Genes involved in centrosome-independent mitotic spindle assembly in *Drosophila* S2 cells

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Animal mitotic spindle assembly relies on centrosome-dependent and centrosome-independent mechanisms, but their relative contributions remain unknown. Here, we investigated the molecular basis of the centrosome-independent spindle assembly pathway by performing a whole-genome RNAi screen in *Drosophila* S2 cells lacking functional centrosomes. This screen identified 197 genes involved in acentrosomal spindle assembly, eight of which had no previously described mitotic phenotypes and produced defective and/or short spindles. All 197 genes also produced RNAi phenotypes when centrosomes were present, indicating that none were entirely selective for the acentrosomal pathway. However, a subset of genes produced a selective defect in pole focusing when centrosomes were absent, suggesting that centrosomes compensate for this shape defect. Another subset of genes was specifically associated with the formation of multipolar spindles only when centrosomes were present. We further show that the chromosomal passenger complex orchestrates multiple centrosome-independent processes required for mitotic spindle assembly/maintenance. On the other hand, despite the formation of a chromosome-enriched RanGTP gradient, S2 cells depleted of RCC1, the guanine-nucleotide exchange factor for Ran on chromosomes, established functional bipolar spindles. Finally, we show that cells without functional centrosomes have a delay in chromosome congression and anaphase onset, which can be explained by the lack of polar ejection forces. Overall, these findings establish the constitutive nature of a centrosome-independent spindle assembly program and how this program is adapted to the presence/absence of centrosomes in animal somatic cells.

mitosis | meiosis | anastral | Aurora B | centrosomin

Chromosome segregation during mitosis/meiosis is mediated by a microtubule (MT)-based bipolar spindle structure. Mitotic spindle assembly in animal somatic cells was initially believed to rely exclusively on the presence of centrosomes, but it is now well established that centrosomes are not essential (1–6). Land plants and many animal oocytes are paradigmatic examples in which an MT-based spindle normally assembles without centrosomes (7, 8). More recently, it was shown that spindle assembly during somatic cell divisions in the early mouse embryo is also independent of centrosomes (9) and that centrosomes are fully dispensable in planarians throughout their development (10). Overall, these data support the existence of centrosome-independent mechanisms that mediate mitotic/meiotic spindle assembly in animals.

Acentrosomal spindle assembly has been particularly well characterized in *Xenopus laevis* egg extracts, in which MTs form in the vicinity of mitotic chromatin due to a stabilizing effect imposed by a Ras-related nuclear protein in the GTP-bound state (RanGTP) gradient. RanGTP is present at highest concentrations around chromosomes, due to the localization of the Ran guanine nucleotide exchange factor regulator of chromosome condensation 1 (RCC1) on chromosomes (11). However, it remains controversial whether the gradient of RanGTP is required for spindle assembly in other systems (12, 13). Some of the downstream effectors of RanGTP include TPX2 and augmin, which promote MT assembly (14, 15). The chromosomal passenger complex (CPC) has also been implicated in acentrosomal spindle assembly/function in *X. laevis* egg extracts, as well as in *Drosophila* and mouse oocytes, and is believed to function independent of RanGTP (16–20). However, despite significant recent progress, a full picture of the molecular mechanisms behind acentrosomal spindle assembly in animal somatic cells is lacking. Moreover, it remains unknown whether the genes involved in acentrosomal spindle assembly are just a subset of those required when centrosomes are present or include specific genes that become essential only when centrosomes are compromised/absent.

Here, we investigated the gene requirements for acentrosomal spindle assembly in *Drosophila* S2 cells by performing a whole-genome RNAi screen. We found that virtually the same set of genes is involved in spindle assembly either with or without centrosomes, although a small subset of genes exhibit a different specific phenotype in the presence or absence of centrosomes.

Results

Whole-Genome RNAi Screen for Identifying Genes That Affect Mitotic Spindle Assembly in *Drosophila* S2 Cells in the Absence of Functional Centrosomes. In a previous study, we performed a whole-genome RNAi screen for identifying genes involved in mitotic spindle

Significance

The mitotic spindle, a structure composed primarily of microtubules, guides the segregation of DNA during cell division. In somatic animal cells, centrosomes (microtubule nucleating structures) reside near the mitotic spindle poles. However, germ cells lack centrosomes, and even somatic cells can execute cell division if centrosome function is compromised. This study on *Drosophila* cells reports a whole-genome RNAi screen for genes involved in spindle assembly in the absence of functional centrosomes. The results show that spindle assembly pathways with and without centrosomes involve an essentially identical set of genes, demonstrating the constitutive nature of centrosome-independent spindle assembly. However, certain gene knockdowns show distinct phenotypes when centrosomes are absent, thus revealing how spindles adapt to the presence or absence of centrosomes.

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The authors declare no conflict of interest.

Data deposition: The CNN spindle screen database is available at http://rnai.ucsf.edu/cnn.

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assembly in *Drosophila* S2 cells (21). In this study, we performed a similar RNAi screen using the same protocol but now in the absence of functional centrosomes by performing systematic RNAi knockdown of centrosomin (Cnn), the centrosome-localized docking factor for the γ-tubulin ring complex (γ-TuRC) that nucleates MT assembly (22). To improve acquisition performance due to the low mitotic index of S2 cells, automated microscopy was initially conducted in a cell division cycle 27 (Cdc27) (a subunit of the anaphase promoting complex) RNAi background as before (21). In the absence of Cnn (Fig. S1*A*), S2 cells form functional mitotic spindles through an acentrosomal pathway. However, we have found a slight but statistically significant delay from nuclear envelope breakdown (NEB) to the alignment of the last chromosome (metaphase) and the subsequent onset of anaphase (Fig. 1 *A*–*C* and Movies S1 and S2). This is consistent with recent findings implicating centrosome-mediated polar ejection forces in chromosome alignment and stabilization of kinetochore–MT attachments in S2 cells (23).

After generating galleries of spindles (typically ∼100) for each gene knockdown, spindles were blindly scored for various phenotypic defects, including shape, size, chromosome alignment, pole focusing, and MT abundance (Fig. 1 *D*–*F*). The initial screen revealed 187 of the 205 genes identified in our earlier study (21) plus 191 potential new hits that were not previously annotated. To rule out common off-target effects (24, 25), these 191 potential new hits were rescreened with new dsRNAs covering different target sequences (Table S1). Forty-five of the 191 rescreens yielded phenotypes in acentrosomal spindle assembly. We then repeated the RNAi for these 45 genes by high-resolution microscopy analysis and without Cdc27 RNAi. From this group, 10 genes displayed a highly consistent spindle phenotype (Table 1). While doing the screen, two of the 10 genes (encoding the newly detected augmin subunit Dgt7 and Mob4) were reported to result in abnormal spindles upon RNAi in the presence of functional centrosomes (26, 27).

Of the remaining eight genes without reported phenotypes, one gene produced a chromosome misalignment phenotype and generally defective spindles and encoded a homolog of mammalian survivin [termed deterin (28)]. The other seven genes displayed short spindles (Table 1 and Fig. S2 *A* and *B*). Two of these genes have previously described functions. One gene (CG5127) is a *Drosophila* Sec1 family member (Vps 33B) implicated in trafficking of proteins from the Golgi to vacuoles (29). The second gene (CG3983 or NS1) is the *Drosophila* homolog of nucleostemin, a nucleolar GTPase involved in ribosome biogenesis (30). The other five short-spindle phenotype genes (CG8801, CG1240, CG7993, CG6619, and CG32667) were poorly described in terms of function. Although the above eight genes did not emerge as hits from our initial mitotic screen (21), we rescreened their RNAi phenotypes in S2 cells without simultaneous Cnn RNAi. In all eight cases, the same phenotype was observed with centrosomes as without; thus, these genes must have been missed in our original RNAi screen. Hence, at the conclusion of our screenings, we did not identify a single gene whose depletion by RNAi specifically affected acentrosomal spindle assembly.

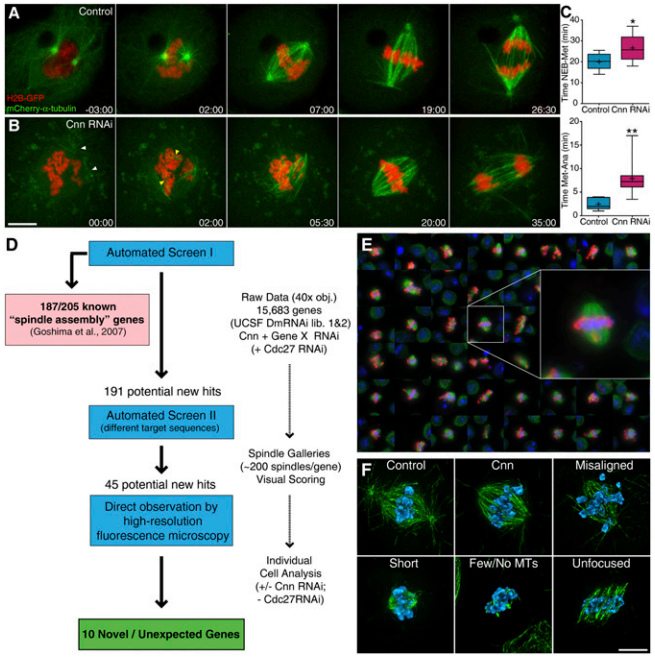

Fig. 1. Genome-wide screen for genes required for acentrosomal spindle assembly in *Drosophila* S2 cells. (*A* and *B*) Live imaging of S2 cells stably expressing H2B-GFP (red) and mCherry–α-tubulin (green). Cnn RNAi cells nucleate MTs in the vicinity of the nuclear envelope (white arrowheads) and near chromatin (yellow arrowheads). Time is expressed as minutes:seconds. (*C*) Chromosome congression and the subsequent onset of anaphase are delayed in Cnn RNAi cells. The boxes on whisker plots shows 75% of the values, and the bars show the total range of values. The line in the middle of the boxes is plotted at the median, and "+" represents the mean ($n = 10$ cells per condition; $^*P < 0.05$, $^{**}P < 0.001$ per t test). (*D*) Flow chart of the screening procedure and summary of results. (*E* and *F*) Examples of a typical spindle gallery and most common spindle phenotypes scored. α-Tubulin (green), DNA (blue), and phosphohistone H3 (red in *B*) are shown. (Scale bars: 5 μm.)

Further Characterization of Spindle Assembly Genes. To characterize the eight spindle assembly genes identified in the screen better, we sought to determine their intracellular localization by GFP tagging. Deterin-GFP localized at centromeres from prophase until early anaphase and at spindle midzone in late anaphase and telophase. This localization is typical for chromosome passenger proteins. This result, together with the sequence similarity with mammalian survivin, indicates that deterin is the survivin ortholog in *Drosophila* (Fig. 2 *A* and *B* and Movie S3).

For four genes, NS1 and three other previously unknown genes (CG8801, CG1240, and CG7993), the GFP-tagged proteins localized in the nucleolus during interphase, without any specific localization into obvious mitotic structures (Fig. S2 *C*–*F* and our unpublished observations). Because of their localization, we renamed CG8801, CG1240, and CG7993 as novel nucleolar proteins 1, 2, and 3 (Non1–3, respectively). For the last three short-spindle phenotype genes (CG6619, CG32667, and CG5127), we did not observe a specific localization in interphase or mitosis for the GFP-tagged genes (our unpublished observations). Following previously proposed nomenclature (21), the two uncharacterized proteins encoded by CG6619 and CG32667 were named short spindle proteins 6 and 7 (Ssp6 and Ssp7, respectively).

In our previous screen (21), we also identified two genes encoding nucleolar proteins whose knockdown results in a short-spindle phenotype. Furthermore, knockdown of numerous genes encoding ribosomal subunits or treatment with the ribosome inhibitor cycloheximide also produces a short-spindle phenotype, perhaps due to limited translation of tubulin and/or other necessary spindle components. Thus, it is plausible that the knockdown of these nucleolar proteins produces their spindle phenotypes by impairing ribosome biogenesis. Thus, the uncharacterized nucleolar proteins that we have identified may be good candidates to investigate in the future for roles in ribosome biogenesis. However, we cannot exclude the possibility that nucleolar genes might have more direct roles in spindle formation.

Chromosomal Passengers Regulate Multiple Centrosome-Independent Aspects Required for Mitotic Spindle Assembly and Maintenance. Given the previous implication of the CPC in acentrosomal

Table 1. Uncharacterized/unexpected genes required for acentrosomal spindle assembly in *Drosophila* S2 cells

Gene name	CG no.	Phenotype	Functional annotations
Deterin	CG12265	Misaligned chromosomes/ generally defective	Negative regulation of apoptosis; *Homo sapiens* survivin, chromosomal passenger protein
Dgt7*	CG2213	Long spindles/unfocused	Augmin subunit (27)
Mob4*	CG3403	Unfocused spindles	Spindle pole focusing (26)
Non1	CG8801	No/defective/short spindles	GTP binding, Ras-like GTPase, NOG domain; *H. sapiens* GTPBP4/CRFG
Non2	CG1240	Short spindles/misaligned chromosomes	DEK C-terminal domain; SWIB/MDM2 domain
Non3	CG7993	No/defective/short spindles	Brix domain; *H. sapiens* RPF2
Nucleostemin 1	CG3983	Short spindles	GTP binding; regulation of insulin receptor signaling; growth regulation
Ssp6	CG6619	Short spindles/misaligned chromosomes	*H. sapiens* "cytohesin binding protein HE"; *Mus musculus* "MT-associated serine/threonine protein kinase MAST205"
Ssp7	CG32667	Short spindles	Multicellular organism reproduction
Vps 33B	CG5127	No/defective/short spindles	Vacuolar protein sorting 33B; vesicle-mediated transport; vesicle docking during exocytosis

CG, computed gene; DEK, acute myeloid leukemia-associated protein; NOG, nucleolar GTP-binding; SWIB/MDM2, SWI/SNF complex, including complex B/murine double minute 2.
*Identified independently during the course of this study.

spindle assembly in meiotic systems (17–19), we sought to investigate the role of deterin and the CPC in mitotic spindle morphogenesis/function and the respective dependence on centrosomes in animal somatic cells. For this purpose, we followed spindle assembly and chromosome behavior at high resolution after depletion of deterin or Aurora B inhibition with the *Drosophila*-specific small-molecule binucleine 2 (31), with or without Cnn, in live S2 cells (Fig. 2 *C*–*F*, Fig. S3*A*, and Movies S4 and S5). We found that upon these perturbations, the vast majority of cells were able to organize MTs and ∼50% of them assembled a bipolar spindle of normal length, regardless of the presence of centrosomes (Fig. 2 *E*, *F*, and *H* and Fig. S3*B*). The other 50% of the cells showed severe spindle abnormalities, such as multiple minispindles or few MTs (Fig. 2 *C*, *D*, and *H*), suggesting that the CPC is important for MT stability and bipolar spindle organization. Strikingly, all cells initiated chromosome decondensation and spindle disassembly despite the presence of spindle abnormalities and incomplete chromosome alignment (Fig. 2 *C*–*F*), presumably due to premature anaphase onset. Indeed, the time from NEB to chromosome decondensation was shorter upon perturbation of deterin or Aurora B and comparable to a loss of SAC function after Mad2 RNAi (Fig. 2*I*). This uncontrolled mitotic exit was fully rescued by preventing anaphase onset with Cdc27 RNAi, without significant changes in the observed spindle phenotypes (Fig. 2 *G* and *H*, Fig. S3*B*, and Movie S6).

To investigate whether the SAC was compromised upon perturbation of deterin or Aurora B, we challenged cells with the MT depolymerizing drug colchicine. We found that codepletion of Cnn and deterin, which prevented recruitment of Aurora B to centromeres (Fig. S3 *C* and *D*), or binucleine 2 treatment (with or without Cnn) significantly accelerated DNA decondensation in the presence of colchicine (Fig. 2*J*), indicating a role for the CPC in SAC response. Interestingly, some mitotic spindle abnormalities prevailed upon Aurora B inhibition even in conditions that prevented mitotic exit in a SAC-independent manner (Fig. 2*H* and Fig. S3 *E*–*G*), suggesting that the CPC regulates additional aspects required for spindle assembly that are not exclusively related to their role in SAC response.

Previous studies in meiotic spindles assembled in vitro have suggested that CPC regulates the activity of the kinesin-13 mitotic centromere-associated kinesin to permit spindle formation via stabilization of chromatin-associated MTs (17). Interestingly, RNAi-mediated depletion of Klp10A, the only *Drosophila* kinesin-13 implicated in spindle assembly (see also ref. 21), rescued the "few MTs" phenotype but was unable to reestablish spindle bipolarity in binucleine-treated cells (Fig. 2*H*). Thus, the CPC orchestrates multiple centrosome-independent processes behind mitotic spindle assembly/maintenance in *Drosophila* somatic cells that go beyond the regulatory roles in the SAC and control of kinesin-13 activity.

Gene Knockdowns That Have Distinct Phenotypes in the Presence or Absence of Centrosomes. Although our genome-wide acentrosomal spindle RNAi screen identified a similar set of "hits" as the screen in the presence of centrosomes, we carefully examined the image galleries of the identified 197 genes to see if there were any obvious differences in spindle phenotypes in the two backgrounds (centrosomes vs. no centrosomes). We excluded obvious phenotypic differences that involve the centrosome directly (e.g., genes required for centrosome formation, monastral bipolar spindles, centrosome-positioning defects). We identified 40 genes whose spindle phenotypes were markedly different with and without centrosomes (Fig. 3*A*). One of the most obvious phenotypic differences was in specific gene knockdowns that produced poorly focused poles in acentrosomal spindles but focused poles for centrosome-containing spindles. These genes included many members of the t-complex polypeptide-1 (TCP-1) chaperone complex [involved in folding various proteins, including actin and tubulin (32)], proteins associated with γ-tubulin (γ-tubulin itself and Dgrips 71 and 128), several proteasome complex subunits, and the phosphatase PP2A 29B (Fig. 3 *A* and *B*). Strikingly, the same phenotype was seen in S2 cells that occasionally went through mitosis with a monastral bipolar spindle (e.g., proteasome β4 subunit RNAi in Fig. 3*B*), demonstrating in a single cell that the activity of these genes in pole focusing can be rescued by an intact centrosome. It is interesting that knockdown of many genes in this category (γ-TuRC, tubulin chaperones, and several proteasome subunits) results in a lower amount of MTs in the spindle (Fig. 3*A*), and perhaps the centrosome plays a more important role in pole focusing under these circumstances. It is unclear how tubulin chaperones and proteasome activity specifically affect pole focusing. However, many tubulin chaperones have been localized to the centrosomes, where they play a role in MT nucleation/organization (33). Moreover, γ-tubulin folding is chaperone-mediated (34), and a fraction of γ-tubulin exists in a complex with TCP-1 (35). Similarly, several proteasome components associate with centrosomes, with the extent of

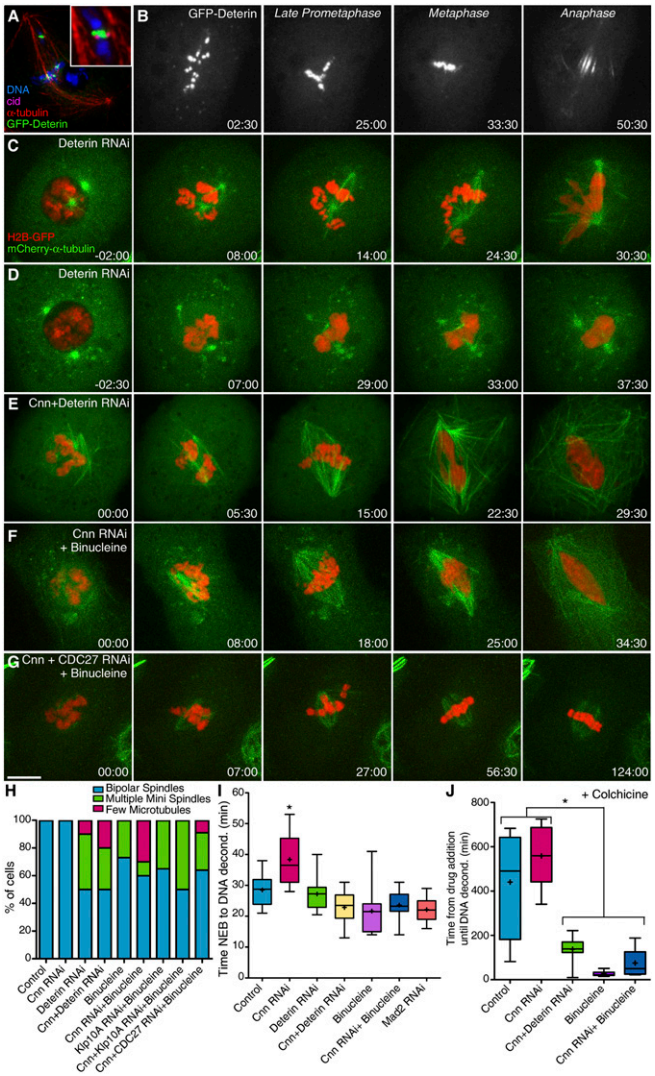

Fig. 2. Chromosomal passengers regulate multiple centrosome-independent processes required for spindle assembly/maintenance in S2 cells. (*A*) Localization of GFP-deterin at inner centromeres. (*B*) Live imaging of S2 cells stably expressing GFP-deterin shows the typical CPC localization. (*C*–*G*) Live imaging of S2 cells stably expressing H2B-GFP (red) and mCherry–α-tubulin (green) after deterin RNAi or Aurora B inhibition, with and without functional centrosomes (Cnn RNAi). In *G*, cells were treated with Cdc27 RNAi to prevent anaphase onset. Time is expressed as minutes:seconds. (Scale bar: 5 μm.) (*H*) Quantification of spindle abnormalities in different experimental conditions. (*I*) Quantification of NEB to DNA decondensation duration in different experimental conditions. (*J*) Quantification of the duration between colchicine addition and chromosome decondensation in different experimental conditions represented as whisker plots. In both *I* and *J*, $n = 10$ cells per condition and the asterisk indicates statistically different conditions relative to controls (*$P < 0.0001$, Dunnett's test).

accumulation being dependent on the level of misfolded proteins (36). Proteasome activity is also necessary to maintain mitotic spindle pole integrity and bipolarity, as well as centrosome-dependent MT nucleation/organization (37, 38). Thus, a picture where proteasome function is required for normal centrosome/spindle pole function is emerging, perhaps in response to the presence of misfolded proteins such as γ-tubulin.

The other clear phenotypic difference was marked by the formation of multipolar spindles exclusively in the presence of centrosomes upon RNAi of a specific cohort of genes associated with cytokinesis (e.g., Pavarotti/kinesin-6, RacGAP50C, CPC), MT focusing at spindle poles (e.g., Ncd/kinesin-14), and kinetochore–MT attachments (e.g., Ndc80, Bub1, CPC) (Fig. 3 *A* and *B*). Multipolar spindles are a common feature of human cancer cells. However, similar to human cancer cells, *Drosophila* S2 cells normally cluster multiple centrosomes into a common pole, assembling a bipolar spindle (39–41). It is interesting that many of the genes associated with spindle multipolarity only in the presence of centrosomes have been previously implicated in centrosome clustering in *Drosophila* and human cells (40, 42), suggesting that the presence of multiple centrosomes drives spindle multipolarity and can be highly disruptive in those specific genetic backgrounds.

Role of a RanGTP Gradient in Mitotic Spindle Assembly/Function in *Drosophila* S2 Cells. A RanGTP gradient has been proposed to play an important role in chromatin-mediated MT nucleation and spindle assembly (reviewed in ref. 43). However, a gene that was notably absent as a hit from our screen was RCC1, the guanine nucleotide exchange factor for Ran, which is localized to chromatin. Thus, we decided to retest the RNAi for RCC1 [Bj1 in *Drosophila* (44)] and to evaluate the level of protein knockdown, as well as the effect on the RanGTP levels and gradient. After a single RNAi pulse for 4 d or two pulses for 7 d, >95% of RCC1 was depleted from different S2 cell lines (Fig. 4*A* and

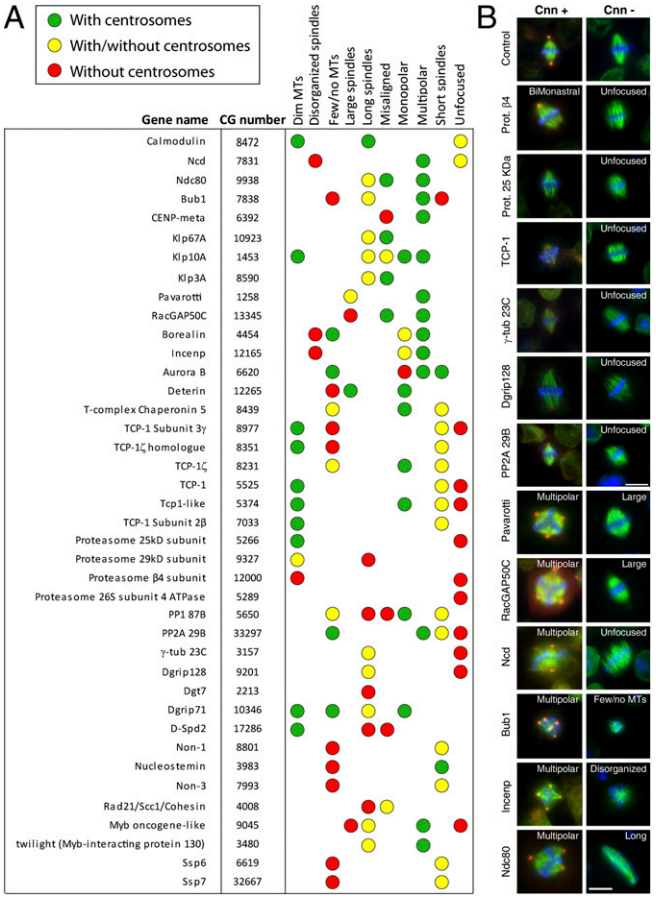

Fig. 3. Comparative analysis of spindle assembly with and without functional centrosomes. (*A*) Summary of phenotypes as a function of the presence of centrosomes upon RNAi of particular genes that showed different phenotypes with/without centrosomes. Large spindles refer to spindles that are wider than controls along the metaphase plate axis. (*B*) Representative spindles illustrate genes whose depletion leads to a different phenotype with (Cnn+) and without (Cnn–) functional centrosomes. MTs (green), DNA (blue), and centrosomes (γ-tubulin; red) are shown (Scale bar: 10 μm.)

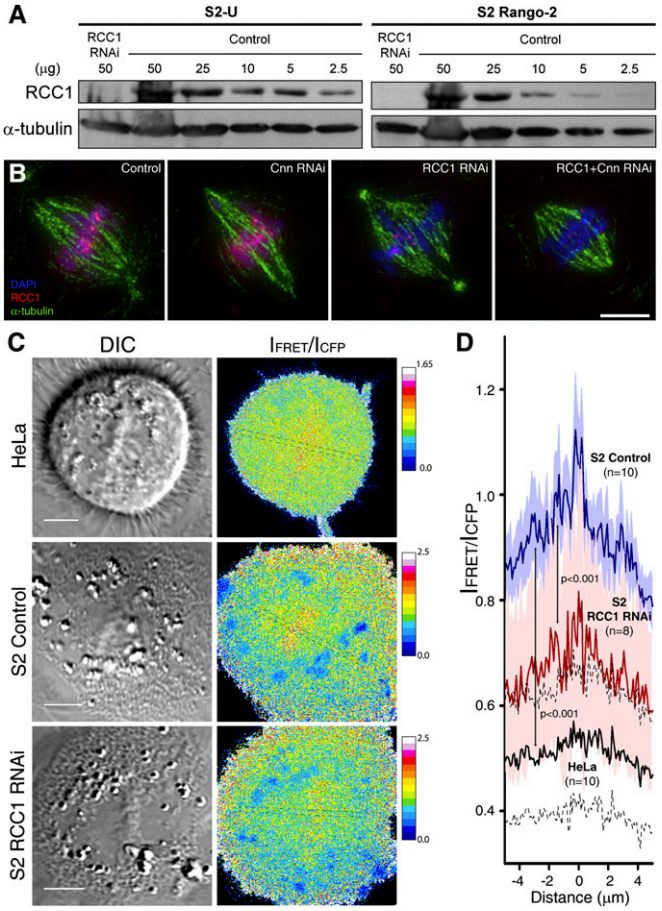

Fig. 4. Steep RanGTP gradient from chromosomes is present in S2 cells and is sensitive to RCC1 levels. (*A*) Quantitative Western blot analysis of RCC1 depletion by RNAi in different S2 cell lines. α-Tubulin was used as a loading control. (*B*) Localization of RCC1 (red) in control mitotic S2 cells and after depletion of Cnn, RCC1, and RCC1 + Cnn. (*C*) Differential interference contrast (DIC) (*Left*) and FRET/CFP emission ratio intensity (*Right*) images of representative mitotic HeLa, control S2, and RCC1-depleted S2 cells are shown. (*D*) Average line scans (solid lines) for each condition were obtained over a width of 10 pixels for FRET/CFP intensity ratios perpendicular to the metaphase plate axis from the indicated cell numbers. The corresponding colored backgrounds or dashed-line scans indicate the variability of the measurements, here represented as the SEM. The difference between the measured FRET/CFP intensity ratios along the spindle in S2 RCC1 RNAi or HeLa cells is statistically significant from S2 control cells ($P < 0.001$; Mann–Whitney test). (Scale bars: 5 μm.)

Fig. S1*A*). RCC1 depletion from chromosomes was also confirmed by immunofluorescence on mitotic cells (Fig. 4*B*).

To test whether the RanGTP gradient was indeed sensitive to RCC1 depletion, we adapted the previously developed Rango-2 FRET sensor (12) for stable and constitutive expression in S2 cells (S2 Rango-2). In this system, increased FRET reports cargo release induced by the binding of RanGTP to importins and correlates with a high local concentration of free RanGTP produced by RCC1 on chromosomes. We found that FRET from the Rango-2 sensor was high around the chromosomes of the metaphase plate and then dissipated toward the poles of the S2 cells. Strikingly, S2 cells showed a much steeper and intense RanGTP gradient from chromosomes compared with HeLa cells (Fig. 4 *C* and *D*), a cell type in which a RanGTP gradient from chromosomes has been previously reported (12, 45). This may be explained by the "semiopen" nature of mitosis in *Drosophila*, which may concentrate RanGTP and associated cargo in the spindle region (46). Importantly, the intensity of this gradient was sensitive (but not completely abolished) to RCC1 depletion in S2 cells ($P < 0.001$, Mann–Whitney test; Fig. 4 *A*, *C*, and *D*), thus validating the Rango-2 sensor as a bona fide reporter for RanGTP in this system. Surprisingly, S2 cells depleted of >95% of RCC1 showed no significant differences in spindle assembly, function, length, mitotic duration, and fidelity relative to controls, with or without the presence of functional centrosomes (Fig. S1 *B* and *C* and Movie S7). Moreover, after challenging S2 cells to repolymerize their MTs after cold-induced depolymerization, we found no major differences in bipolar spindle reassembly upon RCC1 RNAi, with or without Cnn (Fig. S4 *A*–*C*). Finally, depletion of RCC1 did not exacerbate the defective spindle phenotypes observed in cells codepleted with Cnn and treated with binucleine-2 (Fig. S3 *F*–*H*). Overall, we conclude that despite the existence of a steep RanGTP gradient in S2 cells, its experimental attenuation did not compromise mitotic spindle assembly in *Drosophila* S2 cells. With the possible caveat that RNAi-mediated depletion of >95% of RCC1 did not completely abolish the formation of the RanGTP gradient, this result suggests that low levels of RanGTP suffice to drive MT production and spindle assembly in the absence of centrosomes and that there are alternative mechanisms involving the CPC that can produce MTs when both centrosomes and Ran pathways are compromised.

Conclusion

Here, we have identified eight genes involved in spindle assembly in S2 cells. We showed that mitotic spindle organization in the presence/absence of centrosomes is driven by a common set of genes. However, we identified a specific cohort of genes that differentially affect the formation of a bipolar spindle depending upon whether the centrosomes are present or not. In particular, we found that knockdown of γ-TuRC, 26S proteasome, and TCP-1 subunits all produced a much more obvious pole-focusing defect specifically when centrosomes are absent. Finally, we also found that spindle assembly in S2 cells is not affected by >95% depletion of the RanGTP effector RCC1 (see also ref. 47). These results suggest that either *Drosophila* S2 cell spindles are very robust to a decrease in RanGTP or that, alternatively, Ran-independent pathways compensate for the loss of RanGTP. The recently discovered Aurora B phosphorylation gradient along the spindle (48) may provide the necessary spatiotemporal cues for centrosome-independent MT stabilization and bipolar spindle formation. Indeed, previous findings have implicated the human CPC in spindle assembly (49) and in SAC response (50). Our present data link both processes and support that the CPC has an impact on mitotic spindle assembly/maintenance, in part, by regulating the duration of mitosis, regardless of the presence/absence of functional centrosomes.

Collectively, our RNAi screening results in *Drosophila* S2 cells suggest that a centrosome-independent spindle pathway operates constitutively during spindle assembly and does not require a distinct backup genetic mechanism. These data are fully consistent with recent transcriptome profiling studies of acentrosomal cells in *Drosophila* brains and wing discs (51). However, certain cell systems demonstrate a greater dependence on centrosomes for spindle assembly and furrow positioning during early embryonic divisions (52–54) or centrosomes might enhance cell division fidelity in mammalian somatic cells. Nevertheless, our conclusions support the view that centrosomes are not main drivers of spindle assembly during mitosis.

Materials and Methods

Two different *Drosophila* RNAi libraries were used in this screen: University of California, San Francisco (UCSF) DmRNAi library version 1, which only contains genes conserved in *Caenorhabditis elegans* and/or humans (approximately half of the genome), and UCSF DmRNAi library version 2, which contains genes that are not present in library version 1 (total of 14,425

genes). Both libraries were obtained from Open Biosystems. An additional set of 1,250 genes was added to these libraries and used in this screen, for a total of 15,683 genes. Primers for the selected region of each gene were designed with the Primer3 program (http://fokker.wi.mit.edu/primer3/input.htm), and the T7 promoter sequence was added to each primer. Plating of the entire library was performed with the help of the Biomek FX Laboratory Automation Workstation (Biomek FX; Beckman Coulter, Inc.). A detailed description of library generation can be found in the supplemental material in ref. 21. Additional methods used can be found in *SI Materials and Methods*.

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