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Report

Centrocortin Cooperates with Centrosomin to Organize *Drosophila* Embryonic Cleavage Furrows

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Summary

In the *Drosophila* early embryo, the centrosome coordinates assembly of cleavage furrows [1–3]. Currently, the molecular pathway that links the centrosome and the cortical microfilaments is unknown. In *centrosomin* (*cnn*) mutants, in which the centriole forms but the centrosome pericentriolar material (PCM) fails to assemble [4, 5], actin microfilaments are not organized into furrows at the syncytial cortex [6]. Although CNN is required for centrosome assembly and function [4, 6, 7], little is known of its molecular activities. Here, we show the novel protein Centrocortin (CEN), which associates with centrosomes and also with cleavage furrows in early embryos, is required for cleavage furrow assembly. CEN binds to CNN within CNN Motif 2 (CM2), a conserved 60 amino acid domain at CNN's C terminus. The *cnn*^{B4} allele, which contains a missense mutation at a highly conserved residue within CM2, blocks the binding of CEN and disrupts cleavage furrow assembly. Together, these findings show that the C terminus of CNN coordinates cleavage furrow formation through binding to CEN, thereby providing a molecular link between the centrosome and cleavage furrow assembly.

Results and Discussion

The CM2 Domain of CNN Is Required for Cleavage Furrow Assembly Independent of MTOC Activity

The mutant protein encoded by *cnn*^{B4} (Figure 1A and Figure S1A available online) was reported to localize to centrosomes; yet the mutation was maternal effect lethal and embryos were deficient in cleavage furrow assembly [6]. This finding indicated that the conserved domain at the carboxyl-terminus of CNN is necessary for a critical function of centrosomes: the organization of actin into cleavage furrows. We examined the centrosomes in *cnn*^{B4} embryos and neuroblasts to assess the microtubule-organizing center (MTOC) activity, as well as to re-examine CNN localization and actin furrow formation in this point mutant. We found that CNN^{B4} was expressed in embryos at levels similar to those in wild-type (Figure 1B) and localized to centrosomes as previously reported [6], but had a looser centrosome association compared to wild-type CNN (Figures 1C and 1E and Figures S3–S5). Regardless of CNN^{B4} mutant protein localization to centrosomes, *cnn*^{B4} embryos exhibit the linked-spindles phenotype characteristic of *cnn*^{hk21} (null) embryos (Figures 1D and 1E); such a result is further indication of defective cleavage furrows. Furthermore, in *cnn*^{B4} embryos some PCM markers had a looser association with the centrosome in comparison to wild-type (Figures S3–S5). Despite this PCM defect, *cnn*^{B4} centrosomes were very efficient MTOCs, producing robust astral microtubules similar to wild-type centrosomes (Figure 1E and Figures S2 and S4).

To assess the kinetics of microtubule assembly from *cnn*^{B4} centrosomes, we performed a microtubule regrowth assay in larval neuroblasts (Figure S2). The *cnn*^{B4} centrosomes assembled astral microtubules at prophase rapidly, similar to wild-type centrosomes. In contrast, no astral microtubules assembled in *cnn*^{hk21} mutant neuroblasts, where the MTOC activity of the centrosome is severely deficient [4]. Moreover, the microtubule nucleation and assembly factors γ-tubulin, D-TACC, and Msps were localized to *cnn*^{B4} centrosomes (Figures S3–S5). However, CNN^{B4} localization is more disperse than wild-type, and the level of CNN signal at these centrosomes was ~28.8% higher compared to that of wild-type (Figure S3). The dispersed CNN^{B4} PCM may expose more surface area for antigen sites upon antibody staining, making the level of CNN at centrosomes appear artifactually higher in *cnn*^{B4} embryos. Nevertheless, it appears that the level of CNN^{B4} is not less and may even be greater than wild-type CNN at centrosomes. In contrast, the level of γ-tubulin at *cnn*^{B4} centrosomes was ~30.5% less compared to that in the wild-type (Figure S3).

Central spindle and astral microtubules are important for furrow assembly at cytokinesis [8, 9]. Therefore, the role of the centrosome in actin organization at the embryonic cortical membrane may rely on the MTOC activity at centrosomes. However, despite the proficient MTOC activity at *cnn*^{B4} mutant centrosomes, furrow assembly was severely deficient (compare Figure 1F to Figure 1G). Thus, MTOC activity per se is insufficient for the proper assembly of actin into furrows at mitosis; such a finding is consistent with previous experiments indicating that MTOC and actin-organizing activities may be partly separate activities for the centrosome [10]. However, given that microtubules are required at anaphase for cleavage furrow assembly in the following prophase [11], we cannot exclude the possibility that qualitative features of the microtubules or alterations in their dynamics are altered at *cnn*^{B4} centrosomes. Furthermore, in contrast to *cnn*^{hk21} null embryos, which lack any perceptible actin organization at the cortex [6, 12], *cnn*^{B4} centrosomes were partially competent to organize actin into cleavage furrows, attesting to the hypomorphic nature of this mutation.

The CM2 Domain of CNN Interacts with Centrocortin

The *cnn*^{B4} point mutation resides in CM2, the conserved C-terminal 60 amino acid domain of CNN (Figure 1A and Figure S1A). Thus, CM2 is essential for CNN function in vivo, specifically for cleavage furrow assembly. How centrosomes coordinate furrow assembly with the cleavage cycle at the cortex is unknown, but we reasoned that proteins that associate with CM2 may mediate this function. Therefore, we screened for interacting partners of CM2 in order to discern the molecular signal conveyed from the centrosome to the

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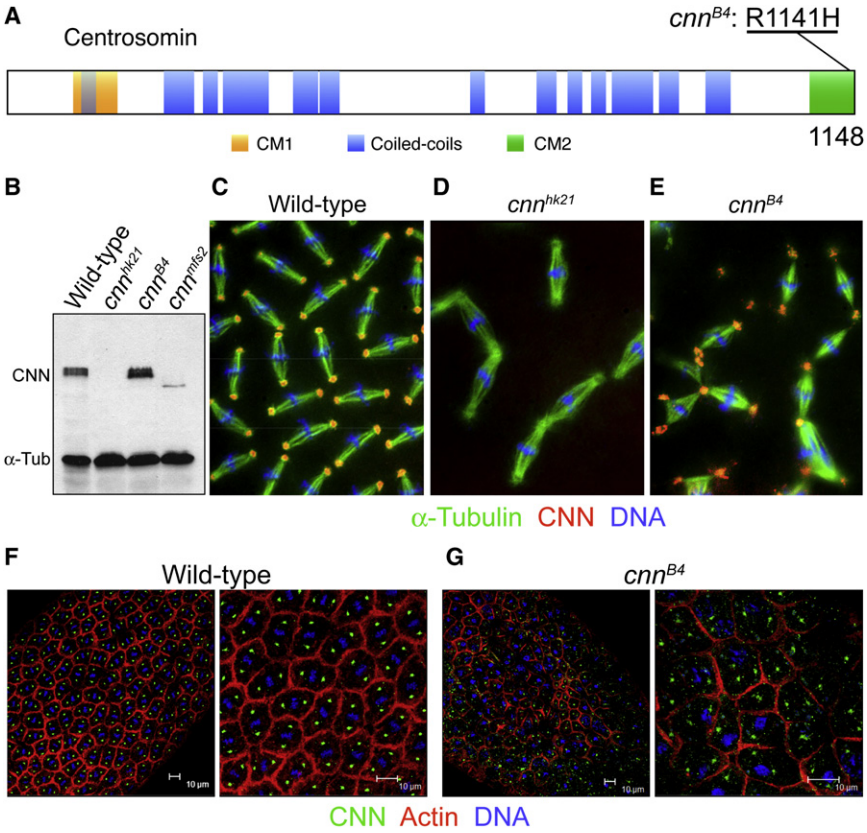

Figure 1. Centrosomin Motif 2 Is Required for Cleavage Furrow Formation Independent of Centrosome Localization

(A) Schematic drawing of CNN showing the positions of predicted coil-coil regions in blue, Centrosomin Motif 1 (CM1) in orange, and Centrosomin Motif 2 (CM2) in green. The position of the missense mutation in *cnn*^{B4} is indicated.

(B) Western blot for CNN shows that CNN^{B4} is expressed at levels similar to wild-type CNN. Proteins expressed by *cnn*^{hk21} and *cnn*^{mfs2}, truncations at amino acid positions 105 and 947, respectively [7], are shown for comparison. α-Tub was used as a loading control.

(C–E) Wild-type and *cnn* mutant embryos were stained for CNN, α-tubulin, and DNA. CNN^{B4} localized to centrosomes, unlike CNN^{hk21}. However, *cnn*^{B4} embryos had the linked-spindles phenotype similar to *cnn*^{hk21} embryos.

(F and G) Wild-type and *cnn*^{B4} embryos were stained for CNN, actin, and DNA. Note that *cnn*^{B4} embryos form partially organized actin structures, unlike *cnn*^{hk21}, which lacks any actin organization [6, 12].

cortex. By using CM2 (the C-terminal 67 amino acids of CNN) as bait in a yeast two-hybrid screen, we identified one interacting partner encoded by a cDNA for the *CG1962* gene, which we hereafter refer to as *centrocortin* (*cen*) because of the association of CEN protein with the centrosome and the cortex (see below). In the two-hybrid assay, the CEN-CNN interaction was reduced when the CNN^{B4} protein was tested (Figures 2A and 2B), suggesting a functional link between CEN-CNN association and CM2 function.

cen is an uncharacterized gene with orthologs in mammals including two in humans: *cerebellar degeneration related-2* (*Cdr2*) and *Cdr2-like* (*Cdr2L*)/*Cdr3* (Figure 2C and Figure S1B). Cdr2 is an autoimmune antigen targeted by "anti-Yo" antibodies associated with paraneoplastic cerebellar degeneration [13]. CEN is a 790 amino acid protein with a highly conserved 110 amino acid domain near its amino terminus. CEN is similar to human Cdr2L and Cdr2 (Figure 2C and Figure S1B), and to orthologs found throughout metazoans (not shown), within this domain. Cdr2 is reported to bind to the transcription factor Myc, sequestering it in the cytoplasm, through the association of the helix-leucine zipper motifs of Cdr2 and Myc [14]. CEN, however, does not contain a predicted leucine zipper in the conserved domain, appearing more similar to Cdr2L in this regard, and therefore may not share the Myc-binding property of Cdr2. Other than Myc inhibition, the function of Cdr2 remains largely unknown, and no activity has been attributed to Cdr2L.

To further verify the interaction between CNN and CEN, we performed coimmunoprecipitation from embryo and S2 cell lysates. We detected a weak association between CNN and CEN with this assay, but this was inefficient regardless of varied attempts and buffer conditions (Figure S6). We suspect that the major association between these partners occurs at embryonic centrosomes (see below) and thus complicates coIP analysis given that centrosomes, which are large organelles, typically pellet at low g forces during the preparation of lysates. Thus, we were unable to detect a strong association between CNN and CEN by IP.

CEN Localizes to Centrosomes and to Cleavage Furrows

We raised antibodies against the amino and carboxyl ends of CEN in order to examine its expression pattern and localization in embryos. CEN is expressed at high levels in ovaries and early embryos and then drops to very low levels in late embryos and in larval stages (Figure S7A). We also examined the localization of CEN during early embryogenesis to determine whether it colocalizes with CNN at centrosomes. To accomplish this, we used affinity-purified CEN antibodies, as well as examined the localization of green fluorescent protein (GFP)-tagged CEN fusion proteins expressed from transgenes. Immunofluorescent staining revealed that CEN is localized to centrosomes in a unique and dynamic pattern. CEN localized to a structure adjacent to centrosomes, with some overlap with CNN at centrosomes (Figures 2D and 2E). At late syncytial cleavage cycles, beginning at cycle 10, CEN localized to a discrete particle between centrosome pairs at interphase or early prophase (Figure 2D). Upon centrosome separation at mitosis, the major CEN puncta segregated asymmetrically with one of the two centrosomes. At mitosis, the CEN dot associated with one centrosome per mitotic spindle. The signal for CEN at this pericentrosomal particle was dispersed at telophase (Figure 2D). The majority of mitotic spindles showed this asymmetric pattern of CEN localization; however, some figures showed localization to both centrosomes, whereas, in others, neither harbored CEN puncta or harbored only a weak signal (Figure 2D and Figure S8A). CEN did not localize to centrosomes or other subcellular structures in early embryos prior to cycle 10. CEN localized at