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13. ABSTRACT (Maximum 200 Words)

Breast tumors are aneuploid and contain high pericentrin levels. The ABC kinases, known individually as protein kinases A, B (akt), and C have each been linked to breast carcinogenesis. Pericentrin has binding sites for ABC kinases and may be a scaffold for their centrosome localization. Since pericentrin expression in vitro causes aneuploidy, it's intriguing to suggest that pericentrin is an oncogene whose oncogenic potential hinges upon its association with one or more ABC kinases. To address this, we asked if endogenous pericentrin binds ABC kinases in-vivo, and whether disruption of pericentrin-kinase interactions would cause aneuploidy. We found that pericentrin binds all three kinases and that disruption of PKC and PKA from the centrosome causes aneupoidy as a result of cytokinetic failure and microtubule disorganization, consistent with these kinases playing a role in tumorigenesis. Overexpression of Akt also results in the production of binucleate cells. Additionally, we discovered active IKK complex—also linked to breast carcinogenesis, at the centrosome and found that it functions in centrosome duplication and cytokinesis (aneuploidy). Perhaps most surprisingly, we have identified a centrosome "damage" G1 checkpoint that is dependent upon intact p53 and p38 signaling. Each of these discoveries has important implications for breast tumor biology.

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Introduction

A feature common to virtually all breast tumors, and cancer in general, is genetic instability, also known as aneuploidy. Aneuploidy is tightly linked to advanced disease states [1, 2] and is the state of an altered chromosome number, while chromosomal instability (CIN) is the rate of change [3]. The molecular mechanisms that give rise to aneuploidy are unknown, however, models for CIN implicate mitotic malfunction, namely, errors in chromosome segregation during anaphase and cytokinesis—which immediately suggest centrosome abnormalities. In fact, our lab and others have reported that centrosome defects in both number and size accompany breast carcinogenesis, and most solid tumors [4-6].

The centrosome consists of two barrel-shaped centrioles arranged perpendicular to one another surrounded by the pericentriolar matrix (PCM). In most cells the centrosome is located at the cell center where it serves to nucleate and organize polarized microtubule arrays and is therefore responsible for meiotic and mitotic spindles. Following cytokinesis, a normal diploid cell inherits one centrosome that replicates during S phase, separates at G2/M, and is found at the spindle poles during mitosis, for reviews see [7-9]. Recent studies have demonstrated that the centrosome is no longer thought of as "just" an organizer of microtubules, but has functions at cell checkpoints, in cell proliferation, and at cytokinesis. Failure in any one of these centrosome functions could contribute to genetic instability, therefore paving the road for carcinogenesis [4, 10, 11]. For example, the presence of more than two centrosomes at mitosis—the result of either cytokinetic failure or centrosome overduplication will almost invariably result in chromosome missegregation and aneuploidy due to the formation of multipolar spindles. However, it is still unclear if genetic alterations precipitate centrosome defects, or if the opposite is true. Recent data from our lab suggests the presence of centrosome defects in pre-cancerous lesions and lends support for the latter hypothesis [5].

There are numerous studies reporting that increased levels of cyclic AMP-dependent kinase (PKA), Akt/PKB, and protein kinase C (PKC), known collectively as the ABC kinases, are found in breast tumors and predict a worse outcome for patients [12-14]. Despite sparse data on how these kinases may relate to tumor biology, there are ongoing trials testing the efficacy of broad-spectrum kinase inhibitors in patients [15-17]. However, the key to unraveling how these kinases may function in tumorigenesis is to take a reductionist approach. We have discovered that the centrosome protein pericentrin serves as a scaffold for ABC kinases PKA, PKC, and Akt *in vivo* (add refs)[18, 19].

Pericentrin was discovered in the Doxsey lab where it was found to be overexpressed in breast and others tumors including pre-invasive carcinomas of the breast [5]. Ectopic expression of pericentrin in cell lines triggers genetic instability and anchorage-independent growth, two hallmarks of tumorigenesis. It is intriguing to speculate that pericentrins tumor-promoting effects may reside in its ability to anchor ABC kinases to the centrosome bringing them into close proximity with their substrate(s). Identifying the function of localizing ABC kinases to the centrosome may be the key to understanding the mechanism by which these kinases contribute to tumorigenesis.

Body

To date, the training I have received in the Doxsey lab has been excellent. Not only have I become proficient in cutting-edge techniques such as the use of small-interfering RNAs (siRNAs) to affect gene expression and microinjections into living cells, but I have learned to focus on those aspects of basic science which may have the greatest impact upon human disease. Our collaborations with Dr. Dario Altieri (our new Cancer Center Director) and Dr. German Pihan (an oncologist and long-time collaborator) continually bridge the gap between basic and clinical research. In addition, we have established productive collaborations with Dr Dirk Dobbelaere (Bern, Switzerland) to investigate the

function of centrosomal IKK localization and with Dr. Philipp Kaldis (NCI) to define the role of cyclin-dependent kinase in centrosome damage induced G1 arrest. I attribute much of the success I have had in the lab to the guidance received and collegiality fostered in the Doxsey lab.

The Umass Medical Center has proven to be an outstanding training facility. The faculty and staff are both diverse and expert, therefore advice or technical assistance is usually available on site. Seminar series offered by the Cancer Center and other departments continually bring leaders in the fields of cancer diagnosis, treatment, and biology to discuss their latest research. This week Dr. Gerald Elfenbein (Roger Williams Medical Center) discussed the use of high dose chemotherapy and autologous stem cell rescue in the management of solid tumors and Dr. Peter Sorger (MIT) was here to talk about chromosome segregation and genomic instability. In addition to attending departmental seminars I have been participating in a weekly journal club dedicated to questions of cell motility, and therefore relevant to metastasis. In the coming year I plan to audit a course dedicated to breast tumor biology, which is offered by the school of medicine and targeted to training medical students.

I had the opportunity of attending the 2003 Annual Meeting of the American Society for Cell Biology (ASCB), where I presented a poster examining the role of centrosome-associated kinases in centrosome duplication. In addition, my abstract detailing a centrosome-damage triggered G1 checkpoint was chosen for presentation at the Organelle Maintenance and Inheritance minisymposium. This was an excellent forum for getting feedback on my work. I also attended numerous cancer-related symposia on topics such as cell cycle regulation, ECM and cancer, cell division, signaling and cancer, and cell motility. Participation in this meeting has bolstered the depth and breadth of my knowledge of tumorigenesis and has provided me with valuable insight I will need to ask critical questions fundamental to human disease.

Unexpected difficulties

We have narrowed pericentrins PKC binding site to residues 494-593 and have determined that the pericentrin-PKC interaction is mediated by PKC BIIs C1A domain. However, we have not identified pericentrin point mutations that fail to bind PKC. Instead, we have shown that expression of the 100 amino acid pericentrin interaction domain completely releases PKC BII from the centrosome [19]. We are planning to make stable cell lines expressing peri494-593 to test their tumorigenic properties. This disrupting fragment will likely be more useful than a pericentrin point mutant since endogenous, rather than overexpressed pericentrin, will be present.

Deviations from the original statement of work

The goal of this project is to understand the relationship between pericentrin and breast carcinogenesis and I have not ventured from this course. Years 1-3 were to be spent examining the pericentrin-PKA/PKC/PKB interactions, respectively. However, three key discoveries have impelled me to work on PKA/PKB in collaboration with Aruna Purohit. However, I will continue the course laid out in my statement of work, with the changes detailed below.

First, I decided to examine pericentrin-PKC interactions in year one since, in the time elapsed between submission of this grant and its funding, my colleague made excellent progress elucidating the functional role of pericentrin-anchored PKC betaII [19]. I was only peripherally involved in the project at this time and judged co-authorship to be unwarranted. This report demonstrates, for the first time, that pericentrin-anchored PKC is essential for normal microtubule organization and cytokinesis. Therefore, disruption of the PKC-pericentrin interaction is likely to exacerbate genetic instability incurred through overexpression of pericentrin.

Second, the novel and unexpected finding that active IKK localizes to the centrosome led us to focus our efforts here since IKK is intimately involved with PKB/Akt and is linked to breast tumorigenesis [20, 21]. This has resulted in a paper that we are currently preparing for submission to *Nature Cell Biology.*

Third, in pursuing the IKK centrosome link, as well as pericentrin overexpression, we discovered that there is a p53-dependent G1 checkpoint that is triggered upon centrosome damage. This exciting finding has led to a patent application, as well a paper currently being prepared for submission to *Nature.*

Key Research Accomplishments

We found that overexpression of Ht31, a peptide that blocks AKAP binding sites without affecting PKA activity, displaces PKA from the centrosome (Fig. 1-2). Ht31-GFP was expressed in Hek293 cells to determine if displacement of PKA from the centrosome would have any phenotype. Hek293 were used because of they are amenable to experimental manipulation and serve as a surrogate for follow-up studies in breast cell lines. Real-time analysis of transfected cells enabled us to determine that PKA disruption from the centrosome results in a 4-fold delay in the transition from anaphase to cytokinesis (Fig. 1-2). Peak PKA disruption occurs approximately 20h after transfection and correlates with a 4-fold increase in cytokinetic failure (Fig. 3). This finding demonstrates a requirement for centrosome-localized PKA activity in normal centrosome function. Construction of cell lines permanently expressing Ht31 may be a useful tool for differentiating

We also tested if PKB overexpression would cause aneuploidy. This is especially important since the interaction data that we have came from studies where pericentrin and PKB were both overexpressed. Since ectopic expression of PKB causes apoptosis we performed our experiments in the presence of an apoptotic inhibitor. Using this strategy we found that overexpression of PKB caused a 6-fold increase in cytokinesis failure (Fig. 4). This is the first report that overexpression of PKB causes aneuploidy and lends support to the hypothesis that PKB may have a function at the centrosome.

IKK is directly linked to breast cancer [21]. IKK has been found to promote tumorigenesis by constituitively activating cell survival signaling and also by making tumors resistant to therapy. IKK complex consists of IKK $\alpha/\beta/\gamma$ which phosphorylates IkB, an inhibitor of NF-kB. NF-kB is thought to be important for tumor necrosis factor-induced apoptosis (add ref). We discovered not only that active IKK is present at the centrosome (Fig. 5), but that siRNA-mediated depletion of IKK components from the centrosome demonstrated that IKK functions in centrosome duplication (Fig. 6) and cytokinetic failure (Fig. 7). It is therefore very likely that centrosome-associated IKK will have a functional role in tumorigenesis. Future studies will be directed towards answering this question.

The advent of siRNA-mediated knockdown of gene expression has made it possible to probe the function of almost any gene. Using this approach we targeted pericentrin, in order to determine if there was any link between pericentrin and the cell cycle, as had been previously postulated. Astonishingly, we found that targeting pericentrin, IKK, and other centrosome genes resulted in G1 arrest (Fig. 8). We went on to discover that this arrest is dependent upon intact p53 (Fig. 9-10).Our results are consistent with a "centrosome checkpoint" operating in G1 whereby an intact fully functional centrosome is required for cell cycle progression. A centrosome checkpoint could function as a guard against genomic instability by preventing cells with compromised centrosomes from entering the cell cycle.

Reportable Outcomes

• Poster presentation 2003, "Centrosome Genes Involoved in Centriole Replication" K. Mikule, C. Bullock, S. J. Doxsey, *43rd annual meeting of the ASCB, San Francisco, CA.*

• Minisymposia presentation 2003, "siRNA-mediated centrosome damage activates a G1 checkpoint", K. Mikule, A. Jurczyk, A. Gromley, S. J. Doxsey, *43rd annual meeting of the ASCB, San Francisco, CA.*

• Patent pending, "A novel cell cycle checkpoint under the control of the centrosome", Inventors: Stephen Doxsey and Keith Mikule.

• Manuscript, "A role for IKK in centrosome duplication and the regulation of mitosis", Sven Rottenberg, Keith Mikule, Rebekka Schwab, Volker T. Heussler, Paula C. Fernandez, Stephen Doxsey, and Dirk A. E. Dobbelaere, in preparation.

• Manuscript, "Centrosome insult activates a p53-dependent G1 checkpoint", Keith Mikule, A. Jurczyk, A. Gromley, and S. J. Doxsey, in preparation.

• Invited review, "Centrosomes and the Cell Cycle", Keith Mikule and Stephen Doxsey, *Trends in Cell Biology,* in preparation.

• Cell lines have been established for imaging living cells for centrosome behavior and chromosome segregation including RPE1-GFP-histone2B, RPE1-GFP-centrin2, RPE1-GFP-EB1, and RPE-GFP-α tubulin, breast lines are currently being made.

Conclusions:

This year has been an exciting one. This excitement stems from novel and significant discoveries that have lead the establishment of a role for ABC kinases in aneuplody, the identification and functional characterization of a new centrosome-associated signaling complex (IKK), and the identification of a new "centrosome-centric" G1 checkpoint. These findings have the potential to significantly impact our understanding of breast tumorigenesis with respect to the role centrosomes may play in this disease process. Ultimately, our discoveries may provide new targets for chemotherapeutic intervention in the treatment of breast cancer.

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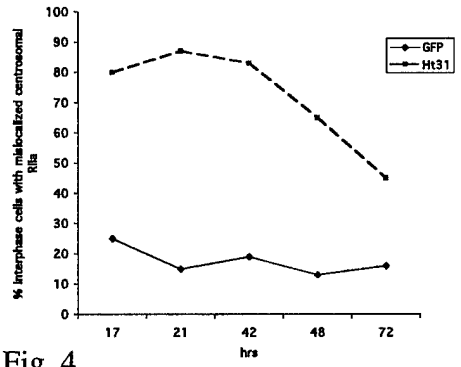
Fig. 1



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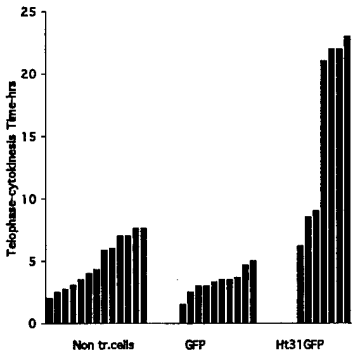

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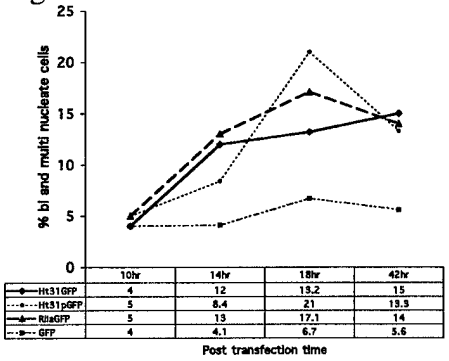

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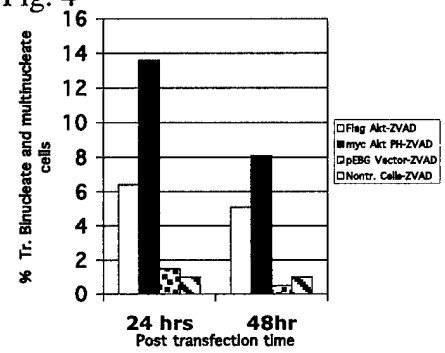
Fig. 4



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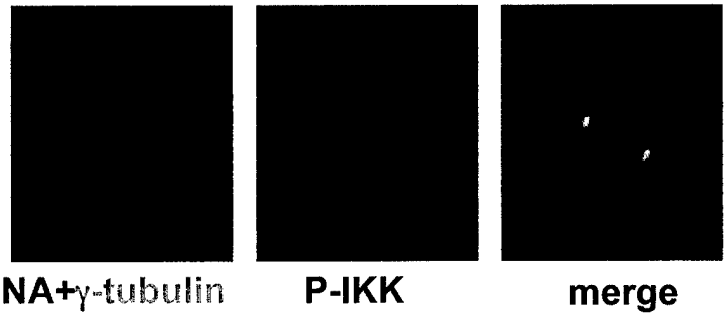

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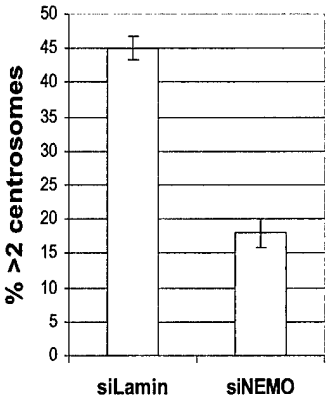

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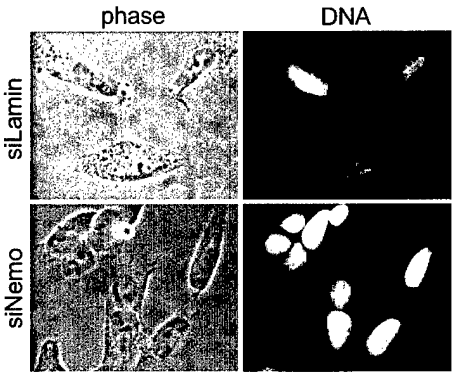

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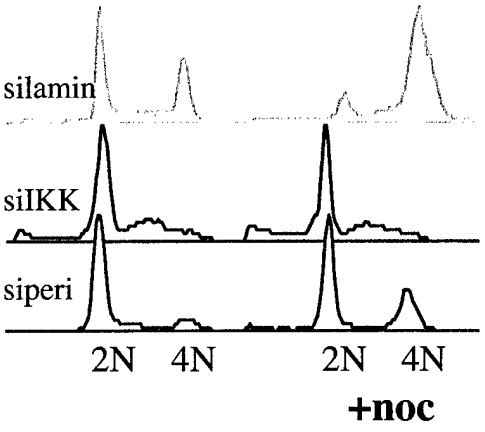

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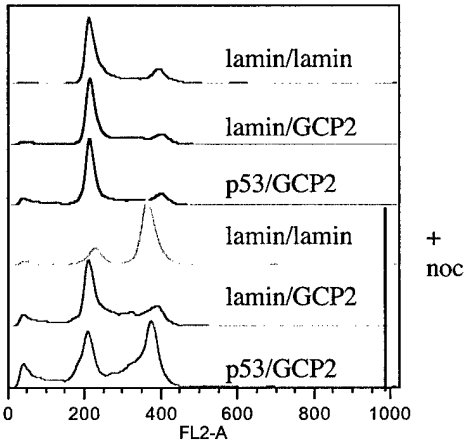

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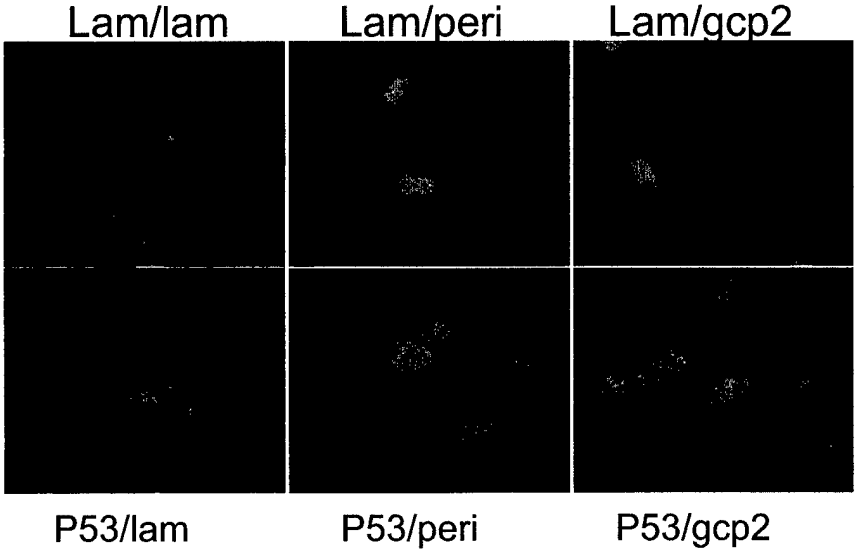

Legends

Figure 1 Quantitation centrosome-mislocalized PKA RIIa in cells expressing Ht31 and GFP (control), note peak of RIIa disruption occurs at 20h post transfection. Pericentrin localization is unaffected in Ht31 expressing cells (not shown). Cells were fixed in methanol and stained with antibodies to RIIa and pericentrin and processed for indirect immunofluorescence.

Figure 2 Quantitation of the telophase to cytokinesis transition interval in cells ectopically expressing either Ht31 or GFP (control), each bar represents a measurement taken from an individual cell. Non-transfected cells are shown for comparison and served as internal controls. Measurements come from real-time imaging of live cells.

Figure 3 Quantitation of multinucleate formation in cells expressing indicated transgene over time. Note that the peak of multinucleation corresponds to the peak of RIIa centrosome disruption (Fig. 1). Cells were methanol-fixed, stained with DAPI to visualize DNA, and GFP expressing cells were scored for the presence of extra nuclei.

Figure 4 Ectopic expression of Akt or the pericentrin interacting domain of Akt (Akt-PH) causes binucleate cell formation. ZVAD, an inhibitor of apoptosis, was used to block apoptosis induced by overexpression of Akt.

Figure 5 P-IKK (active) localizes to centrosomes during mitosis. Cells were fixed in methanol and stained with antibodies against gamma tubulin and P-IKK and processed for indirect immunofluorescence. Note colocalization of P-IKK and gamma tubulin.

Figure 6 Knockdown of NEMO (an IKK complex component) via siRNA inhibited centrosome duplication. Hydroxyurea-treated U2OS cells were methanol fixed after 72 h of siRNA treatment, processed for indirect immunofluoresce to reveal centrosomes, and scored by microscopy. Results are from three independent experiments +/- standard deviation.

Figure 7 siRNA induced knockdown of NEMO causes formation of multinucleate cells (aneuploidy). Cells were treated with the indicated siRNAs for 48h before fixation and immunfluorescent analysis of multinucleation.

Figure 8 IKK and pericentrin knockdown induces G1 arrest. After siRNA induced knockdown, cells were fixed and processed for FACS analysis of DNA content. Nocodazol treatment (12h) activates the spindle checkpoint and results in G2 arrest with a 4N DNA content in cells that are cycling. G1-arrested cells fail accumulate a G2 peak. Note a greater than 70% of siIKK and siPeri treated cells arrest in G1.

Figure 9 p53 knockdown prevents G1 arrest caused by centrosome knockdown. Prior to knockdown of GCP2 (a cenrosome component), cells were treated with either silamin (control) or sip53. Cells were then grown for 72 h before challenge with nocodazol and FACS analysis to determine the cell cycle position. Prior loss of p53 prevented cells from becoming arrested in G1.

Figure 10 Brdu analysis indicates cells are arrested in G1 and that arrest is p53 dependent. Arrested cells fail to incorporate Brdu. Following the knockdown of indicated gene, cells were grown in the presence of Brdu for 24 hours. 80% of arrested cells **failed** to incorporate Brdu. Note that cells knocked down for p53 failed to arrest following centrosome gene knockdown.