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13. ABSTRACT (Maximum 200 words)

Centrosomes are cellular organelles involved in the organization of mitotic spindles and thus, in the segregation of chromosomes during mitosis. When defective, centrosomes may contribute to chromosome missegregation and aneuploidy in human cancers (Pihan et al., 1998; Doxsey, 1998). In this proposal we investigate whether centrosome defects: 1. Are an intrinsic feature of clinically aggressive prostate carcinoma, 2. Provide a prognostic marker for aggressive prostate cancer and 3. Contribute to prostate tumorigenesis. To achieve these goals, we recently developed a high-resolution microscopic method and a quantitative assay to monitor centrosome defects in archival prostate cancer tissue. We used these approaches to demonstrate that both centrosome structure and cellular levels of the centrosome protein pericentrin, are abnormal in most malignant prostate tumors (Pihan et al., 1998 and Appendix). We have also initiated construction of permanent cell lines expressing pericentrin and produced cell lines expressing GFP-histones (to label chromosomes). These cell lines will be used to study the mechanism of chromosome segregation/aneuploidy in tumor cells. Perhaps most exciting are our recent findings showing that: 1. Prostate cancer precursor lesions have centrosome defects and that 2. Overexpression of pericentrin causes mitotic spindle abnormalities, chromosomal instability and aneuploidy in normal cells. Taken together, these results suggest that pericentrin overexpression and centrosome defects may contribute to aneuploidy, a condition that is commonly observed during development and progression of malignant tumors. In future studies, we hope to answer these questions.

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Centrosomes, genetic instability, mitotic spindles.

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INTRODUCTION.

In this proposal, we are testing a novel hypothesis: that centrosomes—key organizers of mitotic spindles--play a role in the genesis and progression of prostate carcinoma. This hypothesis is strongly supported by recent progress made with funds provided by the U.S. Army Medical Research and Materiel Command. We discovered that centrosome defects are present in nearly all in malignant prostate tumors (Pihan et al., 1998, see Appendix) and that a single centrosome gene when overexpressed in normal cells can induce a cancer-like phenotype (Purohit et al., 1999, see Appendix). Based on these and other observations discussed below, our current model is that centrosome abnormalities cause spindle defects and chromosomal missegregation, and through the generation of genomic instability, contribute to prostate cancer progression. We are using newly developed and improved immunohistochemical assays for detection and quantitation of centrosome abnormalities in prostate tumor tissues. We are combining this well documented approach (Pihan et al., 1998) with *in vitro* cell culture studies, biochemical analyses and whole animal experiments.

There is currently no good prognostic indicator for malignant prostate cancer. One goal of this research is to determine whether centrosome defects can provide an early marker for malignant disease. This information could then be used to limit treatment to those at risk for malignant disease, sparing those not at risk from the anguish and side effects of these treatments. We are encouraged by our unexpected recent observation that centrosome defects are found in early neoplastic lesions of the prostate (see below and Appendix). Based on this and other observations, we ultimately ope to exploit defects in centrosome structure, function and molecular composition (see below) to develop tumor-selective therapies.

BODY

Centrosome defects in malignant prostate cancer: In our initial experiments, we established that invasive and metastatic prostate carcinoma have a high incidence of centrosome abnormalities (see Pihan et al., 1998, see appendix). We are extending these studies to include 173 tumors at different stages of progression. Preliminary results suggest that centrosome abnormalities are more prevalent and extensive in lesions with high Gleason and nuclear grade scores--the tumors one would predict to have the worst clinical behavior (see fig.3 of the appendix). With the completion of this work we have accomplished the first subtask of Task 1 as shown in the statement of work (months 1-12)

Development of a high-resolution morphological assay for analyzing centrosomes: We improved the resolution of our immunohistochemistry assays for centrosomal proteins allowing us to more finely map structural centrosomal defects (compare fig 4 and 5 in appendix with fig 1 and 2 in Pihan et al., 1998, in appendix). This high-resolution assay has been critical for two important discoveries:
1. We have been able to determine that centrosomes are abnormal in precancerous lesions of the prostate (see below, prostate intraepithelial neoplasia, PIN). PIN is the only lesion that is unanimously considered to be the precursor of invasive carcinoma.

2. We have discovered a unique and easily detectable centrosome defect present exclusively in tumor cells (and not observed in nontumor cells) that will facilitate rapid and quantitative analysis of centrosome defects in tumors (see below).

Development of a quantitative assay for analyzing centrosomes: We have developed a method for quantifying both centrosomal and non-centrosomal (cytoplasmic) pericentrin in tissue sections (see fig 1 for an example of quantitation of non-centrosomal pericentrin). Using this method we show that pericentrin levels are increased in many tumors (see fig 2 in appendix). This observation has important implications for our work. It demonstrates that pericentrin levels are increased in cells without overt centrosome abnormalities. It also suggests that elevated pericentrin levels are a precursor to (and maybe a cause of) centrosome defects. Interestingly, cellular levels of other centrosome proteins do not appear to be elevated in tumors (G. Pihan and S. Doxsey, unpublished observation). This indicates that pericentrin is uniquely overproduced in tumor cells, and implicates the protein in tumor progression based on the observation that forced overexpression of pericentrin can induce aneuploidy in normal cells (Purohit et al., 1999). This observation also demonstrates that cells with elevated pericentrin levels are more prevalent than cells with centrosome defects, suggesting that this molecular assay will be more sensitive than the morphological assay. Used together, these assays should provide powerful tools to continue our studies on centrosome defects in tumors.

*Centrosome defects in precursor lesions to prostate cancer: Within the last year, we made a major discovery: that centrosome defects are present in about 20% of all precursor prostate cancer lesions (PIN, compare fig.4, normal prostate gland with fig.5, high grade PIN lesion). Centrosome defects in precancerous lesions suggests that centrosome dysfunction may not only be involved in tumor progression but also in tumor genesis. The common occurrence of aneuploidy in both PIN lesions and invasive carcinomas suggests that the latter is derived from the former (4). Since centrosome defects are present in both lesions, it is possible that they contribute to aneuploidy common to both and thus play a role in tumor development. The work described in this section contributes to the analysis of early stage tumors as described in subtask #2 of task 1 of the statement of work (months 6-12).

A cancer-like phenotype can be induced by pericentrin overexpression: We have shown that overexpression of pericentrin in COS cells and human can induce centrosomal defects, mitotic spindle abnormalities, chromosomal missegregation and aneuploidy (Purohit et al., 1999, see appendix). We have shown that similar defects are common in cells of malignant tumors (Pihan et al., 1998). These results lend direct support to our hypothesis that centrosomes are involved in tumorigenesis through the generation of genomic instability.

Pericentrin-expressing prostate epithelial and tumor cell lines: Based on the ability of pericentrin to create aneuploid cells after transient transfection, we have cloned HA- and GFP- tagged pericentrin into tetracycline-regulated plasmids. Transient transfections with these plasmids has shown that pericentrin expresses well upon induction and this produces a phenotype similar to that described in Purohit et al., 1999. We cloned pericentrin into two tetracycline-inducible systems and introduced them into several prostate cell lines that are under selection (12 PC-3, 4 LNCap and 4 DU-145). We are characterizing these cell lines

for inducibility with tetracycline and once a cell line with good dynamic rage of pericentrin expression is identified, we will characterize its in vivo and in vitro behavior. This work has begun to address subtasks 3-5 of Task 2 ahead of schedule (months 12-18).

Exploiting defective checkpoint control to selectively kill tumor cells. Since we made the unexpected and exciting observation that centrosome defects are present in precancerous lesions, we have focused our efforts on this aim. For this reason, we are slightly behind our goals for Aim 3. We will initiate the studies outlined in Aim 3 within the next two months. We will begin by treating prostate tumor cells sequentially with mitotic agents followed by S-phase inhibitors. We hope to selectively kill tumor cells with DNA synthesis inhibitors (S-phase) after they fail to arrest in mitosis (with taxol or nocodazole).

KEY RESEARCH ACCOMPLISHMENTS:

- We provided the first demonstration that centrosomes are abnormal in prostate cancer (see publications: Pihan et al., 1998 Cancer Research; Pihan and Doxsey, Seminars in Cancer Biology, in press; Doxsey, 1998, Nature Genetics). Another group made similar findings in breast cancer (see Lingle et al., 1998). Our work demonstrated that most malignant prostate cancers have abnormal centrosomes and that centrosome abnormalities correlate with chromosomal instability (CIN)

- The topic of centrosomes and cancer has become a very "hot" topic in cancer research. Following our original demonstration that centrosomes are abnormal in tumors (Pihan et al., Cancer Research 1998), several subsequent publications have appeared on this subject (5-13).

- Based on the observations in Pihan et al., 1998, we were asked to write 2 reviews. One on "mitotic machinery in cancer" and one on centrosomes in cancer (see refs 2 and 3 and appendix).

- Based on the observations in Pihan et al, 1998, we have been invited to give several seminars at prominent meetings and at universities around the nation and abroad:

In 1998:
-Gordon research conference, Colby, NH;
-Fred Hutchinson Cancer Research Center, Seattle; WA.
-Universtiy of Utah, Huntsman Cancer Institute, Salt Lake City, UT.
-National Cancer Institute, Bethesda, MD.
-University of Texas M.D. Anderson Cancer Center, Dallas, TX;
-American Society of Cell Biology Meeting, San Francisco, CA;

In 1999:
-Massachusetts Dept. Public Health Symposium:"Progress in Prostate Cancer".
-Second International Symposium on GFP, San Diego, CA
-Symposium: "Cell Division", Research Institute of Molecular Pathology,

Vienna, Austria

- Based on the observations in Pihan et al, 1998 and our current research, we were asked to present our findings in a radio interview WSRS on Sunday, June 14th, 1999 on "Worcester speaks out". Our research will also be highlighted in a newspaper article in the Medicine News section of the Telegram and Gazette (Worcester, MA) in June, 1999.

- We are also proud to announce that we serve on a prostate cancer advocacy group that recently lobbied in congress and the senate to secure $500,000 for prostate cancer research in Massachusetts. The budget previously was $50,000.

- Based on the observations in Pihan et al, 1998, we submitted a grant application to the NCI as part of a Program Grant on aspects of pericentrin as an inducer of a tumor-like phenotype. The grant was given excellent scores and has been resubmitted (6/99).

❖ *Perhaps the single most exciting discovery that we have made while executing the experiments proposed in this grant application is that centrosome defects occur in prostate intraepithelial neoplasia (PIN) a lesion known to be the precursor of invasive prostate cancer. This occurs before PSA increases are detected in serum and before enlarged prostate can be palpated.* The unexpected but exciting observation that structural centrosome abnormalities are present in prostate cancer precursors raises the possibility that *our centrosome assay may have prognositc value*. Our observations also suggest that centrosomes are not only involved in the progression of prostate cancer as we postulated in this grant proposal but may also be involved in tumor genesis. These observations were made possible through the development of a high-resolution assay for centrosome structure in archival paraffin sections of prostate biopsies (see fig. 3-5).

- We made the exciting discovery that a cancer-like phenotype can be artificially induced in normal cells simply by overexpressing the centrosome protein pericentrin (centrosome defects, spindle abnormalities, chromosome missegregation, aneuploidy, see Purohit et al, in appendix). This observation corroborates our model as described in Pihan et al 1998 and Doxsey, 1998.

- We discovered that pericentrin overexpression leads to disruption of dynein (see ref 4). This observation is particularly interesting from a cancer point of view since dynein has previously been shown to be involved in chromosome segregation and to interact with proteins of the mitotic checkpoint, processes that are commonly abnormal in tumors.

- We have found a particular centrosome abnormality that is tumor-specific (fig. 3A, B). This allows us to unequivocally discern between tumor and nontumor cells and will replace our previous, more subjective morphological assay.

- In addition to our improved morphological assay (above) we developed a new and powerful densitometric method to quantify the level of pericentrin in human tissues. This sensitive assay will allow us to obtain quantitative data on pericentrin levels in tumors. Used in combination with our tumor-specific morphological assay (above) we will be

able to carefully monitor pericentrin levels and centrosome defects in the same cells. (see fig. 1, 2)

- We developed a method for detecting changes in chromosome number (bright field) in alternate tissue sections stained for centrosome abnormalities. This will allow us to obtain direct information on the relationship between centrosome defects and chromosome instability in the same tumor.

- We have cloned pericentrin into inducible (tetracycline-controlled) vectors and generated prostate cancer cell lines permanently carrying these vectors.

- We have generated prostate cancer cell lines (LNCap, DU-145 and PC-3) carrying a histone B2-green fluorescent protein fusion protein that will permit studying the process of chromosome segregation in living cells and how pericentrin overexpression affects this process following microinjection of pericentrin overexpressing plasmid.

REPORTABLE OUTCOMES.

Manuscripts: (2 published, 2 in press), See above for details
Abstracts : (5) (Amer. Soc. Cell Biol., Gordon Res. Conf. IMP Conf., GFP Conf.)
Presentations (9), See above for details
Cell lines: (GFP-histone prostate cancer cell lines, others in progress)
Funding applied for: NCI Program Grant (resubmited 6/99)

CONCLUSIONS:

During the first funding year of this grant application we have answered some of the questions posed in this grant application. Centrosomes are numerically and structurally abnormal in malignant prostate cancer, and the extent of centrosome abnormalities correlates with histologic grade of the tumors. Perhaps most interesting is our unexpected observation that centrosome abnormalities are present in PIN, a pre-invasive form of prostate cancer. This indicates that centrosome abnormalities may be involved in the genesis (and progression) of prostate cancer and suggests that the centrosome assay could have prognostic value. This exciting data was made possible through technological improvements and new developments. We also discovered that pericentrin overexpression in normal cells can induce centrosome abnormalities, spindle defects, chromosome missegregation and aneuploidy. This observation, together with the demonstration that pericentrin levels are elevated in tumors, provides proof of principle about the potential oncogenicity of pericentrin.

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Figure Legends.

Figure 1. Pericentrin levels are elevated in tumors. Method used for densitometric quantification of cellular levels of pericentrin. Box represents area of measurement. See Fig. 2 for results.

Figure 2. **Pericentrin levels are elevated in tumors.** Densitometric quantitation of pericentrin in cells from tumors and notumor tissues.

Figure 3. Abnormal centrosomes in invasive prostate carcinoma . A. *Low power view (X100) of an invasive prostate carcinoma* with extensive centrosome abnormalities. Centrosomes were detected by indirect avidin-biotin immunohistochemistry using a polyclonal pericentrin antibody (a centrosomal protein cloned in our laboratory). The brown pigment correspond to the distribution of pericentin. Note the numerous "paracrystalline" inclusions in most of the neoplastic cells (small glands in the center of the field) and the presence of a single, distinct apical dot of immunoreactivity (normal centrosomes) in cells in the surrounding normal epithelium (larger glands toward the corners of the photomicrograph). **Figure 3B. *Malignant tumor-specific abnormal centrosome pheotype.*** High power view of A.

Figure 4. Normal prostate epithelium (compare with Fig. 5). Photomicrograph of a normal prostate gland immunostained with pericentin as in fig. 3. Note both the centrosomes, as round dark-brown structures near the apex of most epithelial cells, and the bland nuclear cytologic features typical of normal epithelium (600X original magnification)

Figure 5. Centrosome abnormlaities are present in precursor lesion in prostate cancer (prostate in situ). Photomicrograph of a high grade PIN (Prostate intraepithelial neoplasia) lesion. Note the many centrosomes with abnormal shapes and size near the lumen of the gland as well as the high grade cytologic features of the nuclei characteristic of high grade PIN lesions (600X original magnification)
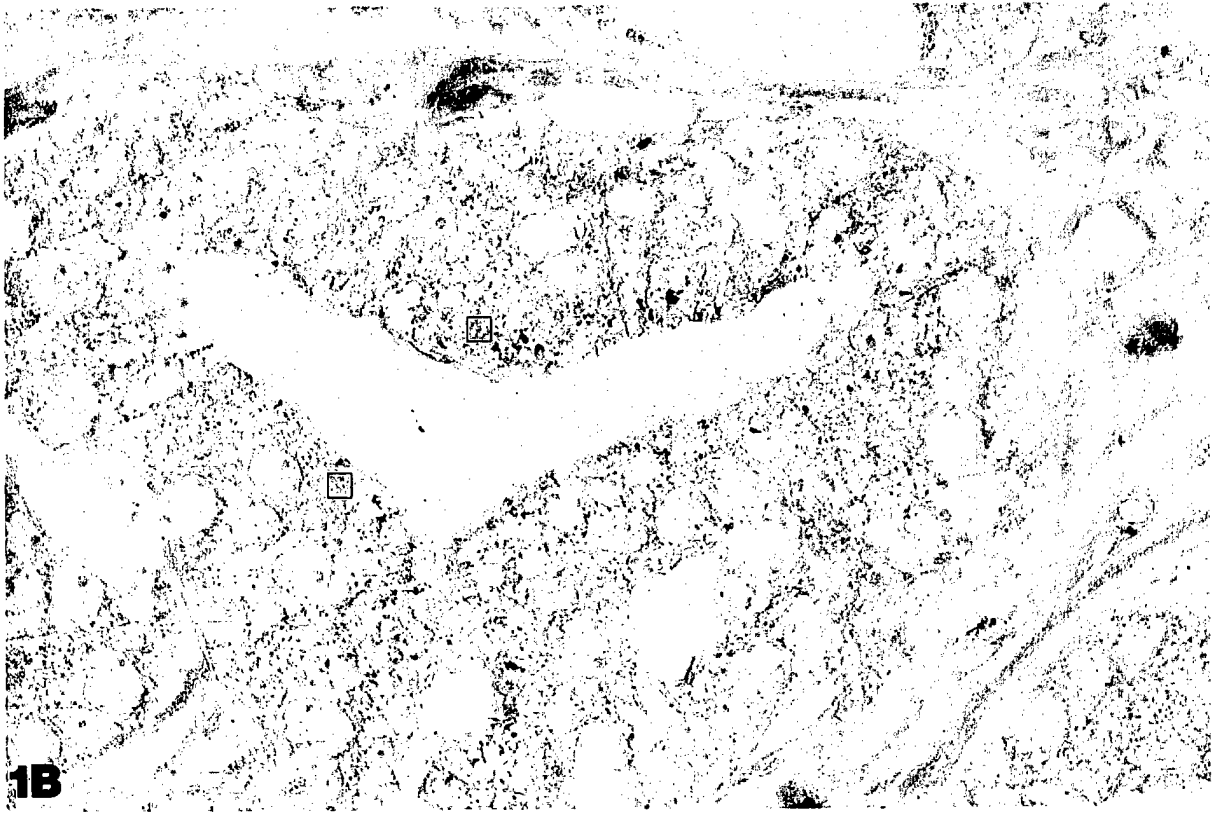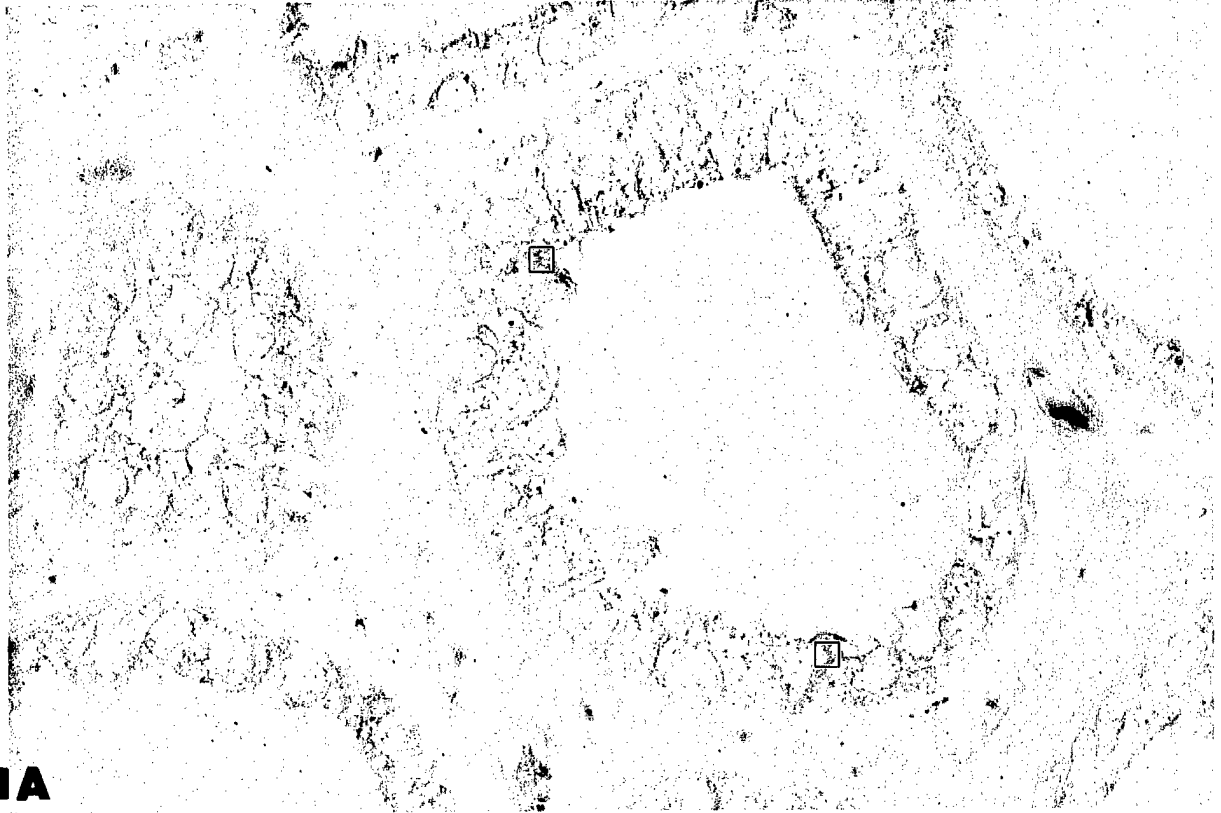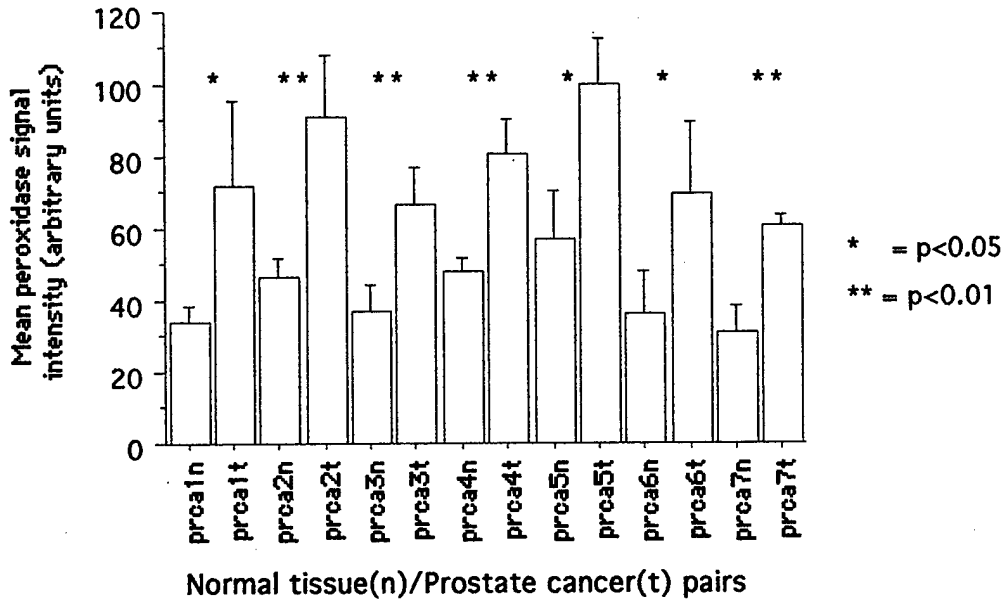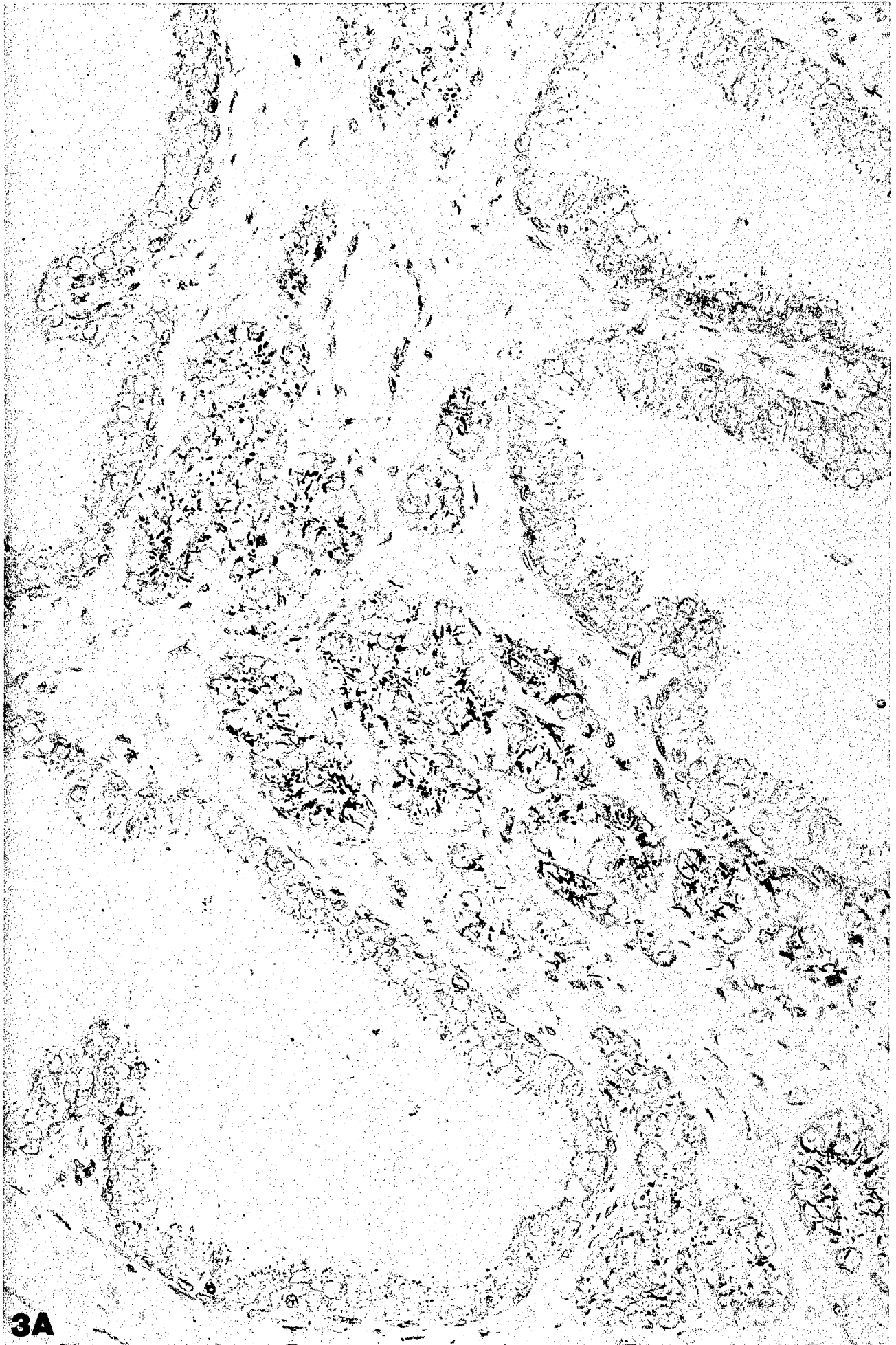
Figure 1

1A

1B

Figure 2

* = p<0.05
** = p<0.01

Mean peroxidase signal intensity (arbitrary units)

Normal tissue(n)/Prostate cancer(t) pairs
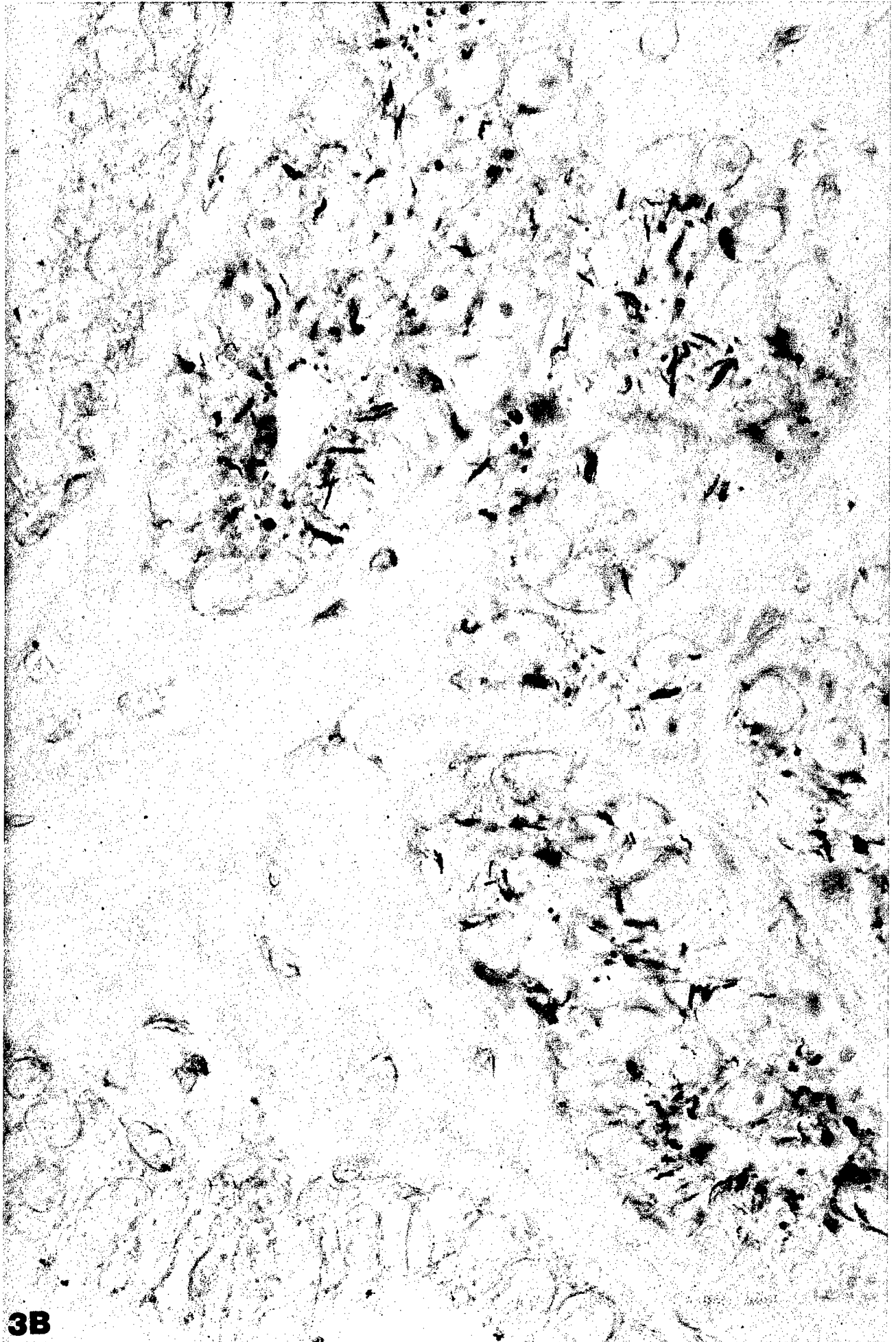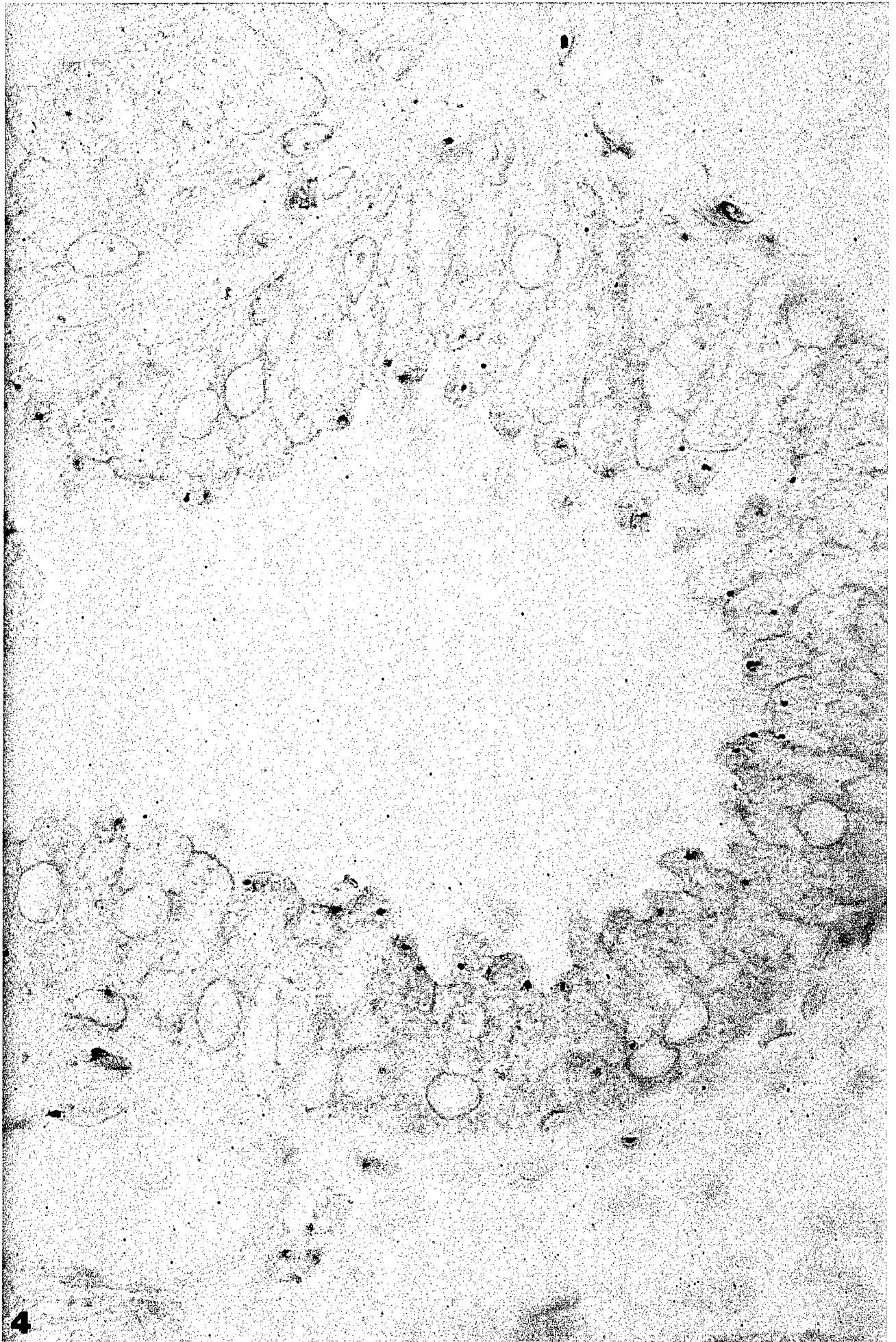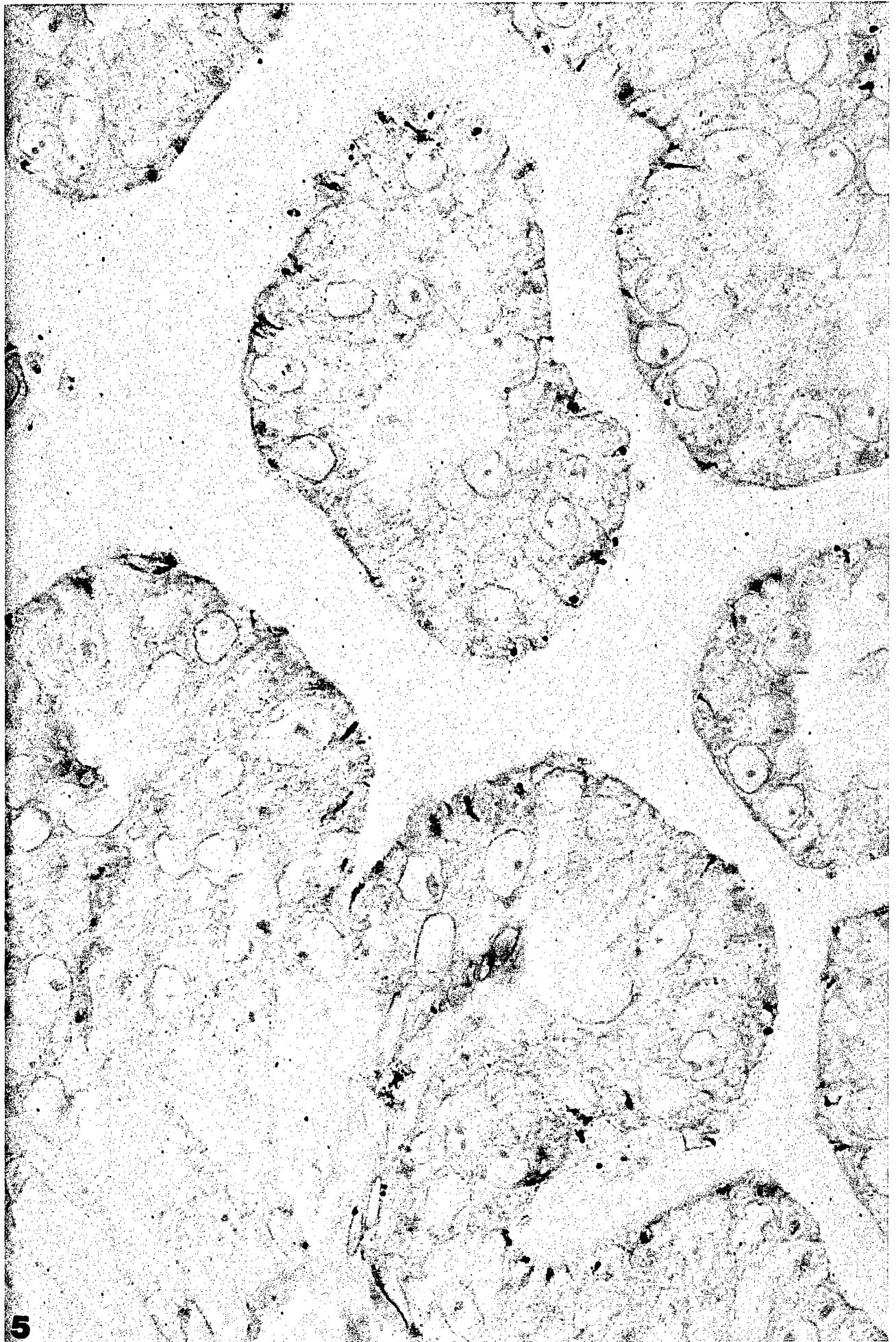
Figure 3A

3A

Figure 3B

3B

Figure 4

17

Figure 5

5

18

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 mm³) 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 × 10⁵ 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.

[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% H₂O₂ 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 MgCl₂, 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 G₂ 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. 3H) and were sometimes interconnected by atypical filaments of pericentrin (Fig. 2O, *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
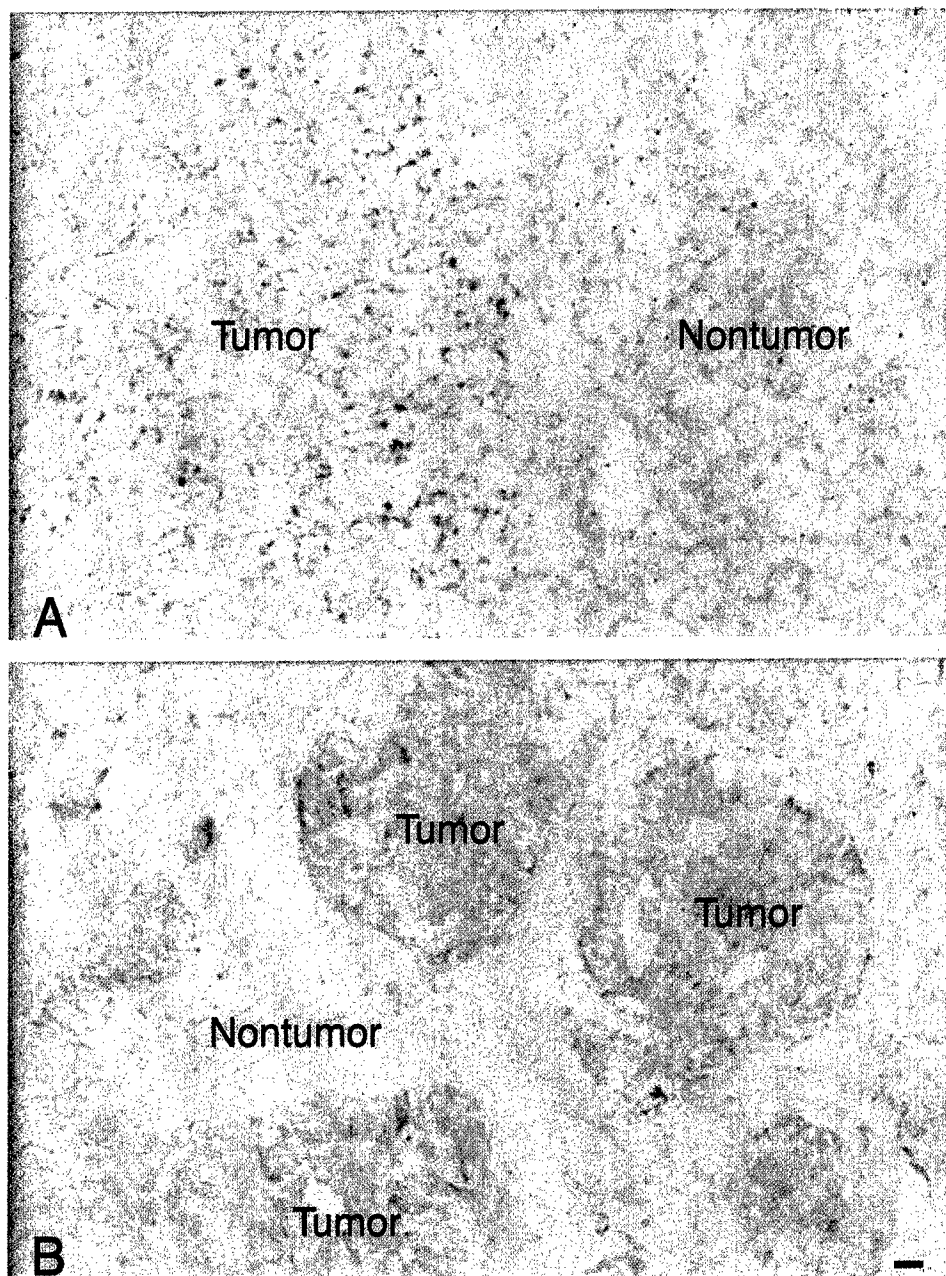
Fig. 1. Low-magnification images of malignant tumors showing amplified pericentrin staining. Paraffin-embedded tissue sections were stained for pericentrin by the immunoperoxidase method (*brown*) and counterstained with hematoxylin (*blue*) to reveal details of tissues and cells. Images show a high 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. 3H). 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. 3F), 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. 3G). 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
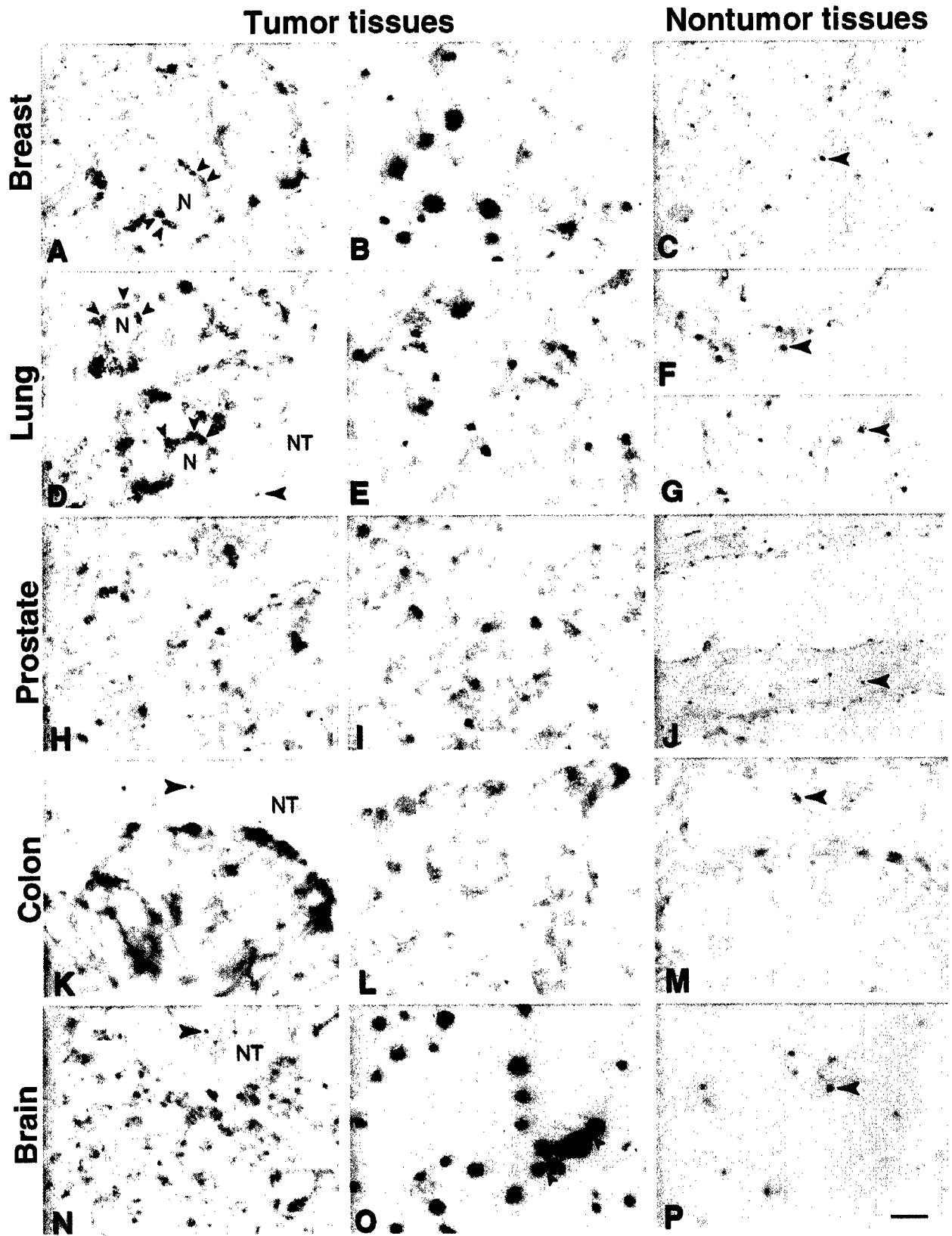
Fig. 2. High-magnification images of malignant tumors showing abnormal pericentrin structures. Tissues were processed for pericentrin staining as in Fig. 1. Cells in nontumor tissues (*NT*), in the same tissue section as tumor cells (*T*), usually have a single small focus of staining, typical of normal 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*).
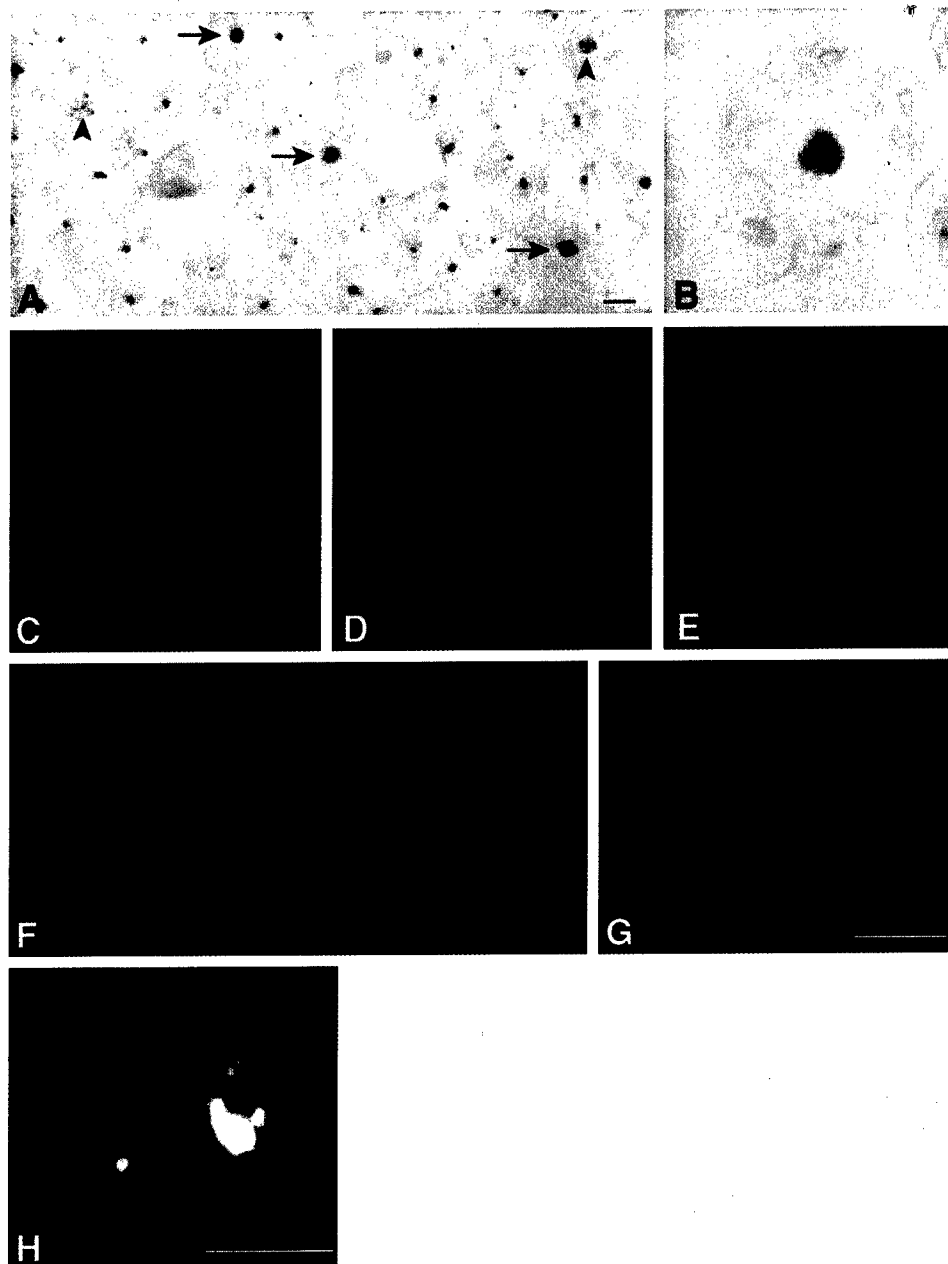
Fig. 3. Abnormal pericentrin structures in tumor-derived cell lines (*A–G*) and cells dissociated from tumors (*H*). *A*, HD cell line (L428) stained by immunoperoxidase (as in Fig. 1), showing several cells with enlarged pericentrin staining structures (*arrows*) and multiple pericentrin staining structures (*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. 5G) 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. 7E, *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 $\chi2$ 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-
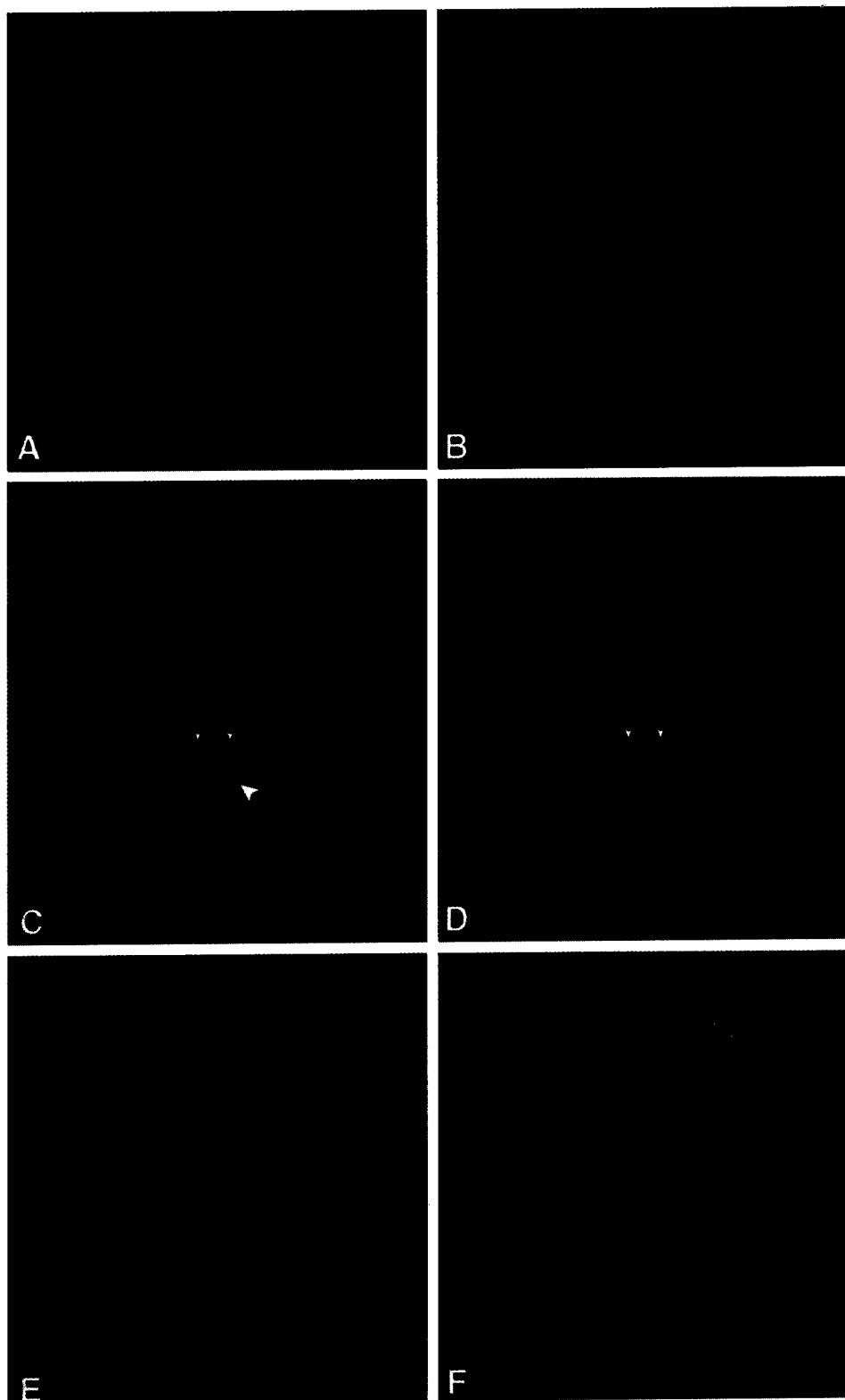
Fig. 4. Supernumerary centrioles and acentriolar structures in tumor-derived cell lines. Centrioles were labeled with an α-tubulin antibody following depolymerization of cytoplasmic microtubules by nocodazole (see "Materials and Methods"). Horizontal series are of the same cell in all cases. In control 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
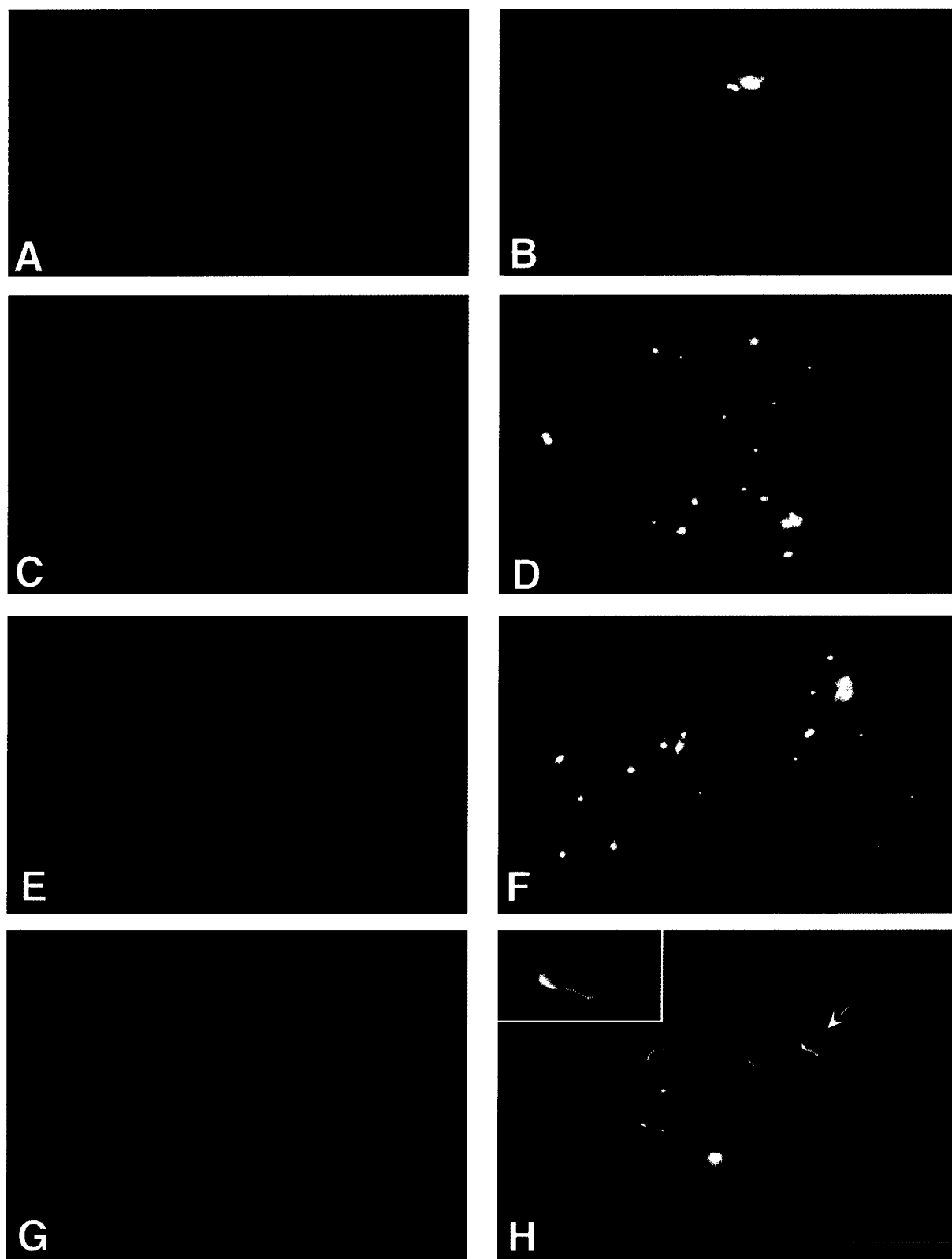
Fig. 5. All pericentrin-staining material nucleates microtubules. Cells from the control cell line B115 (*A* and *B*) and breast cancer-derived cell lines, BT-549 (*C*, *D*, *G*, and *H*) and MDA-MB-436 (*E* and *F*) were treated with nocodazole to depolymerize microtubules, washed, and allowed to regrow 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.
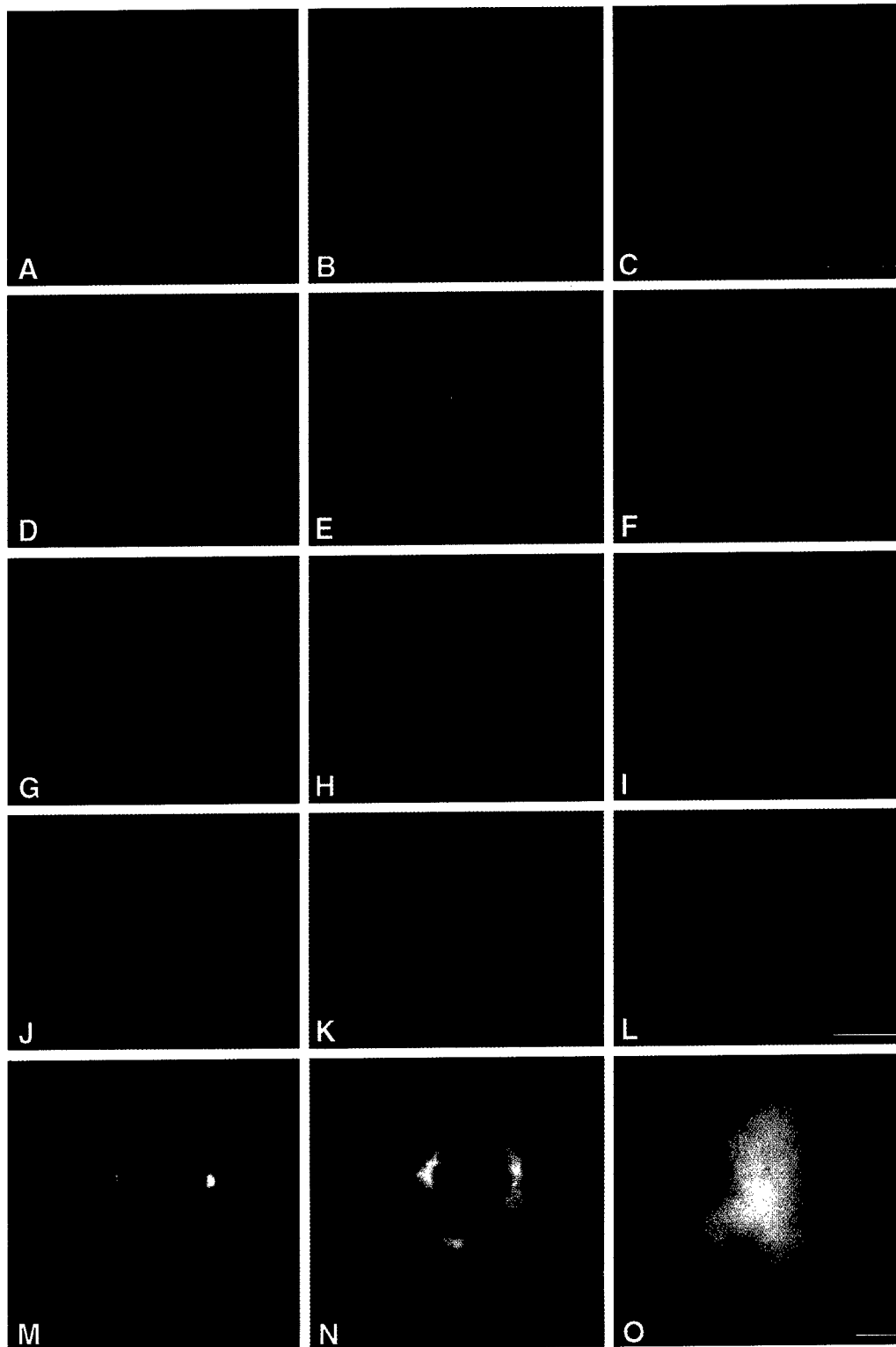
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Fig. 6. Abnormal pericentrin structures are associated with aberrant spindles. Control cell (B115) with two centrosomes (A) at the poles of a normal bipolar spindle (B) and DNA aligned on the metaphase plate (C). Abnormal pericentrin structures and 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-
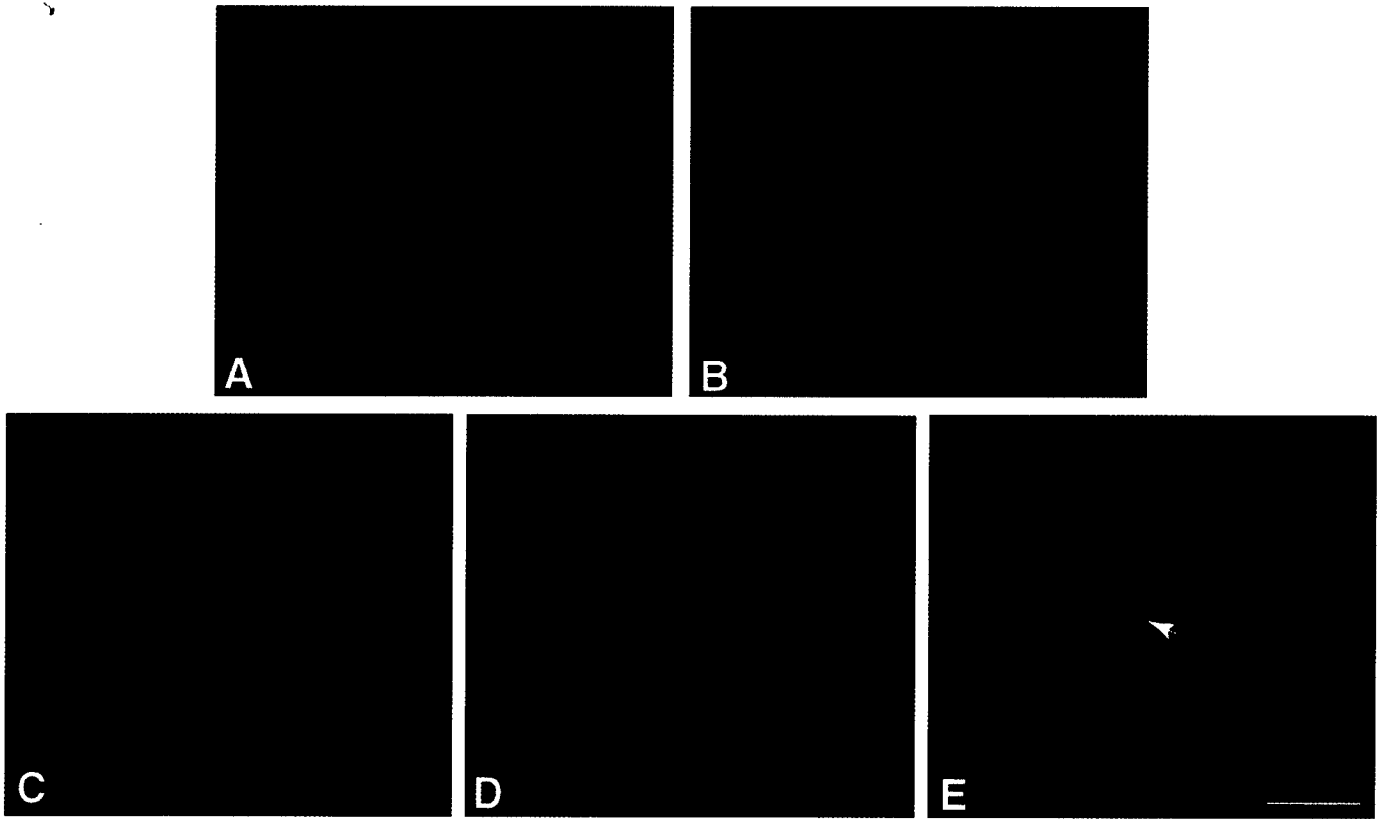
Fig. 7. Aberrant spindles missegregate chromosomes. A telophase cell from the prostate cancer cell line (PC-13) showing a tripolar spindle (*B*) with three spindle poles (*A*), some with multiple pericentrin structures (*A, bottom left* and *top right*). Chromosomes are segregated into three nascent 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 χ^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.
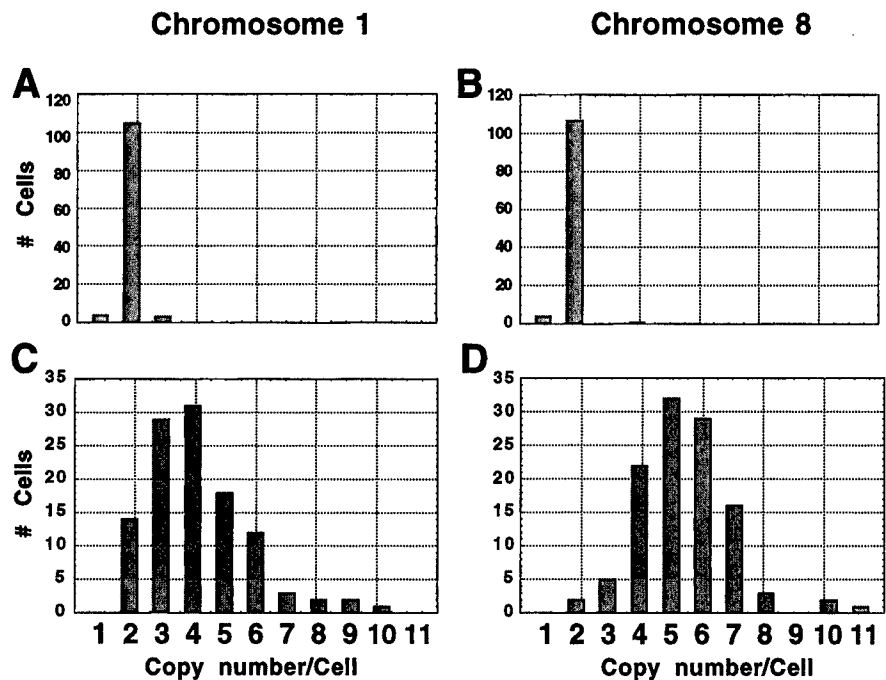
between these two cellular anomalies. In addition, we observed a statistically significant correlation between the level of centrosome defects and the level of chromosome 1 instability in tumor cells ($P < 0.05$, Spearman's rank correlation).

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.

ACKNOWLEDGMENTS

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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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 suc-

cessful 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
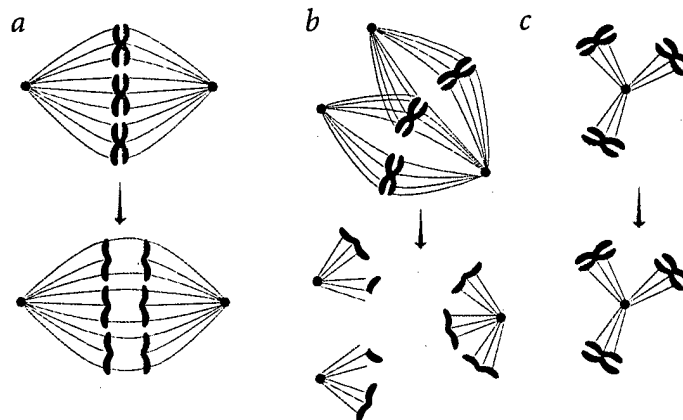
Fig. 1 Mitotic spindles in normal and centrosome-defective cells. **a,** Centrosomes (red) are located at the poles of the metaphase spindle. The replicated chromosomes (blue) are aligned at the spindle centre. Microtubule fibres (green) arise from centrosomes and contact chromosomes at protein plaques 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. 1a). 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. 1b; 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. 1b, 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. 1c). 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.

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

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.

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

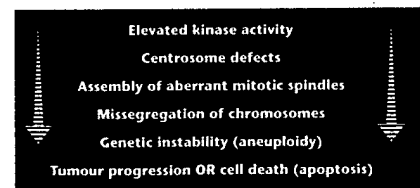**Fig. 3** Model of centrosome-induced tumorigenesis

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.
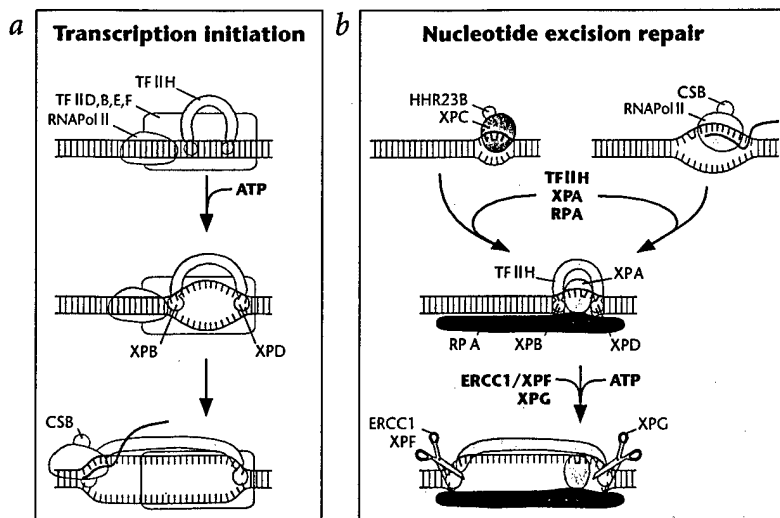
The mitotic machinery as a source of genetic instability in cancer.

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Abstract. 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.

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1. 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 suggests 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 [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 [2?]. Aneuploid-linked immortalization can be induced by oncogenic viruses [22,24], chemical carcinogen [23], ionizing radiation [25-28]. It can also occur spontaneously in fibroblasts cultures derived from L Fraumeni families carrying germline mutations of the p53 gene [25].

Aneuploidy as a cause of tumorigenesis. At the turn of this century, David von Hanseman [29] and later Theodore Boveri [30] postulated that aneuploidy was the cause of cancer. With t discovery of genes, the demonstration that carcinogens such as X-rays and alkylating agents we mutagenic [31], see [32], the identification of germline gene mutations in familial cancer syndromes [3 and the demonstration that cancer genes carried by oncogenic retroviruses are no more than muta:

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copies of endogenous cellular genes (proto-oncogenes) [34], the currently dominant gene mutation hypothesis gained favor. For review of gene mutation and cancer see [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 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. second and popular hypothesis to explain the development of aneuploidy postulates the 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 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 but that tetraploid tumor cell populations may be a reflection of a G2 arrest during progresison of 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 segregate chromosomes in a tumor progenitor cell. This would lead to persistent change chromosome number at every cell division. The predicted outcome of this condition would be that DNA content of tumor cell populations would be heterogeneous and continuously changing. R

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 chromosome has the potential to cause, accelerate or contribute to tumorigenesis by facilitating accumulation o chromosomes with growth promoting genes (oncogenes) and deletion (LOH) of chromosomes wit growth restraining genes (tumor suppressors) [44,59]. In this model, chromosomes would be lost an gained in a stochastic manner and those cells with oncogenes that exhibit a growth advantage woul be selected for. This selection pressure could operate at the level of entire chromosomes, differe fragments of individual chromosomes, and even on the same fragment of a given chromosome [60-6]

2. THE MITOTIC MACHINERY AS A POTENTIAL SOURCE OF ANEUPLOID IN TUMORIGENESIS.

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

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 this section 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 [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. Nonspindle (astral microtubules interact with the cell cortex and are involved in orienting the spindle in the cytoplasm an may also play a role in anaphase B movements. Most of these microtubule interactions are highl dynamic, a consequence of the inherently dynamic nature of microtubule ends, and the activity c microtubule motor proteins (see below).

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

The contribution of microtubule defects to aneuploidy in cancer progression has not yet b addressed although several studies are consistent with such a role. For example, numerous chem compounds that affect microtubule function, can induce aneuploidy (reviewed in [69]. Moreo changes in the expression of the tubulin, the subunit of microtubules [70] and mutations in the tub genes [71], can lead to chromosome missegregation. In addition, a number of proteins that directl indirectly interact with microtubules (in addition to motor proteins, see below), induce chromos

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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 curently unknown. Other proteins that may affect chromosome segregation are microtubule-associated proteins (MAPs) such as stathmin/Op18 [80], Tau and others (for review see [81,82]. Stahmin/Op18 promotes microtubule instability [83] and is overexpessed 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 [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 [88,89]

γ-tubulin and pericentrin are two centrosome matrix 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 [59]). Many other proteins are found at the centrosome [91] including cell cycle regulatory molecules and others implicated in tumorigenesis (for review, see [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 nontumor 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 [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].

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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 [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 nucleate 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 [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 [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 motors 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 [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 [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 (nonkinetochore) 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 [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 [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 [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 [125-130]. Inhibitors of topoisomerase II can induce both numerical and structural chromosomal abnormalities by interfering with chromosome

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 [4,44,97,150,151].

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 [127]. Mutations in several

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genes whose gene products are part of the cytokinetic machinery or regulate late cytokinetic events (ex. 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].

3. 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 [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].

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The metaphase/anaphse 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 [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 cold 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 [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 [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.

FIGURE LEGEND

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

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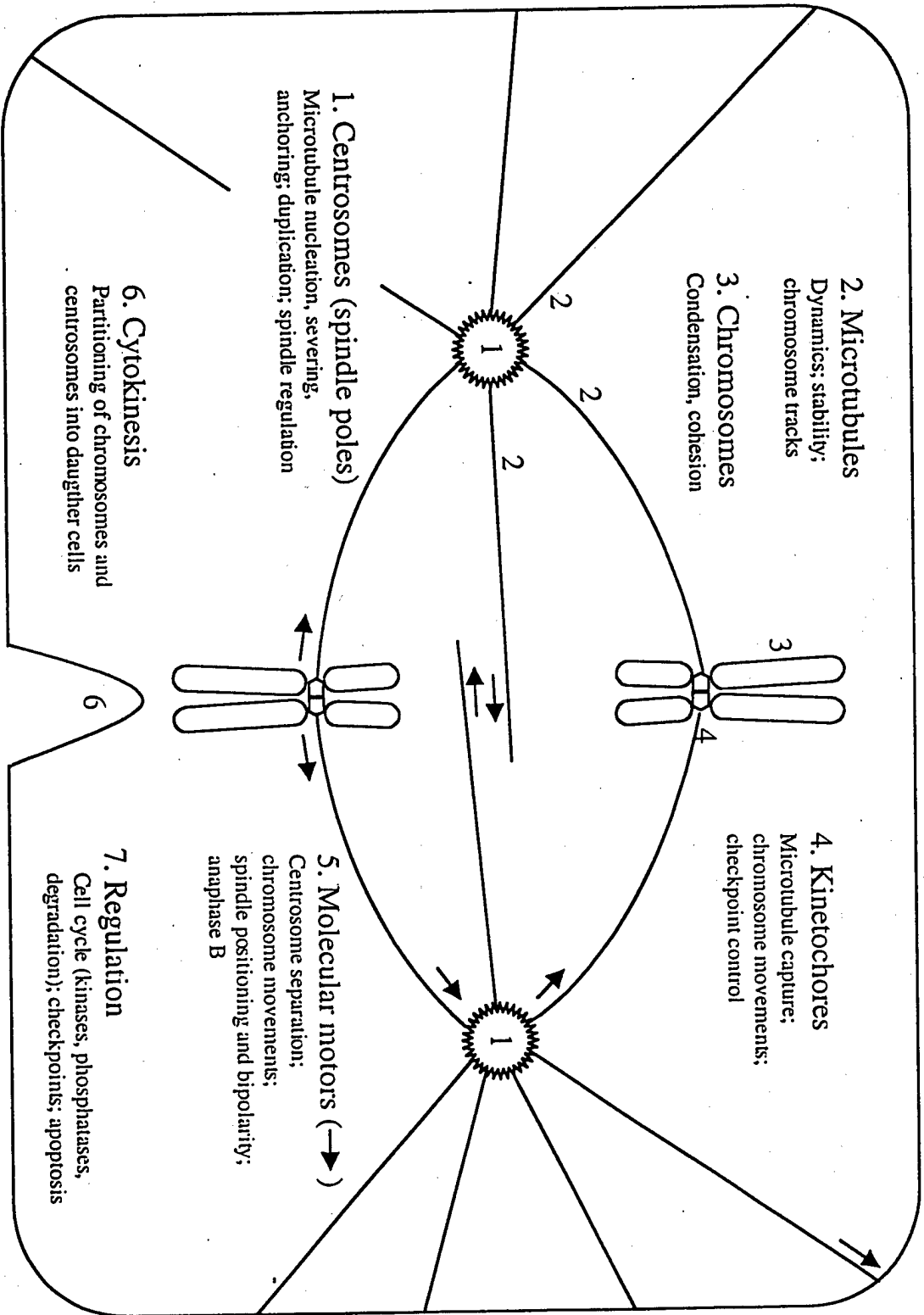127.   Yanagida M: Fission yeast cut mutations revisited: control of anaphase. Trends Cell Bic 8:144-9, 1998

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2. Microtubules
Dynamics; stability;
chromosome tracks

3. Chromosomes
Condensation, cohesion

1. Centrosomes (spindle poles)
Microtubule nucleation, severing,
anchoring; duplication; spindle regulation

6. Cytokinesis
Partitioning of chromosomes and
centrosomes into daughter cells

4. Kinetochores
Microtubule capture;
chromosome movements;
checkpoint control

5. Molecular motors (→)
Centrosome separation;
chromosome movements;
spindle positioning and bipolarity;
anaphase B

7. Regulation
Cell cycle (kinases, phosphatases,
degradation); checkpoints; apoptosis

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Overexpressed pericentrin interacts with dynein, disrupts dynein localization and function and causes spindle defects and aneuploidy.

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Abbreviations:

MTOC, microtubule organizing center

HA, hemagglutinin

MSH, melanocyte stimulating hormone

HA-Pc, hemagglutinin-tagged pericentrin

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 examined centrosomes and mitotic spindles for changes in composition and function. Mitotic spindles in pericentrin overexpressing cells were disorganized and mispositioned and chromosomes were misaligned and missegregated during cell division, giving rise to aneuploid cells. Although centrosome-mediated microtubule nucleation and localization of γ tubulin to the poles of mitotic spindles was normal, we unexpectedly found that levels of the molecular motor cytoplasmic dynein were dramatically reduced at spindle poles. Dynein levels were also diminished at kinetochores, and the dynein-mediated organization of the Golgi complex and centripetal transport of pigment granules in frog melanophores were severely impaired. Dynein and overexpressed pericentrin co-immunoprecipitated from detergent-soluble fractions of both COS cells and frog melanophores indicating that dynein was sequestered by pericentrin in the cytoplasm preventing it from localizing to its cellular targets. These results suggest that a pericentrin-dynein interaction in vivo may contribute to the assembly, organization or function of mitotic spindles and perhaps to the generation of aneuploidy during tumorigenesis (Pihan, G.A, A. Purohit, B.Woda, P. Quesenberry and S.J. Doxsey. 1998. *Cancer Res.* 58, 3974-3985,1998).

Introduction.

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 (Gould and Borisy, 1990; Szollosi et al., 1972). 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., 1995a; 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 prior to its assembly at the centrosome (Dictenberg et al., 1998). Recently, a Drosophila protein called Asp (abnormal spindle protein) has been shown to restore microtubule nucleating activity to salt-stripped centrosomes (Avides and Glover, 1999). This protein and others are likely to be important in regulating the assembly and activity of centrosomes (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)). For example, 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 recently been described (see (Gaglio et al., 1997; Hyman and Karsenti,

1998; Merdes and Cleveland, 1997; Waters and Salmon, 1997)). In these acentrosomal spindle assembly systems, the molecular motor cytoplasmic dynein and the nuclear mitotic apparatus protein (NuMA) play key roles in the organization and focusing of the spindle poles (Gaglio et al., 1996; Heald et al., 1996; Merdes et al., 1996). In one study, a complex of NuMA, dynein and dynactin, a protein complex thought to facilitate the localization and/or function of dynein (Schroer, 1996), was isolated and shown to be required for spindle focusing (Merdes et al., 1996). All three of these proteins are also present at the poles of centrosome-containing spindles and they all appear to affect spindle organization and integrity in these systems as well (Compton, 1993; Echeverri et al., 1996; Vaisberg et al., 1993). Dynein also plays important roles in spindle orientation (Carminati and Stearns, 1997; Cottingham and Hoyt, 1997; Stearns, 1997), chromosome alignment and segregation (Echeverri et al., 1996; Saunders et al., 1995), vesicle/organelle transport (for review see (Karki and Holzbaur, 1999)) and virus infection (Sodeik et al., 1997). Given the multiple sites of dynein action in the cell it is difficult to specifically dissect the role of the motor in spindle function.

One approach that could be used to elucidate individual functions of dynein in spindle organization is to identify dynein-interacting proteins and examine the functional consequences of the protein interactions. The identification of NuMA as one of the first molecular "cargoes" for dynein is an important step in this direction. Discovery of other dynein interacting molecules will provide information on the function of dynein and its binding partners, and will provide insight into spindle organization and function.

The precise role of pericentrin in mitotic spindle function is unclear. 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 embryos disrupt the organization of centrosomes, meiotic and mitotic spindles (Doxsey et al., 1994). Moreover, when added to Xenopus extracts the antibodies inhibit assembly of microtubule asters. Recent results demonstrate that pericentrin levels are elevated in human malignant tumor cells (Pihan et al., 1998). These cells exhibit defects in centrosome structure, spindle organization, chromosome segregation and they ultimately become aneuploid. These observations suggest that elevated pericentrin levels may contribute to the characteristic spindle abnormalities and aneuploidy that accompany tumorigenesis (for review see (Doxsey, 1999)) (Pihan et al., 1998).

In this study, we examined the role of pericentrin in spindle organization by overexpressing the protein in somatic cells. Cells with excess pericentrin formed aberrant mitotic spindles, missegregated chromosomes and became aneuploid. We unexpectedly found that cytoplasmic dynein was displaced from centrosomes and kinetochores, and the dynein-mediated organization of the Golgi complex and aggregation of pigment granules was disrupted. An interaction between dynein and overexpressed pericentrin was observed in detergent-soluble fractions of both COS cells and frog melanophores, suggesting that the mechanism of dynein disruption was via sequestration of the motor in the cytoplasm by the overexpressed pericentrin. These results indicate that pericentrin and dynein may act together to ensure proper spindle organization and function.

MATERIALS AND METHODS

HA-tagged pericentrin 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 the polymerase chain reaction (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' GTTTGGGAGGTAGAGGCT 3') with a PvuI site. The amplified PCR product was digested with EcoRV and PvuI. Plasmid pcDNAI/Amp (Invitrogen, San Diego, CA) was used to construct a vector with 13 amino acids of hemagglutinin protein (MAYPYDVPCYASL, pHAI) (Wilson et al., 1984) inserted at the HindIII site in the polylinker (a gift of Michael Green). 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 a automated sequencer (Biorad, Hercules, CA).

Cell culture, DNA transfection, cell viability and growth. Unless otherwise stated, COS-7 cells (monkey kidney) were used in all experiments described in this manuscript. COS cells were cultured as described (ATCC, Rockville, MD) with 10% fetal bovine serum, 100U/ml Penicillin and 100 µg/ml Streptomycin (Sigma Chem Co., St Louis, MO) respectively. Cells were grown on 12 mm round glass coverslips in 35 mm culture dishes (Falcon, Lincoln park, NJ) and transfected with 2 µg of plasmid DNA (HA-pericentrin, β galactosidase, pHAI, or no DNA) using Lipofectamine (GIBCO/BRL,

Gaithersburg, MD); transfection efficiency was ~15%. Cells were fixed 35-42 hours after transfection and processed for immunofluorescence staining, immunoprecipitation or Western blotting (see below). Cell viability was determined using mitotracker (Sigma) which measures energy-dependent electron transport in mitochondria and 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 hour time period.

Culturing and microinjection of frog melanophores. Immortalized Xenopus melanophores (gift of Dr. M. Lerner, University of Texas Southwestern) were cultured, injected and analyzed as described (Rogers and Gelfand, 1998). Briefly, cells plated on coverslips were microinjected (Graessman et al., 1980) with full length HA-pericentrin cDNA (0.2 mg/ml in 90 mM KCl, 10 mM NaH_2PO_4, pH 7.2) on a Diaphot 200 inverted microscope (Nikon, Japan) equipped with a PLI-188 micromanipulator (Narishige, Japan), a Nikon microinjector (Japan), a long distance 40 X phase-contrast objective lens and borosilicate needles pulled on a micropipette puller P-97 (Sutter Instrument Co., Novato, CA). Twenty four hours later cells were treated to aggregate(10 nM melatonin) or disperse (100 nM MSH) then fixed with 4% formaldehyde and analyzed for pigment distribution. Fully aggregated melanosomes form a tight mass at the cell center; partially dispersed/aggregated melanosomes were released from the central mass and localized at short distances from the cell center; fully dispersed melanosomes were evenly distributed throughout the cytoplasm (Reilein et al., 1998). In each of three experiments, 50-100 cells were counted.

Antibodies. Affinity-purified rabbit IgG was prepared from sera raised against the carboxy-terminus of pericentrin (Doxsey et al., 1994) and used at 1:1000 for immunofluorescence microscopy and Western blotting. Anti-HA monoclonal antibodies (12CA5) were obtained from Babco (Berkely, CA) and anti-HA polyclonal antibodies were a gift from Joanne Buxton (Meisner et al., 1997). Antibodies to α- and γ-tubulin, mouse IgG and rabbit IgG were obtained from Sigma (St. Louis, MO). Antibodies to β galactosidase were from Boehringer Mannheim (Indianapolis, IN). Antibodies to the following proteins were also used in these studies under conditions described in the accompanying references: dynein heavy chain JR-61 (Asai et al., 1994), dynein intermediate chain L5 (Vaughan and Vallee, 1995) and 74.1 (Dillman and Pfister, 1994), dynamitin (Echeverri et al., 1996), p150^{glued} (Waterman-Storer et al., 1995), anti-p58 Golgi protein (Bloom and Brashear, 1989), CENP-E (Lombillo et al., 1995). Fluorescein- (FITC) and cyanine (cy3)-conjugated IgGs were obtained from Jackson ImmunoResearch Laboratories (West Grove, PA). Alkaline phosphatase-conjugated IgGs were obtained from Biorad (Hercules, CA); HRP-conjugated IgGs from Amersham (Arlington heights, Il). Antibodies were used alone or in combination as described in the text.

Immunofluorescence microscopy and quantification.
Immunofluorescence microscopy was performed essentially as described (Doxsey et al., 1994; Sparks et al., 1995). Unless otherwise stated, cells expressing HA-pericentrin constructs, β-galactosidase, pHAI, or mock transfected were fixed in 100% methanol at -20ºC. Cells were stained for HA together with the co-labeling antibody as designated in the figures. In most cases, monoclonal or polyclonal HA antibody was detected with FITC-labeled secondary antibody and the antibody used in the co-labeling experiment was

detected with a cy3 secondary. In all cases, cells were stained with dapi to detect chromatin. Cells were observed using an Axiophot fluorescence microscope with a 100X objective (Zeiss, Germany). Quantification of centrosomal staining in mitotic cells and DNA (dapi) was performed as described (Dictenberg et al., 1998). Briefly, the total fluorescence from centrosomes and nuclei in individual cells was determined. Background values from 3 positions in the cytoplasm and camera noise (dark current) were subtracted (usually <10% of total). For centrosome staining, fluorescence signals were obtained from only one centrosome per mitotic cell to avoid photobleaching.

For detection of melanophores expressing HA-pericentrin, cells were fixed in methanol at -20°C and stained with anti-HA followed by a FITC-conjugated anti-mouse antibody to detect transfected cells. Cells were co-stained with DM-1 alpha antibodies (Sigma Chem Co., St Louis, MO, 1:2000) followed by Texas-Red conjugated goat anti-mouse IgG (Jackson Labs, West Grove, PA) to detect microtubules. Coverslips were mounted in Elvanol and viewed in a Microphot-SA microscope (Nikon). Images were acquired (CCD #CH250, Photometrics, Tucson, AZ) controlled by Oncor Image software (Gaithersburg, MD). Bright field and fluorescence images were overlaid using Adobe Photoshop software to show the pigment distribution in transfected cells.

Immunoprecipitation and immunoblotting. For COS cells immunoprecipitations were performed as previously described with minor modifications (Dictenberg et al., 1998). COS cells were transfected with HA-pericentrin, β-galactosidase or mock transfected (no DNA). Cells were rinsed briefly with PBS, detached from culture dishes with 5mM EDTA in PBS, pelleted and lysed in 1% Triton-X-100,137mM NaCl, 50mM Tris.HCl pH 7.4,

1mM EDTA, 1mM EGTA, protease inhibitors (Dictenberg et al., 1998). Insoluble material was removed by centrifugation. Lysates (400ul, 4mg/ml) were precleared for immunoprecipitation by adding 25 ul of a 50% slurry of protein G fast flow or protein A beads (Pharmacia) and incubated on an orbital rocker at 4° C for 1 hr. Beads were removed and the precleared lysates were incubated with antibody prebound to either protein G or protein A beads for 12 hrs at 4° C. Beads were washed in lysis buffer, resuspended in Laemmli sample buffer (Laemmli, 1970) and heated to 95° C for 3-5 minutes. Proteins were separated by SDS-PAGE on 7% gels, electroblotted to PVDF membranes (Millipore Co., Bedford, MA) and processed for western blotting as described (Sparks et al., 1995).

Frog melanophores were rinsed briefly with PBS and detached with 1mM EDTA in 0.7X PBS, then pelleted and resuspended in 50mM Tris.HCl pH8.0, 150mM NaCl, 1mM EDTA and 1mM EGTA, 1mM β-mercaptoethanol, and protease inhibitors. Cells were lysed in suspension by passing them through a syringe with a 27G needle as previously described (Rogers and Gelfand, 1998). The extract was spun at 16,000g. Triton X-100 was added to the supernatant to a final concentration of 1%, and the extract was spun once more at 16,000g for removal of nonsolubilized membranes. Extracts were precleared for 1.5 h with normal rabbit serum prebound protein A beads (Sigma, Co., St. Louis, MO). The precleared extracts were incubated for 12 h at 4 ° C with antibody and beads. Pelleted beads were washed twice with lysis buffer, once with 0.3M NaCl in lysis buffer, once more with lysis buffer. Beads were resuspended in Laemmli sample buffer. Proteins from immunoprecipitations were separated by 6% SDS-PAGE gels, electroblotted to nitrocellulose. Blots were blocked overnight in 0.1% Tween in TBS (TBST) supplemented with 5% non-fat dry milk. The blots were then incubated for

30min with primary antibodies, washed three times in TBST, incubated for 30min with HRP-conjugated secondary antibodies and washed three more times. SuperSignal (Pierce Chemical Co., Rockford, IL) was used for secondary antibody detection.

RESULTS

We previously demonstrated that functional depletion of pericentrin disrupted centrosome and spindle organization in several systems (Doxsey et al.,). Based on these observations, we reasoned that an artificial elevation of pericentrin levels would provide additional information on protein function. To this end, we constructed and expressed a hemagglutinin-tagged pericentrin in somatic cells and examined centrosome and spindle composition and function.

Biochemical analysis demonstrated that HA-pericentrin in COS cells had an electrophoretic mobility of ~220 kDa and partitioned between Triton X-100-soluble and insoluble fractions (Fig. 1A). Immunofluorescence analysis demonstrated that the more abundant detergent-soluble fraction was distributed throughout the cytoplasm (Fig. 1B) while the detergent-insoluble fraction colocalized with γ tubulin at centrosomes (Fig. 1C, D). Centrosome localization of HA-pericentrin 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 impaired in pericentrin overexpressing cells. The most dramatic consequence of HA-pericentrin expression detected in this study was the disruption of mitotic spindle organization (Fig. 2, 3). A significant proportion of prometaphase and metaphase COS cells at all expression levels exhibited spindle defects (75.7+/-6.1%, average of 48 experiments, n=423 cells) compared with nontransfected cells (2.5+/-1.5%, n=598 cells) and vector DNA transfected

cells (3.0+/-1.0, n=201 cells). We observed defects in spindle structure (36.2% of all cells with spindle defects), spindle positioning (22%) and spindle function (42% of anaphase and telophase cells had missegregated chromosomes); many cells had multiple defects. In many cells, spindle poles were positioned at various angles to the metaphase plate and sometimes formed monopolar attachments with subsets of chromosomes outside the bipolar structure (Fig. 2E, F, arrowheads). Spindles were often elongated in the pole to pole dimension (Fig. 2E) and were organized into monopolar and multipolar structures (Fig. 2H; also see Fig. 7K and L, O and P). In addition to the structural defects, spindles were often displaced from their typical central position in the cell (Fig. 2K; 22% of spindle defects). Mispositioned spindles occupied peripheral regions of cells adjacent to the plasma membrane and within tapered cellular processes, far from the cell center. Consistent with defects in spindle organization, was the observation that chromosomes were often misaligned (Fig. 2F, 3C, F). Misaligned chromosomes, abnormal assemblies of chromosomes and chromosomes with monopolar attachments to the spindle were seen in cells with both normal and structurally abnormal spindles. Spindle defects similar to those described above were observed in CHO cells and 3T3 cells (data not shown).

Improper attachment of chromosomes to microtubules of the mitotic spindle is known to activate the spindle checkpoint and arrest cells in mitosis (Rudner and Murray, 1996; Waters, 1997). Despite the presence of disorganized spindle microtubules, apparently unattached chromosomes and other spindle defects, HA-pericentrin cells progressed through mitosis. The percentage of mitotic figures in the population of HA-pericentrin-expressing cells (3.1+/-0.9%, n=3490) 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=5002). HA-pericentrin expressing cells were frequently observed in later stages of mitosis (anaphase, telophase). They appeared to missegregate chromosomes at a high rate (Fig. 3A-J), although their viability and growth rate appeared unchanged throughout the course of this analysis (~36 hours). The prevalence of chromosome missegregation events indicated that the cells were becoming aneuploid. Quantification of dapi-stained nuclei confirmed this and revealed a remarkably wide variation in DNA content with values ranging from 0 to 5 times those of controls (Fig. 3K, L).

Distribution and function of cytoplasmic dynein is disrupted at multiple cellular sites in HA-pericentrin expressing cells. To identify potential candidates that contribute to the spindle defects observed in HA-pericentrin expressing cells, we examined centrosome and spindle pole composition and function. To our surprise, the ability of centrosomes to nucleate microtubules appeared normal. Figure 4 shows that the quantity of microtubule polymer arising from mitotic centrosomes following microtubule depolymerization and regrowth, was roughly similar in HA-pericentrin and control cells. Moreover, centrosome and spindle pole levels of γ tubulin, the protein thought to mediate microtubule nucleation, were not significantly different from control cells (see Fig. 6C and Fig. 7I-L).

Cytoplasmic dynein has been shown to play a role in spindle positioning in *S. cerevisiae* through interactions with astral microtubules at the plasma membrane (Carminati and Stearns, 1997; Stearns,). Since we commonly observed mispositioned spindles in HA-pericentrin expressing cells, we reasoned that the localization or function of dynein may be impaired.

Immunofluorescence microscopy revealed that cytoplasmic dynein was dramatically reduced at spindle poles in mitotic cells (Fig. 5G-I) although it did not appear to be altered at centrosomes in interphase cells (data not shown). Quantitative analysis demonstrated that dynein levels at spindle poles were reduced 8-12-fold in prometa- and metaphase (Fig. 6A). Several lines of evidence suggested that the reduction in dynein staining resulted from displacement of the protein from spindle poles and not masking of antigenic epitopes. First, diminished dynein staining was detected with two independent antibody preparations raised against the dynein intermediate chain (L5, polyclonal and 74.1, monoclonal). Second, control cells expressing β-galactosidase (Fig. 5A-C) or untransfected cells (Fig. 5D-F, Fig. 6A) had normal levels of dynein at their poles. Third, the distribution and abundance of several other centrosome and spindle pole components, including dynein-interacting proteins, remained unchanged. The spindle pole component NuMA, which forms a protein complex with dynein and helps to maintain the integrity of spindle poles (Merdes et al., 1996), localized normally to spindle poles (Fig. 7E-H). Interestingly, there was no significant difference in the spindle pole localization of either p150^{glued} or dynamitin two components of dynactin, a complex believed to localize dynein to its targets (Fig. 6B, 7A-D). Finally, in addition to its reduction at spindle poles, dynein was displaced from or functionally abrogated at several other cellular sites.

During the analysis of spindle poles, we noticed that dynein staining with two independent dynein intermediate chain antibody preparations was reduced at kinetochores (L5, 74.1, Fig. 5M-O). In contrast, no changes in localization of the kinesin-like protein CENP-E (Lombillo et al., 1995; Yen et al., 1992) to kinetochores was detected (Fig. 7M-P). This observation indicated that the

reduction in dynein was specific and suggested that the overall integrity of kinetochores was unchanged.

The reduction in dynein levels at both centrosomes and kinetochores suggested that dynein localization and/or function was disrupted at multiple cellular sites. To test this, we examined the Golgi complex whose organization is mediated by cytoplasmic dynein (Burkhardt et al., 1997). In a significant proportion of HA-pericentrin expressing cells, Golgi elements were dispersed throughout the cytoplasm (Fig. 8B, 77+/-3.3%, n=251) compared with the tightly-focused characteristic perinuclear structures observed in adjacent nontransfected control cells (Fig. 8B, arrowheads, 95.6%, n=497) (Bloom and Brashear, 1989). Dispersal of Golgi elements did not result from impaired microtubule integrity as there were no detectable changes in the microtubule network (data not shown, see below).

One of the most dramatic examples of dynein-mediated membrane transport is the centripetal movement of pigment organelles toward the minus ends of microtubules in frog melanophores (Nilsson and Wallin, ; Rogers et al.,). In cells expressing HA-pericentrin following microinjection of the cDNA into nuclei, 98% of melanophores were unable to aggregate pigment granules upon stimulation with melatonin (Fig. 9D, F), while cells expressing a control construct (GFP), aggregated granules normally (Fig. 9B, F). The effect of HA-pericentrin on granule aggregation was specific as the kinesin-based granule dispersion process was unaltered in the same cells (Fig. 9C, F). Moreover, the microtubule cytoskeleton in HA-pericentrin expressing cells appeared normal (Fig. 9E) indicating that disruption of granule aggregation resulted specifically from disruption of dynein-mediated transport.

Pericentrin and cytoplasmic dynein co-immunoprecipitate from pericentrin overexpressing cells. The pancellular loss or functional abrogation of cytoplasmic dynein at spindle poles, kinetochores, the plasma membrane, the Golgi complex and pigment granules, suggested that overexpressed pericentrin disrupted dynein by sequestering the motor in the cytoplasm. This idea was supported by the observation that HA-pericentrin and dynein co-immunoprecipitated from detergent lysates of both COS cells and frog melanophores. Figure 10 shows that antibodies to both dynein intermediate chain and dynein heavy chain precipitated HA-pericentrin, while several control IgG preparations did not. Conversely, antibodies to HA but not control IgGs precipitated dynein intermediate chain in the cells. These results demonstrate that HA-pericentrin and multiple subunits of cytoplasmic dynein co-immunoprecipitate from both frog and monkey cells and suggest that HA-pericentrin interacts with the dynein complex in the cytoplasm of these cells preventing it from associating with its normal target sites.

DISCUSSION

We have demonstrated that pericentrin overexpression has profound effects on the organization, positioning and function of mitotic spindles in somatic cells. We also show that these spindles missegregate chromosomes and generate aneuploid cells. During the course of these studies, we unexpectedly found that overexpressed pericentrin is a potent inhibitor of dynein localization and dynein-mediated functions. Overexpressed pericentrin interacts with dynein in both frog and monkey cells suggesting that the dynein-interacting domain of pericentrin is a conserved feature of the molecule. The interaction of HA-pericentrin with dynein appears to sequester the motor in the cytoplasm, preventing it from loading onto spindle poles, kinetochores, Golgi vesicles, pigment granule membranes and the plasma membrane. The perturbation of dynein localization and function is specific for the motor as microtubule nucleation and integrity are preserved in HA-pericentrin expressing cells. The disruption of dynein from spindle poles and kinetochores in HA-pericentrin expressing cells is likely to contribute to some of the observed defects in spindle structure and function.

Molecular mechanism of spindle disruption. Several studies show that dynein plays an important role in many of the same processes that are disrupted in HA-pericentrin expressing cells including spindle organization and positioning, chromosome alignment, and spindle pole integrity (for review see (Karki and Holzbaur, 1999)). Based on these data and the observation that dynein is selectively lost from spindle poles and kinetochores in HA-pericentrin expressing cells, it is likely that dynein contributes to the pericentrin-induced spindle defects. In contrast, proteins

that interact with dynein and perform important spindle functions such as NuMA and dynactin (see (Karki and Holzbaur, 1999)), localize normally to spindle poles and kinetochores in HA-pericentrin expressing cells, and they may not play a role in generating aberrant mitotic spindles. In fact, preliminary results suggest that dynactin does not co-immunoprecipitate with HA-pericentrin (A. Purohit and S. Doxsey, unpublished observations), consistent with the observation that the localization of dynactin to spindle poles and kinetochores is not perturbed by pericentrin overexpression. More work will be required to determine whether the interaction of pericentrin with dynein is direct or is mediated by an intermediate binding partner such as dynactin.

Functional implications for a pericentrin-dynein interaction. The ability of overexpressed pericentrin to cause a pancellular disruption of dynein localization and dynein-mediate functions suggests that native pericentrin and dynein may interact in cells with normal levels of pericentrin. However, in preliminary experiments we were unable to detect the proteins by conventional co-precipitation techniques, indicating that the interaction is either a transient event or that it takes place in the detergent-insoluble fraction at the centrosome. Preliminary observations suggest that both possibilities may be correct. We recently identified a fraction of dynein that remains associated with centrosomes and spindle poles in the absence of microtubules and colocalizes with pericentrin at these sites by quantitative analysis of high resolution fluorescence images obtained by advanced deconvolution methods (A. Young and S. Doxsey, unpublished observations) (Dictenberg et al., 1998). These data indicate that the proteins may interact at this site and may cooperate in spindle organization and function. Dynein also

appears to associate with pericentrin to mediate assembly of pericentrin and γ tubulin onto centrosomes in somatic cells (A. Young, R. Tuft and S. Doxsey, Mol. Biol. Cell 9, 137a, 1998). In this capacity, pericentrin and γ tubulin may serve as cargoes for dynein-mediated transport to centrosomes. More work will be required to understand the relationship of pericentrin and dynein and to document the functional consequences of this relationship.

Pericentrin, genomic instability and cancer. Pericentrin overexpressing cells appear to progress through mitosis despite overt spindle defects and misaligned chromosomes, and they ultimately become aneuploid. This is in contrast to the mitotic arrest observed when dynein mislocalization or function is disrupted by overexpression of dynamitin (Echeverri et al., 1996) or injection of a dynein antibody (Vaisberg et al., 1993). These observations suggest that excess levels of pericentrin may abrogate the spindle checkpoint (see (Rudner and Murray, 1996)). If future studies support this idea, it is likely that the mechanism by which pericentrin affects checkpoint control will be independent of dynein and may define a pathway leading to aneuploidy.

The ability of pericentrin to induce aneuploidy is intriguing in light of the recent observation that pericentrin levels (Pihan et al., 1998) and the levels of other centrosome proteins (Bischoff et al., 1998; Lingle et al., 1998) are elevated in malignant tumors. Tumor cells exhibit features indistinguishable from those observed in pericentrin-overexpressing cells including centrosome defects, spindle abnormalities and aneuploidy (for reviews, see (Doxsey, ; Pihan and Doxsey, 1999)). Moreover, these features are often

observed in the same tumor cell, suggesting a structural or functional relationship between these cellular defects. Based on the ability of elevated levels of pericentrin to induce aneuploidy in normal cells, the increased pericentrin levels observed in aneuploid tumor cells may contribute to genetic instability in cancer.

FIGURE LEGENDS

Figure 1. HA-pericentrin partitions between detergent-soluble and insoluble fractions. (A) Triton-X-100-soluble (lane 1) and insoluble fractions (lane 2) of HA-pericentrin expressing COS cells exposed to SDS-PAGE and Western blotted with anti-HA antibodies. (B) HA-pericentrin expressing COS cell fixed in -20°C methanol and immunostained with HA antibodies reveals significant cytoplasmic staining. The cytoplasmic fraction can be detergent-extracted prior to fixation by treating briefly (1 min) with 0.5% TX-100 in 80mM pipes pH6.8, 5mM EGTA, 1mM MgCl$_2$. This procedure reveals the detergent-insoluble fraction of HA-pericentrin (C) that co-localizes with γ tubulin at the centrosome (D). B, C, anti HA, D, anti-γ tubulin. Bars, 10 μm; bar in C for C and D.

Figure 2. Mitotic spindle organization and positioning is impaired in HA-expressing cells. Immunofluorescence staining of microtubules (or γ tubulin, K) in non-transfected (A-C) and pericentrin overexpressing COS cells (D-L). HA-pericentrin- expressing cells with l o n g e r p o l e t o p o l e distance s (E), compared to a spindle in a control cell (B). A spindle with a subset of chromosomes misaligned and mispositioned on the metaphase plate (E, F, arrowheads). A spindle with multiple poles is shown in H (also see Fig. 7K, L and O, P). A spindle located in the cell periphery near the plasma membrane (J-L). In general, 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. Cells were stained with antibodies to HA (A, D, G, J), α tubulin (B, E, H) or γ tubulin (K) and dapi (C, F , I, L) . Horizontal series are of the same cell. Bar in I, 10 μm for A-I, bar in L, 10 μm for J-L.

Figure 3. Chromosome misaligment, missegregation and aneuploidy in pericentrin overexpressing cells. In panel C, a COS cell chromosome(s) remains unattached to the spindle while all others have aligned on the metaphase plate (C, arrowhead). In panel F, chromosomes appear to be unattached to the spindle (small arrowhead, right) or attached to an extra focus of γ tubulin staining (small arrowhead, left). Large arrowheads in E and F represent the presumed bipolar spindle stained for γ tubulin to mark spindle poles. A cell that has divided but excluded a chromosome(s) from the reforming nuclei (H, arrow). Another cell has undergone a tripolar division (I, J) giving rise to two cells with chromosomes and a third cell with no DNA. The amount of dapi-stained chromatin in pericentrin transfected cells (L) is highly variable compared with control cells (K) (see Materials and Methods). Anti-HA (A, D, G, I) anti-α tubulin (B), anti-γ tubulin (E), dapi (C, F, H, J). Bar in J, 10 μm for all.

Figure 4. Centrosome-mediated microtubule nucleation is unaffected in mitotic HA-pericentrin expressing cells. Mitotic COS cells (prometaphase are shown) expressing HA-pericentrin (C, D) or mock transfected (A, B) were treated with nocodazole (10 μg/ml) for 1 hour at 37°C to depolymerize microtubules. Following removal of the drug, cells were incubated for 3 minutes to allow microtubules to regrow then fixed in methanol and stained with α tubulin to reveal nucleated microtubules as described previously (Brown et al., 1996a; Dictenberg et al., 1998). The amount of microtubule polymer nucleated from pericentrin expressing (C) and control cells (A) was roughly similar. Note that individual microtubules

are not easily observed after these short periods of microtubule regrowth. Inset in C, HA staining. Bar in D, 10 μm.

Figure 5. Dynein immunofluorescence is reduced at spindle poles and kinetochores in mitotic HA-pericentrin-expressing cells. COS cells were fixed and processed for immunofluorscence as described in Fig. 1C using antibodies to the dynein intermediate chain. HA-pericentrin expressing cells show significantly reduced levels of dynein immunofluorescence at spindle poles (G-I) when compared to β-galactosidase-expressing control cells (A-C) or nontransfected control cells (D-F). Kinetochore staining in prometaphase is also reduced in HA-pericentrin-expressing cells (M-O) compared to nontransfected controls (J-L). Staining for β galactosidase staining (A), HA (D, G, J, M), DNA (C, F, I, L, 0), and dynein (B, E, H, K, N). Horizontal series are of the same cell. Metaphase cells (A-I), prometaphase cells (J-O). Bars, 10 μm; bar in C for A-C, bar in O for D-O.

Figure 6. Quantitative analysis of protein levels at spindle poles in pericentrin-overexpressing cells. COS cells were fixed as in Fig. 1C, immunolabeled for the proteins shown and the centrosome-associated fluorescence signals were quantified as described in Materials and Methods. The level of dynein at spindle poles in both prometphase and metaphase HA-pericentrin expressing cells was ~8-12-fold lower (A, filled bars) compared with nonexpressing cells (A, empty bars). In contrast, the levels of the dynactin component p150^{glued} (B) and γ tubulin (C), were not significantly different from nonexpressing control cells. At least 40

centrosomes from 40 individual cells were counted for each bar. Cells with low, intermediate and high expression levels were included in the analysis.

Figure 7. Localization of other essential centrosome and kinetochore proteins are unaltered in pericentrin-expressing cells. COS cells were immunolabeled for proteins involved in dynein localization (dynamitin, A-D), spindle pole integrity (NuMA, E-H), microtubule nucleation (γ tubulin, I-L) and chromosome movement (CENP-E, M-P). The distribution and levels of these proteins in HA-pericentrin-expressing cells (lower panels), did not appear different from adjacent nonexpressing control cells (upper panels). HA stained cells shown in insets in C, G, K, O. Horizontal series the same cell. Bar in P, 10 μm for all .

Figure 8. Golgi complexes are disrupted in pericentrin-overexpressing cells. An HA-pericentrin COS 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.

Figure 9. HA-expressing frog melanophores fail to aggregate pigment. Frog melanophores expressing either HA-pericentrin (C, D) or a control protein (GFP, A, B) were fixed and analyzed for dispersion and aggregation of pigment granules by phase contrast microscopy as described (Nilson, #309). In HA-pericentrin expressing cells, the aggregation of pigment granules in response to melatonin is significantly impaired (D) although pigment dispersion stimulated with MSH appears normal (C). In contrast, cells expressing GFP aggregate and disperse pigment granules

normally (A, B). 98% of pericentrin overexpressing cells did not aggregate pigment (F(d), n=54), while most GFP expressing cells did so (F(b), n=100). There is no detectable difference between organization of the microtubule cytoskeleton in HA-pericentrin expressing cells stained with α tubulin antibodies (E, center) and in surrounding control (nonexpressing) cells. Inset in E shows HA staining of pericentrin expressing cell shown at center of E. Bar, 30 μm.

Figure 10. HA-pericentrin and cytoplasmic dynein co-immunoprecipitate from frog melanophores and COS cells.
Detergent lysates of HA-pericentrin-expressing frog melanophores (lanes 1-3) and COS cells (lanes 4-11) were incubated with antibodies to dynein intermediate chain, dynein heavy chain, HA, nonspecific rabbit IgG or nonspecific mouse IgG as indicated. IgGs were collected with protein A or G beads, exposed to SDS-PAGE and transferred to immobilon. Filters were blotted for dynein intermediate chain (lanes 1-6) or HA-pericentrin (lanes 7-11). The conditions used for immunoprecipitations caused some HA-pericentrin degradation (note lower band in lanes 7-9) that was never observed when cell lysates were blotted directly (see Fig. 1A). ~60kD band is nonspecifically precipitated with HA antibody when mouse IgG is used (lanes 7, 8, 10). Molecular mass markers are indicated (in kDa); markers for lanes 7-11 are same as for lanes 4-6.

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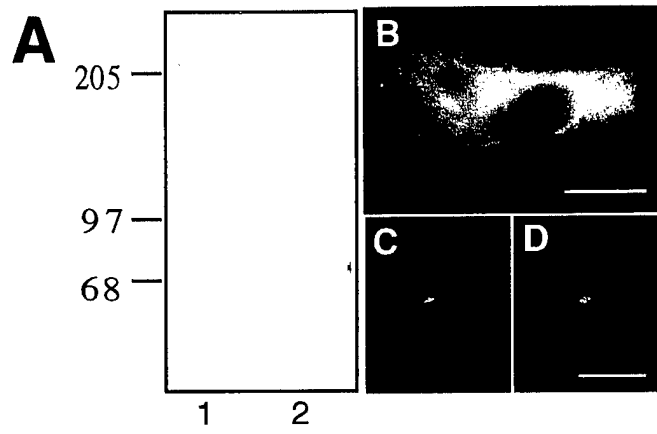
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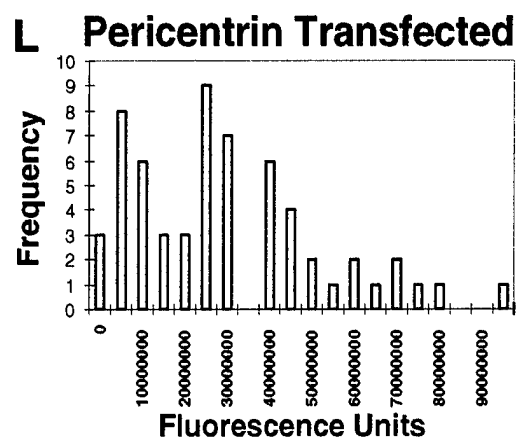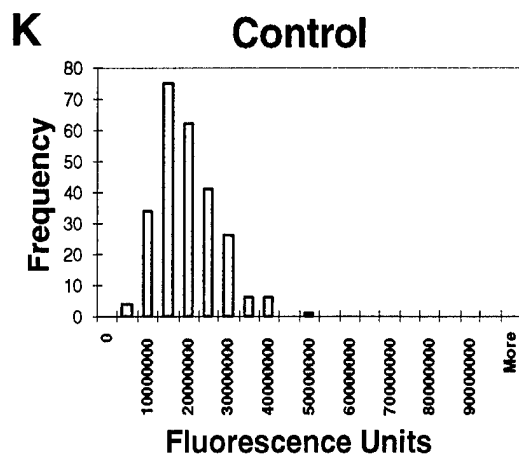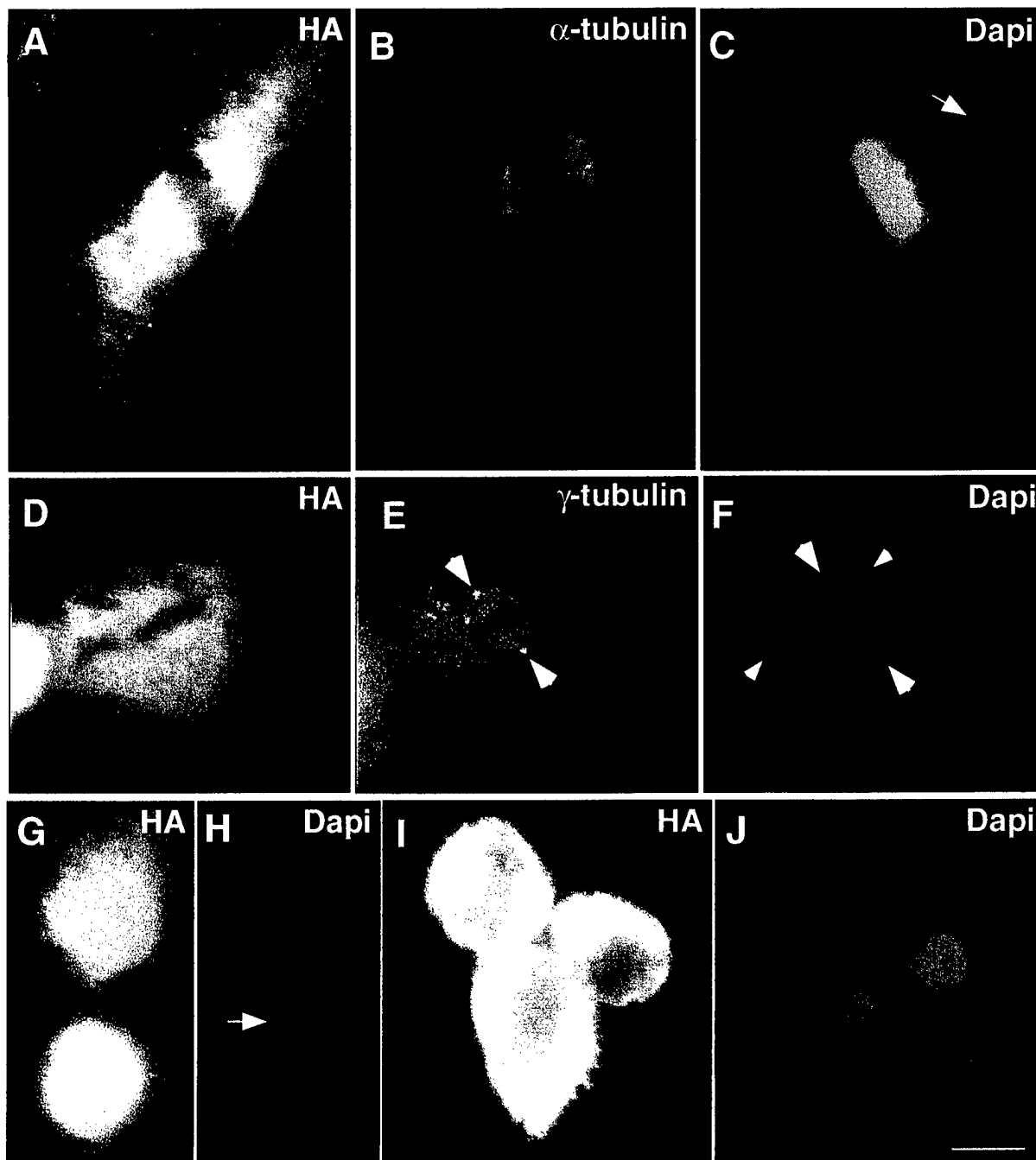
Figure 1

A

205 —

97 —

68 —

1   2

B

C   **D**

HA **Tubulin** **Dapi**

Figure 2

Figure 3

K   Control

L   Pericentrin Transfected
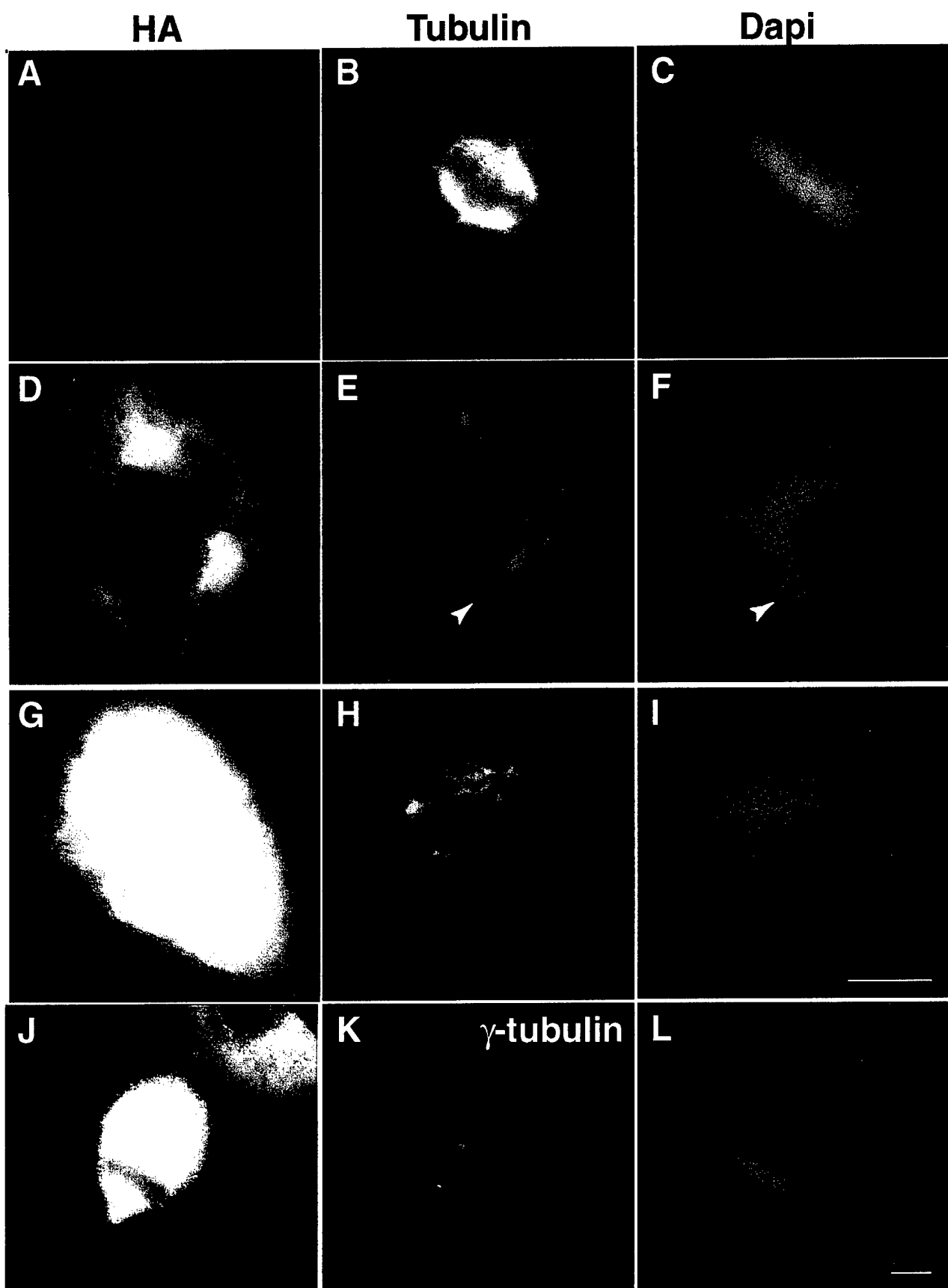
α-tubulin | Dapi

Figure 4

Figure 5

Controls/HA	Dynein	Dapi
A β-gal	B	C
D	E	F
G	H	I
J	K	L
M	N	O

Figure 6

A

Fluorescence Units

$2\ 10^5$

$1.5\ 10^5$

$1\ 10^5$

$5\ 10^4$

0

Prometaphase Metaphase

Dynein

B

$5\ 10^6$

$4\ 10^6$

$3\ 10^6$

$2\ 10^6$

$1\ 10^6$

0

Prometaphase Metaphase

p150/dynactin

C

$1.2\ 10^4$

$1\ 10^4$
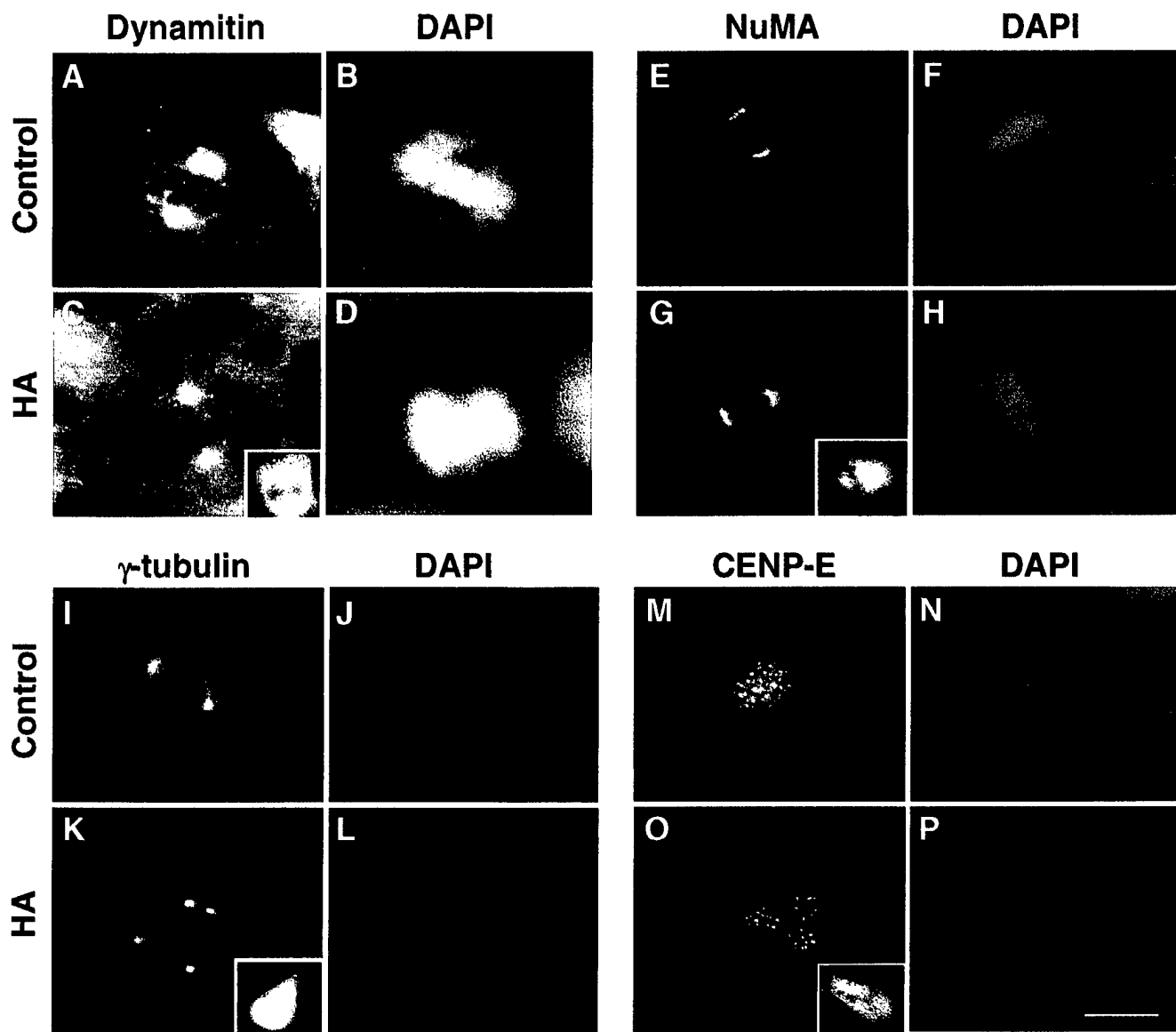
8000

6000

4000

2000

0

Pro- and
Metaphase

γ tubulin

Figure 7

HA **Golgi (p58)** **Dapi**
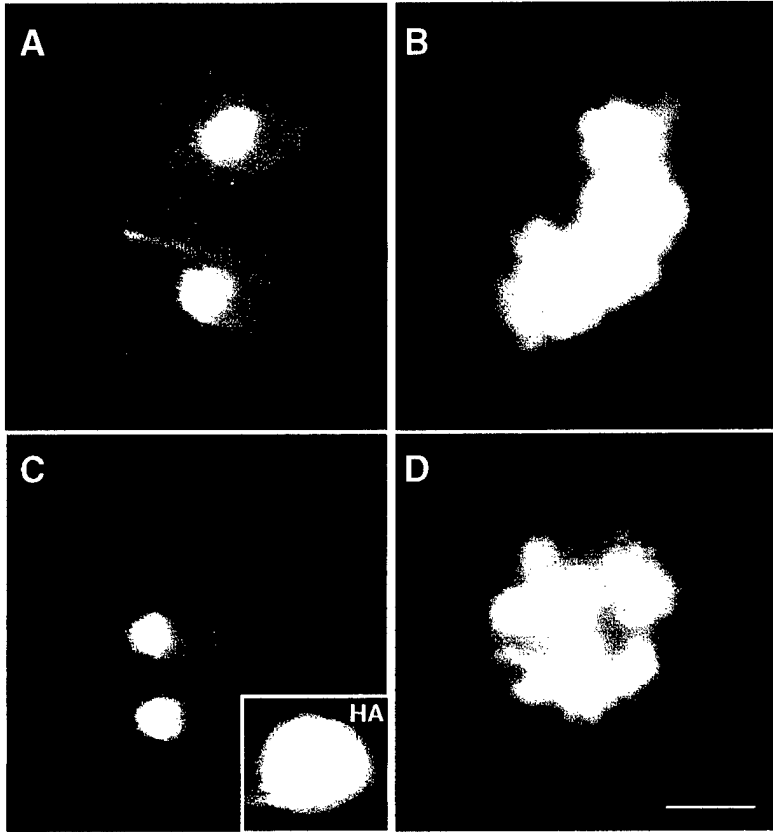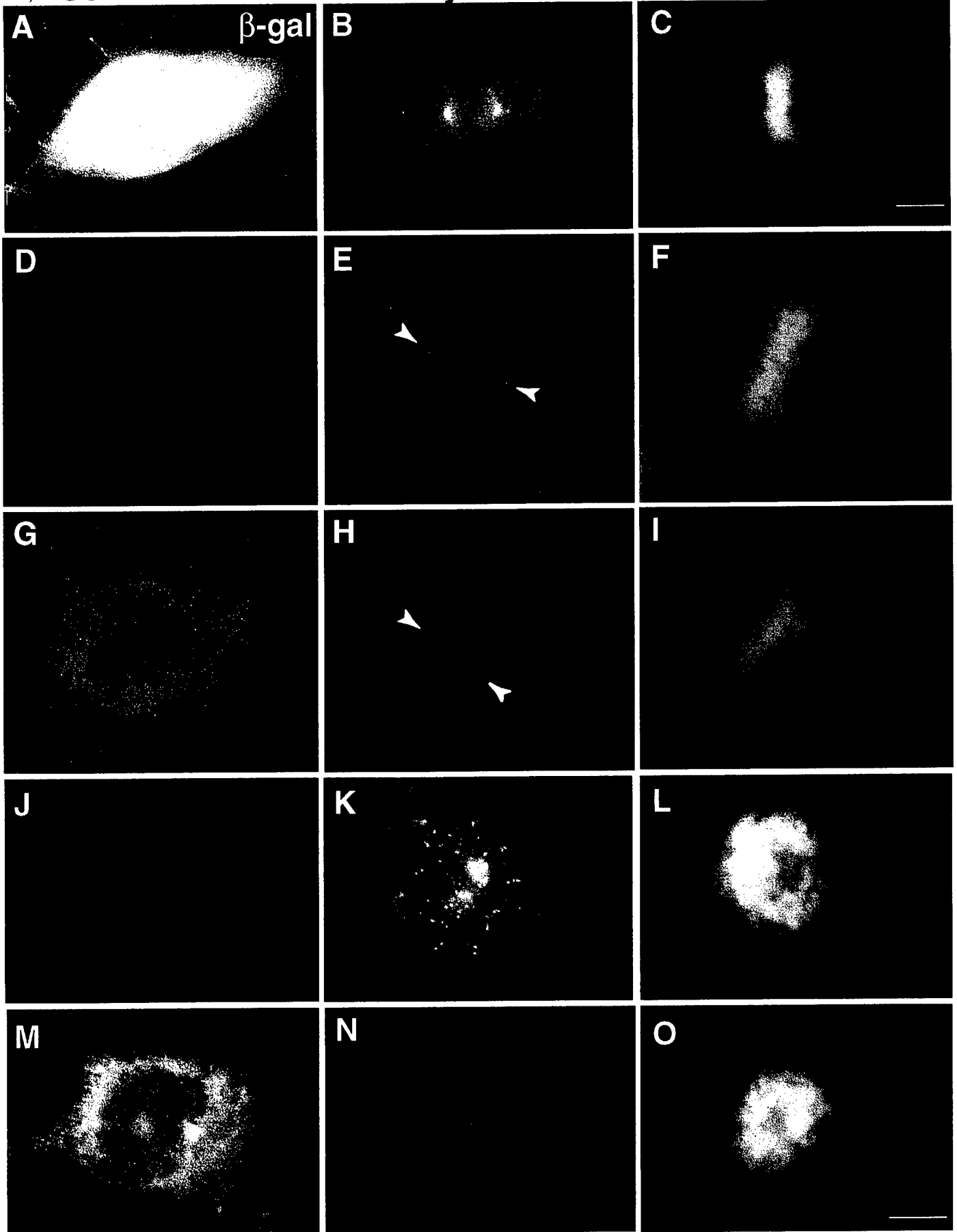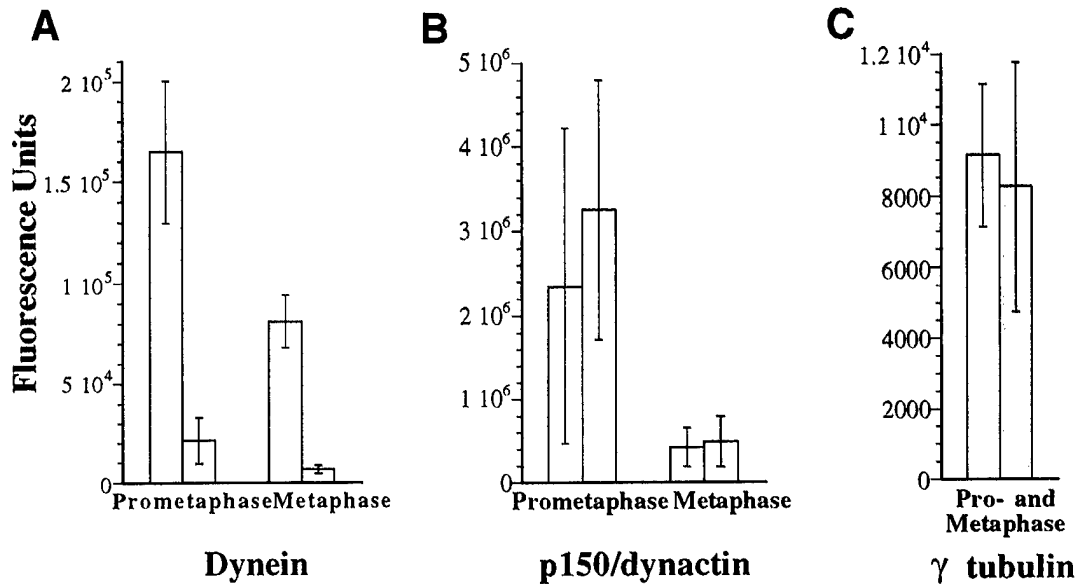
Figure 8

Figure 9

Figure 10

Frog melanophores

COS Cells

HA-pericentrin