstrate that artificially altering CnnIN can alter centrosome size, we wanted to test

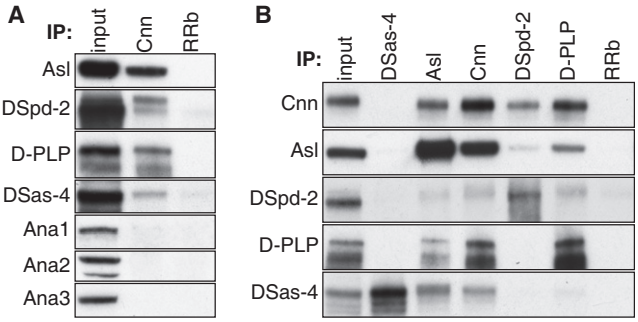

Figure 3. Cnn Interacts Biochemically with Several Conserved Centriolar Proteins

Images show western blots of immunoprecipitation experiments (as indicated above each lane) probed with antibodies against Cnn or several centriolar components (as indicated on the left of each blot).

(A) Note how the anti-Cnn antibodies coimmunoprecipitate Asl, DSpd-2, D-PLP, and, to a lesser extent, DSas-4, but not Ana1, Ana2, or Ana3.

(B) Most interactions were confirmed in reciprocal coimmunoprecipitation experiments, although anti-DSas-4 antibodies did not detectably coimmunoprecipitate Cnn.

whether cells normally regulate CnnIN to control centrosome size. In most proliferating animal cells, centrosomes increase in size in preparation for mitosis. Syncytial fly embryos have an abbreviated cell cycle, consisting entirely of alternating S and M phases, but their centrosomes appear to grow in size in preparation for mitosis [20]. Consistent with this, we observed that the amount of GFP-Cnn in the PCM steadily increased throughout S phase, reaching a plateau just as the embryos entered M phase (Figure 2F). We found that CnnIN remained high throughout S phase but then rapidly decreased by 2-fold as embryos entered M phase (Figure 2G), even though the cytoplasmic concentration of Cnn did not detectably change during the cell cycle (data not shown). We conclude that CnnIN is normally regulated during the cell cycle in *Drosophila* embryos and that changes to CnnIN correlate with changes in centrosome size.

Asl and DSpd-2 Cooperate to Drive Cnn Incorporation into the PCM

To understand why Cnn is only incorporated into the PCM close to the centrioles, we tested whether Cnn could interact biochemically with several previously characterized centriolar proteins in coimmunoprecipitation experiments from embryo extracts. We found that endogenous Cnn interacted strongly with endogenous DSpd2, Asl, and D-PLP and weakly with DSas-4 (Figure 3A), all conserved centriolar proteins implicated in centriole duplication (DSas-4 and Asl) [2, 22] and PCM recruitment (Asl, DSpd-2, and D-PLP) [23–26] in flies. Moreover, we detected several interactions between these proteins in reciprocal coimmunoprecipitation experiments, indicating that they may form higher-order complexes together (Figure 3B). Cnn did not, however, detectably interact with endogenous Ana1, Ana2, or Ana3 (Figure 3A), centriolar proteins implicated in centriole duplication (Ana2) and in the maintenance of centriole structure (Ana1 and Ana3) [27–29]. This demonstrated that the anti-Cnn antibodies were not simply coprecipitating entire centrosomes.

To directly determine whether Asl, DSpd-2, D-PLP, or DSas-4 was required for the incorporation of Cnn into the PCM, we injected fluorescently labeled affinity-purified antibodies raised against each protein into *cnn* null mutant embryos

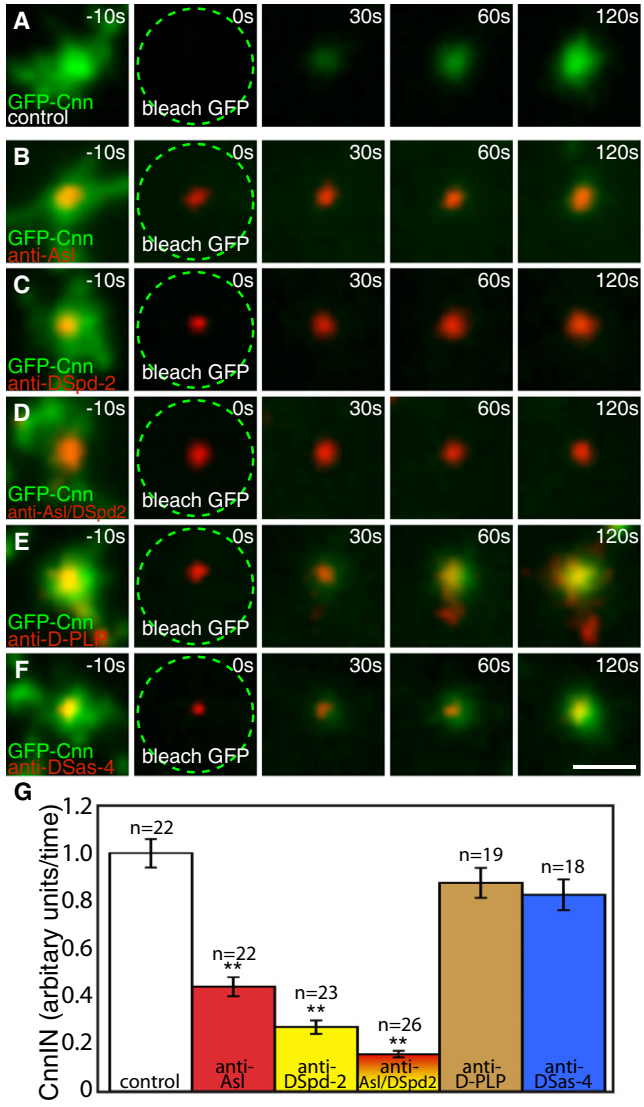

Figure 4. Asl and DSpd-2 Drive the Incorporation of Cnn into the PCM Closest to the Centrioles

Images (A–F) and graph (G) show how CnnIN is affected by inhibiting the function of various centriole components by antibody injection. *cnn* null mutant embryos expressing GFP-Cnn (green) were injected with Texas red-labeled antibodies (red, as indicated). Centrosomes located far from the injection site received a low antibody dose and acted as internal controls (an example from the anti-Asl injection is shown in A), whereas centrosomes located close to the injection site received a high antibody dose (as shown in B–F). Graph in (G) shows CnnIN for centrosomes located far from the injection site (white bar, control) or close to the injection site (colored bars, individual antibodies injected as indicated). ** indicates CnnIN values significantly different from the control and from each other. Error bars denote standard error. Scale bars represent 2 μm. See also Figure S5 and Movie S5.

expressing GFP-Cnn. In this assay, antibodies rapidly bind to centrosomes close to the injection site and locally inhibit protein function, whereas centrosomes located farther away do not bind the antibodies and act as internal controls [2, 30]. By bleaching selected centrosomes, we analyzed CnnIN at close versus distant centrosomes. Anti-DSpd-2 and anti-Asl antibodies both dramatically reduced CnnIN (Figures 4A–4C and 4G; Movie S5), and the combination of both antibodies led to an even greater reduction (Figures 4D and 4G;

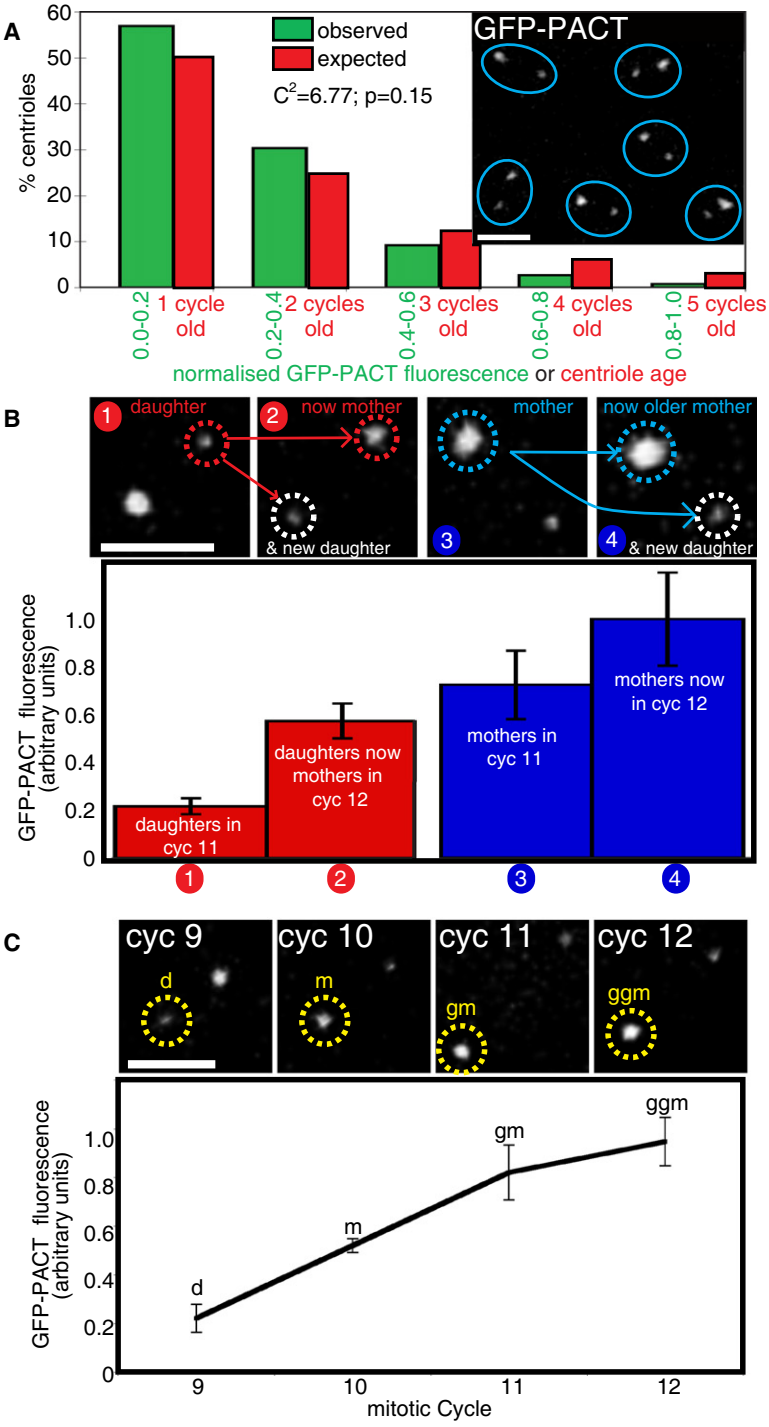

Figure 5. GFP-PACT Fluorescence Intensity Can Be Used to Measure Centriole Age

(A) Green bars show the observed frequency of centrosomes exhibiting different levels of centriolar GFP-PACT fluorescence in fixed embryos; red bars indicate the expected frequency of centriole ages; inset shows a typical field of centrioles (each pair is circled in blue).

(B) Dim daughter centrioles give rise to another dim daughter centriole and a brighter mother centriole in the next mitotic cycle (panels 1 and 2: red bars show the fluorescence intensity of the daughters in cycle 11 and of the mothers they became in cycle 12; n = 6 centrosomes). Brighter mother centrioles give rise to a dim daughter centriole and to an even brighter mother centriole in the next mitotic cycle (panels 3 and 4: blue bars represent the fluorescent intensity of the mothers in cycle 11 and of the older mothers they became in cycle 12; n = 5 centrosomes).

(C) Panels show the GFP-PACT fluorescence associated with a typical daughter centriole (d) as it became a mother (m), grandmother (gm), and great-grandmother (ggm) through successive rounds of division. The graph shows the average GFP-PACT fluorescence of four centrosomes as they passed through these transitions. Error bars denote standard error. Scale bars represent 3 μm.

Movie S5). In these experiments, CnnIN rapidly decreased in the presence of the anti-Asl and/or anti-DSpd-2 antibodies (Figures 4A–4D and 4G; Movie S5), whereas the total amount of Cnn in the PCM decreased more slowly (Figure S5), suggesting that the reduction in CnnIN led to a gradual reduction in the amount of Cnn in the PCM.

In contrast, even though anti-D-PLP and DSas-4 antibodies bound to centrioles, they had relatively little effect on CnnIN or the amount of Cnn in the PCM (Figures 4E–4G; Movie S5). Both antibodies appeared to interfere with their respective protein's function, however, because anti-DSas-4 antibodies blocked centriole duplication (data not show) [2], whereas anti-D-PLP antibodies subtly perturbed PCM organization (data not shown) [25]. Thus, it appears that Asl and DSpd-2 cooperate to drive the incorporation of Cnn into the PCM surrounding the centrioles.

Cnn Associates Preferentially with Mother Centrioles during Centrosome Separation

We noticed that the separating centrosomes in syncytial embryos were always asymmetric in size. In male GSCs, the mother centriole always organizes a larger centrosome than the daughter centriole [8], so we wondered whether the size asymmetry in embryos might also be related to centriole age. To test this, we needed a marker of centriole age. GFP-PACT incorporates irreversibly into *Drosophila* centrioles [25]. Because centrosomes replicate rapidly in syncytial embryos, whereas GFP takes ~1 hr to mature into its fully fluorescent form [31], in principle, constitutively expressed GFP-PACT should provide a measure of the relative age of the centrioles, because older centrioles will contain a higher proportion of mature, fully fluorescent, GFP-PACT.

In living embryos expressing GFP-PACT, the centrioles always separated to give one weakly fluorescent centriole (presumably the original daughter) and one strongly fluorescent centriole (presumably the original mother) (Figure 5A). The presumptive daughters all exhibited a similar level of fluorescence (as expected, because they are all the same age), whereas the presumptive mothers exhibited a range of fluorescence intensities (as expected, because they vary in age); the overall range of fluorescent intensities matched the expected distribution of centriole ages (Figure 5A). Most importantly,

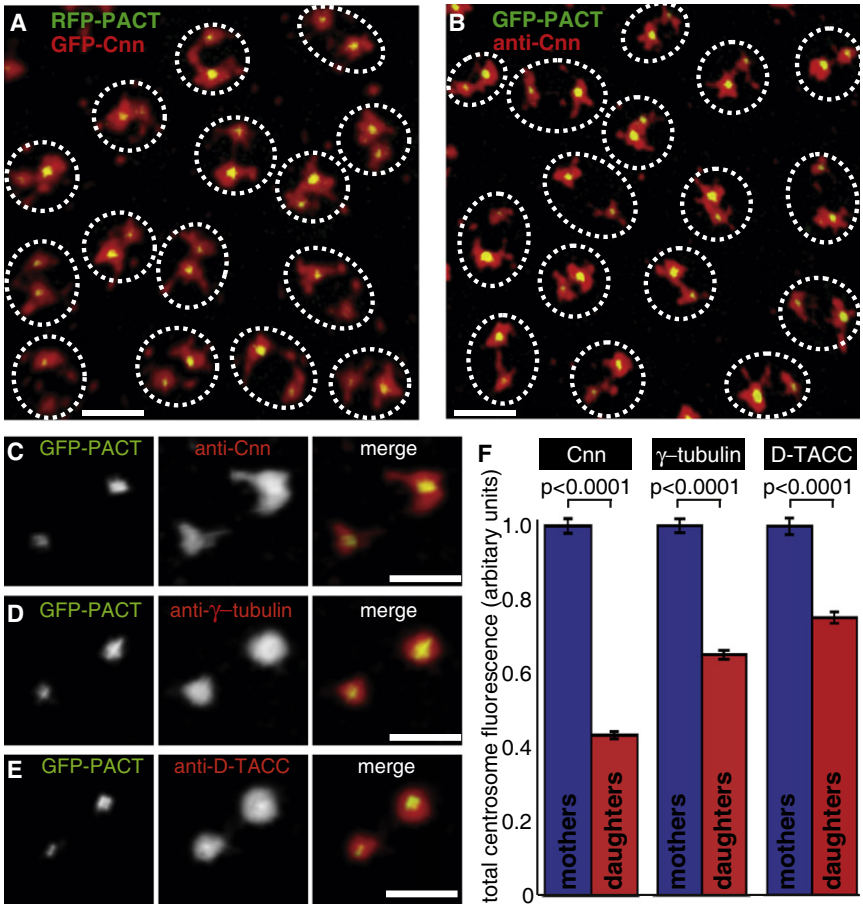

Figure 6. Cnn, γ-tubulin, and D-TACC Are All Asymmetrically Distributed between Separating Mother and Daughter Centrioles

(A and B) Panels show separating centrioles and the Cnn they organize in a living embryo expressing GFP-Cnn (pseudocolored red) and RFP-PACT (pseudocolored green) (A) and in a fixed embryo expressing GFP-PACT (green) stained for endogenous Cnn (red) (B). Each centrosome pair is circled in white.

(C–E) Panels show separating centrioles and the PCM they organize in embryos expressing GFP-PACT (green) that have been fixed and stained to reveal the distribution of Cnn, γ-tubulin, or D-TACC (red in C, D, and E, respectively).

(F) Bar graph shows the average total fluorescence intensity of Cnn, γ-tubulin, or D-TACC organized by separating mother (blue bars) or daughter (red bars) centrioles (n = 130, 106, 95 pairs, respectively). Error bars denote standard error. Scale bars represent 3 μm.

the average fluorescence intensity of both mother and daughter centrioles increased from one mitotic cycle to the next (Figure 5B), whereas the average fluorescence intensity of daughter centrioles increased as they matured into mothers, grandmothers, and then great-grandmothers (Figure 5C). RFP-PACT behaved in a similar manner (data not shown). Thus, centriolar GFP-PACT (or RFP-PACT) fluorescence intensity can be used as a marker of centriole age.

In living embryos expressing RFP-PACT and GFP-Cnn, separating mother centrioles always organized more GFP-Cnn than their daughters (Figure 6A). This was also true for endogenous Cnn in fixed embryos expressing GFP-PACT (Figures 6B, 6C, and 6F). Both γ-tubulin and D-TACC preferentially localized to mother centrosomes, although their asymmetry was not as dramatic as Cnn asymmetry (Figures 6D–6F): whereas 100% of mother centrioles associated with more Cnn than their daughters, this figure was ~95% and ~82% for γ-tubulin and D-TACC, respectively. To quantify the degree of centrosome size asymmetry, we calculated an asymmetry index (AI) for each protein (see Experimental Procedures). Cnn had an AI of +1.45 ± 0.07 (n = 130 pairs), meaning that, on average, mother centrosomes contained ~145% more Cnn than their daughters; γ−tubulin and D-TACC had AIs of +0.58 ± 0.04 (n = 106 pairs) and +0.46 ± 0.05 (n = 106 pairs), respectively. Thus, separating mother centrosomes always contain more Cnn than their daughters, and this appears to lead to a more general centrosome size asymmetry.

Cnn Size Asymmetry Is Generated Because Daughter Centrioles Do Not Start to Incorporate Cnn until They Disengage from Their Mothers

To our surprise, Cnn size asymmetry was not generated by the differential regulation of CnnIN at mother and daughter centrioles (Figure S6A). We therefore considered that it might arise because newly born daughter centrioles only start to incorporate Cnn once they disengage and separate from their mothers (shown schematically in Figure S6B). To test this, we bleached the centrosomal GFP signal in GFP/RFP-Cnn embryos at different times during the cell cycle. We reasoned that if both centrioles were incorporating GFP-Cnn into the PCM at the time of bleaching, they should associate with equal amounts of newly incorporated GFP-Cnn when they separate. Alternatively, if only the mother centriole was incorporating GFP-Cnn into the PCM at the time of bleaching, it should organize more newly incorporated GFP-Cnn than its daughter when they separate.

When we bleached centrosomes during or after anaphase B, the recovering GFP signal was roughly symmetrically distributed between the separating centrosomes (Figure 7A; Movie S6). In contrast, when we bleached centrosomes at any time prior to anaphase B, the separating mother centrosome (recognized because it contained more RFP-Cnn) always associated with more GFP signal than its daughter (Figure 7B; Movie S7). Moreover, as predicted by our model, this asymmetry decreased as the time between bleaching and anaphase B onset decreased (Figures S6C–S6E). Thus, newly born daughter centrioles do not appear to start incorporating Cnn into the PCM until about the time that they disengage from their mothers. Consistent with this, when we expressed a photoactivatable form of GFP-Cnn and photoactivated the centrosomal pool of Cnn prior to anaphase B, only the mother centrosome contained fluorescent GFP-Cnn after centrosome separation (Figure 7C).

Discussion

Centrosome size is tightly regulated during the cell cycle, and centrosome size asymmetry is an important feature of certain

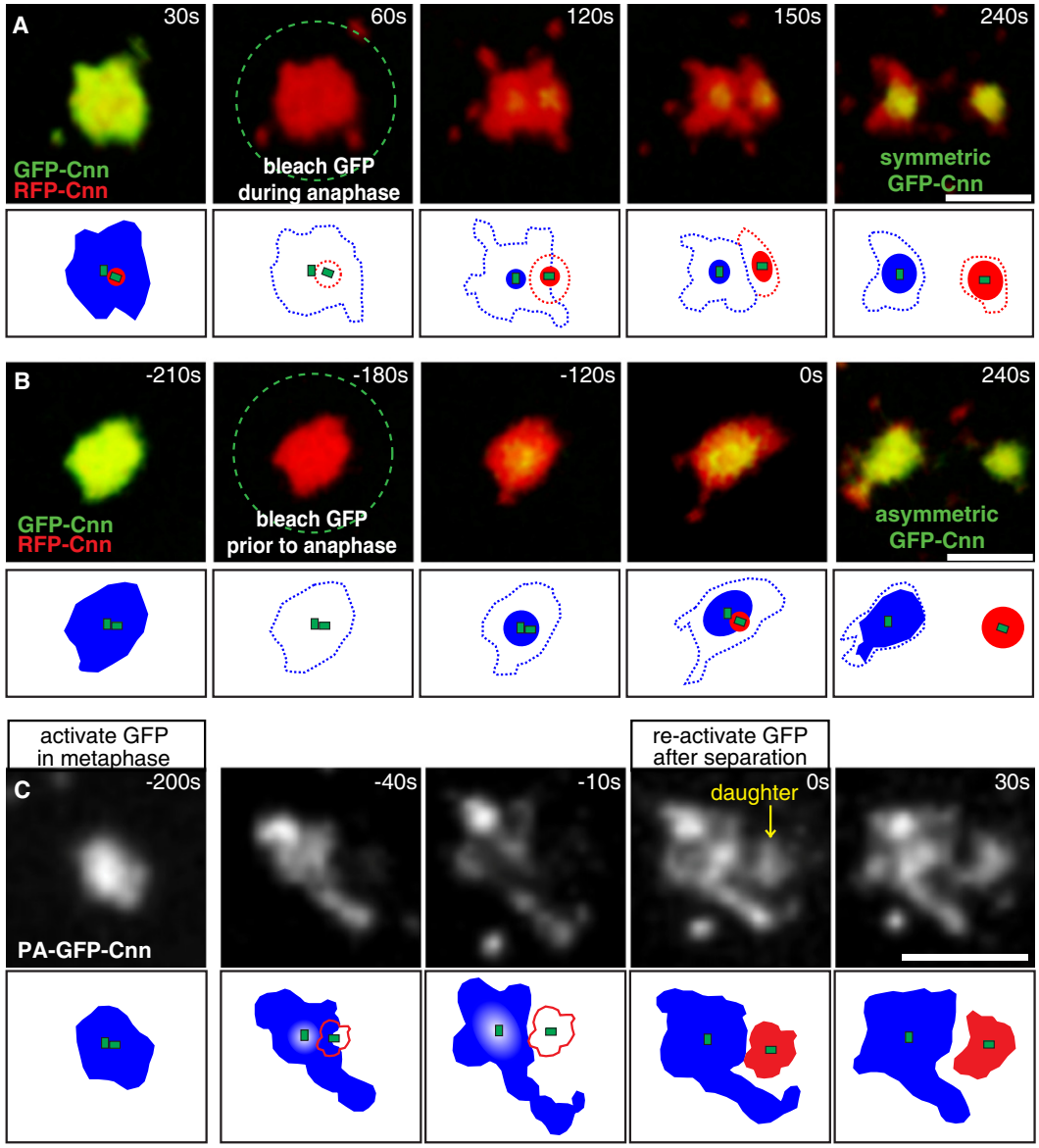

Figure 7. Cnn Dynamics Generate Centrosome Size Asymmetry in Syncytial Embryos

(A and B) Images (top panels) from a fluorescence recovery after photobleaching experiment in GFP/RFP-Cnn embryos testing the hypothesis in Figure S6B; bottom panels show our interpretation of these images (blue denotes fluorescent GFP-Cnn organized by mother centriole; red denotes fluorescent GFP-Cnn organized by daughter centriole). Centrosomes were bleached at different times in mitosis (t = 0 s at anaphase B).

(A) A centrosome bleached after anaphase B. When the centrioles separated, they associated with approximately equal amounts of newly incorporated GFP-Cnn, indicating that both centrioles were incorporating GFP-Cnn at the time of bleaching.

(B) A centrosome bleached prior to anaphase onset. When the centrioles separated, the newly incorporated GFP-Cnn was asymmetrically distributed, indicating that only the mother centriole was incorporating GFP at the time of bleaching.

(C) Photoactivation of PA-GFP-Cnn at a centrosome in metaphase (prior to centriole disengagement) and then again after centrosome separation. Time before and after the second activation event (t = 0 s) is indicated. The fluorescence from the first activation event is only associated with the mother centrosome after centrosome separation. Scale bars represent 3 μm. See also Figure S6, Movie S6, and Movie S7.

stem cells, yet the question of how centrosome size is determined has received little attention. Previous reports have indicated that centrioles play an important part in setting centrosome size [7, 8], but how they might do so has remained unclear. Our observation that Cnn is only incorporated into the PCM closest to the centrioles provides a plausible molecular mechanism to explain how centrioles influence centrosome size.

We propose that the conserved centriolar proteins Asl and DSpd-2 cooperate to incorporate Cnn into the region of the PCM immediately surrounding the centrioles. Once incorporated, the Cnn molecules move away from the centrioles, spreading outward through the rest of the PCM. We postulate that the incorporation of Cnn molecules into the PCM provides a dynamic structural lattice that allows other centrosomal components to be stably retained in the PCM: some of these, like γ-tubulin and Aurora A, may bind directly to Cnn [32], whereas others may bind indirectly via interactions with other Cnn-binding proteins such as D-PLP, which has been shown to interact with several PCM components in several systems

[33]. In this way, the amount of Cnn in the PCM influences the amount of the other proteins in the PCM.

In our model, the centrioles regulate centrosome size by influencing the amount of Cnn incorporated into the PCM per unit time (CnnIN). We envisage that when centrosomes need to grow in size (during centrosome maturation, for example) CnnIN would increase, so that CnnIN > CnnOUT. This would lead to an increase in the amount of Cnn in the PCM, and thus to an increase in centrosome size; if centrosomes need to shrink in size, CnnIN would decrease, leading to a decrease in centrosome size as CnnIN < CnnOUT (Figure S3). This model can potentially explain why larger centrioles organize more PCM [7], because a larger centriole surface area would be expected to drive a larger CnnIN. We stress, however, that regulating CnnIN is unlikely to be the only mechanism that influences centrosome size. Other proteins and regulatory events, including the regulation of CnnOUT, will almost certainly play a part.

An important implication of our model is that mother and daughter centrioles can regulate CnnIN (and so set centrosome size) independently of one another. Indeed, in syncytial embryos, daughter centrioles do not start to organize their own domains of Cnn until they disengage from their mothers. It is tempting to speculate that this switch may be related to the licensing of centriole duplication that also occurs upon centriole disengagement [34], a process that, like centrosome maturation, is dependent on Polo/Plk1 kinase [35]. Intriguingly, this mechanism ensures that separating mother centrioles always initially organize more Cnn than their daughters. In embryos, this size asymmetry gradually decreases as embryos progress through the cell cycle (data not shown), but clearly the differential regulation of CnnIN could be used to generate centrosome size asymmetry in cells such as larval neuroblasts or male GSCs, in which mother and daughter centrioles organize centrosomes of different sizes for sustained periods [5, 6, 8]. Indeed, elsewhere in this issue of *Current Biology*, we provide strong evidence that the differential regulation of CnnIN at mother and daughter centrioles generates centrosome size asymmetry in larval neuroblasts [36].

The molecular mechanisms regulating CnnIN are currently unknown but might include changes to the phosphorylation state of Asl and/or DSpd-2, or to Cnn itself. Cnn is phosphorylated during mitosis, and this is dependent on Polo kinase [10]. The role of Polo kinases in centrosome maturation is highly conserved [37], and Polo and Cnn were found to have the most influence on centrosome maturation in a genome-wide RNAi screen in *Drosophila* S2 cells [10]. Asl and DSpd-2 were also identified as regulators of centrosome maturation in this screen. Thus, in the simplest model, when centrosomes need to grow in size, Polo would phosphorylate Cnn and/or Asl/DSpd2, increasing the strength of the Cnn-Asl/DSpd2 interactions and therefore leading to an increase in CnnIN and thus to an increase in centrosome size. Proteins related to Cnn, Asl, and DSpd-2 are found in many other species, including humans, suggesting that these proteins may form a conserved module that establishes and maintains the structure and size of the PCM.

Experimental Procedures

Generation of Transgenic Lines

P element-mediated transformation vectors were made by introducing the full-length Cnn cDNA into the Ubq-GFPNT [38], Ubq-RFPNT, or Ubq-PA-GFPNT (this study) Gateway vectors. Constructs were injected by BestGene. All other GFP and RFP fusions have been described previously [11, 21, 30, 39, 40].

Antibodies

For immunofluorescence analysis, we used the following antibodies: rabbit anti-Cnn (1:1000) [10], guinea pig anti-Cnn (1:500) [38], mouse anti-γ-tubulin (1:500; GTU88, Sigma), and rabbit anti-D-TACC (1:500) [30]. Secondary antibodies were from Molecular Probes (Invitrogen): Alexa Fluor 488, 568, and 647 (all used at 1:1000). For immunoprecipitation and antibody injection experiments, we used affinity-purified rabbit antibodies raised against Asl (this study), DSpd-2 [23], D-PLP [25], DSas-4 [2], Ana1 (this study), Ana2 [29], and Ana3 [28]. For antibody injection, each antibody was covalently coupled to Texas Red, as described previously [30].

Antibody Injections

Antibodies were injected at the start of a mitotic cycle, and embryos were observed on the spinning disc confocal system described in the Supplemental Experimental Procedures. Centrosomes were bleached in pairs, one centrosome located close to the injection site and one centrosome located far from the injection site. Typically, three centrosome pairs were bleached per embryo. Multiple embryos were injected for each antibody, and the data were collated.

Asymmetry Index Calculation

The total intensity of GFP-PACT was used to identify the mother centrosome. For each mother-daughter centrosome pair, the larger fluorescence value was divided by the smaller fluorescence value and multiplied by V. V = 1 if the larger centrosome was the mother, and V = −1 if the larger centrosome was the daughter. V was then subtracted from this value. Any centrosome pair with a larger mother centrosome would have a score > 0; any pair with a larger daughter centrosome would have a score < 0. This calculation gives equal weight to centrosome pairs in which the mother is larger and to centrosome pairs in which the daughter is larger. The average centrosome pair value was taken as the AI value.

Definition of CnnIN and CnnOUT

We define CnnIN as the amount of Cnn added to the PCM per unit time and CnnOUT as the amount of Cnn lost from the PCM per unit time. CnnIN can be measured (in relative terms) in fluorescence recovery after photobleaching experiments as the initial rate of recovery of fluorescent GFP-Cnn. Both parameters are rates, although they do not reflect the rate of an individual molecular interaction; CnnIN and CnnOUT will depend on the sum of several different molecular interactions.

Supplemental Information

Supplemental Information includes seven figures, Supplemental Experimental Procedures, and seven movies and can be found with this article online at doi:10.1016/j.cub.2010.11.011.

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