Room 14-0551
77 Massachusetts Avenue
Cambridge, MA 02139
Ph: 617.253.5668 Fax: 617.253.1690
Email: docs@mit.edu
http://libraries.mit.edu/docs

DISCLAIMER OF QUALITY

Due to the condition of the original material, there are unavoidable flaws in this reproduction. We have made every effort possible to provide you with the best copy available. If you are dissatisfied with this product and find it unusable, please contact Document Services as soon as possible.

Thank you.

Some pages in the original document contain color pictures or graphics that will not scan or reproduce well.

Investigation of the Role of Chromosome Missegregation in Embryo Development and in Tumorigenesis

by

Aurora A. Burds

B.A. Biology and French
Grinnell College, 1996

Submitted to the Department of Biology in Partial Fulfillment of the Requirements for the Degree of

Doctor of Philosophy in Biology

at the
Massachusetts Institute of Technology

© 2002 Massachusetts Institute of Technology. All rights reserved.

[June 2002]

Signature of Author: ______________________________.
Department of Biology
May 22, 2002

Certified by: ______________________________.
Peter K. Sorger
Associate Professor of Biology
Thesis Supervisor

Accepted by: ______________________________.
Alan Grossman
Professor of Biology
Co-Chairperson, Committee for Graduate Students

MASSACHUSETTS INSTITUTE OF TECHNOLOGY
MAY 2 8 2002
LIBRARIES

ARCHIVES

Investigation of the Role of Chromosome Missegregation in Embryo Development and in Tumorigenesis

by

Aurora A. Burds

Submitted to the Department of Biology at the Massachusetts Institute of Technology in Partial Fulfillment of the Requirements for the Degree of

Doctor of Philosophy in Biology

Abstract

The separation and partitioning of sister chromatids prior to cytokinesis must be tightly regulated to insure that progeny cells receive the correct number of chromosomes. The spindle assembly checkpoint prevents chromosome missegregation by arresting the cell before sister chromatid separation if any one chromatid is not properly attached to the spindle microtubules. Defects in chromosome segregation lead to the creation of aneuploid cells that contain too much or too little genetic material. Some aneuploid cells have gained oncogenes or lost tumor suppressor genes, and these genetic alterations allow the cells to ignore signals that regulate growth and proliferation. A majority of tumors are aneuploid, but it is not yet known whether chromosome instability is a driving force or merely a secondary effect of tumor development. In Chapter 2, I will present work showing that the protein most often altered in colon cancers, Adenomatous Polyposis Coli (AdPC) (Kinzler and Vogelstein 1996), is modified by members of the spindle assembly checkpoint and that a truncating mutation of the AdPC protein leads directly to aneuploidy in mouse cells. Although genetic disruption of any member of the spindle assembly checkpoint signaling cascade has proven lethal for metazoans on both the cellular and organismal level (Basu et al. 1999; Kitagawa and Rose 1999; Dobles et al. 2000; Kalitsis et al. 2000), Chapter 3 will detail the creation and analysis of a Mad2-/- mouse embryonic fibroblast cell line that is viable due to a simultaneous disruption in p53. Together these two bodies of research support the possibility that a chromosome missegregation event can be a defining, early step in tumorigenesis while providing unique tools to investigate this possibility.

Thesis Supervisor: Peter K. Sorger
Title: Associate Professor of Biology

Aurora A. Burds

Education

1996-2002	Massachusetts Institute of Technology, Cambridge, MA Ph.D. June 2002. Department of Biology Thesis: Investigation of the Role of Chromosome Missegregation in Embryo Development and in Tumorigenesis
1992-1996	Grinnell College, Grinnell, IA B.A. in Biology and French. June 1996

Research Experience

1997-2002	Massachusetts Institute of Technology Graduate Student Principle Investigator: Peter Sorger
1995-1996	University of Iowa Hospitals and Clinics Research Technician Principle Investigator: Jeff Murray
1993-1996	Grinnell College Research Assistant Principle Investigator: Leslie Gregg-Jolly
1991	Iowa State University Research Intern Principle Investigator: Peggy Miller-Graber

Publications

Kaplan KB, Burds AA, Swedlow JR, Bekir SS, Sorger PK, Nathke IS. A role for the Adenomatous Polyposis Coli protein in chromosome segregation. Nat Cell Biol. 2001 Apr;3(4):429-32.

Lidral AC, Romitti PA, Basart AM, Doetschman T, Leysens NJ, Daack-Hirsch S, Semina EV, Johnson LR, Machida J, Burds AA, Parnell TJ, Rubenstein JL, Murray JC. Association of MSX1 and TGFB3 with nonsyndromic clefting in humans. Am J Hum Genet. 1998 Aug;63(2):557-68.

Rauch PJ, Palmen R, Burds AA, Gregg-Jolly LA, van der Zee JR, Hellingwerf KJ. The expression of the Acinetobacter calcoaceticus recA gene increases in response to DNA damage independently of RecA and of development of competence for natural transformation. Microbiology. 1996 Apr;142 (Pt 4):1025-32.

Acknowledgements

First, I would like to thank Peter Sorger for your enthusiasm, guidance and support. You had faith in me and in my work even when I was frustrated and confused. Thank you for creating an environment of exciting and stimulating research and for allowing me to be a part of it. If I had to do it all again, I'd sign up to do a project in your lab in an instant.

Thank you to all current and past members of my thesis committee for your assistance with my research and thank you to the present members for your participation in my thesis defense. Thank you to Alan Grossman, Sylvia Sanders, Angelika Amon, Frank Gertler, Graham Walker and David Pellman for your advice in discovering and presenting this exciting body of work. Thank you to Tyler Jacks for your advice and encouragement as an advisor and as a friend and for the use of your microscope for embryo histology.

Thank you very much to my parents for whispering in to my crib to encourage me to go to college and do something that I would love for my whole life. Thank you also for always encouraging me to ask questions, to explore the world and to cherish the wonder of the things around me.

A huge thanks to all past and present members of the Sorger Lab for all of your intellectual and personal support over the years. I could not have chosen a more wonderful, intelligent and fun group of scientists and friends to undertake this journey with. Thank you to Max Dobles for making the Mad2 knockout mouse and training me in the way of the mouse and to Ken Kaplan for the training in microscopy and biochemistry. Thank you especially to Michelle Beaucher and Jessica Tytell for putting up with my music and being all around great bay mates. I am endlessly indebted to those of you who have read and edited my thesis: Andrew McAinsh, Jessica Tytell, Stephanie Xie, Rob Hagan, Brian Hughes, Annegret Schulze-Lutum, and especially Peter Sorger. You have helped to bring order out of the chaos and I could not have done it without you.

Thank you to all of the people in the department of biology who have created and fostered an amazing learning environment. Thank you to Alicia Caron, Roderick Bronson, Mandy Tam and members of the Jacks lab for assistance with embryo histology.

Thank you to Erica, Carrie, Dina, Barb, Janet, Liz, Mitch, Dianne, Patrick, Adrian and all my classmates in Bio96 for being the best friends I have ever had.

And finally, thank you to Chris. For being patient and supportive and loving when I was stressed. For encouraging me when things were not working and for sharing my excitement when they were. For listening attentively while I blabbered on and on about chromosome missegregation and for taking care of me when I was so involved with the experiments that I forgot to take care of myself. I love you.

Table Of Contents

Chapter 1 – 7
Introduction: Cell cycle, checkpoints and tumorigenesis
The Cell Cycle 9
G_1, S and G_2 10
Mitosis 13
Cell Cycle Checkpoints 23
Discovery of Checkpoints 24
DNA-damage Checkpoint 26
Apoptosis 27
Spindle Assembly Checkpoint 28
Checkpoint mutations lead to aneuploidy and cancer 39
Mutations in DNA-damage Checkpoint are Tumorigenic 39
Loss of p53 Results in Aneuploidy 41
Mouse Models of Cancer 42
Mutations in the Spindle Assembly Checkpoint 42
Human Anneuploidy 44
Conclusion 46
Bibliography 47

Chapter 2 – 71
The Adenomatous Polyposis Coli Tumor Suppressor Protein Interacts with the Mitotic Checkpoint Kinases, Bub1 and BubR1, and Plays a Role in Chromosome Segregation
Abstract 74
Introduction 75
Results 78
Discussion 98
Experimental Procedures 100
Bibliography 106

Chapter 3 – 113
The Spindle Assembly Checkpoint is Not Essential for the Viability of Mouse Cells
Abstract 114
Introduction 115
Results 118
Discussion 144
Experimental Procedures 147
Bibliography 153

Chapter 4 – 157
Conclusions and Future Directions

List of Figures

Figure 2.1 87
AdPC is localized to kinetochores in mitotic cells.

Figure 2.2 89
AdPC association with kinetochores requires microtubules.

Figure 2.3 91
Bub kinases specifically phosphorylate AdPC.

Figure 2.4 93
Identification of a Bub1-Bub3-AdPC complex.

Figure 2.5 95
Analyzing the role of AdPC in the spindle assembly checkpoint.

Figure 2.6 97
Cells lacking functional AdPC protein undergo aberrant mitoses.

Figure 3.1 129
The gene dosage of p53 affects the post-gastrulation survival of $Mad2^{-/-}$ embryos.

Figure 3.2 131
A disruption in p53 rescues lethality of Mad2 cultured blastocysts.

Figure 3.3 133
Viable $Mad2^{-/-}$ embryos contain increased levels of apopotic cells.

Figure 3.4 135
Analysis of chromosome missegregation in cells of a $Mad2^{-/-}$ embryo.

Figure 3.5 137
$Mad2^{-/-}$ $p53^{+/-}$ and $Mad2^{-/-}$ $p53^{-/-}$ embryos show developmental retardation.

Figure 3.6 139
Mitotic arrest of cycling mouse embryonic fibroblasts requires Mad2.

Figure 3.7 141
Reduction of Mad2 gene dosage results in chromosome instability.

Figure 3.8 143
Mad2 gene dosage is inversely proportional to the appearance of defective chromosome segregation in $p53^{-/-}$ MEFs.

Chapter 1

Introduction

Cell cycle, checkpoints and tumorigenesis

Introduction Outline

I. The Cell Cycle
    A. G_1, S and G_2
    B. Mitosis
        1. Formation of a Metaphase Spindle
        2. Biochemistry of Dynamic Microtubules
        3. Role of Spindle Dynamics in Mitosis
        4. Components of the Kinetochore
            a. Yeast Kinetochore Proteins
            b. Metazoan Kinetochore Proteins
        5. The Anaphase Promoting Complex Regulates Destruction of Cohesions
        6. Exit from Mitosis

II. Cell Cycle Checkpoints
    A. Discovery of Checkpoints
    B. DNA-damage Checkpoint
    C. Apoptosis
    D. Spindle Assembly Checkpoint
        1. The Kinetochore is Essential for Delay
        2. Signal Transduction Pathway
            a. Discovery of Genes in Yeast
            b. Biochemical Analysis of Components in Yeast
            c. Homologues in Higher Eukaryotes
        3. Attachment vs. Tension
        4. BubR1 and Mad2 Both Inhibit the AnaPC
    E. Summary

III. Checkpoint mutations lead to aneuploidy and cancer
    A. Mutations in DNA-damage Checkpoint are Tumorigenic
    B. Mouse Models of Cancer
    C. Mutations in the Spindle Assembly Checkpoint
    D. Human Anneuploidy
        1. Syndromes
        2. Tumors
    E. Loss of p53 Results in Aneuploidy

IV. Conclusion

I. The Cell Cycle

The cell cycle is a series of biochemical processes that must be successfully completed in a specific order for cell duplication. This includes the accumulation of nutrients for growth in G1, the replication of the genome during S phase, a second growth stage called G2, and chromosome segregation followed by cytokinesis during mitosis. Progression from one stage of the cell cycle to the next requires active cyclin/cyclin-dependent kinase (CDK) complexes. The levels of CDKs remain steady throughout the cell cycle, and their activity is regulated positively by cyclins and negatively by CDK inhibitors (Felix et al. 1989). In budding yeast, the G1 cyclins, Cln1p, Cln2p and Cln3p, bind to and activate the CDK Cdc28p (Cdc2p in fission yeast). Cdc28p also associates with the S phase cyclins, Clb5p and Clb6p, and then with cyclins Clb1p, Clb2p, Clb3p and Clb4p for progression through mitosis (Cross 1995). Higher eukaryotes contain functional homologues of the yeast cyclins that bind to CDKs at similar stages of the cell cycle: cyclins D and E are found during G1, cyclins E and A during S phase, and cyclins A and B during mitosis (Reviewed in Obaya and Sedivy 2002). A cell progresses through this coupled cyclin/cell cycle to become two, genetically identical daughter cells. In this section I discuss the phases of the eukaryotic cell cycle, focusing particularly on the mechanics of chromosome segregation during mitosis.

A. G_1, S and G_2

Before committing to division, cells monitor the internal and external environment and prepare for DNA duplication. If any basic nutrients, such as carbon, nitrogen, oxygen or salts, are in low abundance, cells cannot grow and they remain arrested prior to the commitment of division. *S. cerevisae* grown in minimal medium can spend many hours in G1, accumulating enough nutrients to duplicate, but cells in rich media complete one round of the cell cycle in 90 minutes. Yeast begin the cell cycle by passing through an event called Start, a molecular switch that results in irreversible commitment to cell duplication (Reviewed in Toone et al. 1997). Transcription factors, Swi4p, Swi6p and Mbp1p, stimulate the expression of many genes, including the genes that encode the two G1 cyclins, Cln1p and Cln2p (Siegmund and Nasmyth 1996). The cyclin/CDK complexes accumulate, and the Swi6p/Mbp1p complex also activates transcription of genes that encode proteins necessary for DNA replication in S phase: ribonucleotide reductase, dihydrofolate reductase, thymidine kinase, DNA primase, and DNA polymerase (Noguchi et al. 1983; Tubo and Berezney 1987; Johnston and Lowndes 1992). These proteins join with others, including the origin replication complex, to form pre-replicative complexes at discrete loci, called origins of replication, along the chromosomes. Cells are now competent to initiate DNA synthesis in S phase.

A similar set of events occurs in the G1 cells of multicellular organisms, though most cultured mammalian cells require 24 hours to complete one round of the cell cycle. Because a cell in a multicellular organism is surrounded by other cells, recognition of

adhesion to either the extracellular matrix or to other cells is also important. Mitogenic signals stimulate cell proliferation by activating signaling cascades that promote both the transcription of the D-type cyclins and the elimination of CDK inhibitors (Reviewed in Sherr and Roberts 1999). Cyclin D binds to and activates Cdk4 and Cdk6 to phosphorylate the retinoblastoma protein Rb, and other targets. As the cell progresses through G1, the cyclin E/Cdk2 complex also accumulates and hyperphosphorylates Rb. The hyperphosphorylation of Rb marks the passage through the Restriction Point in metazoans, an event that is analogous to Start and commits a cell to division (Lundberg and Weinberg 1999). Hyperphosphorylated Rb releases the E2F family of transcription factors, allowing E2Fs to upregulate the transcription of genes that are involved in DNA synthesis. As in yeast, the pre-replicative complexes form at the origins of replication and the genome is ready for the next phase of the cell cycle, S phase (Maiorano and Mechali 2002).

During the S phase of the cell cycle, the DNA and structural components of chromosomes must be replicated. In yeast, the origin recognition complex (ORC) binds to DNA replication origins, distorts the structure of an adjacent region of AT-rich DNA and recruits replication proteins, including polymerases, to the replication forks (Reviewed in Borowiec et al. 1990; Baker and Bell 1998). Autoradiography of pulse-labeled DNA spreads have shown that bi-directional DNA replication begins at multiple sites, activating groups of replication forks in batches at different times (Edenberg and Huberman 1975; Hand 1978). The cyclin A/Cdk2 complex localizes to the replication forks and is necessary for the initiation of DNA synthesis (Kaufmann et al. 2001). The

catalytic protein complex found at each of the forks contain several components that perform unique tasks. A helicase unwinds the original DNA strand to provide the other proteins access to the DNA. Polymerases synthesize the new, complementary strands of nucleic acids with the help of accessory proteins that maintain the interaction between polymerases and DNA. Finally, an editing polymerase checks the newly generated strands to ensure that they are exact matches of the originals (Reviewed in Baker and Bell 1998). CAF-1 and PCNA place histones onto the new strands of DNA, creating nucleosomes, and the ATP-dependent chromatin assembly factors restructure the DNA/histones to form chromatin (Krude and Keller 2001). Cohesins fasten the two copies of each chromosome together and this formation of sister chromatid pairs completes S phase.

In G2 the cell pauses in a cyclin-dependent manner to check for errors and to grow. *S. cerevisiae* does not have an obvious G2 phase in a normal round of cell division (Russell and Nurse 1986), but *S. pombe* utilizes the CDK inhibitor Wee1p to hold the cells in this pre-mitotic growth phase (Russell and Nurse 1987). Similarly, Wee1 and Myt1 inhibit cyclin B/Cdk1 to regulate the progression of mammalian cells (Mueller et al. 1995). A cell is driven into mitosis when the Polo-like kinases stimulate Cdc25, the antagonist of Wee1 and Myt1. As active cyclin/Cdc2p (*S. pombe*) and cyclin B/Cdk1 (metazoan) complexes are generated, the CDK creates a positive feedback loop by activating more Cdc25 and inhibiting Wee1/Myt1. This feedback loop maximizes the amount of active mitotic cyclin/CDK in the cell and thereby promotes the exit from G2 and entry into mitosis (Reviewed in O'Farrell 2001).

B. Mitosis

Once a cell has duplicated its genome, its entire contents must be physically partitioned into two daughter cells. Dividing cells accomplish this partitioning in a series of four mitotic stages: prophase, metaphase, anaphase and telophase. At each stage, distinct physical changes take place to ensure accurate transmission of the genome and division of the cell membrane. During prophase, the nuclear envelope breaks down (but not in yeast), replicated sister chromatids condense and a multi-protein complex known as a kinetochore begins to form specifically at each centromere. The paired sister chromatids connect to the newly formed spindle microtubules via kinetochores, attaching one chromatid to one pole and its sister chromatid to the opposite pole. Metaphase is defined as the stage at which all chromatid pairs have achieved bipolar attachment to the mitotic spindle and have moved to the midzone of the cell, which is also known as the metaphase plate. In anaphase A, the cohesin complexes connecting the sister chromatid pairs to each other are cleaved, allowing the chromosomes to separate and be pulled by spindle microtubules to opposite sides of the cell. In anaphase B, the spindle elongates, separating the clusters of chromosomes even further. Finally, during telophase, a cleavage furrow forms to separate the cell into two compartments, and a nuclear envelope forms around the decondensing chromosomes in each new cell.

The activity of the mitotic cyclin kinases is required for many of the key events in mitosis. In prophase, cyclin B/Cdc2 phosphorylates the condensin complex, causing chromosome condensation (Kimura et al. 1998). Likewise, nuclear envelope breakdown

at prophase is also a result of CDK activity (Mendenhall 1993), though both cyclin A and cyclin B are important for the destruction of the nuclear envelope (Peter et al. 1990; Furuno et al. 1999). Cyclin B/Cdc2 contributes to cell rounding at mitosis by phosphorylating the actin-binding protein caldesmon, and weakening the structure of the actin cytoskeleton (Yamashiro et al. 1990). Genetic analysis in *S. cerevisiae* has shown that the mitotic cyclins are also required for the establishment of the mitotic spindle (Surana et al. 1991; Fitch et al. 1992; Schwob and Nasmyth 1993). The Clb5p/Cdc28p complex is necessary for the binding of microtubules to the cell cortex to establish the correct spindle polarity, and Clb3p/Cdc28p and Clb4p/Cdc28p promote spindle assembly (Segal et al. 2000). Thus, the cyclin/CDK complexes contribute to the initiation and execution of the mitotic program in eukaryotic cells.

1. Formation of a Metaphase Spindle

After a cell completes DNA replication, the microtubules and microfilaments of the cytoskeleton disassemble, reorganizing the interphase cell into a spherical morphology. Next, a spindle of microtubule fibers self-assembles between newly separated centrosomes, known in yeast as spindle pole bodies, the microtubule organizing centers of the cell. Dynamic microtubules radiate in all directions from each centrosome, flung from the organizing focus much as a fly fisher casts and retracts his line, hoping to contact one of three desired “prey”: other microtubules, the cellular cortex or a sister chromatid. Some of the microtubules emanating from one centrosome encounter and interact with their counterparts from the other centrosome. The interpolar microtubules

establish the characteristic spindle shape and generate the midzone of the spindle. The interpolar microtubules elongate to separate the spindle pole bodies at anaphase B. Other microtubules, known as astral microtubules, extend away from the interpolar spindle to contact the nuclear membrane and anchor each centrosome to the cellular cortex. Finally, some of the microtubules contact and bind to kinetochores, multi-protein complexes that assemble at centromeric DNA. The microtubule-kinetochore attachment secures the sister chromatid to the spindle apparatus. Due to the undirected nature of the "fishing" by the microtubules during the establishment of the mitotic spindle, the capture of each sister chromatid by a spindle fiber is a random event, and mitosis in each individual cell is therefore a unique and stochastic process (Hyman and Karsenti 1996; Nicklas 1997).

2. Biochemistry of Dynamic Microtubules

The stochastic nature of the attachment of spindle microtubules to sister chromatid pairs is due in large part to the dynamic structure of the microtubules themselves. Microtubules are composed of thirteen strands of parallel protofilaments that form a single, hollow tube measuring 25 nm in diameter (Nogales 2000). Each of the protofilaments is a linear polymer of repeating alpha and beta tubulin heterodimers, assembling in a head to tail fashion that gives the microtubule an intrinsic polarity (Amos and Klug 1974). Spindle pole bodies are microtubule nucleating centers, which anchor and stabilize the (-) end of microtubule protofilaments, which exposes the alpha tubulin subunits and faces into the spindle pole (Fan et al. 1996). The beta tubulin subunit is exposed at the (+) end, which extends into the cell and transitions stochastically between

growth and catastrophe (Allen and Borisy 1974). The dynamic behavior of microtubules is characterized by four rates: the rates of polymerization and depolymerization and the frequencies of catastrophe (the switch from polymerization to depolymerization) and rescue (the switch from disassembly to assembly) (Mitchison and Kirschner 1984). Dynamic instability is caused by GTP hydrolysis and the changing concentrations of tubulin dimers at each end of the microtubule (Mitchison 1993). Only dimers that are bound to GTP can assemble onto a microtubule, but the GTP is hydrolyzed into GDP shortly after the dimer assembles into a protofilament. The structure of the GDP-bound tubulin dimer favors disassembly from the polymer, but if another GTP-bound dimer has already joined the polymer, sandwiching the GDP-bound dimer into the structure, the microtubule will remain stable and continue to grow. Thus, a cap of GTP-bound tubulin dimers stabilizes the filament and allows continuing assembly of GTP-tubulin subunits. On the other hand, if GTP hydrolysis occurs to the very end of the microtubule, the microtubule will switch from growing to shrinking and undergo a catastrophe (Mitchison and Kirschner 1984; Nogales 2000). Microtubules are dynamic because they are constantly alternating between growth and shrinkage, and this property of microtubules allows for the creation of a labile spindle structure.

3. Role of Spindle Dynamics in Mitosis

Many proteins alter microtubule dynamics in a cell cycle-dependent manner. As cells transition from interphase to mitosis, the catastrophe frequency increases ten-fold to break down the cytoskeletal microtubules (Mitchison et al. 1986; Belmont et al. 1990;

Verde et al. 1992). This controlled destruction of microtubules at mitosis may be mediated by Op18/Stathmin, a protein that sequesters tubulin (Belmont and Mitchison 1996; Howell et al. 1999), or by a microtubule motor, MCAK, that disrupts the interaction of protofilaments at the (+) end (Walczak et al. 1996; Maney et al. 2001). On the other hand, several microtubule associated proteins (MAPs) promote microtubule stability by increasing polymerization rates, suppressing catastrophe rates and promoting rescue of microtubules in interphase. Some MAPs are down regulated while others lose functional potency prior to mitosis (Pryer et al. 1992; Andersen 1998; Tournebize et al. 2000). As the cell increases the activity of proteins that destabilize microtubules and depresses the activity of those proteins that stabilize them, the cytoskeletal microtubules are destroyed; new spindle microtubules that emanate from the centrosome are very unstable and disassemble rapidly if they do not contact other proteins to stabilize them. The instability of the spindle microtubules allows them to effectively search the volume of the cell for target structures, such as the cell cortex and the kinetochores, which then stabilize the microtubule structure.

The goal of spindle assembly is the bipolar attachment of every pair of sister chromatids so that they can be segregated in anaphase. A series of live cell microscopy observations in higher eukaryotes has shown that most kinetochores initially contact a microtubule along the length of the fiber rather than at the (+) end. The kinetochore slides along the microtubule towards the centrosome (Rieder and Alexander 1990) where the kinetochore then encounters and attaches to the (+) ends of 20-30 other microtubules (Rieder et al. 1986; McEwen et al. 1997). It is believed that proteins in the kinetochore stabilize the

(+) ends of microtubules because bundles of microtubules that attach to a kinetochore rarely undergo catastrophe. As microtubules assemble, the attached chromatids are propelled towards the opposite spindle pole. Because the kinetochores are situated "back to back" on the sister chromatid pair, the unattached kinetochore is presented in an optimal orientation to engage and stabilize microtubules emanating from the other centrosome. Now that each kinetochore is connected to a centrosome, the balanced forces of both bundles of microtubules pushing and pulling on the joined chromatids bring the sister chromatid pair to the midzone of the spindle, an event called congression. As more and more sister chromatid pairs congress, a distinctive stripe or disk of DNA is observed between the spindle poles at the metaphase plate.

4. Components of the Kinetochore

Kinetochore proteins bind specifically at the centromeric sequences of sister chromatids and may function as microtubule stabilizers in addition to interacting with microtubule associated proteins to solidify the microtubule-DNA connection. Currently, the complete set of proteins that comprises this physical link between the centromeric DNA and the microtubules is not yet known, though a large number of them have been uncovered in yeast and in mammalian cells.

a. Yeast Kinetochore Proteins

In *S. cerevisiae*, the centromeric DNA sequence is a 125-base pair region that interacts with the multi-protein CBF3 complex, with a histone-H3 variant (Cse4p) (Stoler et al.

1995) and the centromere-binding factor Cbf1p (Baker et al. 1990). The assembly of the kinetochore begins with the binding of CBF3 to the centromere, and then many additional proteins build onto this foundation (Reviewed in Kitagawa and Hieter 2001). Among these proteins are complexes containing Spc24p, Spc25p, Ndc80p and Nuf2p as well as complexes of Slk19p, Ctf19p and Mcm21p (He et al. 2001). Other proteins and complexes display the ability to bind microtubules directly, and they have been placed on the outer layer of the yeast kinetochore. Several motors as well as other microtubule associated proteins have been found at kinetochores, including a Dam1p-Dad1p-Duo1p complex that contains at least seven kinetochore proteins (Hofmann et al. 1998; Janke et al. 2002). Although it is not yet know what function the motors have in chromosome segregation, the non-motor proteins appear to link the centromeres to the spindle while the microtubule fiber alternates between periods of growth and shrinkage.

b. Metazoan Kinetochore Proteins

Centromeres in eukaryotes other than budding yeast display much greater variation and complexity, ranging in size from 40–100 kb in the fission yeast *S. pombe* to several megabases in humans. These sequences are comprised of a series of repeated DNA elements (Reviewed in Wiens and Sorger 1998). While some kinetochore proteins show sequence conservation across all levels of the eukaryotic spectrum, most of the components of the mammalian kinetochore have been discovered experimentally. People with the CREST (Calcinosis, Raynaud's phenomenon, Esophageal dysmotility, Sclerodactyly, Telangiectasia) variant of the autoimmune disease scleroderma produce antibodies to centromere associated proteins (CENPs). The use of these anti- centromere

antibodies has facilitated the discovery of three kinetochore proteins, CENP-A, -B and –C, which associate with the centromere throughout the entire cell cycle (Brenner et al. 1981). CENP-A is the homologue to the histone H3 variant budding yeast Cse4p, and is found in centromere-specific nucleosomes (Sullivan et al. 1994). CENP-B forms a homodimer that binds to alpha centromeric DNA (Muro et al. 1992; Kitagawa et al. 1995). CENP-C is the mammalian homologue of yeast Mif2p, and its possession of a non-sequence-specific DNA binding activity suggests it might stabilize the linkage between centromeric DNA and nucleosomes containing CENP-A (Lanini and McKeon 1995; Yang et al. 1996). A functional interaction between CENP-A and CENP-C is likely because a mutant version of CENP-A that is mistargeted to regions of non-centromeric DNA recruits several kinetochore proteins, including CENP-C (Van Hooser et al. 2001). CENP-A, CENP-B and CENP-C appear to be functioning as structural components of the kinetochore because they localize to centromeres throughout the cell cycle.

A larger group of proteins, including several motors and members of the spindle assembly checkpoint signaling cascade, are recruited to the kinetochore after DNA replication to play a more specific role in the segregation of chromosomes in mammalian cells. CENP-E is a kinesin-like motor that may transport the sister chromatid pair to the very tip of the microtubule upon attachment to the length of the fiber, aiding chromosome congression at metaphase (Yen et al. 1991; Wood et al. 1997). CENP-E is necessary for chromosome alignment and may stabilize the kinetochore-microtubule connection (Schaar et al. 1997). Another motor complex, dynein and dynactin, assembles at the

mitotic kinetochore and its (-) end directed locomotion is required for the poleward movement of chromosomes in anaphase (Sharp et al. 2000). A third and final motor, MCAK, is known to be present at mitotic kinetochores, though it contributes to chromosome segregation by depolymerizing microtubules in anaphase rather than shuttling chromatids along the length of the spindle fibers (Walczak et al. 1996; Maney et al. 2001). A number of microtubule binding proteins are also localized at kinetochores, including the tumor suppressor AdPC (Adenomatous Polyposis Coli) and Clip170 (the homologue of *S. cerevisiae* Bik1p). Both AdPC and Clip170 probably play a role in chromosome segregation by securing the connection between the kinetochore complex and the microtubule fiber (Dujardin et al. 1998; Kaplan et al. 2001). A growing list of other proteins, including CENP-F, INCENP, Aurora A+B, as well as hNuf2 and Hec1 (the homologues of yeast Nuf2p and Ndc80p, respectively) also concentrate at the kinetochore, although their functions are less well known (Goepfert and B.R. 2000; Saffery et al. 2000; Wigge and Kilmartin 2001).

5. Anaphase Promoting Complex Regulates Destruction of Cohesions

Each chromosome is replicated during S phase, and the two identical sister chromatids are physically joined together by cohesin until anaphase, when the sister chromatids need to separate and segregate. Cohesin is a multi-subunit complex composed, in budding yeast, of Smc1p, Smc3p, Scc1p and Scc3p (Reviewed in Amon 2001). All of the cohesin proteins are highly conserved in mammalian cells and localize to the centromeres and along the chromatid arms after DNA replication. Cohesin creates a physical link between

the identical chromatids and positions their two kinetochores in a back-to-back orientation, increasing the likelihood that each sister chromatid pair will bind to microtubules from opposite poles. This juxtaposing of the kinetochores ensures that the sister chromatid pair achieves bipolar attachment to the spindle, and therefore each daughter cell will receive one copy of the chromosome at anaphase. Once all chromatid pairs have been correctly captured by the spindle microtubules, Cdc20 binds to and activates the Anaphase Promoting Complex (AnaPC), a multi-subunit ubiquitin ligase. The AnaPC ubiquitinates Pds1p (securin in metazoans), targeting it for destruction by the 26S proteasome (Cohen-Fix et al. 1996; Coux et al. 1996). Pds1p/securin functions in cells by heterodimerizing with and inhibiting Esp1p/separin, a protease. When Pds1p is destroyed, the active Esp1p/separin protease cleaves the cohesin component, Scc1p, releasing the sisters from each other and allowing them to move to opposite spindle poles (Waizenegger et al. 2000). The ubiquitin-ligase function of the AnaPC is therefore required for sister chromatid separation at anaphase.

6. Exit from Mitosis

Interestingly, the Anaphase Promoting Complex also regulates the destruction of mitotic cyclins and other proteins to allow cells to exit mitosis (Schwab et al. 1997; Visintin et al. 1997). When Cdc20p activates the AnaPC, it ubiquitinates some of the mitotic cyclin, but a necessary pool of active mitotic cyclins/Cdc28p complexes remains for spindle elongation in anaphase B. After the sister chromatids have successfully segregated, an unknown signal causes Cfi1p to release the phosphatase Cdc14p from the nucleolus.

Cdc14p now stimulates two pathways to inactivate the remaining cyclin/CDK complexes. Cdc14p activates the CDK inhibitor Sic1p and another protein, Cdh1p, which stimulates the AnaPC to ubiquitinate any mitotic cyclin that remains in the cell (Reviewed in Bardin and Amon 2001). The cell now contains only inactive forms of the mitotic cyclin-dependent kinases, and it progresses through telophase to send two daughter cells into G1 for the next round of the cell cycle. Cdh1p then maintains the activity of the AnaPC throughout the G1 phase to prevent the premature accumulation of the mitotic cyclins. At the G1/S transition, it seems that the S phase cyclin kinases phosphorylate Cdh1p directly or indirectly, inhibiting it until its activity is again required for mitotic exit (Reviewed in Morgan 1999).

II. Cell Cycle Checkpoints

The intrinsic properties of the cell cycle machinery prevent errors and correct damage quickly, but cannot accommodate all possible crises. Checkpoints ensure accurate cell duplication by delaying the transition from one phase of the cell cycle to the next phase, arresting cell cycle progression until upstream tasks have been completed and all problems have been corrected. Genetic experiments in yeast led to the isolation and analysis of a large collection of mutants that arrest the cell cycle at specific points (Hartwell et al. 1970; Nurse 1975). From the analysis of these mutants, the cell cycle was modeled as a domino cascade where each step utilizes a substrate provided by the previous step and, in turn, provides the substrate for the next step. Although cell cycle control is now known to be more complex than initially thought, the core of the model

remains: every step in the cell cycle relies on the full and correct completion of the previous step. Experimental results from organisms at all evolutionary levels have shown that each transition point in the cell cycle is tightly regulated: DNA replication does not occur until damage to the genome is repaired, segregation of the genetic material to the two new daughter cells does not occur until the genome has been replicated, and the two new cells do not separate from each other until the DNA has been completely partitioned (Weinert and Hartwell 1988; Hoyt et al. 1991; Li and Murray 1991; Bardin and Amon 2001; Burkholder et al. 2001). Each round of the cell cycle is successful if and only if an accurate and complete copy of the genome is transmitted to each new cell, and cell cycle checkpoints ensure success. Several of the triggers, signaling cascades and effects of the cell cycle checkpoints will be discussed in this section.

A. Discovery of Checkpoints

The original paradigm for cell cycle checkpoints was developed from the study of the *S. cerevisiae rad9* mutation that regulated the transition from S phase to mitosis. The *RAD9* gene makes the initiation of mitosis dependent upon the absence of DNA damage (Weinert and Hartwell 1988). Mutations in the *RAD9* gene allow cells whose genomes have been damaged by x-irradiation to undergo cell division rather than arresting as wild type cells do. This lead to the discovery of the DNA-damage checkpoint signaling pathway. Included in the pathway are *MEC1* and *RAD53 (MEC2),* genes that are responsible not only for cell cycle arrest, but also for stimulating DNA repair complexes (Weinert 1992). Interestingly, cells that contain only a *rad9* mutation and have not been

exposed to DNA damaging agents do not show compromised growth or viability, although they do exhibit an increased rate of chromosome loss. This result suggested that the DNA damage checkpoint was not essential under normal circumstances, though it was vital under some conditions (Weinert and Hartwell 1988). The first model of cell cycle checkpoints therefore proposed that checkpoints function in normal cells as non-essential, auxiliary control mechanisms that play a role in cell cycle progression only when a relatively rare DNA-damaging event occurs (Hartwell and Weinert 1989).

It is now clear that the cell cycle is controlled by not one checkpoint, but a series of checkpoint signaling pathways that monitor the transitions from one stage of the cell cycle to the next. Checkpoints are quality control pathways that signal for the arrest of cell cycle progression, allowing time to repair lesions in the genome or in the mitotic structures and insuring that the progeny cells will be absolutely identical to the parent. In addition to the yeast cell cycle checkpoint that utilizes Rad9p to halt damaged cells after S phase, checkpoints also assess the integrity and replication status of the genetic material in G1 before a cell commits to chromosome replication (Zhou and Elledge 2000; Abraham 2001; Bartek and Lukas 2001a). Checkpoints also monitor the attachment of sister chromatid pairs to the mitotic spindle and regulate the exit from mitosis (Amon 1999; Bardin and Amon 2001; Taylor and Stark 2001). It has become clear that some proteins involved in checkpoint pathways signal not only for cell cycle arrest, but also for an increase in DNA repair activities, senescence or apoptosis (Lowe et al. 1993; Xu and Baltimore 1996; Cortez et al. 1999). Thus, checkpoints are needed for much more than a rare triggering of cell cycle arrest.

B. DNA-damage Checkpoint

The DNA-damage checkpoint pathway has been conserved from unicellular to multicellular organisms and its molecular components have been characterized extensively because mutations in the DNA-damage checkpoint pathway give rise to cancer in humans. Ionizing radiation and ultraviolet light both create DNA damage that is sensed by members of the phosphatidylinositol-3-OH kinase family that signal through converging pathways to arrest the cell at several points in the cell cycle (Walworth 2000). Ionizing radiation creates double stranded breaks in the DNA, which is sensed by Tel1p in budding yeast and ATM in mammals. ATM/Tel1p phosphorylates Chk2, which in turn places an inhibitory phosphate on Cdc25 to prevent its activation of the cyclin/CDK complexes (Reviewed in Bartek and Lukas 2001b). Similarly, ultraviolet light induces DNA damage, such as single strand breaks and thymine dimers, which is detected by Mec1p in *S. cerevisiae* and ATR in mammals. They signal through Rad53p and Chk1, respectively to inhibit Cdc25 and the cyclin/CDKs (Reviewed in Carr 1997). Mec1p/Rad53p also activates the downstream kinase Dun1p, which leads to the increased transcription of DNA repair genes (Zhou and Elledge 1993; Zhao and Rothstein 2002). Similarly, ATM and ATR phosphorylate BRCA1, activating the BRCA1/Rad51 complex that is required for the repair of double strand breaks (Gatei et al. 2001). In general, it seems that ATM functions predominantly during G1 while ATR is responsible for G2 arrest, though some overlap is observed (Reviewed in Bartek and Lukas 2001a; Smits and Medema 2001). In either stage in metazoans, ATM/ATR also directs Chk2/Chk1 to phosphorylate p53, a modification that releases p53 from the ubiquitin ligase MDM2 and

allows p53 to accumulate in the cell (Caspari 2000). p53 is a transcription factor that induces the expression of genes like p21 and 14-3-3 sigma, two CDK inhibitors, to further ensure that the cell will arrest (Reviewed in Lundberg and Weinberg 1999). In this way, the mammalian cell attacks the cyclin/CDK from two sides; initiating p53-dependent and p53-independent means of inactivating CDKs and thereby preventing cell cycle progression in the presence of DNA damage.

C. Apoptosis

When a cell is placed under stress, such as growth factor deprivation, oncogene expression or DNA damage, it activates signaling pathways to induce either cell cycle arrest or apoptotic cell death (Reviewed in Jimenez et al. 1999). Although the signaling cascades of cell cycle checkpoints are more complex than first imagined, mutated cells can bypass checkpoints and continue to cycle until the mutations or errors lead either to death or to a tolerated alteration in the physiology of the cell (Hoyt et al. 1991; Li and Murray 1991; Bennett et al. 2001; Sidorova and Breeden 2002). While this outcome is acceptable for unicellular organisms like yeast, multicellular organisms have developed a much more stringent network of cell cycle checkpoints to include apoptosis because they cannot allow the survival of altered progeny that are capable of transformation or tumorigenesis. Mammalian cells utilize p53-dependent and p53-independent signaling pathways to activate caspases that carry out the cellular suicide program. One of the p53-dependent pathways results in the release of cytochrome C, a factor that increases the activity of the caspases, from the mitochondria. p53 can regulate the expression of Bax, a

protein that promotes apoptosis (Miyashita and Reed 1995), and Bcl-2, which antagonizes Bax to inhibit cell death (Thomas et al. 2000). Bcl-2 and Bax form homo- and heterodimers in the mitochondrial membrane and regulate the release of cytochrome C (Kluck et al. 1997; Yang et al. 1997; Marzo et al. 1998; Otter et al. 1998). By increasing the ratio of Bax to Bcl-2 in the cell, p53 pulls one of the many triggers that result in programmed cell death. As a cell apoptoses, the cytoplasm shrinks, the nucleus becomes pyknotic, the DNA fragments and the cell eventually disperses into smaller vesicles called apoptotic bodies (Saraste and Pulkki 2000). Apoptosis eliminates cells that have acquired extensive DNA damage, genetic instability or faulty cell cycle machinery before they gain the ability to undergo the unregulated, rapid cell division that is necessary for tumorigenesis (Rinkenberger and Korsmeyer 1997).

D. Spindle Assembly Checkpoint

Due to the dynamic structure of microtubules during mitosis, the attachment of sister chromatid pairs to spindle microtubules occurs stochastically. Therefore, each cell requires a system to monitor the capture of the kinetochores by the spindle microtubules and to ensure that chromosome segregation does not initiate while any kinetochores lack connections to microtubules. Early cytological studies in plant cells found that sister chromatid separation does not occur until all chromatid pairs are attached to the mitotic spindle and properly lined up at the metaphase plate (Bajer and Mole-Bajer 1956; Nicklas 1967; Zirkle 1970). Later studies showed that the destruction of spindle microtubules by drugs such as benomyl, taxol and nocodazole results in a prolonged metaphase arrest

(Fuchs and Johnson 1978; Zieve et al. 1980; Jacobs et al. 1988). These observations supported the model that a checkpoint functions at the metaphase-anaphase transition, monitoring the attachment of chromatids to the spindle and delaying anaphase if even a single kinetochore has not attached to a microtubule fiber (Hartwell and Weinert 1989). The spindle assembly checkpoint monitors bipolar attachment and inhibits the destruction of cohesin if any of the sister chromatids have not properly attached to the mitotic spindle (Reviewed in Amon 1999).

1. The Kinetochore is Essential for Delay

The kinetochore is not merely a structural bridge between the centromere and the spindle; it also monitors the status of microtubule attachment and generates the signal to negatively regulate Cdc20 and delay the initiation of anaphase. Targeted mutations of the *S. cerevisae* centromeric DNA result in the assembly of an incomplete kinetochore and lead to mitotic arrest of the cells (Wang and Burke 1995; Wells and Murray 1996). The injection of antibodies against several structural components of the human kinetochore induces mitotic arrest in Hela cells (Bernat et al. 1990; Tomkiel et al. 1994). However, if some essential components of the yeast kinetochore are severely mutated so that no kinetochore is formed at all, cells no longer arrest even when the spindle microtubules have been destabilized by nocodazole (Goh and Kilmartin 1993). These results led to the deduction that the assembly of the kinetochore is necessary to activate the components of the spindle assembly checkpoint, either by modifying one or more of the checkpoint proteins or by bringing the checkpoint signaling proteins in close proximity to one

another. The checkpoint proteins do not passively diffuse throughout the cell. If two pre-anaphase cells are fused, the arrest signal generated by unattached kinetochores on one spindle cannot delay the cell cycle progression of the other spindle (Rieder et al. 1997). In hamster PtK1 cells that arrested at metaphase due to the presence of only one unattached kinetochore, destruction of that final kinetochore by laser ablation also destroys the cell cycle arrest and allows anaphase to begin (Rieder et al. 1995). Subsequent experiments supported the suggestion that the proteins responsible for the pre-anaphase arrest were actually members of the kinetochore complex itself (Taylor and McKeon 1997; Chen et al. 1998; Martinez-Exposito et al. 1999; Howell et al. 2000). From these results it is clear that the signal for checkpoint-dependent arrest arises from or is transduced by members of the kinetochore complex.

2. Signal Transduction Pathway

a. Discovery of Genes in Yeast

The identification of the molecular components of the spindle assembly checkpoint has been an ongoing process for the past ten years. In 1991, two screens in yeast uncovered a set of genes that are necessary for a pre-anaphase arrest after the introduction of drugs that destabilize microtubules. Without microtubules, wild type cells halt in metaphase, but checkpoint-deficient yeast continue to grow without microtubules for several more cell cycles until they die. The six genes discovered in the two screens were named *BUB1-3* for the Budding Uninhibited in Benzimidazole phenotype and *MAD1-3* for Mitotic Arrest Defective (Hoyt et al. 1991; Li and Murray 1991). Further investigation

of mitotic arrest in yeast has expanded and refined this list of genes: *BUB2* does not, in fact, play a role in the spindle assembly checkpoint, but rather in a mitotic exit checkpoint (Alexandru et al. 1999; Fraschini et al. 1999), and the gene *MPS1* is clearly a member of the spindle assembly checkpoint signaling pathway even though it also plays an essential role in spindle pole duplication (Winey et al. 1991; Weiss and Winey 1996). A mutation in a *BUB* or a *MAD* gene obliterates the mitotic arrest, even in cells that would normally arrest due to the presence of altered centromeres, kinetochores or microtubules (Spencer and Hieter 1992; Guenette et al. 1995; Strunnikov et al. 1995; Wang and Burke 1995; Pangilinan and Spencer 1996). The intrinsic mechanics of sister chromatid capture by the spindle microtubule are so efficient that chromosome loss occurs in only one in 10^5 normal cell divisions (Hartwell et al. 1982), and *mad* cells show a 10-fold increase in the occurrence of chromosome missegregation events (Li and Murray 1991). Although chromosome loss is immediately lethal for haploid yeast, this extreme efficiency of the underlying mechanisms of chromosome segregation means that none of the genes involved in the checkpoint is essential for growth. As the original model of cell cycle checkpoints predicts, the spindle assembly checkpoint does function as an auxiliary mechanism in yeast and is not required for most cell duplications.

b. Biochemical Analysis of Components in Yeast

Biochemical studies of the budding yeast spindle assembly checkpoint have uncovered several interactions that begin to form a model for how the checkpoint pathway halts cell cycle progression in mitosis. Bub1p is a kinase that binds to Bub3p (Roberts et al. 1994).

Mps1p, also a protein kinase, phosphorylates Mad1p only upon activation of the checkpoint (Hardwick and Murray 1995; Weiss and Winey 1996), and over-expression of Mps1p is sufficient to cause metaphase arrest (Hardwick et al. 1996). The presence of Bub1 and Mps1 kinases in the signaling pathway implies there may be a step-by-step signal cascade with ordered protein associations, but the functions of the spindle assembly checkpoint proteins actually seem very interdependent. A complex of Mad1p, Bub1p and Bub3p has been immunoprecipitated from mitotic cells (Brady and Hardwick 2000), and another large complex has been seen that contains Bub3p, Mad3p, Mad2p and Cdc20p (Hardwick et al. 2000). This is the same Cdc20p that activates the Anaphase Promoting Complex for the destruction of cohesin. The formation of these complexes is not unexpected because Bub1p and Mad3p both contain separate binding domains for Bub3p and Cdc20p (Hwang et al. 1998). Additionally, it is believed that Cdc20p and Mad1 compete for a shared or overlapping binding site on Mad2p (Luo et al. 2002). Mutations in Cdc20p that destroy its ability to bind to Mad2p also prevents mitotic arrest (Hwang et al. 1998). This finding suggests that the arrest signals transduced by the proteins of the spindle assembly checkpoint culminates in the sequestration of Cdc20p by Mad2p, preventing the binding of Cdc20p to the Anaphase Promoting Complex.

c. Homologues in Higher Eukaryotes

The discovery of the genes involved in the spindle assembly checkpoint in yeast quickly led to the identification of conserved homologues in other eukaryotes. Most of the proteins show both sequence and functional similarities across many species, though the

Mad3 metazoan homologue, BubR1, actually combines features of both Mad3p and Bub1p. In yeast, the NH_2-terminal residues of Bub1p and Mad3p are very similar, containing defined domains that bind to Bub3p and Cdc20p, respectively (Roberts et al. 1994; Hwang et al. 1998; Taylor et al. 1998). Interestingly, where Mad3p terminates, mammalian BubR1 contains a Bub1-like kinase domain (Taylor et al. 1998). BubR1 is more similar to yeast Mad3p than to yeast Bub1p at the NH_2-terminus, but its kinase domain has earned it the name Bub-Related rather than Mad3-Related. The homologues of Mps1p have been named Esk1 and TTK in mouse and human genomes, respectively, but they will be referred to as Mps1 to avoid confusion. The seven mammalian checkpoint proteins: Mps1, Bub1, BubR1, Bub3, Mad1, Mad2 and Cdc20, all localize to kinetochores in mitosis (Chen et al. 1996; Li and Benezra 1996; Taylor and McKeon 1997; Chen et al. 1998; Jablonski et al. 1998; Kallio et al. 1998; Taylor et al. 1998; Martinez-Exposito et al. 1999; Fisk and Winey 2001). Mad1 is a coiled-coil protein that homodimerizes and recruits Mad2 to unattached kinetochores (Hardwick and Murray 1995; Chen et al. 1998; Chen et al. 1999). Bub3 is necessary for the kinetochore localization of Bub1 and BubR1, although Bub1 appears there early in prophase while BubR1 does not do so until after the nuclear membrane breaks down in late prophase (Jablonski et al. 1998). This difference in the timing of each protein's arrival at the kinetochore is complementary to the finding that only Bub1 binds to CENP-F, a nuclear matrix protein that may participate in the first steps of kinetochore formation (Rattner et al. 1993; Liao et al. 1995; Chan et al. 1998) while only BubR1 binds to CENP-E, a kinesin-like motor protein (Schaar et al. 1997; Chan et al. 1998; Jablonski et al. 1998). Additionally, BubR1 appears to be associated with Cdc20 and the Anaphase Promoting

Complex:, indicating that the spindle assembly checkpoint may signal through BubR1 to inhibit the ubiquitin-ligase functions of the AnaPC (Chan et al. 1999).

Continued research in higher eukaryotes has reinforced the idea that evolution has interwoven the spindle assembly checkpoint pathway with the cell cycle machinery, making the checkpoint proteins essential for cellular division. Genetic analysis of *bub1* in *Drosophila* (Basu et al. 1999), Bub3 in mice (Kalitsis et al. 2000), *mad1* in *C. elegans* (Kitagawa and Rose 1999) and Mad2 in mice (Dobles et al. 2000) confirms that the spindle assembly checkpoint is essential for organismal viability. Instead of acting as a backup system, the spindle assembly checkpoint seems to control the timing of mitosis in each and every cell division. Perturbation of a mitotic checkpoint proteins forces metazoan cells to pass through mitosis more quickly than unaltered cells do. Real-time observations of mitoses in wild type PtK1 cells have revealed that the time period from nuclear envelope breakdown to the initiation of anaphase varies from 30 minutes to over 3 hours (Rieder et al. 1994). The injection of anti-Mad2 antibodies accelerates mitosis so that sister chromatid segregation commences an average of 15 minutes after the nuclear envelope breaks down (Gorbsky et al. 1998). Similarly, human cells expressing a Bub1 mutant that lacks its kinase domain resulted in a 25 minute decrease in the average length of mitosis (Taylor and McKeon 1997). It seems feasible to speculate that the cell cycle machinery intrinsically allocates a limited amount of time for the attachment of sister chromatids to spindle microtubules, and additional time can be provided by the spindle assembly checkpoint.

3. Attachment vs. Tension

If an unattached kinetochore is sufficient to inhibit cohesin destruction and anaphase initiation, what physical event turns off the checkpoint? Some findings suggest it is the tension created when the kinetochores in a sister chromatid pair have securely attached to microtubules from opposite centrosomes and are being pulled to opposite sides of the spindle. Other results support the theory that the physical attachment of the microtubule to the kinetochore allows Cdc20 to activate the AnaPC. An elegant series of experiments in mantid spermatocytes demonstrated that tension without microtubule attachment is sufficient to overcome metaphase arrest and that the cells respond biochemically to the generation of tension at kinetochores. If only one kinetochore remains unattached to the spindle microtubules, pulling this free kinetochore towards the opposite centrosome with a microneedle results in anaphase initiation (Li and Nicklas 1995). In yeast, tension cannot be generated between the kinetochores of *scc1* strains because the defective cohesin complexes fail to form any connections between the sister chromatid pairs. Even when all kinetochores attach to microtubules, the *scc1* cells remain in a pre-anaphase arrest and Pds1p is not ubiquitinated (Biggins and Murray 2001). This experiment demonstrates that the spindle assembly checkpoint senses the lack of tension and inhibits cell cycle progression. In mammalian cells, kinetochores that are not under tension are chemically distinct from those that are being tugged by microtubules. The 3F3/2 monoclonal antibody recognizes an unknown phospho-epitope(s) that is present only at kinetochores that have not achieved bipolar attachment to the spindle (Gorbsky and Ricketts 1993), and tension applied by a microneedle also eliminates 3F3/2 reactivity

(Nicklas et al. 1995). More specifically, kinetochore-localized Bub1 is also phosphorylated only until the sister chromatids achieve bipolar attachment, and Bub1-phosphorylation reappears if the microtubules, and therefore tension, are disrupted (Taylor et al. 2001). *Drosophila* cells that lack Bub1 still react to the 3F3/2 antibody (Basu et al. 1999), so the two phosphorylation events are separate from one another. These results show that the forces exerted by spindle microtubules cause chemical modifications of the kinetochore components (Nicklas et al. 1995). Bipolarly attached kinetochore pairs in yeast and in mammalian cells are significantly stretched in metaphase, demonstrating that structural modifications to the kinetochore also occur (Waters et al. 1998; Martinez-Exposito et al. 1999; He et al. 2000). Collectively, these data show that the spindle assembly checkpoint is affected by the tensile stresses generated between the kinetochores of a sister chromatid pair.

On the other hand, changes in the amounts of checkpoint proteins at the kinetochores during mitosis suggest that the attachment of microtubules dislodges some of the signaling components, disrupting physical interactions that are necessary to transduce the checkpoint arrest signal. The attachment of microtubules to a kinetochore dislodges the Bub1, Bub3 and Mad2 proteins (Waters et al. 1998; Martinez-Exposito et al. 1999; Taylor et al. 2001). Studies in PtK1 cells showed that Mad2 concentrates at the kinetochore until it contacts a microtubule. Mad2 is then transported along the microtubule fiber to the centrosomes, and the cell begins sister chromatid separation approximately 10 minutes after the last kinetochore has released its Mad2 (Howell et al. 2000). If only one of the two kinetochores of a sister chromatid pair has attached to the

spindle, Bub1, Bub3 and Mad2 are several fold more abundant at the unattached kinetochore than at the attached kinetochore (Waters et al. 1998; Martinez-Exposito et al. 1999). Low doses of the drug taxol stabilize microtubules, preserving kinetochore attachment but eliminating tension between the sister chromatids. Loss of tension does not result in the relocalization of Mad2 or Bub3 to kinetochores that have lost those proteins, though Bub1 and the 3F3/2 phospho-epitope do reappear at all of the kinetochores in taxol-treated cells (Waters et al. 1998). Overall, these results suggest that the binding of microtubules displaces Mad2 and Bub3 from the kinetochore, destroying the Mad2-Cdc20 complex, and releasing Cdc20 to activate the Anaphase Promoting Complex.

4. BubR1 and Mad2 Both Inhibit the AnaPC

Results published in the last year has offered the unexpected possibility that the spindle assembly checkpoint signaling cascade utilizes Mad2 to monitor spindle attachment and BubR1 to sense tension. BubR1, with its Mad3-like N-terminus and its Bub1-like kinase domain, is produced only in higher eukaryotes and it has been shown to inhibit the Anaphase Promoting Complex much more potently than Mad2 does (Sudakin et al. 2001; Tang et al. 2001). Because the presence of only one unattached kinetochore is sufficient to inhibit the AnaPC throughout the cell, the spindle assembly checkpoint must be able to send a diffusible signal out from the kinetochore (Nicklas 1997). Approximately 5% of total cellular Mad2 localizes to unattached kinetochores with an unexpectedly swift turnover rate of 24-28 seconds (Howell et al. 2000), so it seems likely that Mad2 is a

diffusible Cdc20 inhibitor. Diffusible Mad2 may transiently associate with unattached kinetochores to become activated for binding to and sequestering Cdc20. When microtubules attach to a kinetochore, the highly dynamic population of Mad2 appears to translocate to the spindle poles, a relocalization that may prevent its ability to bind to Cdc20 (Howell et al. 2000). On the other hand, BubR1 levels are maintained similarly on free and attached kinetochores until anaphase (Taylor et al. 2001), perhaps sensing tension rather than microtubule attachment. While Cdc20 is phosphorylated by BubR1 (Wu et al. 2000), this chemical modification does not appear to be necessary for mitotic arrest (Tang et al. 2001, Fang, 2002 #573), and the direct role of BubR1 in the signaling cascade remains unknown.

E. Summary

Checkpoint signaling encompasses a network of stringent controls to regulate fundamental aspects of the cell cycle. Checkpoints are responsible for more than just detecting cellular abnormalities and arresting the cell cycle. Checkpoints also monitor the timing of the transitions from one cell cycle stage to the next, initiate the repair of small and large errors and trigger cellular death if unacceptable or irreparable damage is detected. The inactivation of even one checkpoint gene can lead to an increased mutation rate, chromosome loss or changes in ploidy, depending on the gene (Paulovich et al. 1997). Traditional cell cycle checkpoints ensure the genomic integrity of cycling cells and protect the organism from the proliferation of damaged cells by incorporating cellular suicide into the cell cycle. Programmed cell death acts as the final backup, or checkpoint,

system to ensure that metazoan cells containing an imperfect genome are not allowed to propagate.

III. Checkpoint mutations lead to aneuploidy and cancer

The components of the checkpoint signaling pathways have been studied very extensively because alterations in checkpoint proteins are associated with diseases that predispose people to cancer (Caspari 2000, O'Connell 2000). The role of the DNA-damage checkpoint in tumorigenesis has been uncovered with much success in the last decade and many researchers are currently interested in discovering the connections between other checkpoint signaling cascades and cancer progression. Many of the proteins in the DNA-damage checkpoint pathway also function as tumor suppressors or as proto-oncogenes. In this section I will briefly describe some of the links that have between established between tumor development and aberrations in the DNA damage checkpoint and then discuss the possible role of the spindle assembly checkpoint in tumorigenesis.

A. Mutations in DNA-damage Checkpoint are Tumorigenic

Cells taken from tumors often display one of two types of genetic instability: microsatellite instability (MIN) or chromosome instability (CIN). An increased rate of fluctuation at the nucleotide level within regions of repetitive sequence, called microsatellites, has been termed MIN (Ionov et al. 1993). Only a small fraction of all

solid tumors are MIN, and they maintain a diploid karyotype (Lengauer et al. 1998). Non-MIN tumors, however, fail to regulate their chromosome number and classified as CIN. Cell lines rarely display both types of instabilities, suggesting that the causes or effects of either MIN or CIN are sufficient to drive tumorigenesis (Lengauer et al. 1997). Much evidence supports the notion that MIN and CIN cells arise and proliferate when mutations in either the DNA damage checkpoint or the DNA repair machinery occur (Cahill et al. 1999). People with ataxia telangectasia are extremely sensitive to ionizing radiation, and usually carry mutations in the *ATM* gene. They are particularly prone to breast carcinomas, lymphomas and leukemias that arise from cells undergoing double strand breaks. (Reviewed in Shiloh and Rotman 1996; Meyn 1999; Finkel 2002). Sporadic mutations in *CHK1* or *CHK2* have been found in osteosarcomas as well as ovarian and breast carcinomas (Codegoni et al. 1999; Bougeret et al. 2001; Miller et al. 2002). Inherited mutations in *CHK2* or p53 are associated with Li-Fraumeni syndrome, a condition that predisposes patients to cancer (Bell et al. 1999), and at least half of all human tumors contain cells that have lost p53 functions (Reviewed in Walworth 2000; Wahl and Carr 2001). The overexpression of other DNA damage checkpoint proteins, such as Cdc25 and cyclin E has been associated with carcinomas of the breast and ovaries, and gene amplification at the cyclin D locus has been seen in many types of cancer (Reviewed in Bartek and Lukas 2001a).

E. Loss of p53 Results in Aneuploidy

Cell lines lacking functional p53 become aneuploid at a very high rate. While it is clear that p53 is active in both G1 and G2, monitoring different types of cellular stress and then orchestrating multiple responses, its role in chromosome segregation during mitosis and in the prevention of aneuploidy is less clear. Initial studies of the mitotic functions of p53 found that it localized to the centrosomes (Brown et al. 1994), was required for mitotic arrest in the presence of microtubule poisons, and may be a member of the spindle assembly checkpoint (Cross et al. 1995). It was observed that cells treated with nocodazole arrest at metaphase for several hours, but eventually exit mitosis without undergoing cytokinesis (Kung et al. 1990; Rieder and Palazzo 1992), and subsequent research showed that p53 was upregulated in the nocodazole-treated cells after they exited mitosis (Minn et al. 1996). Lanni and Jacks (1998) undertook a careful characterization of p53 in cells treated with microtubule poisons and found that p53 did not play a role in the mitotic checkpoint and did not contribute to the length of time that the cells remained in mitosis. Instead of monitoring spindle assembly, p53 and p21 were both required for arrest of the tetraploid cells after they had exited mitosis, and aneuploid cells lacking either gene initiated DNA replication in an apparent attempt to continue propagation (Lanni and Jacks 1998). It is not known if chromosome breakage occurs in the aberrant exit from mitosis to activate the DNA damage checkpoint in a wild type cell, or if a separate upstream signaling cascade monitors chromosome number and then feeds into p53 to arrest and apoptose the aneuploid cell.

B. Mouse Models of Cancer

Ongoing research using mouse models of human cancers has confirmed that the mutation of checkpoint genes results in the tolerance of genetic lesions that predispose cells to malignancy. The complexity of the DNA damage checkpoint is apparent in the wide range of phenotypes caused by a deletion of a signaling gene. In mice, ATM deficiencies result in growth retardation, sensitivity to gamma-irradiation and the development of lymphomas within the first 2-4 months after birth (Xu and Baltimore 1996; Barlow et al. 1999). In contrast, the ATR knockout is inviable and the ATR heterozygous mouse has a tumor load that is four times larger than that of the ATM heterozygous mouse (Brown and Baltimore 2000). Deletion of Chk1 also kills the embryo, but the heterozygous mice have a normal lifespan and no increase in tumor formation (Liu et al. 2000). Complete inactivation of p53 results in a maximum lifespan of nine months, due to high tumor loads, and mice that are heterozygous for the disrupted allele of p53 develop thymic lymphomas and several types of sarcomas within a year from birth (Donehower et al. 1992; Jacks et al. 1994).

C. Mutations in the Spindle Assembly Checkpoint

Cells containing mutations in spindle assembly checkpoint genes or in other genes involved in chromosome segregation have lost their ability to maintain the integrity of their genomes. Genetic analysis of the spindle assembly checkpoint pathway has proven difficult because loss of any one of the mitotic checkpoint genes is lethal in metazoans.

Experiments utilizing either the injection of anti-Mad2 antibodies or the expression of a dominant-negative mutant of Bub1 have shown that interference with the signaling pathway decreases the duration of mitosis and increases the incidence of chromosome missegregation events (Taylor and McKeon 1997; Gorbsky et al. 1998). Targeted disruptions of *C. elegans mad1*, *Drosophila bub1*, and mouse Mad2 or Bub3 result in embryonic lethality in the homozygous knockout progeny (Basu et al. 1999; Kitagawa and Rose 1999; Dobles et al. 2000; Kalitsis et al. 2000). Mouse embryos lacking either Mad2 or Bub3 are viable at the blastocyst stage, but they cannot survive in culture; the inner cell mass of the null embryos degenerates and dies within five days of harvesting. Careful examination of null blastocysts confirms that nullizygous cells proceed through anaphase in the presence of lagging chromosomes that have not attached to the spindle microtubules. This premature initiation of anaphase results in non-disjunction, chromosome breakage and translocations. Observations of nocodazole-treated cells suggest that aneuploid cells that are produced from an aberrant mitosis undergo apoptosis (Lanni and Jacks 1998). Although the precise mechanism that links errors in chromosome segregation to apoptosis is not known, any lagging chromosomes that are crushed by the cytokinetic furrow incur double strand breaks that will trigger the p53-dependent DNA damage and apoptotic pathways. Indeed, Mad2 -/- embryos analyzed *in utero* at E7.0 display high levels of TUNEL staining, suggesting that programmed cell death is the most immediate cause of the embryonic lethality (Dobles et al. 2000).

D. Human Anneuploidy

1. Syndromes

Perhaps it is not surprising that mutations leading to chromosome missegregation are embryonically lethal in mice, because aneuploidy clearly causes developmental defects and embryonic lethality in humans. The majority of miscarried fetuses display an improper number of chromosomes, and nearly all human monosomies and trisomies are lethal (Carr 1971; Boue et al. 1975). Only three variations in the number of autosomes allow the fetus to survive to birth, and all of these trisomies result in severe physical and mental abnormalities (Reviewed in Scarbrough et al. 1982). Babies born with either trisomy 18, known as Edward's Syndrome or trisomy 13, known as Patau Syndrome, have mental deficiencies, growth retardation and many physical defects, so they rarely survive early infancy. People can survive to adulthood if they have trisomy 21, commonly known as Down Syndrome, but they, too show decreased mental capabilities and physical defects. Surprisingly, triploidy babies, whose cells contain three copies of each chromosome instead of the normal two copies, are born alive, although it is a very rare event and triploidy babies usually live for a few hours (Leisti et al. 1974). How is it that three copies of almost any one chromosome is lethal to an embryo while three copies of each of the 22 chromosomes can be viable? Again, this seems to suggest that gene dosage is very important and is in a fragile balance in the cell. Too many or too few of only a subset of genes may result in physiological changes that renders the cell unable to contribute to the life of the organism.

2. Tumors

Genetic instability is widely recognized as an integral component of tumorigenesis and a majority of tumors contain an abnormal number of chromosomes (Loeb 1991; Lengauer et al. 1998). This association between aneuploidy and neoplasias suggests that chromosome missegregation may play a causative role in tumorigenesis (Kinzler and Vogelstein 1996). Indeed, people with Down Syndrome are fifteen times more likely to develop leukemia than people in the general population (Mertens et al. 1998). Each chromosome contains approximately 1000 genes (Consortium 2001), so not only does each chromosome missegregation event lead to inbalances in the gene doses of oncogenes and tumor suppressors, it may also accentuate growth-promoting mutations that already exist in the cell. For example, if a cell contains a loss of function mutation in one copy of a tumor suppressor gene, but the chromosome containing the functional copy of the gene falls victim to chromosome missegregation, the non-functional allele is uncovered in one of the daughter cells. Unmasking the mutations in a recessive tumor suppressor is thought to be a critical step towards tumorigenesis. Chromosome loss can also uncover mutator phenotypes or other genetic alterations that actually open the door to further mitotic errors. In fact, cells from many tumor cell lines have been shown to constantly gain and lose chromosomes, some at a rate as high as 10^{-2} per chromosome per generation. This characteristic of cancer cells is referred to as chromosome instability or CIN (Lengauer et al. 1997). Mice carrying only one functional copy of Mad2 develop late-onset lung tumors (Michel et al. 2001), further supporting the idea that a decrease in the fidelity of chromosome transmission may be tumorigenic.

IV. Conclusion

The eukaryotic cell cycle is controlled by the activity of cyclin dependent kinases and their regulators, cyclins and CDK inhibitors. Cell cycle transitions are regulated by checkpoints that monitor the extracellular environment, the integrity of the genome, the status of DNA replication and the attachment of chromosomes to the mitotic spindle. Many of the members of checkpoint signaling pathways are tumor suppressor proteins, the inactivation of which leads to uncontrolled cellular proliferation, genomic instability and tumorigenesis. It appears that a two-fold safety net of cell cycle checkpoints prevents the accumulation of aneuploid cells. First, the spindle assembly checkpoint delays progression through mitosis if all chromosomes are not properly attached to the spindle. If this security system fails, unsegregated chromosomes are trapped and broken by the cytokinetic furrow. The broken chromosome activates the second, DNA-damage checkpoint in G1 to trigger apoptosis of the aneuploid cell.

Bibliography

Abraham, R.T. 2001. Cell cycle checkpoint signaling through the ATM and ATR kinases. *Genes Dev* 15: 2177-96.

Alexandru, G., W. Zachariae, A. Schleiffer, and K. Nasmyth. 1999. Sister chromatid separation and chromosome re-duplication are regulated by different mechanisms in response to spindle damage. *Embo J* 18: 2707-21.

Allen, C. and G.G. Borisy. 1974. Structural polarity and directional growth of microtubules of Chlamydomonas flagella. *J Mol Biol* 90: 381-402.

Amon, A. 1999. The spindle checkpoint. *Curr Opin Genet Dev* 9: 69-75.

Amon, A. 2001. Together until separin do us part. *Nat Cell Biol* 3: E12-4.

Amos, L. and A. Klug. 1974. Arrangement of subunits in flagellar microtubules. *J Cell Sci* 14: 523-49.

Andersen, S.S. 1998. Xenopus interphase and mitotic microtubule-associated proteins differentially suppress microtubule dynamics in vitro. *Cell Motil Cytoskeleton* 41: 202-13.

Bajer, A. and J. Mole-Bajer. 1956. Cine-micrographic studies on mitosis in endosperm. II. Chromosome, cytoplasmic, and brownian movements. *Chromosoma* 7: 558-607.

Baker, R.E., D.C. Masison, and . 1990. Isolation of the gene encoding the Saccharomyces cerevisiae centromere-binding protein CP1. *Mol Cell Biol* 10: 2458-67.

Baker, T.A. and S.P. Bell. 1998. Polymerases and the replisome: machines within machines. *Cell* 92: 295-305.

Bardin, A.J. and A. Amon. 2001. Men and sin: what's the difference? *Nat Rev Mol Cell Biol* 2: 815-26.

Barlow, C., M.A. Eckhaus, A.A. Schaffer, and A. Wynshaw-Boris. 1999. Atm haploinsufficiency results in increased sensitivity to sublethal doses of ionizing radiation in mice. *Nat Genet* 21: 359-60.

Bartek, J. and J. Lukas. 2001a. Mammalian G1- and S-phase checkpoints in response to DNA damage. *Curr Opin Cell Biol* 13: 738-47.

Bartek, J. and J. Lukas. 2001b. Pathways governing G1/S transition and their response to DNA damage. *FEBS Lett* 490: 117-22.

Basu, J., H. Bousbaa, E. Logarinho, Z. Li, B.C. Williams, C. Lopes, C.E. Sunkel, and M.L. Goldberg. 1999. Mutations in the essential spindle checkpoint gene bub1 cause chromosome missegregation and fail to block apoptosis in Drosophila. *J Cell Biol* 146: 13-28.

Bell, D.W., J.M. Varley, T.E. Szydlo, D.H. Kang, D.C. Wahrer, K.E. Shannon, M. Lubratovich, S.J. Verselis, K.J. Isselbacher, J.F. Fraumeni, J.M. Birch, F.P. Li, J.E. Garber, and D.A. Haber. 1999. Heterozygous germ line hCHK2 mutations in Li-Fraumeni syndrome. *Science* 286: 2528-31.

Belmont, L.D., A.A. Hyman, K.E. Sawin, and T.J. Mitchison. 1990. Real-time visualization of cell cycle-dependent changes in microtubule dynamics in cytoplasmic extracts. *Cell* 62: 579-89.

Belmont, L.D. and T.J. Mitchison. 1996. Identification of a protein that interacts with tubulin dimers and increases the catastrophe rate of microtubules. *Cell* 84: 623-31.

Bennett, C.B., L.K. Lewis, G. Karthikeyan, K.S. Lobachev, Y.H. Jin, J.F. Sterling, J.R. Snipe, and M.A. Resnick. 2001. Genes required for ionizing radiation resistance in yeast. *Nat Genet* 29: 426-34.

Bernat, R.L., G.G. Borisy, N.F. Rothfield, and W.C. Earnshaw. 1990. Injection of anticentromere antibodies in interphase disrupts events required for chromosome movement at mitosis. *J Cell Biol* 111: 1519-33.

Biggins, S. and A.W. Murray. 2001. The budding yeast protein kinase Ipl1/Aurora allows the absence of tension to activate the spindle checkpoint. *Genes Dev* 15: 3118-29.

Borowiec, J.A., F.B. Dean, P.A. Bullock, and J. Hurwitz. 1990. Binding and unwinding—how T antigen engages the SV40 origin of DNA replication. *Cell* 60: 181-184.

Boue, J., A. Bou, and P. Lazar. 1975. Retrospective and prospective epidemiological studies of 1500 karyotyped spontaneous human abortions. *Teratology* 12: 11-26.

Bougeret, C., S. Jiang, I. Keydar, and H. Avraham. 2001. Functional analysis of Csk and CHK kinases in breast cancer cells. *J Biol Chem* 276: 33711-20.

Brady, D.M. and K.G. Hardwick. 2000. Complex formation between Mad1p, Bub1p and Bub3p is crucial for spindle checkpoint function. *Curr Biol* 10: 675-8.

Brenner, S., D. Pepper, M.W. Berns, E. Tan, and B.R. Brinkley. 1981. Kinetochore structure, duplication, and distribution in mammalian cells: analysis by human autoantibodies from scleroderma patients. *J Cell Biol* 91: 95-102.

Brown, C.R., S.J. Doxsey, E. White, and W.J. Welch. 1994. Both viral (adenovirus E1B) and cellular (hsp 70, p53) components interact with centrosomes. *J Cell Physiol* 160: 47-60.

Brown, E.J. and D. Baltimore. 2000. ATR disruption leads to chromosomal fragmentation and early embryonic lethality. *Genes Dev* 14: 397-402.

Burkholder, W.F., I. Kurtser, and A.D. Grossman. 2001. Replication initiation proteins regulate a developmental checkpoint in Bacillus subtilis. *Cell* 104: 269-79.

Cahill, D.P., K.W. Kinzler, B. Vogelstein, and C. Lengauer. 1999. Genetic instability and darwinian selection in tumours. *Trends Cell Biol* 9: M57-60.

Carr, A.M. 1997. Control of cell cycle arrest by the Mec1sc/Rad3sp DNA structure checkpoint pathway. *Curr Opin Genet Dev* 7: 93-8.

Carr, D.H. 1971. Chromosomes and Abortion. In *Advances in Human Genetics* (ed. H. Harris and K. Hirschhorn). Plenum, New York.

Caspari, T. 2000. How to activate p53. *Curr Biol* 10: R315-7.

Chan, G.K., S.A. Jablonski, V. Sudakin, J.C. Hittle, and T.J. Yen. 1999. Human BUBR1 is a mitotic checkpoint kinase that monitors CENP-E functions at kinetochores and binds the cyclosome/APC. *J Cell Biol* 146: 941-54.

Chan, G.K., B.T. Schaar, and T.J. Yen. 1998. Characterization of the kinetochore binding domain of CENP-E reveals interactions with the kinetochore proteins CENP-F and hBUBR1. *J Cell Biol* 143: 49-63.

Chen, R.H., D.M. Brady, D. Smith, A.W. Murray, and K.G. Hardwick. 1999. The spindle checkpoint of budding yeast depends on a tight complex between the Mad1 and Mad2 proteins. *Mol Biol Cell* 10: 2607-18.

Chen, R.H., A. Shevchenko, M. Mann, and A.W. Murray. 1998. Spindle checkpoint protein Xmad1 recruits Xmad2 to unattached kinetochores. *J Cell Biol* 143: 283-95.

Chen, R.H., J.C. Waters, E.D. Salmon, and A.W. Murray. 1996. Association of spindle assembly checkpoint component XMAD2 with unattached kinetochores. *Science* 274: 242-6.

Codegoni, A.M., F. Bertoni, G. Colella, G. Caspani, L. Grassi, M. D'Incalci, and M. Broggini. 1999. Microsatellite instability and frameshift mutations in genes involved in cell cycle progression or apoptosis in ovarian cancer. *Oncol Res* 11: 297-301.

Cohen-Fix, O., J.M. Peters, M.W. Kirschner, and D. Koshland. 1996. Anaphase initiation in Saccharomyces cerevisiae is controlled by the APC-dependent degradation of the anaphase inhibitor Pds1p. *Genes Dev* 10: 3081-93.

Consortium, I.H.G.S. 2001. Initial sequencing and analysis of the human genome. *Nature* 409: 860-921.

Cortez, D., Y. Wang, J. Qin, and S.J. Elledge. 1999. Requirement of ATM-dependent phosphorylation of brca1 in the DNA damage response to double-strand breaks. *Science* 286: 1162-6.

Coux, O., K. Tanaka, and A.L. Goldberg. 1996. Structure and functions of the 20S and 26S proteasomes. *Annu. Rev. Biochem.* 65: 801-47.

Cross, F.R. 1995. Starting the cell cycle: what's the point? *Curr Opin Cell Biol* 7: 790-7.

Cross, S.M., C.A. Sanchez, C.A. Morgan, M.K. Schimke, S. Ramel, R.L. Idzerda, W.H. Raskind, and B.J. Reid. 1995. A p53-dependent mouse spindle checkpoint. *Science* 237: 1353-6.

Dobles, M., V. Liberal, M.L. Scott, R. Benezra, and P.K. Sorger. 2000. Chromosome missegregation and apoptosis in mice lacking the mitotic checkpoint protein Mad2. *Cell* 101: 635-45.

Donehower, L.A., M. Harvey, B.L. Slagle, M.J. McArthur, C.A. Montgomery, J.S. Butel, and A. Bradley. 1992. Mice deficient for p53 are developmentally normal but susceptible to spontaneous tumours. *Nature* 356: 215-21.

Dujardin, D., U.I. Wacker, A. Moreau, T.A. Schroer, J.E. Rickard, and J.R. De Mey. 1998. Evidence for a role of CLIP-170 in the establishment of metaphase chromosome alignment. *J Cell Biol* 141: 849-62.

Edenberg, H.J. and J.A. Huberman. 1975. Eukaryotic chromosome replication. *Annu Rev Genet* 9: 245-84.

Fan, J., A.D. Griffiths, A. Lockhart, R.A. Cross, and L.A. Amos. 1996. Microtubule minus ends can be labelled with a phage display antibody specific to alpha-tubulin. *J Mol Biol* 259: 325-30.

Felix, M.A., J. Pines, T. Hunt, and E. Karsenti. 1989. Temporal regulation of cdc2 mitotic kinase activity and cyclin degradation in cell-free extracts of Xenopus eggs. *J Cell Sci* 12: 99-116.

Finkel, E. 2002. Consortium piecing together role of ATM gene in breast cancer. *J Natl Cancer Inst* 94: 158-9.

Fisk, H.A. and M. Winey. 2001. The mouse Mps1p-like kinase regulates centrosome duplication. *Cell* 106: 95-104.

Fitch, I., C. Dahmann, U. Surana, A. Amon, K. Nasmyth, L. Goetsch, B. Byers, and B. Futcher. 1992. Characterization of four B-type cyclin genes of the budding yeast Saccharomyces cerevisiae. *Mol Biol Cell* 3: 805-18.

Fraschini, R., E. Formenti, G. Lucchini, and S. Piatti. 1999. Budding yeast Bub2 is localized at spindle pole bodies and activates the mitotic checkpoint via a different pathway from Mad2. *J Cell Biol* 145: 979-91.

Fuchs, D.A. and R.K. Johnson. 1978. Cytologic evidence that taxol, an antineoplastic agent from Taxus brevifolia, acts as a mitotic spindle poison. *Cancer Treat Rep* 62: 1219-22.

Furuno, N., N. den Elzen, and J. Pines. 1999. Human cyclin A is required for mitosis until mid prophase. *J Cell Biol* 147: 295-306.

Gatei, M., B.B. Zhou, K. Hobson, S. Scott, D. Young, and K.K. Khanna. 2001. Ataxia telangiectasia mutated (ATM) kinase and ATM and Rad3 related kinase mediate phosphorylation of Brca1 at distinct and overlapping sites. In vivo assessment using phospho-specific antibodies. *J Biol Chem* 276: 17276-80.

Goepfert, T.M. and B. B.R. 2000. The centrosome-associated Aurora/Ipl-like kinase family. *Curr Top Dev Biol* 49: 331-42.

Goh, P.Y. and J.V. Kilmartin. 1993. NDC10: a gene involved in chromosome segregation in Saccharomyces cerevisiae. *J Cell Biol* 121: 503-12.

Gorbsky, G.J., R.H. Chen, and A.W. Murray. 1998. Microinjection of antibody to Mad2 protein into mammalian cells in mitosis induces premature anaphase. *J Cell Biol* 141: 1193-205.

Gorbsky, G.J. and W.A. Ricketts. 1993. Differential expression of a phosphoepitope at the kinetochores of moving chromosomes. *J Cell Biol* 122: 1311-21.

Guenette, S., M. Magendantz, and F. Solomon. 1995. Suppression of a conditional mutation in alpha-tubulin by overexpression of two checkpoint genes. *J Cell Sci* 108: 1195-204.

Hand, R. 1978. Eucaryotic DNA: organization of the genome for replication. *Cell* 15: 317-25.

Hardwick, K.G., R.C. Johnston, D.L. Smith, and A.W. Murray. 2000. MAD3 encodes a novel component of the spindle checkpoint which interacts with Bub3p, Cdc20p, and Mad2p. *J Cell Biol* 148: 871-82.

Hardwick, K.G. and A.W. Murray. 1995. Mad1p, a phosphoprotein component of the spindle assembly checkpoint in budding yeast. *J Cell Biol* 131: 709-20.

Hardwick, K.G., E. Weiss, F.C. Luca, M. Winey, and A.W. Murray. 1996. Activation of the budding yeast spindle assembly checkpoint without mitotic spindle disruption. *Science* 273: 953-6.

Hartwell, L., S. Dutcher, J. Wood, and B. Garvik. 1982. The fidelity of mitotic chromosome reproductuion in *S. cerevisiae*. *Rec. Adv. Yeast Mol. Biol.* 1: 28.

Hartwell, L.H., J. Culotti, and B. Reid. 1970. Genetic control of the cell-division cycle in yeast. I. Detection of mutants. *Proc Natl Acad Sci U S A* 66: 352-9.

Hartwell, L.H. and T.A. Weinert. 1989. Checkpoints: controls that ensure the order of cell cycle events. *Science* 246: 629-34.

He, X., S. Asthana, and P.K. Sorger. 2000. Transient sister chromatid separation and elastic deformation of chromosomes during mitosis in budding yeast. *Cell* 101: 763-75.

He, X., D.R. Rines, C.W. Espelin, and P.K. Sorger. 2001. Molecular analysis of kinetochore-microtubule attachment in budding yeast. *Cell* 106: 195-206.

Hofmann, C., I.M. Cheeseman, B.L. Goode, K.L. McDonald, G. Barnes, and D.G. Drubin. 1998. Saccharomyces cerevisiae Duo1p and Dam1p, novel proteins involved in mitotic spindle function. *J Cell Biol* 143: 1029-40.

Howell, B., N. Larsson, M. Gullberg, and L. Cassimeris. 1999. Dissociation of the tubulin-sequestering and microtubule catastrophe-promoting activities of oncoprotein 18/stathmin. *Mol Biol Cell* 10: 105-18.

Howell, B.J., D.B. Hoffman, G. Fang, A.W. Murray, and E.D. Salmon. 2000. Visualization of Mad2 dynamics at kinetochores, along spindle fibers, and at spindle poles in living cells. *J Cell Biol* 150: 1233-50.

Hoyt, M.A., L. Totis, and B.T. Roberts. 1991. S. cerevisiae genes required for cell cycle arrest in response to loss of microtubule function. *Cell* 66: 507-17.

Hwang, L.H., L.F. Lau, D.L. Smith, C.A. Mistrot, K.G. Hardwick, E.S. Hwang, A. Amon, and A.W. Murray. 1998. Budding yeast Cdc20: a target of the spindle checkpoint. *Science* 279: 1041-4.

Hyman, A.A. and E. Karsenti. 1996. Morphogenetic properties of microtubules and mitotic spindle assembly. *Cell* 84: 401-10.

Ionov, Y., M.A. Peinado, S. Malkhosyan, D. Shibata, and M. Perucho. 1993. Ubiquitous somatic mutations in simple repeated sequences reveal a new mechanism for colonic carcinogenesis. *Nature* 363: 558-61.

Jablonski, S.A., G.K. Chan, C.A. Cooke, W.C. Earnshaw, and T.J. Yen. 1998. The hBUB1 and hBUBR1 kinases sequentially assemble onto kinetochores during prophase with hBUBR1 concentrating at the kinetochore plates in mitosis. *Chromosoma* 107: 386-96.

Jacks, T., L. Remington, B.O. Williams, E.M. Schmitt, S. Halachmi, R.T. Bronson, and R.A. Weinberg. 1994. Tumor spectrum analysis in p53-mutant mice. *Curr Biol* 4: 1-7.

Jacobs, C.W., A.E. Adams, P.J. Szaniszlo, and J.R. Pringle. 1988. Functions of microtubules in the Saccharomyces cerevisiae cell cycle. *J Cell Biol* 107: 1409-26.

Janke, C., J. Ortiz, T.U. Tanaka, J. Lechner, and E. Schiebel. 2002. Four new subunits of the Dam1-Duo1 complex reveal novel functions in sister kinetochore biorientation. *EMBO J* 21: 181-93.

Jimenez, G.S., S.H. Khan, J.M. Stommel, and G.M. Wahl. 1999. p53 regulation by post-translational modification and nuclear retention in response to diverse stresses. *Oncogene* 18: 7656-65.

Johnston, L.H. and N.F. Lowndes. 1992. Cell cycle control of DNA synthesis in budding yeast. *Nucleic Acids Res* 20: 2403-10.

Kalitsis, P., E. Earle, K.J. Fowler, and K.H. Choo. 2000. Bub3 gene disruption in mice reveals essential mitotic spindle checkpoint function during early embryogenesis. *Genes Dev* 14: 2277-82.

Kallio, M., J. Weinstein, J.R. Daum, D.J. Burke, and G.J. Gorbsky. 1998. Mammalian p55CDC mediates association of the spindle checkpoint protein Mad2 with the cyclosome/anaphase-promoting complex, and is involved in regulating anaphase onset and late mitotic events. *J Cell Biol* 141: 1393-406.

Kaplan, K.B., A.A. Burds, J.R. Swedlow, S.S. Bekir, P.K. Sorger, and I.S. Nathke. 2001. A role for the Adenomatous Polyposis Coli protein in chromosome segregation. *Nat Cell biol* 3: 429-32.

Kaufmann, H., R. Marone, M.A. Olayioye, J.E. Bailey, and M. Fussenegger. 2001. Characterization of an N-terminally truncated cyclin A isoform in mammalian cells. *J Biol Chem* 276: 29987-93.

Kimura, K., M. Hirano, R. Kobayashi, and T. Hirano. 1998. Phosphorylation and activation of 13S condensin by Cdc2 in vitro. *Science* 282: 487-90.

Kinzler, K.W. and B. Vogelstein. 1996. Lessons from hereditary colorectal cancer. *Cell* 87: 159-70.

Kitagawa, K. and P. Hieter. 2001. Evolutionary conservation between budding yeast and human kinetochores. *Nat Rev Mol Cell Biol* 2: 678-87.

Kitagawa, K., H. Masumoto, M. Ikeda, and T. Okazaki. 1995. Analysis of protein-DNA and protein-protein interactions of centromere protein B (CENP-B) and properties of the DNA-CENP-B complex in the cell cycle. *Mol Cell Biol* 15: 1602-12.

Kitagawa, R. and A.M. Rose. 1999. Components of the spindle-assembly checkpoint are essential in Caenorhabditis elegans. *Nat Cell Biolog* 1: 514-521.

Kluck, R.M., E. Bossy-Wetzel, D.R. Green, and D.D. Newmeyer. 1997. The release of cytochrome c from mitochondria: a primary site for Bcl-2 regulation of apoptosis. *Science* 275: 1132-6.

Krude, T. and C. Keller. 2001. Chromatin assembly during S phase: contributions from histone deposition, DNA replication and the cell division cycle. *Cell Mol Life Sci* 58: 665-72.

Kung, A.L., S.W. Sherwood, and R.T. Schimke. 1990. Cell line-specific differences in the control of cell cycle progression in the absence of mitosis. *Proc Natl Acad Sci U S A* 87: 9553-7.

Lanini, L. and F. McKeon. 1995. Domains required for CENP-C assembly at the kinetochore. *Mol Biol Cell* 6: 1049-59.

Lanni, J.S. and T. Jacks. 1998. Characterization of the p53-dependent postmitotic checkpoint following spindle disruption. *Mol Cell Biol* 18: 1055-64.

Leisti, J.T., K.O. Raivio, M.H. Rapola, E.J. Saksela, and P.P. Aula. 1974. The phenotype of human triploidy. *Birth Defects Orig Artic Ser* 10: 248-53.

Lengauer, C., K.W. Kinzler, and B. Vogelstein. 1997. Genetic instability in colorectal cancers. *Nature* 386: 623-7.

Lengauer, C., K.W. Kinzler, and B. Vogelstein. 1998. Genetic instabilities in human cancers. *Nature* 396: 643-9.

Li, R. and A.W. Murray. 1991. Feedback control of mitosis in budding yeast [published erratum appears in Cell 1994 Oct 21;79(2):following 388]. *Cell* 66: 519-31.

Li, X. and R.B. Nicklas. 1995. Mitotic forces control a cell-cycle checkpoint [see comments]. *Nature* 373: 630-2.

Li, Y. and R. Benezra. 1996. Identification of a human mitotic checkpoint gene: hsMAD2. *Science* 274: 246-8.

Liao, H., R.J. Winkfein, G. Mack, J.B. Rattner, and T.J. Yen. 1995. CENP-F is a protein of the nuclear matrix that assembles onto kinetochores at late G2 and is rapidly degraded after mitosis. *J Cell Biol* 130: 507-18.

Liu, Q., S. Guntuku, X.S. Cui, S. Matsuoka, D. Cortez, K. Tamai, G. Luo, S. Carattini-Rivera, F. DeMayo, A. Bradley, L.A. Donehower, and S.J. Elledge. 2000. Chk1 is an essential kinase that is regulated by Atr and required for the G(2)/M DNA damage checkpoint. *Genes Dev* 14: 1448-59.

Loeb, L.A. 1991. Mutator phenotype may be required for multistage carcinogenesis. *Cancer Res* 51: 3075-9.

Lowe, S.W., E.M. Schmitt, S.W. Smith, B.A. Osborne, and T. Jacks. 1993. p53 is required for radiation-induced apoptosis in mouse thymocytes. *Nature* 362: 847-9.

Lundberg, A.S. and R.A. Weinberg. 1999. Control of the cell cycle and apoptosis. *Eur J Cancer* 35: 1886-94.

Luo, X., Z. Tang, J. Rizo, and H. Yu. 2002. The Mad2 spindle checkpoint protein undergoes similar major conformational changes upon binding to either Mad1 or Cdc20. *Mol Cell* 9: 59-71.

Maiorano, D. and M. Mechali. 2002. Many roads lead to the origin. *Nat Cell Biol* 4: E58-9.

Maney, T., M. Wagenbach, and L. Wordeman. 2001. Molecular dissection of the microtubule depolymerizing activity of mitotic centromere-associated kinesin. *J Biol Chem* 276: 34753-8.

Martinez-Exposito, M.J., K.B. Kaplan, J. Copeland, and P.K. Sorger. 1999. Retention of the BUB3 checkpoint protein on lagging chromosomes. *Proc Natl Acad Sci U S A* 96: 8493-8.

Marzo, I., C. Brenner, N. Zamzami, J.M. Jurgensmeier, S.A. Susin, H.L. Vieira, M.C. Prevost, Z. Xie, S. Matsuyama, J.C. Reed, and G. Kroemer. 1998. Bax and adenine nucleotide translocator cooperate in the mitochondrial control of apoptosis. *Science* 281: 2027-31.

McEwen, B.F., A.B. Heagle, G.O. Cassels, K.F. Buttle, and C.L. Rieder. 1997. Kinetochore fiber maturation in PtK1 cells and its implications for the mechanisms of chromosome congression and anaphase onset. *J Cell Biol* 137: 1567-80.

Mendenhall, M.D. 1993. An inhibitor of p34CDC28 protein kinase activity from Saccharomyces cerevisiae. *Science* 259: 216-9.

Mertens, A.C., W. Wen, S.M. Davies, M. Steinbuch, J.D. Buckley, J.D. Potter, and L.L. Robison. 1998. Congenital abnormalities in children with acute leukemia: a report from the Children's Cancer Group. *J Pediatr* 133: 617-23.

Meyn, M.S. 1999. Ataxia-telangiectasia, cancer and the pathobiology of the ATM gene. *Clin Genet* 55: 289-304.

Michel, L.S., V. Liberal, A. Chatterjee, R. Kirchwegger, B. Pasche, W. Gerald, M. Dobles, P.K. Sorger, V.V. Murty, and R. Benezra. 2001. MAD2 haplo-

insufficiency causes premature anaphase and chromosome instability in mammalian cells. *Nature* 409: 355-9.

Miller, C.W., T. Ikezoe, U. Krug, W.K. Hofmann, S. Tavor, V. Vegesna, K. Tsukasaki, S. Takeuchi, and H.P. Koeffler. 2002. Mutations of the CHK2 gene are found in some osteosarcomas, but are rare in breast, lung, and ovarian tumors. *Genes Chromosomes Cancer* 33: 17-21.

Minn, A.J., L.H. Boise, and C.B. Thompson. 1996. Expression of Bcl-xL and loss of p53 can cooperate to overcome a cell cycle checkpoint induced by mitotic spindle damage. *Genes Dev* 10: 2621-31.

Mitchison, T., L. Evans, E. Schulze, and M. Kirschner. 1986. Sites of microtubule assembly and disassembly in the mitotic spindle. *Cell* 45: 515-27.

Mitchison, T. and M. Kirschner. 1984. Dynamic instability of microtubule growth. *Nature* 312: 237-42.

Mitchison, T.J. 1993. Localization of an exchangeable GTP binding site at the plus end of microtubules. *Science* 261: 1044-7.

Miyashita, T. and J.C. Reed. 1995. Tumor suppressor p53 is a direct transcriptional activator of the human bax gene. *Cell* 80: 293-9.

Morgan, D.O. 1999. Regulation of the APC and the exit from mitosis. *Nature Cell Biol* 1: E47-E53.

Mueller, P.R., T.R. Coleman, A. Kumagai, and W.G. Dunphy. 1995. Myt1: a membrane-associated inhibitory kinase that phosphorylates Cdc2 on both threonine-14 and tyrosine-15. *Science* 270: 86-90.

Muro, Y., H. Masumoto, K. Yoda, N. Nozaki, M. Ohashi, and T. Okazaki. 1992. Centromere protein B assembles human centromeric alpha-satellite DNA at the 17-bp sequence, CENP-B box. *J Cell Biol* 116: 585-96.

Nicklas, R.B. 1967. Chromosome micromanipulation. II. Induced reorientation and the experimental control of segregation in meiosis. *Chromosoma* 21: 17-50.

Nicklas, R.B. 1997. How cells get the right chromosomes. *Science* 275: 632-7.

Nicklas, R.B., S.C. Ward, and G.J. Gorbsky. 1995. Kinetochore chemistry is sensitive to tension and may link mitotic forces to a cell cycle checkpoint. *J Cell Biol* 130: 929-39.

Nogales, E. 2000. Structural Insights into Microtubule Function. *Annu. Rev. Biochem* 69: 277-302.

Noguchi, H., G. Prem veer Reddy, and A.B. Pardee. 1983. Rapid incorporation of label from ribonucleoside disphosphates into DNA by a cell-free high molecular weight fraction from animal cell nuclei. *Cell* 32: 443-51.

Nurse, P. 1975. Genetic control of cell size at cell division in yeast. *nature* 256: 547-51.

Obaya, A.J. and J.M. Sedivy. 2002. Regulation of cyclin-Cdk activity in mammalian cells. *Cell Mol Life Sci* 59: 126-42.

O'Farrell, P. 2001. Triggering the all-or-nothing switch into mitosis. *Trends Cell Biol* 11: 512-9.

Otter, I., S. Conus, U. Ravn, M. Rager, R. Olivier, L. Monney, D. Fabbro, and C. Borner. 1998. The binding properties and biological activities of Bcl-2 and Bax in cells exposed to apoptotic stimuli. *J Biol Chem* 273: 6110-20.

Pangilinan, F. and F. Spencer. 1996. Abnormal kinetochore structure activates the spindle assembly checkpoint in budding yeast. *Mol Biol Cell* 7: 1195-208.

Paulovich, A.G., R.U. Margulies, B.M. Garvik, and L.H. Hartwell. 1997. RAD9, RAD17, and RAD24 are required for S phase regulation in Saccharomyces cerevisiae in response to DNA damage. *Genetics* 145: 45-62.

Peter, M., J. Nakagawa, M. Doree, J.C. Labbe, and E.A. Nigg. 1990. In vitro disassembly of the nuclear lamina and M phase-specific phosphorylation of lamins by cdc2 kinase. *Cell* 61: 591-602.

Pryer, N., R. Walker, V. Skeen, B. Bourns, M. Soboeiro, and E. Salmon. 1992. Brain microtuble-associated proteins modulate microtubule dynamic instability in vitro. Real-time observations using video microscopy. *Journal of Cell Science* 103: 965-76.

Rattner, J.B., A. Rao, M.J. Fritzler, D.W. Valencia, and T.J. Yen. 1993. CENP-F is a .ca 400 kDa kinetochore protein that exhibits a cell-cycle dependent localization. *Cell Motil Cytoskeleton* 16: 214-26.

Rieder, C.L. and S.P. Alexander. 1990. Kinetochores are transported poleward along a single astral microtubule during chromosome attachment to the spindle in newt lung cells. *J Cell Biol* 110: 81-95.

Rieder, C.L., R.W. Cole, A. Khodjakov, and G. Sluder. 1995. The checkpoint delaying anaphase in response to chromosome monoorientation is mediated by an inhibitory signal produced by unattached kinetochores. *J Cell Biol* 130: 941-8.

Rieder, C.L., E.A. Davison, L.C. Jensen, L. Cassimeris, and E.D. Salmon. 1986. Oscillatory movements of monooriented chromosomes and their position relative

to the spindle pole result from the ejection properties of the aster and half-spindle. *J Cell Biol* 103: 581-91.

Rieder, C.L., A. Khodjakov, L.V. Paliulis, T.M. Fortier, R.W. Cole, and G. Sluder. 1997. Mitosis in vertebrate somatic cells with two spindles: implications for the metaphase/anaphase transition checkpoint and cleavage. *Proc Natl Acad Sci U S A* 94: 5107-12.

Rieder, C.L. and R.E. Palazzo. 1992. Colcemid and the mitotic cycle. *J Cell Sci* 102: 387-92.

Rieder, C.L., A. Schultz, R. Cole, and G. Sluder. 1994. Anaphase onset in vertebrate somatic cells is controlled by a checkpoint that monitors sister kinetochore attachment to the spindle. *J Cell Biol* 127: 1301-10.

Rinkenberger, J.L. and S.J. Korsmeyer. 1997. Errors of homeostasis and deregulated apoptosis. *Curr Opin Genet Dev* 7: 589-96.

Roberts, B.T., K.A. Farr, and M.A. Hoyt. 1994. The Saccharomyces cerevisiae checkpoint gene BUB1 encodes a novel protein kinase. *Mol Cell Biol* 14: 8282-91.

Russell, P. and P. Nurse. 1986. Schizosaccharomyces pombe and Saccharomyces cerevisiae: a look at yeasts divided. *Cell* 45: 781-2.

Russell, P. and P. Nurse. 1987. Negative regulation of mitosis by wee1+, a gene encoding a protein kinase homolog. *Cell* 49: 559-67.

Saffery, R., D.V. Irvine, B. Griffiths, P. Kalitsis, L. Wordeman, and K.H. Choo. 2000. Human centromeres and neocentromeres show identical distribution patterns of

>20 functionally important kinetochore-associated proteins. *Hum Mol Genet* 9: 175-185.

Saraste, A. and K. Pulkki. 2000. Morphologic and biochemical hallmarks of apoptosis. *Cardiovasc Res* 45: 528-37.

Scarbrough, P.R., W.H. Finley, and S.C. Finley. 1982. A review of trisomies 21, 18, and 13. *Ala J Med Sci* 19: 174-88.

Schaar, B.T., G.K. Chan, P. Maddox, E.D. Salmon, and T.J. Yen. 1997. CENP-E function at kinetochores is essential for chromosome alignment. *J Cell Biol* 139: 1373-82.

Schwab, M., A.S. Lutum, and W. Seufert. 1997. Yeast Hct1 is a regulator of Clb2 cyclin proteolysis. *Cell* 90: 683-93.

Schwob, E. and K. Nasmyth. 1993. CLB5 and CLB6, a new pair of B cyclins involved in DNA replication in Saccharomyces cerevisiae. *Genes Dev* 7: 1160-75.

Segal, M., D.J. Clarke, P. Maddox, E.D. Salmon, K. Bloom, and S.I. Reed. 2000. Coordinated spindle assembly and orientation requires Clb5p-dependent kinase in budding yeast. *J Cell Biol* 148: 441-52.

Sharp, D.J., G.C. Rogers, and J.M. Scholey. 2000. Cytoplasmic dynein is required for poleward chromosome movement during mitosis in Drosophila embryos. *Nat Cell Biol* 2: 922-30.

Sherr, C.J. and J.M. Roberts. 1999. CDK inhibitors: positive and negative regulators of G1-phase progression. *Genes Dev.* 13: 1501-12.

Shiloh, Y. and G. Rotman. 1996. Ataxia-telangiectasia and the ATM gene: linking neurodegeneration, immunodeficiency, and cancer to cell cycle checkpoints. *J Clin Immunol* 16: 254-60.

Sidorova, J.M. and L.L. Breeden. 2002. Precocious s-phase entry in budding yeast prolongs replicative state and increases dependence upon rad53 for viability. *Genetics* 160: 123-36.

Siegmund, R.F. and K.A. Nasmyth. 1996. The Saccharomyces cerevisiae Start-specific transcription factor Swi4 interacts through the ankyrin repeats with the mitotic Clb2/Cdc28 kinase and through its conserved carboxy terminus with Swi6. *Mol Cell Biol* 16: 2647-55.

Smits, V.A. and R.H. Medema. 2001. Checking out the G(2)/M transition. *Biochim Biophys Acta* 1519: 1-12.

Spencer, F. and P. Hieter. 1992. Centromere DNA mutations induce a mitotic delay in Saccharomyces cerevisiae. *Proc Natl Acad Sci U S A* 89: 8908-12.

Stoler, S., K.C. Keith, K.E. Curnick, and M. Fitzgerald-Hayes. 1995. A mutation in CSE4, an essential gene encoding a novel chromatin-associated protein in yeast, causes chromosome nondisjunction and cell cycle arrest at mitosis. *Genes Dev* 9: 573-86.

Strunnikov, A.V., J. Kingsbury, and D. Koshland. 1995. CEP3 encodes a centromere protein of Saccharomyces cerevisiae. *J Cell Biol* 128: 749-60.

Sudakin, V., G.K. Chan, and T.J. Yen. 2001. Checkpoint inhibition of the APC/C in HeLa cells is mediated by a complex of BUBR1, BUB3, CDC20, and MAD2. *J Cell Biol* 154: 925-36.

Sullivan, K.F., M. Hechenberger, and K. Masri. 1994. Human CENP-A contains a histone H3 related histone fold domain that is required for targeting to the centromere. *J Cell Biol* 127: 581-92.

Surana, U., H. Robitsch, C. Price, T. Schuster, I. Fitch, A.B. Futcher, and K. Nasmyth. 1991. The role of CDC28 and cyclins during mitosis in the budding yeast S. cerevisiae. *Cell* 65: 145-61.

Tang, Z., R. Bharadwaj, B. Li, and H. Yu. 2001. Mad2-Independent inhibition of APCCdc20 by the mitotic checkpoint protein BubR1. *Dev Cell* 1: 227-37.

Taylor, S.S., E. Ha, and F. McKeon. 1998. The human homologue of Bub3 is required for kinetochore localization of Bub1 and a Mad3/Bub1-related protein kinase. *J Cell Biol* 142: 1-11.

Taylor, S.S., D. Hussein, Y. Wang, S. Elderkin, and C.J. Morrow. 2001. Kinetochore localisation and phosphorylation of the mitotic checkpoint components Bub1 and BubR1 are differentially regulated by spindle events in human cells. *J Cell Sci* 114: 4385-95.

Taylor, S.S. and F. McKeon. 1997. Kinetochore localization of murine Bub1 is required for normal mitotic timing and checkpoint response to spindle damage. *Cell* 89: 727-35.

Taylor, W.R. and G.R. Stark. 2001. Regulation of the G2/M transition by p53. *Oncogene* 20: 1803-15.

Thomas, A., T. Giesler, and E. White. 2000. p53 mediates bcl-2 phosphorylation and apoptosis via activation of the Cdc42/JNK1 pathway. *Oncogene* 19: 5259-69.

Tomkiel, J., C.A. Cooke, H. Saitoh, R.L. Bernat, and W.C. Earnshaw. 1994. CENP-C is required for maintaining proper kinetochore size and for a timely transition to anaphase. *J Cell Biol* 125: 531-45.

Toone, W.M., B.L. Aerne, B.A. Morgan, and L.H. Johnston. 1997. Getting started: regulating the initiation of DNA replication in yeast. *Annu Rev Microbiol* 51: 125-49.

Tournebize, R., A. Popov, K. Kinoshita, A.J. Ashford, S. Rybina, A. Pozniakovsky, T.U. Mayer, C.E. Walczak, E. Karsenti, and A.A. Hyman. 2000. Control of microtubule dynamics by the antagonistic activities of XMAP215 and XKCM1 in Xenopus egg extracts. *Nat Cell Biol* 2: 13-19.

Tubo, R.A. and R. Berezney. 1987. Identification of 100 and 150 S DNA polymerase alpha-primase megacomplexes solubilized from the nuclear matrix of regenerating rat liver. *J Biol Chem* 262: 5857-65.

Van Hooser, A.A., I.I. Ouspenski, H.C. Gregson, D.A. Starr, T.J. Yen, M.L. Goldberg, K. Yokomori, W.C. Earnshaw, K.F. Sullivan, and B.R. Brinkley. 2001. Specification of kinetochore-forming chromatin by the histone H3 variant CENP-A. *J Cell Sci* 114: 3529-42.

Verde, F., M. Dogterom, E. Stelzer, E. Karsenti, and S. Leibler. 1992. Control of microtubule dynamics and length by cyclin A- and cyclin B-dependent kinases in Xenopus egg extracts. *J Cell Biol* 118: 1097-108.

Visintin, R., S. Prinz, and A. Amon. 1997. CDC20 and CDH1: a family of substrate-specific activators of APC- dependent proteolysis. *Science* 278: 460-3.

Wahl, G.M. and A.M. Carr. 2001. The evolution of diverse biological responses to DNA damage: insights from yeast and p53. *Nat Cell Biol* 3: E277-86.

Waizenegger, I.C., S. Hauf, A. Meinke, and J.M. Peters. 2000. Two distinct pathways remove mammalian cohesin from chromosome arms in prophase and from centromeres in anaphase. *Cell* 103: 399-410.

Walczak, C.E., T.J. Mitchison, and A. Desai. 1996. XKCM1: a Xenopus kinesin-related protein that regulates microtubule dynamics during mitotic spindle assembly. *Cell* 84: 37-47.

Walworth, N.C. 2000. Cell-cycle checkpoint kinases: checking in on the cell cycle. *Curr Opin Cell Biol* 12: 697-704.

Wang, Y. and D.J. Burke. 1995. Checkpoint genes required to delay cell division in response to nocodazole respond to impaired kinetochore function in the yeast Saccharomyces cerevisiae. *Mol Cell Biol* 15: 6838-44.

Waters, J.C., R.H. Chen, A.W. Murray, and E.D. Salmon. 1998. Localization of Mad2 to kinetochores depends on microtubule attachment, not tension. *J Cell Biol* 141: 1181-91.

Weinert, T.A. 1992. Dual cell cycle checkpoints sensitive to chromosome replication and DNA damage in the budding yeast Saccharomyces cerevisiae. *Radiat Res* 132: 141-3.

Weinert, T.A. and L.H. Hartwell. 1988. The RAD9 gene controls the cell cycle response to DNA damage in Saccharomyces cerevisiae. *Science* 241: 317-22.

Weiss, E. and M. Winey. 1996. The Saccharomyces cerevisiae spindle pole body duplication gene MPS1 is part of a mitotic checkpoint. *J Cell Biol* 132: 111-23.

Wells, W.A. and A.W. Murray. 1996. Aberrantly segregating centromeres activate the spindle assembly checkpoint in budding yeast. *J Cell Biol* 133: 75-84.

Wiens, G.R. and P.K. Sorger. 1998. Centromeric chromatin and epigenetic effects in kinetochore assembly. *Cell* 93: 313-6.

Wigge, P.A. and J.V. Kilmartin. 2001. The Ndc80p complex from Saccharomyces cerevisiae contains conserved centromere components and has a function in chromosome segregation. *J Cell Biol* 152: 349-60.

Winey, M., L. Goetsch, P. Baum, and B. Byers. 1991. MPS1 and MPS2: novel yeast genes defining distinct steps of spindle pole body duplication. *J Cell Biol* 114: 745-54.

Wood, K.W., R. Sakowicz, L.S. Goldstein, and D.W. Cleveland. 1997. CENP-E is a plus end-directed kinetochore motor required for metaphase chromosome alignment. *Cell* 91: 357-66.

Wu, H., Z. Lan, W. Li, S. Wu, J. Weinstein, K.M. Sakamoto, and W. Dai. 2000. p55CDC/hCDC20 is associated with BUBR1 and may be a downstream target of the spindle checkpoint kinase. *Oncogene* 19: 4557-62.

Xu, Y. and D. Baltimore. 1996. Dual roles of ATM in the cellular response to radiation and in cell growth control. *Genes Dev* 10: 2401-10.

Yamashiro, S., Y. Yamakita, R. Ishikawa, and F. Matsumura. 1990. Mitosis-specific phosphorylation causes 83K non-muscle caldesmon to dissociate from microfilaments. *Nature* 344: 675-8.

Yang, C.H., J. Tomkiel, H. Saitoh, D.H. Johnson, and W.C. Earnshaw. 1996. Identification of overlapping DNA-binding and centromere-targeting domains in the human kinetochore protein CENP-C. *Mol Cell Biol* 16: 3576-86.

Yang, J., X. Liu, K. Bhalla, C.N. Kim, A.M. Ibrado, J. Cai, T.I. Peng, D.P. Jones, and X. Wang. 1997. Prevention of apoptosis by Bcl-2: release of cytochrome c from mitochondria blocked. *Science* 175: 1129-32.

Yen, T.J., D.A. Compton, D. Wise, R.P. Zinkowski, B.R. Brinkley, W.C. Earnshaw, and D.W. Cleveland. 1991. CENP-E, a novel human centromere-associated protein required for progression from metaphase to anaphase. *Embo J* 10: 1245-54.

Zhao, Z. and R. Rothstein. 2002. The Dun1 checkpoint kinase phosphorylates and regulates the ribonucleotide reductase inhibitor Sml1. *Proc Natl Acad Sci U S A* 99: 3746-51.

Zhou, B.B. and S.J. Elledge. 2000. The DNA damage response: putting checkpoints in perspective. *Nature* 408: 433-9.

Zhou, Z. and S.J. Elledge. 1993. DUN1 encodes a protein kinase that controls the DNA damage response in yeast. *Cell* 75: 1119-27.

Zieve, G.W., D. Turnbull, J.M. Mullins, and J.R. McIntosh. 1980. Production of large numbers of mitotic mammalian cells by use of the reversible microtubule inhibitor nocodazole. Nocodazole accumulated mitotic cells. *Exp Cell Res* 126: 397-405.

Zirkle, R.E. 1970. Ultraviolet-microbeam irradiation of newt-cell cytoplasm: spindle destruction, false anaphase, and delay of true anaphase. *Radiat Res* 41: 516-37.

Chapter 2

The Adenomatous Polyposis Coli Tumor Suppressor Protein Interacts with the Mitotic Checkpoint Kinases, Bub1 and BubR1, and Plays a Role in Chromosome Segregation

Note:

The following chapter has been adapted, with permission, from the following publication:

Kaplan KB, Burds AA, Swedlow JR, Bekir SS, Sorger PK, Nathke IS. A role for the Adenomatous Polyposis Coli protein in chromosome segregation. Nat Cell Biol. 2001 Apr;3(4):429-32.

All mouse blastocyst experiments and genotyping (Figure 2.5) were performed by me.

All kinase assays for Figure 2.3 were performed by Ken Kaplan.

Experiments with Embryonic Stem cells presented in Figure 2.6 were performed by S. Bekir and I. Nathke .

The remainder of the experiments were performed in collaboration with Inke Nathke, Ken Kaplan and Jason Swedlow.

Abstract

Aneuploidy and chromosome instability (CIN) are common properties of tumor cells, implying that chromosome missegregation plays a role in tumor progression (Lengauer et al. 1998). The mutations responsible for CIN phenotypes are unknown, but lesions in the Bub1 and BubR1 checkpoint kinases have been implicated in colon cancer cells (Cahill et al. 1998). Bub kinases localize to kinetochores early in mitosis and to lagging chromosomes in metaphase (Martinez-Exposito et al. 1999), transducing a signal delays the onset of anaphase until all kinetochores have achieved bivalent microtubule attachment (Roberts et al. 1994; Taylor and McKeon 1997). In this chapter I show that Bub kinases interact with the adenomatous polyposis coi (AdPC) protein, a tumor suppressor whose inactivation is a critical, early stage in the development of familial and sporadic colon cancers (Levy et al. 1994). I have also found that AdPC localizes during mitosis to the ends of microtubules embedded in kinetochores and that AdPC is bound to, and efficiently phosphorylated by both Bub1 and BubR1 kinases. AdPC has been shown to stabilize the dynamic (+) ends of microtubules (Zumbrunn et al. 2001), and I propose that Bub kinase-mediated interactions between the kinetochore and AdPC are critical for securing sister chromatids to the mitotic spindle.

Introduction

The epithelial cells that line the colon and give rise to these tumors are the progeny of rapidly dividing stem cells located at the bottom of crypts. These cells must migrate from the pit to the lumen of the intestine, rapidly changing both location and morphology over a matter of days to be shed and replaced by new cells (Reviewed in (Dikovskaya et al. 2001)). It is no surprise that the inactivation of AdPC, a protein whose roles encompass nearly all aspects of the biology of a cell, results in rapid tumorigenesis for these colon cells. The Adenomatous Polyposis Coli protein has been shown to contribute to (1) the binding and stabilization of cytoskeleton and spindle microtubules (Munemitsu et al. 1994; Smith et al. 1994), (2) interactions with the actin cytoskeleton (Rosin-Arbesfeld et al. 2001), (3) cell adhesion (Barth et al. 1997; Hamada and Bienz 2002) (4) cell migration (Nathke et al. 1996), (5) cell cycle regulation (Baeg et al. 1995), and (6) the regulation of apoptosis (Reviewed in (Nathke 1999)).

The best understood activity of the 310kD AdPC protein is its association with Axin and glycogen synthase kinase 3B (GSK3B) to regulate the stability of β-catenin, a cadherin binding protein and a transcription regulator (Reviewed in (McCartney and Peifer 2000; Peifer and Polakis 2000). β-catenin binds to a set of phosphorylated repeats in AdPC (Rubinfeld et al. 1996) and its recruitment into an AdPC-Axin-GSK3B complex is regulated by Wnt signaling. The binding of β-catenin to AdPC-Axin-GSK3B results in its phosphorylation by GSK3B and subsequent ubiquitination and degradation (Munemitsu et al. 1995; Aberle et al. 1997). A majority of colon cancer tumor cells

contain deletions or truncation mutations in the AdPC gene and the resulting AdPC proteins are lacking several if not all of the β-catenin binding sites in the center of the AdPC protein (Kinzler and Vogelstein 1996). Similarly, mice containing the truncating Min mutation at a position similar to that found in many human colonic tumors develop multiple intestinal adenomas (Moser et al. 1990; Su et al. 1992). Cells carrying inherited or sporadic truncating mutations in AdPC cannot form the necessary AdPC/AXIN/GSK3B/β-catenin complex. The free β-catenin increases the transcription of many genes, including the c-myc oncogene and the proliferation promoting cyclin D1 gene (He et al. 1998; Tetsu and McCormick 1999). Indeed, expression of the β-catenin binding domain of AdPC (amino acids 958-2075) was recently shown to be sufficient for down-regulation of such target genes and tumor suppression (Shih et al. 2000).

The GSK3B protein kinase regulates the AdPC/β-catenin interaction by phosphorylating sites within the β-catenin -binding repeats located in the central portion of AdPC (Rubinfeld et al. 1996). While this phosphorylation appears constitutive throughout the cell cycle, hyperphosphorylation of AdPC is seen in mitosis. This suggests that another kinase is playing a role in AdPC regulation, and this theory is supported by the fact that as many as 10% of colorectal tumors lack mutations in AdPC, β-catenin or GSK3B (Sparks et al. 1998). Such tumors are likely to carry mutations in other genes that encode regulators or modifiers of AdPC. Two kinases, Bub1 and BubR1, have been found to be mutated in several colon cancer cell lines, placing them on the list of potential AdPC regulators (Cahill et al. 1998). These kinases associate with the Bub3 protein and localize to kinetochores, a multi-protein complex that forms at

centromeric DNA and links the chromosomes to the spindle microtubules in mitosis. The Bub proteins are components of the spindle assembly checkpoint signaling pathway, transmitting a signal that delays the onset of anaphase until all sister chromatids have formed bipolar attachments to the spindle microtubules (Taylor et al. 1998). This cell cycle checkpoint functions to prevent the loss of chromosomes during cytokinesis and the resulting CIN phenotype.

Analysis of the tumor-promoting effects of AdPC has naturally focused on its role in regulating β-catenin. However, an increasing body of literature points to microtubule-related functions (Lu et al. 1998; McCartney et al. 1999; Yu et al. 1999). In interphase human cells, AdPC localizes to the (+) ends of microtubules near the plasma membrane (Nathke et al. 1996; Mimori-Kiyosue et al. 2000), and a C-terminal fragment of AdPC binds directly to microtubules (Munemitsu et al. 1994; Smith et al. 1994). During mitosis, the microtubule cytoskeleton undergoes a dramatic reorganization and the (+) ends of spindle microtubules become linked to chromosomes via kinetochores. We find that AdPC localizes in mitosis to these kinetochores-associated microtubule (+) ends and that AdPC is also a potent substrate of the Bub kinases. These findings suggest that AdPC may play a role in kinetochore-microtubule attachment. Consistent with this, we find that embryonic stem cells that carry truncating mutations in the murine homologue of the AdPC gene (*Min* ES cells) show increased rates of chromosome missegregation even though AdPC does not appear to a member of the spindle assembly checkpoint signaling pathway. Thus, loss of Bub kinase function or truncations in AdPC that eliminate microtubule-binding may underlie the CIN phenotype of colon cancer cells by

directly affecting the physical connection between chromosomes and the spindle microtubules in mitosis.

Results

To determine the localization of AdPC in mitosis, we co-stained asynchronous PtK and HeLa cells with antibodies against microtubules, AdPC and core kinetochore proteins (CREST antisera (Earnshaw and Rothfield 1985). Accurate 3-D views of nuclear morphology were generated by optical sectioning microscopy followed by iterative deconvolution (using an Applied Precision Deltavision microscope). In mitotic cells, we observed discrete foci of AdPC staining at kinetochores, centrosomes (spindle poles) and, to a varying extent, in the cytoplasm (Figure 2.1 and data not shown). Affinity purified antisera from four different rabbits showed a similar pattern of staining, but preimmune sera and anti-AdPC sera depleted by incubation with antigen did not (data not shown). AdPC staining at kinetochores was particularly bright and these foci were typically found immediately adjacent to sites of CREST staining at the ends of microtubules (Figure 2.1c+d). It appears as though most, if not all, microtubule-bound kinetochores are associated with AdPC. Because CREST antisera recognize kinetochore proteins that are in close association with DNA, AdPC is likely to lie on the external face of kinetochores where microtubules attach. This interpretation is supported by images in which kinetochore-bound microtubules are seen to terminate in AdPC-foci immediately adjacent to CREST staining (Figure 2.1d). Neither β-catenin nor plakoglobin staining

could be detected near CREST foci (data not shown), suggesting that kinetochore-bound AdPC is not in a complex with either of these proteins.

To determine when AdPC binds to kinetochores, we examined cells at various stages of mitosis. As a kinetochore marker sensitive to the state of chromosome-microtubule attachment, we used Bub3-GFP (Martinez-Exposito et al. 1999). Previous observations with the Bub3-GFP fusion protein and immunolocalization have shown Bub3 is present at high levels on kinetochores during prophase, decrease about 3-fold as microtubules become attached during prometaphase and then dissociates late in anaphase (Martinez-Exposito et al. 1999). Bub3 is tightly associated with the two Bub kinases that have been identified in animal cells, Bub1 and BubR1 (Roberts et al. 1994; Jablonski et al. 1998; Taylor et al. 1998; Hardwick et al. 2000). For co-localization studies among kinetochores, spindle microtubules and AdPC, Bub3 was fused to GFP and expressed at approximately wild type levels from an MLV provirus (Morgenstern and Land 1990). Distances and relative signal intensities of AdPC staining and Bub3-GFP were then determined from 3-D optical reconstructions. In prophase cells, strong dots of kinetochore-bound Bub3 were present, but they were rarely associated with the AdPC foci, which were found scattered throughout the cell. (Figure 2.2a+b; the distance between Bub3 and AdPC foci was at least 0.8 to 1.0 μm). In prometaphase cells, two populations of AdPC foci were seen: those that were far from Bub3 (greater than 1 μm away) and those that were very close (in the examples circled in Figure 2.2e, the center to center distances were 0.3 ± 0.1 μm). The latter class included AdPC foci that were adjacent to low intensity Bub3-GFP signals, indicating that the kinetochores were bound

to microtubules. Consistent with this idea, AdPC could be seen in metaphase cells to bind to the ends of microtubules, immediately adjacent to Bub3-GFP (Figure 2.2f). From these data we conclude that AdPC is present along the lengths of the spindle microtubules (Figure 2.2f, white arrows) and concentrates at kinetochores.

To determine whether AdPC binds to core kinetochore components or to microtubules attached to kinetochores, we treated cells with the microtubule-poison nocodazole. This caused microtubules to depolymerize and cells to arrest in mid-metaphase. We observed that AdPC and Bub3-GFP foci were no longer adjacent at kinetochores (Figure 2.2g+h). Quantitation yielded an average separation of 0.8 to 1.2 μm, similar to that observed in prophase. We conclude that the binding of AdPC to kinetochores requires intact microtubules and thus, that AdPC is not a core kinetochore protein.

The close physical proximity of Bub3 and AdPC in mitotic cells prompted us to ask whether AdPC was a substrate for mitotic checkpoint kinases. We co-expressed Bub3 and His_{10}-HA tagged Bub1 or BubR1 in insect cells, isolated the kinases on HA-antibody beads, and incubated the beads with ^{32}P-γATP and a central fragment of AdPC (M-AdPC, Figure 2.3a). Both Bub1-Bub3 and BubR1-Bub3 complexes phosphorylated AdPC as did Bub1-Bub3 isolated by immunoprecipitation from HeLa cells (with no significant difference in activity between the recombinant and native complexes; Figure 2.3b and data not shown). To control for contaminating kinases, we generated recombinant Bub1/R1 kinases carrying lysine to arginine mutations in the ATP binding

pocket (hBub1-K821R or hBubR1-K795R) (Roberts et al. 1994; Hanks and Hunter 1995) and showed that they were much less active in AdPC phosphorylation than wild type kinases (Figure 2.3b, lane 3 and data not shown). We conclude that AdPC is a substrate of the Bub kinases. However, in vitro kinase reactions are notoriously non-specific, and we therefore compared AdPC's ability to act as a Bub kinase substrate to two classical kinase substrates, casein and mylein basic protein (MBP). We observed extraordinary specificity for AdPC as a substrate. Bub1 phosphorylation of AdPC was half-maximal at a substrate concentration of 5 nM, and was at least 500-1000 fold more efficient (as measured by moles of phosphate incorporated per mole of substrate) than phosphorylation of either casein or MBP over a range of substrate concentrations between 0.5 nM to 100 nM (Figure 2.3c). Further evidence that AdPC is an excellent Bub1/R1 substrate is the finding the phosphorylation of AdPC proceeded to 2-4 mol phosphate per mol substrate. Efficient phosphorylation was observed with both a middle and C-terminal fragment of AdPC (Figure 2.3 and data not shown), suggesting that Bub kinase sites, like GSK3B sites, are found at multiple locations in the AdPC polypeptide. No significant difference in activity could be detected between Bub1-Bub3 and BubR1-Bub3 (data not shown) and we conclude that both kinase complexes can phosphorylate AdPC efficiently and with very high specificity in vitro.

Among the kinases that have previously been shown to modify AdPC, GSK3B is particularly interesting because its activity is regulated by the Wnt signal transduction pathway (Rubinfeld et al. 1996; Peifer and Polakis 2000). To investigate the relationship between Bub1 and GSK3B-mediated modification of AdPC, the recombinant, His-tagged

middle fragment of AdPC (M-AdPC) was bound to a nickel column, dephosphorylated with lambda phosphatase and used as a Bub1 substrate directly or pretreated with GSK3B before the addition of Bub1 (as described in (Rubinfeld et al. 1996). Over a 20-fold concentration range, GSK3B-phosphorylated AdPC was a five-fold better substrate for Bub1 than phosphatase-treated AdPC (Figure 2.3d). We conclude that GSK3B phosphorylation promotes subsequent phosphorylation by Bub1. These data are consistent with the notion that only a subset of AdPC in cells can be phosphorylated efficiently by Bub kinases, and that this subset is modulated by Wnt signaling.

Many kinases are observed to bind to their substrates in cell extracts (Rubinfeld et al. 1996; Heilker et al. 1999), and we reasoned that a physiological interaction between Bub kinases and AdPC would be reflected in physical association between the proteins. We therefore immunoprecipitated Bub kinases from HeLa cell lysates using either anti-Bub1 or anti-Bub3 antibodies and then probed western blots of immune complexes with anti-AdPC antibodies (similar experiments with BubR1 await better reagents). IP-western assays revealed that AdPC is associated with Bub1 in mitotic but not in interphase cells (Figure 2.4a). The fraction of AdPC that was Bub1-associated was relatively small, between 2% and 5% of total cellular AdPC, but this is not unexpected for a complex between a kinase and its substrate (Figure 2.4a, compare lane 8 to lanes 11 and 13). To demonstrate the existence of a Bub1-Bub3-AdPC complex by independent means, we reconstituted it by co-expressing the three recombinant proteins in insect cells. We observed that protein fragments comprising the amino terminal residues of AdPC (N-AdPC) or the middle residues (M-AdPC) could both be co-immunoprecipitated with anti-

Bub1 and anti-Bub3 antibodies (Figure 2.4b and data not shown). Bub1 and AdPC appeared to be present at roughly equal stoichiometry, but Bub3 - because it is very efficiently expressed - was present in excess. In summary, we conclude that a fraction of the total, cellular AdPC complexes with Bub1-Bub3 in mitotic, but not in interphase cells, and that a three-way complex can be reconstituted by expressing recombinant proteins in insect cells.

The association of AdPC with kinetochores in mitosis, and its modification by the kinetochore-bound checkpoint kinases Bub1/R1, suggested that AdPC might be involved in checkpoint control. To test this hypothesis, we examined whether *Min/Min* embryos lacking functional AdPC (Moser et al. 1990; Fazeli et al. 1997) had a functional checkpoint. Similar experiments performed with embryos carrying knockouts of the Mad2 (Dobles et al. 2000) or Bub3 (Kalitsis et al. 2000) checkpoint genes have shown that Mad2 and Bub3 are essential for the arrest of embryonic cells in mitosis following microtubule depolymerization. We isolated blastocysts from intercrosses of *Min/+* animals at embryonic day 3.5, grew them in vitro until E5.5, treated them with nocodazole for 6 hours and then stained them with antibodies against phosphorylated Histone H3 to determine the mitotic index. As measure of S-phase index, the extent of BrdU incorporation was also determined. A total of 16 embryos from 4 females were challenged with nocodazole, of which three were unambiguously *Min/Min* by PCR genotyping. The fraction of phospho-H3 positive cells was similar in these three knockout blastocysts and in wild-type littermate controls (Figure 2.5a-c). These data indicate that *Min/Min* embryonic cells can arrest in mitosis in response to microtubule

depolymerization and, within the resolution of this rather crude assay, that the mitotic checkpoint is functional.

As a further test of checkpoint response, we examined the ability of colonic tumor cell lines containing wild type or mutant AdPC genes to arrest in response to nocodazole and taxol. The cell cycle stage of cells was determined by measuring DNA content using FACS (Figure 2.5d) or by western blotting using anti-phospho H3 antibodies (data not shown). Following the treatment of cells with a low concentration of taxol we observed that HT29 and SW480 tumor cell lines, which contain two truncated AdPC alleles, arrested with a 4N DNA content to a similar extent as HCT116 cells with two wild type AdPC alleles. HT29 and SW480 also arrested in response to nocodazole treatment, but to a lesser extent than HCT116 cells (data not shown). Overall, data from multiple experiments with different drugs consistently show that tumor cells carrying truncated AdPC alleles do arrest in response to anti-microtubule drugs, but consistently 10-20% fewer arrested cells are found with AdPC mutant lines than with AdPC wild type lines. Many factors appear to control the responsiveness of tumor cells to taxol and nocodazole (Kung et al. 1990), and our data constitute only preliminary evidence that AdPC may play a modulatory, non-essential role in the checkpoint response.

Having found that full-length AdPC is not required for the mitotic checkpoint response, we asked more generally whether AdPC might play a role in chromosome segregation. Early passage, clonal cultures of wild-type and *Min* ES cells (Moser et al. 1990; Fazeli et al. 1997) (Figure 2.6) were propagated for 10 or more generations and

their karyotypes then examined. In *Min* but not in control wild-type ES cells, we observed a significant number of chromosomes near the spindle equator, some of which appeared torn, even when the bulk of the chromosomes had moved to the spindle poles (Figure 2.6b+c). Moreover, the chromosome number of *Min* ES cells was highly variable, indicating that aneuploid cell divisions had occurred (Figure 2.6d). In contrast, the autosome number in wild type cells clustered tightly around 38 (the diploid number). We conclude that *Min* mutations promote genome instability in ES cells and thus, that functional AdPC is probably required for accurate chromosome segregation.

Figure 2.1

AdPC is localized to kinetochores in mitotic cells.

a-c. Projections of 3-D reconstructions of mitotic PTK cell stained with CREST to visualize kinetochores (green), anti-AdPC (red) and DAPI (blue). Staining of AdPC was also observed at centrosomes, which were located to the left and right of the chromosomes in these images. Similar staining was observed in HeLa cells, but the levels of cytoplasmic AdPC were generally higher. **d** A 32x magnification of the area outlined by the yellow box in "c". Tubulin staining (magenta) is also shown.

Figure 2.1

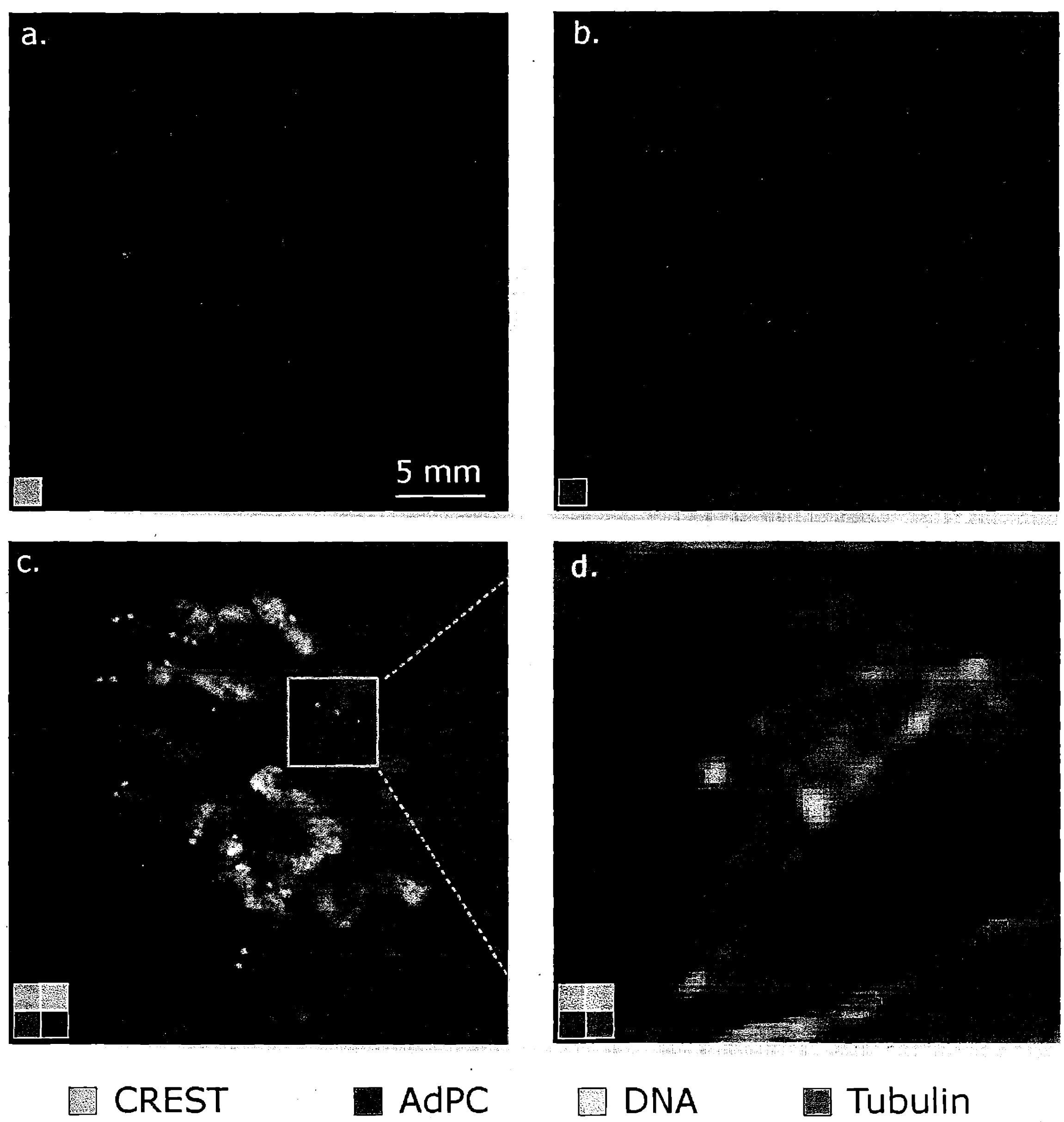


Figure 2.2

AdPC association with kinetochores requires microtubules.

3T3 cells expressing GFP-Bub3 (green) were stained with anti-AdPC (red), DAPI (blue) and anti-tubulin (dark blue) as indicated. **a-b** A prophase cell and **c-d.** prometaphase cell at two magnifications. **e.** The same prometaphasecell with the DAPI channel suppressed and sites of AdPC-Bub3 co-localization circled in yellow. The mean distance between AdPC and Bub3 foci was 0.3 ± 0.1 μm (N=19). **f.** A metaphase cell in which Bub3-GFP and AdPC are seen to co-localize at the ends of microtubules, but AdPC is also visible along the length of microtubule fibers (white arrows). **g-h**. A cell arrested in pro-metaphase by treatment with 30 μM nocodazole for 1 hour. Sites of AdPC and GFP-Bub3 co-localization are absent, as evidenced by an increase in the mean distance between AdPC and GFP-Bub3 signals to 1 ± 0.2μm (N=56). Scale bars are identical for panels a, c+ g and for panels b,d,e,f+h.

Figure 2.2

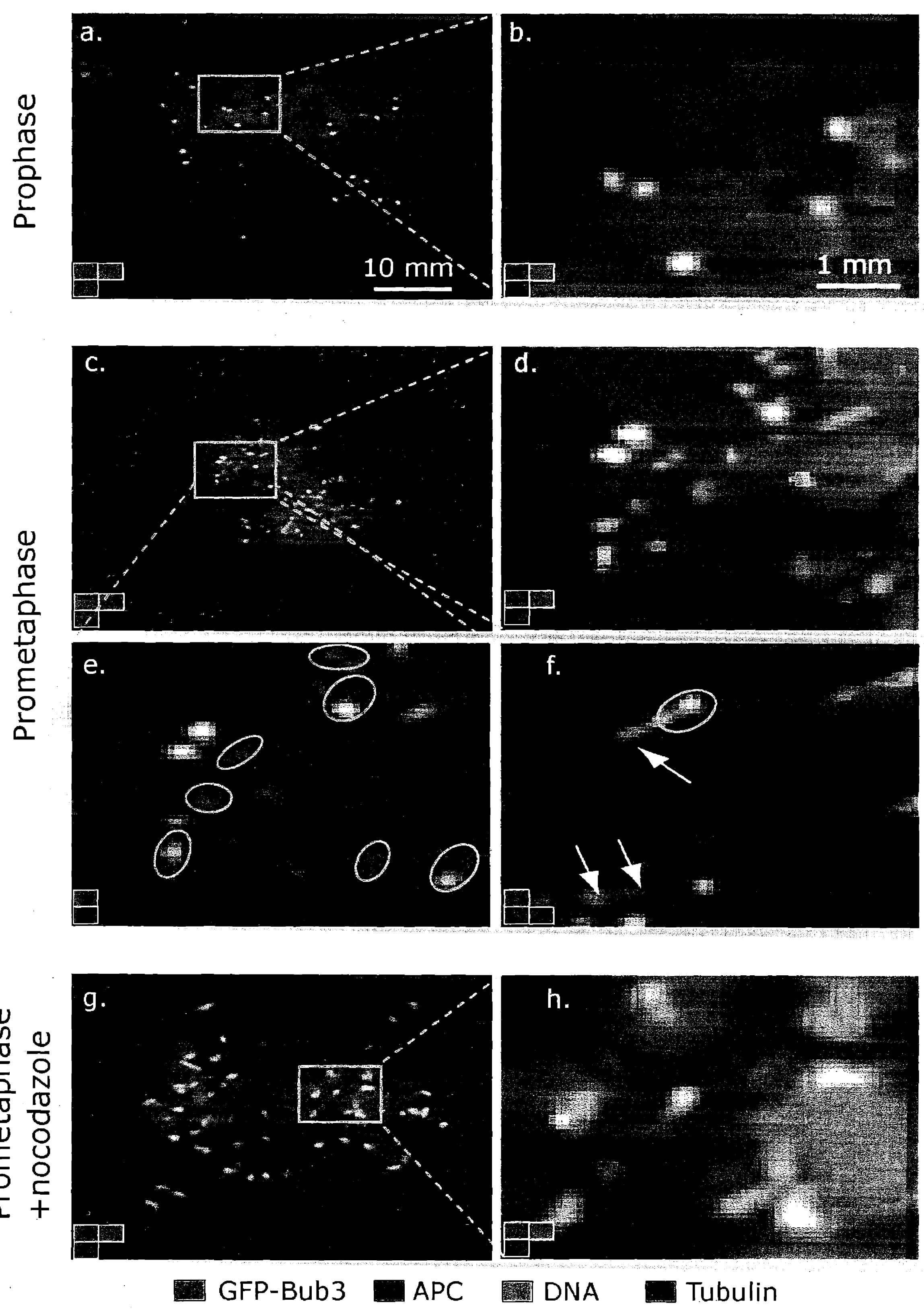
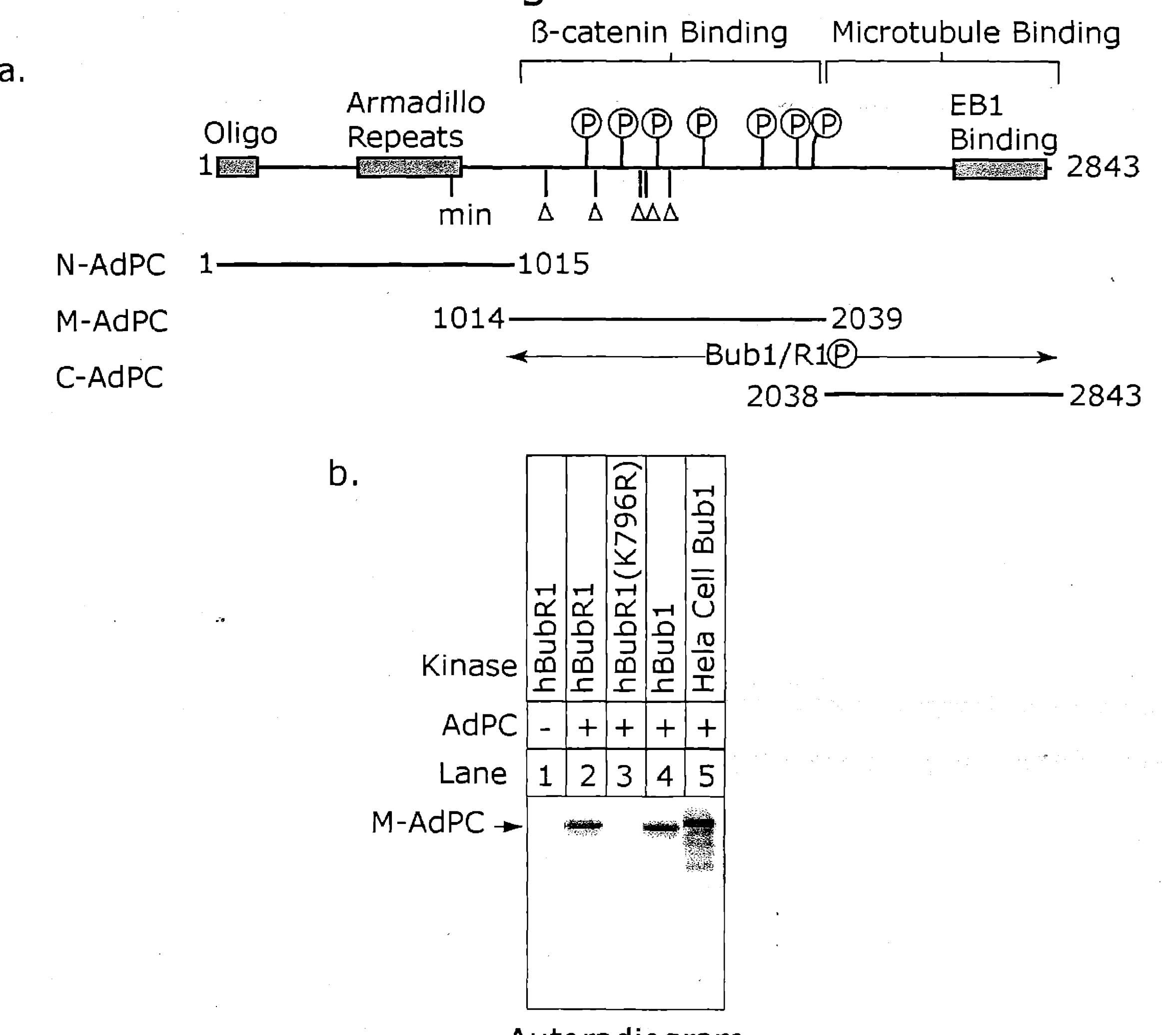


Figure 2.3

Bub kinases specifically phosphorylate AdPC.

a. Schematic of the AdPC sequence with functional domains outlined. "Oligo" denotes the oligomerization domain. Circled "P" indicates predicted sites of GSK3B phosphorylation, the five truncations most commonly found in colon cancers (based on the AdPC mutation database http://perso.curie.fr/Thierry.Soussi/AdPC.html) are indicated by a "Δ", and the nonsense mutation at codon 850 in the *Min* mouse (Su et al. 1992) is indicated by "min". Fragments of AdPC used for phosphorylation and binding studies are shown below the full-length protein. **b**. Phosphorylation by Bub1 and BubR1. In vitro kinase reactions were performed with recombinant M-AdPC (see part a above) and recombinant Bub1-Bub3 or BubR1-Bub3 complexes or HeLa cell Bub1-Bub3 as indicated. As a demonstration of kinase specificity, mutants altered in residues critical for phosphate transfer (Hanks and Hunter 1995) were analysed (hBubR1K796R – lane 3 and hBub1K821R– data not shown). **c.** Bub kinase substrate specificity. In vitro kinase reactions (20 μl) were performed as in "b" using casein, myelin basic protein (MBP) or M-AdPC as substrates. **d.** AdPC phosphorylated by GSK3B is a better substrate for Bub kinase. Recombinant M- AdPC generated in insect cells was dephosphorylated using lambda-phosphatase and either used directly, or first phosphorylated with purified PKA and GSK3B as described (Rubinfeld et al. 1996). AdPC concentrations varied from 1 to 20 nM and the amount of phosphorylation was determined as in "c".

Figure 2.3



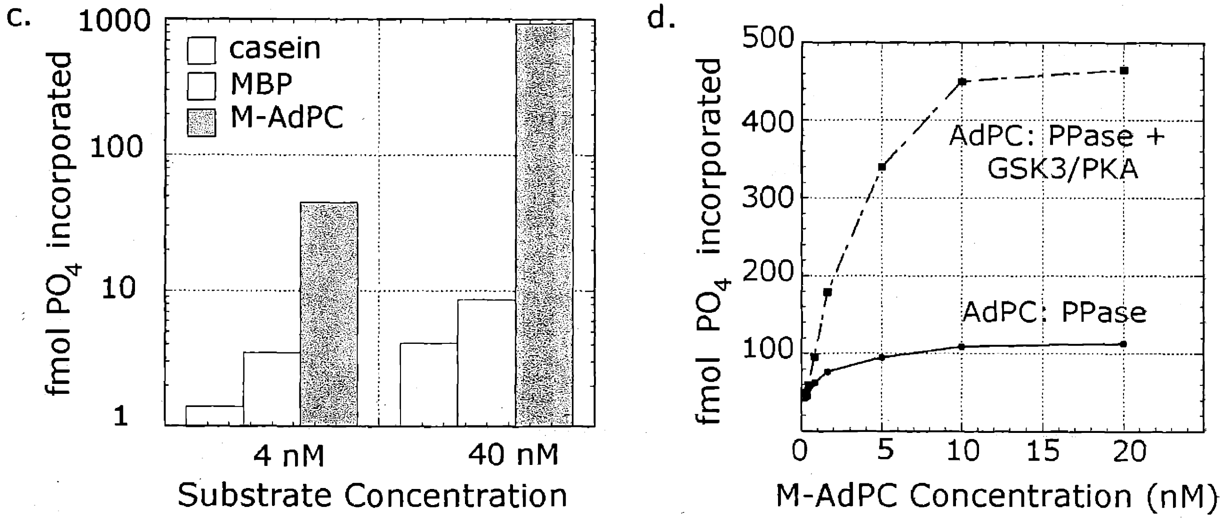


Figure 2.4

Identification of a Bub1-Bub3-AdPC complex.

a. AdPC co-immunoprecipitates with Bub1 and Bub3 in extracts from mitotic HeLa cells. Hela cells arrested in 200 ng/ml nocodazole for 12 hr were lysed in detergent, and analyzed by immunoprecipitation using anti-AdPC, anti-Bub1 and anti-Bub3 antibodies. Immune complexes were analyzed on western blots with anti-AdPC antibodies. **b.** Reconstitution of Bub1-Bub3-AdPC complexes in insect cells. HA-tagged AdPC (residues 1-1015), Bub3 and Bub1 were expressed in insect cells. Lysates were generated and analyzed by immunoprecipitation using anti-Bub3 and anti-AdPC antibodies. Bub3 was present in immune complexes in 6-fold excess because it is highly expressed but Bub1 and AdPC are present in roughly equal amounts.

Figure 2.4

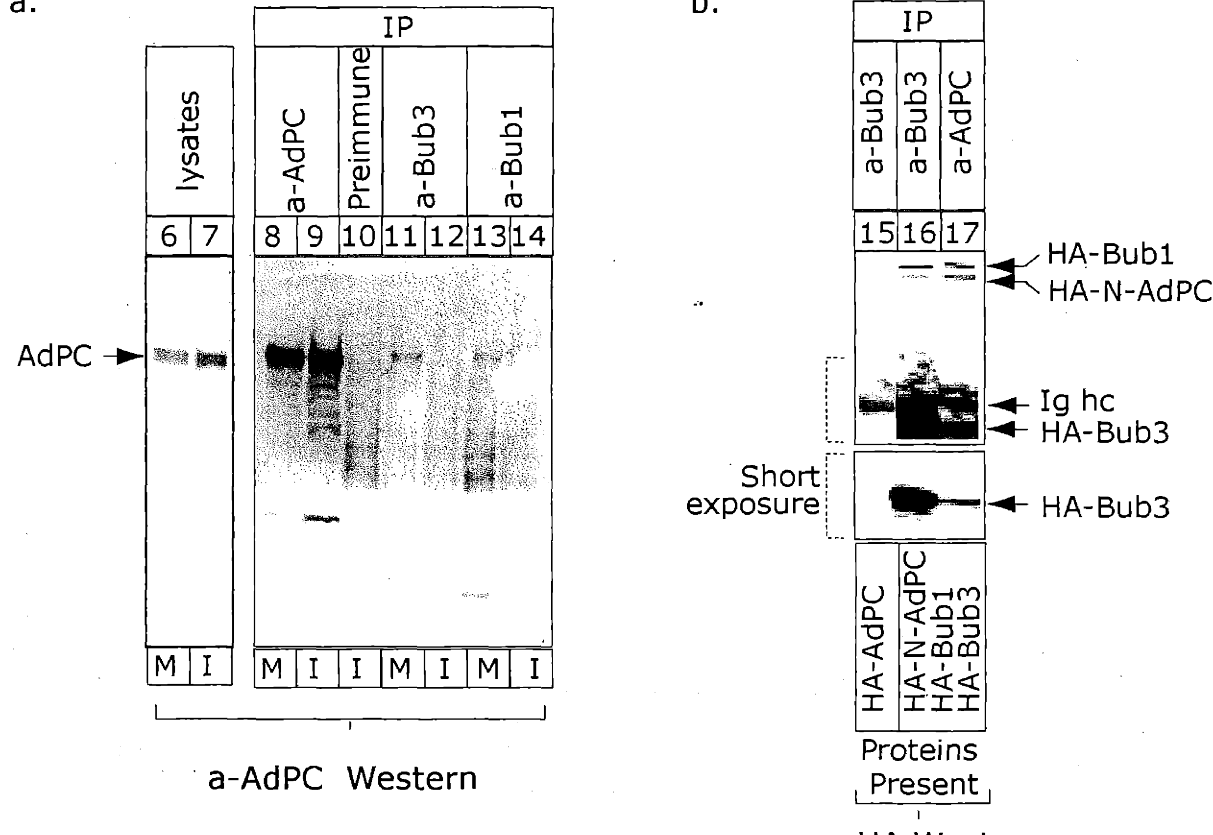


Figure 2.5

Analyzing the role of AdPC in the spindle assembly checkpoint.

a,b. Analysis of mitotic arrest in whole mounts of embryos with the indicated genotypes. Blastocysts were isolated from *Min*/+ intercrosses and cultured in vitro to E5.5, treated with nocodazole and BrdU prior to being fixed, stained and then imaged by deconvolution microscopy as described. Anti-BrdU in red shows S-phase index, anti-phospho H3 in yellow shows mitotic index and DAPI in blue shows all nuclei. **c.** Genotyping by PCR of embryos in b and c using allele–specific primers. DNA isolated from blastocysts was subjected to PCR using allele specific primers to distinguish wild type from *Min/Min* blastocysts. **d.** Cell cycle analysis by FACS of tumor cell lines carrying full length AdPC (HCT116), or truncated AdPC (HT29, SW480). Cells were treated for 6-9 hours with 5 ng/ml taxol prior to analysis.

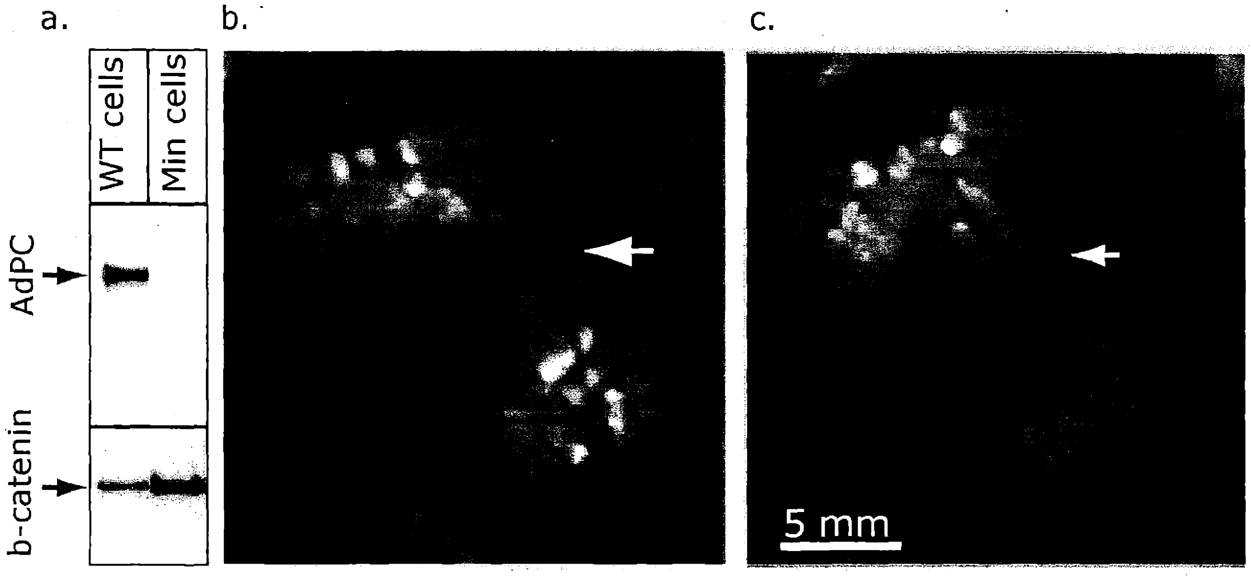
Figure 2.5

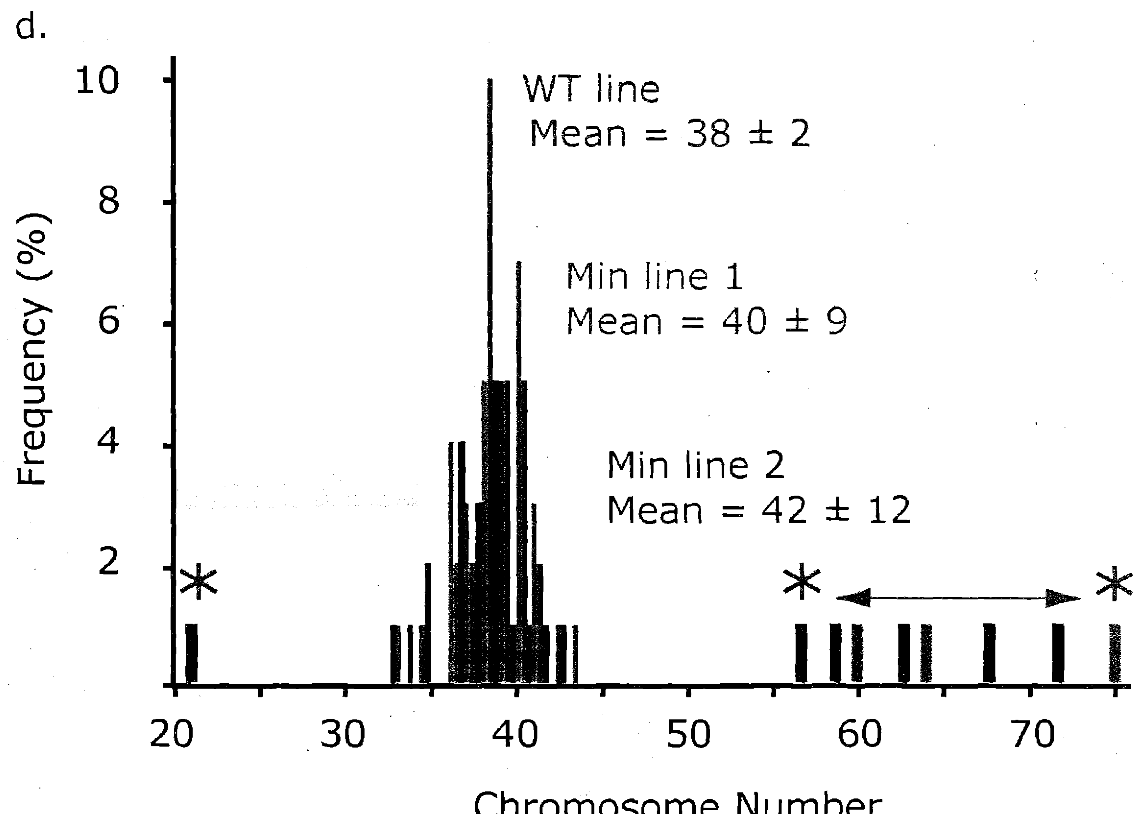
Figure 2.6

Cells lacking functional AdPC protein undergo aberrant mitoses.

a. *Min* ES cells lack full-length AdPC. Whole cell lysates were prepared from stem cells containing mutations in both copies of AdPC (*Min* ES cells), or from wild-type ES cells (Fazeli et al. 1997), and equal amounts of protein analyzed by western blotting with anti-AdPC and anti-b-catenin antibodies. *Min* cells have elevated levels of b-catenin, as expected. **b,c.** Mitotic errors in *Min* ES cells as visualized by DAPI. In 7 of 21 *Min* ES cells (but only 1 of 25 wild type cells) in which the majority of chromosomes had segregated to the poles, some chromosomes remained at the midzone (indicated by white arrows). **d.** Aneuploidy in *Min* ES cell. A clonal, wild type ES cell line and two independent, clonal *Min* lines were cultured for 10 passages and the number of autosomes determined for 20-30 cells from each line. Asterisks denote highly aneuploid cells.

Figure 2.6





Discussion

The finding that chromosome number is unstable in a significant fraction of colorectal cancer cells (a CIN phenotype) has generated great interest because it suggests the existence of tumor-promoting mutations that facilitate chromosome instability (Lengauer et al. 1998). It has been proposed that one cause of the CIN phenotype in human colon cancer cells is mutation of a Bub checkpoint kinase (Cahill et al. 1998), but the kinase substrates whose misregulation might cause the CIN phenotype are not known. In this paper we show that AdPC, a tumor suppressor gene mutated in a majority of human colon cancers, is a potent and specific substrate of Bub1/R1 in vitro, and that AdPC and Bub kinases co-localize to kinetochores in mitotic cells. ES cells carrying a *Min* mutation (a truncation in AdPC) exhibit chromosome instability when grown in culture and are characterized by anaphase bridges typical of fusion chromosomes. Fodde et al. have recently analyzed *Min* ES cells in considerable detail and found similar instability (Fodde et al. 2001). Previous studies have shown that AdPC binds to microtubules via a C-terminal domain that is deleted in many transforming alleles, including the *Min* mutation, (Polakis 1995) and recently it has been reported that AdPC stabilizes microtubule ends (Zumbrunn et al. 2001). We therefore propose that AdPC associated with kinetochore microtubules plays a role as a plus-end microtubule regulator. The *Min* mutation, in this view, causes chromosome instability by subtly impairing chromosome-microtubule attachment.

Our biochemical experiments indicate that AdPC is a potent substrate of Bub kinases in vitro but we have not proven that this is the case in vivo. AdPC is known to be phosphorylated at multiple sites by several different kinases including GSK3B (Rubinfeld et al. 1996) and the cyclin dependent kinase Cdc2 (Trzepacz et al. 1997). Therefore, determining the biological role of Bub-mediated AdPC phosphorylation is likely to be a complex experiment requiring the mapping of four or more phosphorylation sites on AdPC, mutating them and then reintroducing mutated AdPC into cells. Nevertheless, several lines of evidence suggest that the phosphorylation of AdPC by Bub1/R1 is likely to occur in vivo. First, Bub1/R1 and AdPC colocalize at kinetochores in mitotic cells. Second, Bub1 and AdPC form a complex that can be isolated from human cells by immunoprecipitation and can be reconstituted in insect cells using recombinant components. Third, AdPC is a very high affinity (nanomolar) substrate of both Bub1 and BubR1 and is phosphorylated to high stoichiometry under conditions in which Bub1/R1 exhibit high (1000:1) substrate specificity. We therefore hypothesize that Bub1 and/or BubR1 plays a direct role in AdPC regulation. However, experiments with mutant AdPC are clearly required to prove this hypothesis.

If, as we propose, AdPC is regulated by Bub kinases, does this mean that AdPC is a component of the mitotic checkpoint? Our experiments with tumor cell lines and mutant embryos show that AdPC, unlike Mad2 (Dobles et al. 2000) and Bub3 (Kalitsis et al. 2000), is not required for the response of cells to microtubule depolymerization. Instead, we propose that the microtubule-binding functions of AdPC are regulated by kinetochore-bound Bub kinases. Microtubule-associated AdPC may even target

kinetochore proteins for ubiquitin-mediated degradation, just as Axin-GSK3 associated AdPC targets β-catenin. It is also possible that AdPC plays a non-essential role in checkpoint control, since tumor cells carrying mutant AdPC proteins respond slightly less well to nocodazole than tumor cells with wild type AdPC. At our current level of understanding, however the distinction between microtubule-binding and checkpoint roles for AdPC may be specious; in the case of Mad2, an elaborate and highly dynamic interaction clearly occurs between kinetochores, microtubules and the checkpoint (Howell et al. 2000). Similarly, Bim1p, a budding yeast homologue of the AdPC binding protein EB1, has been implicated in both checkpoint control (Muhua et al. 1998; Adames et al. 2001) and in the regulation of microtubule dynamics (Tirnauer et al. 1999; Lee et al. 2000). An important implication of our findings is that the loss of Bub kinase activity or truncations in AdPC that eliminate microtubule binding may underlie the CIN phenotype of colon cancer cells because they directly affect chromosome-spindle attachment. Mapping and then disrupting interaction between Bub kinases and AdPC will make a test of this hypothesis possible.

Experimental Procedures

Immunofluorescence. Coverslips were coated with polybrene and plated with Ptk1 cells or Bub3:GFP expressing HeLa cells at 25-30% confluency. These cells were allowed to grow at 37°C overnight in DME medium supplemented with 10% FCS. The next day the cells were fixed in fresh paraformaldehyde (3.7%in 1mM KOH, pH 6.9 at 37oC, Sigma) for 15minutes at 37oC. Cells were washed twice in PBS and permeablized/blocked for 30 minutes in PBS with 0.1% Triton-X 100, 2% Bovine Serum Albumin Fraction V

(Boehringer Mannheim), 2% normal goat serum (Vector laboratories). Cells were then incubated for 45 minutes with rabbit polyclonal anti-AdPC antisera (Nathke et al. 1996), human CREST serum (Earnshaw and Rothfield 1985) and mouse monoclonal anti-Tubulin antisera (DM 1A or TUB 2.1, Sigma) at 1:1000 in blocking solution. Cells were then washed at least three times with 0.1% TritonX-100 in PBS and then incubated for 20 minutes in secondary antibody (affinity purified FITC, Cy5 or Texas Red-conjugated goat anti-rabbit, goat anti-mouse or goat anti-human antisera, Santa Cruz Inc.) Cells were washed three times with 0.1% TritonX-100 in PBS and then twice in PBS, followed by 1minute of incubation with 1ug/ml of DAPI (Sigma) in PBS and two more washes with PBS. A fusion of EGFP (Clonetech) to the N-terminus of Bub3 was introduced into 3T3 cells, or HeLa cells expressing ecotrophic virus receptor, by infection with a retrovirus constructed from a pBabe-puromycin cassette (Morgenstern and Land 1990). Immunostained specimens were examined using a Ziess-Applied Precision Delta Vision Restoration microscope as described by (Martinez-Exposito et al. 1999)).

Recombinant Proteins and Kinase Assays. Human Bub1 and BubR1 were cloned into baculovirus vectors as His_{10}HA-fusions and co-expressed with murine Bub3 (which differs from human Bub3 at a single residue) in insect cells. Recombinant and endogenous HeLa cell Bub1/R1-Bub3 complexes were isolated by immunoprecipitation using protein A-sepharose beads and anti-HA, anti-Bub3 or anti-Bub1 antibodies (Taylor and McKeon 1997). Beads were washed into 20μl kinase buffer (20mM Tris, pH 7.2, 5mM $MgCl_2$ and 1mM DTT) and incubated at 25°C for 20 minutes in kinase buffer supplemented with 50μM ATP, 30 μCi ^{32}P-γ-ATP and myelin basic protein or casein

purchased from Sigma. Fragments of AdPC were produced in insect cells. The extent of phosphorylation was determined by phosphorimager analysis of SDS-PAGE gels.

Co-immunoprecipitation of AdPC and Bub proteins from Hela cells.

Hela cells were treated with 200ng/ml of nocodazole for 14 hours to arrest them in mitosis. Cell lysates were prepared in 50mM Tris, pH 7.5, 100mM NaCl, 5mM EDTA, 5mM EGTA, 40mM b-glycerophosphate, 0.5% NP-40 (MEBC buffer) supplemented with 10μg/ml of each, leupeptin, pepstatin, and chymostatin, 10mM NaVO4, 10mM NaF. Insoluble material was removed by centrifugation. Cell lysate corresponding to 3mg of cellular protein was immunoprecipitated with 5μl anti Bub 1 or Bub3 polyclonal antisera bound to affiprep beads (Biorad). Beads were washed four times with MEBC buffer and once with 2mM EDTA, 10mM Tri pH 7. Bound material was eluted with 50mM dithethyl amine pH 11.0 and separated on a 3-8% gradient gel (Novex) and transferred to Nitrocellulose (0.1μm pore size, Schleicher & Schuell) in 50mM Tris, 380mM Glycine, 0.02% SDS 7% Methanol for 18 hours. The blot was probed with anti-AdPC antiserum diluted 1:750 and developed using ECL. The relative amount of AdPC that co-immunoprecipitates with Bub 1 or Bub3 is less than 5% of the total AdPC in the lysate. This is comparable to the amount of AdPC that co-immunoprecipiates with B-catenin antiserum under these conditions.

Embryonic Stem Cells. Embryonic stem cells (kind gift of Robert Weinberg and Amin Fazeli) were cultured on gelatin-coated coverslips in Knock-Out D-MEM media (GIBCO-BRL) supplemented with 2mM L-Glutamine (Gibco-BRL), 100 units/ml

Penicillin (Sigma), 100μg/ml Streptomycin (Sigma) and 103units/ml of LIF (Gibco-BRL). They were treated with 10nM taxol (Sigma) in media for 2 hours. Cells were then treated with 0.075M KCl for 16 minutes and fixed with Methanol/acetic acid (volume ratio 3:1) for 1 hour and air-dried overnight. Cells were treated with 50μg/ml trypsin (Sigma) for 1 minute and stained with Giemsa stain (BDH) for 3-5 minutes. After extensive washes in PBS and water, coverslips were allowed to dry and mounted in DPX (BDH). Chromosomes in arrested cells were visualised with a Leica DMR1A microscope using Openlab imaging software (Improvision). The number of chromosomes is plotted against the number of cells containing that number of chromosomes. For the immunoblot of the ES cells, equivalent amounts of total cell lysate (200μg) was loaded (established by protein assay) and probed with anti-AdPC and anti- B-catenin antisera (polyclonal, published previously, (Nathke et al. 1996))

Embryo culture and whole mounts. E3.5 blastocysts from natural matings were harvested into chambered microscopy slides (Nalge Nunc International 177445). Embryos were cultured in ES cell media lacking LIF with 15% fetal bovine serum for 5 days, digested with 0.04% collagenase 3 (Worthington) for 20 min. at 37°C to remove the basal lamina, and fixed for 10 min with 4% paraformaldehyde (EM Science). Cells were permeabilized with 0.5% Triton X-100 for 10 min, the DNA denatured and the embryos blocked (Leonhardt et al., 1992) and reacted with anti-BrdU antibodies (Becton Dickinson, 1:3.5 dilution) and anti-phospho-Histone H3 antibodies (Upstate BioTechnology, 1:400 dilution) for 1 hr at 37°C, followed by 45 min with anti-rabbit or anti-mouse secondary antibodies (Jackson ImmunoResearch). Embryos were viewed and

photographed on a Zeiss-DeltaVision deconvolution microscope (Applied Precision), and then genotyped by PCR.

Genotyping PCR protocol.

Following immunofluorescence analysis, coverslips were removed and embryos were washed 3X5 min in PBS, immersed in PBS, and scraped loose using glass needles (Sutter Instrument Co. BF100-78-10, pulled to the same fineness as used for mouse pronuclei injections). Embryos were placed in 0.2ml thin-wall PCR tubes and digested overnight at 58°C in a total of 20μl 1mg/ml Proteinase K in Tail Digest Buffer (67mM Tris pH 8.8, 16.6mM Ammonium Sulfate, 6.5mM MgCl2, 0.5% Triton-X 100, 143mM β-mercaptoethanol). A Stratagene RobocyclerPCR machine was used to amplify a region of 961bp surrounding the Min locus using oAB53 and oAB59. One μl of digested blastocyst was in 25μl PCR reactions with 200μM deoxyribonucleotides, 1μM each primer and PCR reaction buffer (50mM KCl, 10mM Tris-HCl [pH8.8], 3.5mM $MgCl_2$, 0.1% Triton-X 100). 20μl of a room temperature mix containing all components except Taq (Gibco) were added to 1μl DNA at room temperature, preheated to 95°C for 3min, and then brought to 75°C for addition of Taq (diluted to 4μl on ice). Reactions were subjected to 35 cycles (94°C 30 sec, 64°C 1.min, 72°C 1.5min), and then extended at 72°C for 10min.

for each PCR reaction.

oAB53: 5'-GCACTTGAAATCTCACAGCTTG

oAB59: 5'-GGTTTCATTTGGCCTCTTTTACC

This was followed by re-amplification using nested, allele-specific primers. Accurate genotyping was ensured by repeating amplifications twice

The wild type primer set included oAB50: 5'- ACC TCg CTC TCT CTC CA and oIMR033: 5'- gCC ATC CCT TCA CgT Tag (from the Jackson Laboratory WebSite- http://lena.jax.org/resources/documents/imr/protocols/Apc(Min)_Chem.html). One μl of the first PCR reaction was amplified in a PCR reaction that required buffer containing 2.0mM $MgCl_2$ and subjected to 35 cycles (94°C 30 sec, 54°C 1.min, 72°C 1.5min), and then extended at 72°C for 10min.

The Min-specific primer set is oIMR034: 5'- TCC CAC TTT ggC ATA Agg C and oIMR758; 5'- TCC TgA gAA AgA Cag Aag TTA (also The Jackson Laboratory WebSite). . One μl of the first PCR reaction was amplified in a PCR reaction that required buffer containing no $MgCl_2$ and subjected to 35 cycles (94°C 30 sec, 58°C 1.min, 72°C 1.5min), and then extended at 72°C for 10min.

Bibliography

Aberle, H., A. Bauer, J. Stappert, A. Kispert, and R. Kemler. 1997. beta-catenin is a target for the ubiquitin-proteasome pathway. *Embo J* **16**: 3797-804.

Adames, N.R., J.R. Oberle, and J.A. Cooper. 2001. The surveillance mechanism of the spindle position checkpoint in yeast. *J Cell Biol* **153**: 159-68.

Baeg, G.H., A. Matsumine, T. Kuroda, R.N. Bhattacharjee, I. Miyashiro, K. Toyoshima, and T. Akiyama. 1995. The tumour suppressor gene product APC blocks cell cycle progression from G0/G1 to S phase. *EMBO J* **14**: 5618-25.

Barth, A.I., A.L. Pollack, Y. Altschuler, K.E. Mostov, and W.J. Nelson. 1997. NH2-terminal deletion of beta-catenin results in stable colocalization of mutant beta-catenin with adenomatous polyposis coli protein and altered MDCK cell adhesion. *J Cell Biol* **136**: 693-706.

Cahill, D.P., C. Lengauer, J. Yu, G.J. Riggins, J.K. Willson, S.D. Markowitz, K.W. Kinzler, and B. Vogelstein. 1998. Mutations of mitotic checkpoint genes in human cancers. *Nature* **392**: 300-3.

Dikovskaya, D., J. Zumbrunn, G.A. Penman, and I.S. Nathke. 2001. The adenomatous polyposis coli protein: in the limelight out at the edge. *Trends Cell Biol* **11**: 378-84.

Dobles, M., V. Liberal, M.L. Scott, R. Benezra, and P.K. Sorger. 2000. Chromosome missegregation and apoptosis in mice lacking the mitotic checkpoint protein Mad2. *Cell* **101**: 635-45.

Earnshaw, W.C. and N. Rothfield. 1985. Identification of a family of human centromere proteins using autoimmune sera from patients with scleroderma. *Chromosoma* **91**: 313-21.

Fazeli, A., R.G. Steen, S.L. Dickinson, D. Bautista, W.F. Dietrich, R.T. Bronson, R.S. Bresalier, E.S. Lander, J. Costa, and R.A. Weinberg. 1997. Effects of p53 mutations on apoptosis in mouse intestinal and human colonic adenomas. *Proc Natl Acad Sci U S A* **94**: 10199-204.

Fodde, R., J. Kuipers, C. Rosenberg, R. Smits, M. Kielman, C. Gaspar, J.H. van Es, C. Breukel, J. Wiegant, R.H. Giles, and H. Clevers. 2001. Mutations in the APC tumour suppressor gene cause chromosomal instability. *Nat Cell Biol* **3**: 433-8.

Hamada, F. and M. Bienz. 2002. A Drosophila APC tumour suppressor homologue functions in cellular adhesion. *Nat Cell Biol* **4**: 208-13.

Hanks, S.K. and T. Hunter. 1995. Protein kinases 6. The eukaryotic protein kinase superfamily: kinase (catalytic) domain structure and classification. *Faseb J* **9**: 576-96.

Hardwick, K.G., R.C. Johnston, D.L. Smith, and A.W. Murray. 2000. MAD3 encodes a novel component of the spindle checkpoint which interacts with Bub3p, Cdc20p, and Mad2p. *J Cell Biol* **148**: 871-82.

He, T.C., A.B. Sparks, C. Rago, H. Hermeking, L. Zawel, L.T. da Costa, P.J. Morin, B. Vogelstein, and K.W. Kinzler. 1998. Identification of c-MYC as a target of the APC pathway. *Science* **281**: 1509-12.

Heilker, R., F. Freuler, M. Vanek, R. Pulfer, T. Kobel, J. Peter, H.G. Zerwes, H. Hofstetter, and J. Eder. 1999. The kinetics of association and phosphorylation of

IkappaB isoforms by IkappaB kinase 2 correlate with their cellular regulation in human endothelial cells. *Biochemistry* **38**: 6231-8.

Howell, B.J., D.B. Hoffman, G. Fang, A.W. Murray, and E.D. Salmon. 2000. Visualization of Mad2 dynamics at kinetochores, along spindle fibers, and at spindle poles in living cells. *J Cell Biol* **150**: 1233-50.

Jablonski, S.A., G.K. Chan, C.A. Cooke, W.C. Earnshaw, and T.J. Yen. 1998. The hBUB1 and hBUBR1 kinases sequentially assemble onto kinetochores during prophase with hBUBR1 concentrating at the kinetochore plates in mitosis. *Chromosoma* **107**: 386-96.

Kalitsis, P., E. Earle, K.J. Fowler, and K.H. Choo. 2000. Bub3 gene disruption in mice reveals essential mitotic spindle checkpoint function during early embryogenesis. *Genes Dev* **14**: 2277-82.

Kinzler, K.W. and B. Vogelstein. 1996. Lessons from hereditary colorectal cancer. *Cell* **87**: 159-70.

Kung, A.L., S.W. Sherwood, and R.T. Schimke. 1990. Cell line-specific differences in the control of cell cycle progression in the absence of mitosis. *Proc Natl Acad Sci U S A* **87**: 9553-7.

Lee, L., J.S. Tirnauer, J. Li, S.C. Schuyler, J.Y. Liu, and D. Pellman. 2000. Positioning of the mitotic spindle by a cortical-microtubule capture mechanism. *Science* **287**: 2260-2.

Lengauer, C., K.W. Kinzler, and B. Vogelstein. 1998. Genetic instabilities in human cancers. *Nature* **396**: 643-9.

Levy, D.B., K.J. Smith, Y. Beazer-Barclay, S.R. Hamilton, B. Vogelstein, and K.W. Kinzler. 1994. Inactivation of both APC alleles in human and mouse tumors. *Cancer Res* **54**: 5953-8.

Lu, B., L.Y. Jan, and Y.N. Jan. 1998. Asymmetric cell division: lessons from flies and worms. *Curr Opin Genet Dev* **8**: 392-9.

Martinez-Exposito, M.J., K.B. Kaplan, J. Copeland, and P.K. Sorger. 1999. Retention of the BUB3 checkpoint protein on lagging chromosomes. *Proc Natl Acad Sci U S A* **96**: 8493-8.

McCartney, B.M., H.A. Dierick, C. Kirkpatrick, M.M. Moline, A. Baas, M. Peifer, and A. Bejsovec. 1999. Drosophila APC2 is a cytoskeletally-associated protein that regulates wingless signaling in the embryonic epidermis. *J Cell Biol* **146**: 1303-18.

McCartney, B.M. and M. Peifer. 2000. Teaching tumour suppressors new tricks. *Nat Cell Biol* **2**: E58-60.

Mimori-Kiyosue, Y., N. Shiina, and S. Tsukita. 2000. Adenomatous polyposis coli (APC) protein moves along microtubules and concentrates at their growing ends in epithelial cells. *J Cell Biol* **148**: 505-18.

Morgenstern, J.P. and H. Land. 1990. Advanced mammalian gene transfer: high titre retroviral vectors with multiple drug selection markers and a complementary helper-free packaging cell line. *Nucleic Acids Res* **18**: 3587-96.

Moser, A.R., H.C. Pitot, and W.F. Dove. 1990. A dominant mutation that predisposes to multiple intestinal neoplasia in the mouse. *Science* **247**: 322-4.

Muhua, L., N.R. Adames, M.D. Murphy, C.R. Shields, and J.A. Cooper. 1998. A cytokinesis checkpoint requiring the yeast homologue of an APC- binding protein. *Nature* **393**: 487-91.

Munemitsu, S., I. Albert, B. Souza, B. Rubinfeld, and P. Polakis. 1995. Regulation of intracellular beta-catenin levels by the adenomatous polyposis coli (APC) tumor-suppressor protein. *Proc Natl Acad Sci U S A* **92**: 3046-50.

Munemitsu, S., B. Souza, O. Muller, I. Albert, B. Rubinfeld, and P. Polakis. 1994. The APC gene product associates with microtubules in vivo and promotes their assembly in vitro. *Cancer Res* **54**: 3676-81.

Nathke, I.S. 1999. The adenomatous polyposis coli protein. *Mol Pathol* **52**: 169-73.

Nathke, I.S., C.L. Adams, P. Polakis, J.H. Sellin, and W.J. Nelson. 1996. The adenomatous polyposis coli tumor suppressor protein localizes to plasma membrane sites involved in active cell migration. *J Cell Biol* **134**: 165-79.

Peifer, M. and P. Polakis. 2000. Wnt signaling in oncogenesis and embryogenesis--a look outside the nucleus. *Science* **287**: 1606-9.

Polakis, P. 1995. Mutations in the APC gene and their implications for protein structure and function. *Curr Opin Genet Dev* **5**: 66-71.

Roberts, B.T., K.A. Farr, and M.A. Hoyt. 1994. The Saccharomyces cerevisiae checkpoint gene BUB1 encodes a novel protein kinase. *Mol Cell Biol* **14**: 8282-91.

Rosin-Arbesfeld, R., G. Ihrke, and M. Bienz. 2001. Actin-dependent membrane association of the APC tumour suppressor in polarized mammalian epithelial cells. *EMBO J* **20**: 5929-39.

Rubinfeld, B., I. Albert, E. Porfiri, C. Fiol, S. Munemitsu, and P. Polakis. 1996. Binding of GSK3beta to the APC-beta-catenin complex and regulation of complex assembly. *Science* **272**: 1023-6.

Shih, I.M., J. Yu, T.C. He, B. Vogelstein, and K.W. Kinzler. 2000. The beta-catenin binding domain of adenomatous polyposis coli is sufficient for tumor suppression. *Cancer Res* **60**: 1671-6.

Smith, K.J., D.B. Levy, P. Maupin, T.D. Pollard, B. Vogelstein, and K.W. Kinzler. 1994. Wild-type but not mutant APC associates with the microtubule cytoskeleton. *Cancer Res* **54**: 3672-5.

Sparks, A.B., P.J. Morin, B. Vogelstein, and K.W. Kinzler. 1998. Mutational analysis of the APC/beta-catenin/Tcf pathway in colorectal cancer. *Cancer Res* **58**: 1130-4.

Su, L.K., K.W. Kinzler, B. Vogelstein, A.C. Preisinger, A.R. Moser, C. Luongo, K.A. Gould, and W.F. Dove. 1992. Multiple intestinal neoplasia caused by a mutation in the murine homolog of the APC gene [published erratum appears in Science 1992 May 22;256(5060):1114]. *Science* **256**: 668-70.

Taylor, S.S., E. Ha, and F. McKeon. 1998. The human homologue of Bub3 is required for kinetochore localization of Bub1 and a Mad3/Bub1-related protein kinase. *J Cell Biol* **142**: 1-11.

Taylor, S.S. and F. McKeon. 1997. Kinetochore localization of murine Bub1 is required for normal mitotic timing and checkpoint response to spindle damage. *Cell* **89**: 727-35.

Tetsu, O. and F. McCormick. 1999. Beta-catenin regulates expression of cyclin D1 in colon carcinoma cells. *Nature* **398**: 422-6.

Tirnauer, J.S., E. O'Toole, L. Berrueta, B.E. Bierer, and D. Pellman. 1999. Yeast Bim1p promotes the G1-specific dynamics of microtubules. *J Cell Biol* **145**: 993-1007.

Trzepacz, C., A.M. Lowy, J.J. Kordich, and J. Groden. 1997. Phosphorylation of the tumor suppressor adenomatous polyposis coli (APC) by the cyclin-dependent kinase p34. *J Biol Chem* **272**: 21681-4.

Yu, X., L. Waltzer, and M. Bienz. 1999. A new Drosophila APC homologue associated with adhesive zones of epithelial cells. *Nat Cell Biol* **1**: 144-51.

Zumbrunn, J., K. Kinoshita, A.A. Hyman, and I.S. Nathke. 2001. Binding of the Adenomatous Polyposis Coli Protein to Microtubules Stabilizes Microtubules in vitro and invivo and is regulated by GSK3B phosphorylation. *Current Biology* **11**: 44-9.

Chapter 3

The Spindle Assembly Checkpoint is Not Essential for the Viability of Mouse Cells

The work presented in this chapter was completed solely by me and is now being prepared for publication.

Abstract

Tumor cells often contain an abnormal number of chromosomes and the prognosis for a cancer patient gets worse as the degree of aneuploidy in their tumor increases (Baba et al. 2002). Previous work has shown that the spindle assembly checkpoint prevents aneuploidy (Hoyt et al. 1991; Li and Murray 1991). Thus, investigating how members of the spindle assembly checkpoint arrest mitotic cells to prevent chromosome missegregation is an important step in understanding the cellular mechanisms that affect tumorigenesis. Previous work with $Mad2^{-/-}$ mice has suggested that metazoan cells lacking a spindle assembly checkpoint suffer from DNA damage incurred at cytokinesis and undergo apoptosis (Dobles et al. 2000). Supporting this theory are the findings that DNA damage due to irradiation or aberrant exit from mitosis leads to p53-dependent cell cycle arrest and apoptosis in gastrulating embryos and fibroblast cell line, respectively (Lanni and Jacks 1998; Heyer et al. 2000). These results prompted me to generate mice containing disruptions in both Mad2 and p53. In this chapter I provide evidence that heterozygous or nullizygous disruptions of p53 rescue $Mad2^{-/-}$ blastocyst lethality in culture. I also show that some $Mad2^{-/-}$ embryos survive to E10.5, allowing for both the histological analysis of the nullizygous mice and for the generation and analysis of a mouse embryonic cell line that lacks the spindle assembly checkpoint. These results demonstrate that the spindle assembly checkpoint is not essential for the viability of mammalian cells, though cultured $Mad2^{-/-}$ $p53^{-/-}$ cells are aneuploid and undergo aberrant chromosome segregation during 80% of the observed mitoses.

Introduction

One hallmark of tumor cells is genomic instability. A small fraction of solid tumors displays increased levels of instability in the sequences of the repetitive DNA, or microsatellites, that are distributed throughout the genome. Tumor cells with microsatellite instability (MIN) maintain a diploid karyotype, but most tumor cells are not MIN. The majority of solid tumors are aneuploid, frequently gaining or losing numerous chromosomes. The two forms of genomic instability, one within microsatellites and the other at the chromosomal level, appear to be mutually exclusive (Loeb 1991; Lengauer et al. 1998). Human colorectal cancer cells with chromosome instability (CIN) fail to arrest at mitosis in the presence of microtubule-destabilizing drugs, suggesting that CIN may be due to mutations in the spindle assembly checkpoint (Cahill et al. 1998). This checkpoint makes the initiation of chromosome segregation dependent upon the successful completion of kinetochore-microtubule attachment, thereby ensuring that the chromosomes will be equally partitioned into daughter cells (see Chapter 1, section II.D. for a detailed explanation of this cell cycle checkpoint). Mutations, immunodepletions and RNA interference experiments have shown that treatments which compromise the spindle assembly checkpoint signaling pathway reduce the average time a cell spends in mitosis, eliminate arrest in response to microtubule-depolymerizing drugs, and result in an aberrant exit from mitosis (Li and Benezra 1996; Taylor and McKeon 1997; Cahill et al. 1998; Chen et al. 1998; Gorbsky et al. 1998; Waters et al. 1998; Basu et al. 1999).

Experiments in budding yeast have shown that the spindle assembly checkpoint is not essential for cellular viability. In contrast, all targeted disruptions of spindle assembly checkpoint genes in metazoans have proven lethal thus far, suggesting that the model of yeast checkpoints as auxiliary regulators of the cell cycle does not apply to higher eukaryotes (Basu et al. 1999; Kitagawa and Rose 1999; Dobles et al. 2000; Kalitsis et al. 2000). Efforts to study this signaling pathway through genetic approaches have proven difficult due to the early embryonic lethality of mice that contain homozygous deletions of either the Bub3 or Mad2 gene (Dobles et al. 2000; Kalitsis et al. 2000). The dissection of embryos at day E9.5-E13.5 from Mad2 heterozygous crosses and embryos at day E8.5 from Bub3 heterozygous crosses yielded no homozygous null progeny. Therefore, these nullizygous organisms could only be analyzed as E3.5 blastocysts that were explanted for growth in culture. Nullizygous blastocysts that lacked either Mad2 or Bub3 died within four days of harvesting. This suggested that mice lacking a spindle assembly checkpoint protein die during embryonic gastrulation, a developmental stage that requires rapid cell division (Dobles et al. 2000; Kalitsis et al. 2000). Mouse embryos have been shown to be hypersensitive to DNA damage during gastrulation, triggering p53-dependent apoptosis that kills the entire embryo (Heyer et al. 2000). Consistent with the role of the spindle assembly checkpoint proteins in monitoring kinetochore-microtubule attachment and preventing the premature initiation of chromosome segregation, cells from embryos lacking Mad2 or Bub3 undergo cytokinesis with lagging chromosomes present at the spindle midzone (Dobles et al. 2000; Kalitsis et al. 2000). It is thought that cytokinesis breaks the lagging chromosome, activating the hypersensitive DNA-damage checkpoint at gastrulation to trigger

apoptosis. In this chapter I will describe the extensive harvesting of embryos from Mad2 heterozygous crosses to retrieve a small number of embryos that do progress through gastrulation without the aid of the spindle assembly checkpoint. These embryos are smaller than their littermates, show regions of increased apoptosis and contain cells that proceed through anaphase even when some chromosomes are not attached to the mitotic spindle.

The p53 pathway has also been shown to affect the viability of cells undergoing abnormal mitoses in culture. A careful characterization of the role of p53 following treatment with microtubule destabilizing drugs showed that p53 is necessary for the G1 arrest and apoptosis of cells that have exited mitosis without properly segregating their chromosomes (Lanni and Jacks 1998). In this chapter I show that $Mad2^{-/-}$ $p53^{+/-}$ and $Mad2^{-/-}$ $p53^{-/-}$ (Donehower et al. 1992) blastocysts survive indefinitely in culture, though $Mad2^{-/-}$ $p53^{+/+}$ blastocysts die within 5 days of harvesting. A disruption in p53 also increases the survival rate of $Mad2^{-/-}$ embryos at day 10.5, and a $Mad2^{-/-}$ $p53^{-/-}$ (double knockout) embryo was used to generate a viable, immortal cell line that lacks a spindle assembly checkpoint. I observe that 80% of cells lacking Mad2 fail to segregate their chromosomes properly during any given round of cell division. This results in the generation of cells with multiple nuclei that are connected by a DNA bridge in G1 as well as many aneuploid progeny cells.

Results

p53 disruption is sufficient to rescue cultured Mad2 $^{-/-}$ blastocysts

Previous work has shown that Mad2$^{-/-}$ blastocysts die within 5 days of being placed in culture, a time point equivalent to gastrulation, and that viable Mad2 nullizygous embryos *in utero* cannot be recovered after gastrulation (Dobles et al. 2000). To study the possibility that the introduction of homozygous p53 deletions would rescue this lethality, I interbred the Mad2$^{-/-}$ mice with mice containing deletions in p53 (Donehower et al. 1992). Two types of breeding pairs were established to recover blastocysts with each possible combination of Mad2 and p53 genotype. The first type of breeding scheme intercrossed mice that are heterozygous for both the Mad2 and p53 gene disruptions. To increase the likelihood of recovering Mad2 nullizygous blastocysts that contain one or two disrupted copies of p53, the second type of cross bred Mad2$^{+/-}$ p53$^{-/-}$ male mice to females that were heterozygous for both Mad2 and p53 gene disruptions. The later strategy is more efficient because females lacking p53 are born at only 1/3 the expected frequency and are often infertile.

Consistent with an earlier report (Dobles et al. 2000), I observed that the yield of Mad2$^{-/-}$ blastocysts was lower than Mendelian frequencies would predict (Figure 3.1). The inner cell mass of Mad2$^{-/-}$ p53$^{+/+}$ blastocysts that are explanted to culture stops proliferating after 3 days , and very few cells persist to day 5 (Figure 3.2b). However,

death of the cultured nullizygous blastocysts can be rescued by a disruption in the p53 gene (Figure 3.2 c + d). Wild type blastocysts can survive for many weeks in culture, and I notice that any Mad2$^{-/-}$ blastocysts containing either heterozygous or homozygous deletions in p53 grow at a rate that is comparable to wild type blastocysts for at least four weeks (data not shown and Figure 3.2 e + f). Rescue of Mad2$^{-/-}$ blastocysts by a p53 heterozygous mutation was unexpected, but not unprecedented, because the embryonic lethality of mice lacking either DNA ligase IV or Xrcc4 proteins can be rescued by p53 heterozygosity (Frank et al. 2000; Gao et al. 2000). DNA ligase 4 and Xrcc4 are both components of the non-homologous, DNA end-joining apparatus, and the rescue of these lethalities emphasize the role of p53 in the destruction of cells that incur double strand breaks.

The Mad2$^{-/-}$ p53-disrupted blastocysts develop, as wild type blastocysts do, into large embryoid bodies (Figure 3.2 e + f). I conclude that a reduction in the cellular levels of p53 rescues blastocyst lethality caused by the lack of Mad2. The inner cell mass of a Mad2$^{-/-}$ p53$^{+/+}$ does not survive in culture. In contrast, Mad2$^{-/-}$ blastocysts proliferate efficiently , forming a mass of cells that measures approximately 2mm in diameter after four weeks of growth in culture. Although the lack of p53 has been shown to confer immortality to cell lines, a heterozygous disruption of p53 does not. Therefore, the extended survival of Mad2$^{-/-}$ p53$^{+/-}$ blastocysts demonstrates that immortalization is not required for their rescue. Additionally, I believe these results reinforce the original

model of cell cycle checkpoints that had been developed in yeast; the spindle assembly checkpoint is not essential for cell viability or for proliferation (Hartwell and Weinert 1989).

Mad2$^{-/-}$ embryos display dilated tissues, elevated levels of apoptosis and chromosome missegregation at E 10.5

To determine the impact of p53 mutations on Mad2$^{-/-}$ embryonic lethality in vivo, I recovered embryos from Mad2 heterozygous, p53 heterozygous intercrosses. In contrast to previous reports, I found that some Mad2 nullizygous embryos do survive through gastrulation, though their yield decreases dramatically at each developmental stage (Figure 2.1). From heterozygous intercrosses, the ratio of nullizygous progeny to wild type progeny should be 1:1. Only 72 % of the expected Mad2$^{-/-}$ pre-implantation blastocysts can be recovered at E3.5, suggesting that a Mad2 deficiency can cause lethality within the first cell divisions of embryogenesis. Gastrulation begins at day E6.5, and 23 % of the expected embryos are still viable at day E10.5, but no Mad2$^{-/-}$ pups have been born. Thus far, nearly 600 pups have been born from Mad2$^{+/-}$ intercrosses, and the observed 1:2 ratio of wild type to heterozygous pups (data not shown) from these crosses demonstrates that the decreased recovery of Mad2$^{-/-}$ blastocysts is not due to a reduced fitness of the Mad2-null gametes.

Survival of $Mad2^{-/-}$ embryos to E10.5 does not absolutely require a disruption in p53; of the 42 $p53^{+/+}$ embryos recovered, two are Mad2 nullizygous. I fixed one of these $Mad2^{-/-}$ $p53^{+/+}$ embryos as well as its $Mad2^{+/+}$ $p53^{+/+}$ littermate for histological analysis (Figures 3 and 4). The nullizygous embryo is approximately half the size of its littermates, and the thinner walls of the heart, liver and brain suggest that its tissues are not being generated at the same rate as the organs and tissues of embryos containing Mad2 protein (Figure 3.3a + b). Consistent with this theory, nervous tissues of the $Mad2^{-/-}$ embryo are apoptotic, and these dead cells are recognizable after haematoxylin and eosin staining as dark, solid circles of pyknotic nuclei (Figure 3.3e+f). In the control embryos, regions of the nervous tissues are undergoing mitosis, (Figure 3.3b and Figure 3.4b+c), and cells in these tissues are rarely apoptotic. The Mad2 knockout embryo, on the other hand, does not display even one area of tissue that contains more than a few mitotic cells within the field of view, and even this was a rare occurrence (data not shown). Several mitotic cells in the Mad2 nullizygous embryo exhibit a bridge of DNA connecting the two clusters of pole-proximal chromosomes (Figure 3.4e+f). Chromosome bridges occur when a chromosome does not attach to the spindle microtubules and is left floating in the midzone at anaphase. This type of mitotic error has previously been seen in presumptive Mad2 null embryos at E6.5 (Dobles et al. 2000). From these and previous data, I postulate that highly proliferative embryonic tissues cannot successfully complete the necessary rounds of rapid cell division without a functional spindle assembly checkpoint. The cells initiate anaphase before all sister

chromatids have attached to the spindle microtubules, and the resulting chromosome missegregation events trigger p53-dependent programmed cell death.

Deletion of only one copy of p53 increases survival of Mad2 embryos *in utero*

While mutations in p53 are not required for the post-gastrulation survival of Mad2$^{-/-}$ embryos, I find that the yield of Mad2$^{-/-}$ p53$^{+/-}$ embryos is 4 times that of Mad2$^{-/-}$ p53$^{+/+}$ embryos at E 10.5. After harvesting 150 embryos from Mad2 heterozygous intercrosses (which include all possible p53 genotypes), I collected 22 Mad2$^{+/+}$ p53$^{+/-}$ embryos and 9 Mad2$^{-/-}$ p53$^{+/-}$ embryos. Thus, approximately 41% of the expected Mad2 nullizygous embryos survive in the presence of a p53 heterozygous deletion. This contrasts with the approximate 14% survival of Mad2 nullizygous p53$^{+/+}$ embryos (14 wild type embryos vs. 2 knockout embryos). Due to the small numbers in these populations, extensive statistical analysis to determine the significance of this data is still in progress. It should be noted that the increased survival of Mad2$^{-/-}$ p53$^{+/-}$ embryos is not a complete rescue of the developmental defects. The Mad2 nullizygous embryos have progressed through gastrulation and have begun developing organs as well as the basic structural features of an embryo, but they are generally one-fifth to one-third the size of their Mad2$^{+/+}$ and Mad2$^{+/-}$ littermates. A significant number of the Mad2$^{-/-}$ p53$^{+/-}$ embryos show gestational retardation (Figure 3.5a lanes 1, 3+4) that is more

severe than that of the Mad2$^{-/-}$ p53$^{+/+}$ embryo in Figures 3.3d and 3.4d. The Mad2$^{-/-}$ p53$^{+/-}$ embryos often resemble a healthy, E9-E10 embryo. These data suggest that either the Mad2$^{-/-}$ embryos are developmentally delayed, or that the Mad2$^{-/-}$ embryos are dying between day E9 and E10 to provide smaller corpses rather than viable embryos. Both of the Mad2$^{-/-}$ p53$^{+/+}$ embryos that were recovered had beating hearts when they were removed from the placenta, demonstrating their systemic viability at E10.5. None of the Mad2$^{-/-}$ p53$^{+/-}$ embryos had progressed to a gestational stage that would generate a beating heart, so there was no immediate way to know if the embryos were still viable.

Generation of an immortal MEF line lacking Mad2 and p53

To determine if Mad2$^{-/-}$ p53$^{+/-}$ embryos are still viable upon recovery at E10.5, embryos from Mad2$^{+/-}$ p53$^{+/-}$ females who were crossed to Mad2$^{+/-}$ p53$^{-/-}$ males were harvested at E10.5, disaggregated and placed in culture. PCR genotyping was performed on the yolk sacs from each of the 106 harvested embryos (Figure 3.5b+c). Cells from nine Mad2$^{-/-}$ p53$^{+/-}$ embryos were placed in culture, but all of these cells underwent senescence after 2-3 passages (data not shown). The recovery and passage of viable cells from Mad2$^{-/-}$ p53$^{+/-}$ embryos confirms that the embryos are probably gestationally retarded rather than un-absorbed embryos that had died at an earlier developmental stage.

Only one Mad2$^{-/-}$ p53$^{-/-}$ embryo was discovered at E10.5 (Figure 3.5 lane 4), and it was disaggregated to generate an immortal cell line AB98. Control cell lines AB100 (Mad2$^{+/-}$ p53$^{-/-}$, Figure 3.5 lane 5) and AB103 (Mad2$^{+/+}$ p53$^{-/-}$, Figure 3.5 lane 8) were generated from littermates of the double knockout embryo and cultured in parallel with AB98. All three cell lines are p53 nullizygous (Figure 3.5c) and proliferate at similar rates (data not shown). To confirm the genotype of each cell line, trypsinized cells were collected from dishes of AB98, AB100 and AB103 and genotyped by PCR (Figure 3.6a). To confirm the abrogation of the spindle assembly checkpoint in AB98, I treated each cell line with 200 ng/mL nocodazole for 12 hours and looked for rounded cells (Figure 3.6 b – g). To quantitate the percentage of rounded cells in each dish, I collected the floating cells and the trypsinized adherent cells separately and counted the cells with a hemocytometer. Mad2 wild type and heterozygous cell lines treated with nocodazole displayed mitotic indexes of nearly 70% while only 4% of the Mad2 nullizygous cells rounded up following nocodazole treatment. Thus, the study of Mad2$^{-/-}$ embryos at E10.5 provides observations that parallel the results seen in cultured blastocysts. Mutations in p53 increase the survival of Mad2$^{-/-}$ embryos and allow for the extended culturing of Mad2 nullizygous cell even though these cells do not contain a functional spindle assembly checkpoint.

Chromosome instability in Mad2-deficient cells

It has been shown that the heterozygous disruption of Mad2 by targeted deletion is sufficient to induce chromosome instability (CIN) in the chromosomally stable HCT116 human colon carcinoma cell line (Michel et al. 2001). Mice that are heterozygous for a Mad2 disruption also develop lung tumors at a high frequency, suggesting that Mad2 halpoinsufficiency may lead to CIN and tumorigenesis in living organisms as well (Michel et al. 2001). To examine whether heterozygous or homozygous deletion of the Mad2 gene alters the rate of chromosome loss, cultures of AB98, AB100 and AB103 were collected at passage 8 and analyzed by flow cytometry for DNA content. Consistent with the previous observations, I find that the Mad2$^{+/-}$ cell line is highly aneuploid. In fact, a majority of cells from AB100 appear to be at least tetraploid (Figure 3.7, green line).

Although the deletion of p53 has been reported to induce aneuploidy, a majority of the Mad2$^{+/+}$ p53$^{-/-}$ cells contain a normal chromosome complement. Therefore, the chromosome instability seen in the Mad2$^{+/-}$ p53$^{-/-}$ cell line is not due to its p53 status. This data suggests that a Mad2$^{+/-}$ genotype may result in haploinsufficiency in the spindle assembly checkpoint signaling pathway. Additionally, AB98, which is nullizygous for Mad2, presents a DNA profile that suggests these cells are typically 6N in G1 and then 12N following DNA replication (Figure 3.7, pink line). Like most tumor

cells, AB98 and AB 100 show chromosome instability and these data support the view that a reduction in the activity of the spindle assembly checkpoint provides a mechanism for the generation of cells that have an increased probability of losing tumor suppressor genes or gaining oncogenes.

Mad2-/- p53-/- cells undergo anaphase with unattached chromosomes

To examine the process of chromosome segregation in the absence of the spindle assembly checkpoint, I grew cells from lines AB98, AB 100 and AB103 on coverslips, fixed the cells and stained their DNA. The mitotic index of each of the three cell lines is roughly 3% as determined by counting the number of cells with and without condensed chromosomes. I then located every anaphase cell on each of the coverslips. In Mad2$^{+/+}$ cells, anaphase is almost always characterized by a sharp separation between bipolar clusters of chromosomes (Figure 3.8a). In contrast, nearly 80% of Mad2 nullizygous cells fail to separate their metaphase chromosomes properly (Figure 3.8b). In most cases, one chromosome or a piece of a chromosome appears between the DNA masses, lagging behind the others. Strikingly, some Mad2 nullizygous cells contain multiple chromosomes that have not segregated with the bulk of the DNA (Figure 3.8c). It appears that the cytokinetic furrow constricts around the lagging chromosomes, giving rise to cells with a dumbbell morphology (Figure 3.8d), and the cytokinetic furrow may sever the DNA to allow for cytokinesis. This phenotype mimics that of fission yeast cut mutations, which cause cytokinesis in the absence of normal nuclear division (Uemura

and Yanagida 1984). At least two Mad2 nullizygous cells also contain joined sister chromatids that will both remain in one cell rather than being partitioned to each daughter cell (Figure 3.8e). This phenomenon is called non-disjunction, and results in the creation of daughter cells that are trisomic and monosomic for that chromosome. The appearance of these sister chromatids that remain attached by cohesin after the initiation of anaphase suggests that other pairs of sister chromatids may also experience non-disjunction, but are located near the bulk of the DNA and are therefore indistinguishable with this assay. Consistent with the FACS analysis of DNA content, cell line AB100, which is Mad2 heterozygous, displays an intermediate rate of chromosome missegregation. Mad2$^{+/-}$ p53$^{-/-}$ cells show a 6-fold increase in the appearance of abnormal anaphases as compared to the Mad2$^{+/-}$ p53$^{-/-}$ control cells (Figure 3.8). This data supports the view that Mad2 heterozygosity reduces the ability of the spindle assembly checkpoint to monitor kinetochore-microtubule attachment during mitosis.

Figure 3.1

The gene dosage of p53 affects the post-gastrulation survival of $Mad2^{-/-}$ embryos.

* The observed ratio of 2 $Mad2^{+/-}$ pups for each 1 $Mad2^{-+/+}$ pup in litters from Mad2 heterozygous intercrosses (regardless of p53 genotype) demonstrates that the Mad2- and Mad2+ gametes are produced at similar levels and are equally fit. $Mad2^{-/-}$ blastocysts are recovered at approximately 73% of the expected rate, regardless of p53 genotype (n=72). Mad2 $^{-/-}$ $p53^{+/-}$ embryos display increased survival rates of 41% at day E10.5 as compared to Mad2 nullizygous embryos that are either $p53^{+/+}$ or $p53^{-/-}$, which are recovered at rates of 14% and 6%, respectively (n=150).

Figure 3.1

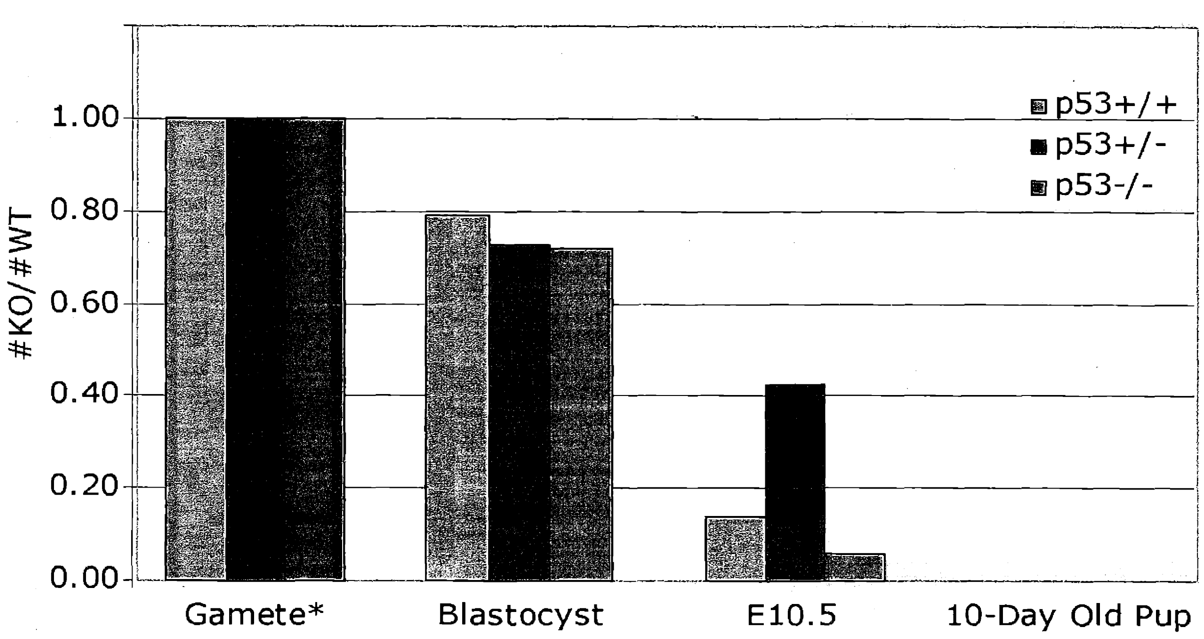


Figure 3.2

A disruption in p53 rescues lethality of Mad2 cultured blastocysts.

E3.5 blastocysts from Mad2 heterozygous intercrosses were cultured in vitro for 5 or 28 days and then photographed using phase contrast microscopy. Genotypes were determined by PCR. **b.** The inner cell mass of Mad2$^{-/-}$ p53$^{+/+}$ cultured blastocysts fails to proliferate. **c,d.** Heterozygous or nullizygous disruptions in p53 rescue the lethality of Mad2$^{-/-}$ cultured blastocysts, allowing them to grow at rates similar to those of Mad2$^{+/+}$ blastocysts (**a**). **e,f.** In 28-day outgrowths, Mad2$^{-/-}$ cultured blastocysts that have been rescued by a mutation in p53 form large embryoid bodies.

Figure 3.2

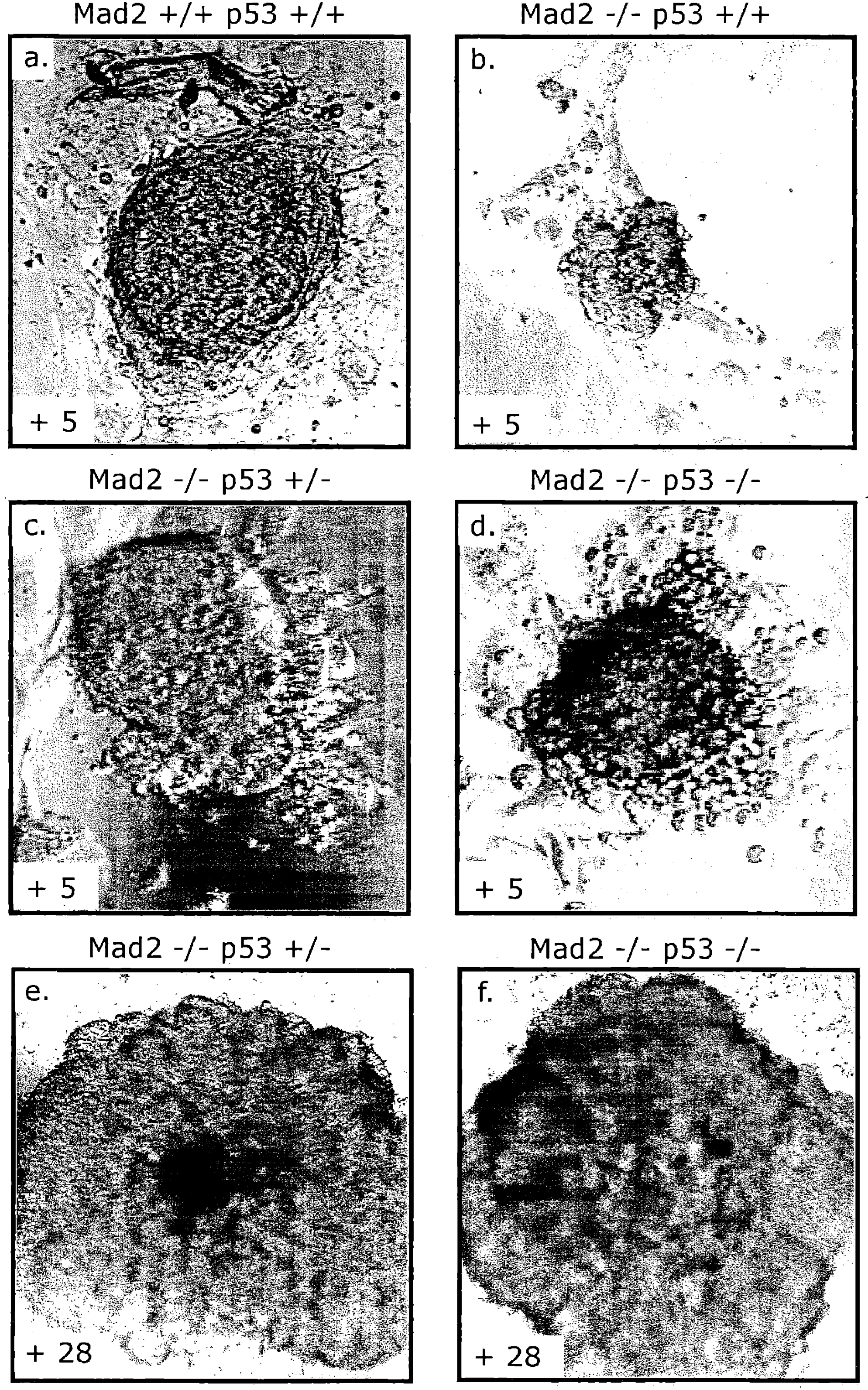


Figure 3.3

Viable Mad2$^{-/-}$ embryos contain increased levels of apopotic cells.

Histological analysis of progeny from a Mad2 heterozygous intercross. A Mad2$^{+/+}$ p53$^{+/+}$ embryo (**a.**) and its smaller Mad2$^{-/-}$ p53$^{+/+}$ littermate (**b.**) were harvested at E10.5, fixed in 4% paraformaldehyde, sectioned and stained with haematoxylin and eosin. Yellow boxes in **a** and **b** designate the areas of 25x magnification seen in b,c,e+f. **c.** Mitotic cells are identified by the presence of elliptical areas of dense haematoxylin stain, indicated by 'M'. **d,f.** Apoptosis was identified by the presence of pyknotic nuclei (dense circles of haematoxylin stain).

Figure 3.3

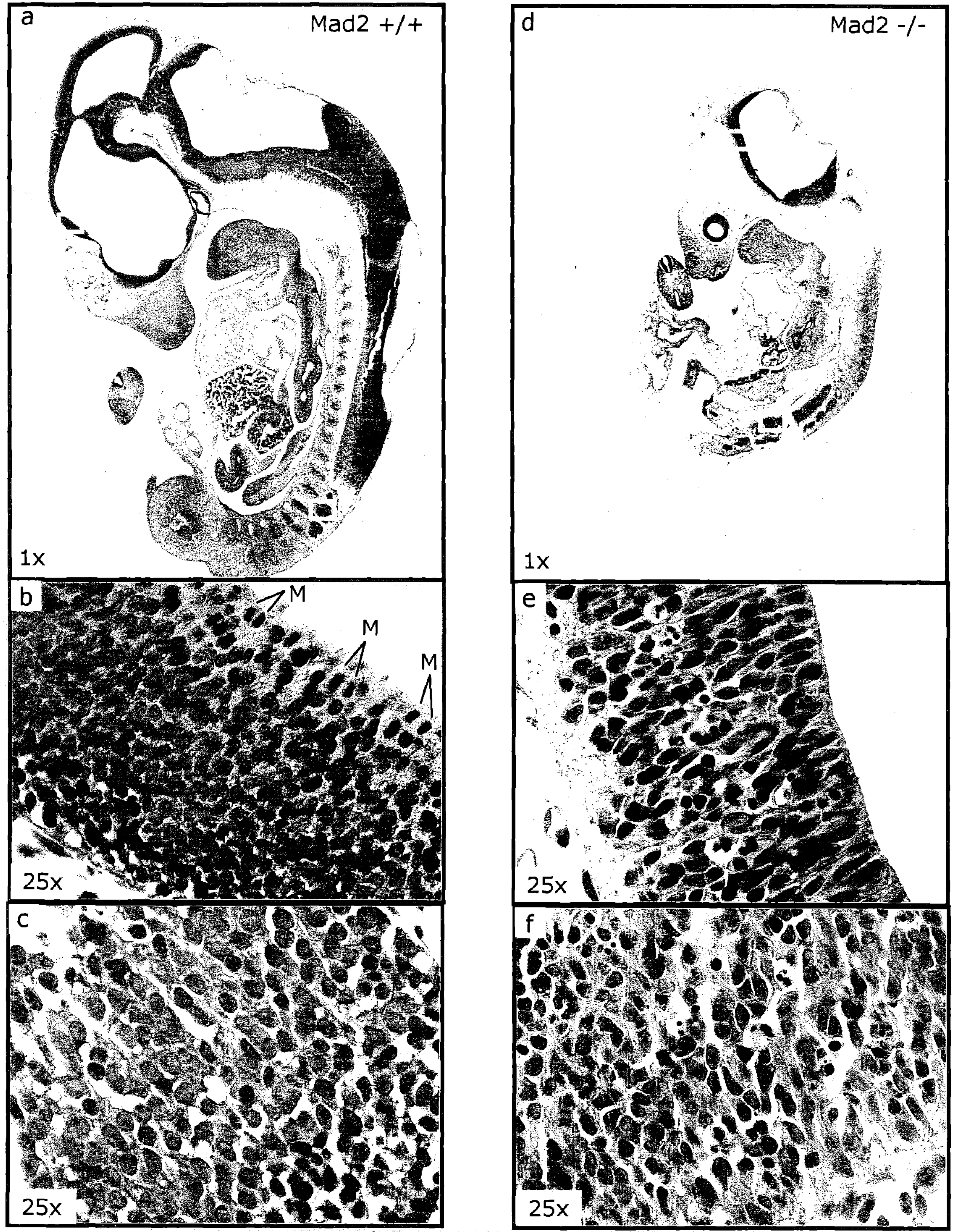


Figure 3.4

Analysis of chromosome missegregation in cells of a Mad2$^{-/-}$ embryo.

Histological analysis of progeny from a Mad2 heterozygous intercross. **a.** A Mad2$^{+/+}$ p53$^{+/+}$ embryo and its smaller Mad2$^{-/-}$ p53$^{+/+}$ littermate **(b.)** were harvested at E10.5, fixed in 4% paraformaldehyde, sectioned and stained with haematoxylin and eosin. The yolk sac of each embryo was digested in proteinase K and genotyped by PCR. Yellow boxes in **a** and **b** designate areas of 25x magnification seen in **b,c,e+f** for the presentation of spindle morphologies. Mitotic cells are identified by the presence of elliptical areas of dense haematoxylin stain. **a,b.** Cells in the wild type embryo had apparently normal spindle morphologies. **e,f.** A significant fraction of anaphase cells (indicated by arrows) in the Mad2 nullizygous embryo contain one or more chromosomes clearly separated from the bulk of DNA.

Figure 3.4

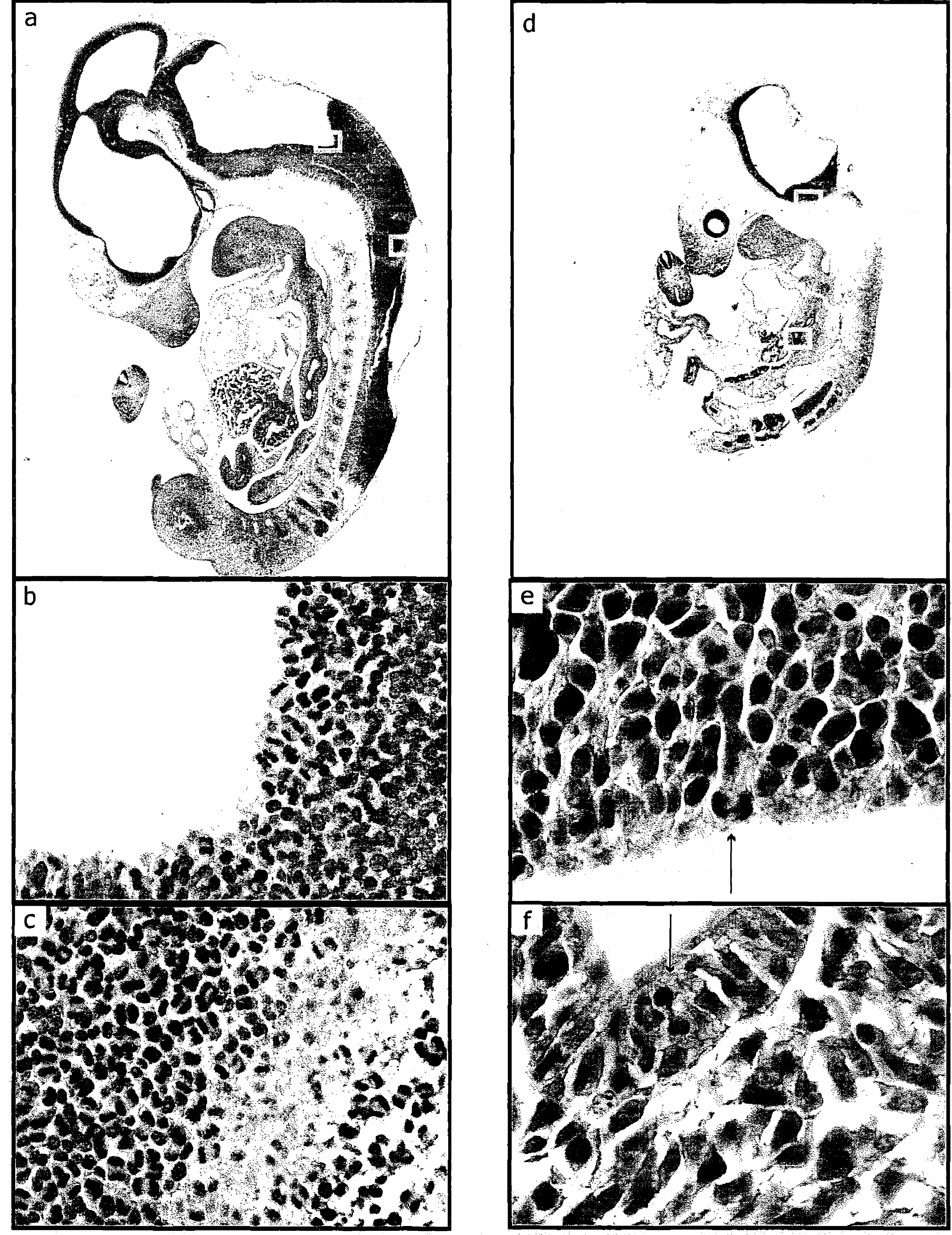


Figure 3.5

Mad2$^{-/-}$ p53$^{+/-}$ and Mad2$^{-/-}$ p53$^{-/-}$ embryos show dramatic developmental retardation.

Embryos from Mad2$^{+/-}$ p53$^{+/-}$ x Mad2$^{+/-}$ p53$^{-/-}$ matings were harvested at E10.5, placed in 24- well tissue culture plates and photographed. **a.** Embryos recovered from one pregnant mother. Genomic DNA was prepared from the yolk sacks of each embryo and subjected to PCR analysis at the Mad2 (**b.**) and p53 (**c.**) loci. Following mechanical disaggregation in trypsin, the cells from each embryo were placed in culture in 24- or 96-well plates, depending on the size of the embryo, and given a cell culture number (**d.**).

Figure 3.5

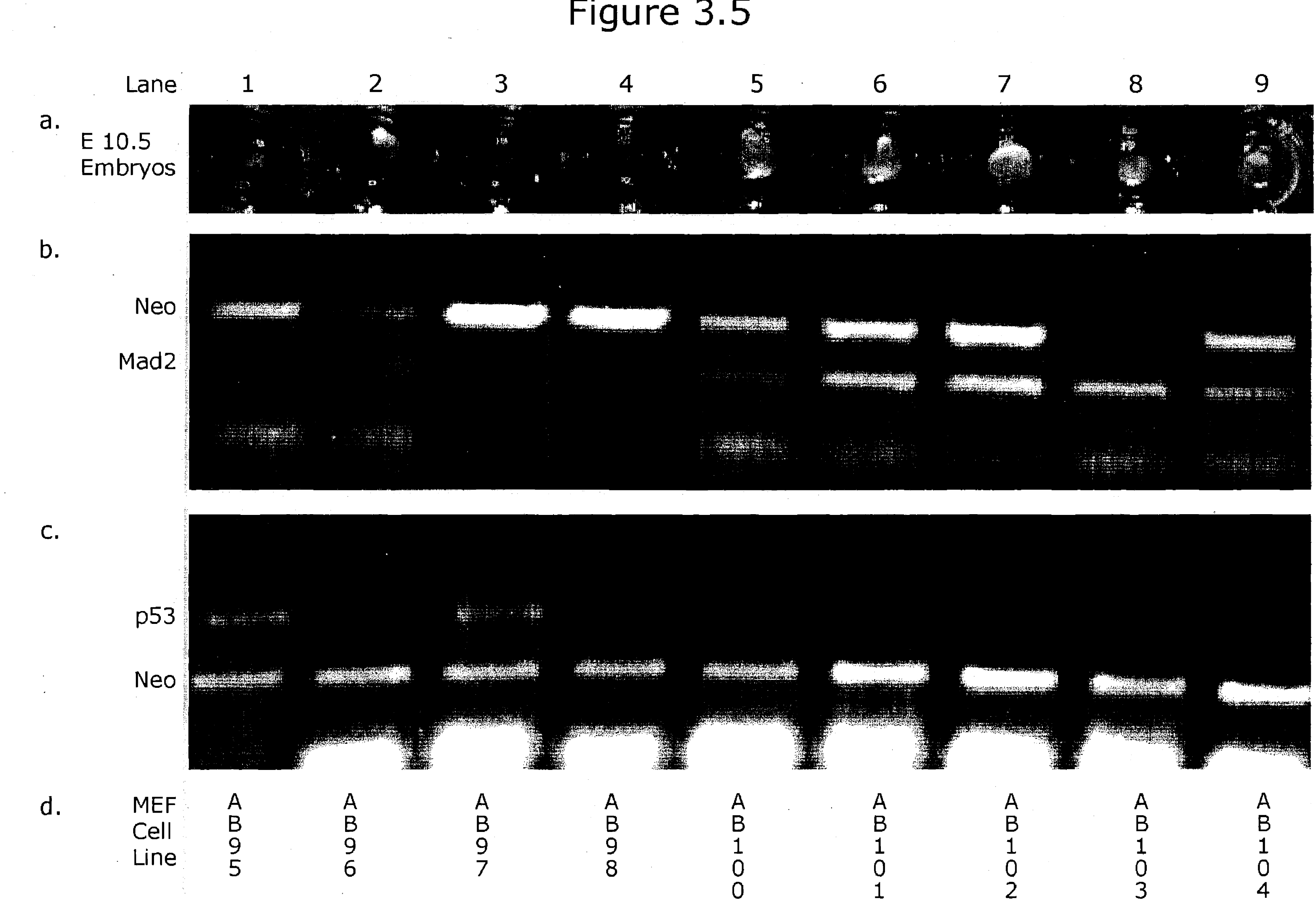
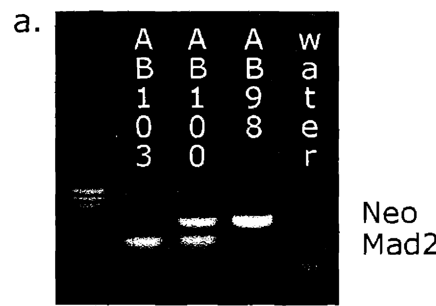


Figure 3.6

Mitotic arrest of cycling mouse embryonic fibroblasts requires Mad2.

a. Genomic DNA was prepared from cycling fibroblast cells of AB103, AB100 and AB98 and subjected to PCR analysis at the Mad2 locus. As a negative control, PCR amplification was performed on a water sample. **b-d.** Three 10cm dishes of each cell line were plated at approximately 50% confluency, photographed 8 hours later and placed in fresh media containing 200ng/mL nocodazole. **e-g.** After 18 hours of nocodazole treatment, the same dishes were photographed using phase contrast microscopy. Representative images are shown here.

Figure 3.6



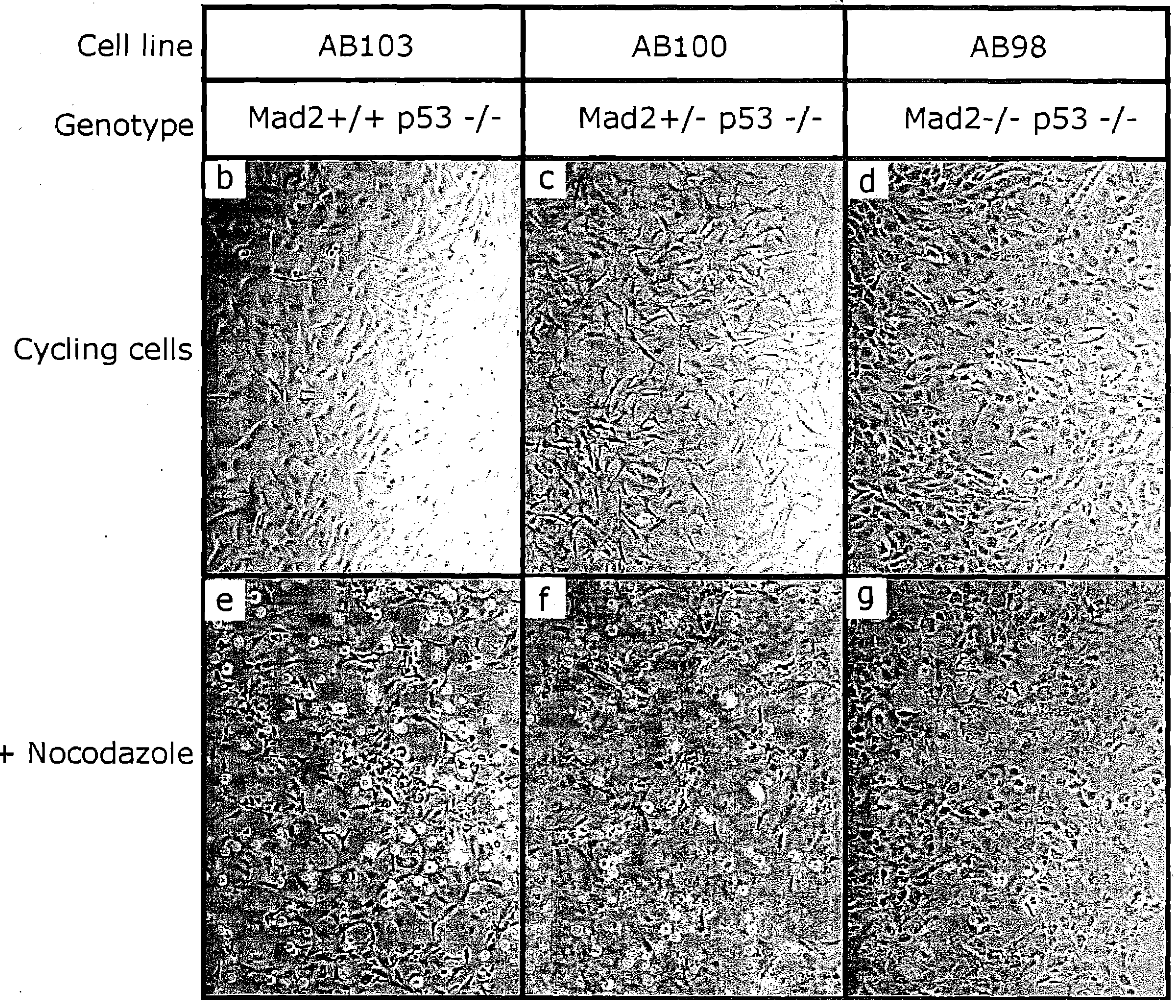


Figure 3.7

Reduction of Mad2 gene dosage results in chromosome instability.

FACS profile of unsynchronized Mad2$^{+/+}$ p53$^{-/-}$ (**purple**), Mad2$^{+/-}$ p53$^{-/-}$ (**green**), and Mad2$^{-/-}$ p53$^{-/-}$ (**pink**) mouse embryonic cell lines stained with propidium iodide. DNA content is represented on the x axis; number of cells is represented on the y axis.

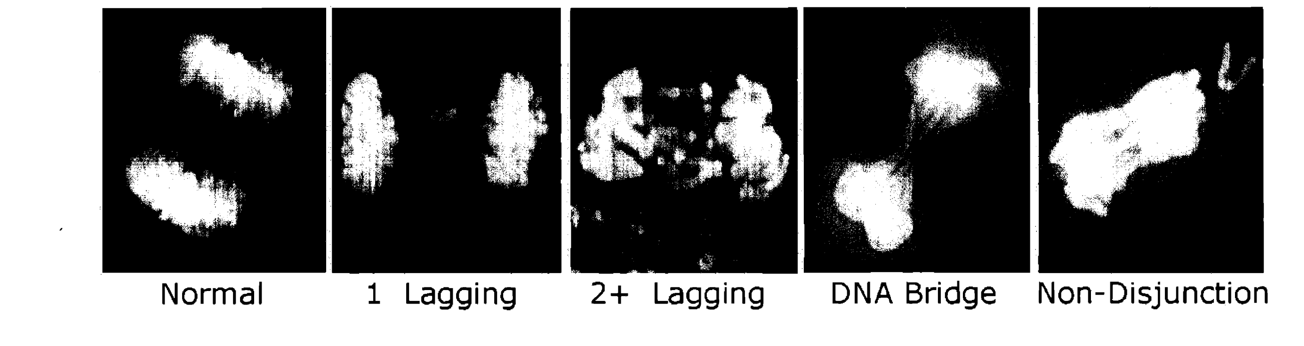
Figure 3.7

Figure 3.8

Mad2 gene dosage is inversely proportional to the appearance of defective chromosome segregation in $p53^{-/-}$ MEFs.

$Mad2^{+/+}$ $p53^{-/-}$, $Mad2^{+/-}$ $p53^{-/-}$, and $Mad2^{-/-}$ $p53^{-/-}$ mouse embryonic cell lines were grown on coverslips, fixed, and stained with DAPI. Every anaphase cell on each coverslip was categorized and recorded. **a.** Normal, complete separation of sister chromatids and migration to opposite poles. **b.** One chromatid or portion of a chromatid is clearly separated from the bulk of the segregating DNA. **c.** Two or more lagging chromatids remain at the metaphase plate. **d.** Cytokinetic furrow constricts around unsegregated DNA in telophase cells, creating a dumbbell or bridge morphology. **e.** Joined sister chromatids are located far from the metaphase plate, separate from the bulk of the DNA.

Figure 3.8



Cell Line	Mad2	Normal	1 Lagging	2+ Lagging	DNA Bridge	Non-Disjunction	n=
AB103	wt	24	1	0	0	0	25
AB100	het	14	2	2	0	0	18
AB98	ko	6	14	3	3	2	28

Discussion

I have shown that the Mad2 protein is not essential for the survival of gastrulating embryos or for the survival of mouse embryonic cells in vitro. These observations demonstrate that the mammalian spindle assembly checkpoint conforms to the classic definition of a checkpoint. That is, checkpoints are non-essential, auxiliary components of the cell cycle (Hartwell and Weinert 1989). Previous analyses of the embryonic lethality of the Mad2- and Bub3- deleted mice have inspired the modeling of the mammalian spindle assembly checkpoint as an essential pathway that is required for proper chromosome segregation (Dobles et al. 2000; Kalitsis et al. 2000). It was believed that mouse embryos lacking a mitotic checkpoint could not successfully progress through gastrulation (E6.5-E7.5) because cells in gastrulating embryos must complete several rounds of rapid cell division (Snow 1977; Hogan et al. 1994) and this accelerated progression though the cell cycle would depend more heavily on the checkpoints to ensure successful cell duplication. My recovery of viable Mad2$^{-/-}$ p53$^{+/+}$ embryos at E10.5 shows that even the very short cell cycles of gastrulating embryos do not necessarily require the spindle assembly checkpoint. In fact, a Mad2$^{-/-}$ cell can successfully replicate several dozen times, generating tens of thousands of cells that form tissues and organs.

Lethality of Mad2 embryos is rescued by disruptions in p53

The lack of a mitotic checkpoint in embryos is associated with the appearance of cells that contain lagging chromosomes and an increase in the levels of apoptosis in the embryo. It has been shown that the addition of microtubule-destabilizing drugs to cycling cells eventually leads to p53-mediated apoptosis (Cross et al. 1995; Lanni and Jacks 1998) supporting the possibility that p53 may contribute to the death of the $Mad2^{-/-}$ embryos. I postulate that lagging chromosomes are cleaved by the cytokinetic furrow, generating double strand DNA breaks that trigger the DNA damage pathway and induce p53-mediated apoptosis. I used two separate methods to study the effect of p53-mediated apoptosis on the survival of $Mad2^{-/-}$ embryos. Cultured blastocysts that are wild-type for p53, but nullizygous for Mad2, fail to proliferate and they begin to die within several days of explantation from the uterus. This lethality is rescued by deficiencies in p53. Similarly, a reduction in the gene dosage of p53 increases the survival rate of $Mad2^{-/-}$ embryos at E10.5. These results are consistent with the findings that the embryonic lethality of mice that are prone to double strand DNA breaks is rescued by a heterozygous disruption of p53 (Frank et al. 2000; Gao et al. 2000).

Postgastrulation $Mad2^{-/-}$ $p53^{-/-}$ embryos are under-represented

Interestingly, the complete elimination of p53 appears to reduce the viability of $Mad2^{-/-}$ embryos. Only one $Mad2^{-/-}$ $p53^{-/-}$ (double knockout) embryos was recovered at E10.5, from a total of 106 harvested embryos. These embryos resulted from crosses between $Mad2^{+/-}$ $p53^{+/-}$ females and $Mad^{+/-}$ $p53^{-/-}$ males, so the recovery of nine $Mad2^{-}$

$^{/-}$ p53$^{+/-}$ embryos implies that many more Mad2$^{-/-}$ p53$^{-/-}$ embryos should be recovered. Equal numbers of p53$^{+/-}$ and p53$^{-/-}$ embryos with Mad2 wild type or heterozygous genotypes were recovered from these matings, so the reduction in the number of Mad2$^{-/-}$ p53$^{-/-}$ embryos cannot be attributed to a reduced fitness of p53$^{-/-}$ embryos at this stage. Additionally, the numbers of Mad2$^{-/-}$ p53$^{-/-}$ and Mad2$^{-/-}$ p53$^{+/-}$ blastocysts at E3.5 are similar, suggesting that the double knockout embryos are eliminated during gastrulation. Heyer et al. have shown that p53 is required for apoptosis in mouse embryos after E5.5, but not before then (Heyer et al. 2000), supporting the hypothesis that the p53-mediated apoptotic pathway does not function in blastocysts. I speculate that the intermediate level p53-mediated apoptosis that is provided by p53 heterozygosity can eliminate cells that undergo mitosis in spite of numerous chromosome segregation errors, but that the more frequent, minor errors that occur in cells lacking a spindle assembly checkpoint may not be detected.

Mad2$^{+/-}$ and Mad2$^{-/-}$ cells show chromosome instability

To quantitate the effect of Mad2 disruptions on the range and occurrence of chromosome missegregation errors, I generated fibroblast cell lines from E10.5 embryos and observed the positions of chromosomes in fixed cells during anaphase. Dobles et al. had previously shown that that the spindle assembly checkpoint is required for accurate chromosome segregation in mouse cells (Dobles et al. 2000), but were unable to quantitate this phenotype definitively. I find that the deletion of one copy of Mad2

causes the rate of chromosome missegregation to increase at least 6 fold and the homozygous deletion of Mad2 increases this error rate 20 fold, as compared to Mad2 in cells on a p53$^{-/-}$ background. Because only one Mad2$^{-/-}$ cell line could be generated, the possibility exists that these cells contain other mutations, in addition to being Mad2 p53 double knockout, that may affect the viability or chromosome stability of these cells. To address the possibility of that other mutations in this cell line may have compromised chromosome stability, it has been shown that transient expression of Mad2 in these cells is sufficient to restore the induction of mitotic arrest following treatment of the cells with nocodazole (Robert Hagan, personal communication). I was unable to generate a Mad2$^{-/-}$ p53$^{+/-}$ MEF line to analyze the possibility that p53 heterozygosity reduces that appearance of severe defects, but this experiment could be performed in cell lines that are derived from differentiated blastocysts.

Experimental Procedures

Growth and Analysis of Mouse Blastocysts in Culture. E3.5 blastocysts from natural matings were flushed from the uterus of the mother mouse with M2 media and transferred into chambered microscopy slides (Nalge Nunc International 177445) by mouth aspiration using a pulled capillary tube (Kimax-51 ART # 34502, size 0.8-1.10 x 100mm). Embryos were cultured in ES cell media lacking LIF with 15% fetal bovine serum for a total of 2-28 days at 37°C in a humidified incubator under 5% CO_2 and examined by phase contrast microscopy prior to genotyping by PCR.

Mad2 PCR Genotyping of Mice

All of the genotyping protocols listed below were developed by Max Dobles and required minimal alterations from me.

DNA was prepared from tails by overnight digestion at 55°C with 1mg/ml Proteinase K (Worthington) in Tail Digest Buffer (67mM Tris pH 8.8, 16.6mM Ammonium Sulfate, 6.5mM MgC12, 0.5% Triton-X 100, 143mM β-mercaptoethanol). A Stratagene Robocycler PCR machine was used to amplify Mad2 and neo genes in 25μl PCR reactions with 200μM deoxyribonucleotides, 1μM each primer (MOL 232, 233, 242, 243 – sequences below), and PCR reaction buffer (50mM KCl, 10mM Tris-HCl [pH8.8], 1.5mM $MgCl_2$, 0.1% Triton-X 100). 20μl of a room temperature mix containing all components except Taq were added to 1μl DNA at room temperature, preheated to 95°C for 3min, and then brought to 75°C for addition of Taq (diluted to 4μl on ice). Reactions were subjected to 35 cycles (94°C 1min, 64°C 1.5min, 72°C 1.5min), and then extended at 72°C for 5min.

MOL 232, Agg CTg AgC Cgg gCC TTA ggA C

MOL233, gTA ACC gTg TAA TAA CgT TTA AgT CTC

MOL242, CgC TgT TCT CCT CTT CCT CAT CTC

MOL243, CCC CTg ATg CTC TTC gTC CAg ATC

MOL 232-233 amplify a 295bp band from intron 1 of Mad2.

MOL 242-243 amplify a 547bp band from the neomycin gene

p53 PCR Genotyping of Mice

p53 genotyping was performed in parallel with Mad2 genotyping. Reactions containing primers MOL290, MOL293 and MOL295 were subjected to 40 cycles (94°C 1min, 65°C 1.5min, 72°C 1.5min), and then extended at 72°C for 5min.

MOL 290, CTT CCC TCA CAT TCC TTC TTg g

MOL 293, ACA ggg CAC gTC TTC ACC Ag

MOL 295, gAg CCC Tgg CgC TCg ATg T

MOL 290-293 amplify a 595 bp band spanning exon 4 and exon 5 of p53.

MOL 290-295 amplify a 306 bp band spanning the site of the neo cassette insertion in Allen Bradley's p53 mice (Donehower et al. 1992).

Genotyping of Blastocysts. Following phase contrast microscopy, embryos were washed 3X5 min in PBS, immersed in PBS, and scraped loose using glass needles (Sutter Instrument Co. BF100-78-10, pulled to the same fineness as used for mouse pronuclei injections). Embryos were placed in 0.2ml thin-wall PCR tubes and digested overnight at 58°C in a total of 20μl 1mg/ml Proteinase K in Tail Digest Buffer.

Mad2. 1μM each of MOL236 and 237 (sequences below) were used to amplify Mad2 from 1μl of DNA in 25μl reactions using 0.625U AmpliTaq (Perkin Elmer) with AmpliTaq Buffer and 200μM deoxyribonucleotides in a Stratagene Robocycler Gradient 96. As with the tail DNA PCR protocol, 20μl of a master mix was mixed into 1μl DNA at

room temperature, and this was then preheated to 95°C for 3min, and brought to 75°C for addition of AmpliTaq (diluted to 4µl on ice). Reactions were subjected to 35 cycles (94°C 1 min, 61°C 2 min, 72°C 3 min), and then extended at 72°C for 10 min. neo reactions were performed in separate reaction tubes using 1µM each of MOL242 and 243 (sequences below) with identical reaction conditions as those used for Mad2, except the annealing step was performed at 63°C.

1µl from each of the Mad2 and neo reactions was used in a second round PCR reaction with nested primers to amplify specific Mad2 and neo PCR products. Both sets of reactions were done with reaction conditions identical to those described above, with the exceptions that the Mad2 reaction used MOL252 and 255 (sequences below), the neo reaction used MOL257 and 259 (sequences below), and 40 cycles were performed (94°C 1 min, 60°C 2 min, 72°C 2 min) followed by a 5 min extension.

MOL236, ACg TTg gCC AgC TCT Cgg TCT gC

MOL237, Tgg gCC TCA CTA TTg AAC CAC CTC

MOL242, CgC TgT TCT CCT CTT CCT CAT CTC

MOL243 CCC CTg ATg CTC TTC gTC CAg ATC

MOL252 TCA ATA AAg TgA AAg CAC AgC Tg

MOL255 CCA CCT CTT gCT AgA AAg gTA g

MOL257 ggC TgC AgC TAT ggg ATC

MOL259 gAC AAg ACC ggC TTC CAT CC

MOL 236-237: 291bp

MOL 242-243: 547bp

MOL 252-255: 249bp

MOL 257-259: 480bp

p53. Nested PCR was unnecessary for the p53 locus, and the protocol for genotyping p53 mice was follows using 1μl of DNA from each digested blastocyst.

Genotyping of Embryos. DNA was prepared from the yolk sacs of harvested embryos by overnight digestion in 0.2ml thin-wall PCR tubes at 58°C with 1mg/ml Proteinase K (Worthington) in 25μl, 50μl or 100μl Tail Digest Buffer, depending on the amount of yolk sac tissue that was collected. Each PCR reaction (Mad2 and p53) required 1-2μl of digested yolk sac to provide visible product bands in an agarose gel.

Immunofluorescence. Coverslips were coated with poly-L-lysine (Sigma) and plated with AB98, AB100 or AB103 cells at 25-30% confluency. These cells were allowed to grow at 37°C overnight in DME medium supplemented with 10% FCS. The next day the cells were fixed in fresh paraformaldehyde (4% solution in PBS from a 16% stock solution, Electron Microscopy Sciences) for 10 minutes at room temperature. Cells were washed 2x5min in PBS and permeablized/blocked for 10 minutes in PBS with 0.5% Triton-X 100, 2% Bovine Serum Albumin Fraction V (Boehringer Mannheim), 2% normal goat serum (Vector laboratories). Cells were washed three times with 0.1% TritonX-100 in PBS and then twice in PBS, followed by a 10 incubation with 1ug/ml of DAPI (Sigma) in PBS and two more washes with PBS. Coverslips were mounted with VectaShield (Vector laboratories) and the cells were examined using a Ziess-Applied

Precision Delta Vision Restoration microscope as described by (Martinez-Exposito et al. 1999).

Bibliography

Baba, H., D. Korenaga, Y. Kakeji, M. Haraguchi, T. Okamura, and Y. Maehara. 2002. DNA ploidy and its clinical implications in gastric cancer. *Surgery* **131**: S63-70.

Basu, J., H. Bousbaa, E. Logarinho, Z. Li, B.C. Williams, C. Lopes, C.E. Sunkel, and M.L. Goldberg. 1999. Mutations in the essential spindle checkpoint gene bub1 cause chromosome missegregation and fail to block apoptosis in Drosophila. *J Cell Biol* **146**: 13-28.

Cahill, D.P., C. Lengauer, J. Yu, G.J. Riggins, J.K. Willson, S.D. Markowitz, K.W. Kinzler, and B. Vogelstein. 1998. Mutations of mitotic checkpoint genes in human cancers. *Nature* **392**: 300-3.

Chen, R.H., A. Shevchenko, M. Mann, and A.W. Murray. 1998. Spindle checkpoint protein Xmad1 recruits Xmad2 to unattached kinetochores. *J Cell Biol* **143**: 283-95.

Cross, S.M., C.A. Sanchez, C.A. Morgan, M.K. Schimke, S. Ramel, R.L. Idzerda, W.H. Raskind, and B.J. Reid. 1995. A p53-dependent mouse spindle checkpoint. *Science* **237**: 1353-6.

Dobles, M., V. Liberal, M.L. Scott, R. Benezra, and P.K. Sorger. 2000. Chromosome missegregation and apoptosis in mice lacking the mitotic checkpoint protein Mad2. *Cell* **101**: 635-45.

Donehower, L.A., M. Harvey, B.L. Slagle, M.J. McArthur, C.A. Montgomery, J.S. Butel, and A. Bradley. 1992. Mice deficient for p53 are developmentally normal but susceptible to spontaneous tumours. *Nature* **356**: 215-21.

Frank, K.M., N.E. Sharpless, Y. Gao, J.M. Sekiguchi, D.O. Ferguson, C. Zhu, J.P. Manis, J. Horner, R.A. DePinho, and F.W. Alt. 2000. DNA ligase IV deficiency in mice leads to defective neurogenesis and embryonic lethality via the p53 pathway. *Cell* **5**: 993-1002.

Gao, Y., D.O. Ferguson, W. Xie, J.P. Manis, J. Sekiguchi, K.M. Frank, J. Chaudhuri, J. Horner, R.A. DePinho, and F.W. Alt. 2000. Interplay of p53 and DNA-repair protein XRCC4 in tumorigenesis, genomic stability and development. *Nature* **404**: 897-900.

Gorbsky, G.J., R.H. Chen, and A.W. Murray. 1998. Microinjection of antibody to Mad2 protein into mammalian cells in mitosis induces premature anaphase. *J Cell Biol* **141**: 1193-205.

Hartwell, L.H. and T.A. Weinert. 1989. Checkpoints: controls that ensure the order of cell cycle events. *Science* **246**: 629-34.

Heyer, B.S., A. MacAuley, O. Behrendtsen, and Z. Werb. 2000. Hypersensitivity to DNA damage leads to increased apoptosis during early mouse development. *Genes Dev.* **14**: 2072-84.

Hogan, B., R. Beddington, F. Constantini, and E. Lacy. 1994. Manipulating the Mouse Embryo. Cold Spring Harbor Press, New York.

Hoyt, M.A., L. Totis, and B.T. Roberts. 1991. S. cerevisiae genes required for cell cycle arrest in response to loss of microtubule function. *Cell* **66**: 507-17.

Kalitsis, P., E. Earle, K.J. Fowler, and K.H. Choo. 2000. Bub3 gene disruption in mice reveals essential mitotic spindle checkpoint function during early embryogenesis. *Genes Dev* **14**: 2277-82.

Kitagawa, R. and A.M. Rose. 1999. Components of the spindle-assembly checkpoint are essential in Caenorhabditis elegans. *Nat Cell Biolog* **1**: 514-521.

Lanni, J.S. and T. Jacks. 1998. Characterization of the p53-dependent postmitotic checkpoint following spindle disruption. *Mol Cell Biol* **18**: 1055-64.

Lengauer, C., K.W. Kinzler, and B. Vogelstein. 1998. Genetic instabilities in human cancers. *Nature* **396**: 643-9.

Li, R. and A.W. Murray. 1991. Feedback control of mitosis in budding yeast [published erratum appears in Cell 1994 Oct 21;79(2):following 388]. *Cell* **66**: 519-31.

Li, Y. and R. Benezra. 1996. Identification of a human mitotic checkpoint gene: hsMAD2. *Science* **274**: 246-8.

Loeb, L.A. 1991. Mutator phenotype may be required for multistage carcinogenesis. *Cancer Res* **51**: 3075-9.

Martinez-Exposito, M.J., K.B. Kaplan, J. Copeland, and P.K. Sorger. 1999. Retention of the BUB3 checkpoint protein on lagging chromosomes. *Proc Natl Acad Sci U S A* **96**: 8493-8.

Michel, L.S., V. Liberal, A. Chatterjee, R. Kirchwegger, B. Pasche, W. Gerald, M. Dobles, P.K. Sorger, V.V. Murty, and R. Benezra. 2001. MAD2 haplo-insufficiency causes premature anaphase and chromosome instability in mammalian cells. *Nature* **409**: 355-9.

Snow, M. 1977. Gastrulation in the Mouse: Growth and regionalization of the epiblast. *J. Embryol. Exp. Morphol.* **42**: 293-303.

Taylor, S.S. and F. McKeon. 1997. Kinetochore localization of murine Bub1 is required for normal mitotic timing and checkpoint response to spindle damage. *Cell* **89**: 727-35.

Uemura, T. and M. Yanagida. 1984. Isolation of type I and II DNA topoisomerase mutants from fission yeast: single and double mutants show different phenotypes in cell growth and chromatin organization. *EMBO J* **3**: 1737-44.

Waters, J.C., R.H. Chen, A.W. Murray, and E.D. Salmon. 1998. Localization of Mad2 to kinetochores depends on microtubule attachment, not tension. *J Cell Biol* **141**: 1181-91.

Chapter 4

Conclusions and Future Directions

Although the incidence of new cancer cases and the rates of cancer mortality are declining in the United States (Howe et al. 2001), cancer is still the second leading cause of death (Minino and Smith 2001). Although we tend to think that death occurs when the cells, tissues or organs of the body wear out, cancer develops because some cells of the body are actually more fit than those around them. The treatment and prevention of cancer hinges on the identification of the molecular changes that enable a cell to gain a fitness advantage as well as an understanding of the cellular mechanisms that are already in place to prevent the generation and proliferation of rogue, tumorigenic cells. Current models predict that a cell must acquire three to seven mutations during the progression from being a normal cell to being a malignant one (Vogelstein and Kinzler 1993; Weinberg 1996). One hallmark of cancer cells that distinguishes them from normal cells is aneuploidy, a deviation in the number of chromosomes in a cell. The importance of this difference is still under debate. Is chromosome instability an early event in tumorigenesis that may establish a mutator phenotype, or is it a later side effect of the deregulation of the cell cycle? Several studies have shown that allelic imbalances are present in cells from very early stage tumors, suggesting that chromosomal instability might be playing a causal or initiating role in tumorigenesis (Giaretti 1994; Bardi et al. 1997; Stoler et al. 1999; Shih et al. 2001). While it is clear that the generation of aneuploid cells is a result of an error in either chromosome replication or chromosome segregation, the molecular basis of these errors is not well understood. Mutations in spindle assembly checkpoint genes are rarely found in tumors, suggesting that inactivation of this checkpoint is not a frequent cause of tumorigenesis. However, in my thesis I present two bodies of research that demonstrate connections among chromosome

missegregation, apoptosis and tumor suppressors. First, the investigation into the biochemistry of the Bub kinases uncovered a new mechanism for the generation of aneuploid cells, namely the inactivation of the Adenomatous Polyposis Coli tumor suppressor. Subsequently, my research has focused on the role of the spindle assembly checkpoint and, to a lesser extent, the DNA-damage checkpoint in the generation and elimination of aneuploid cells. I have shown that neither a disruption of the spindle assembly checkpoint nor the resulting aneuploidy are directly lethal for mice, although chromosome missegregation does appear to trigger p53-dependent apoptosis, which then leads to gestational retardation and eventually to death in the embryo. This chapter presents the results of my research in the context of the entire field of the spindle assembly checkpoint and in the context of the field of research relating chromosome instability to tumorigenesis, and follows with suggestion for approaches and directions for future investigations.

Conclusions

Elimination of AdPC functions results in chromosome missegregation

Although the mutation of spindle assembly checkpoint genes appears infrequently in tumors, the discovery of interactions between kinetochore proteins and a known tumor suppressor reinforces the importance of this checkpoint in tumorigenesis. It is now believed that the elimination of AdPC may cause chromosome missegregation at an early stage of tumorigenesis, providing an increased probability that other tumor suppressors will be lost as chromosomes are lost (Hartwell and Kastan 1994). Because the spindle

assembly checkpoint is functional in cells lacking AdPC, the chromosome missegregation may occur after the initiation of anaphase – perhaps due to a weakened connection between kinetochore and microtubule, such that the chromosome falls off the spindle after sister chromatid separation. Alternatively, the phosphorylation of AdPC by the Bub kinases may reflect a change in the transduction of the checkpoint signal. AdPC is a very potent substrate for the Bub kinases and may out-compete the substrates that can transmit the signal for the inhibition of the Anaphase Promoting Complex.

The spindle assembly checkpoint is not required for the viability of mouse cells.

The disruption of spindle assembly genes has proven consistently lethal to metazoan organisms (Basu et al. 1999; Kitagawa and Rose 1999; Dobles et al. 2000; Kalitsis et al. 2000), but my research has shown that the mammalian spindle assembly checkpoint does, in fact, fall within the classical definition of checkpoints as non-essential, monitoring systems. The ability of Mad2 nullizygous embryos to survive gastrulation and develop into highly-organized fetuses demonstrates that the spindle assembly checkpoint is not required for the proliferation or differentiation of mouse embryos. The decreased recovery of $Mad2^{-/-}$ embryos with the progression of embryogenesis does suggest that the spindle assembly checkpoint is required in the event of a problem. Due to the dynamic nature of spindle assembly and the random attachment of kinetochores to spindle microtubules, it follows that chromosome missegregation events occur stochastically and therefore lead to a steady rate of embryonic death over time. Experiments in budding yeast showed that Mad2p is only required for the prevention of chromosome loss once in every 10,000 cell divisions (Li and Murray

1991). Although the chromosome loss rate has not been measured in metazoan organisms, the recovery of Mad2 embryos at E10.5 may cause a re-evaluation of the projected rates.

Disruptions in p53 rescue some Mad2$^{-/-}$ lethality

Embryonic lethality in both Mad2$^{-/-}$ mice and *bub1*-mutant *Drosophila* is associated with widespread apoptosis (Basu et al. 1999; Dobles et al. 2000). The rescue of Lig4$^{-/-}$ and Xcrr4$^{-/-}$ lethalities in mice suggests that p53 is required for a large percentage of the apoptosis that follows double stranded DNA breaks (Frank et al. 2000; Gao et al. 2000). Research by Lanni and Jacks (1998) has shown that p53 is required for cell cycle arrest and apoptosis of mouse embryonic cell lines that undergo cytokinesis in the presence of chromosomes that have not attached to the mitotic spindle (Lanni and Jacks 1998). Based on these results, I hypothesized that a chromosome missegregation event in a developing embryo triggers p53-mediated apoptosis, and eventually the increased level of apoptosis overwhelms either the embryo or a critical tissue and results in embryonic lethality. Although I have not addressed whether apoptosis directly follows a chromosome missegregation event, I have shown that disruption of p53 results in the survival of cultured Mad2$^{-/-}$ blastocysts and in the increased recovery of post-gastrulation Mad2$^{-/-}$ embryos.

Future Directions

The appearance of aneuploidy in tumor cells has been documented for several decades, and the phenomenon of mitotic arrest in response to microtubule destabilization has been known for nearly fifty years, but the discovery of the metazoan spindle assembly checkpoint genes began less than a decade ago (Li and Benezra 1996; Taylor and McKeon 1997; Cahill et al. 1998; Jin et al. 1999; Fisk and Winey 2001). Identification of these genes has allowed for biochemical and genetic analyses of this signaling pathway, improving our understanding of how a cell responds to improperly formed spindles and to chromosome missegregation. As these analyses continue, the number of proteins involved in the accurate partitioning of chromosomes grows, and a complete description of how cells link spindle assembly to the initiation of anaphase remains elusive. It seems clear that eukaryotes monitor both microtubule-kinetochore attachment as well as tension across the sister kinetochores, but the molecular link between these events and the activation of the Anaphase Promoting Complex remains patchy at best.

Further genetic analysis of the mammalian spindle assembly checkpoint will require more sophisticated and subtle methods. The knockouts of *Mad2* and *Bub3* in mice have proven useful for an initial study of the consequences of complete loss of the mitotic checkpoint, but their embryonic lethality limits their usefulness. Although the addition of p53 disruptions to the Mad2 mice has proven quite valuable, the Mad2$^{-/-}$ p53$^{-/-}$ cell lines carry many limitations as investigative reagents. This cell line began as a very limited population of cells and may have acquired numerous additional mutations before

it could be analyzed. Furthermore, cells outside the context of an embryo may not have the same requirements for successful chromosome segregation. Use of Cre-lox (Kuhn et al. 1995) or Flp-frt (Dymecki 1996) technologies to generate conditional knockouts of the spindle assembly genes will circumvent the obstacles of embryonic lethality and allow for the investigation of the immediate consequences of the disruption of the spindle assembly checkpoint.

Because genetic approaches to understanding the spindle assembly checkpoint in metazoans have proven difficult due to their lethality at the organismal level, investigations of the biochemical interactions among the signaling proteins becomes all the more critical. We have attempted to uncover one step in the signaling pathway by identifying a substrate for the Bub kinases. Although AdPC is important for chromosome segregation, it is not necessary for the propagation of the checkpoint signal. However, it can be used as a positive control in the search for the substrate that does affect the onset of anaphase. Another substrate for BubR1, Cdc20, has already been discovered (Wu et al. 2000). Although the phosphorylation of Cdc20 does not appear to be necessary for checkpoint signaling (Tang et al. 2001; Fang 2002), it will be interesting to learn how this protein compares to AdPC as a substrate for kinase activity. As more substrates are identified, and as the phosphorylation sites on these proteins are mapped, a consensus sequence for the Bub kinases may be uncovered, permitting a database search to identify other potential substrates.

The discovery that AdPC interacts with spindle assembly proteins and contributes to proper chromosome segregation without transducing the checkpoint signal emphasizes the importance of the structural integrity of the complexes that ensure the fidelity of mitotic chromosome transmission. It is well accepted that the maintenance of the physical structure of the cohesin complex is essential for successful chromosome segregation; the gradual degradation of meiotic cohesins is believed to be partially responsible for the age-dependent risk of trisomic births in people (Michaelis et al. 1997; Nicolaidis and Petersen 1998). Conversely, a cell line lacking securin cannot activate separin to cleave the cohesins, and these cells display a high incidence of chromosome missegregation (Jallepalli et al. 2001). AdPC may also serve as a structural component that partitions the sister chromatids, strengthening or reinforcing the physical connection between the kinetochores and the spindle microtubules. In this capacity, AdPC would not be necessary for kinetochore-microtubule attachment, but rather for the maintenance of the connection as stress is placed upon the segregating chromosomes. Thus, the lagging chromosomes appear in Min/Min cells because the kinetochore-microtubule connection is lost after the initiation of anaphase, at a time point that is beyond the regulation of the spindle assembly checkpoint. The mouse APC1638T truncating mutation creates a protein that lacks the C-terminal portion of AdPC that is responsible for microtubule binding, and homozygous APC1638T mice are viable and tumor free (Smits et al. 1999). Observations of cells from these mice, especially cell lines that contain homozygous mutations in both the AdPC and p53 genes, may clarify the influence of AdPC on chromosome segregation. These experiments will require an examination of the dynamic process of spindle assembly and chromosome segregation in

live cells. This is currently feasible with the introduction of either of two fluorescently-labeled proteins: Histone 2B:GFP labels the entire length of all of the sister chromatids in a cell (Kanda et al. 1998), and CENP-A:GFP labels only kinetochores (Sugimoto et al. 2000). Each system has both advantages and drawbacks; H2B:GFP labeling does not allow for the discovery of most non-disjunction events, but CENP-A:GFP labeling will not allow for the identification of cells that contain chromosome fragments at the metaphase plate. Therefore, I believe both methods must be utilized for a complete analysis of live-cell chromosome segregation.

Due to the juvenile status of our understanding of the spindle assembly checkpoint, the fundamental connections among the spindle assembly checkpoint, chromosome instability, apoptosis and tumorigenesis are still being established. The possibility that p53 plays a direct role in chromosome segregation or mitotic apoptosis has been proposed repeatedly, even though careful studies of p53-dependent apoptosis following treatment with microtubule poisons suggest that p53 is not acting during mitosis (Minn et al. 1996; Lanni and Jacks 1998). Intercrosses with the $Mad2^{+/-}$ mice and mice that contain disruptions in genes that regulate the function of p53, such as ATM (Xu et al. 1996), CHK1 (Liu et al. 2000) or $p19^{ARF}$ (Kamijo et al. 1997), may aid in elucidating the connection between chromosome missegregation and aneuploidy. Currently, the most favored hypothesis for this connection is that lagging chromosomes are cleaved by the cytokinetic furrow, resulting in double strand breaks in the DNA, which trigger the DNA-damage checkpoint. It is not known if such damage occurs, or how often, but Histone 2AX localizes specifically to double stranded DNA breaks,

marking the location of this type of DNA damage (Paull et al. 2000). The use of anti-Histone 2AX antibodies may provide an assay to quantitate the rate at which double strand breaks are generated, especially in cell lines such as the Mad2$^{-/-}$ p53$^{-/-}$ MEF line that will presumably show a high incidence of DNA damage.

Summary

As the number of known kinetochore-associated proteins increases as a result of innovative research in chromosome segregation from budding yeast to human tumors, our understanding of the connections between spindle assembly, cell cycle progression, apoptosis and tumorigenesis will also increase. The work presented in this thesis describes a significant contribution to this field, adding a known tumor suppressor to the list of kinetochore proteins and revealing that the spindle assembly checkpoint is not absolutely required for the survival of either gestating mouse embryos or cultured cells. Additionally, the increased survival of Mad2$^{-/-}$ p53$^{+/-}$ blastocysts and embryos suggests that even a heterozygous mutation in p53 may be sufficient to establish chromosome instability in cells, accelerating the likelihood that other genes responsible for cell cycle regulation will be lost. Although we have only begun to test the models that describe the role of chromosome missegregation in human disease, as our understanding of the molecular basis of the spindle assembly checkpoint grows, so will the potential for prevention and treatment of disease.

Bibliography

Bardi, G., L.A. Parada, L. Bomme, N. Pandis, R. Willen, B. Johansson, B. Jeppsson, K. Beroukas, S. Heim, and F. Mitelman. 1997. Cytogenetic comparisons of synchronous carcinomas and polyps in patients with colorectal cancer. *Br J Cancer* **76**: 765-9.

Basu, J., H. Bousbaa, E. Logarinho, Z. Li, B.C. Williams, C. Lopes, C.E. Sunkel, and M.L. Goldberg. 1999. Mutations in the essential spindle checkpoint gene bub1 cause chromosome missegregation and fail to block apoptosis in Drosophila. *J Cell Biol* **146**: 13-28.

Cahill, D.P., C. Lengauer, J. Yu, G.J. Riggins, J.K. Willson, S.D. Markowitz, K.W. Kinzler, and B. Vogelstein. 1998. Mutations of mitotic checkpoint genes in human cancers. *Nature* **392**: 300-3.

Dobles, M., V. Liberal, M.L. Scott, R. Benezra, and P.K. Sorger. 2000. Chromosome missegregation and apoptosis in mice lacking the mitotic checkpoint protein Mad2. *Cell* **101**: 635-45.

Dymecki, S.M. 1996. Flp recombinase promotes site-specific DNA recombination in embryonic stem cells and transgenic mice. *PNAS* **93**: 6191-6.

Fang, G. 2002. Checkpoint Protein BubR1 Acts Synergistically with Mad2 to Inhibit Anaphase-promoting Complex. *Mol Biol Cell* **13**: 755-66.

Fisk, H.A. and M. Winey. 2001. The mouse Mps1p-like kinase regulates centrosome duplication. *Cell* **106**: 95-104.

Frank, K.M., N.E. Sharpless, Y. Gao, J.M. Sekiguchi, D.O. Ferguson, C. Zhu, J.P. Manis, J. Horner, R.A. DePinho, and F.W. Alt. 2000. DNA ligase IV deficiency in mice leads to defective neurogenesis and embryonic lethality via the p53 pathway. *Cell* **5**: 993-1002.

Gao, Y., D.O. Ferguson, W. Xie, J.P. Manis, J. Sekiguchi, K.M. Frank, J. Chaudhuri, J. Horner, R.A. DePinho, and F.W. Alt. 2000. Interplay of p53 and DNA-repair protein XRCC4 in tumorigenesis, genomic stability and development. *Nature* **404**: 897-900.

Giaretti, W. 1994. A model of DNA aneuploidization and evolution in colorectal cancer. *Lab Invest* **71**: 904-10.

Hartwell, L.H. and M.B. Kastan. 1994. Cell cycle control and cancer. *Science* **266**: 1821-8.

Howe, H.L., P.A. Wingo, M.J. Thun, L.A.G. Ries, H.M. Rosenberg, E.G. Feigal, and B.K. Edwards. 2001. Annual Report to the Nation on the Status of Cancer (1973 Through 1998), Featuring Cancers With Recent Increasing Trends. *J Natl Cancer Inst* **93**: 824-42.

Jallepalli, P.V., I.C. Waizenegger, F. Bunz, S. Langer, M.R. Speicher, J.M. Peters, K.W. Kinzler, B. Vogelstein, and C. Lengauer. 2001. Securin is required for chromosomal stability in human cells. *Cell* **105**: 445-57.

Jin, D.Y., C.A. Kozak, F. Pangilinan, F. Spencer, E.D. Green, and K.T. Jeang. 1999. Mitotic checkpoint locus MAD1L1 maps to human chromosome 7p22 and mouse chromosome 5. *Genomics* **55**: 363-4.

Kalitsis, P., E. Earle, K.J. Fowler, and K.H. Choo. 2000. Bub3 gene disruption in mice reveals essential mitotic spindle checkpoint function during early embryogenesis. *Genes Dev* **14**: 2277-82.

Kamijo, T., F. Zindy, M.F. Roussel, D.E. Quelle, J.R. Downing, R.A. Ashmun, G. Grosveld, and C.J. Sherr. 1997. Tumor suppression at the mouse INK4a locus mediated by the alternative reading frame product p19ARF. *Cell* **91**: 649-659.

Kanda, T., K.F. Sullivan, and G.M. Wahl. 1998. Histone-GFP fusion protein enables sensitive analysis of chromosome dynamics in living mammalian cells. *Curr Biol* **8**: 377-85.

Kitagawa, R. and A.M. Rose. 1999. Components of the spindle-assembly checkpoint are essential in Caenorhabditis elegans. *Nat Cell Biolog* **1**: 514-521.

Kuhn, R., F. Schwenk, M. Aguet, and K. Rajewsky. 1995. Inducible gene targeting in mice. *Science* **269**: 1427-9.

Lanni, J.S. and T. Jacks. 1998. Characterization of the p53-dependent postmitotic checkpoint following spindle disruption. *Mol Cell Biol* **18**: 1055-64.

Li, R. and A.W. Murray. 1991. Feedback control of mitosis in budding yeast [published erratum appears in Cell 1994 Oct 21;79(2):following 388]. *Cell* **66**: 519-31.

Li, Y. and R. Benezra. 1996. Identification of a human mitotic checkpoint gene: hsMAD2. *Science* **274**: 246-8.

Liu, Q., S. Guntuku, X.S. Cui, S. Matsuoka, D. Cortez, K. Tamai, G. Luo, S. Carattini-Rivera, F. DeMayo, A. Bradley, L.A. Donehower, and S.J. Elledge. 2000. Chk1 is an essential kinase that is regulated by Atr and required for the G(2)/M DNA damage checkpoint. *Genes Dev* **14**: 1448-59.

Michaelis, C., R. Ciosk, and K. Nasmyth. 1997. Cohesins: chromosomal proteins that prevent premature separation of sister chromatids. *Cell* **91**: 35-45.

Minino, A.M. and B.L. Smith. 2001. Deaths: Preliminary data for 2000. *National Vital Statistics Report* **49**.

Minn, A.J., L.H. Boise, and C.B. Thompson. 1996. Expression of Bcl-xL and loss of p53 can cooperate to overcome a cell cycle checkpoint induced by mitotic spindle damage. *Genes Dev* **10**: 2621-31.

Nicolaidis, P. and M.B. Petersen. 1998. Origin and mechanisms of non-disjunction in human autosomal trisomies. *Hum Reprod* **13**: 313-9.

Paull, T.T., E.P. Rogakou, V. Yamazaki, C.U. Kirchgessner, M. Gellert, and W.M. Bonner. 2000. A critical role for histone H2AX in recruitment of repair factors to nuclear foci after DNA damage. *Curr Biol* **10**: 886-95.

Shih, I.M., W. Zhou, S.N. Goodman, C. Lengauer, K.W. Kinzler, and B. Vogelstein. 2001. Evidence that genetic instability occurs at an early stage of colorectal tumorigenesis. *Cancer Res* **61**: 818-22.

Smits, R., M.F. Kielman, C. Breukel, C. Zurcher, K. Neufeld, S. Jagmohan-Changur, N. Hofland, J. van Dijk, R. White, W. Edelmann, R. Kucherlapati, P.M. Khan, and R. Fodde. 1999. Apc1638T: a mouse model delineating critical domains of the adenomatous polyposis coli protein involved in tumorigenesis and development. *Genes Dev* **13**: 1309-21.

Stoler, D.L., N. Chen, M. Basik, M.S. Kahlenberg, M.A. Rodriguez-Bigas, N.J. Petrelli, and G.R. Anderson. 1999. The onset and extent of genomic instability in sporadic colorectal tumor progression. *Proc Natl Acad Sci U S A* **96**: 15121-6.

Sugimoto, K., R. Fukuda, and M. Himeno. 2000. Centromere/kinetochore localization of human centromere protein A (CENP-A) exogenously expressed as a fusion to green fluorescent protein. *Cell Struct Funct* **25**: 253-61.

Tang, Z., R. Bharadwaj, B. Li, and H. Yu. 2001. Mad2-Independent inhibition of APCCdc20 by the mitotic checkpoint protein BubR1. *Dev Cell* **1**: 227-37.

Taylor, S.S. and F. McKeon. 1997. Kinetochore localization of murine Bub1 is required for normal mitotic timing and checkpoint response to spindle damage. *Cell* **89**: 727-35.

Vogelstein, B. and K.W. Kinzler. 1993. The multistep nature of cancer. *Trends Genet* **9**: 138-41.

Weinberg, R.A. 1996. How cancer arises. *Sci Am* **275**: 62-70.

Wu, H., Z. Lan, W. Li, S. Wu, J. Weinstein, K.M. Sakamoto, and W. Dai. 2000. p55CDC/hCDC20 is associated with BUBR1 and may be a downstream target of the spindle checkpoint kinase. *Oncogene* **19**: 4557-62.

Xu, Y., T. Ashley, E.E. Brainerd, R.T. Bronson, M.S. Meyn, and D. Baltimore. 1996. Targeted disruption of ATM leads to growth retardation, chromosomal fragmentation during meiosis, immune defects, and thymic lymphoma. *Genes Dev* **10**: 2411-22.