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13. ABSTRACT *(Maximum 200 Words)*

Centrosomes are essential organelles that control mitotic spindle organization, chromosome segregation, cell shape and cell polarity--all features of epithelial gland integrity. In the studies supported by this grant, we (and another group) discovered that centrosomes were structurally and numerically abnormal in nearly all malignant human tumors. Moreover, artificial induction of centrosome defects--by elevating the levels of a single centrosome protein called pericentrin--caused cellular disorganization and genomic instability in nontumor cells. Examination of prostate tumor tissues showed that pericentrin levels were elevated and the levels increased during tumor progression. We consistently found that cells with elevated pericentrin levels had centrosome defects and genomic instability. Finally, centrosome defects were detected in a percentage of precursor lesions of prostate carcinoma (~20% of PIN lesions) together with genetic instability. Taken together, these resutls suggest that centrosome defects and elevated pericentrin levels contribute to rather than result from the tumorigenic process. Centrosome defects may thus prove to be a prognostic indicator of aggressive disease.

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Table of Contents

INTRODUCTION/ABSTRACT

Centrosomes are essential organelles that control a multitude of cellular functions. They are critical elements in the organization of the mitotic spindle and the accurate segregation of chromosomes during mitosis. They also control cell shape and cell polarity, which are fundamental properties of epithelial gland organization. We (and another group) were the first to discover that centrosomes are structurally and numerically abnormal in nearly all malignant human tumors. This striking observation has important implications for cancer progression since it suggests that centrosome defects might contribute to cytologic anaplasia and genomic instability that so often accompany these advanced cancers. Support for this idea came from our observation that centrosome defects, cytologic anaplasia and genomic instability could be artificially induced in nontumor cells by elevating the levels of a single centrosome protein called pericentrin. Based on these observations, and the knowledge that clinically aggressive prostate carcinoma (high Gleason grade) exhibits significant anaplasia, epithelial de-differentiation and genomic instability, we proposed an innovative hypothesis: that centrosome dysfunction may be a critical factor in prostate cancer progression. The most exciting aspect of our hypothesis and the rationale for this project, is that progressive centrosome dysfunction is the first biologic factor identified that can fully explain most of the phenotypic changes characteristic of prostate carcinomas during their progression from clinically indolent forms (majority) to clinically aggressive forms (minority). The specific aims of the original proposal were designed to test several features of this model. 1. Are centrosome defects present in malignant prostate carcinoma and in early prostate cancer where they have the potential to serve as prognostic markers for aggressive disease? 2. Can normal prostate epithelial cells be induced to express the tumor-like phenotype following overexpression of pericentrin and can these cells form prostate tumors in mice? 3. Can prostate tumor cells be selectively killed by exploiting the possibility that the tumor cells have defective cell cycle control mechanisms resulting from centrosome dysfunction? In this final progress report, we are excited to report that we have achieved most of the objectives stated in our proposal and we made several unexpected discoveries. To achieve these goals, we developed high-resolution imaging methods and quantitative assays to monitor centrosome defects and pericentrin protein levels in tissue sections of prostate cancers. Moreover, we subjected our results to rigorous statistical analysis and interpretation by establishing a collaboration with members of the Biostatistics Core at our Cancer Center. Thus far, our data unequivocally support our centrosome-mediated model for prostate cancer progression. We have shown that centrosome defects are present in malignant prostate cancer, increase with increasing histologic (Gleason) grade and directly correlate with genomic instability. We are most excited about two unexpected findings. 1. Centrosome defects and elevated pericentrin levels are found in some precursor lesions of prostate carcinoma even earlier than the most common diagnostic marker, prostate specific antigen (see Fig. 1). 2. Artificial elevation of pericentrin induces a prostate tumor-like phenotype in normal prostate cell lines and exacerbates this phenotype in prostate tumor cell lines (PC-3, Fig. 2). Importantly, both lines exhibit a very severe form of genetic instability. These unique observations are consistent with the idea that centrosome defects and elevated pericentrin levels contribute to rather than result from the tumorigenic process. We are currently testing the ability of recently constructed pericentrin-expressing cell lines to form tumors in mice. This work is highly relevant to prostate cancer biology because it has the potential to uncover a unique pathway for prostate cancer progression that may also be involved in the genesis of prostate cancer. Moreover, elucidation of the mechanisms and molecules (e.g. pericentrin) that contribute to severe centrosome dysfunction may identify new and powerful prognostic markers as well as novel cancer-specific therapeutic targets for clinically aggressive prostate cancer, the form of prostate carcinoma that is clinically most critical in terms of diagnosis, treatment and health care expenditure. We are continuing this work in a Phase II grant from the Department of Defense (PC000018).

REPORT BODY

Statement of Work (summarized from original grant application):

Task 1. Analysis of aggressive prostate tumors for centrosome defects ***(DONE)***
Clone pericentrin into vector with inducible promoter ***(DONE)***
Test prostate cell lines and control cells for the ability to overcome cell cycle checkpoints ***(DONE)***
Task 2. Analysis of early stage prostate tumors for centrosome defects ***(DONE)***
Establish permanent prostate cell lines expressing pericentrin ***(DONE)***
Determine relationship between centrosome defects and aneuploidy ***(DONE)***
Test ability of pericentrin-expressing cells using in vitro assays ***(underway, to be completed by 3/00)***
Test ability of pericentrin-expressing cells to form tumors in mice ***(underway, to be completed by 9/00)***
Test prostate cell lines and control cells for the ability to be selectively killed in S-phase after failure to undergo mitotic arrest ***(to be completed by 9/00 or in Phase II)***

Summary: During the funding period of this grant we made significant progress toward our research goals including several discoveries that were unexpected and particularly exciting. This work provides strong support for our centrosome-mediated model for prostate cancer progression (see manuscripts # 1-3, below) and raises the possibility that centrosome defects may be involved in the genesis of prostate tumors. In our model, progressive centrosome dysfunction leads to genomic instability (chromosome missegregation), cellular anaplasia and loss cell polarity and glandular organization. Using immunohistochemical methods to stain paraffin sections, we demonstrated that centrosome defects were present in nearly all malignant prostate tumors ($n=71$ tumors, 2556 individual measurements, $p<0.007$) and that they increased with increasing histologic grade (Gleason grade, $n=36$, $p<0.001$; Cancer Res. 58, 3974-3985, 1998; Pihan et al., in press., see below #6). We developed a high resolution imaging method, and used it to identify a unique structural defect in centrosomes that was tumor-specific. We also demonstrated that there was a close correlation between centrosome defects and genomic instability in prostate tumors. If centrosome defects contribute to the disease, they should be present early in the disease process. In fact, we unexpectedly found that precancerous lesions of the prostate (prostate intraepithelial neoplasia, PIN) had significant centrosome abnormalities (Fig. 1). Interestingly, only a fraction of PIN lesions had centrosome defects (~15%), raising the possibility that this fraction represents patients that develop aggressive disease. Another prediction of our model is that induction of centrosome defects in normal cells should produce tumor-like features. This was indeed the case. By elevating the levels of the centrosome protein pericentrin we artificially induced centrosome defects, genetic instability and anaplasia in normal COS cells (see manuscripts #4 and 5, below) and more recently in normal prostate cell lines ($p<0.007$, Pihan et al., in prep., see below #6). Elevated pericentrin levels also exacerbated these defects in a prostate tumor cell line (PC-3, $p< 0.001$, Fig. 2). To test whether pericentrin levels were altered in tumors, we developed a method for quantifying protein levels in paraffin sections and found that pericentrin levels were higher than in nontumor cells ($n=57$, $p<0.005$). More importantly, pericentrin levels appeared to be elevated in PIN lesions ($n=14$, $p<0.011$, Pihan et al., in prep., see below #7) suggesting that this is an early event in tumorigenesis. Taken together, this work supports our centrosome-mediated model for prostate cancer progression and raises the intriguing possibility that centrosome dysfunction may occur early in the disease. To directly test whether increased pericentrin levels can contribute to tumor development *in vivo*, we constructed tetracycline-inducible pericentrin-expressing prostate cell lines and introduced them into mice. We will examine the potential of these cells to induce (1542-NPTX) or exacerbate (PC-3, DU-145, LN-Cap) prostate tumor formation during the final months of this proposal. Based on the novelty of our findings, we have focused our efforts on these research areas. Consequently, we have had less time to address issues proposed in Aim 3, and we now agree with the reviewer's assessment that our original proposal was over-ambitious. Work on Aim 3 (killing tumor cells that harbor cell cycle checkpoint defects) will be continued in Phase II (if funded). We are encouraged about the potential success of this Aim as we discovered that pericentrin overexpression abrogates the mitotic checkpoint in normal cells (J. Rosa, S. Doxsey, unpublished). Elucidation of the mechanism of

prostate cancer progression would positively impact the lives of many individuals. As potential prognostic indicators, therapeutic targets and contributors to tumor progression, centrosome defects deserve further study.

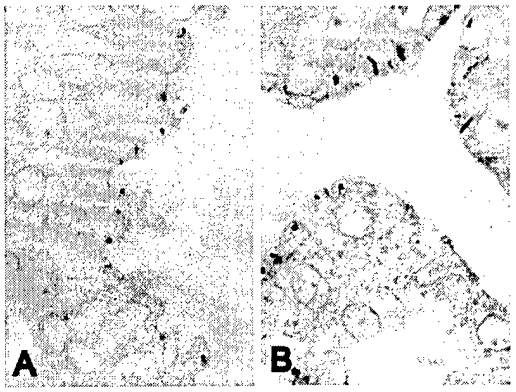

Fig. 1. Centrosome defects in PIN lesions (B) are not in normal tissue (A). Defects include enlarged and elongated centrosomes. A, B, same magnification.

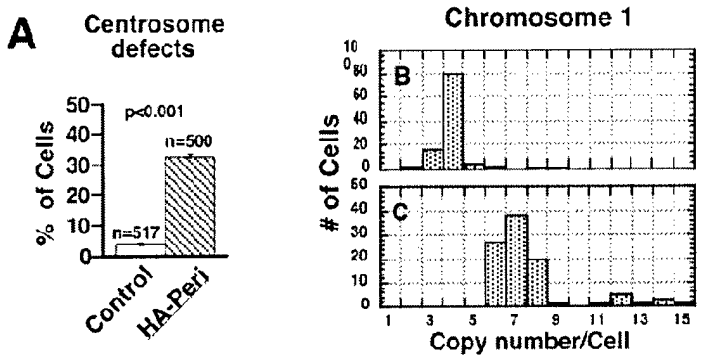

Fig. 2. Defects in centrosomes (A) and chromosomes (B, C) in a prostate tumor cell line (PC-3) +/- HA-pericentrin expression. Copy number of chromosome 1 in parent (B) and pericentrin-expressing cells (C) by *in situ* hybridization with centromeric probes.

RESEARCH ACCOMPLISHMENTS

- Centrosome defects are present in nearly all malignant prostate tumors.
- The centrosome protein pericentrin is elevated in prostate tumors.
- Pericentrin levels and centrosome defects increase with increasing histologic (Gleason) tumor grade.
- Centrosome defects directly correlate with genomic instability.
- Centrosome defects are found in some precursor lesions of prostate carcinoma (~20%) together with genetic instability.
- Artificial elevation of pericentrin induces a prostate tumor-like phenotype in normal prostate cell lines and exacerbates this phenotype in prostate tumor cell lines.

REPORTABLE OUTCOMES.

- **Manuscripts relating to this proposal (5 published, 1 in press, 1 in preparation):**

1. Pihan, G., Purohit, A., Knecht, H. Woda, B. Quesenberry, P. and Doxsey, S.J. Centrosome defects and genetic instability in malignant tumors. Cancer Res., 58, 3974-3985, 1998.
2. Pihan, G., Doxsey, S. The mitotic machinery as a source of genetic instability in cancer. Sem. Cancer Biol. 9, 289, 1999.
3. Doxsey, S.J. The centrosome--a tiny organelle with big potential. Nature Genet. 20, 104-106,1998.
4. Purohit, A. and Doxsey, S. Direct interaction of pericentrin with dynein light intermediate chain contributes to mitotic spindle organization. J. Cell Biol. 147, 481-491, 1999.
5. Diviani, D., Langeberg. L., Doxsey, S. and Scott, J. Pericentrin anchors protein kinase A at the centrosome through a newly identified RII-biding domain. Curr. Biol. 10, 417-420, 2000.
6. Pihan, G, Purohit,A, Doxsey S. Centrosome defects correlate with Gleason grade in prostate cancer. Cancer Res., in press for 3/2000.
7. Pihan, G., Wallace, J., Doxsey, S. Centrosome defects in precancerous lesions of the prostate [in prep].

- **Abstracts relating to Prostate Cancer (9 total, presented by S. Doxsey, G. Pihan, A. Purohit):**

1-4. Amer. Soc. Cell Biol. (4 total, 1998-1999, San Francisco, Washington, D.C.), **5.** Gordon Res. Conf. (Colby, N.H.), **6.** IMP Conf. (Research Inst. Molecular Pathol., Vienna, Austria), **7.** GFP Conf. (Second Intl. Symp. on GFP, San Diego, CA), **8, 9.** Conf. of the Intl. Acad.of Pathologists (1999, Boston; New Orleans, 2000), **10.** Amer. Soc. Cell Biol. (2000, San Francisco—***chosen for Press book release***).

♦ **Presentations on Prostate Cancer (12 total, presented by S. Doxsey, G. Pihan):**
In 1998: 1. Gordon research conference, Colby, NH.; **2.** Fred Hutchinson Cancer Research Center, Seattle, WA ***(Host: Brian Reid); 3.*** Universtiy of Utah, Huntsman Cancer Institute, Salt Lake City, UT ***(Host: Ray White); 4.*** National Cancer Institute, Bethesda, MD ***(Host: Lance Liotta); 5.*** University of Texas M.D. Anderson Cancer Center, Dallas, TX; **6.** American Society of Cell Biology Meeting, San Francisco, CA.
In 1999: 7. Massachusetts Dept. Public Health Symp.: *Progress in Prostate Cancer*; **8.** Second Intl. Symposium on GFP, *Centrosome and chromosome dynamics in living cells,* San Diego, CA; **9.** Symposium: *Centrosomes and chromosome segregation* Research Inst. of Molecular Pathol., Vienna, Austria.
Planned for 2000: 10. *Third Annual Prostate Cancer Symposium* Mass. Dept. Public Health Marlboro, MA (5/00), **11.** Symposium: *Colon Cancer Development and Progression*, Montreal, Canada (5/00); **12.** American Heart Association Established Investigator Research Conference, Dallas (5/00); Symposium: *Spindle poles,* Amer. Soc. Cell Biol. (12/2000); FASCEB conference: *Cell cycle and cancer (7/2001).*

6. Patents. We previously obtained a patent for: *Cancer Detection by Centrosome Abnormality* (#5,972,626) and we are applying for another: *Detection of Centrosome Defects and Elevated Pericentrin Levels in PIN.*

♦ **Clinical Translational Research.** We ultimately hope to develop prognostic tests and therapeutics for detection of centrosome defects in PIN and for the treatment of aggressive prostate cancer, respectively.

6. **Development of Permanent Cell Lines (Prostate Tumor and Nontumor Lines):**
1. HA-pericentrin-expressing cells (tetracycline-inducible) to study oncogenic effect of pericentrin. We constructed 3 tumor lines (PC-3, DU-145, LN-Cap) and 1 normal line (1542-NPTX, normal prostate tissue cells transformed w/ HPV, gift Lance Liotta; Cancer Res. 57, 995-1002, 1997).
2. GFP-histone (H2B)-expressing cells for studying segregation of GFP-labeled chromosomes in living cells: 1542-NPTX, PC-3, DU-145, LN-Cap. Others under selection include PEPC-2.

♦ **Development of methods for high-resolution imaging and quantitation.** We developed and improved methods for increased resolution of centrosomes and for quantifying proteins in tissue sections (Fig. 1).

1. **Promotion obtained based on research supported by this award.** The P.I. (SJD) was promoted to Associate Professor (7/99) based in part on work accomplished in this proposal (see letter in proposal). Dr. Aruna Purohit was promoted to Instructor based on her work on prostate cancer (10/99).

♦ **Other Relevant Items:**
Media presentation 1: Based on our current research on prostate cancer, we were recruited to present our findings in a radio interview on WSRS on June 14th, 1999 on the medical program, *Worcester speaks out.*
Media presentation 2: Our research on prostate cancer was highlighted in a newspaper article in the *Health* section of the Worcester newspaper *Telegram and Gazette* on June 28th 1999 (see article in grant proposal).
Prostate cancer advocacy: We are active members of a prostate cancer advocacy group (see letter in proposal). We lobbied in (congress & senate) to increase prostate cancer research funds from $50,000 to $500,000 in MA.
Press Release: Our work was highlighted at the recent Amer. Soc. of Cell Biol. Meeting, 12/2000. Our abstract was released to the press based on its high potential for clinical applications.

CONCLUSIONS.

We believe that our work on centrosome dysfunction will have a significant impact on our understanding of prostate cancer progression and possibly etiology. It also has the potential to improve our ability to detect and treat the more aggressive and devastating forms of this disease. One compelling reason for this assertion is that our hypothesis examines a fundamentally different and unexplored mechanism for prostate cancer development and progression: that centrosome defects cause genetic instability and cytologic anaplasia, and thus underlie the genesis of malignant disease. For this reason, the reviewers of the original proposal described it as unique and highly innovative. Insights gained from this approach should yield novel information on cellular processes, structures (centrosomes) and molecules (pericentrin) that have the potential to serve as therapeutic targets and prognostic indicators of malignant disease. Data obtained during the first 18 months of this funding period supports these ideas. The discovery that centrosome defects are present in precancerous lesions of the prostate and the identification of a tumor-specific centrosome abnormality indicates that centrosome defects may serve as prognostic indicators of malignant disease. The presence of centrosome defects at the earliest disease stages also suggests that centrosomes may play an active role in the tumorigenic process and thus, may serve as prime therapeutic targets. Moreover, pericentrin may be a novel molecular target for prostate cancer therapeutics since artificial elevation of pericentrin in normal prostate cells induces a tumor-like phenotype and since pericentrin levels are specifically elevated in tumors and precancerous lesions. To accomplish the goals outlined in this final progress report, we developed and optimized techniques for histochemical staining of centrosome antigens on paraffin sections and developed methods for high resolution imaging of centrosomes and quantification of protein levels in tissue sections. We plan to patent the use of this technology for identification of centrosome defects and elevated pericentrin levels in prostate cancer and PIN lesions and for potential use in prostate cancer prognosis and treatment. In conclusion, we have produced compelling data in support of our unique centrosome-based model for prostate cancer progression. We believe this research will provide novel and more discriminating tools for prostate cancer prognosis and treatment.

APPENDICES

1. Pihan, G., Purohit, A., Knecht, H. Woda, B. Quesenberry, P. and Doxsey, S.J. Centrosome defects and genetic instability in malignant tumors. Cancer Res., 58, 3974-3985, 1998.
2. P ihan, G., Doxsey, S. The mitotic machinery as a source of genetic instability in cancer. Sem. Cancer Biol. 9, 289, 1999.
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4. Zimmerman, W., Sparks, C. and Doxsey, S. Amorphous no longer: the centrosome comes into focus. Curr. Opin. Cell Biol., 11, 122-128, 1999.
5. Purohit, A. and Doxsey, S. Direct interaction of pericentrin with dynein light intermediate chain contributes to mitotic spindle organization. J. Cell Biol. 147, 481-491, 1999.
6. Pihan, G, Purohit,A, Doxsey S. Centrosome defects correlate with Gleason grade in prostate cancer. Cancer Res., in press for 3/2000.

[CANCER RESEARCH 58, 3974–3985, September 1, 1998]

Centrosome Defects and Genetic Instability in Malignant Tumors[1]

German A. Pihan, Aruna Purohit, Janice Wallace, Hans Knecht, Bruce Woda, Peter Quesenberry, and Stephen J. Doxsey[2]

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ABSTRACT

Genetic instability is a common feature of many human cancers. This condition is frequently characterized by an abnormal number of chromosomes, although little is known about the mechanism that generates this altered genetic state. One possibility is that chromosomes are missegregated during mitosis due to the assembly of dysfunctional mitotic spindles. Because centrosomes are involved in spindle assembly, they could contribute to chromosome missegregation through the organization of aberrant spindles. As an initial test of this idea, we examined malignant tumors for centrosome abnormalities using antibodies to the centrosome protein pericentrin. We found that centrosomes in nearly all tumors and tumor-derived cell lines were atypical in shape, size, and composition and were often present in multiple copies. In addition, virtually all pericentrin-staining structures in tumor cells nucleated microtubules, and they participated in formation of disorganized mitotic spindles, upon which chromosomes were missegregated. All tumor cell lines had both centrosome defects and abnormal chromosome numbers, whereas neither was observed in nontumor cells. These results indicate that centrosome defects are a common feature of malignant tumors and suggest that they may contribute to genetic instability in cancer.

INTRODUCTION

Faithful segregation of chromosomes into daughter cells is essential for maintaining the genetic stability of most organisms. Chromosome segregation is mediated by the mitotic spindle, which has a complex structural organization and precisely timed movements that ensure the accuracy of this process (reviewed in Refs. 1–5). In normal cells, the metaphase spindle is a bipolar structure comprised of microtubules that emanate from centrosomes at each pole with chromosomes aligned at the spindle center (6, 7). Although it is not completely understood how spindles are assembled, the centrosome appears to play an important role in the process (reviewed in Refs. 4 and 8). Spindle assembly and spindle-mediated movements during chromosome segregation are controlled, in part, by cell cycle regulators. These include a system of biochemical checkpoints, feedback controls, and degradation events that ensure the stepwise progression of mitotic events and, ultimately, the fidelity of chromosome segregation and the maintenance of genetic stability (1–3, 9–11). Genetic instability is a common feature of malignant tumors. It is frequently characterized by an abnormal number of chromosomes, a condition known as aneuploidy (12–14). Furthermore, recent results demonstrate that aneuploid cells exhibit continuous changes in chromosome number throughout their lifetimes, suggesting that this CIN[3] may contribute to aneuploidy (15). These defects in chromosome number are thought to occur through missegregation of chromosomes (1, 15), but the mechanism by which this occurs has not been elucidated. It is easy to envision how defects in mitotic spindle organization and function could directly lead to chromosome missegregation (2, 3, 5, 16). Furthermore, because spindles are organized in part by centrosomes (4, 8, 17), it is possible that abnormal centrosome function could contribute to CIN. Support for this idea comes from a recent observation suggesting that centrosome number is amplified in genetically unstable cells mutant for the tumor suppressor p53 (18).

Centrosomes are comprised of a pair of centrioles, the duplication of which occurs once and only once during the normal cell cycle, and the surrounding pericentriolar material, the substance involved in microtubule nucleation (see Ref. 7). As an initial test of the idea that centrosome dysfunction may lead to chromosome missegregation through the organization of aberrant mitotic spindles, we examined centrosomes in malignant tumors and cell lines derived from tumors. We found that centrosomes immunolabeled with antibodies to pericentrin (19) were abnormal in structure, number, and function in a wide range of malignant tumors and tumor cell lines. Furthermore, tumor cell lines with abnormal centrosomes exhibited spindle abnormalities and high levels of CIN.

MATERIALS AND METHODS

Preparation of Archival Tissues. Archival tissue consisted of paraffin-embedded biopsy material fixed for 4–24 h in 10% formaldehyde in PBS. Samples used in this study were 2 weeks to 4 years old. Five-mm-thick tissue sections were cut on a conventional microtome used for paraffin-embedded tissue sectioning. Sections were floated on a water bath kept at 37°C, picked up on glass slides, allowed to air-dry, and baked at 60°C overnight. Sections were deparaffinized in xylenes (twice for 3 min each at room temperature) and placed in 100% ethanol. Sections were rehydrated in a descending gradient of ethanol-water to 70% ethanol, transferred to PBS, and kept at 4°C until immunostaining (see below).

Preparation of Cells from Fresh Tissues by Collagenase/DNase Digestion. Cell suspensions were prepared from surgical resection specimens of carcinomas and sarcomas by removing small samples (5 mm^3) and mincing with a razor blade in PBS at room temperature. Minced tissue was washed in PBS and resuspended on an 1-ml aliquot of fresh PBS containing 1.0 unit/ml collagenase (Sigma Chemical Co.) and 0.1 unit/ml of DNase I (Sigma; Ref. 20). Tissue was rotated end-over-end for 2 h at room temperature. Samples were then strained in a 100-mm nylon filter (Nytex; Small Parts, Inc.). Cells were pelleted and washed in PBS by sequential centrifugation at 325 × *g* and then cytospun onto slides.

Cytospinning of Cells onto Slides. Suspension cells were collected by various methods (see below). Approximately 2×10^5 cells were resuspended in 100 ml of PBS at room temperature and placed in a cytospin funnel (Shandon, Inc.). Cytofunnels were attached to slides and spun at room temperature for 5 min at 65 rpm in a clinical cytocentrifuge (Cytp 2; Shandon, Inc.). Cells on slides were fixed, processed, and mounted as described below.

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[2] To whom requests for reprints should be addressed, at Program in Molecular Medicine, University of Massachusetts Medical School, 373 Plantation Street, Worcester, MA 01605. Phone: (508) 856-1613; Fax: (508) 856-4289; E-mail: stephen.doxsey@ummed.edu.

[3] The abbreviations used are: CIN, chromosomal instability; DAPI, 4′,6-diamidino-2-phenylindole; HD, Hodgkin's disease; pen/strep, 100 units/ml penicillin-0.1 mg/ml streptomycin; FISH, fluorescence *in situ* hybridization; MTOC, microtubule organizing center.

Antibodies. Antibodies to pericentrin (5 mg/ml, rabbit polyclonal) and α-tubulin (2 mg/ml, mouse monoclonal) were used as described (19, 21). To label spindles, we mixed antibodies to both proteins and incubated them with different secondary antibodies (Jackson ImmunoResearch Laboratories). DNA was visualized by staining with DAPI (Sigma).

Cell Lines. Tumor-derived cell lines were grown on coverslips (19) or fixed in formalin, embedded in paraffin, and sectioned. Most cell lines were obtained from American Type Culture Collection and grown as described. L428, KHM2, and JC are HD cell lines. They were obtained from the German Collection of Microorganisms and Cell Cultures (L428 and KHM2) and from an immunocompromised patient with a HD-like lymphoma (JC; grown in our laboratory). B115 and B218 are early-passage EBV-transformed lymphoblastoid B-cell lines from peripheral blood B lymphocytes (gift of J. Sullivan, University of Massachusetts Medical School, Worcester, MA). All lines listed above were grown in RPMI (Hyclone Laboratories), 20% FCS, and pen/strep. Breast carcinoma cell lines MDA-MB-436 and MDA-MB-157 were grown in Leibovitz L-15 medium with 20% FCS, insulin (0.25 units/ml), glucose (45 mg/ml), and pen/strep; BT-549 and HS578T were grown in RPMI 1640 with 10% FCS-pen/strep. The prostate cell line PC-13 was grown in RPMI with 10% FCS-pen/strep. Colon carcinoma cell lines HT-29 and Lovo were grown in McCoy's 5A medium with 10% FCS-pen/strep. All other cell lines above were grown in RPMI with 20% FCS-pen/strep (American Type Culture Collection).

Immunoperoxidase Labeling of Tissues and Cells for Centrosomes. Sections or cells on slides were pressure-heated in antigen retrieval solution (1 mM EDTA in water) in a microwaveable pressure cooker (Nordic Ware) for 20 min, allowed to cool to room temperature, and transferred to PBS (22). Slides were immersed in 3% H_2O_2 in PBS for 15 min to block endogenous peroxidase. Cells were then blocked in 2-nitro-5-thiobenzoate blocking buffer (TSA-Indirect kit; NEN Life Science Products) for 1 h, followed by standard indirect immunohistochemistry. Briefly, pericentrin antibody was diluted 1:1000 in TBB (*See* TSA-Indirect kit) and added to slides in 100-ml aliquots for 1 h at room temperature. Slides were washed in TNT (*See* NEN Life Science Products kit) 3 times for 5 min each. Biotinylated secondary antibody against rabbit immunoglobulins (Ventana Medical Systems) diluted 1:1000 was applied for 1 h and incubated as above. Slides were washed in TNT 3 times for 5 min each. Signals were amplified by catalyzed reporter deposition Tyramine signal amplification (Ref. 23; TSA-Indirect kit), following manufacturer's instructions. Slides were washed in TNT, counterstained in hematoxylin, and mounted in Permount (Sigma), as described by manufacturer.

Immunofluorescence Labeling of Tissues and Cells. Cells were grown on 12-mm glass coverslips or cytospun onto glass slides. Cells were washed in PBS by placing coverslips into 12-well plates (Costar) with 1–2 ml of PBS or by immersing slides in Coplin jars filled with PBS. Cells were permeabilized to release soluble proteins and better visualize centrosome staining (19). PBS was then aspirated, and permeabilization buffer [80 mM PIPES (pH 6.8), 5 mM EGTA, 1 mM $MgCl_2$, and 0.5% Triton X-100] was added to plates or Coplin jars and incubated for 60 s at room temperature. Coverslips or slides were transferred to new container/plate with −20°C methanol and incubated for 5 min. Samples were sometimes stored for days in methanol at −20°C. Cells were washed 5 times in PBS by replacing half of the volume and aspirating half of the volume. Blocking solution (1× PBS, 0.5% Triton X-100, and 2% BSA) was added, and cells were incubated for 10 min. Coverslips or slides were prepared for immunofluorescence microscopy as described (19). Immunofluorescence images were recorded on a Zeiss Axiophot using a ×100 objective on a Xillix charge-coupled device camera with a Kodak (KAF 1400) chip and then pseudocolored and merged using ITEX-IPL software. Immunoperoxidase images were recorded using ×60 and ×100 objectives in real color on an Olympus Vanox-S photomicroscope equipped with a Kodak CDS 460 digital camera.

Microtubule Nucleation and Centriole Labeling. To depolymerize and regrow microtubules, cells were treated with nocodazole and washed free of the drug as described (19, 21). To visualize centrioles, cells were treated with nocodazole, permeabilized with detergent (above) and processed for immunofluorescence using an α-tubulin antibody. Similar results were obtained with an antibody that selectively stains the polymerized form of tubulin (tyrosinated; gift of C. Bulinski, Columbia University).

FISH. Chromosome numbers were determined by FISH on interphase cells using centromeric probes specific for chromosomes 1 and 8, labeled directly with Spectrum Green (Vysis, Chicago, IL) or Spectrum Red as described (15, 24). Evaluation of chromosome numbers by FISH rather than conventional metaphase analysis was used so that cells could be examined at all cell cycle phases. Cells were grown on coverslips or cytospun onto glass slides, permeabilized in detergent, and fixed as for centrosome immunofluorescence. Probe hybridization and washes were as recommended by the manufacturer (Vysis). Nuclei were counterstained by DAPI (20 ng/ml, Sigma) in PBS, mounted (Vectashield, Vector Laboratories), and analyzed on a Zeiss epifluorescence microscope equipped with a triple-band pass filter cube, allowing the simultaneous visualization of Spectrum Green, Spectrum Red, and DAPI signals. Centromeric signals appeared as discrete dots in most cells or as elongated dots in cells presumed to be in the G_2 phase of the cell cycle. The numbers of red and green signals per cell were determined in 100–150 cells in each cell line in two separate experiments.

RESULTS

Defects in Pericentrin Organization in Tumors. We examined malignant tumors from a variety of tissues for the presence of centrosome defects. These included primary tumors of the breast, prostate, lung, colon, and brain, as well as metastatic tumors of the breast, lung, and colon. Tissue sections from archival formalin-fixed, paraffin-embedded material were reacted with antibodies to the centrosome protein pericentrin (19), and antibodies were detected by the amplified immunoperoxidase technique (23). The pericentrin antibody used in this analysis has been shown to specifically label centrosomes in a wide variety of cell types when used in combination with the immunofluorescence technique (19). We confirmed that the antibody produced a similar staining pattern by the immunoperoxidase technique in tissue sections and cells in culture. In normal interphase cells, a single brown dot was observed (the product of the immunoperoxidase reaction), and in mitotic cells, a pair of dots was detected, one at each pole of the spindle.

When tumors were analyzed at low magnification by immunoperoxidase staining, the tumor tissue could easily be delineated from adjacent nontumor tissue by the significantly higher level of pericentrin staining (Fig. 1). Higher magnification revealed that the pericentrin staining was organized into structures that were abnormal in size, shape, and number (Fig. 2, *Tumor tissues*). Most tumor cells had a single focus of pericentrin that was significantly greater in diameter than centrosomes in nontumor cells (3–10-fold greater). Tumor cells often had multiple pericentrin foci suggesting that supernumerary centrosomes were present in these cells (see below). Multiple foci were detected in both paraffin sections [Fig. 2, *small arrowheads* in *A* and *D*] and freshly prepared samples (Fig. 3*H*) and were sometimes interconnected by atypical filaments of pericentrin (Fig. 2*O*, *arrowheads*). These structural defects occurred together with variable levels of diffuse and patchy pericentrin material in the cytoplasm of tumor cells (Fig. 2, most panels).

The abnormal distribution of pericentrin staining seen in malignant tumors was not observed in nontumor tissues. We examined over 12 cell types in tissues adjacent to tumors including cells of tumor origin, resident cells in metastatic tumors, and cells in stroma, ducts, blood vessels, and smooth muscle [Fig. 2, *Nontumor tissues* (*NT*), *arrowheads* and *large arrowheads* in *D, K,* and *N*]. In all cases, a single discrete focus of pericentrin staining was detected, typical of the centrosome pattern in normal cells. A low level of diffuse staining was sometimes detected in nontumor tissues, which most likely represented the modest level of cytoplasmic pericentrin known to be present in normal cells. The absence of pericentrin anomalies in the many different types of nonneoplastic cells within tumor sections (for example, proliferating and nonproliferating cells, epithelial and endothelial cells, and so on) strongly suggests that this phenotype is tumor

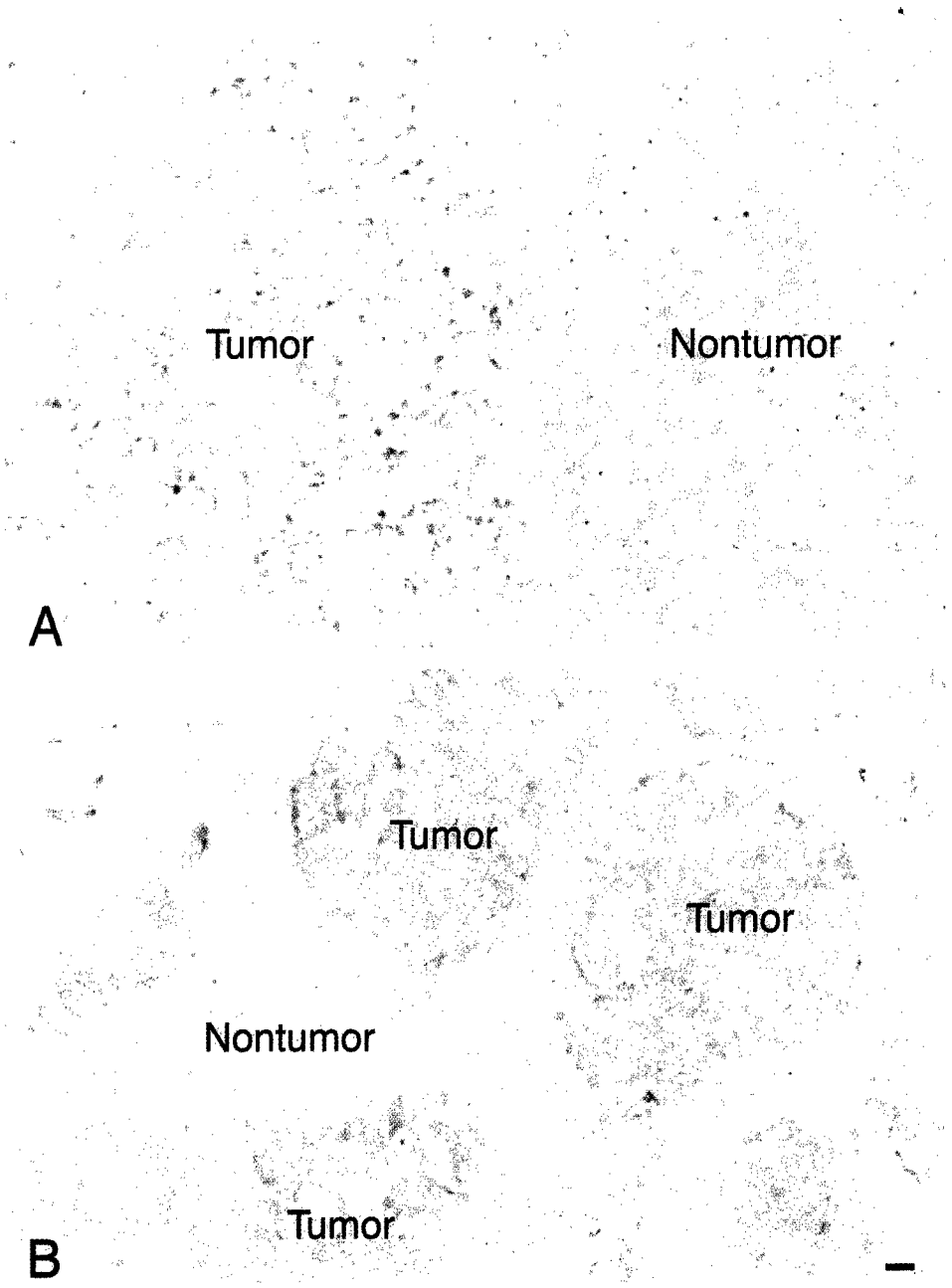

Fig. 1. Low-magnification images of malignant tumors showing amplified pericentrin staining. Paraffin-embedded tissue sections were stained for pericentrin by the immunoperoxidase method (*brown*) and counterstained with hematoxylin (*blue*) to reveal details of tissues and cells. Images show a high level of pericentrin staining in tumor tissue compared to nontumor tissue. *A*, breast adenocarcinoma metastasized to lymph node. *B*, lung tumor *in situ*. *Scale bar* (in *B*), 20 μm (for both *A* and *B*).

related and does not simply reflect the stage of differentiation, differences in cell type, or proliferation rate.

The presence of defective pericentrin structures in tumors was significantly higher than in nontumor tissues (Table 1, $P < 0.0001$, two-sided Fisher's exact test). Although nontumor tissues appeared normal in all cases, 93% of the tumors examined (81 of 87) showed one or more defects. Up to 95% of the cells in some tumors exhibited the abnormal phenotype. In some tumors, the abnormal phenotype was not observed. This could reflect a lower stage of tumor progression, the inability of our assay to detect subtle abnormalities in pericentrin organization, or the lack of centrosome abnormalities in these tumors. It appears that insensitivity of the archival tissue assay may be partially responsible for the apparent lack of defects in some tumors because pericentrin organization appeared to be more severely perturbed in freshly isolated cells from a limited number of tumors ($n = 5$; for example, see Fig. 3*H*). These data indicate that many malignant tumors have higher levels of pericentrin and that pericentrin is organized into atypical and supernumerary structures in the cytoplasm of tumor cells.

Defects in Pericentrin Organization in Tumor-derived Cell Lines. The observed defects in pericentrin organization in malignant tumors were also found in permanent cell lines established from tumors. These included cell lines derived from colon, breast, and prostate and from patients with HD (Fig. 3). Using both immunoperoxidase and immunofluorescence methods, we detected pericentrin structures of abnormal size and shape (Fig. 3, *A*, *arrows*, *B*, and *D–G*) and supernumerary structures (Fig. 3, *A*, *arrowheads*, and *D–F*). Over 25 centrosomes were detected in some tumor-derived cells (Fig. 3*F*), and they varied in size from tiny flecks of material a fraction of the size of normal centrosomes to large aggregates (Fig. 3, *D* and *F*) or long linear arrays up to ten times larger than normal centrosomes (Fig. 3*G*). Diffuse cytoplasmic material was also observed in tumor cells and was usually found together with other centrosome defects (data not shown; see "Materials and Methods"). Up to 67% of the cells in

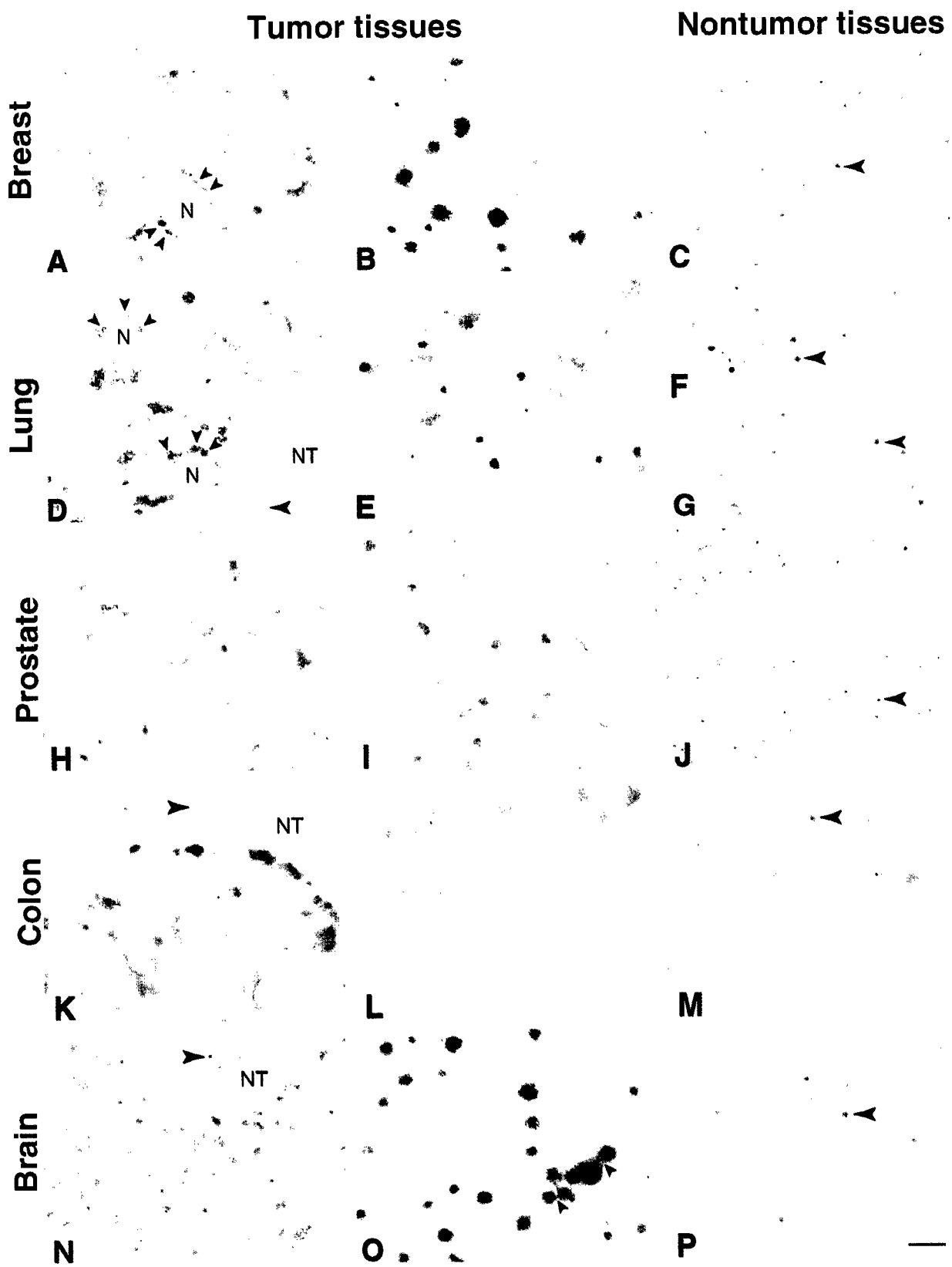

Fig. 2. High-magnification images of malignant tumors showing abnormal pericentrin structures. Tissues were processed for pericentrin staining as in Fig. 1. Cells in nontumor tissues (*NT*), in the same tissue section as tumor cells (*T*), usually have a single small focus of staining, typical of normal centrosomes (*large arrowheads*, *Nontumor tissues*, and *NT* in *D*, *K*, and *N*). Pericentrin-staining structures in tumor cells are usually larger in diameter (most panels) and often abnormal in number (*A* and *D*, *small arrowheads*). In addition, most tumor cells contain increased levels of pericentrin within the cytoplasm (most panels). Occasionally, structures with abnormal morphology are observed (*O*, see linear elements at arrowheads). Tissues were from the following: *A–C*, lymph node with metastatic breast tumor; *D–G*, lung; *H–J*, prostate; *K–M*, colon; *N–P*, brain. Nontumor tissues were from the following: *C*, lymph node; *D* (*NT*), stroma in lung; *F*, alveolar wall; *G*, bronchial epithelium; *J*, prostate gland; *K* (*NT*), stroma in intestine; *M*, intestinal epithelium; *N* (*NT*), blood vessel; *P*, brain white matter. *NT*, nontumor tissue; *N*, nucleus. All images are same magnification. *Scale bar* (in *P*), 10 μm (for *A–P*).

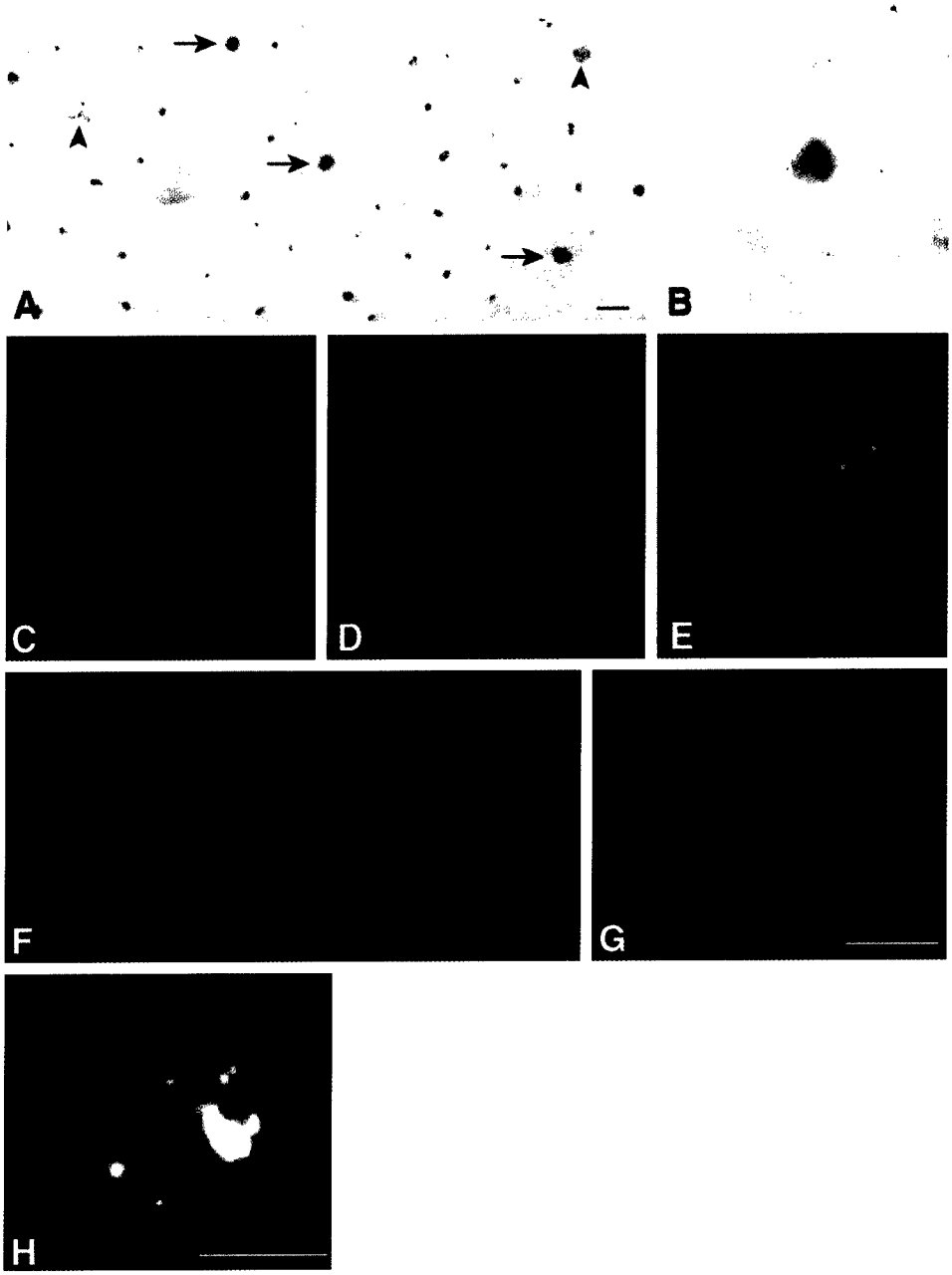

Fig. 3. Abnormal pericentrin structures in tumor-derived cell lines (*A–G*) and cells dissociated from tumors (*H*). *A*, HD cell line (L428) stained by immunoperoxidase (as in Fig. 1), showing several cells with enlarged pericentrin staining structures (*arrows*) and multiple pericentrin staining structures (*arrowheads*). *B*, enlargement of cell in *A*, showing large pericentrin structure at center of multiple nuclei. *C*, cell from a nontumor cell line (B218) processed for immunofluorescence with antipericentrin antibodies and showing a single dot (*red*) next to the nucleus (*blue*), typical of centrosome staining in normal cells. Cells from breast tumor cell lines (*D*, MDA-MB-157; *F*, BT-549) and a prostate tumor cell line (*E* and *G*, PC-13) showing multiple pericentrin staining structures (5 to >25). In addition, the structures are variable in size (*D* and *F*), linked together by strands of pericentrin-staining material (*E*) and organized into string-like arrays (*G*, nucleus out of view). *H*, cell dissociated from a human breast tumor showing multiple fluorescent foci of pericentrin staining (*white/yellow*). *Scale bars*, 10 μm (*scale bar* in *G* for *B–G*. *A* and *B*, immunoperoxidase labeling; *C–H*, immunofluorescence labeling. *A–G*, cell lines; *H*, cell dissociated from tumor.

some tumor-derived lines had defective pericentrin structures, whereas most cells from nontumor lines had single fluorescent dots of uniform size, typical of pericentrin staining in normal cells (Fig. 3*C*). Statistical analysis demonstrated that the defects observed in all eight tumor lines examined were significantly greater than those in nontumor cell lines (Table 2, all *P*s <0.001, Pearson's $\chi 2$ test). Nontumor cells rarely exhibited multiple pericentrin foci. It is possible that nontumor cells in both established lines and primary tumors exhibit a

Table 1 *Centrosome abnormalities in malignant tumors*[a]

Abnormal centrosomes[b]	Tumor type[c]				
	Breast	Prostate	Brain	Lung	Colon
In tumor cells	18/19	16/18	19/20	15/15	13/15
In nontumor cells[d]	0/21	0/25	0/18	0/23	0/20

[a] For all samples in this analysis, paraffin-embedded tissues were sectioned, reacted with pericentrin antibodies and immunoperoxidase methods, and examined by light microscopy. Defects in centrosomes were statistically higher in tumors as compared to nontumor cells. Statistical analyses were described in "Results" and "Discussion."

[b] Centrosomes were considered abnormal if they had diameters >2 times the diameter of centrosomes in nontumor control cells in the same section; if they lacked centrioles; if they were present in more than two copies per cell; or if they were organized into elongated structures >3 μm long, string-like elements, or large patchy aggregates. Most tumor cells had more than one defect. Similar results were obtained by immunofluorescence analysis (data not shown).

[c] Tumors were identified by architectural and nuclear cytological features on hematoxylin-counterstained immunoperoxidase preparations.

[d] Nontumor cells had none of the centrosome abnormalities described above. They were used as internal controls for each tumor and included stromal cells, lymphocytes, astroglia, endothelial cells, and mature nonneoplastic epithelial cells present within the same tumor tissue sections. Centrosomes in nontumor cells were indistinguishable from those of normal cells.

Table 2 *Aberrant centrosomes, nuclei, and chromosome numbers in tumor- and non-tumor-derived cell lines*[a]

Cells and cell lines[b]	Tissue of origin	Abnormal centrosomes[c]	Abnormal Nuclei[d]	Chromosomal instability (Chr1/Chr8)[e]
Tumor-derived cell lines				
HT-29	Colorectal	24%	12%	57%/43%[f]
Lovo	Colorectal	9%	11%	ND[g]/27%[f]
HS578T	Breast	22%	15%	66%/70%
BT-549	Breast	67%	50%	73%/72%
MDA-MB-436	Breast	14%	17%	36%/40%
L428	HD	16%	26%	33%/29%
KHM2	HD	45%	29%	37%/28%
JC	HD	13%	11%	29%/29%
Non-tumor-derived cell lines				
B115	Lymphoblastoid	3%	2%	6%/5%
B218	Lymphoblastoid	2%	3%	7%/4%
COS 7	Monkey kidney	0.3%	0.5%	ND

[a] Defects in centrosomes, nuclei, and chromosome number were all statistically higher in tumor cells as compared to nontumor cells. Statistical analyses were performed as described in "Results" and "Discussion."

[b] Cell lines were described in "Materials and Methods."

[c] The percentage of cells with three or more discrete pericentrin-staining foci, pericentrin structures without centrioles, long linear structures (>3 μm long), and structures much smaller (<50%) or larger (>300%) in diameter than in control cells. All cells were examined by immunofluorescence methods. At least 500 cells were counted for each cell line. Values represent the average of three independent experiments. Similar results were obtained by immunoperoxidase labeling (data not shown).

[d] The percentage of cells with nuclei exhibiting defects in morphology and/or size (multilobed or multinucleate), as observed by DAPI staining. At least 300 cells were counted for each cell line and values represent the average of two experiments.

[e] Percentages represent the fraction of cells with chromosome numbers that were different from the mode (a gain or loss), as described (15). We used directly labeled chromosome-specific centromeric probes to chromosome 1 (Chr1) and chromosome 8 (Chr8). Between 100 and 150 cells were counted for each value, which is the average of two staining reactions.

[f] Previously determined values for chromosomal instability (15).

[g] ND, not determined.

basal level of pericentrin abnormalities that is corrected through appropriate cell cycle checkpoints or eliminated by activation of appropriate apoptotic pathways (see Refs. 1 and 11).

Supernumerary Centrioles and Acentriolar Structures in Tumor-derived Cell Lines. If the atypical pericentrin structures described above were centrosomes with normal architecture, they should possess centrioles (see Ref. 7). To detect centrioles, cells were stained with antibodies to α-tubulin following the selective depolymerization of cytoplasmic microtubules with nocodazole (7, 19). To our surprise, centrioles in tumor cells were sometimes absent from pericentrin structures, especially those of variable size and irregular shape (Fig. 4, *C* and *D*, *large arrowhead*). However, pericentrin structures of normal size and morphology usually had centrioles, even when they were present in multiple copies in the cytoplasm of tumor cells (Fig. 4, *E* and *F*) and cells dissociated from fresh tumors (data not shown). Control cells typically had a pair of centrioles at the focus of pericentrin staining, as expected for normal cells (Fig. 4, *A* and *B*).

Quantitative analysis showed a good correlation between centrioles and pericentrin foci in control cells (100%, $n = 214$), whereas centrioles were absent from pericentrin structures in a significant percentage of cells in a breast line (11.2%, $n = 223$, BT-549), a HD line (14.1%, $n = 227$, L428), and others (data not shown). This demonstrates that, although many pericentrin-staining structures observed in tumors and tumor-derived cell lines are canonical centrosomes, a proportion of them lack centrioles. Because pericentrin is found in centrosomes and other MTOCs that lack centrioles (19), we examined all pericentrin structures in tumor cells for the ability to nucleate microtubules.

All Pericentrin Structures in Tumor Cell Lines Nucleate Microtubules. To test for microtubule nucleation, cells were treated with nocodazole to depolymerize microtubules and were washed free of the drug to allow microtubule regrowth from centrosomes. Under these conditions, essentially all pericentrin foci nucleated the growth of new microtubules regardless of their number, size, morphology, and the presence of centrioles (Fig. 5). Even the smallest detectable specks of material (Fig. 5, *C* and *E*) and the long linear arrays (Fig. 5*G*) nucleated microtubules (Fig. 5, *D*, *F*, and *H*). These additional MTOCs significantly increased the nucleating capacity of tumor cells compared to control cells, in which a single centrosome (one or two dots) nucleated a single microtubule aster (Fig. 5, *A* and *B*). The presence of multiple MTOCs suggested that tumor cells might form abnormal spindles during cell division.

Defects in Mitotic Spindle Organization and Chromosome Segregation in Tumor Cell Lines. Spindle defects were observed in cells of all tumor-derived lines (Fig. 6) and cells freshly dissociated from tumors (data not shown). Although control cells had a typical bipolar spindle with a single pericentrin focus at each pole (Fig. 6, *A–C*), tumor cells often had misshapen spindles and spindles with poorly focused poles or multiple poles (Fig. 6, *E*, *H*, *K*, and *N*). Most abnormal spindles were associated with pericentrin structures that were aberrant in number (Fig. 6, *D–F*, *G–I*, and *M–O*), shape (Fig. 6, *M–O*), and orientation (Fig. 6, *D–F*, *G–I*, and *J–L*).

In many tumor cells, unequal numbers of chromosomes were aligned between multiple poles of abnormal spindles (Fig. 6, *I* and *O*), and they appeared to be missegregated as cells divided (Fig. 7). We often observed telophase cells undergoing multipolar divisions and segregating their genomes unequally into more than two progeny (Fig. 7, *A* and *B*). In other telophase cells, chromosomes appeared to remain at the metaphase plate after others had been segregated to the poles (Fig. 7*E*, *arrow*) or they segregated part way but did not appear to be included in reforming nuclei (data not shown). Abnormalities in spindle organization and function were detected in up to 36% of mitotic cells in some tumor cell lines (for example, BT-549, $n = 143$). These observations demonstrate that defects in pericentrin organization, spindle structure, and chromosome segregation often occur together in the same tumor cell, and they suggest that centrosome and spindle defects contribute to abnormal partitioning of chromosomes. To obtain a more accurate measure of chromosome missegregation, we examined the copy number of individual chromosomes in tumor cells.

CIN and Nuclear Abnormalities in Tumor Cell Lines. To assay for CIN in tumor cells, we examined chromosomes in individual cells by FISH (24) using probes for chromosomes 1 and 8. In all malignant tumor cell lines examined, we found a dramatic variability in chromosome copy number among individual cells in the population. One such example is shown in Fig. 8, where the frequency distribution of chromosomes 1 and 8 in a malignant breast carcinoma cell line (Fig. 8, *C* and *D*, BT-549) clearly demonstrates a highly variable number of chromosomes per cell. In contrast, a nontumorigenic cell line (Fig. 8, *A* and *B*, B115) has only two copies of each chromosome in most cells. The variability in chromosome number observed in tumor cells has recently been termed CIN (15) and is thought to result from chromosome missegregation during mitosis. Over 70% of the cells in some lines exhibited CIN of chromosomes 1 and 8, with copy numbers ranging from 1 to 22 per cell (Fig. 8 and Table 2). The level of CIN in all tumor cell lines examined (27–73%) was statistically higher than that in control cells (Table 2; 4–7%, $P < 0.001$, Pearson's $\chi 2$ test). Control cells used in this study had CIN levels similar to those of uncultured lymphocytes and to those used in other studies (15) and, thus, appeared to represent the intrinsic error rate of the FISH methodology. Despite the fact that the number of tumor cell lines used in this analysis was low ($n = 8$), we found a positive correlation between abnormal pericentrin organization and insta-

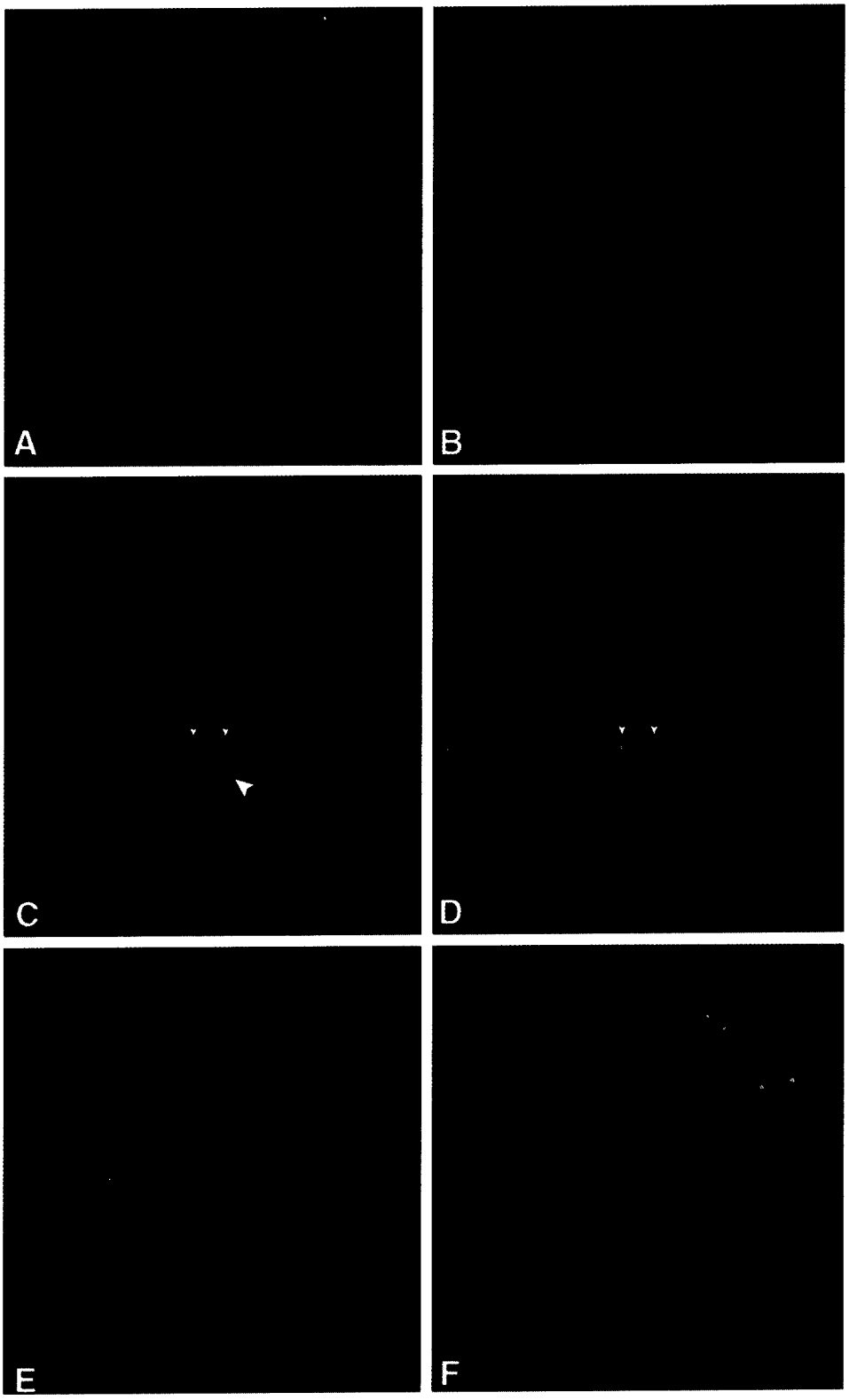

Fig. 4. Supernumerary centrioles and acentriolar structures in tumor-derived cell lines. Centrioles were labeled with an α-tubulin antibody following depolymerization of cytoplasmic microtubules by nocodazole (see "Materials and Methods"). Horizontal series are of the same cell in all cases. In control cells (B115), a pair of centrioles (*B*) is found at the focus of pericentrin staining (*A*). In the HD cell line (L428) the two separated centrioles (*D*, *arrowheads*) are coincident with some pericentrin staining foci (*C*, *small arrowheads*) but not with others (*C*, *large arrowhead*). A cell from a breast tumor cell line (BT-549) with multiple foci of pericentrin staining is shown in *E*, each coincident with centriole staining (*F*). A few microtubules that were incompletely depolymerized are present in *F*. *Scale bar* (in *F*), 10 μm (for *A–F*).

bility of chromosome 1 ($P < 0.05$, Spearman's rank correlation) but not chromosome 8 ($P = 0.204$, see "Discussion"). In addition, aberrant nuclei (multilobed or multinucleate) were observed in all tumor cell lines (Fig. 3, *E* and *F*, and Table 2), and their presence correlated with abnormal pericentrin organization ($P < 0.001$, Pearson's $\chi 2$ test). Taken together, these results indicate that centrosome defects and CIN occur together in most malignant tumor cell lines.

DISCUSSION

Using immunoperoxidase and immunofluorescence labeling techniques and antibodies to pericentrin, we have identified widespread defects in centrosomes in the most common human malignant tumors and tumor-derived cell lines. These tumor cells missegregate chromosomes on aberrant mitotic spindles and exhibit variability in chromosome number. Given the important role of

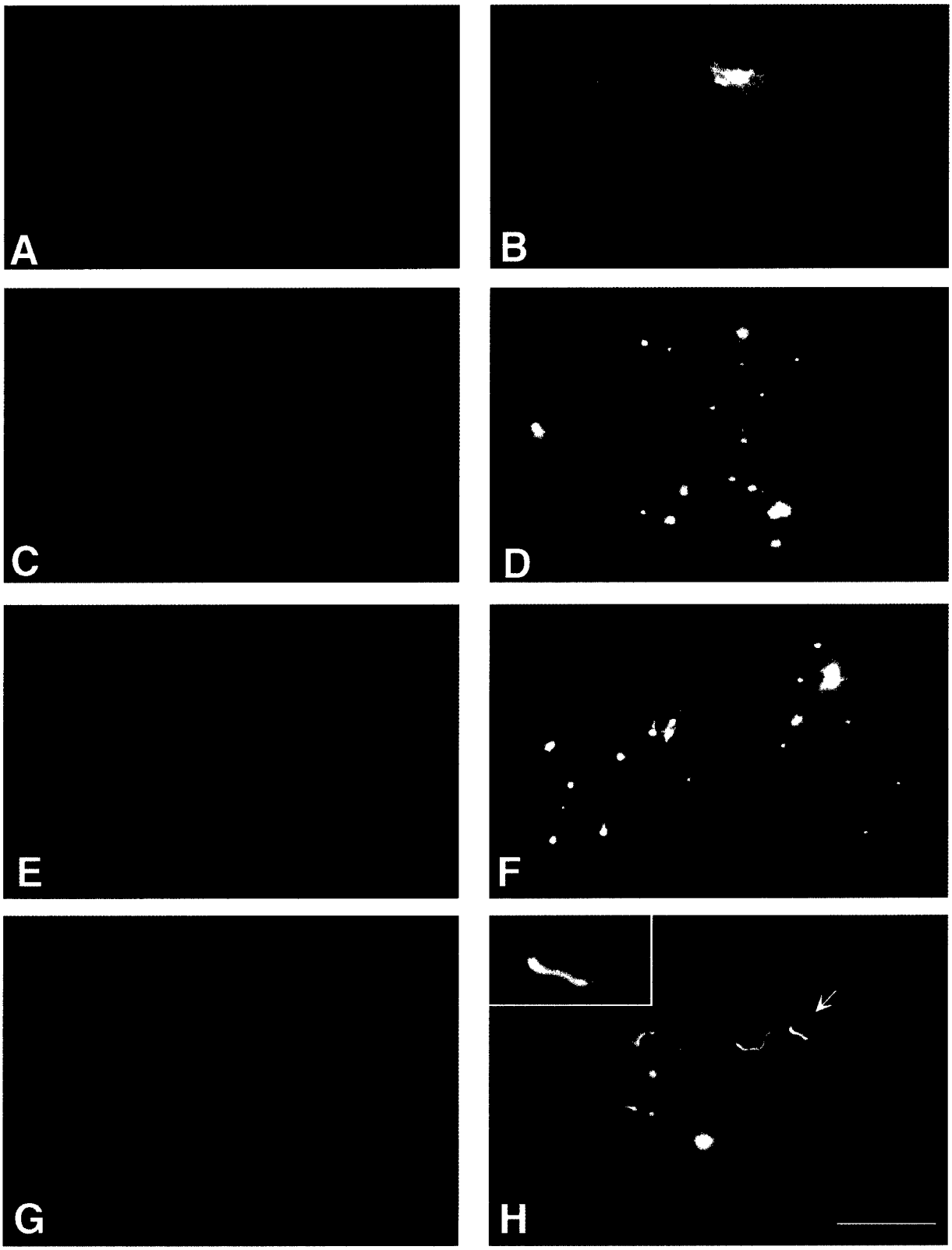

Fig. 5. All pericentrin-staining material nucleates microtubules. Cells from the control cell line B115 (*A* and *B*) and breast cancer-derived cell lines, BT-549 (*C*, *D*, *G*, and *H*) and MDA-MB-436 (*E* and *F*) were treated with nocodazole to depolymerize microtubules, washed, and allowed to regrow microtubules. Cells were triple-labeled for pericentrin (*red* or *yellow*), microtubules (*green*), and DNA (*blue*). All foci of pericentrin staining (*A*, *C*, *E*, and *G*) nucleated the growth of microtubules (*B*, *D*, *F*, and *H*). Even the very small foci seen in *C*, *E*, and *G* and the atypical elongated elements in *G* nucleated microtubules (*D*, *F*, and *H*). *Inset* (in *H*), higher magnification of region at *arrow*. All ectopic nucleating centers are in a single cell as determined by phase contrast microscopy (data not shown). *A*, *C*, *E*, and *G*, pericentrin staining; *B*, *D*, *F*, and *H*, triple-channel overlay showing pericentrin (*yellow*), microtubules (*green*), and DAPI (*blue*). *Scale bar* (in *H*), 10 μm (for *A–H*). Horizontal series (*A* and *B*; *C* and *D*; *E* and *F*; *G* and *H*) are of the same cell.

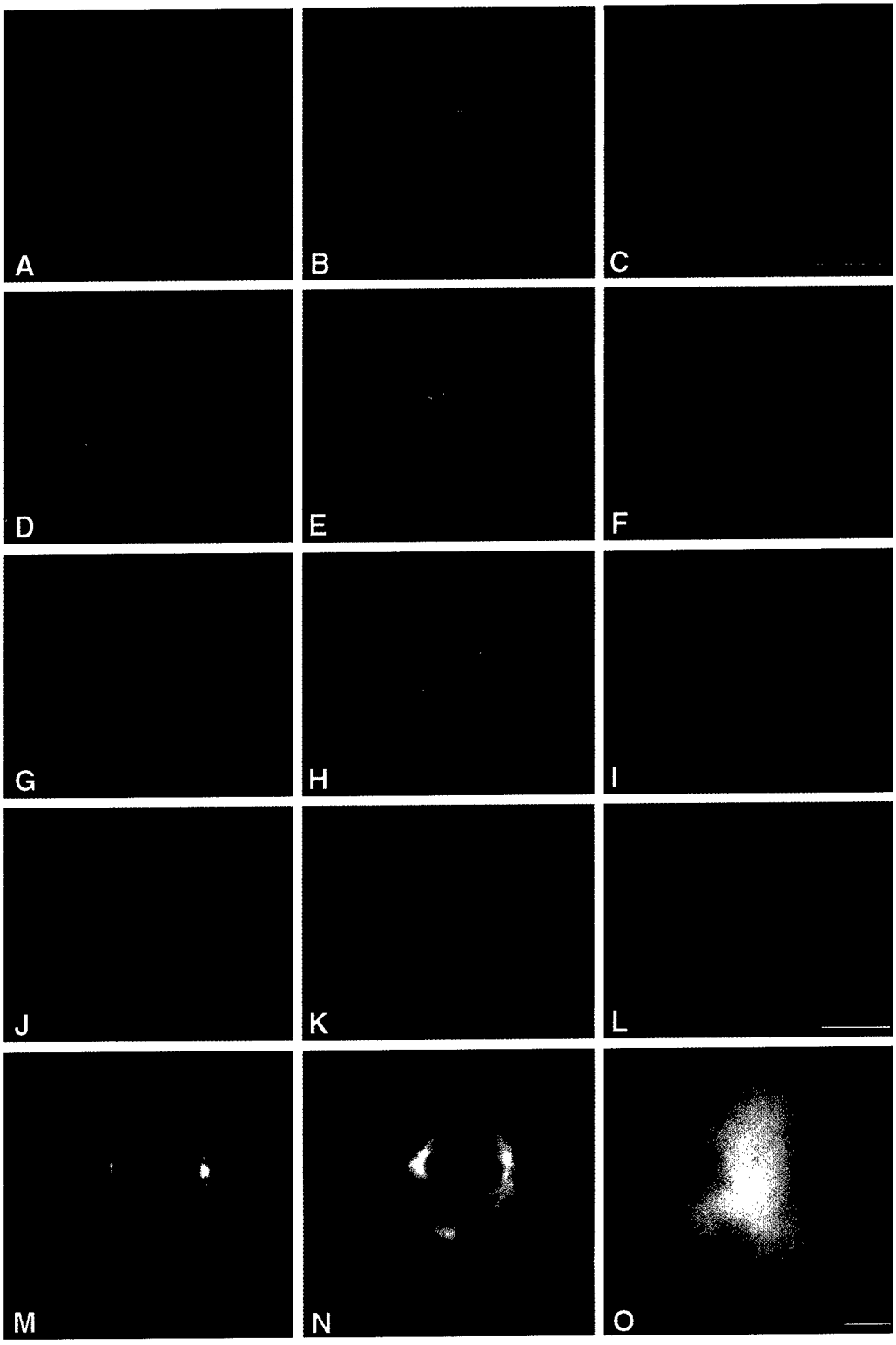

Fig. 6. Abnormal pericentrin structures are associated with aberrant spindles. Control cell (B115) with two centrosomes (*A*) at the poles of a normal bipolar spindle (*B*) and DNA aligned on the metaphase plate (*C*). Abnormal pericentrin structures and spindle defects in cell lines derived from a breast tumor (BT-549, *D–I*), a prostate tumor (PC-13, *J–L*), and an individual with HD (*M–O*). Cells with pericentrin structures of variable sizes, shapes, and numbers participate in the formation of multipolar spindles (*G*, *H*, *M*, and *N*) and spindles with unfocused or misshapen poles (*D*, *E*, *J*, and *K*). Some pericentrin structures do not localize to the poles of aberrant spindles (*D*, *E*, *G*, and *H*). *A*, *D*, *G*, and *J*, pericentrin structures; *B*, *E*, *H*, and *K*, microtubules; and *C*, *F*, *I*, and *L*, DNA. Horizontal series (*A–C*; *D–F*; *G–I*; *J–L*) are of the same cell. *Scale bars*, 10 μm (*scale bar* in C for *A–C*, in *L* for *D–L*; in *O*, for *M–O*).

centrosomes in mitotic spindle organization, it is possible that centrosome defects contribute to this CIN and, ultimately, to the neoplastic phenotype.

Although tumor cells derived from different tissue sources have defects in different biochemical pathways (25), it is remarkable that nearly all malignant tumors examined in this study exhibited abnormal centrosomes. Abnormal centrosome features included structural defects, the absence of centrioles, elevated levels of pericentrin staining, super-

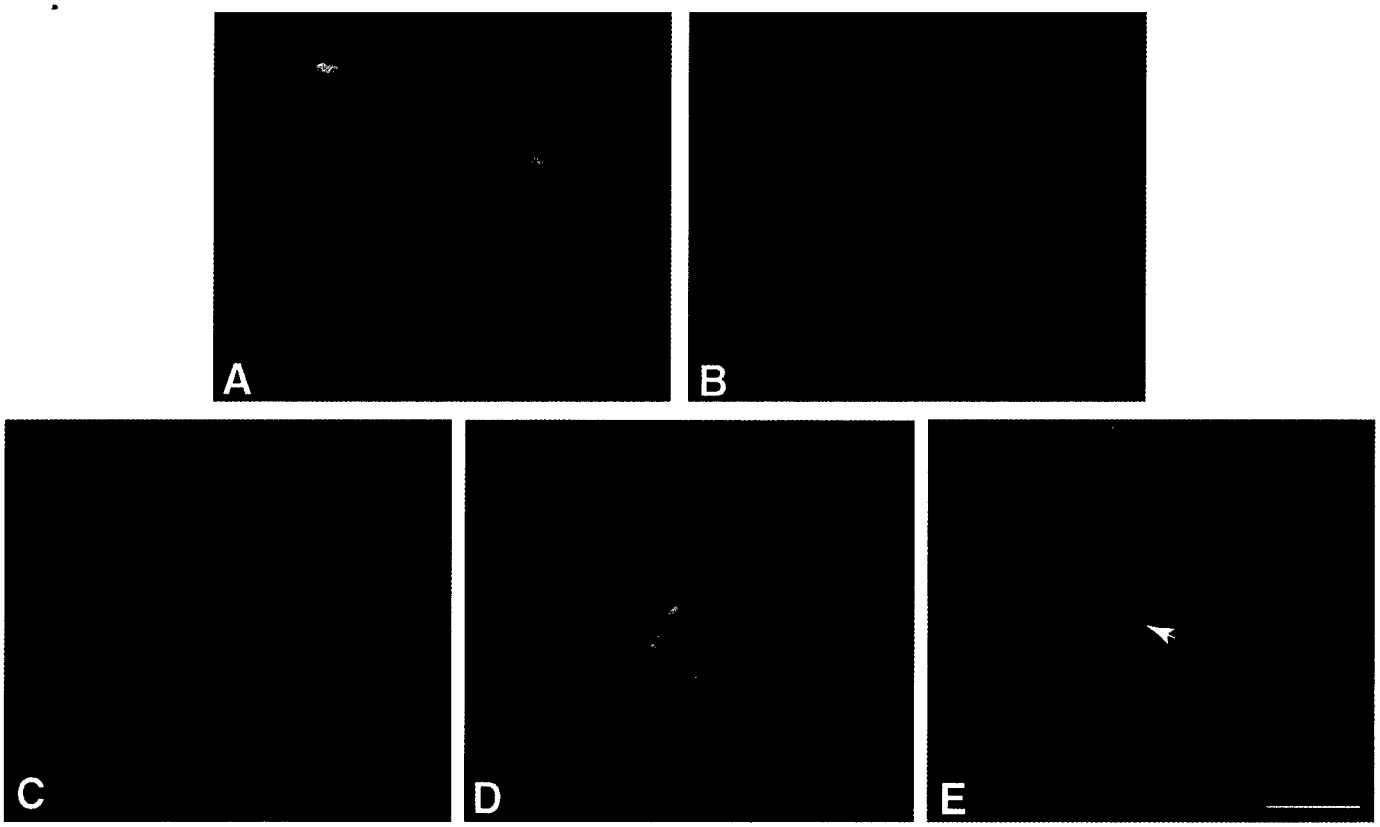

Fig. 7. Aberrant spindles missegregate chromosomes. A telophase cell from the prostate cancer cell line (PC-13) showing a tripolar spindle (*B*) with three spindle poles (*A*), some with multiple pericentrin structures (*A*, *bottom left* and *top right*). Chromosomes are segregated into three nascent daughter cells (*B*; note midbodies, the remnants of the spindle). Another telophase cell from a breast cancer cell line (BT-549, *C–E*) with multiple centrosomes at both poles (*C*) and typical midbody staining of microtubules (*D*) is shown. Missegregated chromosome(s) remain between reforming nuclei of daughter cells (*E*, *arrow*). *Green/yellow*, centrosomes; *red*, microtubules; *blue*, DNA. *A*, superposition of centrosomes and microtubules; *B*, superposition of microtubules and DNA. *Bar* (in *E*), 10 μm (for *A–E*). Horizontal series (*A* and *B*; *C–E*) are of the same cell.

numerary structures, and increased microtubule nucleation. In contrast, centrosomes in nontumor cells were consistent in size, shape, and number and indistinguishable from those of other normal cells (19). These observations clearly demonstrate that the centrosome-defective phenotype is tumor related.

The presence of centrosome defects correlated remarkably well with chromosome instability because both were significantly higher in tumor *versus* nontumor cells ($P < 0.001$, Pearson's $\chi 2$ test). Furthermore, we often observed missegregated chromosomes and defective centrosomes in the same mitotic cells, suggesting a direct relationship

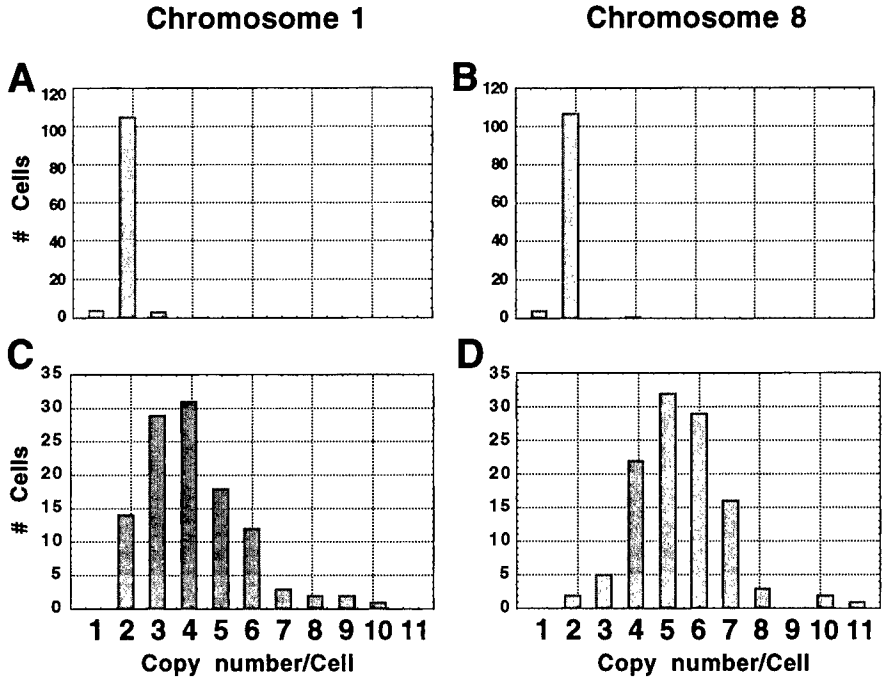

Fig. 8. CIN in tumor and nontumor cell lines. Frequency distribution of chromosomes 1 (*A* and *C*) and 8 (*B* and *D*) in a control cell line (B115, *A* and *B*) and in the breast cancer cell line (BT-549, *C* and *D*), as determined by quantitative analysis of cells stained by FISH. The copy number of chromosomes 1 and 8 are different from the mode in ~70% of the cells in BT-549 and <5% in B115. B115, mode = 2 for both chromosomes; BT-549, mode = 4 for chromosome 1 and mode = 5 for chromosome 8.

between these two cellular anomalies. In addition, we observed a statistically significant correlation between the level of centrosome defects and the level of chromosome 1 instability in tumor cells ($P < 0.05$, Spearman's rank correlation).

Although these data show a correlation between centrosome defects and CIN in tumor cell lines, they do not demonstrate that centrosomes play a direct role in the generation of CIN. Perhaps the most compelling data supporting a role for centrosomes in this process comes from transient transfection experiments showing that overexpression of a single centrosome protein (pericentrin) induces the formation of abnormal centrosomes, assembly of disorganized spindles and variability in chromosome numbers (CIN; Ref. 26). These aberrant features of pericentrin overexpressing cells are strikingly similar to those observed in malignant tumor cells. We are currently analyzing the pericentrin overexpressing cells for tumorigenic properties *in vitro* and *in vivo* (27–29).

It is easy to envision how a primary centrosome defect could contribute to CIN and, perhaps, to the development of the neoplastic phenotype. We propose a model in which centrosome defects alter the normal assembly, organization, and function of mitotic spindles, leading to the missegregation of chromosomes. These events could result in gains and losses of chromosomes that, together with the growth-selection pressure that tumors experience, provide a mechanism by which cells could accumulate tumor-promoting genes (activated oncogenes) and lose normal copies of tumor suppressor genes. Cells with these genetic defects would be predisposed to the acquisition of additional genetic lesions that could lead to the malignant neoplastic phenotype (1, 15). If centrosome defects are involved in tumorigenesis, they should appear early in tumorigenesis. We are currently examining early-stage cancers for centrosome anomalies.

The ability to induce chromosome instability through the artificial elevation of pericentrin (and perhaps other centrosome proteins) raises the possibility that a similar mechanism may be operating in tumor cells. Consistent with this idea is the universally higher levels of pericentrin staining observed in malignant tumors. Assembly of this excess protein could induce the formation of the ectopic microtubule nucleating centers and aberrant mitotic spindles that are commonly observed in tumor cells. Assembly of these multiple atypical MTOCs could occur without invoking multiple rounds of centriole duplication (18) because structures that lack centrioles and retain the capacity to nucleate and organize microtubules are found in cells of many organisms (30–33).

The centrosome defects observed in tumor cells could also arise indirectly through disruption of other cellular processes such as cytokinesis or through abrogation of cell cycle regulatory pathways such as cell cycle checkpoints that allow mitosis to proceed even when DNA is damaged or when chromosomes are improperly aligned on the spindle (see below; Refs. 1, 2, 9–11, and 34). Although cytokinesis failure may occur in some tumor cells, we believe that it cannot account for the centrosome defects observed in this study. Multiple rounds of failed cytokinesis should produce cells with structurally normal centrosomes, the numbers of which reflect multiple doublings (2 to 4 to 8, and so on; Ref. 35). However, centrosomes in tumor cells were highly variable in number and had numerous structural defects. Furthermore, cells that fail in cytokinesis should exhibit strict duplications of the genome (tetraploid, octaploid, and so on) rather than the enormous variability in chromosome number observed in this study (Fig. 8). These observations indicate that cytokinesis failure alone is insufficient to explain the defective centrosome phenotype observed in tumor cells.

Little is known about how the mammalian centrosome duplicates and assembles to form a functionally mature organelle. Results from embryonic systems have shown that centrosome duplication and assembly continues when the cell cycle is blocked (36, 37) and when DNA replication is arrested (7, 35). However, recent work suggests that the centrosome duplication cycle may be controlled by the tumor suppressor gene *p53*, which is involved in regulating cell cycle checkpoints at both G_1-S and G_2-M (18, 38, 39). In addition, other genes are likely to control this process (see Refs. 7 and 30). It does not appear that the centrosome abnormalities observed in this study result from abrogation of p53 function because some cancer cell lines used in our analysis (Lovo) exhibit centrosome defects and CIN but have normal levels of functional p53 (15). Duplication of centrioles in mammalian cells and the spindle pole body in yeast begins around the time of the G_1-S transition (start, restriction point; see Refs. 7 and 30). Although the regulatory pathways that control this transition are likely to play a role in centriole duplication in mammalian cells, it is not until late in G_2 that two functionally active centrosomes appear. This suggests that additional regulatory controls are involved in the assembly and functional maturation of centrosomes. A more detailed analysis of the centrosome-defective phenotype in malignant tumors using high-resolution microscopy (40) and other methods may provide insights into the mechanisms of centrosome assembly and maturation and may also provide a better understanding of the relationship between centrosome defects and chromosome missegregation in cancer.

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Note Added in Proof

Similar centrosome defects were recently described in breast carcinoma (W. Lingle, W. H. Lutz, J. Ingle, N. J. Maihle, and J. L. Salisbury, Proc. Natl. Acad. Sci. USA, *95:* 2950–2955, 1998.)

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The mitotic machinery as a source of genetic instability in cancer

*German A. Pihan and Stephen J. Doxsey**

Development and growth of all organisms involves the faithful reproduction of cells and requires that the genome be accurately replicated and equally partitioned between two cellular progeny. In human cells, faithful segregation of the genome is accomplished by an elaborate macromolecular machine, the mitotic spindle. It is not difficult to envision how defects in components of this complex machine—molecules that control its organization and function and regulators that temporally couple spindle operation to other cell cycle events—could lead to chromosome missegregation. Recent evidence indicates that the persistent missegregation of chromosomes result in gains and losses of chromosomes and may be an important cause of aneuploidy. This form of chromosome instability may contribute to tumor development and progression by facilitating loss of heterozygocity (LOH) and the phenotypic expression of mutated tumor suppressor genes, and by favoring polysomy of chromosomes that harbor oncogenes. In this review, we will discuss mitotic defects that cause chromosome missegregation, examine components and regulatory mechanisms of the mitotic machine implicated in cancer, and explore mechanisms by which chromosome missegregation could lead to cancer.

Key words: aneuploidy / genetic instability / chromosome missegregation / mitosis

Aneuploidy in tumorigenesis

THE UBIQUITOUS NATURE OF aneuploidy in most malignant tumors and in many early stage carcinomas suggests that this condition is intimately involved in the tumorigenic process. Recent compelling data suggest that aneuploidy develops from defects in the process of chromosome segregation during mitosis. Below we discuss the relationships between cancer, aneuploidy and chromosome missegregation.

Aneuploidy in tumors

The presence of abnormal amounts of DNA in cancer cells, first discovered in 1936,[1] has become one of the identifying characteristics of cancer cells (reviewed in refs 2–4). Aneuploidy is defined as cells with chromosome numbers that are greater or smaller than the diploid complement, and is a constant feature of solid tumors. A variety of methods have been used to demonstrate aneuploidy over the years including karyotyping,[5,6] flow cytometry,[2,3,7,8] image analysis,[3,8,9] and fluorescence *in situ* hybridization (FISH).[10–13] Importantly, aneuploidy appears to develop early during tumor progression as seen in carcinoma *in situ* of the cervix,[14,15] breast,[16,17] prostate,[18] urothelium,[19] and esophagus.[20] The appearance of aneuploidy in early stage tumors suggests that the altered DNA content, and its underlying cause, may play a role in both the development and progression of tumors. Consistent with this idea is the observation that most malignant tumors are aneuploid, have an aggressive clinical behavior and a poor outcome, while most benign tumors are diploid and curable by surgical resection[2,3,21] (see aneuploidy in solid tumors in this issue).

In support of the clinical observations are *in vitro* studies in human and animal cells indicating that aneuploidy is required for neoplastic transformation.[22,23] As in tumors, the development of aneuploidy in experimental systems occurs at an early stage[22,23] and appears to be required for cell immortalization, a critical and rate-limiting step that precedes transformation (reviewed in ref 22). Aneuploid-linked immortalization can be induced by oncogenic viruses,[22,24] chemical carcinogens,[23] ionizing radiation.[25–28] It can also occur spontaneously in fibroblast cultures derived from Li-Fraumeni families carrying germline mutations of the p53 gene.[25]

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Aneuploidy as a cause of tumorigenesis

At the turn of this century, David von Hansemann[29] and later Theodore Boveri[30] postulated that aneuploidy was the cause of cancer. With the discovery of genes, the demonstration that carcinogens, such as X-rays and alkylating agents were mutagenic,[31] see ref 32, the identification of germline gene mutations in familial cancer syndromes,[33] and the demonstration that cancer genes carried by oncogenic retroviruses are no more than mutated copies of endogenous cellular genes (proto-oncogenes),[34] the currently dominant gene mutation hypothesis gained favor. For review of gene mutation and cancer see refs 35,36 and this issue. Since aneuploidy is often accompanied by mutations, it has been difficult to determine the contribution of aneuploidy to tumorigenesis independent of genetic and other cellular changes that accompany the aneuploid state. For example, aneuploidy would be expected to alter such fundamental processes as genetic imprinting,[37,38] allelic dominance[39] and gene dosage,[40] which could all be tumorigenic. However, aneuploidy is almost always accompanied by structural chromosomal abnormalities and widespread loss of heterozygocity (LOH).[41] Both of these genetic lesions are by themselves, strongly associated with tumorigenesis. Studies designed to dissect the respective contributions of aneuploidy and genetic anomalies in tumorigenesis are difficult to interpret. For example, it has recently been shown that 'nongenotoxic' drugs are able to induce profound and near complete aneuploidy and transformation in populations of embryonic fibroblasts.[23,42] Since the effects of the drugs on genetic alterations and other cellular processes were not monitored, it is difficult to conclude that aneuploidy occurred in the absence of genetic mutations. To elucidate the roles of chromosomal missegregation and aneuploidy in tumorigenesis, future studies must be designed to specifically alter molecular components of the mitotic machinery, and examine their effects on chromosome segregation and tumorigenesis *in vivo* and *in vitro*.

Chromosome missegregation as a cause of aneuploidy in cancer

On theoretical grounds, aneuploidy could arise by at least three different mechanisms. Aneuploidy could result from sporadic missegregation of chromosomes in tumor progenitor cells leading to a stable cell population with a relatively homogeneous DNA content that is perpetuated by normal mitotic divisions. Tumors, such as chronic lymphocytic leukemia with trisomy 12 are probably good examples of this category. A second and popular hypothesis to explain the development of aneuploidy postulates that polyploidization—whether caused by multiple rounds of S-phase in the absence of mitosis (endoreduplication), mitotic failure (spindle failure, cytokinesis failure) or other mechanisms (cell fusion, etc.)—precedes aneuploidy. The subsequent and progressive loss of chromosomes from the original polyploid progenitor cell would generate aneuploid cells.[4] This condition would give rise to tumor cells with relatively stable chromosome numbers and less than tetraploid chromosome content. However, it has recently been shown that polyploidization alone does not give rise to aneuploidy,[43] but that tetraploid tumor cell populations may be a reflection of a G2 arrest during progression of a normal cell cycle, rather that true polyploidization. A third possible mechanism for generating aneuploidy—and the one we favor—involves acquisition of a permanent defect in the ability to segregate chromosomes in a tumor progenitor cell. This would lead to persistent changes in chromosome number at every cell division. The predicted outcome of this condition would be that the DNA content of tumor cell populations would be heterogeneous and continuously changing. Recent work by Vogelstein and collaborators has shown that this is likely true in the most common forms of colon carcinomas, such as those associated with APC mutations.[43] These tumors have a high degree of chromosomal instability (CIN) and a missegregation rate in excess of 10^{-2} per chromosome per generation. This dynamic form of aneuploidization appears to be common as it is found in cancers of the colon,[43] breast, lung, prostate and brain.[44] This form of instability would explain the extreme variability in karyotypes both between and within solid tumors.[5,6] It would also provide an explanation for the cell to cell variability in DNA content within malignant tumors detected by flow and image cytometry,[45–49] interphase cytogenetic analysis[50–55] and DNA content measurements of late mitotic figures in tumors.[56,57] This mechanism of aneuploidization is also consistent with the observation that virtually all malignant tumor cell lines in the American Type Culture Collection (ATCC) are aneuploid and show CIN, whereas many non-tumor diploid cell lines do not show CIN. It is also important to note that chromosome instability occurs early in malignant tumors[16,17,54] and may parallel the development of aneuploidy,[58] suggesting that CIN is not simply a late

consequence of aneuploidization. Moreover, diploid tumors[21] (G. Pihan and S. Doxsey, unpublished observations), including those resulting from microsatellite instability,[43] and diploid cell lines,[43,44] do not exhibit significant chromosome instability.

In summary, recent evidence strongly supports the notion that aneuploidy in cancer develops in most cases from the persistent missegregation of chromosomes in mitosis. In this model of aneuploidization and tumorigenesis, we believe that the persistent missegregation of chromosomes has the potential to cause, accelerate or contribute to tumorigenesis by facilitating accumulation of chromosomes with growth promoting genes (oncogenes) and deletion (LOH) of chromosomes with growth restraining genes (tumor suppressors).[44,59] In this model, chromosomes would be lost and gained in a stochastic manner and those cells with oncogenes that exhibit a growth advantage would be selected for. This selection pressure could operate at the level of entire chromosomes, different fragments of individual chromosomes, and even on the same fragment of a given chromosome.[60-63]

The mitotic machinery as a potential source of aneuploidy in tumorigenesis.

The metaphase spindle can be simply viewed as a bipolar structure with microtubules extending from the spindle poles to the chromosomes at the spindle center (Figure 1). Assembly and function of the spindle requires a complex series of temporally and spatially linked events. In addition, there are a series of regulatory pathways that control these events and ensures that they are properly timed during cell-cycle

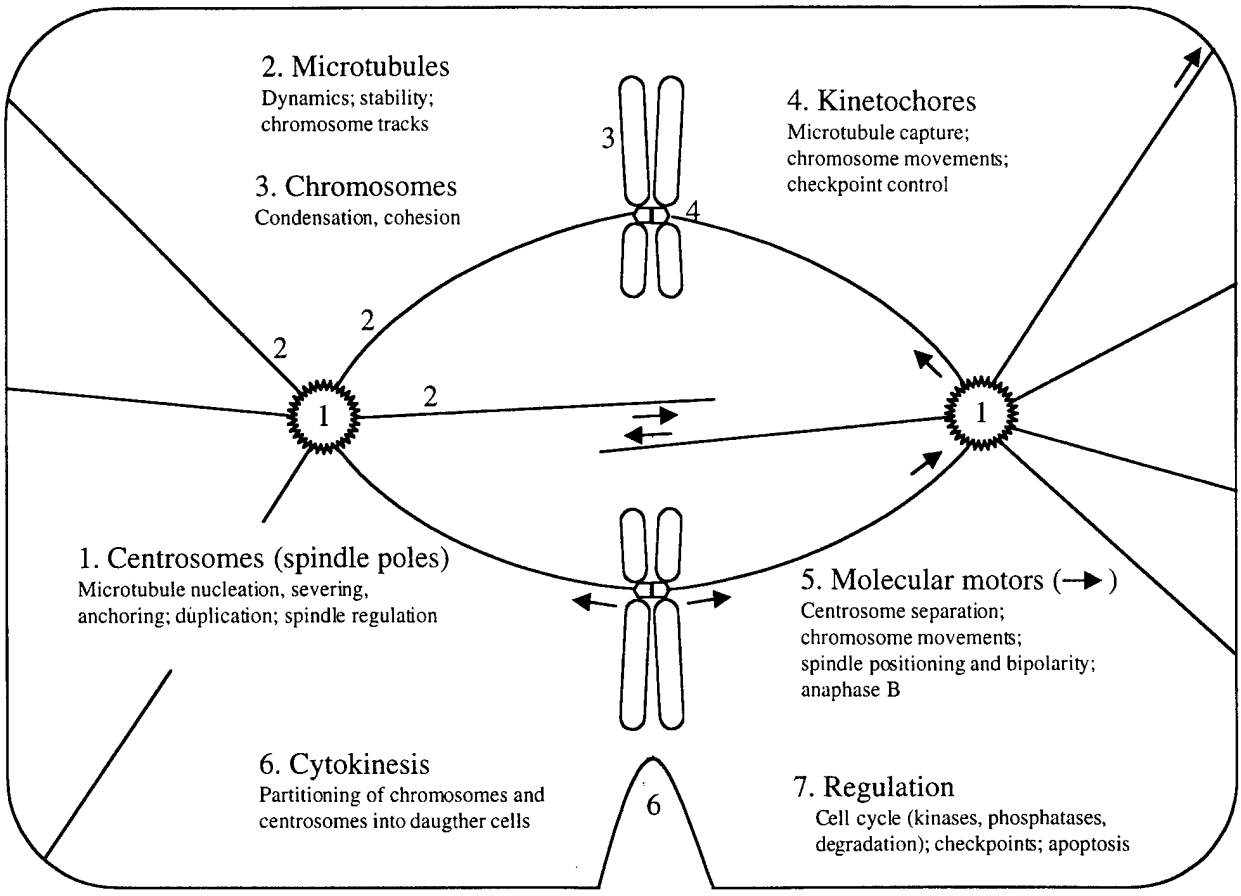

Figure 1. Components of the mitotic machinery and their functions. Defects in several mitotic functions have the potential to contribute to chromosome missegregation, aneuploidy and tumorigenesis.

progression. The events of spindle assembly and function in most human cells include nuclear envelope breakdown, depolymerization of interphase microtubules, centrosome-mediated nucleation of new microtubules, centrosome separation, chromosome condensation, congression, cohesion and movement, and cytokinesis (see Figure 1).

In Section 2 we will discuss the contribution of components and regulators of the mitotic machinery to chromosome missegregation and aneuploidy. While there is not yet a direct link between defects in the mitotic machine and aneuploidy in cancer, there is a growing list of molecular components and processes that cause chromosome missegregation *in vitro* and *in vivo*, making them prime candidates for tumorigenesis.

A. Microtubules

The microtubule spindle provides the structural framework for many of the processes that take place during mitosis (for review see ref 64). For example, kinetochore microtubules that arise from centrosomes and contact chromosomes, provide the tracks for chromosome segregation during mitosis. Polar microtubules that interconnect the two poles of the spindle through lateral interactions at the spindle center, participate in moving the spindle poles apart during anaphase. Non-spindle (astral) microtubules interact with the cell cortex and are involved in orienting the spindle in the cytoplasm and may also play a role in anaphase B movements. Most of these microtubule interactions are highly dynamic, a consequence of the inherently dynamic nature of microtubule ends, and the activity of microtubule motor proteins (see following).

Microtubules are essential for spindle function as is perhaps best illustrated by the widespread and effective use of anti-microtubule drugs in cancer therapy.[65–67] Anti-microtubule drugs effectively block mitosis and thus, cell proliferation. At the molecular level, these agents work by depolymerizing microtubules or by modifying microtubule dynamics.[68] By analogy, cellular components that affect microtubule polymerization, dynamics and stability could contribute to spindle malfunction. It is conceivable that minor changes in these microtubule properties could lead to chromosome missegregation and aneuploidy.

The contribution of microtubule defects to aneuploidy in cancer progression has not yet been addressed although several studies are consistent with such a role. For example, numerous chemical compounds that affect microtubule function, can induce aneuploidy (reviewed in ref 69). Moreover, changes in the expression of the tubulin, the subunit of microtubules[70] and mutations in the tubulin genes,[71] can lead to chromosome missegregation. In addition, a number of proteins that directly or indirectly interact with microtubules (in addition to motor proteins, see below), induce chromosome missegregation when overexpressed, mutated or functionally abrogated. These include the *Saccaromyces cerevisiae* genes Stu2p,[72] rhc21p,[73] CIN1, CIN2 and CIN4,[74] the Schizosaccaromyces pombe genes rad21[75] and Mal3,[76] the *Drosophila* gene product Sup35p[77] and the *Xenopus* XMAP215, XMAP230 and XMAP310.[78] Human homologs for most of these proteins have been identified and some, such as TOGp, a homolog of XMAP215, is overexpressed in some cancers.[79] However, the contributions of these genes to chromosome missegregation in cancer is currently unknown. Other proteins that may affect chromosome segregation are microtubule-associated proteins (MAPs), such as stathmin/Op18,[80] Tau and others (for review see refs 81,82). Stathmin/Op18 promotes microtubule instability[83] and is overexpressed in leukemias and lymphomas[84,85] making it a good candidate for a chromosome instability (CIN)-promoting factor. Consistent with this view, is the observation that overexpression of a dominant negative mutant form of stathmin/Op18 induces chromosome segregation abnormalities.[86]

B. Centrosomes

Recent studies indicate that centrosome defects may contribute to spindle abnormalities, aneuploidy and tumor development and progression (for review see ref 59). Centrosomes are comprised of a pair of centrioles (microtubule barrels) surrounded by a protein matrix known as the pericentriolar material or centrosome matrix. Centrosomes play a vital role in organizing both the microtubule network in interphase cells and the mitotic spindle during cell division. While centrioles may play a role in organizing the centrosomal material,[87] it is the centrosome matrix that nucleates microtubules. In addition to nucleation, the centrosome appears to be involved in other important processes during mitosis including severing, movement and anchoring of microtubules, and they appear to provide a scaffold for localization of mitotic regulatory activities. For more information on centrosome structure and function see refs 88,89.

γ-Tublin and pericentrin are two centrosome ma-

trix proteins involved in microtubule nucleation. They appear to colocalize at the centrosome and are both part of a cytoplasmic complex.[89,90] It has been proposed that assembly of the complex onto centrosomes regulates microtubule nucleation at the centrosome,[89,90] and that the regulation of centrosome-mediated microtubule nucleation controls spindle assembly and function in mammalian cells (see ref 59). Many other proteins are found at the centrosome[91] including cell cycle regulatory molecules and others implicated in tumorigenesis (for review, see ref 59).

Centrosome defects in tumors

Over 100 years ago Theodor Boveri hypothesized that centrosome abnormalities lay at the origin of cancer.[30] Recent studies support this hypothesis. Using antibodies to pericentrin and γ-tubulin, it has been shown that the vast majority of malignant tumors exhibit abnormal centrosomes.[44] These include carcinomas of the prostate, breast, lung and colon as well as tumors of the brain. Centrosome abnormalities included: supernumerary centrosomes, acentriolar centrosomes and centrosomes of aberrant size and shape.[44] Some tumor cells had no immunostainable centrosomes (G. Pihan and S. Doxsey, unpublished observations). Centrosome abnormalities were accompanied by dramatic changes in the number and distribution of nucleated microtubules. They emanated from multiple cellular sites instead of a single site (the centrosome), and collectively they constituted a much greater number than in non-tumor cells. In another study in which high grade breast carcinomas were analyzed using antibodies to another centrosome protein centrin, multiple large centrosomes and aberrant phosphorylation of centrosome proteins was observed.[92]

Centrosome abnormalities in tumors and tumor-derived cell lines induced two phenomena that could contribute to tumorigenesis.[44] First, all centrosomes regardless of size, shape or number, were able to participate in the formation of structurally and functionally aberrant mitotic spindles. Second, cells with abnormal centrosomes missegregated chromosomes at a high rate producing aneuploid cells with dramatically different chromosome numbers (i.e. chromosome instability[43]). Based on these observations, we propose a model in which centrosome abnormalities induce spindle defects that lead to chromosome missegregation and aneuploidy. Aneuploidy is a form of genetic instability that is likely to contribute to tumor development and progression (see above and refs 44,59).

Mechanisms for generating centrosome defects in tumors

The mechanism(s) by which centrosome abnormalities are generated in malignant tumors has not yet been determined. Below we discuss how malfunction of three cellular processes—centrosome assembly, centrosome duplication, and cytokinesis—either singly or in combination, could produce an abnormal centrosome phenotype.

It is possible that the increased levels of pericentrin and γ-tubulin observed in tumor cells, leads to ectopic assembly of the proteins into aberrant and supernumerary structures.[44] Consistent with this idea is the observation that tumor cells that have high levels of the proteins form supernumerary and gigantic centrosomes, while tumor cells with low protein levels appear unable to form centrosomes at all. Further support for this idea comes from studies showing that overexpression of the centrosome proteins pericentrin (A. Purohit and S. Doxsey, unpublished observations), γ-tubulin[93] and a Ran binding protein,[94] all lead to ectopic assembly of acentriolar structures that nucleate microtubules. Perhaps more compelling is data showing that forced expression of pericentrin induces spindle defects and anueploidy, features indistinguishable from those seen in tumor cells (A. Purohit and S. Doxsey, unpublished observations). It should be noted that many of the pericentrin overexpressing cells form structurally normal bipolar spindles that nevertheless missegregate chromsomes. This suggests that in addition to gross spindle defects induced for example by multiple centrosomes,[44] more subtle defects, such as missegregation of single chromosomes could be caused by centrosome defects that are undetectable by conventional imaging techniques. Among the subtle defects are single spindle poles that have multiple centrosomes, a feature common to many tumor cells (Pihan and S. Doxsey, unpublished observations) and other cell types.[95,96] The presence of excess centrosomal material at spindle poles may contribute to defects in spindle function that have yet to be uncovered.

A second potential mechanism for generating centrosome defects in tumor cells is through misregulation of centrosome duplication. Centrosomes are duplicated once and only once during each cycle in normal cells, and the two resulting centrosomes (each with two centrioles) form the poles of the mitotic spindle and contribute to spindle assembly (see ref 88). We believe that this pathway would not generate centrosome defects, such as those observed in tumors for two reasons. Abnormal centrosome duplication would not produce acentriolar structures that nucle-

ate microtubules and it would not induce assembly of centrosomes that are structurally, biochemically and functionally abnormal, such as those seen in tumor cells.[44]

Another mechanism by which the number of centrosomes could be increased in tumor cells is through failed cytokinesis.[97] Failure of cells to divide would create tetraploid cells with twice the number of centrosomes. However, as with centrosome duplication, cytokinesis failure would result in cells with structurally normal centrosomes that contained centrioles, a phenotype not observed in tumor cells. For more details on cytokinesis, see below.

Misregulation of centrosome structure and function in tumors

Several centrosome-associated regulatory molecules have been implicated in centrosome function. These include kinases that are believed to regulate centrosome assembly and integrity, such as the human homologue of Polo (Plk), the human homologue of *Drosophila aurora* (aurora2/Stk15) and NEK 2 (for reviews see refs 59,89). Moreover, forced expression of the aurora2 is able to transform fibroblasts *in vitro* and produce tumors *in vivo*.[98,99] Recent studies indicate that centrosome duplication is controlled by the centrosome-associated cdk2-cyclin E protein complex,[100] although the status of this complex in tumor cells is unknown. Supernumerary centrosomes are observed in $p53^{-/-}$ cells,[101] suggesting that this regulatory molecule may affect centrosome duplication. One caveat of this study is that centrosome abnormalities can also occur in cells with wild type p53 (G. Pihan and S. Doxsey, unpublished observations) suggesting that abrogation of p53 function is not an absolute requirement for generating supernumerary centrosomes. Aurora2, Plk and other kinases and regulatory molecules have also been implicated in the regulation of centrosome duplication (see ref 59).

C. Molecular motors and spindle movements

Disruption of microtubule motor proteins can induce chromosome missegregation and aneuploidy although it is still unclear if motors play a role in tumorigenesis. Motors provide much of the power for chromosome and spindle movements during mitosis. They also provide directionality to these movements —one class of motor moves toward the minus ends of microtubules (toward the spindle pole) and the other toward the plus ends. Motor proteins provide the force for centrosome separation, chromosome congression and segregation, spindle elongation in anaphase B and spindle positioning. Motors may also serve to anchor microtubules, depolymerize microtubules[102] and focus the poles of the spindle.[103] Given their involvement in multiple mitotic functions, motors have the potential to dramatically affect chromosome segregation. Below we give some examples of mitotic processes driven by molecular motors and indicate how they may perturb chromosome segregation. For more information on motor proteins and their mitotic functions see refs 104–106.

Centrosome separation

The separation of centrosomes in mitosis is mediated by motor proteins that interact with intercentrosomal microtubules. Several motor proteins participate in this process including HsEg5[107] and Xklp2.[108] Failure of centrosome separation during mitosis may produce monopolar spindles that do not progress through mitosis giving rise to polyploid and aneuploid cells. Pertubation of centrosome separation may also produce functionally impaired bipolar spindles.

Chromosome movements

Perhaps the most important function of microtubules and microtubule motors in mitosis is the movement of chromosomes. Microtubule motors are involved in the movement of chromosomes toward and away from the spindle poles during congression of chromosomes to the metaphase plate,[109–111] and in the poleward movement of chromosomes during chromosome segregation at anaphase[112] (for review, see ref 113). In addition, microtubule motors drive the separation of spindle poles (and chromosomes) in anaphase B by inducing the anti-parallel sliding of microtubules at the central spindle. Defects in motor proteins that move chromosomes can affect chromosome alignment and segregation. For example, microinjection of antibodies against the kinetochore motor, CENP-E or overexpression of a transdominant negative form of CENP-E, both abolish chromosome alignment at the spindle equator.[111] Similarly, overexpression of dynamitin, a subunit of the dynactin complex, disrupts chromosome alignment by dissociating the dynein motor from kinetochores.[110] In contrast, disruption of (non-kinetochore) chromosome-associated kinesin-like proteins (Klps), such as Nod, lead to precocious movement of chromosomes to the poles.[114–116] Similar experiments have demonstrated that dynein and CENP-E are also involved in chromosome segregation during anaphase and when disrupted, cause chromosome missegregation.[117] Given

their importance in partitioning of chromatin in normal cells, it will be interesting to determine whether these and other motor proteins contribute to chromosome missegregation in cancer. In this regard, it is interesting that the human kinesin-like protein, chromokinesin appears deregulated in retinoblastoma cells,[118] and that another Klp, KIF4 binds to murine leukemia virus Gag proteins.[119]

D. Chromosomes

Cellular structures and processes that remodel chromosomes and facilitate chromosome movement could have profound affects on the fidelity of chromosome partitioning during mitosis. These include kinetochore structure and function and chromosome condensation and cohesion. For more information on chromosome structure and function see refs 113,120,121.

Centromeres and kinetochores

Although there is little direct evidence that kinetochore defects play a role in tumorigenesis, centromere DNA lacking kinetochore proteins have been observed in tumor cells.[122,123] Kinetochores are complex multiprotein structures assembled around specialized regions of chromosomes (centromeres) that play multiple essential roles during mitosis.[113] First, they provide unique sites for attachment of spindle microtubules during mitosis (kinetochore fibers). Second, they anchor the microtubule motors required for moving chromosomes both toward and away from the spindle poles during chromosome congression and segregation[109–112] (for review, see ref 113). Third, kinetochores possess proteins involved in the metaphase to anaphase transition checkpoint (spindle checkpoint, see below) which ensures that anaphase is initiated only after all chromosomes have established bipolar attachments to the spindle.[124] From this discussion, it is clear that kinetochore malfunction could induce missegregatation of chromosomes by interfering with any of the functions described above. Some of these potential defects in kinetochores are discussed in the context of molecular motors (see above) and spindle checkpoints (see below).

Chromosome condensation and cohesion

Chromosome condensation is a fundamental mitotic event that solves two topological problems of chromosome segregation: entanglement between replicated chromatids and excessive length of a interphase chromosome. For reviews on chromosome condensation and decondensation see ref 121.

Recent results suggest that defects in chromosome condensation can induce chromosome missegregation. One class of molecules required to relieve the topological constrains resulting from chromosome entanglements during DNA replication and from supercoiling during chromosome condensation are the topoisomerases (for review see refs 125–130). Inhibitors of topoisomerase II can induce both numerical and structural chromosomal abnormalities by interfering with chromosome condensation/decatenation.[131] A similar phenotype could arise from mutations in topoisomerase II and other molecules involved in chromosome remodeling, and could thus contribute to genetic instability in tumor cells. Some tumor cells have mutations in genes involved in chromosome condensation, such as topoisomerase II, although it is not yet clear if they play a role in tumorigenesis[132] other than conferring resistance to topoisomerase II inhibitors. Another class of molecules involved in chromosome condensation are members of the SMC (structural maintenance of chromosomes) family of proteins (for review see refs 133–135). These proteins associate with a large complex called condensin[135–137] and together with topoisomerase I, can supercoil DNA *in vitro*. This activity is regulated by the mitotic kinase cdc2-cyclin B through phosphorylation of three of the condensin protein subunits.[138] Mutations in some of these genes prevent chromatin condensation resulting in fragmentation of chromosomes and chromosome missegregation during mitosis.[127,135,139]

The cohesion of replicated sister chromatids is mediated by protein–protein interactions (reviewed in ref 140). Cohesion is established during chromosome replication[141] and is not lost until the subsequent metaphase/anaphase transition. Mutations in several yeast genes cause early dissolution of cohesion leading to premature separation of sister chromatids or prevent dissolution and lead to chromosome nondisjunction.[142] Both defects can lead to chromosome missegregation and both have been postulated as potential causes of aneuploidy in human tumors.[122,143] In yeast, and possibly in animal cells since homologs exist, two SMC genes (SMC1 and SMC3) and two SCC (sister chromatin cohesion) genes, SCC1 and SCC2, are required for this process.[140] Untimely separation and replication of chromatids (at centromeres) have been documented in cancer,[144] in cancer predisposition,[145] and are suspected in the generation of aneu-

ploidy. In yeast, dissolution of cohesins and separation of sister chromatids is achieved by proteolysis of Scc1p/Smc1 (reviewed in ref 146). This and other proteolytic events required for sister chromatid separation are accomplished by the anaphase promoting complex, a mitotic proteolytic machine that is itself highly regulated[146–149] and a potential contributor to chromosome missegregation in cancer (see below).

E. Cytokinesis

Although not directly implicated in tumorigenesis, cytokinesis—the physical division of one cell into two daughter cells—has the potential to contribute to tumor development and progression.[4,44] For example, failure of cytokinesis would create a cell with two sets of chromosomes and two centrosomes, thus setting the stage for centrosome-mediated formation of dysfunctional spindles and the generation of aneuploid cells (see Section B, above).

Cytokinesis is the last step of mitosis and arguably the least understood of all. In mammalian cells, cytokinesis involves the ingression of the equatorial cell membrane of the dividing cell, driven by an actin–myosin contractile ring, until the equator congresses to a single point where the daughter cells can separate. Cytokinesis is composed of five discrete events: cleavage plane specification, contractile ring assembly, furrow ingression, midbody formation and cell separation. Malfunction at any stage could potentially cause problems in the ability of the cell to divide properly. For more information on cytokinesis see refs 4,97,150,151.

Cytokinesis and tumorigenesis

Cytokinesis failure has been observed in tumor cells. The maintenance of a sustained G2 cell cycle arrest after DNA damage in tumor cells appears to be dependent on the presence of functional p53/p21 pathway.[152] In cells with abrogated p53 and/or p21, cells escape G2 arrest, enter mitosis, segregate chromosomes but fail to undergo cytokinesis leading to a doubling of the DNA complement.

Cytokinesis failure has also been implicated in polyploidization, a condition common in tumors.[4] However, cytokinesis failure on its own—in the absence of other cell cycle or spindle anomalies—would be expected to produce cells with two diploid nuclei (not a single tetraploid nucleus), since it occurs only after chromosomes have been properly segregated. Recently, it has been shown that overexpression of AIM-1, an aurora-related mid-body protein in human cells induces multiple nuclei and increased ploidy providing a direct connection between a cytokinesis protein and a cancer-like phenotype.[153] In addition two known oncogenes, Vav-2[154] and Mos[155] have been shown to induce cytokinesis abnormalities.

Although multinucleate cells are commonly observed in yeast cytokinesis mutants (see below), they are infrequently observed in tumors with near tetraploid DNA content, indicating that cytokinesis failure by itself is unlikely to be a major cause of polyploidization in tumors. However, this does not preclude the possibility that cytokinesis failure may occur in tumor cells together with other mitotic and cell cycle defects that result perhaps from malfunction of an upstream regulator of multiple cellular processes.

Mechanism and regulation of cytokinesis: potential role in tumorigenesis

Cytokinesis can also be affected directly through functional abrogation of components of the cytokinetic machinery or molecules that regulate cytokinesis, and can result in the generation of aneuploid and polyploid cells. Cut mutants ('cell untimely torn') in *S. cerevisiae* undergo premature cleavage and 'cut' the DNA randomly at any time during the cell cycle. These events usually give rise to cells with fragmented chromosomes or cells with different chromosome numbers (see ref 127). Mutations in several genes whose gene products are part of the cytokinetic machinery or regulate late cytokinetic events (e.g. ring constriction), block cytokinesis and produce polyploid cells. This group of molecules also includes proteins involved in coupling cytokinesis to other mitotic events that appear to serve as cytokinesis checkpoint genes.[156] Such molecules and mechanisms that directly affect cytokinesis have not yet been linked to tumorigenesis and thus represent important areas for future investigation.[97]

Regulating the mitotic machine: regulatory circuits, mitotic checkpoints and control of apoptosis.

The many components of the mitotic machine (Figure 1) appear to be regulated by specific pathways and are also integrated through common pathways. Defects in the regulation of mitosis would thus be expected to affect individual processes in some cases

and multiple processes in others. Below we discuss the regulatory mechanisms that control mitosis and those implicated in tumorigenesis.

Regulatory circuitry

Entry into and exit from mitosis is controlled by multiple cell cycle regulatory pathways including the p34cdc2/cyclin B kinase cascade and ubiquitin-dependent proteolysis.[146,157] Deregulated expression of p34cdc2 has been observed in several cancers[158-161] and has been associated with polyploidization in megakaryocytes,[158-164] tumor cell lines[165,166] and virally infected cells.[167] Anaphase is triggered by activation of the anaphase promoting complex or cyclosome (APC/C), a multiprotein complex that ubiquitinates cyclin B and proteins involved in sister chromatid cohesion, and targets them for destruction by the proteosome.[146-149,168] Recently, it has been shown that the APC can be activated in a substrate-specific manner, and can thus control the tempo of different anaphase/telophase events (reviewed in ref 146). Mutations of some APC components in yeast are known to cause chromosome missegregation.[169,170] In humans, a fusion protein from Ewing's sarcoma (EWS/FLI1), up-regulates a ubiquitin conjugating enzyme involved in cyclin B destruction.[171] Moreover, human CDC23, another APC component, is a candidate tumor suppressor gene on chromosome 5q31, an area often deleted in many hematological malignancies.[172] These observations indicate that alterations in mitotic regulators occur in tumors and may contribute to chromosome instability.

Mitotic checkpoints

Progression through mitosis is monitored by at least two checkpoints: one that operates in early prophase and controls mitotic entry and one that controls the metaphase/anaphase transition. It is also possible that additional checkpoints control anaphase progression or cytokinesis in mammalian cells as they do in yeast.[156,173]

The metaphase/anaphase transition checkpoint is activated by kinetochores that remain unattached to the spindle and delays the cell cycle at metaphase until all chromosomes have established bipolar attachment. This information is communicated through an elaborate kinetochore protein complex composed of Mps1p and several Mad and Bub proteins, as well as components of the anaphase-promoting complex (for review see refs 124,174). Injection of antibodies to proteins of this complex triggers premature anaphase onset even in the presence of unattached kinetochores.[175] Recent work by Cahill and collaborators has implicated defective mitotic checkpoint control in the development of chromosome instability (CIN) in colorectal cancers.[176] They found that cell lines with CIN do not maintain a metaphase arrest when subjected to anti-microtubule disruption of the mitotic spindle, while diploid cell lines without CIN do arrest.[176] One checkpoint gene, hBub1, was found to be mutated in a low proportion of colorectal cell lines. Interestingly, transfer of the mutated gene to a diploid, CIN-negative cell line abrogated the checkpoint and induced CIN.[176]

Oncogenic viruses often cause aneuploidy and express proteins that can interact with the cell cycle machine.[177,178] Recently it has been demonstrated that viral oncoproteins may target components of mitotic checkpoints. The HTLV-I TAX oncoprotein, targets the mitotic checkpoint protein MAD1 (see below), interferes with its function and leads to multinucleation and aneuploidy.[179] Papillomavirus E2, E6 and E7 proteins and SV-40 large T antigen have been shown to interfere with mitotic checkpoints although the targets have not been delineated.[180-182] The adenovirus E2 protein can abolish the mitotic spindle checkpoint after colcemid treatment.[183] These observations are consistent with data showing that viral oncoproteins, such as LMP-1, and SV-40 large T antigen, induce multinucleate cells when overexpressed.[184,185] Together these observations suggest that destabilization of mitotic checkpoints may be a more common pathway for viral oncogenesis than is currently appreciated.

Apoptosis in mitosis

Given the complexity of mitosis in normal cells, it is likely that aberrant mitoses occur at some frequency and produce cells with abnormal chromosome numbers. These cells could either die due to lack of life sustaining genes, grow as normal, become tumorigenic or die by apoptosis. Recent studies indicate that there may be a default pro-apoptotic pathway in mitosis that needs to be actively overcome for the successful completion of mitosis.[186] This pathway appears to involve survivin, an inhibitor of apoptosis[187] expressed in G2/M and associated with microtubules. Survivin is overexpressed in many cancers[188] and may constitute an important mechanism whereby cancer cells progress through aberrant mitoses and fail to undergo apoptosis.

Apoptosis in mitosis can be induced by aberrant microtubule function. In fact, the apoptotic activity of anti-microtubule agents may be the reason for their therapeutic efficacy (reviewed in ref 189). High doses of antimicrotubule agents induce p53-independent apoptosis during mitosis[190] and may be mediated through mitotic checkpoint proteins. For instance, expression of a dominant-negative mutant Bub1 protein (see above), leads to a reduction in the number of apoptotic cells after treatment with nocodazole, a microtubule depolymerizing agent.[191] Bcl-2 appears to be involved in this pathway as well. Disruption of microtubules leads to bcl-2 phosphorylation and inactivation, initiating apoptosis (reviewed in ref 192). Low doses of antimicrotubule agents lead to apoptosis via an apparently different mechanism. Some cells ($p53^{-/-}$) continue to cycle, become large and multinucleated and eventually die, while others ($p53^{+/+}$) arrest in G1 and undergo apoptosis.[68,190]. These and other data suggest that apoptosis during mitosis is a safeguard mechanism much like those enacted in other phases of the cell cycle. The data also suggest that abrogation of these mechanisms may have an important permissive role in the development and progression of cancer by allowing cells to progress through abnormal mitoses that could generate genetically unstable progeny.

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regulated *SH2D1A* could be introduced into autologous haematopoietic stem cells, as proposed for other X-linked immunodeficiencies, thus bypassing the substantial risks of allogeneic transplantation. Nevertheless, a number of theoretical and practical questions must be addressed before such an approach can be undertaken. The function of SH2D1A and the consequences of its dysregulation should be investigated in more detail. The putative existence of dominant-negative SH2D1A proteins also must be recognized, as their presence can undermine complementation strategies.

The lack of skewed mosaicism in *SH2D1A* mutant heterozygotes suggests that competitive repopulation by corrected stem cells would not take place, thus requiring myeloablation to achieve successful engraftment. Alternative strategies to stem cell replacement are therefore desirable. The genetic modification of autologous T cells or their more immediate precursors represents an attractive option. Indeed, the infusion of EBV-reactive donor T cells can be effective against EBV-associated lymphoproliferative disease in allogeneic bone marrow transplant recipients[10]. If wild-type *SH2D1A* expression restores the ability of T cells to effectively contain EBV infection, genetically corrected T cells, generated *in vitro* prior to EBV infection, could be useful in a prophylactic or therapeutic setting. In providing cellular rather than humoral immunity, T cell-based therapy could provide a layer of immune protection that passive immunization with immunoglobulins cannot achieve.

Alternatively, active immunization might be envisaged to attenuate the fatal spiral of events set off by EBV infection. However, the findings of Coffey *et al.* and Sayos *et al.* also raise the possibility that such an intervention would stoke the fire of the aberrant response in XLP patients.

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The centrosome—a tiny organelle with big potential

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Centrosomes were observed over 100 years ago by Theodor Boveri who believed they represented the "material of inheritance"[1]. While they have not lived up to Boveri's original claim, centrosomes have re-entered centre stage as structures involved in partitioning the material of inheritance—now commonly known as chromosomes—into daughter cells during cell division. Boveri later understood the importance of centrosomes in segregating chromosomes and proposed that chromosome missegregation (aneuploidy) in human malignant tumours could arise from defects in centrosome function[2]. The work described by Hongyi Zhou and co-workers on page 189 (ref. 3), together with recent data from other laboratories, provides a more direct link between centrosomes and tumorigenesis.

The centrosome is an inconspicuous organelle, about 1 μm in diameter, that occupies a position at the centre of interphase cells (hence the term *centro-some* or *central body*, assigned by Boveri; ref. 4). The best known function of the centrosome is its ability to nucleate the growth of microtubules, cellular fibres that form astral arrays in interphase and undergo a dramatic reorganization to form the mitotic spindle during cell division. Spindle assembly and organization is orchestrated in part by the centrosome. Prior to mitosis, the centrosome duplicates by an intriguing process that is poorly understood, and each new centrosome acquires an increased ability to nucleate microtubules through the recruitment of microtubule-nucleating proteins[5-7]. The microtubules nucleated

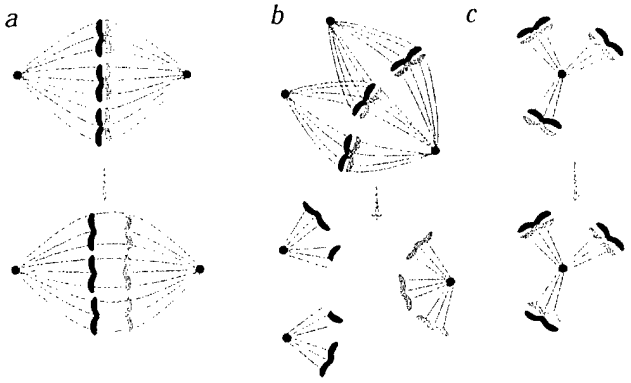

Fig. 1 Mitotic spindles in normal and centrosome-defective cells. ***a***, Centrosomes (red) are located at the poles of the metaphase spindle. The replicated chromosomes (blue) are aligned at the spindle centre. Microtubule fibres (green) arise from centrosomes and contact chromosomes at protein plaques known as kinetochores that bind to specific DNA sequences at the chromosome centromeres. Upon exit from mitosis, the replicated chromosomes are partitioned equally between the two resulting daughter cells and ultimately come to reside within the newly-formed nuclei of the nascent cells. Centrosome anomalies can lead to spindle disorganization and aneuploidy. Excessive duplication or ectopic assembly of centrosome proteins could lead to multipolar spindles (***b***) and segregation of two sets of chromosomes into more than two progeny. The forces generated from pulling a single chromosome toward more than one spindle pole could also create chromosome breaks. Failure to duplicate or separate centrosomes could lead to monopolar spindles that are unable to segregate chromosomes (***c***).

from centrosomes at the poles of the nascent spindle bind to chromosomes and position them at the spindle centre (Fig. 1*a*). As the cell exits mitosis, chromosomes move towards the spindle poles along tracks formed by microtubules, leading to the segregation of chromosomes into two daughter cells following cell division.

It is not difficult to envision how spindle abnormalities could result from perturbations in centrosome assembly or function. Excessive duplication of centrosomes or ectopic assembly of microtubule nucleating proteins could lead to the formation of spindles with multiple poles (Fig. 1*b*; Fig. 2). Multipolar spindles could segregate the replicated sets of chromosomes into more than two daughter cells. Moreover, multidirectional forces exerted on a single chromosome in a multipolar spindle could create chromosome breaks (Fig. 1*b*, bottom). Failure to duplicate or separate centrosomes could lead to the formation of monopolar spindles that would be unable to segregate chromosomes, resulting in stalled cell division (Fig. 1*c*). Mitotic failure could also result from the inability of centrosomes to recruit microtubule nucleating proteins required for the formation of spindle fibres. In all of these scenarios, daughter cells would receive abnormal numbers of chromosomes and become aneuploid.

Fig. 2 Multiple abnormal centrosomes in a cancer cell. Normal interphase cells have a single centrosome (bottom left) that is near the nucleus (blue) and nucleates a single microtubule aster (green). Cells from malignant tumours often contain multiple centrosomes (yellow) that are highly variable in size and shape and distributed randomly throughout the cytoplasm. Despite their size variability, all centrosome-like structures are able to nucleate microtubules (green) and contribute to the assembly of multipolar spindles.

STK15, centrosome amplification and transformation

The work presented by Zhou *et al.* shows that a human serine/threonine kinase, STK15, associates with centrosomes and is amplified in multiple human cancers known to be aneuploid. Similar results were obtained by Plowman and co-workers who call the identical kinase aurora2 (ref. 8), named after a homologous protein originally identified in *Drosophila*. *Drosophila* aurora is a centrosome-associated kinase that has a role in centrosome maturation and spindle assembly[9]. Consistent with this is the fact that the *Saccharomyces cerevisiae* homologue of aurora has been shown to regulate chromosome segregation, although the precise mechanism was not determined[10]. Taken together, these early studies on *Drosophila* and yeast proteins suggest a possible connection between centrosome dysfunction and chromosome segregation.

The data of Zhou *et al.* and the Plowman group show that the aurora2/STK15 kinase is associated with cancers. The gene maps to chromosome 20q13.2, a 'hot spot' frequently amplified in human cancers. Furthermore, it is amplified and the levels of the RNA, protein and kinase activity increased in many malignant human tumours. As molecular oncologists know all too well, however, such data are merely suggestive of an oncogenic role; it could well be that the elevated kinase levels are a result of the tumorigenic process and do not directly contribute to the cancer phenotype.

To address whether the kinase is oncogenic, Zhou *et al.* overexpressed the protein in nonmalignant cultured cells. Cells expressing the kinase acquired altered growth characteristics and formed colonies in soft agar, features of cell transformation typical of tumour cells. Tumorigenic potential was further demonstrated by showing that cells expressing the kinase (but not the kinase-dead mutant) were able to induce tumour formation in rats[8].

While these data demonstrate that the centrosome-associated kinase has oncogenic properties, they do not address the role of the centrosome in oncogenesis. Recent work has shown that centrosomes are abnormal in number, form and function in a wide range of human malignant tumours[11,12], although the mechanism by which centrosome anomalies arise is unknown. Zhou *et al.* have provided compelling evidence for amplification of centrosome number in cultured cells overexpressing STK15. Moreover, they show that chromosomes are mis-segregated in these cells and that the cells become aneuploid—suggesting a role for STK15 in the regulation of centrosome number and function and the proper partitioning of chromosomes during mitosis.

A simple model can be proposed to explain the mechanism by which an inappropriate increase in STK15 activity could contribute to oncogenesis (Fig. 3). In this model[11], an increase in kinase levels causes centrosome dysfunction, leading to the assembly of aberrant spindles and the improper segregation of chromosomes. Chromosome mis-segregation could result in gains and losses of genes that confer tumorigenic potential or predispose cells to additional tumorigenic lesions. The precise mechanism by which STK15 alters centrosome function and how it contributes to tumour progression has yet to be elucidated.

As with most biological processes and particularly with tumorigenesis, the story is more complicated than appears at first sight. It is likely that STK15 is one kinase in a complex pathway (or parallel pathways) that controls centrosome assembly and function. Support for this idea comes from the study of other potentially oncogenic molecules that have similar effects on centrosomes. For example, the centrosome-associated human kinase, PLK1 (a homologue of *Drosophila* polo) has properties akin to that of STK15; it regulates centrosome function[13], transforms cells *in vitro*[14] and is elevated in tumours[15]. Genetic alterations in the ataxia-telangiectasia and rad3-related kinase gene (*ATR*) may contribute to the development of

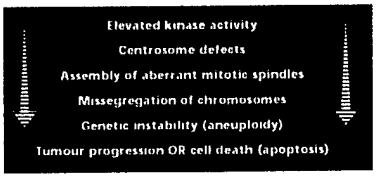

Fig. 3 Model of centrosome-induced tumorigenesis

rhabdomyosarcomas by preventing muscle differentiation and producing a population of undifferentiated proliferating cells that are aneuploid and have amplified centrosomes[16]. Another oncogenic molecule that appears to have a role in the regulation of centrosome function is the tumour suppressor p53, which is mutant or diminished in most human tumours[17].

Identification of the molecular targets of centrosome kinases and elucidation of the pathways that regulate centrosome function and contribute to tumour formation represent unique approaches to determine the origin of malignancies and provide novel opportunities for therapeutic intervention. A potential target of these kinases and other centrosome effectors is pericentrin, a centrosomal phosphoprotein that has a role in microtubule nucleation[6], induces spindle abnormalities and aneuploidy when overexpressed in nontumour cells *in vitro* (S.D., manuscript submitted), and is elevated in malignant cancers[11].

A number of converging studies on tumorigenesis and centrosome biology now suggest that the centrosome may provide a venue for many oncogenic activities, and that these activities may impact directly on centrosome function as part of the tumorigenic process. Indeed, it would seem that the answers to some of the big questions of tumorigenesis are hiding in small places like the centrosome. □

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From a DNA helicase to brittle hair

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A perplexing example of the complexity of genotype-phenotype relationships is provided by XPD, a DNA repair/transcription helicase encoded by the xeroderma pigmentosum (XP) group D gene (*ERCC2*). It is part of the TFIIH complex, which binds to the promoters of genes and facilitates the initiation of transcription and at the same time is involved in repair of damages DNA. As XPD is required for basal transcription, any mutation that severely compromises its function is lethal. Rare mutations with milder effect are viable and translate into a bewildering heterogeneity of phenotypes, involving at least three distinct disorders. Depending on the mutation, the consequence can either be the cancer-prone condition XP, or XP in addition to either the neuro-developmental disease Cockayne syndrome (CS) or the brittle hair disorder trichothiodystrophy (TTD). In a first effort to disentangle this complex genotype-phenotype jumble, a study presented by Frédéric Coin and colleagues on page 184 provides a detailed account of the biochemical defects caused by *ERCC2* mutations[1]. A crucial factor appears to be the interaction of XPD with p44, another TFIIH subunit which stimulates XPD's helicase activity. This interaction is compromised by disease-associated mutations, and as a consequence, XPD helicase activity is reduced. This, however, is only part of the story.

The 9-subunit TFIIH complex has an essential function in two processes: transcription initiation of protein-encoding genes and nucleotide excision repair (NER; refs 2–4). The first process involves a cascade of events at the promoter—eventually culminating in the departure of the RNA polymerase for transcription elongation. The NER system, on the other hand, removes a wide variety of lesions, including UV-induced photoproducts, in a multistep 'cut-and-paste' reaction involving 20–30 proteins. The XPB and XPD helicase subunits of TFIIH supply a bi-directional unwinding capacity required for local helix opening to form an open DNA intermediate in both processes[5,6] (Fig. 1).

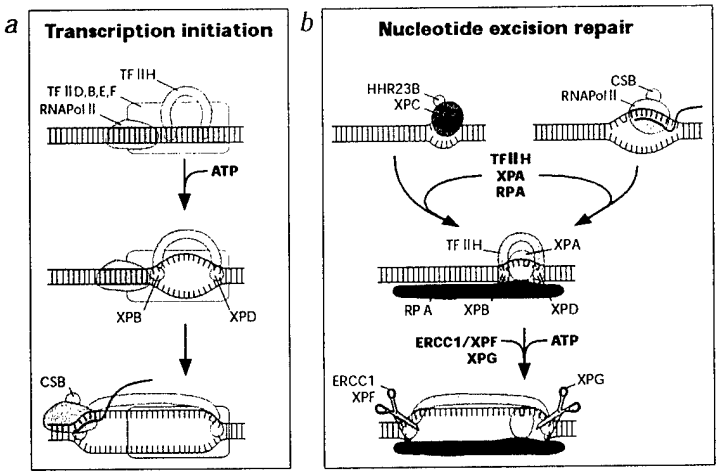

Fig. 1 The role of TFIIH in transcription and repair. ***a***, TFIIH in transcription initiation of RNA polymerase II. After assembly of the pre-initiation complex—consisting of five basal transcription factors and RNA polymerase—the promoter region is opened by the XPB and XPD helicases of TFIIH. This allows formation of the first phosphodiester bond, promoter escape of RNA polymerase and transcription elongation. ***b***, TFIIH in NER. Recognition of DNA damage can occur by either the XPC-HR23B complex or by RNA polymerase and Cockayne syndrome B protein. Subsequently, DNA around the lesion is opened by the concerted action of RPA, XPA and the bidirectional XPB/XPD helicase of TFIIH. This allows incisions of the damaged strand on both sides of the injury by the repair-endonucleases ERCC1-XPF and XPG, excision of the lesion-containing oligonucleotide and gap-filling DNA synthesis.

Amorphous no longer: the centrosome comes into focus

Wendy Zimmerman, Cynthia A Sparks and Stephen J Doxsey*

Recent genetic and biochemical studies have provided new insights into the molecular basis of centrosome-mediated microtubule nucleation. In addition, molecules and mechanisms involved in microtubule severing and stabilization at the centrosome, assembly of proteins onto centrosomes and regulation of centrosome duplication and separation are being defined. Characterization of centrosome function, together with studies implicating centrosomes in tumorigenesis and demonstrating that centrosomes are highly organized, are beginning to bring into focus an organelle once viewed as an 'amorphous cloud'.

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Abbreviations

cdk	cyclin-dependent kinase
C-Nap1	NEK associated protein
EM	electron microscopy
MTOC	microtubule organizing center
NEK	Nima related kinase
PCM	pericentriolar material
PP4	protein phosphatase 4
SPB	spindle pole body
γTuRC	γ tubulin ring complex

Introduction

Microtubule organizing centers (MTOCs) represent a class of organelles that are structurally diverse but share the common ability to nucleate and organize microtubules. They include the centrosome of animal cells, the spindle pole body (SPB) of yeasts and the blepharoplast of some lower plant cells, to name a few. Together with the microtubule cytoskeleton, the centrosome is involved in a number of important cellular functions including spindle function, the organization and transport of cytoplasmic organelles, morphogenesis and determination of cell shape. Although we know that the molecular composition of animal cell centrosomes is complex, we know little about how individual components contribute to the organization and function of the organelle. The recent identification of molecular components that are conserved between MTOCs of different organisms, however, provides an opportunity to dissect the underlying functional significance of these components, rather than focus on structural differences between MTOCs. In addition, the ever increasing number of regulatory molecules that associate with centrosomes such as kinases, phosphatases and proteins of degradation pathways (see Figure 1), suggests that centrosomes will provide a fertile area for future discovery. In this review, we discuss recent work on MTOCs from several systems with an emphasis on the composition, assembly, organization and regulation of components involved in microtubule nucleation. For previous reviews and additional information on MTOCs in various organisms see [1–5]. We apologize for work not referenced in this review due to space limitations.

Anatomy of microtubule nucleating structures

In this section we will discuss microtubule nucleation at three levels: the general organization of the nucleating material at the centrosome, γ tubulin complexes that appear to play a direct role in microtubule nucleation and core components of the protein complexes.

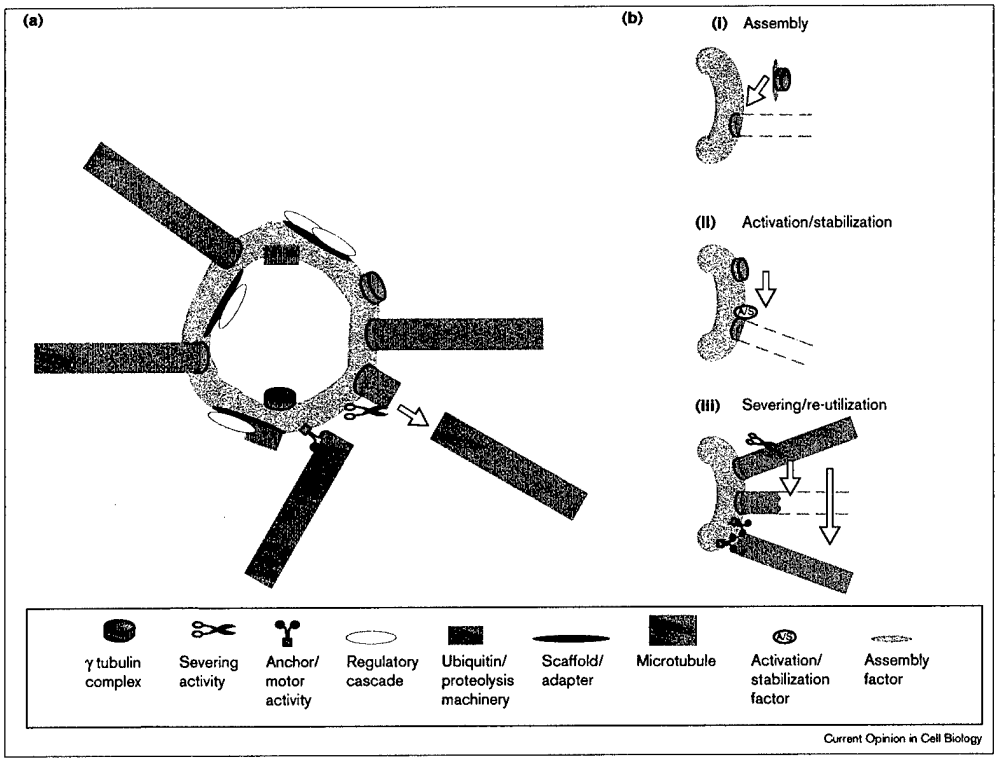
Organization of microtubule nucleating material at the centrosome

Over 100 years ago, the centrosome was described by Theodor Boveri as a "pair of centrioles surrounded by a differentiated cytoplasm" (see [6]). Since that time we have learned a great deal about the structure of centrioles although, until recently, little progress has been made in understanding the structure of the 'differentiated cytoplasm' or pericentriolar material (PCM). Advances in immunofluorescence image deconvolution have been used to demonstrate that the centrosome protein pericentrin [7] is organized into a highly-ordered lattice structure within the PCM [8••]. In this same study, fluorescence resonance energy transfer showed that γ tubulin, the protein thought to interact with the α/β tubulin heterodimer during microtubule nucleation [9], colocalized with pericentrin to the lattice. Centrosome lattice structures have also been revealed using electron microscopy (EM) techniques in *Drosophila melanogaster* [10], the surf clam, *Spistula solidissima* [11] and the sea urchin, *Strongylocentrotus purpuratus* [12]. Taken together, these results suggest that the pericentrin and γ tubulin lattice observed by immunofluorescence imaging may represent the general architectural framework of the PCM as observed by EM techniques.

γ tubulin rings at the centrosome

Higher magnification EM imaging of the PCM revealed ring-like structures that contained γ tubulin and had diameters roughly similar to those of microtubules [9]. Rings were not detectable after microtubule nucleation, suggesting that they served as templates for nucleated microtubules. This idea was supported by the observation that removal of γ tubulin and ring structures from the lattice by salt treatment abrogated microtubule nucleation, whereas re-association of γ tubulin and re-appearance of rings accompanied restoration of nucleating activity [11,13••]. Further support came from the observation that the ends of centrosome-nucleated microtubules contacted elements of the pericentrin and γ tubulin lattice [8••]. These results suggest that γ tubulin rings are organized into a centrosome

Figure 1



(a) Centrosome-based activities. In addition to microtubule nucleating sites, centrosomes have microtubule severing activity and perhaps microtubule anchoring sites. Kinases, phosphatases, components of the ubiquitin degradation pathway and other regulatory molecules have been localized to centrosomes. They may regulate centrosome and spindle function, and provide signals for other cellular processes. Multiple activities may be organized at discrete sites in the centrosome by scaffolding proteins. **(b)** Possible mechanisms for regulating microtubule nucleation at the centrosome. (i) Regulated assembly of microtubule nucleating proteins. (ii) Activation of preassembled nucleating sites or stabilization of nascent nucleated microtubules. (iii) Re-utilization of preassembled microtubule nucleating sites by severing and regrowth. See text for detailed descriptions.

lattice that may provide the structural and biochemical basis for microtubule nucleation.

Multiple γ tubulin complexes and their composition

γ tubulin ring complexes (γ TuRCs) have been purified from cytoplasmic extracts of *Xenopus laevis* eggs and shown to nucleate microtubules *in vitro*, suggesting that they may be the soluble form of centrosome-associated γ tubulin rings [14]. At present, several soluble protein complexes containing γ tubulin have been identified in a variety of organisms. Complexes of 2–3 MDa containing seven or eight protein species have been identified in *Xenopus* [14], *Drosophila* [13••] and mammals [8••,15,16]. Smaller γ tubulin complexes have also been identified in *Drosophila* [13••], *Aspergillus nidulans* [16] and *Saccharomyces cerevisiae* (see [17]). The *S. cerevisiae* complex is composed of three protein species (see below) and appears to be the only γ tubulin complex in yeast. The smaller of the two *Drosophila* complexes has been shown to nucleate microtubules, albeit at a reduced efficiency compared to the γ TuRC [13]. A 3.5–4 MDa γ tubulin complex has been identified in *Xenopus* extracts that may represent an assembly-competent form of microtubule nucleating material in animal cells [8••]. It appears to be composed of two subcomplexes: a γ tubulin complex that is similar in molecular mass to the γ TuRC and a pericentrin complex that has not been previously described. The relationship between the various γ tubulin complexes and their respective roles in microtubule nucleation and centrosome assembly is currently unknown. For views on

how γ tubulin complexes may mediate microtubule nucleation see [18].

Recent work has focused on determining the molecular composition of γ tubulin complexes by biochemical and genetic analyses in several systems. On the basis of genetic interactions with the *S. cerevisiae* homologue of γ tubulin, *tub4*, two additional proteins of the γ tubulin complex were identified, Spc97p and Spc98p (see [17]). The three proteins form a stable cytoplasmic complex and localize to sites of microtubule nucleation at the SPB [19••]. Loss of function of any of the three proteins produces the same result, reduced microtubule nucleation and perturbation of microtubule organization (for review see [17]). Homologues of Spc97p and Spc98p have been identified in higher eukaryotes using biochemical purification methods [20] and human expressed sequence tags (ESTs) [15,21], and the interaction between the Spc98p homologue and γ tubulin was confirmed in these studies.

Assembly of microtubule-nucleating components

It is generally believed that cytoplasmic protein complexes containing γ tubulin represent precursors of nucleating sites at the centrosome in embryonic systems [13••,14], mammalian cells [8••] and yeast [22••]. A better understanding of how these complexes assemble onto and disassemble from centrosomes will be important in understanding how microtubule nucleation is controlled in cells and may have important implications for human cancer (see below).

Nucleated microtubules in *S. cerevisiae* arise from two surfaces of the SPB, the electron dense plaque facing the cytoplasm and a similar plaque facing the nuclear interior (see [2] for review of SPB structure). Binding of the *S. cerevisiae* γ tubulin complex to the nuclear face of the SPB appears to be mediated by the spindle pole component, Spc110p. Spc110p interacts directly with Spc97p and/or Spc98p in the γ tubulin complex, but not with Tub4p [19••,23•]. Taken together, these results indicate that Spc110p serves as the receptor for γ tubulin complexes on the nuclear face of the yeast SPB. On the cytoplasmic side, a different protein, Spc72p interacts with Spc97p and Spc98p and thus appears to be the receptor for γ tubulin complexes at this site [22••]. In cells carrying temperature-sensitive mutations of Spc72p, cytoplasmic microtubules are absent or unattached to the SPB [24••]. Interestingly, a fusion protein containing the amino terminus of Spc110p and the carboxyl terminus of Spc72p will function as the cytoplasmic receptor for the γ tubulin complex, showing that this binding function is conserved between the two proteins [22••]. Thus, in *S cerevisiae* there are two independent, site-specific receptors for the γ tubulin complex. Two receptors may be required in organisms that undergo closed mitoses such as yeasts, since they have two nucleating surfaces in distinct, membrane bound compartments (nucleus and cytoplasm). In animal cells, one receptor may be sufficient as the centrosome is cytoplasmic throughout the cell cycle and the nuclear envelope breaks down in mitosis.

Studies in a number of higher eukaryotic systems have recently demonstrated that assembly of nucleating proteins onto centrosomes is required for microtubule nucleation and appears to require factors in addition to the γ tubulin complex. Reconstitution of microtubule nucleation on salt-stripped centrosomes from *Drosophila* embryos requires γ tubulin complexes and an additional fraction of ~220 kDa [13••]. One candidate for the additional activity is pericentrin, which is roughly the same molecular mass and has been implicated in the assembly of γ tubulin complexes in *Xenopus* eggs [8••]. In mammalian cells, pericentrin and γ tubulin assemble progressively at the centrosome lattice from G_1 until metaphase, and this assembly occurs concomitant with increased microtubule nucleating activity. Time-lapse imaging of green fluorescent protein (GFP) tagged pericentrin and antibody microinjection experiments have shown that pericentrin and γ tubulin assembly requires microtubules and the molecular motor dynein and is necessary for normal microtubule nucleation (Young A, Doxsey S, unpublished data). It is possible that dynein-mediated transport is a common mechanism for centrosome assembly, although it may not be utilized in embryonic systems where numerous copies of centrosome proteins are stockpiled [25], and are able to assemble onto centrosomes in a microtubule-independent manner [26]. The recently developed assays for centrosome assembly described above should provide powerful approaches to dissect the molecular basis of centrosome assembly and function.

Mechanisms regulating microtubule nucleation

At present, little is known about how microtubule nucleating activity at the centrosome is controlled. Microtubule nucleation could be regulated by one or more mechanisms including: assembly of nucleating proteins from cytoplasmic pools, activation and/or stabilization of preassembled nucleating proteins and re-utilization of existing nucleating sites (see Figure 1b). In addition, the total microtubule nucleating activity may be affected by the number of MTOCs in the cell.

The presence of centrosome proteins in both soluble and centrosome-associated forms (above) indicates that centrosome assembly must be a regulated process (Figure 1bi). Among the molecules that could potentially regulate centrosome assembly is the centrosome-associated Polo kinase of *Drosophila* and the human homologue Plk1 (for reviews see [27,28]). Functional abrogation of these molecules by mutational analysis or antibody injection results in small centrosomes with reduced levels of centrosome components (see [26,27]). Another protein recently implicated in control of centrosome assembly is the *Drosophila* protein phosphatase 4 (PP4) whose reduced expression results in decreased γ tubulin staining at centrosomes and diminished microtubule nucleation [29•]. These and other regulatory

molecules could control assembly of centrosome proteins via signaling pathways that have yet to be identified.

The nucleation capacity of centrosomes could also be regulated by activation of preassembled nucleating sites or by the stabilization of nascent nucleated microtubules (Figure 1bii). One candidate for microtubule stabilization is Stu2p, an essential protein of the *S. cerevisiae* SPB that appears to bind laterally to microtubules [30•]. Stu2p also interacts with the cytoplasmic γ tubulin receptor Spc72p [24••]. The ability to bind both γ tubulin receptor and microtubules suggests that Stu2p may play a role in the stabilization of the nucleating site, and could thus be involved in regulating microtubule nucleation at the SPB. Other studies have shown that the nucleation capacity of isolated centrosomes could be reduced by pretreatment with phosphatases [31] and increased by treatment with cyclin A *in vitro* [32]. One candidate for deactivation of nucleating sites is PP4 (above) [29•]. In addition to its affect on centrosome assembly, PP4 appears to reduce microtubule nucleation from centrosomes, suggesting that the phosphatase may affect centrosome-mediated microtubule nucleation at multiple levels. These results suggest that preassembled nucleating sites may be turned on and off by kinase/phosphatase cascades through modification of target proteins.

Another way to increase the nucleating capacity of centrosomes is to re-utilize existing nucleating sites (Figure 1biii). This could be accomplished by severing nucleated microtubules and reusing the severed sites for microtubule growth. Severed microtubules could be subsequently anchored at the centrosome, at other cellular sites or released into the cytoplasm [33•]. Microtubule severing and anchoring has been proposed to explain the increased number and dynamics of microtubules in mitotic cells [33•,34•,35], and to explain the genesis of apical/basal microtubule arrays adjacent to centrosomes in specialized epithelial cells [36]. Katanin is currently the best candidate for mitosis-specific microtubule severing since immunodepletion of the protein abrogates severing activity specifically in mitotic *Xenopus* extracts [34•]. Katanin is a bimolecular protein complex with a 60 kDa subunit that has severing activity and an 80 kDa subunit that mediates localization of the protein to centrosomes and spindle poles [37•]. The katanin subunits associate to form ring-shaped complexes that are slightly smaller than the diameter of microtubules (20 nm). The mechanism by which the protein complex severs microtubules has not been determined.

Centrosome duplication, separation and integrity

Centrosome duplication has been traditionally defined by the appearance and growth of nascent centrioles during the G_1/S transition, culminating in the separation of the two resulting centriole pairs and associated PCM in mitosis.

The duplication of centrosomes occurs once and only once during each cell cycle and the two resulting centrosomes contribute to the organization of the poles of the mitotic spindle and thus, to the proper segregation of chromosomes in mitosis. While the process of centrosome duplication is temporally coupled to the cell cycle under normal conditions, it does not appear to be controlled by the mitosis-specific cyclin-dependent kinase (cdk)1–cyclinB complex [38•]. Two recent studies indicate that centrosome duplication is regulated by the G_1-specific cdk2–cyclin E complex [39•,40•]. In these studies, inhibition of cyclin E in *Xenopus* embryos [40•] and extracts [39•,40•] was accomplished using cdk inhibitors and their inhibitory domains (p21, p27, Δ34Xic1) or by immunodepletion of cdk2–cyclins. All treatments blocked centrosome duplication as assayed indirectly by microtubule aster doubling [39•,40•], γ tubulin staining [40•] and centriole separation [40•]. Importantly, addition of baculovirus-expressed cdk2–cyclin E to duplication-defective extracts restored centrosome duplication activity. As cdk2–cyclin E has previously been shown to regulate the initiation of DNA synthesis, it may serve to couple DNA replication and centrosome duplication during cell cycle progression. Interestingly, multiple centrosomes and acentriolar MTOCs can be induced through misexpression of regulatory molecules implicated in tumorigenesis, suggesting that these molecules, like cdk2-cyclin E, may affect centrosome duplication, assembly and/or integrity (see below).

The separation of centrosomes in mitosis requires microtubule motors (see [41] for review) although the biochemical events involved in this process are poorly understood. Nima related kinase (NEK2) and its substrate NEK-associated protein (C-Nap1), have recently been implicated in the biochemical modification of centrosomes during their separation at mitosis [42•,43•]. C-Nap1 was isolated in a screen for NEK2 interacting proteins and is a substrate for the NEK2 kinase *in vitro* [43•]. C-NAP1 is concentrated at centrioles ends where they are joined together, and it becomes reduced during mitosis when centrosomes separate. Overexpression of NEK2 leads to premature centrosome splitting and apparent fragmentation of the PCM, suggesting that these proteins play a role in centriole/centrosome separation during mitosis. PCM fragmentation or ectopic assembly of PCM components is also observed in tumor cells, although the mechanism by which these centrosome defects arise are unknown (see below).

Centrosomes and cancer

At the beginning of the 20th century, Theodor Boveri speculated that centrosomes could contribute to the chromosome missegregation and aneuploidy that was commonly observed in tumor cells [44]. As we near the turn of the 21st century, we are getting our first glimpses of alterations in molecular determinants of centrosomes that accompany and may contribute to tumorigenesis. The centrosome components pericentrin, γ tubulin and centrin all appear to be overexpressed and ectopically assembled in many malignant tumors [45,46] (for reviews see [47]). Centrosomes in these tumors exhibit aberrant

features including: dramatic alterations in size and shape, absence of centrioles and excessive numbers. Regardless of their morphology, most of these structures nucleate microtubules and contribute to assembly of abnormal spindles.

The mechanism by which structural and functional centrosome defects arise in tumor cells is unknown. While recent studies suggest that overexpression of pericentrin alone can induce centrosome defects and aneuploidy (Purohit A, Doxsey S, unpublished data), other studies indicate that centrosome-associated kinases and other regulatory molecules may affect centrosomes in tumors (for reviews see [47,48]). It is possible that aberrant centrosomes assemble dysfunctional spindles and contribute to genetic instability and tumorigenesis. Elucidation of the molecular mechanisms that control centrosome duplication, assembly and integrity in normal cells will provide insight into the role of this organelle in tumorigenesis.

Future directions

Several recent provocative observations suggest that centrosomes may be involved in much more than the nucleation and organization of microtubules. An increasing number of regulatory molecules have been localized to centrosomes (kinases, cyclins, cdks, ubiquitin enzymes), suggesting that centrosome proteins may serve as scaffolds for the organization of specific biochemical regulatory pathways (see Figure 1) [49]. In this regard, pericentrin shares homology with scaffold proteins involved in anchoring regulatory kinases [50].

Among the regulatory components localized to centrosomes are proteins of the degradation machinery [51]. Degradation of cyclin B in *Drosophila* appears to be initiated at the centrosome and spreads into the spindle (Raff J, personal communication). In mutants whose centrosomes detach from spindles, the ability to degrade GFP–cyclin B within the spindle is lost, although centrosome-associated degradation is retained. In other studies, the centrosome-associated Polo-like kinases and protein kinase A have recently been shown to serve as activators of and substrates for the anaphase promoting complex of the ubiquitin-mediated degradation pathway [52]. These examples illustrate centrosome-associated activities that may regulate processes other than microtubule nucleation.

Biochemical differences between the two poles of the mitotic spindle have recently been observed. The Cdc7 protein kinase and the active from of Spg1 GTPase are present at one pole in the fission yeast *Schizosaccharomyces pombe*, and this asymmetry appears to be required for the proper initiation of cytokinesis [53•]. In the nematode *Caenorhabditis elegans*, there appears to be selective loss of the transcription factor pie-1 from one spindle pole, and this event is thought to be important for the generation of somatic cells during development [54]. The mechanism by which asymmetric localization of proteins is achieved is unknown, although it is possible that protein degradation may play a role.

Another unanswered question in centrosome biology is whether centrioles are passengers at the centrosome or participants in centrosome function. Centrioles may be vestigial structures retained during development to ensure that specialized structures such as cilia and flagella can be assembled when required. Alternatively, they may have acquired centrosome-specific functions, perhaps serving as templates for recruitment and focusing of PCM components and thus, they may limit the number of MTOCs in the cell to two. Consistent with this idea, is the observation that centrioles at the base of sperm flagellum (called basal bodies), assemble the first centrosome and microtubule aster during fertilization in many organisms (see above). Moreover, recent work has shown that defective centrosome and microtubule aster assembly at the sperm centriole during human fertilization may contribute to male infertility [55]. Further support for the idea that centrioles serve as templates for assembly of centrosome components comes from a study in which centrioles were disrupted by injection of an antibody to glutamylated tubulin, a posttranslationally modified form of tubulin found predominantly at centrioles in non-neuronal cells [56]. In the injected cells, components of centrosomes were no longer focused but somewhat dispersed, although some cells were able to progress through mitosis. Alternative pathways for assembly of spindles in the absence of centrosomes have recently been described (for review see [57]). A full understanding of the spindle assembly mechanism will require a careful analysis of the respective roles of soluble microtuble nucleating complexes and chromatin.

There are several systems that offer great promise for future identification and functional analysis of centrosome and SPB components. The molecular composition of enriched *S. cerevisiae* SPB fractions has recently been determined [58•] and will serve to expedite this process. The *S. pombe* SPB is a potentially important model for future study as it has features of both *S. cerevisiae* SPBs and mammalian centrosomes — perinuclear and amorphous during interphase and nuclear-associated and plaque-like during mitosis [59•] — and the genome should be sequenced within a year. The genome sequence of *C. elegans* will also soon be available. *C. elegans* is a multicellular system that may reveal additional roles for the centrosome, and a rapid gene disruption technique using double stranded RNA has been developed [60•] which will provide a powerful combination for molecular dissection of centrosome function.

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Direct Interaction of Pericentrin with Cytoplasmic Dynein Light Intermediate Chain Contributes to Mitotic Spindle Organization

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Abstract. Pericentrin is a conserved protein of the centrosome involved in microtubule organization. To better understand pericentrin function, we overexpressed the protein in somatic cells and assayed for changes in the composition and function of mitotic spindles and spindle poles. Spindles in pericentrin-overexpressing cells were disorganized and mispositioned, and chromosomes were misaligned and missegregated during cell division, giving rise to aneuploid cells. We unexpectedly found that levels of the molecular motor cytoplasmic dynein were dramatically reduced at spindle poles. Cytoplasmic dynein was diminished at kinetochores also, and the dynein-mediated organization of the Golgi complex was disrupted. Dynein coimmunoprecipitated with overexpressed pericentrin, suggesting that the motor was sequestered in the cytoplasm and was prevented from associating with its cellular targets. Immunoprecipitation of endogenous pericentrin also pulled down cytoplasmic dynein in untransfected cells. To define the basis for this interaction, pericentrin was coexpressed with cytoplasmic dynein heavy (DHCs), intermediate (DICs), and light intermediate (LICs) chains, and the dynamitin and $p150^{Glued}$ subunits of dynactin. Only the LICs coimmunoprecipitated with pericentrin. These results provide the first physiological role for LIC, and they suggest that a pericentrin–dynein interaction in vivo contributes to the assembly, organization, and function of centrosomes and mitotic spindles.

Key words: pericentrin • centrosomes • mitotic spindle • cytoplasmic dynein light intermediate chains • aneuploidy

THE centrosome is the major microtubule nucleating organelle in animal cells (Kellogg et al., 1994; Zimmerman et al., 1999). It is usually composed of a pair of centrioles surrounded by a protein matrix from which microtubules are nucleated (Szollosi et al., 1972; Gould and Borisy, 1990). The centrosome proteins, pericentrin and γ tubulin, are localized to the matrix material where they form a unique lattice-like network (Dictenberg et al., 1998). The lattice appears to represent the higher order organization of γ tubulin rings, structures comprised of γ tubulin and several other proteins that appear to provide the templates for nucleation of microtubules at the centrosome (Moritz et al., 1995; Zheng et al., 1995; Schnackenberg et al., 1998). γ Tubulin and pericentrin are also part of a large cytoplasmic protein complex that may represent the fundamental subunit of microtubule nucleation before its assembly at the centrosome (Dictenberg et al., 1998). In addition, the *Drosophila melanogaster* protein, Asp (abnormal spindle protein), has been shown to play a role in the centrosomal recruitment of γ tubulin (Avides and Glover, 1999). However, the precise role of this protein and others in the assembly, organization, and activity of centrosomes is unknown (see Zimmerman et al., 1999).

The assembly and molecular organization of the centrosome is important for bipolar spindle assembly during mitosis (for review see Waters and Salmon, 1997). Functional abrogation or depletion of pericentrin or γ tubulin disrupts centrosome assembly and organization, and creates structural defects in microtubule asters and spindles (Doxsey et al., 1994; Felix et al., 1994; Stearns and Kirschner, 1994). Alternative pathways for assembly of microtubule asters and spindles in the absence of centrosomes have been described (Gaglio et al., 1997; Merdes and Cleveland, 1997; Waters and Salmon, 1997; Hyman and Karsenti, 1998). In these acentrosomal spindle assembly systems, the molecular motor cytoplasmic dynein and the nuclear mitotic apparatus protein (NuMA)[1] play key

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1. *Abbreviations used in this paper:* DAPI, 4′,6-diamidino-2-phenylindole; DHC, dynein heavy chain; DIC, dynein intermediate chain; GFP, green fluorescent protein; HA, hemagglutinin; HA-Pc, hemagglutinin-tagged pericentrin; LIC, dynein light intermediate chain; NuMA, nuclear mitotic apparatus protein.

roles in the organization and focusing of the spindle poles (Heald et al., 1996; Merdes et al., 1996; Gaglio et al., 1997). These proteins are also involved in the organization of spindle poles in the presence of centrosomes (Merdes and Cleveland, 1997; Karki and Holzbaur, 1999).

The precise role of pericentrin in spindle function is currently unknown. The protein has been shown to contribute to the organization of microtubule arrays in both interphase and mitosis. Pericentrin antibodies introduced into mouse oocytes and *Xenopus laevis* embryos disrupt the organization of centrosomes and meiotic and mitotic spindles (Doxsey et al., 1994). Moreover, when added to *Xenopus* extracts, the antibodies inhibit assembly of microtubule asters. Recently, it has been shown that pericentrin levels are elevated in human tumor cells that exhibit defects in centrosome structure, spindle organization, and chromosome segregation (Pihan et al., 1998; Pihan, G., and S. Doxsey, unpublished observations). This suggests that pericentrin may contribute to tumorigenesis through the organization of dysfunctional spindles that missegregate chromosomes and generate aneuploid cells (for review see Doxsey, 1998; Pihan and Doxsey, 1999).

To further examine the role of pericentrin in spindle organization, we overexpressed the protein in somatic cells. Cells with excess pericentrin formed aberrant mitotic spindles, missegregated chromosomes, and became aneuploid. We found that cytoplasmic dynein was displaced from centrosomes and kinetochores, and the dynein-mediated organization of the Golgi complex was impaired. An interaction between cytoplasmic dynein and pericentrin was identified and shown to be mediated specifically by light intermediate chain (LIC) subunits (Gill et al., 1994; Hughes et al., 1995) of the motor protein. These results indicate that pericentrin and dynein act together to ensure proper organization and function of centrosomes and spindles.

Materials and Methods

cDNA Constructs

A full-length mouse pericentrin was constructed using a three piece cloning strategy. Pericentrin clone λpc1.2 (Doxsey et al., 1994) was excised with restriction enzymes PvuI and EcoRV. The 5′ end of the final clone was amplified by PCR using VENT polymerase from clone PCR 1 (Doxsey et al., 1994) using a 5′ primer (5′-CCGATATCAGATGGAAGACG-3′) with an EcoRV restriction enzyme site and a 3′ primer (5′-GTTTGG-GAGGTAGAGGCT-3′) with a PvuI site. The amplified PCR product was digested with EcoRV and PvuI. Plasmid pcDNAI/Amp (Invitrogen Corp.) was used to construct a vector with 13 amino acids of hemagglutinin (HA) protein (MAYPYDVPCYASL, pHAI; Wilson et al., 1984) inserted at the HindIII site in the polylinker (a gift of Michael Green, UMass Medical School, Worcester, MA). The vector was linearized with EcoRV and ligated to form the full-length pericentrin, as described (Sambrook et al., 1989). The correct orientation of the fragments was confirmed by PCR using the T7 vector primer and the 5′-directed pericentrin primer. The sequence of the clone was confirmed using an automated sequencer (Bio-Rad Laboratories). The preparation of cDNAs encoding full-length rat $p150^{Glued}$ (Vaughan et al., 1999), the human dynamitin (Echeverri et al., 1996), rat myc-tagged cytoplasmic dynein intermediate chain (DIC) 2C (IC-2C; Vaughan and Vallee, 1995), and rat FLAG-tagged cytoplasmic heavy chain (Mazumdar et al., 1996) have been described previously.

Cell Culture, DNA Transfection, Cell Viability, and Growth

COS-7 cells were cultured as described (American Type Culture Collection) with 10% FBS, 100 U/ml penicillin, and 100 μg/ml streptomycin (Sigma Chemical Co.). Cells were grown on 12-mm round glass coverslips in 35-mm culture dishes (Falcon Plastics) and transfected with 2 μg of plasmid DNA (HA-tagged pericentrin [HA-Pc], β-galactosidase, pHAI, or no DNA) using lipofectamine (GIBCO BRL); transfection efficiency was ~15%. Cells were fixed 35–42 h after transfection and processed for immunofluorescence staining, immunoprecipitation, metabolic labeling, or Western blotting. Cell viability was determined using mitotracker (Sigma Chemical Co.), which measures energy-dependent electron transport in mitochondria. Cell growth was determined by measuring the ratio of transfected cells to the total cell population; there was little change in this ratio over a 50 h time period.

Antibodies

Affinity-purified rabbit IgG was prepared from sera raised against the COOH terminus of pericentrin (Doxsey et al., 1994) and used at 1:1,000 for immunofluorescence microscopy and Western blotting. Anti-HA mAbs (12CA5) were obtained from Berkeley Antibody Co., Inc., and anti-HA polyclonal antibodies were a gift from Joanne Buxton (UMass Medical School, Worcester, MA; Meisner et al., 1997). Antibodies to α and γ tubulin, mouse IgG, and rabbit IgG were obtained from Sigma Chemical Co. Antibodies to β-galactosidase were from Boehringer Mannheim Corp. Antibodies to the following proteins were also used in these studies under conditions described in the accompanying references: dynein heavy chain (DHC; JR-61, Asai et al., 1994), DIC L5 (Vaughan and Vallee, 1995), 74.1 (Dillman and Pfister, 1994), dynamitin (Echeverri et al., 1996), $p150^{glued}$ (Vaughan and Vallee, 1995; Vaughan et al., 1999), anti-p58 Golgi protein (Bloom and Brashear, 1989), and CENP-E (Lombillo et al., 1995). Fluorescein (FITC) and cyanine (cy3)-conjugated IgGs were obtained from Jackson ImmunoResearch Laboratories, Inc. HRP-conjugated IgGs were from Nycomed Amersham Inc. Antibodies were used alone or in combination as described in the text.

Immunofluorescence Microscopy and Quantification of Protein and DNA

Immunofluorescence microscopy was performed essentially as described (Doxsey et al., 1994; Dictenberg et al., 1998). Unless otherwise stated, COS-7 cells expressing HA-Pc, β-galactosidase, pHAI, or mock transfected were fixed in 100% methanol at −20°C. Where indicated, cells were detergent-extracted to remove cytoplasmic protein before fixation (0.5% TX-100 in 80 mM Pipes, pH 6.8, 5 mM EGTA, 1 mM $MgCl_2$, for 1 min). In most cases, monoclonal or polyclonal HA antibody was detected with FITC-labeled secondary antibody, and antibodies used in colabeling experiments were detected with cy3 secondary antibodies. In all cases, cells were stained with 4′,6-diamidino-2-phenylindole (DAPI) to detect chromatin. Cells were observed using an Axiophot fluorescence microscope with a 100× objective (Carl Zeiss Inc.).

Quantification of centrosomal staining in mitotic cells and DNA (DAPI) was performed as described (Dictenberg et al., 1998). In brief, the total fluorescence from centrosomes and nuclei in individual cells was determined. Background values from three positions in the cytoplasm and camera noise (dark current) were subtracted (<10% of total). For centrosome staining, fluorescence signals were obtained from only one centrosome per mitotic cell, to avoid photobleaching. Cells with low, intermediate, and high expression levels were included in all analyses.

For coexpression studies (see Fig. 8), HA-Pc and dynein, or dynactin cDNAs were cotransfected into COS-7 cells and processed 38–46 h later. Cells were washed in PBS, lysed in modified RIPA buffer at 4°C for 20 min (150 mM NaCl, 50 mM Tris, pH 8.0, 1 mM EGTA, 1% IGEPAL) with leupeptin, aprotinin, and AEBSF (Boehringer Mannheim Corp.), and precleared. Monoclonal anti-HA bound to protein G beads (Pharmacia Biotech) was added to lysates at 4°C for 12 h, and beads were collected and washed five times with modified RIPA buffer. Proteins were exposed to SDS-PAGE and transferred to PVDF membranes (Millipore Corp.). The presence of dynein/dynactin subunits was assayed by Western blot with anti-myc, anti-p50, and anti-p150 antibodies.

^{35}S-Labeling of Cells

COS-7 cells were transferred to methionine- and serum-deficient DME (GIBCO BRL) containing 50–100 uCi of [^{35}S]methionine (New England Nuclear). They were labeled for 4 (see Fig. 8 B) or 18 h (see Fig. 7 B), washed in PBS, and lysed in 50 mM Tris, pH 7.5, 137 mM NaCl, 1% Triton X-100, 1 mM EDTA, 1 mM EGTA, 1 mM PMSF, 4 μg/ml aprotinin, 4 μg/ml leupeptin, 4 μg/ml antipain, 12.5 μg/ml chymostatin, 5 mM iodoacetamide, 130 μg/ml caproic acid, 12 μg/ml pepstatin, 200 μg/ml P-amino-benzamidine, and 1 mg/ml BSA. Protein G beads preblocked with COS-7 cell extract made from untransfected, unlabeled cells (4 h at 4°C), were added to precleared ^{35}S-labeled extracts with primary antibody, and immunoprecipitates were processed as described above. Dried gels (see Fig. 7) or membranes (see Fig. 8) were exposed to X-OMAT film (Kodak) for 24–48 h.

Microtubule Nucleation

COS-7 cells expressing HA-Pc or mock transfected were treated with nocodazole (10 μg/ml) for 1 h at 37°C to depolymerize microtubules. After removal of the drug, cells were incubated for 3 min to allow microtubules to regrow, then fixed in methanol and stained with α tubulin to reveal nucleated microtubules, as described previously (Brown et al., 1996; Dictenberg et al., 1998).

Results

Previously, we demonstrated that functional abrogation of pericentrin disrupts centrosome and spindle organization in several systems (Doxsey et al., 1994). Based on these observations, we reasoned that an artificial elevation of pericentrin levels would provide additional information on protein function and interaction. To this end, we constructed and expressed an HA-Pc in COS-7 cells, and examined centrosome and spindle composition and function.

As expected, HA-Pc had an electrophoretic mobility of ~220 kD and was found in both Triton X-100 soluble and insoluble fractions (Fig. 1 A). Immunofluorescence analysis demonstrated that the more abundant detergent soluble fraction was distributed throughout the cytoplasm (Fig. 1 D, inset), whereas the detergent insoluble fraction colocalized with γ tubulin at centrosomes (Fig. 1, B and C). Centrosome localization of HA-Pc was unaltered when microtubules were depolymerized, suggesting that the protein was an integral component of centrosomes and not simply bound there by microtubules (data not shown).

Mitotic Spindles Are Structurally and Functionally Disrupted in Pericentrin-overexpressing Cells

The organization of microtubules in interphase HA-Pc expressing cells was indistinguishable from control cells (Fig. 1 D). Moreover, there was no detectable difference in microtubule nucleation from centrosomes (Fig. 1, E–H). The most dramatic consequence of HA-Pc expression was disruption of mitotic spindle organization (Figs. 2 and 3). A significant fraction of mitotic COS-7 cells at all expression levels exhibited spindle defects (75.7 ± 6.1%, n = 423), compared with nontransfected cells (2.5 ± 1.5%, n = 598) and vector DNA transfected cells (3.0 ± 1.0, n = 201). Three categories of spindle defects were observed. Spindles with structural defects were detected in 36.2% of transfected cells and included multipolar, monopolar, and distorted spindles (Fig. 2, D–I, also see Fig. 5, L, M, Q, and R). Mispositioned spindles were observed in 22.0% of the cells, and were often positioned far from the cell center (Fig. 2, J–L). Spindles with misaligned, missegregated, and

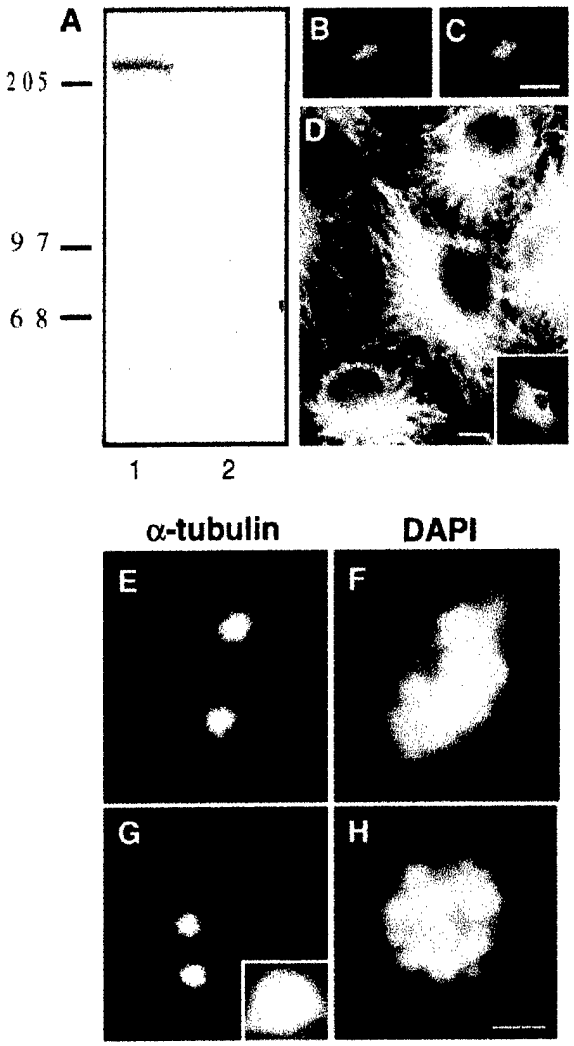

Figure 1. HA-Pc overexpression has no detectable effect on microtubule nucleation or organization. A, Triton X-100 soluble (lane 1) and insoluble fractions (lane 2) of HA-Pc–expressing COS-7 cells immunoblotted with anti-HA antibodies. Detergent extracted COS-7 cell showing centrosome-associated HA-Pc (B), which colocalizes with γ tubulin (C). Microtubule organization in a pericentrin-expressing interphase cell (D, inset) is similar to surrounding control cells. The extent of microtubule regrowth from prometaphase centrosomes after nocodazole-induced depolymerization is similar in an HA-Pc–expressing cell (G and H) and a control cell (E and F). Inset in G, HA stain. DAPI staining shows prometaphase chromosomes (F and H). Note that individual microtubules are not easily observed (E and G) after short periods of microtubule regrowth. Bars: (C, for B and C) 1 μm; (D) 5 μm; (H, for E–H) 10 μm.

mono-oriented chromosomes were commonly observed (42.5%; Fig. 3, also see Fig. 5, L, M, Q, and R). Spindle defects occurred alone or in combination.

Despite the presence of improperly attached chromosomes, HA-Pc cells progressed through mitosis and were

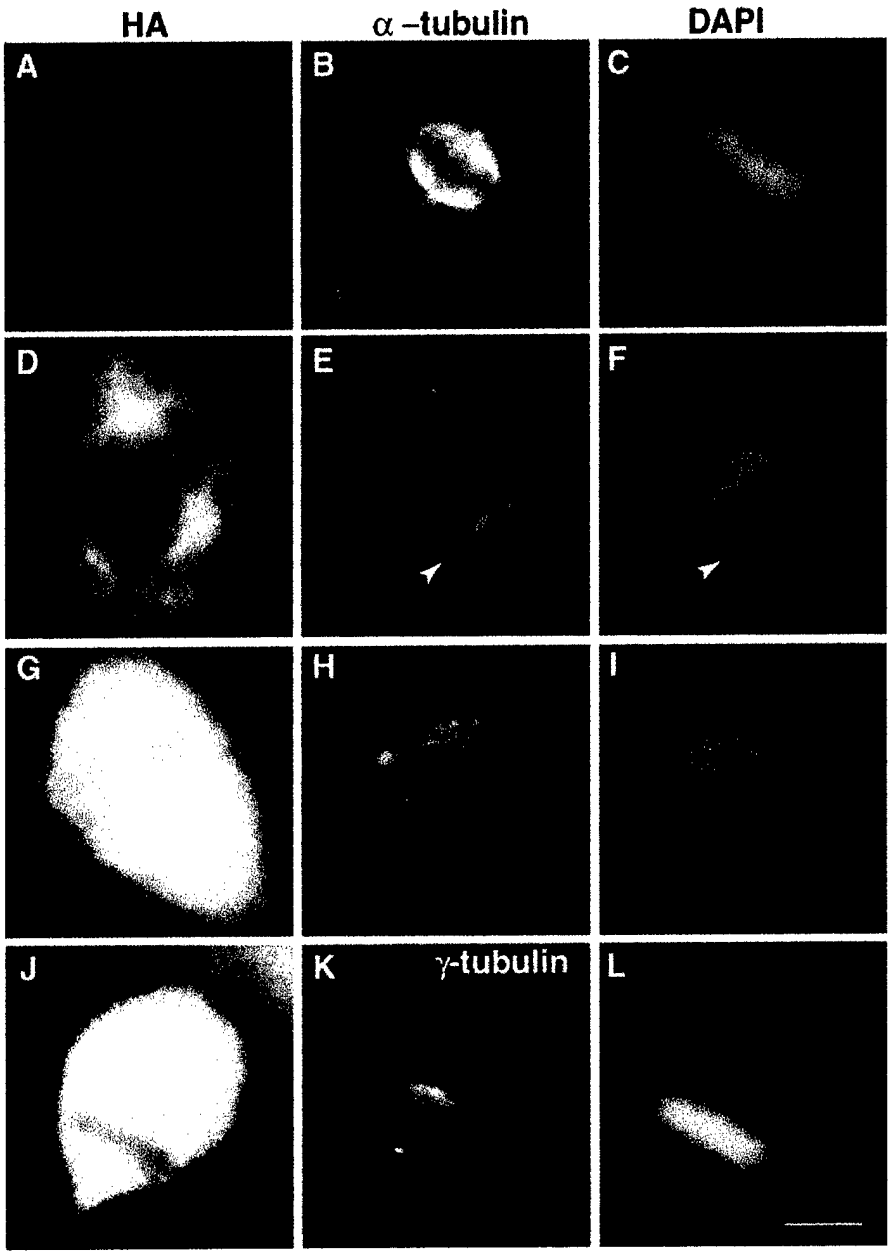

Figure 2. Mitotic spindle organization and positioning is impaired in HA-expressing cells. Immunofluorescence staining of microtubules (or γ tubulin; K) in nontransfected (A–C) and HA-Pc–overexpressing COS-7 cells (D–L). HA-Pc–expressing cell with a spindle elongated in the pole to pole dimension (D–F) and a subset of chromosomes misaligned on the metaphase plate (arrowheads). A spindle with multiple poles is seen in G–I (also see Fig. 5, L, M, Q, and R). A mispositioned spindle located adjacent to the plasma membrane is shown in J–L. Spindles were considered mispositioned if the metaphase chromosomes did not contact the intersection of two lines drawn in the cell at its shortest and longest dimensions. Horizontal series are of the same cell. Bar, 10 μm.

frequently observed in later stages of mitosis with missegregated chromosomes (Fig. 3, C and D). The percentage of mitotic figures in the population of HA-Pc–expressing cells (3.1 ± 0.9%, $n = 3{,}490$) was not significantly different from control cells transfected with other constructs or mock transfected cells (2.9 ± 1.0 to 4.4 ± 2.1%, $n = 5{,}002$), and the cell viability and growth rate appeared unchanged. Nuclei exhibited a remarkably wide variation in DNA content. Values ranged from zero to five times those of controls (Fig. 3, E and F), demonstrating that the cells were becoming aneuploid. From this analysis, we conclude that pericentrin overexpression causes multiple mitotic spindle defects leading to chromosome missegregation and aneuploidy.

Cytoplasmic Dynein Is Dissociated from Multiple Cellular Sites in HA-Pc–expressing Cells

The spindle defects in pericentrin-overexpressing cells were similar to those previously observed in cells overexpressing the dynamitin subunit of dynactin (Echeverri et al., 1996; Burkhardt et al., 1997). Dynactin is a protein complex which regulates the function of cytoplasmic dynein, a minus end microtubule motor protein involved in

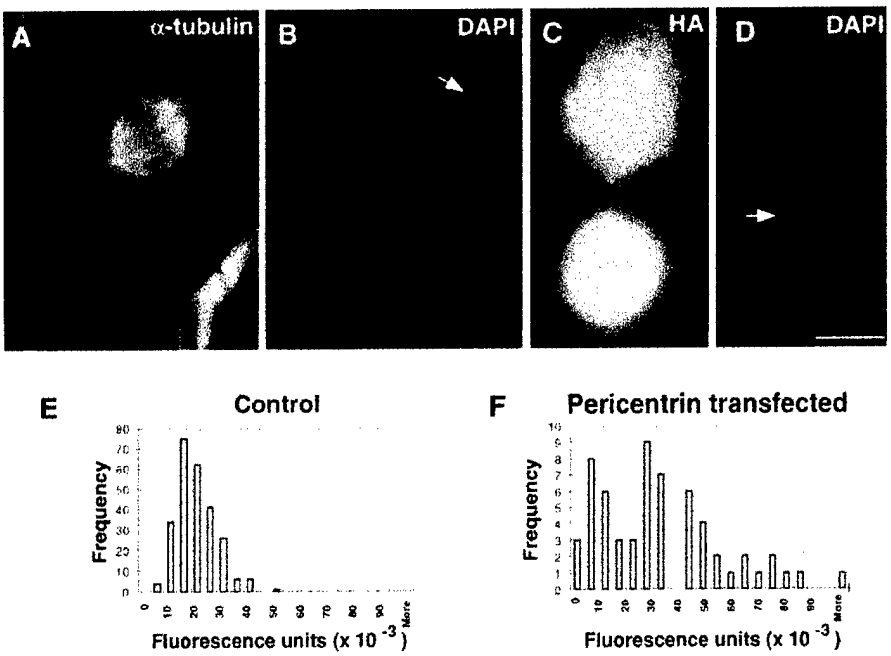

Figure 3. Chromosomes in pericentrin-overexpressing cells are misaligned and missegregated, creating aneuploidy. A pericentrin-overexpressing COS-7 cell (A) with a chromosome that is not aligned on the metaphase plate (B, arrow). Note metaphase DNA overexposed to highlight misaligned chromosome. Inset in A, HA staining. A late telophase cell (C) with chromosome(s) excluded from a reforming nucleus (D, arrow). Quantification of DAPI-stained chromatin in nuclei of pericentrin-overexpressing cells (F) reveals significant variability in DNA content, compared with control cell nuclei (E). Bar, 10 μm.

numerous physiological processes (reviewed in Vallee and Sheetz, 1996; Karki and Holzbaur, 1999). Dynein and dynactin have been localized to prometaphase kinetochores, centrosomes, spindle poles, and the plasma membrane (Pfarr et al., 1990; Steuer et al., 1990; Clark and Meyer, 1992; Echeverri et al., 1996; Busson et al., 1998). Overexpression of dynamitin disrupts the dynactin complex, releases cytoplasmic dynein from mitotic kinetochores, disrupts mitosis, and alters the distribution of membranous organelles, including the Golgi complex (Echeverri et al., 1996; Burkhardt et al., 1997).

To test the possibility that cytoplasmic dynein or dynactin contributed to the pericentrin overexpression phenotype, we examined the distribution of these protein complexes in pericentrin-overexpressing cells. The level of cytoplasmic dynein immunoreactivity in mitotic cells was dramatically reduced at spindle poles (8–12-fold; Fig. 4, G–J). The motor appeared to be specifically displaced from spindle poles and not simply masked from antibody access for several reasons. First, diminished dynein staining was detected with two independent antibody preparations raised against the DIC (L5, polyclonal and 74.1, monoclonal). Second, control cells expressing β-galactosidase (Fig. 4, A–C) or untransfected cells (Fig. 4, D–F) had normal levels of dynein at their poles (Fig. 4 J). Third, there was no detectable change in the distribution and abundance of several other centrosome and spindle pole components. The centrosome localization and levels of the dynactin subunits, dynamitin (Fig. 5, A–D) and p150^{glued}, did not appear to be altered, although there was some variability in p150^{glued} levels in prometaphase (Fig. 5 E). There was no apparent change in the level of γ tubulin at individual spindle poles, even in cells with multiple poles (Fig. 5, J–N). This suggests that multipolar spindles have normal centrosomes at their poles, each with the appropriate amount of γ tubulin (see Discussion). The spindle pole protein, NuMA, also appeared to be localized normally to poles of mitotic spindles (Fig. 5, F–I).

Cytoplasmic dynein was also dramatically reduced at kinetochores (Fig. 4, K–P). In contrast, kinetochore localization and levels of dynactin (Fig. 5, A–D, arrowheads; and data not shown) and the kinesin-related protein, CENP-E (Fig. 5, O–R; Yen et al., 1992; Lombillo et al., 1995), both appeared unchanged.

Consistent with defects in the Golgi complex induced by overexpression of the dynamitin subunit of dynactin (Burkhardt et al., 1997), HA-Pc overexpression caused dispersal of Golgi elements. This was observed by immunostaining with antibodies to the Golgi protein, p58 (Bloom and Brashear, 1989; Fig. 6; 77 ± 3.3%, n = 251). In adjacent nontransfected control cells, Golgi complexes had the characteristic tightly focused appearance and were found in the perinuclear region of the cells (Fig. 6 B, arrowheads; 95.6%, n = 497). Disruption of the Golgi complex was also observed using a green fluorescent protein (GFP)-tagged N-acetylglucosamine transferase in cotransfection experiments with pericentrin (data not shown). Golgi complex dispersal did not appear to result from impaired microtubule integrity, as no detectable changes in the microtubule network were observed (see Fig. 1 D).

Pericentrin Interacts Directly with Cytoplasmic Dynein through the Light Intermediate Chain

The loss of cytoplasmic dynein from spindle poles and kinetochores, and the abrogation of cellular functions mediated by dynein (spindle positioning, Golgi complex organization) suggested that overexpressed pericentrin sequestered the motor in the cytoplasm. This was tested directly by coimmunoprecipitation assays. Antibodies to

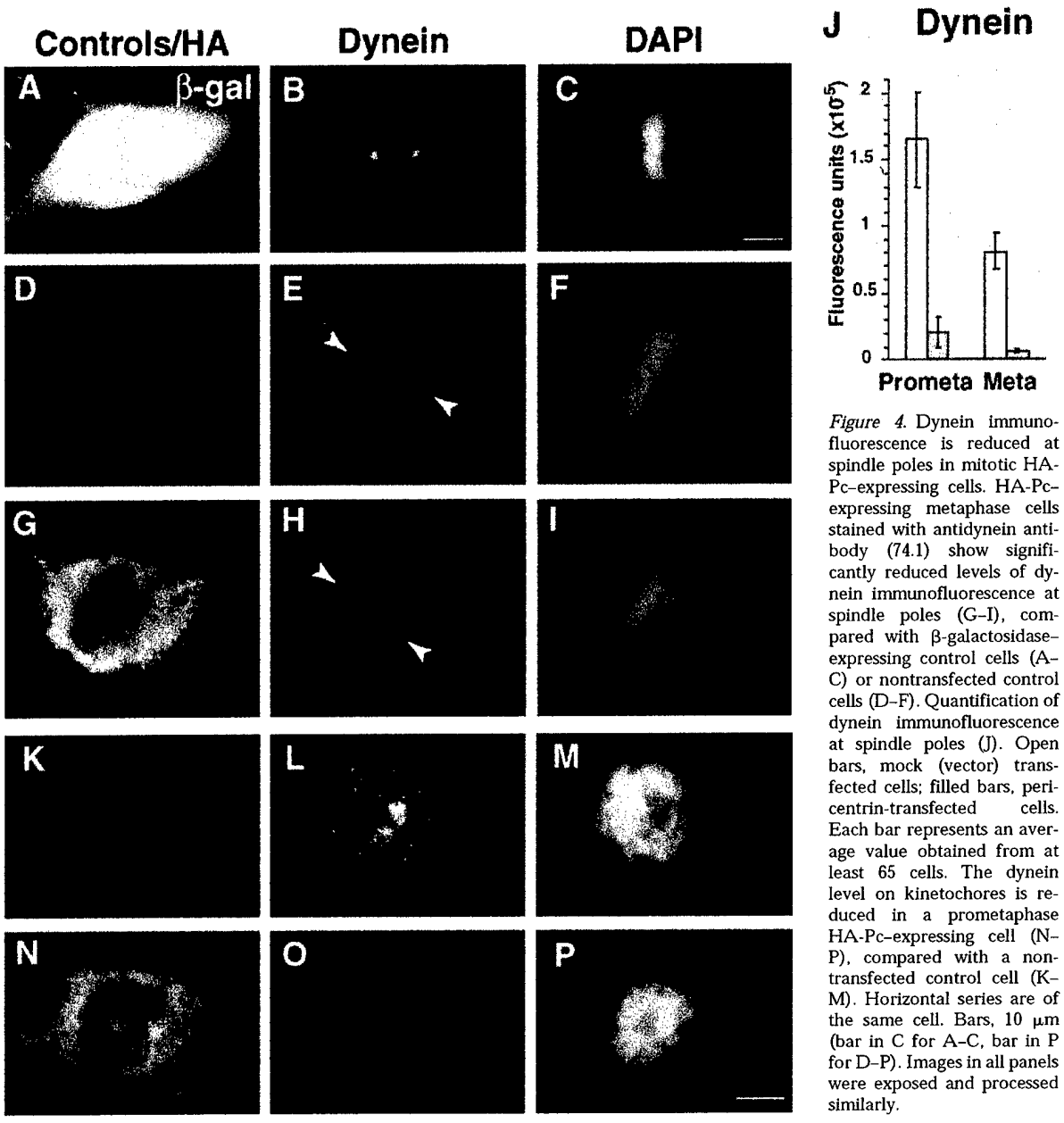

Figure 4. Dynein immunofluorescence is reduced at spindle poles in mitotic HA-Pc–expressing cells. HA-Pc–expressing metaphase cells stained with antidynein antibody (74.1) show significantly reduced levels of dynein immunofluorescence at spindle poles (G–I), compared with β-galactosidase–expressing control cells (A–C) or nontransfected control cells (D–F). Quantification of dynein immunofluorescence at spindle poles (J). Open bars, mock (vector) transfected cells; filled bars, pericentrin-transfected cells. Each bar represents an average value obtained from at least 65 cells. The dynein level on kinetochores is reduced in a prometaphase HA-Pc–expressing cell (N–P), compared with a nontransfected control cell (K–M). Horizontal series are of the same cell. Bars, 10 μm (bar in C for A–C, bar in P for D–P). Images in all panels were exposed and processed similarly.

both DIC and DHC precipitated HA-Pc (Fig. 7 A, lanes 5 and 6), whereas a control IgG preparation did not (Fig. 7 A, lane 7). Conversely, antibodies to HA, but not to control IgGs, precipitated DIC (Fig. 7 A, lanes 1–3). Under the same conditions, antibodies to dynactin components (dynamitin and $p150^{glued}$) did not precipitate detectable amounts of HA-Pc (Fig. 7 A, lanes 8 and 9), although they immunoprecipitated other proteins of the dynactin complex (Fig. 7 A, lanes 11, 12). In cells metabolically labeled with [^{35}S]methionine, HA-Pc was specifically immunoprecipitated with antibodies to DHC, but not to preimmune sera (Fig. 7 B). Moreover, despite very low levels of endogenous pericentrin in nontransfected control cells (Doxsey et al., 1994), we were able to specifically detect DHC after immunoprecipitation of pericentrin from lysates prepared from large numbers of cells (Fig. 7 C). These results suggest that overexpressed pericentrin binds to and sequesters dynein in the cytoplasm, and prevents it from associating with its cellular targets.

To determine whether the dynein–pericentrin interaction was direct or indirect, we cotransfected cells with HA-Pc and individual dynein and dynactin subunits, and performed a series of immunoprecipitation and immunoblot analyses. Immunoprecipitation of HA-pericentrin failed to

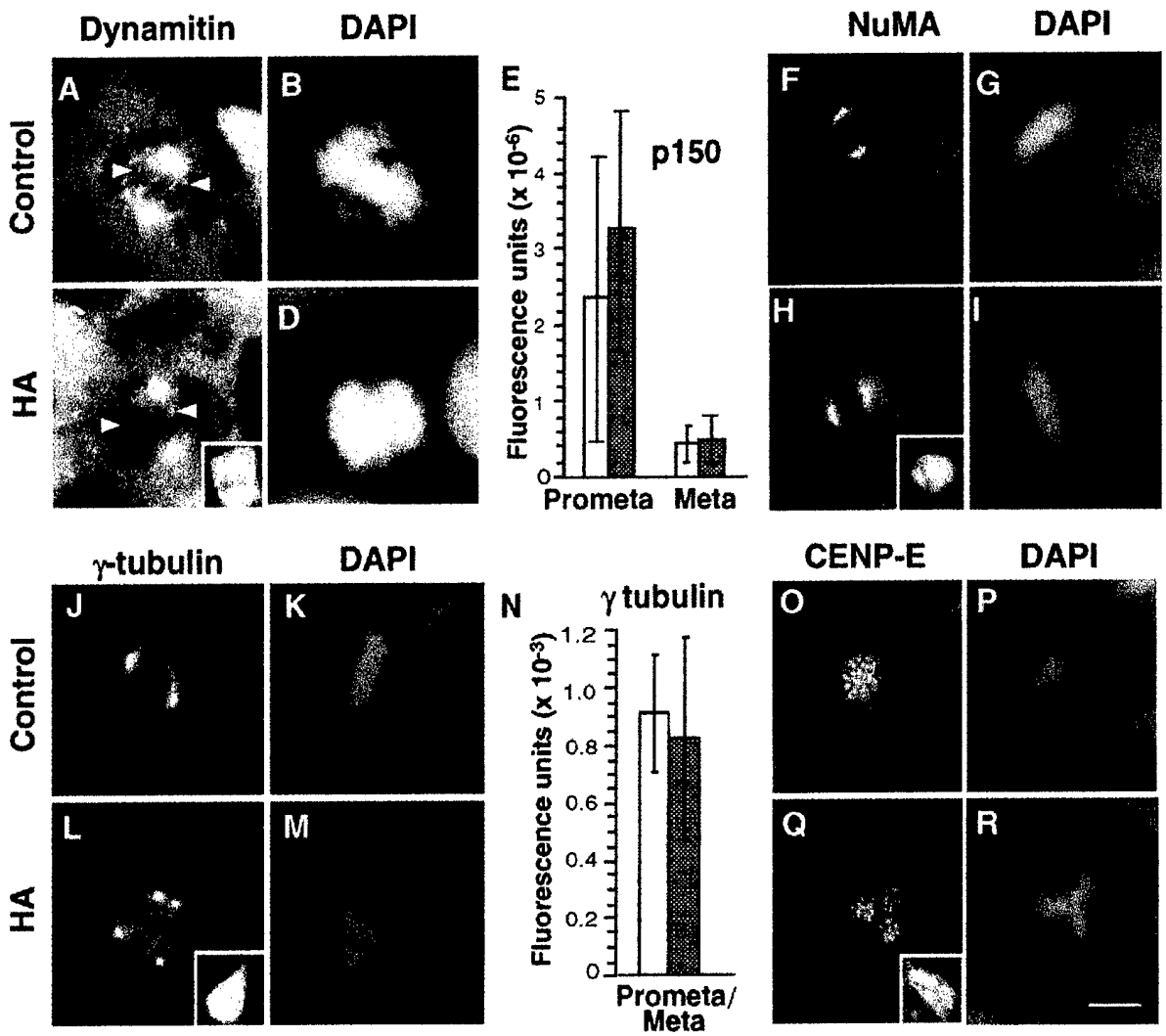

Figure 5. Localization of several centrosome and kinetochore proteins are unaltered in pericentrin-expressing cells. HA-expressing COS-7 cells were immunolabeled for dynactin subunits (dynamitin, A–D; $p150^{glued}$, E) or proteins involved in spindle pole integrity (NuMA, F–I), microtubule nucleation (γ tubulin, J–N), and kinetochore function (CENP-E, O–R). The distribution and levels of these proteins in HA-Pc–expressing cells (HA panels, white bars) did not appear to be significantly different from nonexpressing control cells (control panels, filled bars). HA stained cells shown in insets in C, H, L, and Q. Horizontal series are of the same cell. Bar, 10 μm. In E and N the fluorescence intensity of individual centrosomes/spindle poles is shown ($n > 40$ centrosomes/bar). Cells with low, intermediate, and high expression levels were included in the analysis.

pull down the DHCs and DICs or the dynactin subunits $p150^{Glued}$ and dynamitin (Fig. 8 A). However, a myc-tagged rat cytoplasmic dynein light intermediate chain (Hughes, S., A. Purohit, S. Doxsey, and R. Vallee, manuscript in preparation) and its COOH-terminal fragment N174 clearly coimmunoprecipitated with HA-pericentrin (Fig. 8 A). When cells cotransfected with the LIC N174 fragment and HA-Pc were labeled with [^{35}S]methionine, the only bands specifically immunoprecipitated with anti-HA antibodies were HA-Pc and N174 (Fig. 8 B). Dynactin did not appear to be required for the pericentrin–LIC interaction since overexpression of dynamitin had no effect on the ability of the proteins to coimmunoprecipitate (data not shown). These results provide strong evidence for a direct interaction between HA-Pc and the light intermediate chain of cytoplasmic dynein.

Discussion

We have found that pericentrin overexpression has profound effects on the organization, positioning, and function of mitotic spindles, and on the organization of the Golgi complex. Several studies show that cytoplasmic dynein is involved in processes affected by pericentrin overexpression (for reviews see Holzbaur and Vallee, 1994; Vallee and Sheetz, 1996; Karki and Holzbaur, 1999). Con-

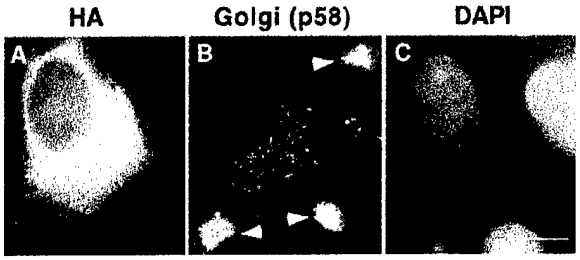

Figure 6. Golgi complexes are disrupted in pericentrin-overexpressing cells. An HA-Pc COS-7 cell (A) showing dispersal of the Golgi complex as revealed by staining with anti-p58 antibodies (B, center). In adjacent nontransfected cells, the Golgi complexes are well organized (B, arrowheads) and found in the typical juxtanuclear region (C, DAPI). Bar, 10 μm.

sistent with a role for cytoplasmic dynein in mediating the pericentrin overexpression phenotype is the reduction of dynein staining intensity at the prometaphase kinetochore and the centrosome/spindle pole. Our immunoprecipitation data further support an interaction between pericentrin and cytoplasmic dynein. Our data indicate that the interaction is direct and specifically mediated by the light intermediate chains of the motor protein complex. Thus, this study provides the first evidence for a dynein–pericentrin interaction, and identifies the first functional role for LICs.

Mechanism of Dynein–Pericentrin Interaction

The function of the light intermediate chains has been obscure. They have only been identified in cytoplasmic forms of dynein and contain well-conserved P-loop elements of unknown function near their NH_2 termini (Paschal et al., 1987; Gill et al., 1994; Hughes et al., 1995). Previous studies have implicated a different class of dynein subunit, the intermediate chains, in subcellular targeting. The intermediate chains reside at the base of the dynein complex and interact with the $p150^{Glued}$ subunit of the dynactin complex (Karki and Holzbaur, 1995; Vaughan and Vallee, 1995). Dissociation of the dynactin complex by dynamitin overexpression was found to release dynein from prometaphase kinetochores. Together, these data supported a role for dynactin in anchoring dynein to at least one form of subcellular cargo through the intermediate chains (Echeverri et al., 1996). This mechanism has received further support from evidence that mutations in zw10, a dynactin-anchoring kinetochore component, also release dynein from the kinetochore (Starr et al., 1998).

The current studies identify an additional and previously unsuspected mechanism for linking dynein to its cargo. The presence of cytoplasmic dynein, but not dynactin, in pericentrin immunoprecipitates, strongly suggests that dynactin is not necessary for the pericentrin/dynein interaction. Coexpression of recombinant dynein and dynactin subunits with pericentrin reveal a direct interaction with the light intermediate chains, further supporting a dynactin-independent mechanism. Thus, these results identify the light intermediate chains as an additional class of dynein-anchoring or -targeting subunit. Whether these polypeptides serve in a subset of dynein-mediated processes, such as interactions with soluble protein complexes versus membranous organelles or kinetochores, remains to be determined.

Whether light intermediate chain-mediated dynein in-

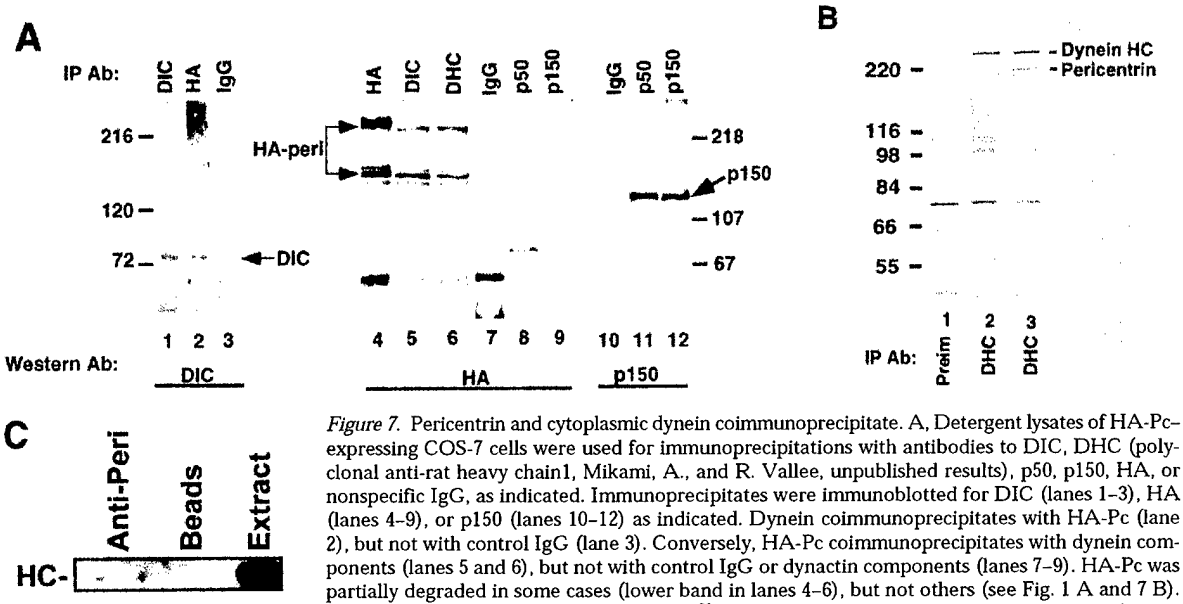

Figure 7. Pericentrin and cytoplasmic dynein coimmunoprecipitate. A, Detergent lysates of HA-Pc–expressing COS-7 cells were used for immunoprecipitations with antibodies to DIC, DHC (polyclonal anti-rat heavy chain1, Mikami, A., and R. Vallee, unpublished results), p50, p150, HA, or nonspecific IgG, as indicated. Immunoprecipitates were immunoblotted for DIC (lanes 1–3), HA (lanes 4–9), or p150 (lanes 10–12) as indicated. Dynein coimmunoprecipitates with HA-Pc (lane 2), but not with control IgG (lane 3). Conversely, HA-Pc coimmunoprecipitates with dynein components (lanes 5 and 6), but not with control IgG or dynactin components (lanes 7–9). HA-Pc was partially degraded in some cases (lower band in lanes 4–6), but not others (see Fig. 1 A and 7 B). An ~60-kD band is nonspecifically precipitated by IgG (lanes 4, 5, and 7). B, [^{35}S]methionine-labeled cells expressing HA-Pc (lanes 1 and 3) or mock transfected (lane 2) immunoprecipitated with antibodies to DHC or preimmune sera (Preim) as indicated. HA-Pc is only detected in HA-Pc–expressing cells after DHC immunoprecipitation (lane 3). C, Lysates from nontransfected control cells were used for immunoprecipitation with antipericentrin antibodies (lane 1) or no antibody (beads, lane 2), and proteins were immunoblotted with anti-DHC antibody. Molecular mass markers are indicated (in kD × 10^{-3}).

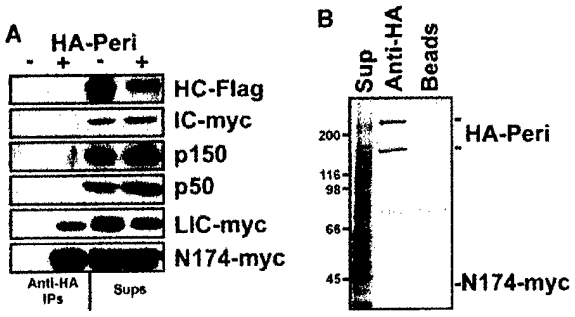

Figure 8. HA-Pc interacts directly with the light intermediate chain of dynein. A, Dynein and dynactin components were expressed alone or together with HA-Pc in COS-7 cells (+ or −). Cells were lysed, HA-Pc was immunoprecipitated, and blots were probed with myc, Flag, or dynactin antibodies, as indicated on right. LIC (LIC-myc) and a COOH-terminal fragment of LIC (N174-myc) coprecipitated with HA-Pc, whereas other dynein components, DHC (HC-Flag), DIC (IC-myc), and dynactin components (p50 and $p150^{glued}$) did not coprecipitate. Supernatants (Sups) from immunoprecipitations are shown on right. HA-Pc expression was similar in all samples, as confirmed by Coomassie blue staining (data not shown). B, [^{35}S]methionine-labeled COS-7 cells coexpressing HA-Pc and N174-myc were used for immunoprecipitation with anti-HA antibodies or no antibody (beads). HA-Pc (HA-Peri) and N174-myc coprecipitated specifically with HA antibodies. The identity of N174 was confirmed by immunoblotting (data not shown). The ~60-kD band represents a nonspecific protein that precipitates with HA antibodies in COS-7 cells.

teractions are completely independent of dynactin also remains to be resolved. Examination of the behavior of GFP-pericentrin in living cells has revealed clear centripetal transport of pericentrin-containing particles to the centrosome (Young, A., R. Tuft, J. Dictenberg, A. Purohit, and S. Doxsey, manuscript submitted for publication). This behavior is correlated with a cell cycle-dependent accumulation of pericentrin and γ tubulin at the centrosome, which is strongly inhibited by nocodazole, antibody to cytoplasmic DIC, or overexpressed dynamitin. These data, together with the identification of a pericentrin–dynein interaction (this study), demonstrates that recruitment of pericentrin and γ tubulin to centrosomes involves dynein-mediated transport. Since pericentrin previously has been shown to interact with the γ tubulin complex (Dictenberg et al., 1998), and more recently with protein kinase A (Diviani, D., L. Langeberg, A. Purohit, A. Young, S. Doxsey, and J. Scott, manuscript submitted for publication), we currently believe that pericentrin functions as a molecular scaffold that transports important activities to the centrosome and anchors them at this site.

The ability of dynamitin overexpression to inhibit centrosome protein recruitment suggests a role for dynactin in pericentrin-mediated transport, despite the lack of evidence in the current study for a role for dynactin in the dynein–pericentrin interaction. It is conceivable that dynactin disruption affects pericentrin accumulation via a mechanism unrelated to direct pericentrin transport, such as the disruption of the microtubule cytoskeleton. Alternatively, dynactin could regulate dynein-mediated pericentrin motility independent of a role in linking pericentrin to dynein. Such a model contrasts with an obligatory role for dynactin in the attachment of dynein to kinetochores (Echeverri et al., 1996), but is consistent with our current evidence for an involvement of alternative dynein targeting mechanisms in different cellular processes. Finally, it is possible that pericentrin interacts with dynein by a bivalent mechanism involving both the light intermediate chains and dynactin, but that the latter interaction is poorly preserved in vitro.

Molecular Basis for the Pericentrin Overexpression Phenotype

Our data support a cytoplasmic dynein sequestration model to explain the effects of pericentrin overexpression. Dynein is removed from at least two of the sites where it is normally found, the kinetochore and the spindle pole (Fig. 4). The association of dynein with membranous structures is more difficult to assess because of the profusion of such structures in the cytoplasm, but the dispersal of the Golgi apparatus that we observe is strongly consistent with a loss of dynein from this organelle as well. Thus, we imagine that soluble pericentrin binds to the light intermediate chains and interferes with normal dynein targeting interactions. Interference of light intermediate chain localization or function by overexpressed pericentrin could result from competition with other light intermediate chain interactions in the cell. Alternatively, it could be due to steric interference by overexpressed pericentrin with the intermediate chain/dynactin interaction. Mapping studies have, in fact, shown the binding sites for the intermediate and light intermediate chains to be in close proximity within the DHCs (Tynan, S., and R. Vallee, unpublished results). Further work will be required to identify the full range of light intermediate chain functions.

One distinction between the pericentrin and dynamitin overexpression effects is that there is no detectable change in the mitotic index of pericentrin-overexpressing cells. This result is puzzling in view of the similarity in mitotic defects observed in the two cases, including the production of multipolar mitotic spindles. The latter structures are suggestive of mitotic failure (i.e., cytokinesis failure) which typically occurs after a delay in mitosis. Although pericentrin-overexpressing cells do not exhibit a mitotic delay, they appear to grow and divide normally. This suggests a defect in the checkpoint that regulates the transition from metaphase to anaphase (Rudner and Murray, 1996), an idea we are currently testing.

Pericentrin previously has been shown to be part of a large protein complex that includes γ tubulin (Dictenberg et al., 1998). Thus, it is possible that disruption of γ tubulin in pericentrin-overexpressing cells contributes to the spindle defects. However, we believe this is unlikely because recruitment of γ tubulin to spindle poles is not noticeably different than in control cells. Moreover, the ability of individual mitotic spindle poles to nucleate microtubules, a function thought to be mediated by γ tubulin, appears unchanged in pericentrin-overexpressing cells. Some pericentrin-overexpressing cells have multiple γ tubulin staining structures that seem to contribute to the formation of multipolar spindles. Since each of the multiple poles has

approximately the same amount of γ tubulin as normal spindle poles, we believe that they represent bona fide centrosomes (with centrioles). We are currently investigating how these multiple foci of γ tubulin are generated and whether they contribute to aneuploidy in pericentrin-overexpressing cells.

It is unclear why recruitment of γ tubulin and NuMA to spindle poles appear to be unaffected by the HA-Pc-induced dynein disruption since the evidence suggests that both proteins may also interact with, or be under the control of, cytoplasmic dynein. One possibility is that cell cycle variability in the localization and levels of these proteins (Compton and Cleveland, 1993; Dictenberg et al., 1998), together with variability in the level of pericentrin overexpression, make it difficult to detect significant differences. Another possibility is that the proposed HA-Pc-induced sequestration of dynein may be less than complete, allowing some dynein-mediated transport to occur. This may be sufficient to localize the spindle pole proteins examined in this study, but insufficient to maintain Golgi complex organization or localize dynein to spindle poles and kinetochores. Alternatively, dynein may interact with many different cargoes (e.g. vesicles, protein complexes) whose localization is differentially affected by pericentrin overexpression. This could explain why NuMA and dynactin, which form a discrete complex with dynein in *Xenopus* extracts (Merdes et al., 1996), appear to accumulate to normal levels at spindle poles.

A final interesting feature of the pericentrin-overexpressing phenotype is the generation of aneuploid cells. In fact, pericentrin-overexpressing cells have chromatin levels both below and above diploid, suggesting that they undergo persistent chromosome missegregation as described (Lengauer et al., 1997). Since little is known about how aneuploid cells are generated, this cell system provides a powerful model to study this phenomenon. This system may also prove useful in understanding human tumorigenesis since pericentrin levels are elevated in most aneuploid tumors (Doxsey, 1998; Pihan et al., 1998; Pihan, G., and S. Doxsey, unpublished observations). For these reasons, it is important to determine the precise contributions of dynein and other pericentrin-interacting molecules in the generation of aneuploidy and spindle defects in pericentrin-overexpressing cells.

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Centrosome defects can account for cellular and genetic changes that characterize prostate cancer progression.

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ABSTRACT

Factors that determine the biologic and clinical behavior of prostate cancer are largely unknown. Prostate tumor progression is characterized by increased changes in cellular architecture, glandular organization and genomic composition. These features are reflected in the Gleason grade of the tumor and in the development of aneuploidy. Cellular architecture and genomic stability are controlled in part by centrosomes, organelles that organize microtubule arrays including mitotic spindles. Here we demonstrate that centrosomes are structurally and numerically abnormal in the majority of prostate carcinomas. Centrosome abnormalities increase with increasing Gleason grade and with increasing levels of genomic instability. Selective induction of centrosome abnormalities by elevating levels of the centrosome protein pericentrin in prostate epithelial cell lines reproduce many of the phenotypic characteristics of high-grade prostate carcinoma. Cells that transiently or permanently express pericentrin exhibit severe centrosome and spindle defects, cellular disorganization, genomic instability and enhanced growth in soft agar. Based on these observations, we propose a model in which centrosome dysfunction contributes to the progressive loss of cellular and glandular architecture and genomic instability that accompany prostate cancer progression, dissemination and lethality.

INTRODUCTION.

Prostate carcinoma is the most common gender-specific cancer in the United States, accounting for nearly one third of all cancers affecting men (1). The lifetime risk of developing invasive prostate carcinoma in the United States is ~20% (2-5), while that of octogenarians based on histopathologic examination of the prostate at autopsy, approaches 80% (6). Despite the high incidence of prostate carcinoma, the lifetime risk of dying from the disease is much lower, currently estimated to be around 3.6% (1/28, Surveillance Epidemiology & End Results, NCI, 2,000). These epidemiological trends, which may intensify in the coming decades due to the aging of the Baby Boom generation and our increasing ability to recognize tumors at earlier stages, mean that 180,000 new cases of prostate cancer will be diagnosed in the coming year in the United States.

Radical prostatectomy is the most common therapy for the small group of patients with high grade tumors. However, there currently are no sound medical facts to direct treatment of the majority of patients that present with lower grade tumors (7, 8). Because a subgroup of patients with low grade carcinoma ultimately develop aggressive, often lethal cancers, current therapeutic recommendations are to treat all patients with intent of cure (7, 8). Thus, the most pressing need in the management of prostate carcinoma is to develop a non-invasive test to distinguish clinically indolent (low grade) carcinoma from potentially fatal disease (see discussion) (9). This test would spare the majority of patients with indolent prostate cancer from unnecessary prostatectomy. Reducing such surgeries would result in significant cost savings in health care; decreased therapy-related

morbidity and more focused therapy on the more homogeneous group of patients with aggressive disease where the efficacy of newer therapies could be assessed more quickly (9).

One of the best predictors of prostate cancer progression is the Gleason score, a numerical measure compiled from the two most prevalent histologic Gleason grades. The Gleason grade reflects cytoarchitectural features that become increasingly aberrant with tumor progression (10, 11). Recent results indicate that the parameter with the greatest predictive power is the *proportion* of tumor with the highest Gleason grades (4 and 5) (12). An intimate relationship between Gleason grade, aneuploidy and unfavorable clinical outcome has long been known (13-17). This suggests that the molecular components and subcellular structures that control cell and tissue architecture and genetic fidelity are likely to contribute to tumor progression. These parameters have the potential to dictate the clinical behavior of tumors and thus serve as predictors of aggressive cancer.

In a search for cellular elements that contribute to the constellation of cellular and genetic features found in high Gleason grade prostate carcinoma, we focused on centrosomes (18). Centrosomes are tiny cellular organelles that nucleate microtubule growth and organize the mitotic spindle for segregating chromosomes into daughter cells (for reviews see (19, 20)). As organizers of microtubules, centrosomes also play an important role in many microtubule-mediated processes such as establishing cell shape and cell polarity, processes essential for epithelial gland organization (21-24). Centrosomes also coordinate

numerous intracellular activities in part by providing docking sites for regulatory molecules including those that control cell cycle progression, centrosome and spindle function and cell cycle checkpoints (20, 24-29). Because high Gleason grade prostate cancer is characterized by defects in the same set of cellular processes controlled by centrosomes, we hypothesized that centrosome dysfunction may be the biologic basis for these phenotypic abnormalities.

In this report we show that centrosome defects are found in essentially all high grade prostate cancers. Moreover, centrosome defects are present in low grade tumors and they increase with increasing Gleason grade and with increasing genomic instability. Artificial induction of centrosome abnormalities in cultured prostate cells by overexpression of the centrosome protein pericentrin reproduces many features of aggressive prostate cancer. We discuss our results in terms of a centrosome-mediated mechanism for tumor progression. Centrosome abnormalities in prostate cancer could be exploited to develop markers for tumor virulence and selective therapies that target tumor-specific centrosome abnormalities thus circumventing the greatest limitation of current chemotherapy--its lack of tumor selectivity.

MATERIALS AND METHODS

Immunohistochemical detection of centrosomes in archival tissue sections of prostate carcinoma. Archived cases of invasive prostate carcinoma treated by radical prostatectomy were selected from the files of the Department of Pathology of the University of Massachusetts Medical Center accrued between the months of July 1995 and June of 1997. Criteria for inclusion were availability of archival tissue blocks from which good quality histology sections could be prepared. Sections with the highest Gleason grade from each radical prostatectomy were selected since there is good indication that the highest Gleason grade is the best indicator of clinical outcome (12). We analyzed only high quality tissue sections (109 total) from radical prostatectomies with invasive carcinoma representing Gleason grades 2-5 and from metastatic prostate carcinoma (31 cases). Immunostaining for pericentrin was judged satisfactory when the characteristic single or paired centrosome pattern (30) was detected in non-neoplastic cells within adjacent to the tumor (Table 1).

Immunohistochemistry for pericentrin (18) was performed on serial paraffin sections (5 μm thick) attached to positively charged glass slides (Ventana Medical Systems). The first section of each series was stained with hematoxylin and eosin to confirm the presence and grade of the carcinoma and to map the tumor within the section. Parallel sections were processed for centrosome staining by immunohistochemistry. Sections were first heated in a microwave pressure cooker for thirty min in a solution containing 0.2 mM EDTA (18) to render centrosome antigens immunoreactive to

pericentrin antibodies (30-32). Antibody was diluted 1:1000 in TBST (50 mM Tris pH 6.5, 150 mM NaCl, 0.5% Tween-20), added to sections at room temperature and incubated for 1hr. Biotinylated secondary antibody (Vector laboratories, Burlingame, CA) was applied in TBST for 1hr and amplified by the Avidin-Biotin-Complex method as described (ABC, Vector laboratories, Burlingame, CA). To block endogenous biotin- and avidin-binding sites, sections were treated with a solution of biotin followed by a solution of avidin before application of the primary antibody. To avoid nonspecific binding by primary and secondary antibodies, washing solutions contained 5% w/v bovine serum albumin (BSA) and 5% v/v goat serum. Endogenous peroxidase was blocked by pre-incubation in a solution of 3% H2O2. After immunostaining sections were lightly counterstained in Hematoxylin.

Criteria for centrosome defects. We considered centrosomes abnormal if they had a diameter at least twice that of centrosomes in normal prostate gland epithelium, if they were present in numbers greater than 2 and if they were structurally abnormal as previously described (18). In some cases, we analyzed levels of the centrosome protein pericentrin at centrosomes and in the cytoplasm by quantifying the opacity/translucence of immunoperoxidase staining. Briefly, bright-field immunoperoxidase images of tumor and normal prostate glands taken a 1000X magnification were digitally color-inverted so the immunoperoxidase product was a bright signal whose luminosity was proportional to the intensity of the original brown signal. Signals were measured as the integral of a 5 μm area about 5 times the size of a centrosome as delineated with the marquee function of

Photoshop. Signal emanating from the neighboring cytoplasm was subtracted from the respective centrosome measurement. For cytoplasmic pericentrin measurements, background signal emanating non-tissue sources were subtracted. Inclusion of internal controls (normal glands present within the same section) allowed us to obtain semiquantitatively measurements of pericentrin levels within and between tumors. This approach has been used to establish differences in protein levels of other proteins (33). Members of our Biostatistics core (Dr. Chung Cheng, UMass Medical School) performed statistical analysis.

In situ hybridization with chromosome specific centromere probes.

For in situ hybridization studies, tissue sections parallel to those stained for centrosomes were deparaffinized and heated in a microwave pressure cooker for twenty min in a solution containing 0.01M sodium citrate (pH 6.0). After cooling to room temperature sections were treated with a solution of pepsin (40 μg/ml) in 0.1 N HCl for 10 min. Pepsin digestion was stopped by washing the sections several times in 2XSSC at room temperature and slides were dehydrated in a series of alcohols and air-dried. Biotinylated probes to the centromeric regions of chromosomes 1 or 8 were added in hybridization buffer and slides were mounted sealing coverslips with rubber cement. Target DNA and probes were codenatured in a Hybrite oven (Vysis, Downers Grove, IL) (18) and slides were washed several times in SSC buffers for maximum stringency (Vysis, Downers Grove, IL), processed to detect signals (NEN Life Science Products, Boston, MA) and lightly counterstained with hematoxylin to reveal nuclei. Data is shown for chromosome 8 (Figs. 5 and 7) and is similar to that observed with probes to chromosome 1 (not

shown).

A total of 100-120 nuclei in tumor and non-tumor areas of the section (identified by hematoxylin counterstain) were scored for centromere signals. Chromosomal instability (CIN) was determined by computing the fraction of cells with signals greater than the mode (34), a parameter known to underestimate the true CIN level (18, 34). To avoid the compounding effect of nuclear truncation artifact in tissue sections, we computed only chromosome gains. Cells in G2 phase of the cell cycle, which normally have four copies of each chromosome were distinguishable from cells with supernumerary chromosomes because sister chromosomes (and centromere signals) in these cells occur in pairs.

Pericentrin transfections into normal or tumor-derived prostate cell lines.

Full length HA-tagged pericentrin in pcDNA I (2 ug) (32) (Invitrogen) was used for transient transfection (Lipofectamine, GIBCO/BRL, Gaithersburg, MD) of the 1542-NPTX cell line derived from normal prostate epithelium by transformation with E6 and E7 from HPV16 (35). Cells transfected with vector alone served as controls. Permanent pericentrin-expressing PC-3 cells were constructed by cloning full length HA-pericentrin into the pRetroON vector (Clonetech) which codes for a reverse tetracycline transactivator protein and contains tetracycline transactivator responsive elements that drive transcription of the gene of interest. The transactivator is reported to bind and activate the promoter in the presence of tetracycline/doxycycline. Following sequence confirmation, the cDNA was introduced into PC-3 cells (ATCC) by transient transfection (as above) and 24 permanent lines were obtained after antibiotic selection (Clontech); cell

lines expressing vector alone served as controls. We found that HA-pericentrin in these lines was expressed in the absence of doxycycline and did not significantly increase in the presence of doxycycline. The pericentrin expressing cells exhibited dramatically different features than control cells in the absence of the drug, these features did not noticeably increase in the presence of drug and they were indistinguishable from features observed in transiently transfected 1542 NPTX cells (Fig. 6) and COS cells (32). Protein expression in the absence of induction from the pRetro-ON vector and the lack of inducibility of the vector has been noted by Clonetech (personal communication) and they have discontinued its sale. Imperfections in the inducibility of the vector did not impact on our study since we obtained several permanent pericentrin-expressing cell lines. In this study, we present data from cells treated with doxycycline for 48 hours.

Immunoflourescence analysis of cell lines. Pericentrin-expressing 1542 NPTX cells (48 hrs post-transfection) and PC-3 cells were fixed in cold methanol and co-stained for γ tubulin to label centrosomes and HA to locate transfected cells (1542 NPTX) as described (32). DNA was stained with DAPI and levels were quantified as described (32).

Growth in agarose of prostate cells lines permanently expressing pericentrin. To study the in vitro behavior of cells with deregulated expression of pericentrin we used the agarose colony assay of Bishop et al (36) with minor modifications. One hundred thousand HA-pericentrin expressing cells or empty vector cells were plated in duplicate in 6 well plates in 0.35% low melting point agarose over a cushion of 0.7% agarose. Cells

were fed full growth medium (10% fetal calf serum, 90% RPMI plus antibiotics and glutamine) and assessed for growth at 3 and 7 days using an inverted microscope equipped with a film camera. Images were then taken at 40X magnification and colonies were counted and sized following an additional 10X projection onto a screen. A total of 10 images per cell line were analyzed (between 500-1,000 colonies).

RESULTS

Centrosome abnormalities in prostate carcinoma.

In this study, we analyzed prostate tumors of different cytologic grades for the presence of centrosome defects. We selected the area with the highest Gleason *grade* within each radical prostatectomy because this parameter appears to be the single most important determinant of clinical outcome (12). We avoided the breakdown of data by Gleason *score,* as customarily done in clinical data representations, because it represents a compound measure of multiple Gleason grades and may thus obscure the significance of our observations. We examined paraffin sections from radical prostatectomies containing tumors ranging from Gleason grades 2 to 5 (n = 103). Gleason grade 1 tumors were not included because they are rare and relatively difficult to recognize, and because they may have a different ontogenic derivation than more common carcinomas (37). We also analyzed a group of metastatic prostate carcinomas comprised primarily of lymph node and bone marrow metastases (n = 31).

Three parameters were initially used to monitor centrosome abnormalities: larger diameter, elevated number and abnormal structure (Figs. 1, 2). These parameters were previously used by our group to provide the first evidence for centrosome abnormalities in malignant tumors of multiple tissue origin (18). Analysis of metastatic carcinomas using these criteria demonstrated that all had abnormal centrosomes (31/31, Table 1). The proportion of tumor with centrosome defects varied from 15% to virtually 100% of tumor

cells. These results confirm our previous results showing that centrosomes are abnormal in prostate tumors (18) and extend these observations to demonstrate that centrosome abnormalities in metastatic tumors appear to be universally present and severe. The majority of carcinomas confined to the prostate (Gleason grades 2-5) also had abnormal centrosomes (101/109, Table 1, Fig. 1). However, abnormalities in this heterogeneous group of tumors were more variable than those observed in metastatic carcinomas. Some exhibited defects in only 1 or 2 of the 3 parameters and the proportion of tumor tissue with centrosome abnormalities were generally lower than in metastatic tumors. In no instance did we observe centrosome abnormalities in nontumor tissues adjacent to tumors (Table 1, Fig. 1).

We reasoned that variability in centrosome defects in this heterogeneous mix of tumors might reflect differences in biologic behavior and Gleason grade. To test this, we analyzed 6 cases each of tumors with Gleason grades 2 through 5 for the 3 parameters of centrosome defects. In addition, we examined in detail the distribution and levels of pericentrin, a highly conserved integral centrosome protein involved in centrosome and spindle organization and chromosome segregation (30-32). Since our previous work had strongly suggested shown that levels of the centrosome protein pericentrin were higher in tumor versus nontumor tissues (18)], we analyzed pericentrin levels using a quantitative method established for tissues processed for immunohistochemistry (33) (see Fig. 2C, D). Of the 5 parameters measured, four were significantly higher in tumors of high Gleason grades (combined 4 and 5) compared with those of low Gleason grades (combined 2 and 3) (Figs. 3, 4). Centrosome size and number were two-fold higher in tumors of high

Gleason grade (Fig. 4A, B) and pericentrin levels at the centrosome and in the cytoplasm were significantly higher in high-grade tumors (Fig. 4C, D). In contrast, neither the severity nor prevalence of structural abnormalities in centrosomes increased with higher Gleason grade (data not shown). Among the structural defects were elongated centrosomes (length to width ratio >5, Fig. 1E, Fig. 3I) that were never observed in normal human prostate cells. This suggested that elongated centrosomes were tumor-specific and had potential to serve as a diagnostic marker (see Discussion). For all five categories of centrosome defects, the distribution within tumors was somewhat heterogeneous, a pattern reminiscent of that reported for tumor DNA content (38-41).

Relationship between centrosome abnormalities and chromosomal instability.

Because centrosomes play a role in the maintenance of genomic stability through control of mitotic chromosome segregation, we asked if there was a correlation between abnormal centrosomes and genomic instability, specifically chromosomal numerical instability (CIN). CIN as first described by Vogelstein and colleagues, is a measure of the nonmodal distribution of chromosomes that is thought to result from persistent missegregation of chromosomes during mitosis (34). In this study, we examined the nonmodal distribution of chromosome 8 in prostate tumors of different Gleason grades using centromere-specific nucleotide probes and in situ hybridization (see Materials and Methods and Fig 5). As expected, the extent of CIN in tumor tissues was significantly greater than in nontumor tissues (Fig. 5). Interestingly, the extent of CIN was significantly greater in Gleason grade 4/5 than in Gleason grade 2/3 (Fig 5C). Finally, the

extent of CIN correlated with the extent of centrosome abnormalities in parallel sections from the same set of tumors (Fig 5D), suggesting a relationship between centrosome defects and genomic instability in prostate tumor progression.

Induction of centrosome defects in prostate cells by ectopic expression of the centrosome protein pericentrin induces a prostate tumor-like phenotype.

If elevated pericentrin levels and centrosome defects observed in prostate tumor tissues contribute to cellular and genetic changes that occur during tumor progression, they may have the potential to induce similar changes when experimentally induced in cultured cells. To directly test this idea, we induced centrosome defects in prostate cells in vitro. We expressed a hemagglutinin-tagged (HA) pericentrin protein in cell lines derived from normal prostate epithelium (1542-NPTX) (35) and from metastatic prostate cancer (PC-3) both by transient transfection and by construction of permanent cell lines (Fig 6, 7).

Elevation of pericentrin levels induced or exacerbated genetic instability and cellular changes in 1542-NPTX and PC-3 cells, respectively. 1542-NPTX cells transiently transfected with the HA-pericentrin construct exhibited numerous defects in centrosome size, shape and number (Fig. 6E) as revealed by immunofluorescence staining for the centrosome protein γ tubulin (42). Defective centrosomes were usually associated with structurally disorganized mitotic spindles and chromosomes associated with these abnormal spindles were often misaligned and missegregated, indicating that the cells were undergoing aberrant mitoses (data not shown). Consistent with this idea were

dramatic changes in nuclear morphology observed in interphase cells (lobate and misshapen nuclei, micronuclei, multiple nuclei). Moreover, DNA levels were elevated in a large proportion of HA-pericentrin cells but not in control cells, demonstrating that pericentrin expression induced aneuploidy/polyploidy (Fig. 6B-D). Control cells included cells transfected with vector alone (Fig. 6), a truncated pericentrin construct (43) and β-galactosidase (data not shown). Similar results were observed in GFP-pericentrin transfected cells (data not shown) indicating that this phenotype was due to pericentrin overexpression and unrelated to the expression tag. These studies demonstrate that tumor-like changes in cellular architecture and genetic composition can take place within 1-3 cell cycles following HA-pericentrin expression.

To examine the long-term effects of HA-pericentrin expression, we constructed permanent prostate tumor-derived cell lines (PC-3, see Materials and Methods). The pericentrin expressing PC-3 cell lines (24 total) exhibited several abnormal features compared to control PC-3 cells containing empty vector (Fig. 7). Six cell lines were examined in detail and all gave a similar phenotype; below we present data from one line (PeriPC-3-4.1). The presence of the HA-pericentrin construct was confirmed by PCR analysis (data not shown) and the HA-tagged pericentrin protein was detected by Western blot (Fig. 7A). Defects in centrosomes, spindles and nuclei were significantly higher than in control cells and were strikingly similar to defects observed in transiently transfected 1542-NPTX cells (Fig. 6) and in prostate tumors (Figs. 1, 2). DNA content analyzed by flow cytometry (Fig. 7E, F) and chromosomal instability assayed by in situ hybridization with centromere probes for chromosome 8 (Fig. 7C, D) were significantly higher in

pericentrin expressing PC-3 cells. Moreover, the cellular architecture of pericentrin-PC-3 cells was dramatically altered (Fig. 7G, H) and the cells grew more rapidly in soft agar compared to controls (Fig. 7I-K). Taken together, these data demonstrate that expression of a single centrosome protein in normal and prostate tumor cells can induce or exacerbate abnormalities in centrosome number and structure, cellular architecture, nuclear morphology, cell growth and genomic stability, features that are characteristically altered in aggressive prostate tumors.

DISCUSSION

The results presented here demonstrate that centrosomes are structurally and numerically abnormal in the vast majority of metastatic and invasive prostate carcinomas. These abnormalities are frequent and usually occur together in the same tumor. The extent of centrosome abnormalities in invasive prostate carcinoma correlates with the Gleason grade in that tumors with the highest Gleason grade have more extensive centrosome abnormalities. The extent of chromosome instability correlates with the extent of centrosome abnormalities, both increasing with increasing Gleason grade. These observations are consistent with the idea that centrosome defects contribute to genomic instability during prostate cancer progression. Support for this idea comes from data showing that artificial induction of centrosome defects by pericentrin overexpression can induce genetic instability, loss of cellular architecture and rapid cell growth in prostate cells.

The in vivo and in vitro data presented in this manuscript implicates centrosomes in the progression of prostate cancer. In our current model (Fig. 8), centrosome dysfunction causes modification of the microtubule cytoskeleton and contributes directly to cellular and glandular disorganization and genomic instability, creating cells that are predisposed to additional changes that lead to aggressive tumor development. We do not know whether centrosome abnormalities develop in a progressive manner (Fig 8, solid arrow) or in a discontinuous fashion (Fig 8, segmented arrows). Elucidation of the mechanisms by which centrosome changes occur may provide insights into the evolutionary pathway

of the cytoarchitectural features that occur during prostate cancer progression (44, 45).

Our observations of CIN in prostate carcinoma are consistent with those previously made by Vogelstein and collaborators in colon carcinoma cells and they suggest that CIN may be the most important cause of aneuploidy in colon and prostate tumors (34). Because centrosome abnormalities are found in essentially all carcinomas examined to date (18), they may be a major cause of aneuploidy/CIN in solid tumors (18, 46). Consistent with this idea is data implicating centrosome dysfunction in CIN and aneuploidy in colon carcinoma cell lines (47).

Our work has important implications for prostate cancer progression, prognosis and treatment. Our observations suggest that progressive dysfunction of centrosomes occurs in prostate carcinoma and that this can have far reaching effects on cell morphology and genetic composition. Elucidating the mechanism(s) that lead to centrosome dysfunction in prostate carcinoma and the fundamental differences between centrosomes of low and high grade tumors could lead to the development of markers for tumor virulence. Such markers could play a critical role in identifying the subset of patients destined to develop aggressive, lethal prostate carcinoma. For example, elevated levels of centrosome proteins could provide a potential marker for early prostate lesions. If released into the circulation like PSA, these proteins could provide a non-invasive method to detect early lesions that lead to aggressive disease. Centrosome abnormalities also constitute an attractive novel therapeutic target because they are tumor-specific. It may be possible to develop chemical inhibitors against molecular components of centrosomes such as

pericentrin that could correct or reverse centrosome defects, genetic instability and tumor progression.

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Table 1. Centrosome abnormalities in prostate carcinoma

Tumor type[c]	Abnormal centrosomes[a, b] non-tumor epithelium[d]	tumor epithelium
Metastatic prostate carcinoma	0/31[e]	31/31
Prostate carcinoma confined to the prostate	0/97	101/109

a, For all samples in this analysis, paraffin-embedded tissues were sectioned, reacted with pericentrin antibodies and immunoperoxidase methods, and examined by light microscopy. The centrosome defects in tumor tissue were statistically higher than control non-tumor glands ($p<0.0001$, Fisher's exact test).

b, Centrosomes were considered abnormal if they had a diameter at least twice that of centrosomes in normal prostate gland epithelium, if they were structurally abnormal (elongated), if they were present in more than two copies per cell and if they had increased cytoplasmic and or centrosomal staining levels of the centrosome protein pericentrin (18).

c, Tumors were identified by cellular, glandular and nuclear features in hematoxylin and eosin stained sections. Parallel sections were stained for pericentrin by the immunoperoxidase technique to detect centrosomes and counterstained with hematoxylin only.

d, Non-tumor epithelium was present within the same section that contained the tumor in the majority of the cases (97/109).

e, Control cells in metastatic organs included cells of that organ (lymphocytes and hematopoietic cells in lymph nodes and bone marrow) as described previously (18).

FIGURE LEGENDS.

Fig. 1. Centrosome abnormalities in invasive prostate carcinoma compared with adjacent normal tissue. Sections from radical prostatectomies were stained for pericentrin (brown color) as described in Materials and Methods. (A) Prominent centrosome abnormalities are seen in small tumor glands (between arrowheads) compared with centrosomes in three large normal glands (upper left, upper and lower right). Image is 100x. Images B-G are higher magnification (1000x) of centrosomes in tumor cells (C-G) and nontumor cells (B). Centrosomes in tumor cells (at arrowheads) are larger in diameter (C, G, arrowheads), elongated (E, arrowheads), multiple and apparently fragmented (D, F) and contain more pericentrin (C-G) than control centrosomes (arrowheads in B). Most cells had combinations of centrosome defects.

Fig. 2. Centrosome diameter and number are increased and pericentrin levels are elevated in prostate carcinoma. Measurements from a single Grade 3 prostate carcinoma are shown. A-D. Densitometric measurements of centrosomes and cytoplasm were performed on tumor tissues and nontumor tissues as described in Materials and Methods (see [33]). Immunoperoxidase reaction product was quantified by measuring translucence in boxes shown in A and B. Insets in A and B represent higher magnification of centrosomes in measuring boxes to show dramatic differences in centrosome size. In panel A, left box is cytoplasmic pericentrin, right box is centrosomal pericentrin. In panel B, left box is centrosomal pericentrin, right box is cytoplasmic pericentrin. An average of eight measurements of centrosomal and cytoplasmic

pericentrin in nontumor (normal) and tumor tissues is shown in C and D, respectively. Centrosome size (E) and number (F, normalized to nuclei) were determined as described in Materials and Methods. Each column in E and F represents the average of over 100 measurements taken from one tumor. p values in C-F obtained by paired student T test.

Fig. 3. Centrosome abnormalities increase with increasing Gleason grade: Pictorial view. Histologic features of a normal prostate gland (A) and prostate carcinoma of Gleason grades 3 (D) and 5 (G) on hematoxylin and eosin stained sections. Areas similar to those imaged in the left column were stained for pericentrin at 100X (B, E, H) and 1000X (C, F, I). With advancing Gleason grade centrosomes become progressively larger, structurally more abnormal and greater in number.

Fig. 4. Centrosome abnormalities and pericentrin levels increase with increasing Gleason grade: Quantitative analysis. Centrosome diameter (A), centrosome # (centrosome/nuclei ratio, B), centrosomal pericentrin (C) and cytoplasmic pericentrin (D) were determined as described in Materials and Methods. The first column in A-D represent the means of measurements made on six tumors of grade 2 and six of grade 3 combined; the second column represent similar numbers of grades 4 and 5. All values are represented as percent increase above nontumor cells present within the tissue sections (y axis). The data demonstrates that abnormal centrosome features are statistically greater in tumors of higher Gleason grade. p values obtained by paired student T test.

Fig 5. Chromosome instability increases with increasing Gleason grade in invasive

prostate carcinoma. In situ hybridization with a chromosome 8 specific centromeric probe in a normal gland (A) and Gleason grade 4 prostate carcinoma (B). Inset in A is a low power (original 40x) view of a hematoxylin and eosin stained parallel section showing a normal gland (A) and high grade prostate carcinoma (B). The figure shows that significant numbers of tumors cells have greater that 3 signal per nuclei (B, arrowheads) whereas no cell shows more than 2 signals in the normal epithelium (A). The extent of CIN (as the fraction of cells with chromosome 8 copy number greater than 2) is greater in tumors of combined Gleason grades 4 and 5 than those of Gleason grades 2 and 3 (C), and correlates with the cumulative extent of centrosome abnormalities (D, correlation coefficient, R=0.445).

Fig. 6. Transient expression of pericentrin in "normal" near diploid prostate cells induces centrosome defects, nuclear abnormalities and aneuploidy. 1542-NPTX cells were transfected with the HA-pericentrin construct or vector alone and grown for an additional forty hours. Western blot (A) following immunoprecipitation of HA-pericentrin from cell lysates. Microspectrofluorometric quantification of DNA stained with DAPI shows that most HA-pericentrin expressing cells (C) had higher or lower nuclear DNA content than control cells (B). The average nuclear DNA content of individual cells (D) was three times greater than that of control cells (D, n>100 cells/column). Centrosome defects detected in cells stained for γ tubulin were > 20-fold higher in HA-pericentrin expressing cells (E).

Fig. 7. Permanent prostate tumor cell lines expressing pericentrin have tumor-like

features. Western blot (A) following immunoprecipitation of HA-pericentrin from cell lysates. Centrosome defects detected and quantified as described in Fig. 6 (B). In situ hybridization with centromere probes to chromosome 8 to evaluate chromosome instability (C, D) or with propidium iodide to determine DNA content by flow cytometry (E, F); y axis, propidium iodide fluorescence. Changes in cellular architecure are observed in HA-pericentrin cells (H) compared to controls (G, note larger cells with larger nuclei). HA-pericentrin expressing cells exhibit enhanced growth in agarose (J) compared with controls (I) as shown by a significant increase in colony size (K, peri+) but not number (L, peri+).

Fig. 8. Centrosome-based model for prostate cancer progression. Diagram of normal (above) and neoplastic prostate glands (below) showing the most salient cytoarchitectural features of tumors with increasing Gleason grade. In our model, centrosomes (represented by asters) become increasingly abnormal and misallocated during tumor progression concurrent with abnormalities in nuclei and nucleoli (represented by a filled dot), cellular and glandular disorganization and chromosome instability. Filled arrows represent currently favored evolutionary pathways; segmented arrows represent possible alternative pathways. Neither pathway has been convincingly demonstrated for prostate carcinoma. Gleason grade I and PIN lesions are not represented.