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The Role of Cdk4 in Her2 Driven Centrosome Amplification

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B.S., Sewanee: The University of the South, 2007

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Abstract

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Centrosome amplification (CA) is a contributor to carcinogenesis, generating aneuploidy and chromosome instability. Previous work shows that breast adenocarcinomas have a higher frequency of centrosome defects compared to normal breast tissues. Abnormal centrosome phenotypes are found in pre-malignant lesions, suggesting an early role in breast carcinogenesis. However, the role of CA in breast cancers remains elusive. The long-term goal of the work presented here is to assess the role of CA in mammary cancers by identifying how specific oncogenes signal CA. Identification of pathways and regulatory molecules involved in the generation of CA is essential to understanding its role in breast tumorigenesis. We established a breast cancer model of CA using Her2+ cells. Our goal was to identify centrosome cycle molecules that are deregulated by aberrant Her2 signaling and the mechanisms driving CA. Our results show some Her2+ breast cancer cell lines harbor both CA and binucleation. Abolishing the expression of Cdk4 abrogated both CA and binucleation in these cells. We also found the source of binucleation in these cells to be defective cytokinesis that is normalized by downregulation of Cdk4. Protein levels of Nek2 diminish upon Cdk4 knockdown, suggesting a molecular connection between Cdk4 and Nek2. Knockdown of Nek2 reduces CA and binucleation in this model while its overexpression further enhances CA. We conclude that CA is modulated through Cdk4 and Nek2 signaling, and that binucleation is a likely source of CA in Her2+ breast cancer cells.

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Chapter 1

Introduction

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In 2012, the United States diagnosed an estimated 1,638,910 new cases of cancer. Excluding skin cancer, breast cancer is the most common cancer among women, accounting for 1 in 3 cancer diagnoses in US women. Since 1990 there has been a steady decrease in breast cancer deaths due to early detection and advancements in treatment. Furthermore, the five-year relative survival rate for women with localized or locally invasive disease is 99% and 84% respectively. Unfortunately, this number drops to only 23% for women with late stage breast cancer(1). Continuing research and better understanding of the molecular mechanisms driving breast cancer are desperately needed to better the outcome of this disease.

Cancer Genetics

At its most basic level, cancer is a disease of uncontrolled cell growth. In 2000, Hanahan proposed six acquired capabilities, or hallmarks, to outline the complex development of a human tumor (2). These included, sustained proliferative signaling, evasion of growth suppressors, activation of invasion and metastasis, enabling replicative immortality, induction of angiogenesis, and resistance to cell death (2). Radiating out from each of these founding principles, like the spokes of a wheel, are a myriad of cellular processes that may contribute to a cell's transformation by impinging on one of the six hallmarks of cancer. A revision of the original model shows that genomic instability is an enabler of abnormalities leading to cancer (3). Centrosome amplification (CA) may facilitate genomic instability and carcinogenesis by generating aneuploidy. This

dissertation focuses on just one of these processes, how a deregulated centrosome duplication cycle and the kinases that affect it mediate CA in Her2+ breast cancer cells.

Through the traits outlined by Hanahan (2), cells acquire alterations in two categories of genes: oncogenes and tumor suppressors. Oncogenes are functionally defined as regulatory genes having dominant transforming properties. Proto-oncogenes are genes normally involved in regulating signal transduction, cell cycle progression, and cell differentiation; these genes give rise to oncogenes through various mechanisms, including mutations, chromosomal rearrangements, or gene amplification. Originally identified in viruses, the first oncogene was discovered in the early 1900s from the avian sarcoma virus and was named *src*. Since then, many oncogenes have been discovered including *myc, ras,* and *ErbB*, which will be further discussed in this dissertation. Tumor suppressor genes (TSG), by contrast, typically inhibit cell growth and/or promote apoptosis can be mutated, physically lost, or epigenetically silenced. Usually, both alleles of a tumor suppressor gene are inactivated in tumor development; however, haploinsufficiency, wherein a single functional copy of a TSG is not able to produce wild-type levels of a gene product has also been described. One important aspect in cancer research is the identification and targeting of genes with genetic and epigenetic alterations, regardless of classification, that drive cancer initiation and progression (4).

Her2

Her2/Neu, also known as ErbB2, is a receptor tyrosine kinase; members of the ErbB family dimerize upon ligand stimulation and transduce their signals by autophosphorylation. They mediate a complex signaling network upon binding their co-receptors, among these activated signals is the well-studied Ras-activated mitogen activated protein kinase (MAPK) pathway (5). While rarely mutated in human cancers, wild-type Her2 is often found amplified at the gene level or overexpressed at the protein level. The oncoprotein is overexpressed in approximately 20-30% of breast tumors, and acutely so (90%) in forms of Ductal Carcinoma *in situ* (DCIS) that have not progressed beyond the basement membrane barrier (6). The mechanism behind why Her2 overexpression potentiates transformation proves an interesting question. The high level of basal autophosphorylation could be instigated by the spontaneous formation of homodimers, or by an increased availability for heterodimerization. Regardless, there is strong downstream interaction with both the MAPK and PI3K pathways, suggesting that cell proliferation and survival pathways are triggered in these cancers (6). Overall, high levels of Her2 predict lower disease free and survival in patients, suggesting a significant functional role for Her2 in breast cancer initiation and progression.

Cyclin D1/Cdk4

Among those targets deregulated by hyperactive Her2 signaling are the G_1/S cell cycle regulators, cyclin D/Cdk4/6 complexes. Overexpressed in 40% of

breast cancers, cyclin D1 has been shown to be required in several different subtypes of breast cancer, especially those in which Her2 plays a role (6). Cyclin D1 and its catalytic partners Cdk4/Cdk6 have been shown to be required for Her2-induced transformation (7-10). There is clear-cut evidence that overexpression of cyclin D1, dependent or independent of gene amplification, are significant drivers of human breast cancer. The importance of cyclin D1 as a breast cancer oncogene is well established, but the mechanism through which cyclin D1 exerts its oncogenic function still requires further research. Studies have shown that the deviation from normal function can be Cdk-dependent or independent, mediating the cell cycle and transcription.

There are many studies in mice showing the importance of cyclin D1 in mediating tumorigenesis. A pivotal paper by Sicinski in 2001 showed the mice lacking cyclin D1 were impervious to tumorigenesis induced by Ras and Her2 (9). They also showed that c-neu/erbB2 and Ha-ras induced tumorigenesis by activating the cyclin D1 promoter. A paper from 2006 showed similar results concerning Cdk4; upon knockdown of Cdk4 by RNAi in c-neu induced mammary tumor cells the oncogenic properties were abrogated when reintroduced into mammary fat pads (11). The demonstration that cyclin D1-Cdk4 deficient mice develop normally but are resistant to mammary tumors triggered by Her2 (12) points to differential roles for this complex in development and tumorigenesis. These findings point to an interesting dichotomy that demands further study to elucidate therapeutic targets.

Nek2

Nek2 is a serine/threonine protein kinase in the NIMA-related kinase family that is regulated in a cell cycle dependent manner. While Nek2 is present at the centrosome throughout the cell cycle, the abundance and activity of Nek2 peaks in S and G_2 phases, where it regulates centrosome separation during mitotic entry (13), mediating the severance of linkages connecting the centrosomes during by phosphorylating centriolar proteins (14). This is critical in the establishment of the mitotic spindle.

The question of Nek2's contribution to cancer progression is being actively studied. Nek2 is overexpressed 2-5 fold in cell lines derived from various cancer types and is significantly up-regulated in the majority of breast tumors, including preinvasive tumors (15). The effect that overexpression has on cells is not fully understood; however, cells expressing recombinant active Nek2 show premature separation of the centrosome at any phase in the cell cycle (16, 17) and aneuploidy in cells transformed by the SV40 T antigen (15). In fact, recent data showed that inhibition of Nek2 suppresses tumorigenesis of various breast cancer cells (14, 18), and mediates chemotherapeutic drug resistance of triple negative breast cancer cells and multiple myeloma (19, 20). This, in concert with data showing that the MAPK pathway can activate Nek2, suggests that Nek2 could be a major player in Her2 induced centrosomal abnormalities, aneuploidy, and tumorigenesis.

G_1 phase Cdks, centrosomal kinases, and phosphatases regulate the centrosome cycle.

The centrosome duplication cycle

Faithful segregation of chromosomes into daughter cells during mitosis is essential to maintain genetic stability in most if not all organisms. The interplay between centrosomes and mitotic microtubules results in the accurate segregation of chromosomes into daughter cells. Following cytokinesis each daughter cell receives only one centrosome; this centrosome, like DNA, must duplicate only once prior to the next mitosis. Centrosome duplication must be tightly regulated, because the generation of more than one procentriole per mother centriole results in CA (21, 22) and contributes to tumorigenesis (23, 24). The different phases of the centrosome cycle were originally assigned based on the morphology of the centriole pair throughout the cell cycle, as established by electron microscopy (25). More recently, establishment of centriole duplication assays in Xenopus egg extracts (26) and cultured mammalian cells (27, 28) markedly improved the dissection of the centrosome cycle. Additionally, the development of centrin-2-GFP constructs has allowed following the centrosome duplication cycle relative to the different cell cycle phases in real-time (29), and allows the assessment of unregulated centrosome cycles (30).

Throughout early G_1 phase, normal cells have one mature centrosome. During late G_1 and S phase, the structure of the mother and daughter centrioles differs, the mother centriole contains appendages, whereas the daughter centriole grows throughout these phases. At the beginning of S phase, centriole

duplication starts with the appearance of short daughter centrioles, or procentrioles, at right angles to the two original centrioles (31, 32). Procentrioles are observed approximately 4 hours after the beginning of S phase (33). This process culminates in the acquisition of appendages by the daughter centriole in G_2 (34) and the recruitment of PCM (31, 32). By late G_2, two mature centrosomes are generated (Figure 1).

Recent studies identifying several centrosome-associated proteins, protein kinases and phosphatases have provided new insights into the regulation of centrosome structure and function, including their ability to control centriole duplication. Because unregulated expression of proteins controlling the synthesis of daughter centrioles can cause centriole reduplication and CA, these proteins are potential targets of oncogenes and altered tumor suppressors, and will be thoroughly discussed in the following sections.

The G_1 phase Cdks coordinate the cell and centrosome cycles

The centrosome duplication cycle must occur in coordination with the cell cycle; otherwise, deregulated centrosome duplication may culminate in CA. Because DNA and centrosomes undergo semi-conservative duplication once every cell cycle, mammalian cells are equipped with a mechanism that coordinates these two events, so that they are duplicated only once (21). This coordination is in part accomplished because cell cycle regulatory proteins also regulate the centrosome duplication cycle. The cell cycle is regulated as follows: The temporal overexpression of cyclins D, E, and A sequentially activates the G_1

phase Cdks, Cdk4/Cdk6 and Cdk2, to trigger entry and progression through S phase (35-44). The G_1 phase Cdks initiate DNA duplication in part through the phosphorylation of the retinoblastoma (Rb) protein and the activation of the E2F transcriptional program (42, 45-66). The Rb/E2F transcription program is essential for the correct expression and regulation of copious genes involved in DNA replication, DNA repair, mitosis, and centrosome duplication (67-69).

Other studies have shown a close relationship between cell cycle regulatory molecules and the regulation of centrosome duplication. For example, ectopic expression of the cyclin-dependent kinase inhibitors $p21^{Waf1/Cip1}$ and $p27^{Kip1}$ blocked centrosome duplication in Xenopus dividing embryos at the blastomere stage (70). In support of those studies, inhibition of cyclin E/Cdk2 in Xenopus egg extracts caused arrest in S phase and thus prevented centriole re-duplication; re-introduction of cyclin E/Cdk2 restored that reduplication (26). It was then suggested, using the same system, that inhibition of Cdk2 activity prevents multiple rounds of centriole duplication, but it does not prevent the initial round of duplication (71). However, there is other more recent evidence suggesting that Cdk2 is also involved in the initial round of centriole duplication. In Xenopus egg extracts, separase causes disengagement of centrioles during anaphase, and cyclin E/Cdk2 activity is required for the synthesis of a daughter centriole following disengagement (72).

Although various data obtained in Xenopus provided a strong correlation between Cdk2 activity and centrosome duplication, gene knockout experiments done in mammalian cells uncovered a much different scenario. Previous studies

demonstrating that Cdk2-deficient mice develop normally (73, 74) raised the question of the requirement of Cdk2 in other processes, such as its ability to regulate DNA and centrosome duplication (73-75). A surprising result was that cells derived from these mice can proliferate and undergo centrosome duplication with moderate defects (73-75), indicating that the function of Cdk2 for proliferation and initiation of the centrosome duplication can be readily and functionally replaced by other Cdks or other centrosome regulatory proteins. Likewise, ablation of the Cdk2 activating partners cyclin E1 and E2 in MEFs was not associated with any centrosomal defects (76). In support of studies done in mammalian cells, various combinatorial knockdowns of two mitotic cyclins (CycA, CycB, and CycB3), and reduction of the dosage of the remaining cyclins in Drosophila embryonic syncytial divisions allows centrosomes to duplicate, while cells do not enter mitosis (77).

Recent experiments have revealed both redundancy and specificity, in regards to the G_1 Cdks regulating centrosome duplication in eukaryotes. For example, chicken DT40 mutants were generated in which an analog-sensitive mutant cdk1 replaced the endogenous Cdk1. In those cells, Cdk1 could be inactivated using bulky ATP analogs (78). In DT40 cells that also lack Cdk2, Cdk1 activity is essential for DNA replication initiation and for centrosome duplication. In addition, the relative contributions of the G_1 Cdks (Cdk2 and Cdk4) to regulate normal centrosome duplication were explored (79). During these studies, experiments used to measure the centrosome cycle at various time points throughout the cell cycle in $Cdk2^{-/-}$ and $Cdk4^{-/-}$ mouse embryonic

fibroblasts (MEFs), as well as transient down-regulation of Cdk2 and Cdk4 using RNA-mediated interference, uncovered distinct centrosome cycle defects, suggesting that Cdk2 and Cdk4 do not have redundant functions. For example, while Cdk2 deficiency allowed the separation and duplication of centrosomes, absence of Cdk4 favored the accumulation of cells with centrosomes that were slow to separate and duplicate.

The centrosome and cancer

It has been well established that CA is a distinct feature of most cancer cells. With this observation came the hypothesis that this phenotype can drive genomic instability and subsequent tumorigenesis. Abnormal centrosome biology, including CA and structural abnormalities frequently occurs in most types of solid tumors, as well some leukemias and lymphomas. Specifically, those cancer types include testicular germ cell, liposarcoma, adrenocortical, bronchial, bladder, cerebral primitive neuroectodermal, cervical, prostate, breast, squamous cell carcinomas of the head and neck, myeloma, and T-cell leukemia (80-92). Work done in hematopoietic malignancies demonstrates that CA in myelomas correlates with a specific gene expression signature, and can serve as a prognostic factor in patients (93).

The role of CA in transformation is unclear. Mammalian transformation is complex, involving the concomitant activation of oncogenes and inactivation of tumor suppressors. Proto-oncogenes, tumor suppressors, the G_1/S phase cell cycle regulatory machinery, and the centrosome-specific machinery regulate the

centrosome cycle to maintain normal centrosome numbers. This is a double-edged sword because deregulated oncogenes and altered tumor suppressors result in CA and aneuploidy. While the evidence suggesting that CA is involved in breast cancers is extensive, no one to date has shown that CA can drive transformation of mammary epithelial cells or that inhibition of CA influences the behavior of breast cancer cells. In order to better understand this problem, oncogenic signals directly contributing to CA must be identified. Centrosome regulatory proteins downstream of those oncogenic alterations must also be found.

One tumor type in which the relationship between CA and cancer is better understood is breast cancer. The vast majority (80-100%) of breast tumors display CA (94). Breast adenocarcinoma cells have a much higher frequency of centrosome defects, including amplification of number (94, 95), increased volume and supernumerary centrioles, when compared to normal breast tissue (95). Similar phenotypes can also be found in pre-invasive in situ ductal carcinoma, and in pre-malignant breast lesions, suggesting that these aberrations occur early in breast carcinogenesis (83, 94, 96). In support of this data, molecular analyses have found that the centrosome pathway is highly enriched for SNPs that are associated with risk of breast cancer (97). In addition to being involved in initiation, having extensive areas of CA in breast tumors correlates with axillary lymph node involvement, suggesting that CA also contributes to the most malignant characteristics of breast cancer cells (98).

There are many correlative studies that link centrosomal abnormalities and cancer, and there are even more studies working to discover the causal link and mechanism behind this correlation. The most direct evidence showing that CA is involved in tumorigenesis was obtained in Drosophila. In a study that specifically addressed the relationship between abnormal centrosome biology and tumorigenesis, Basto et al. assayed the long term consequences of an organism having supernumerary centrosomes. Allotransplantation of Plk4/SAK overexpressing Drosophila neuronal stem cells is sufficient to induce tumors in flies (23). Also, transplanted cells expressing aur-a, plk, asl and dsas4 resulted in tumors with varying efficiency (24). Aurora A, one of the first oncogenes shown to induce CA in mammalian cells (99), proved to be the most efficient at inducing tumors (24). These important experiments and observations are the first step in defining the link between CA and tumors.

Deregulated G_1 Cdks, centrosome amplification, and cancer

Oncogene-dependent centrosome amplification correlates with hyperactive Cdk2 and Cdk4

Because the centrosome cycle is regulated in part by cell cycle machinery, when the cell cycle becomes deregulated by oncogenes and altered tumor suppressors, the centrosome can also be susceptible to deregulation. This can ultimately lead to CA, aneuploidy, and unregulated cell cycling (100, 101).

The first altered tumor suppressor shown to be directly associated with CA was p53, as its genetic deletion in MEFs promoted that abnormal process (102).

Similarly, alterations that affected p53 function resulted in CA. One of the most important functions of the p53 pathway is to trigger cell cycle arrest to allow repair of DNA damage, or cell death if the damage is unrepaired (103). p53 exerts some of its cell cycle regulatory functions through promoting the transcription of $p21^{Waf1/CIP1}$, a CKI that negatively regulates both Cdk2 and Cdk4 activities (104, 105). p53 prevents CA through direct binding to the centrosome, and also in part through its ability to regulate $p21^{Waf1/CIP1}$ (106). Several groups have presented data supporting a role of $p21^{Waf1/CIP1}$ in centrosome biology. For example, introduction of $p21^{Waf1/CIP1}$ into $p53^{-/-}$ cells harboring CA restored normal centrosome duplication and abrogated CA (107). Moreover, knockdown of $p21^{Waf1/CIP1}$in murine myeloblasts stimulates excessive centriole numbers in the presence of only one mature centriole (108) and $p21^{Waf1/CIP1}$ null human hematopoietic cells display elevated frequencies of CA (109).

Consequent to the discovery that CA in p53-null cells correlated with deregulated Cdk2 activity, many other studies began showing similar correlations. For example, when E2F3a/b, transcription factors critical to S phase entry, are ablated, elevated cyclin E-dependent Cdk2 activity correlates with constitutive centriole separation, duplication, and CA (Figure 2) (30). It is to note that this function is specific to E2F3-null cells, as MEFs lacking E2F1, E2F2, E2F4 or E2F5 do not display CA.

Following the discovery that tumor suppressors maintained normal centrosome numbers, various laboratories showed that certain proto-oncogenes displayed the same activity. Some of the first observations that proto-

oncogenes, including tyrosine kinase receptors, controlled the centrosome cycle were made in CHO cells cultured in the presence of hydroxyurea (HU) or aphidicolin. Addition of dialyzed serum to these cells stopped centriole reduplication, while addition of EGF re-initiated the process (110). Additionally, when $PTEN^{-/-}$ neural precursor cells were infected with retrovirus encoding constitutively active EGFRvIII, CA, genomic instability and glial tumors developed (111). Furthermore, it has been shown that other EGFR family members may play a role in this story. Her2/neu (ErbB2) was first described as an oncogene when isolated from neuroglioblastomas that developed in rats treated with ethylnitrosourea (ENU) (112). Her2 mutations are relatively rare in human cancers; however wild-type Her2 is amplified at the genomic level or overexpressed at the protein level (6) in approximately 30% of invasive ductal breast cancers (113). It has been shown that overexpression of this protein correlates with tumor size, spread to lymph nodes, high grade, increased percentage of S phase cells, and aneuploidy (113). A study of mice expressing activated Her2/neu in the mammary epithelium demonstrated its ability to induce chromosomal aberrations as well as CA in cell lines derived from primary tumors (114). Also, analysis of fine-needle aspirations of the breast found a significant correlation between the percentage of cells with CA, overexpression of HER2/neu and negative ER status (94). The molecules downstream of Her2 can also become deregulated upon overexpression. Her2 induces cyclin D1 through the Ras/Rac/Rho pathway in which the ERK, JNK and p38MAPK cascades are distal mediators.

A PubMed search for “Ras and Cancer” returns twenty-four thousand hits for articles and reviews discussing the oncogenic potential of Ras and the many cellular phenotypes that it affects. Probably one of the most thoroughly studied of the many Ras-mediated pathways is the MAP kinase (MAPK) cascade, a critical signaling cascade regulating cell proliferation by exerting control over the cell cycle. It has been shown that constitutive activation of MAPK induces defects in the normal mitotic processes of the cell (115). For example, transduction of v-ras into NIH 3T3 cells induced CA and inhibition of this phenotype was possible with the introduction of MAPK inhibitors (115). Expression of the H-Ras^{G12V} or the H-Ras^{G12V} & c-Myc oncogenes in non-transformed MCF10A human mammary epithelial cells results in elevated frequencies of CA (116). Activation of this pathway is relevant in vivo, as ectopic expression of the K-Ras^{G12D} oncogene in mouse mammary epithelial cells resulted in CA that greatly preceded tumorigenesis (116).

Transcription of the cyclin D1 gene and subsequent interaction with its kinetically active partner, Cdk4, depends on receptor mediated Ras signaling. Various upstream and downstream effectors of the MAPK pathway up-regulate the transcription of cyclin D1 so that when it is bound to Cdk4 it is able to sequester p27^{Kip1} and thus activate cyclin E-Cdk2 complex (117). Upon this activation, both cyclin-Cdk complexes are free to phosphorylate RB family proteins and cells may progress from G_1 to S phase of the cell cycle (117). In normal cells mitogenic growth factors are responsible for inducing cyclin D1; however, overexpression of cyclin D1, independent of growth factor signaling, is

a common feature of many tumors (117). For example, a great majority of small cell lung cancers, breast cancers, glioblastomas, and mantle cell lymphomas display overexpression of cyclin D1 or its catalytic partner, Cdk4. In fact, aberrant overexpression of cyclin D1 occurs in 70-100% of breast tumor cell lines and most breast cancers and seems to be required for neu and Ras-induced mammary epithelial transformation (7). Along the same line, cyclin D and Cdk4 are required for neu and ras induced mammary tumorigenesis (8, 9), demonstrating that the cyclin D1/Cdk4 complex is needed for mammary transformation.

Direct evidence demonstrating involvement of the G_1 phase Cdks in centrosome amplification

Although the evidence associating hyperactive G_1 phase cyclin/Cdks and CA is convincing, it is nevertheless correlative. This is due to the fact that some of the proto-oncogenes, tumor suppressors, and transcription factors that control G_1 phase Cdk activities, such as Her2, Ras, E2f3 and p53, also regulate a plethora of other gene products (67, 69, 118, 119). Table I lists a subset of oncogenes and altered tumor suppressors, and the G_1 phase Cdk they may hyperactivate to signal CA. How do G_1 phase Cdks signal oncogene-dependent CA? Research showing that inhibition of specific Cdks blocks centriole reduplication was the first direct evidence of a relationship between Cdks and CA. In HU-arrested cells, cells treated with butyrolactone I or roscovitine -inhibitors of Cdk2, Cdc2 and Cdk5 activity- (120, 121), and cells treated with the

Cdk2/Cdk4 inhibitor $p21^{Waf1/Cip1}$ centriole reduplication was blocked (27). Following these initial experiments, combinatorial cyclin E/A/p53 gene knockout analyses demonstrated that the G_1 phase cyclins and Cdks play pivotal roles in signaling CA. For example, in $p53^{-/-}$ cells arrested in early S phase, cyclin E, but not cyclin A, is important in centriole reduplication and CA, but in the absence of cyclin E, cyclin A can drive the abnormal phenotype (122). In $p53^{-/-}$ cells, Cdk2 mediated HU-induced centriole reduplication (123). In another study, centriole reduplication triggered by the peptide vinyl sulfone proteasome inhibitor Z-L(3)VS is dependent on cyclin E/Cdk2, as well as Polo-like kinase 4 (124). Furthermore, inhibitors of Cdk2, dominant negative mutants of Cdk2 and DP1, siRNA-mediated silencing of Cdk2, or genetic deletion of Cdk2 abrogate CA triggered by ectopic expression of E7 (75). These studies provided direct support to the role played by E2Fs and Cdk2 in CA associated with the inactivation of Rb by its conditional loss (125), the acute loss of pRb by adenovirus carrying shRNA against Rb (126), or through the expression of the E7 viral protein from the HPV16 virus (127).

Even though most evidence demonstrated that Cdk2 was the central mediator of oncogene-induced CA, our group demonstrated that Cdk4 is also an important mediator. For example, genetic ablation of Cdk2 and Cdk4 abrogated CA in p53-null cells (79) by restricting NPM-dependent excessive licensing of the centrosome cycle, as well as by restricting centriole reduplication in p53-null MEFs treated with HU. Also, we showed that siRNA-mediated silencing of cyclin D1 or Cdk4 suppressed H-Ras^{G12V} or H-Ras^{G12V}/c-Myc-dependent CA in

MCF10A human mammary epithelial cells, while inhibition of cyclin E or cyclin B did not prevent CA (116).

An important molecule downstream of Cdk2 that restricts centrosome separation and duplication is NPM phosphorylated at residue T199 (128-130). Reasoning that this mode of deregulation was an important intermediate to CA, our group showed that when E2F3a/b is ablated, cyclin E/Cdk2 activity is elevated, leading to the hyperphosphorylation of NPM^{T199} (30). Hyperphosphorylation of NPM^{T199} by Cdk2 strongly correlated with constitutive centrosome duplication cycle and CA. The role of NPM as a negative regulator of centrosome duplication was confirmed genetically through a gene knockout approach, as cells heterozygous for NPM displayed CA (131). Silencing of NPM in $p53^{-/-}p19Arf^{-/-}Mdm2^{-/-}$ MEFs also resulted in CA (132). In the same system, ectopic expression of NPM^{T198A} could not rescue the CA phenotype in $p53^{-/-}$ $p19Arf^{-/-}Mdm2^{-/-}$ MEFs. In contrast, our group used a similar mutant of NPM, NPM^{T199A} (which cannot be phosphorylated by Cdk2 or Cdk4) to demonstrate that this mutant prevented CA in p53-null cells to the same extent as ablated Cdk2 or Cdk4 (79). These experiments demonstrated that the G_1 phase Cdks signal CA in p53-null cells through NPM. In terms of other mechanisms linking the G_1 phase Cdks and CA, the Fry group demonstrated that nuclear export is required for centriolar satellite formation and centrosome overduplication in p53-null cells, with export inhibitors causing a Cdk2-dependent accumulation of nuclear centrin granules (123). This group proposed an interesting model of regulation of centriole reduplication: Centrosome precursors arise in the nucleus,

providing a novel mechanistic explanation for how nuclear Cdk2 can promote centrosome overduplication in the cytoplasm.

Other than the hyperphosphorylation and inactivation of NPM and the nuclear accumulation of centrin intermediates, processes that are dependent on Cdk2, the centrosomal targets controlled by oncogenes and altered tumor suppressors directly responsible for CA are largely unknown. The sole exception is Nek2; it has been observed that silencing Nek2 abrogated CA in human mammary epithelial cells expressing H-Ras^{G12D} and H-Ras^{G12D}/c-Myc (116).

Conclusions and future directions

The observations that various pre-malignant lesions harbor CA first mapped CA to tumor initiation. Recent evidence demonstrating that low level aneuploidy caused by interference with spindle assembly components results in various tumors in mouse models (133, 134), together with observations that merotelic attachments cause that same kind of aneuploidy (135, 136) helped to bridge the gap between CA, aneuploidy, and tumor initiation. Furthermore, two recent manuscripts showed that ectopic expression of centrosome regulatory proteins leads to benign tumors in transplanted Drosophila brain stem cells, suggesting for the first time a direct relationship between CA and tumorigenesis (23, 24). However, unlike mammalian cancers, which are grossly aneuploid, the benign tumors in Drosophila harboring CA displayed neither aneuploidy nor detectable gross chromosomal aberrations (24). The classic Weinberg experiments may help shed some light on the kind of genomic changes that may

be needed to transform a human epithelial cell. For example, they showed that transformation of a primary human mammary epithelial cell required ectopic expression of telomerase to protect from senescence induced by telomere shortening (137). Ectopic expression of Ras and c-Myc as well as inactivation of p53 and Rb (via the SV40 large T antigen) was also required for transformation, suggesting that some cooperation is necessary to transform primary cells. It is to note that most of the genes that were required to transform those mammary epithelial cells affect CA, or allow the generation of chromosome breaks and recombination (115, 116, 125, 138-143). This suggests that the CA and genomic instability triggered by those oncogenes, combined with their ability to affect proliferation provide those cells selective advantages to initiate mammary tumors. Future experiments are needed to understand how CA transforms cells, and whether it eventually causes ectopic proliferation and decreases apoptosis, or whether it contributes to tumorigenesis by altering other processes, such as the orientation of cells within a tissue, a concept postulated by the Gonzalez group in their Drosophila model (24). Another pressing issue is to establish, using proteomics and transcriptomics, the centrosomal targets that are deregulated by various oncogenic and altered tumor suppressive pathways. This will allow for the ectopic expression or inactivation of various centrosome regulatory proteins in primary cell lines to more directly assess the role of CA in transformation.

Objective

Because CA is present in the vast majority of human tumors, and since supernumerary centrosomes may generate aneuploidy and genomic instability, centrosome dysfunction is a potentially important contributor to cancer biogenesis. However, the centrosome biology field has yet to demonstrate a causal relationship between CA and mammalian tumorigenesis.

At the inception of this dissertation the majority of the field of centrosome biology was involved in research concerning centrosome duplication and cell cycle regulation. This focus on cell biology provided detailed information on how the centrosome duplication cycle worked within the framework of the well understood cell cycle. As more came to be known about the centrosome, evidence emerged of deregulated oncogenes and tumor suppressors impinging on the centrosome cycle. The first demonstration of the disruption of normal centrosome biology by loss of a tumor suppressor – p53 – was published in 1996 (102), while the first demonstration that an oncogene – Ras – caused CA, was published by my dissertation advisor in 1999 and 2000 (141, 144). Gradually work began associating genomic instability and aneuploidy with centrosomal abnormalities. The seminal work that suggesting a centrosomal/mitotic kinase – Aurora A – caused CA in the mammary epithelium preceding tumorigenesis was published in 2006 (145). A study published in 2008 first showed that overexpression of a centrosomal protein caused tumorigenesis in a Drosophila model (23). Subsequently, the field has not only worked to advance the

understanding of the molecular mechanisms of centrosome biology, but also resolve the contribution of centrosomal proteins to tumorigenesis.

Work in our lab has been no different. We have worked to establish a regulatory function for G_1 phase Cdks in the centrosome cycle, as well as to better understand oncogene specific drivers of CA using mammalian cancer genetic models. Based on our work showing that the Ras oncogene could drive CA through cyclinD /Cdk4 and Nek2 in mammary epithelial cells, we sought a more clinically relevant model, and more importantly, a mechanism. The goal of this dissertation was to further our understanding of the role of G_1 Cdks in CA in a Her2-driven model (Figure 3). Our main objectives included determining a role for Cdk4 in CA and elucidating the molecular mechanism by which Cdk4 impinges on the centrosome duplication cycle in Her2+ breast cancer cells.

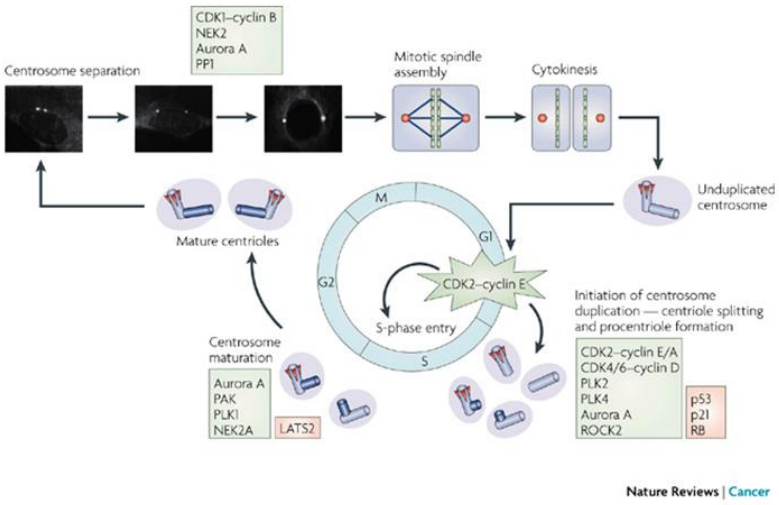


Figure 1. The coordinated cell and centrosome duplication cycle. The cell and centrosome cycles are coordinated by numerous regulators such that genetic material and centrosomes are only duplicated once per cell cycle (101). Reprinted by permission from Macmillan Publishers Ltd.

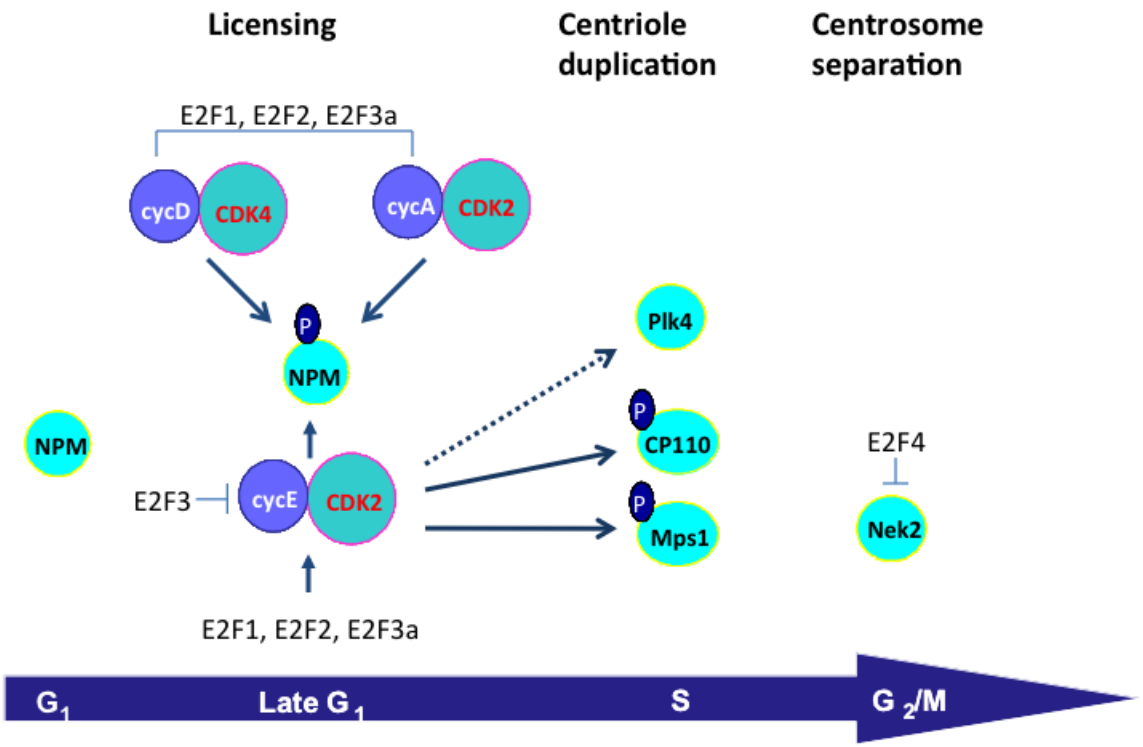


Figure 2. The G_1 phase Cdks and the E2Fs regulate various steps in the centrosome duplication cycle. Evidence suggests that the G_1 phase Cdks directly phosphorylate NPM, CP110 and Mps1 to regulate centrosome licensing and duplication. The dotted line reflects the fact that even though Plk4 is not a direct target of Cdk2, introduction of a dominant-negative Cdk2 construct renders it ineffective in triggering centriole reduplication. The figure reflects how the E2F activators E2F1, E2F2 and E2F3 influence the centrosome duplication cycle by controlling the transcriptional levels of cyclins E, A, D, and Cdk2. The figure also reflects how E2F3 and E2F4 repress cyclin E and Nek2 to influence the centrosome cycle.

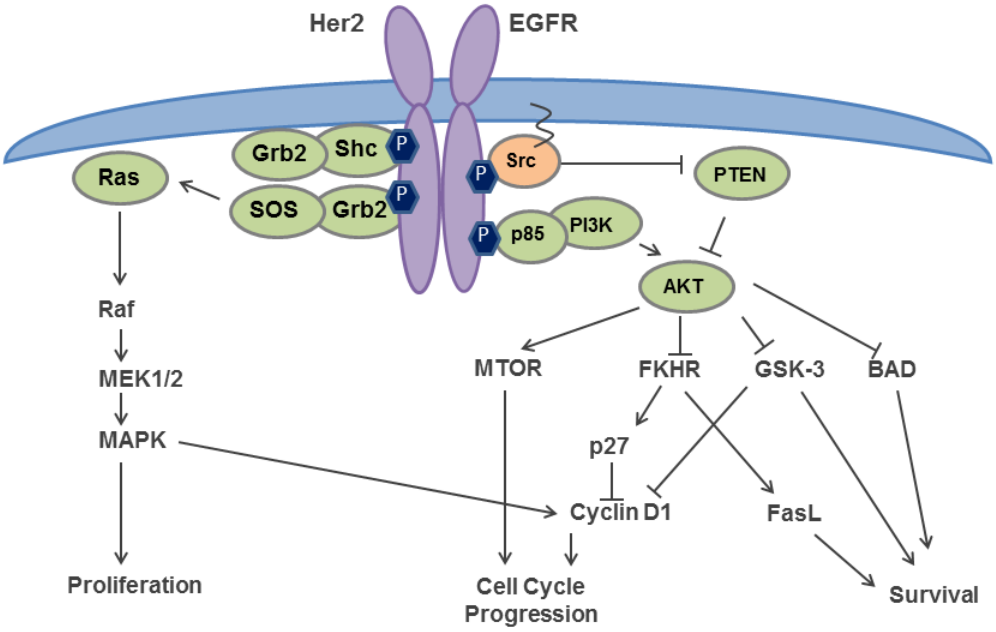


Figure 3. Her2 and associated downstream signaling. Her2 heterodimerization drives multiple downstream signaling cascades. The influence of Her2 overexpression on the Ras/MAPK pathways, specifically its control of Cyclin D1, are discussed in this dissertation.

Table 1. Oncogenes and inactive tumor suppressors and the Cdk they may activate to signal centrosome amplification.

Genetic Alteration	Deregulated Cdk	Reference
Oncogenes		
Cyclin D1	Cdk2, Cdk4	(146, 147)
ErbB2	Cdk4	(8)
Ras	Cdk4	(9, 116)
Tumor Suppressors		
E2F3a/b	Cdk2	(30)
MEK2	Cdk4, Cdk6	(148)
$p16^{INK4A}$	Cdk4, Cdk6	(149, 150)
$p21^{Waf1/CIP1}$	Cdk2, Cdk4	(104, 105, 107, 108)
p53	Cdk2, Cdk4	(79, 106, 107)
Skp2	Cdk2	(151)
Rb	Cdk2	(75)

Chapter 2

Cdk2 and Cdk4 regulate the centrosome cycle, and are critical mediators of centrosome amplification in p53-null cells.

Portions of this chapter have been published in Adon AM, Zeng X, **Harrison MK**, Sannem S, Kiyokawa H, Kaldis P, et al. Mol Cell Biol. 2010 Feb;30(3):694-710. PubMed PMID: 19933848. Epub 2009/11/26. Harrison MK contributed to data presented in Figure 4.

Introduction

The centrosome maintains genomic integrity by enforcing euploidy (21). A centrosome consists of two centrioles containing proteins such as α-tubulin, structural proteins including pericentrin, gamma-tubulin and centrin-2, and cell cycle regulatory proteins that include p53 and cyclin E/Cdk2. Normal cells have one mature centrosome during early G_1 (21). At late G_1, each of the centrioles composing the mature centrosome separate and duplicate a new (or daughter) centriole between late G_1 and late S phase, culminating in two fully mature centrosomes at G_2. The two mitotic centrosomes associate with spindle fibers and migrate towards opposite sides of the spindle pole to establish bi-polarity. This ensures that sister chromatids segregate toward each spindle pole. Following cytokinesis, each daughter cell receives one centrosome, and an equal complement of chromatids. Normal centrosome duplication must be strictly controlled, and strictly coordinated to S phase initiation and progression (107). When this control fails, CA occurs, leading to aberrant and multipolar mitotic spindles, increased frequency of chromosome segregation errors, aneuploidy and chromosome instability (21, 152). CA, aneuploidy, and chromosome instability contribute to cancer biogenesis and progression by triggering reduced expression of tumor suppressors and overexpression of proto-oncogenes.

One of the pathways contributing to CA is deregulated centrosome duplication triggered by the G_1 Cdks (30, 122, 128). The cyclin-dependent kinases (Cdks), a family of serine/threonine protein kinases, control the onset of the major cell cycle events such as DNA synthesis and mitosis (153). Cdk

activities are positively regulated by association with different cyclins, which are temporally expressed at specific phases of the cell cycle; they are negatively regulated by a variety of Cdk inhibitors, CKIs (153). Individual and combinatorial gene-knockouts of the cyclins and Cdks uncovered redundancy in the regulation of DNA synthesis and specificity in their ability to control development and tumorigenesis (8, 9, 73, 74, 76, 154-159). However, how the cyclins and Cdks individually or cooperatively impinge on centrosome duplication is poorly understood. Biochemical and pharmacological evidence pointed to Cdk2 as the only Cdk coordinating the centrosome duplication and the cell cycles (26-28, 70, 160). Cdk2 was proposed to coordinate the cell and the centrosome duplication cycles by phosphorylating Rb to promote S phase (161), and by phosphorylating various centrosomal proteins to regulate the centrosome duplication cycle (128, 162, 163). Cyclin E/Cdk2 phosphorylates NPM/B23 in T199 to regulate centrosome licensing; this phosphorylation allows separation of centrioles and permits commencement of centriole duplication (129). Cdk2 directly promotes centrosome duplication by phosphorylating Mps-1 and CP110, and by modulating the activity of Plk4 (162-164). However, gene knockout approaches dethroned Cdk2 as the sole Cdk coordinating the cell and the centrosome duplication cycles, as MEFs deleted for *Cdk2* (75) or for *cyclins E1* and *E2* (76) only showed a minor deviation in normal centrosome ratios and proliferated. These results implied that as in the cell cycle, there is redundancy amongst the Cdks regulating the centrosome duplication cycle. These results were unexpected, given the involvement of Cdk2 in the regulation of two central steps

in the centrosome duplication cycle: licensing and duplication. To date, the identity of the Cdks supporting Cdk2 in regulating normal centrosome duplication is unknown.

As the cyclins, Cdks and CKIs control centrosome duplication, altered tumor suppressors and oncogenes deregulate those cell cycle regulatory molecules, leading to CA (100, 152). Ablated genes that result in elevated Cdk2 activity and elevated frequencies of CA include *E2F3*, *p53*, *Skp2* and $p21^{Waf\text{-}1}$; similarly, ectopically expressed cyclins E and A result in elevated Cdk2 activity and CA in $p53^{-/-}$ MEFs (28, 30, 107, 108, 122, 151). Likewise, oncogenes and altered tumor suppressors that hyper-activate Cdk4 and result in high frequencies of CA include ectopically expressed Her2 (114), $H\text{-}Ras^{V12}$, v-Mos (115), $MEK1^{Glu\text{-}217/Glu221}$ (141), cyclin D1 (146) and silenced MEK2 (148). Conversely, p16 restricts excessive centriole re-duplication (28, 150). However, the relationships between altered genes, ectopic activities of specific Cdks and CA are correlational, as they deregulate cyclin/Cdk activities as well as complex signal transduction cascades that control a plethora of transcripts.

The ability of the cell cycle and centrosomal checkpoints -signaling pathways which monitor the integrity and replication status of the genome and the centrosome- to inhibit entry into S phase and centrosome duplication are closely associated with the function of the p53 tumor suppressor (165, 166). The p53 transcription factor is inactivated in approximately 50% human cancers (167). P53 regulates the transcription of a large number of genes to prevent entry into S phase in the presence of DNA or centrosome damage (165, 166).

Indeed, ablated *p53* allows CA, aneuploidy, and chromosome instability (102). A gene product central to centrosome duplication control is $p21^{Waf1}$, expressed at low levels in a p53-dependent manner (168) to inhibit the cyclin E/ Cdk2 complex (153). In addition, $p21^{Waf1}$ has been implicated in the assembly of the cyclin D1/Cdk4 complex and its overexpression inhibits it at higher concentrations (105, 169, 170). The continual presence of $p21^{Waf1}$ guards against premature activation of cyclin E/Cdk2 and perhaps cyclin D/Cdk4, ensuring the coordinated initiation of centrosome and DNA duplication. In $p21^{Waf1-/-}$ MEFs initiation of centrosome and DNA duplication is uncoupled, much like cells with constitutively active cyclin E/Cdk2 (107, 108, 168). Importantly, observations that the re-introduction of wild-type p53 into $p53^{-/-}$ cells results in nearly complete restoration of the centrosome duplication cycle while ectopic expression of $p21^{Waf1}$ into $p53^{-/-}$ cells only partially restored that cycle (107), suggests that p53 controls centrosome duplication through multiple pathways, one of which is mediated by the negative regulation of Cdk2 by $p21^{Waf1}$.

As direct evidence linking Cdk2 or Cdk4 to CA in $p53^{-/-}$ MEFs was lacking, we used a genetic approach to test whether Cdk4 and Cdk2 mediate that abnormal process. Our results uncovered that *p53* knockout does not signal CA and chromosome instability exclusively through Cdk2, as suggested previously (100). We propose a new paradigm: ablated *p53* requires both Cdk2 and Cdk4 activities to be present in order to induce high frequencies of CA and chromosome instability.

Results

Mouse embryonic fibroblasts proliferate despite the absence of Cdk2 and Cdk4

To investigate how Cdk2 and Cdk4 individually or synergistically contribute to the regulation of normal centrosome duplication and how they mediate CA, wild-type (Wt), *p53*$^{-/-}$, *Cdk2*$^{-/-}$, *Cdk4*$^{-/-}$, *Cdk2*$^{-/-}$*Cdk4*$^{-/-}$, *p53*$^{-/-}$*Cdk2*$^{-/-}$, *p53*$^{-/-}$*Cdk4*$^{-/-}$ and *p53*$^{-/-}$*Cdk2*$^{-/-}$*Cdk4*$^{-/-}$ E13.5 MEFs were generated. Genotypes were determined by allele-specific PCR (Figure 1A). Western blots confirmed the presence or absence of p53, Cdk2 and Cdk4 (Figure 1B). To establish whether Cdk2 and Cdk4 were up-regulated as compensatory mechanisms for their loss, expression levels of Cdk2 in *Cdk4*$^{-/-}$ cells, and Cdk4 in *Cdk2*$^{-/-}$ cells using wild-type cells as controls were examined (Figure 1C). There is no compensatory up-regulation of Cdk2 or Cdk4 in *Cdk4*$^{-/-}$ and *Cdk2*$^{-/-}$ MEFs, as their levels are identical to wild-type controls.

Published observations indicated that early-passage *Cdk2*$^{-/-}$ and *Cdk4*$^{-/-}$ MEFs proliferated, and that the kinetics of entry into S phase in *Cdk2*$^{-/-}$ or *Cdk4*$^{-/-}$ are moderately delayed relative to wild-type MEFs (74, 159). Additionally, *Cdk2*$^{-/-}$*Cdk4*$^{-/-}$ MEFs senesce at earlier passages than the individual knockouts (73, 74, 156, 159). Ablation of *p53* abrogates senescence associated with single, or combined loss of Cdk2 and Cdk4 at late passages (171). To rule out that any reductions in frequency of CA in *p53*$^{-/-}$ MEFs lacking Cdks associate with major changes in frequencies of proliferation, the G_1/S transition in each genotype was investigated. The indicated MEFs were plated, serum starved, stimulated with serum, and harvested at the indicated time points for S phase entry

measurement by BrdU incorporation. All MEFs peaked with 30 to 50 % of cells in S phase at 12 hrs (Figure 2A). In contrast, *Cdk2*$^{-/-}$*Cdk4*$^{-/-}$ MEFs entered S phase with delayed kinetics, and less than 10% of the cells were actively proliferating at 16 hrs. Likewise, all MEFs lacking p53 and the Cdks reached their peak frequency of cells in S phase at 12 hrs (Figure 2B).

Our goal was to identify deregulated cell cycle regulatory molecules that may trigger the CA observed in *p53*$^{-/-}$ MEFs. Various defects in the cell cycle regulatory machinery result in deregulated Cdk activities and CA. Those include overexpression of cyclins A, E and D (30, 122, 146, 151, 168, 172). To further investigate the molecular consequences of Cdk loss to regulatory molecules governing the G_1/S transition, and whether deregulation of various cyclins accounted for CA in p53-null cells, Western blots were performed to analyze the expression of cyclin A, cyclin D1 and cyclin E at the indicated time points (Figure 2C). Cyclin E levels were robust throughout the cell cycle, but its accumulation was decreased in *Cdk2*$^{-/-}$ and *Cdk2*$^{-/-}$*Cdk4*$^{-/-}$ MEFs from 4 to 12 Hrs post-serum addition. Cyclin D1 levels reached maximal accummulation at 12 and 16 hrs. No major changes in cyclin D1 levels were observed, except moderately diminished levels of cyclin D1 in *Cdk4*$^{-/-}$, *Cdk2*$^{-/-}$*Cdk4*$^{-/-}$ *p53*$^{-/-}$*Cdk4*$^{-/-}$, and *p53*$^{-/-}$*Cdk2*$^{-/-}$*Cdk4*$^{-/-}$ MEFs relative to other genetic groups at 12 and 16 hrs. Cyclin A expression was low in all the MEFs before 8 Hrs post-serum; its expression levels peaked at 16 hrs post-serum addition, with similar expression in all MEFs. We conclude that except for minor changes in the expression of cyclins E and cyclin D1 when their respective catalytic partners, Cdk2 and Cdk4 are ablated, the temporal cyclin

expression patterns are what we would expect of a normal cell cycle, consistent with the similar kinetics of entry into S phase in all genetic groups.

Reduced expression of certain CKIs can also result in elevated frequencies of CA or suppress CA when overexpressed (107, 150, 173). For example, ectopically expressed $p16^{INK4A}$, a Cdk4-specific inhibitor, prevents centriole duplication and CA (28, 150). On the other hand, $p27^{Kip1}$, recently reported to be a dual Cdk2 and Cdk4 inhibitor (174) prevents CA triggered by gamma-irradiation (70, 173). In addition, ablation of $p21^{Waf1}$, a Cdk2-specific inhibitor at physiological levels and a Cdk4 inhibitor at higher levels (104, 105, 170) results in CA; its ectopic expression partly suppresses CA in *p53*$^{-/-}$ MEFs (107-109). To identify whether CKIs may deregulate Cdk2 and Cdk4 in *p53*$^{-/-}$ MEFs leading to CA, we probed Western Blots with antibodies against $p21^{Waf-1}$, $p27^{Kip1}$, $p57^{Kip2}$, and $p16^{INK4A}$ (Figure 2D). To detect constitutive signaling triggered by the absence of p53, Western blots were performed on serum-starved cells. Those analyses showed that in *p53*$^{-/-}$ MEFs, endogenous $p21^{Waf1}$ was undetectable. In contrast, $p27^{Kip1}$, levels were unchanged in *p53*$^{-/-}$ MEFs relative to wild-type and other MEFs. On the contrary, $p57^{Kip2}$ and $p16^{INK4A}$ were overexpressed in *p53*$^{-/-}$ MEFs.

We also examined the expression level of $p57^{Kip2}$ and $p16^{INK4A}$ by siRNA-mediated silencing of p53 in wild-type MEFs (Figure 2E). P53-specific siRNA duplex sequences were synthesized and used to knock down p53 gene in three independent wild-type MEFs. Western blot analysis revealed that depletion of p53 in wild-type cells did not lead to a major elevation of $p57^{Kip2}$, but that steady-

state $p16^{INK4A}$ levels were moderately increased. We conclude that the only major alteration in a cell cycle regulatory molecule associated with CA in *p53*$^{-/-}$ MEFs is the absence of $p21^{Waf1}$, consistent with published results (107). We also conclude that the reported high frequencies of CA in *p53*$^{-/-}$ MEFs (102) occured despite robust levels of CKIs controlling Cdk2 and Cdk4 activities, including $p16^{INK4A}$ and $p27^{Kip1}$, potent inhibitors of CA and centriole duplication (28, 70, 150, 173).

Cells lacking Cdk2 and Cdk4 display abnormal centrosome cycles

MEFs devoid of Cdk2 undergo minor defects in centriole duplication (75). This suggested to us that either the Cdks regulating the centrosome duplication cycle are redundant, such as is the case with the Cdks regulating S phase entry (73, 74, 155-159), or that other Cdks or centrosomal kinases are solely responsible for orchestrating normal centrosome duplication. As centrosome duplication licensing must be coordinated with entry into S phase (the later initiated by cyclin D/Cdk4 and continued by cyclin E/Cdk2), and based on the brief expression overlap between cyclin D/Cdk4 and cyclin E/Cdk2 activities at the G_1/S transition (175), we hypothesized that cyclin D1/Cdk4 is involved in that coordination. To assess the involvement of Cdk4 in normal centrosome duplication, and to explore whether Cdk2 and Cdk4 cooperate to affect centrosome duplication, we measured frequencies of cells with 1, 2 or ≥3 centrosomes in early passage (passage 2) MEFs devoid of Cdk2 and/or Cdk4 using immuno-histochemistry with antibodies against pericentrin and gamma-

tubulin, core components of the centrosome (Figure 3A). Previous studies showed that normal ratios of centrosomes in wild-type MEFs are 60% cells with one centrosome: 40% cells with two centrosomes (107). Any deviation in the ratios of centrosomes within a population is indicative of defects in the various steps driving the centrosome duplication cycle: licensing, separation of centrioles and duplication of centrioles (30, 107). A centrosome ratio favoring cells with one centrosome is indicative of defective licensing of the centrosome cycle, or centriole separation, while a centrosome ratio of two is indicative of premature centriole separation and duplication. Consistent with published results, C*dk2*$^{-/-}$ MEFs did no display a statistically significant deviation in centrosome ratios relative to wild-type MEFs (48:46% relative to 58:38%, respectively). In contrast, *Cdk4*$^{-/-}$ MEFs showed a significant variation in the ratios of cells with one to two centrosomes relative to wild-type MEFs (77:20% relative to 58:38%). This accummulation of cells with one centrosome is not due to a longer G_1, as *Cdk4*$^{-/-}$ MEFs entered S phase with similar kinetics as wild-type MEFs (as presented in Figure 2A). In addition, *Cdk2*$^{-/-}$*Cdk4*$^{-/-}$ *MEFs* also displayed a severe deviation in centrosome ratios relative to wild-type MEFs (35:55% relative to 58:38%).

To assess the phase in the cell cycle in which the various centrosome defects occurred, we dissected the centrosome duplication kinetics in cells by comparative analysis of synchronized wild-type, *Cdk2*$^{-/-}$, *Cdk4*$^{-/-}$ and *Cdk2*$^{-/-}$*Cdk4*$^{-/-}$ cells (Figure 3B). MEFs of the indicated genoypes were grown in duplicate, followed by serum starvation for 60 hours. Quiescent cells were stimulated with serum, and BrdU was included to the medium to monitor S phase entry. Every 4

hrs for a period of 16 Hrs, BrdU incorporation, and the number of one and two centrosomes per cell were scored. Following kinetics of entry into S phase, wild-type MEFs reached peaks in cells with two centrosomes at 12 hrs post-serum stimulation. *Cdk2*$^{-/-}$ MEFs reached maximal ratios of cells with 2 centrosomes earlier than wild-type MEFs, at 8 hrs post-stimulation. In contrast to wild-type or *Cdk2*$^{-/-}$ MEFs, percentages of cells with 2 centrosomes in *Cdk4*$^{-/-}$ MEFs decreased throughout the cell cycle, as the cells steadily accummulated a centrosome content of one. Another intriguing defect was in *Cdk2*$^{-/-}$*Cdk4*$^{-/-}$ MEFs, in which ablation of *Cdk2* overrides the defect in centrosome ratios in *Cdk4*$^{-/-}$ MEFs. Even though *Cdk2*$^{-/-}$*Cdk4*$^{-/-}$ MEFs were unable to replicate their DNA efficiently, their centrosome duplication maxed out at 8 hrs post-serum stimulation; thus, the centrosome and cell cycles are uncoupled in those cells.

We then explored whether transient down-regulation of Cdk2 and Cdk4 with siRNAs in wild-type MEFs recapitulated the centrosome cycle defects in *Cdk2*$^{-/-}$, or *Cdk4*$^{-/-}$ MEFs (Figure 3C, D). Wild-type MEFs were transfected with siRNA duplexes against Cdk2 or Cdk4. Seventy-two hours after transfection, cell lysates were obtained, and centrosome analyses performed. Western blot analysis of the extracts from the transfected cells showed significant depletion of Cdk2 and Cdk4 (Figure 3C). In contrast to the *Cdk2*$^{-/-}$ centrosome profile, which showed normal ratios of centrosomes, siRNA-mediated down-regulation of Cdk2 led to the accummulation of cells with two centrosomes; however, as in *Cdk4*$^{-/-}$ MEFs, depletion of Cdk4 promoted more cells with one centrosome (Figure 3D). Our results have identified one of the major triggers for normal centrosome

duplication, which involves Cdk4, as well as the cooperation of Cdk2 and Cdk4 activities. Experiments that knocked-out and knocked-down Cdk2 or Cdk4 suggested that the functions of those Cdks are unique. To address whether ectopic expression of Cdk2 rescued the accummulation of cells with one centrosome observed in *Cdk4*$^{-/-}$ MEFs, we overexpressed Cdk2 in C*dk4*$^{-/-}$ cells. As shown in Figure 3E, Cdk2 significantly rescued centrosome defects imposed by ablated *Cdk4*, demonstrating that the centrosome defect imparted by ablated *Cdk4* is reversed by overexpression of Cdk2; in this scenario Cdk2 and Cdk4 are redundant.

Individual ablation of Cdk2 or Cdk4 abolishes centrosome amplification in p53$^{-/-}$ *MEFs by preventing excessive centriole duplication*

The current models attempting to explain how absence of *p53* allows CA propose that elevated Cdk2 activity is primarily responsible for CA in those MEFs (100, 107). This was suggested by observations that *p21*$^{Waf1-/-}$ cells have elevated frequencies of CA, or that ectopic expression of p21^{Waf1} partly restored normal centrosome frequencies in *p53*$^{-/-}$ MEFs (107, 176). Since p21^{Waf1} influences cyclinD/Cdk4, positively by promoting its assembly and inhibits it at higher concentrations (104, 105, 170, 177), we speculated that Cdk4 may also mediate CA in *p53*$^{-/-}$ MEFs and set out to explore the relative contributions of Cdk2 and/or Cdk4 to CA in *p53*$^{-/-}$ MEFs. While wild-type cells did not display elevated frequencies of CA, loss of *p53* resulted in 40% of the cells displaying CA (Figure 4A and B). As predicted, ablation of *Cdk2* in *p53*$^{-/-}$ MEFs prevented

CA (by approximately 70%). To our surprise, as no one has reported elevated Cdk4 activity in *p53*$^{-/-}$ MEFs, and as our Western blots presented in Fig 2 did not reveal any changes in any cyclins or CKIs that may promote deregulated Cdk4, ablated *Cdk4* suppressed CA in *p53*$^{-/-}$ MEFs to the same extent as ablated *Cdk2*. To establish whether Cdk2 and Cdk4 cooperated to further decrease CA, we calculated frequencies of CA in *p53*$^{-/-}$*Cdk2*$^{-/-}$*Cdk4*$^{-/-}$ MEFs. Indeed, *p53*$^{-/-}$*Cdk2*$^{-/-}$ *Cdk4*$^{-/-}$ MEFs displayed similar frequencies of CA as *p53*$^{-/-}$*Cdk2*$^{-/-}$ or *p53*$^{-/-}$*Cdk4*$^{-/-}$ MEFs. We next siRNA silenced the expression of Cdk2 or Cdk4 in *p53*$^{-/-}$ MEFs (Figure 4 C, D). Western blots indicated that most of Cdk2 or Cdk4 were depeleted compared to the controls (Figure 4C). Indeed, as in the combinatorial knockouts, siRNA-mediated inhibition of Cdk2 or Cdk4 suppressed CA in *p53*$^{-/-}$ MEFs (Figure 4D). We conclude that Cdk2 and Cdk4 are individually required to mediate CA.

In normal centrosome duplication, cells enter G_1 with one single centrosome, composed of two centrioles: the mother centriole (older) and the daugther centriole (newer). To directly test that Cdk2 and Cdk4 regulate centriole duplication, we performed a centriole re-duplication assay. This assay involves challenging cells with HU for 48 hrs, which inhibits DNA synthesis at late G_1/early S phase. As centrosomal checkpoints are functional in wild-type cells, centrosome reduplication is predicted to be absent in those cells (28). On the other hand, cells lacking certain checkpoint controls, such as those cells ablated for *p53* continue duplicating their centrioles within the centrosomes, resulting in multiple centrioles. For the centriole reduplication assay, proliferating MEFs of

the indicated genotypes were untreated or treated with HU for 48 hrs. To determine the presence of centrioles, the cells were subjected to cold-treatment and brief extraction prior to fixation. This treatment destabilizes microtubules nucleated at centrosomes; hence, centrioles can be microscopically visualized by immunostaining for α-tubulin, a major component of centrioles, at a high magnification. Co-immunostaining of cells subjected to cold treatment and brief extraction with anti-γ-tubulin (which detects the peri-centriolar material, PCM) and anti-α-tubulin revealed that wild-type cells stopped centrosome duplication after 48 hrs in culture in the presence of HU, while *p53*$^{-/-}$ cells continued doing so (Figure 4E and F). On the other hand, ablation of either *Cdk2* or *Cdk4* in *p53*$^{-/-}$ cells completely halted centriole re-duplication. The same experiment was repeated with 48 hrs cultures in the presence of mimosine, or HU, with immuno-stainings done with with gamma-tubulin, and the results were identical: we detected the accummulation of centrosomes in *p53*$^{-/-}$ MEFs and ablated *Cdk2* or *Cdk4* prevented that accummulation (data not shown). These experiments demonstrated that ablated *Cdk2* and *Cdk4* suppress CA by normalizing the centrosome re-duplication defect triggered by ablation of *p53*. Importantly, this result identified one of the specific steps in the centrosome cycle affected by the absence of Cdk2 or Cdk4: centriole duplication.

Genetic ablation of Cdk2 or Cdk4 suppresses chromosome instability in p53$^{-/-}$ *MEFs*

Ablated *p53* generates chromosome instability through CA (102, 178, 179) and by allowing the generation of reactive oxygen species, which are predicted to be the result of double-strand DNA breaks (180). To establish how the absence of Cdk2 and Cdk4 modulate active chromosome instability in *p53*$^{-/-}$ MEFs, we used two assays: the micronucleus assay and gamma□H2AX immunostaining. One of the initial cellular responses to the introduction of double-strand breaks is the phosphorylation of serine 139 of the carboxy-terminal tail of H2AX (181). The number of phosphorylated H2AX (gamma-H2AX) molecules increases linearly with the severity of damage. Therefore, this assay represents a way to mark double-strand DNA breaks, a precursor to the chromosome breaks and recombinations leading to structural chromosomal abnormalities, the second major form of chromosome instability. Control experiments revealed that Adriamycin, a chemical that promotes DNA breaks, resulted in most cells in the population containing gamma-H2AX foci (not shown). As shown in Figure 5A and B, loss of *p53* resulted in a significant elevation of gamma-H2AX foci over wild-type controls. On the contrary, ablation of *Cdk2* or *Cdk4* in *p53*$^{-/-}$ cells inhibited the number of cells containing gamma□H2AX to those observed in wild-type cells, consistent with published observations that loss of *Cdk2* reduces DNA repair (182).

Our second assay to detect active genomic instability was the micronucleus assay. A micronucleus is a chromosome or chromosome fragment mis-segregated during mitosis as a consequence of spindle damage or as a consequence of lost centromeric sequences (acentric chromosomes are unable

to bind mitotic fibers and are excluded from the segregating chromatids) (183-185). Following cytokinesis, micronuclei appear in the cytoplasm as DNA-containing spheres surrounded by a nuclear membrane (Figure 5C, arrows). The extent of micronucleus formation reflects the frequency of cells in a population actively losing whole chromosomes, a type of chromosome instability dependent on CA, as well as fragmented chromosomes, which arise as a result of DNA breaks (115, 141). Our results indicated that ablation of *Cdk2* or *Cdk4* reduced micronuclei formation in *p53*$^{-/-}$ MEFs to wild-type levels (Figure 5D). We conclude that absence of Cdk2 or Cdk4 prevents chromosome instability, an abnormal phenotype strongly associated with tumor biogenesis and progression (21, 186-189).

Cdk2 and Cdk4 signal centrosome amplification and chromosome instability through a common phosphorylation site in nucleophosmin, Thr199

The nucleophosmin (NPM) protein located at the centrosome prevents premature centriole separation and duplication during early G_1 in a similar fashion as nuclear Rb prevents premature entry from early G_1 into S phase. Both proteins are phosphorylated in late G_1 by the Cdks to relieve negative regulation (128, 175). Un-phosphorylated NPM binds to unduplicated centrosomes (centrosomes with closely associated centrioles) to prevent premature centriole separation and duplication (128, 129). Upon phosphorylation by Cdk2 at the G_1/S transition on T199, NPM disassociates from the centrosome, allowing centriole separation and duplication (128, 129). Our laboratory previously

correlated unregulated cyclin E/Cdk2 activity with phosphorylation of NPM in T199 (30). We demonstrated that cyclin E/Cdk2 phosphorylation of NPM^{T199} in G_0 rather than in late G_1 rendered NPM functionally inactive, as it no longer had the ability to bind centrosomes at G_0. This inability to bind centrosomes allowed a constitutive centrosome duplication cycle, where centrioles separated and duplicated uncontrollably, resulting in CA. As loss of *Cdk4* prevented CA in *p53*$^{-/-}$ MEFs as efficiently as ablated *Cdk2*, we set out to establish whether they shared the same phosphorylation site in NPM to signal CA and chromosome instability. Specifically, we tested whether suppression of CA by ablated *Cdk2* and *Cdk4* correlated with restoration of normal phosphorylation of NPM^{T199}. MEFs were serum-starved to mimic G_0, and the phosphorylation status of NPM^{T199} assessed by Western blots (Figure 6A). The following results provided important clues as to the mechanism by which ablated *Cdk2* or *Cdk4* prevented CA in *p53*$^{-/-}$ MEFs. Firstly, while wild-type cells displayed a baseline level of NPM phosphorylation, ablation of *Cdk2* resulted in lower phosphorylation relative to wild-type cells. Relative phosphorylation of NPM in *Cdk4*$^{-/-}$ MEFs was identical to that in wild-type MEFs. Secondly, ablation of *p53* resulted in constitutive phosphorylation of NPM^{T199}; importantly, the absence of Cdk2 or Cdk4 reduced the hyper-phosphorylation of NPM^{T199} in *p53*$^{-/-}$ MEFs to wild-type levels. The results suggested that ablated *Cdk2* or *Cdk4* abrogated CA in *p53*$^{-/-}$ MEFs by restoring the normal phosphorylation of NPM^{T199} and re-establishing normal centrosome duplication licensing. To test whether the Thr-199 phosphorylation of NPM in *p53*$^{-/-}$ MEFs was reduced by ablation of *Cdk2* or *Cdk4* in the conditions used for

the centriole reduplication assay, MEFs were treated with HU for 48 Hrs followed by proteins extraction. As shown in Figure 6B, Western blots indicated that in HU-arrested cells, ablation of *Cdk2* and/or *Cdk4* reduced NPM^{T199} phosphorylation compared to $p53^{-/-}$ MEFs.

NPM/B23 is a direct substrate of Cdk2 (128). To establish the status of NPM^{T199} phosphorylation in wild-type, $Cdk2^{-/-}$, $Cdk4^{-/-}$ MEFs throughout the cell cycle, we performed Western blots at various time-points following release from serum starvation (Figure 6C). Phosphorylation of NPM^{T199} was moderately lower at 4 and 8 hrs. in $Cdk4^{-/-}$relative to wild-type MEFs. In contrast, phosphorylation of NPM^{T199} in $Cdk2^{-/-}$ MEFs between 0 and 12 hrs. was severely diminished. Because Western blots suggested that NPM was indeed a target of Cdk4, we set out to demonstrate that Cdk4 can directly phosphorylate NPM. To that end, wild-type, $Cdk2^{-/-}$ and $Cdk4^{-/-}$ MEFs were serum starved, stimulated with addition of serum, and harvested at different time points followed by protein extraction. The presence of Cdk4 (34 KD) and its co-factor cyclin D1 (36 KD) in Ip-Cdk4 was demonstrated by Western blot analysis probed with anti-cyclin D1 and Cdk4 antibodies (Figure 6D). As expected, IPs using Cdk4 and cyclin D1 antibodies in $Cdk4^{-/-}$ MEFs did not pull down any Cdk4 and cyclin D1, demonstrating the specificity of the antibodies. After showing that the antibodies specifically pulled down Cdk4, we performed luminescent kinase assays with the Kinase Glo kit (Promega), as previously described (190), immuno-precipitating with Cdk4 and incubating with purified NPM (Figure 6E). In this assay, reduction of luminescence by the addition of kinase buffer and purified NPM peptide to the

Cdk4 immuno-precipitates indicates kinase activity. To rule out that any contaminating Cdk2 activity would result in phosphorylation of NPM, we did immunoprecipitates in synchronized *Cdk2*$^{-/-}$ MEFs. As an additional control, we performed kinase assays in *Cdk4*$^{-/-}$ MEFs. In extracts of wild-type or *Cdk2*$^{-/-}$ MEFs from 4 and 8 Hrs postserum stimulation, considerable kinase activity was detected in samples containing NPM peptide as compared to samples without substrate (No NPM), Figure 6E. We then validated our experiment by including Ip-Cdk4 from extracts of *Cdk4*$^{-/-}$ MEFs at the indicated time points; the extract from the *Cdk4*$^{-/-}$ MEFs where no Cdk4 was precipitated showed no kinase activity compared to samples without substrate (No NPM). These results demonstrated that NPM is a Cdk4 target, and that the maximal phosphorylation corresponded at the point of the cell cycle where centrosome cycle licensing occurs: mid/late G_1. However, unlike the phosphorylation of NPM by Cdk2, which occurs in the nucleus and in the cytoplasm (30), due to a centrosome localization signal in cyclin E (191), phosphorylation of NPM by Cdk4 is predicted to be nuclear, because localization of Cdk4 and of cyclin D1 was strictly nuclear, and we did not find any Cdk4 or cyclin D1 within centrosomes.

To gather more direct evidence that the phosphorylation of NPM^{T199} by Cdk2 and Cdk4 is critical to CA, plasmids encoding FLAG epitope-tagged wild-type NPM and the mutant NPM/B23 lacking the T199 phosphorylation site (NPM^{T199A}) were transfected into E13.5 MEFs derived from p53 mutant embryos (Figure 7). As control, the vector alone was transfected. Following neomycin selection, cells were examined for the level of expression of exogenous

NPM/B23 by Western blot analysis using anti-FLAG antibody. This analysis revealed that both NPM- and NPM^{T199A}-transfected cells expressed similar protein levels (Figure 7A). In addition, we set forth to establish whether wild-type NPM or NPM^{T199A} modulated frequencies of CA in *p53*$^{-/-}$ MEFs (Figure 7B). This analysis revealed that while *p53*$^{-/-}$ MEFs expressing vector control and those expressing wild-type NPM had identical frequencies of CA, the mutant lacking the G_1 Cdk phosphorylation site displayed greatly reduced frequencies of CA. The extent of inhibition was almost identical to the one in *p53*$^{-/-}$*Cdk2*$^{-/-}$ or *p53*$^{-/-}$ *Cdk4*$^{-/-}$ MEFs (as presented in Figure 4B). In summary, we have demonstrated that Cdk2 and Cdk4 hyper-phosphorylate NPM, and that this hyper-phosphorylation is critical to CA.

In addition, we established whether the suppression of CA by NPM^{T199A} restored genomic stability. Cells expressing vector and wild-type NPM had identical frequencies of micronucleus formation, while the expression of NPM^{T199A} had reduced frequencies (Figure 7C). Likewise, NPM^{T199A} inhibited gamma-H2AX double-stranded foci (Figure 7D). We conclude from these experiments that ablation of either *Cdk2* or *Cdk4* prevents the generation of chromosome breaks and chromosome losses in *p53*$^{-/-}$ MEFs. We propose a new paradigm for how CA and chromosome instability arises in *p53*$^{-/-}$ MEFs: the presence of both Cdk2 and Cdk4 is absolutely required to hyper-phosphorylate NPM^{T199} to generate CA and chromosome instability (Figure 8).

Discussion

In this manuscript, we investigated the relative contributions of the G_1 Cdks -Cdk2 and *Cdk4*- to CA in *p53*$^{-/-}$ MEFs, and in regulating the centrosome cycle. How do Cdk2 and Cdk4 affect the centrosome cycle? Ablation of *Cdk2* leads to moderate defects in centrosome duplication, suggesting redundancy in the control of this process (75); our results in asynchronously-growing *Cdk2*$^{-/-}$ MEFs confirmed those results. However, experiments measuring the centrosome cycle at various time-points throughout the cell cycle in *Cdk2*$^{-/-}$ and *Cdk4*$^{-/-}$ MEFs, as well as transient down-regulation of Cdk2 and Cdk4 using siRNAs, uncovered distinct centrosome cycle defects, suggesting that their functions are non-redundant. For example, while *Cdk2* deficiency promoted the early separation and duplication of centrosomes, absence of *Cdk4* promoted the accumulation of cells with one centrosome that failed to separate and duplicate. The accumulation of cells with one centrosome in *Cdk4*$^{-/-}$ MEFs was not compensated with passage, as the accumulation of cells with once centrosome was also observed in MEFs silenced with siRNA directed against Cdk4. The accumulation of cells with one centrosome in *Cdk4*$^{-/-}$ MEFs was not due to a block in G_1, as they entered S phase with similar kinetics as wild-type, and *Cdk2*$^{-/-}$ MEFs. On the other hand, inhibition of Cdk2 with siRNAs led to many cells in the population harboring two centrosomes, suggesting that that defect was compensated for in *Cdk2*$^{-/-}$ MEFs by Cdk4, other Cdks, or centrosomal kinases. Our observations showed that concomitant ablation of *Cdk2* and *Cdk4* cause a severe accumulation of cells with two centrosomes; experiments following the cell and centrosome cycles in *Cdk2*$^{-/-}$*Cdk4*$^{-/-}$ MEFs confirmed that centriole separation and

duplication were premature. Because the centrosome cycle defect *Cdk2*$^{-/-}$*Cdk4*$^{-/-}$ MEFs was much more severe relative to other genetic groups suggest that Cdk2 and Cdk4 cooperate to regulate the centrosome cycle. The accumulation of cells with two centrosomes in asynchronously growing *Cdk2*$^{-/-}$*Cdk4*$^{-/-}$ MEFs was not a complete block, since 40% of cells still contained one centrosome. Upon serum arrest, the ratios of *Cdk2*$^{-/-}$*Cdk4*$^{-/-}$ MEFs with one and two centrosomes were similar relative to wild-type MEFs, and upon serum addition centrosomes separated and duplicated prematurely, indicating an active centrosome cycle in *Cdk2*$^{-/-}$*Cdk4*$^{-/-}$ MEFs. In those MEFs the cell and centrosome cycles were uncoupled, as centriole separation and duplication preceded entry into S phase. Thus, as in the cell cycle, in the absence of Cdk2 and Cdk4, other Cdks or centrosome duplication kinases are also playing a role in driving a centrosome duplication cycle. That redundancy is also supported by the baseline phosphorylation of NPM^{T199} in *p53*$^{-/-}$*Cdk2*$^{-/-}$*Cdk4*$^{-/-}$ MEFs cultured under HU, demonstrating that NPM^{T199} is phosphorylated by another kinase in the absence of Cdk2 and Cdk4. Those results indicated that the same redundancy operating within the kinases controlling S phase also exists in the regulation of the centrosome cycle.

Because NPM phosphorylation in Thr199 by Cdk2 is essential to signal entry into the centrosome cycle (128, 129), we assessed whether the centrosome defects observed in *Cdk2*$^{-/-}$ and *Cdk4*$^{-/-}$ MEFs were due in part through changes in NPM^{T199} phosphorylation. As reported previously, NPM^{T199} is a canonical target of Cdk2; our Western blots revealed that at early time-points

(0-8hrs) following serum addition to *Cdk2*$^{-/-}$ MEFs, phosphorylation of NPM^{T199} was greatly diminished relative to wild-type MEF. Phosphorylation of NPM^{T199} increased in *Cdk2*$^{-/-}$ MEFs at 12 hrs. following serum addition. Because cyclin D1 levels peaked at 12 hrs. of serum addition in *Cdk2*$^{-/-}$ MEFs, indicated that that perhaps cyclin D1/Cdk4 was responsible for that phosphorylation. On the other hand, levels of NPM^{T199} were moderately reduced in *Cdk4*$^{-/-}$ MEFs at early time-points after serum addition (0-8 hrs.). Our Cdk4 kinase assays using NPM as a substrate confirmed that NPM is indeed a Cdk4 target, and that that phosphorylation is cell-cycle-regulated. In serum-arrested cells we did not detect phosphorylation of NPM; that phosphorylation commenced at 4 and 8 hrs. following serum addition, and was detected up to 16 hrs. after serum addition. However, because the reduction in NPM^{T199} phosphorylation was not nearly as reduced in *Cdk4*$^{-/-}$ MEFs relative to *Cdk2*$^{-/-}$ MEFs, suggests that in *Cdk4*$^{-/-}$ MEFs the Cdk2 present in the cell actively phosphorylates NPM^{T199}, and that the phosphorylation in NPM^{T199} is more efficiently carried by Cdk2 than by Cdk4. We speculate that cells have evolved compensatory mechanisms where when they are devoid of Cdk2, Cdk4 can indeed license the centrosome cycle. However, at this point we cannot rule out that Cdk2 phosphorylates NPM^{T199} to prime Cdk4 phosphorylation of NPM in other sites that would control centrosome licensing at late G_1. Indeed, there is evidence in the literature that phosphorylation of NPM in Thr234 and Thr237 by Cdk1 stabilizes NPM in the centrosome during M phase (192). A second possibility as to why *Cdk4*$^{-/-}$ MEFs display cells with one centrosome is that Cdk4 may directly phosphorylate and regulate the activity of a

centriole separase, or regulate the Rb/E2F-dependent transcription of a centriole separase. Centriole separases are poorly understood; one centrosome separase normally active at M phase that displays centriole separase activity when ectopically expressed during interphase is Nek2A (193). A third possibility is that Cdk4 modulates other licenser factors besides NPM. The only other known centrosome regulatory protein that may display licensing activity is CP110. Like NPM, CP110 is a phosphorylation target of Cdk2, and inhibition of its activity causes CA (163). However, unlike NPM, that disassociates from centrosomes upon Cdk phosphorylation, CP110 is present in the centrosome to cap the synthesis of a new centriole (22). Thus, we think is unlikely that Cdk4 utilizes CP110 to license the centrosome cycle. Nevertheless, the activity inhibited by ablation of Cdk4 that leads to the accumulation of cells with one centrosome, is reversible, as ablation of Cdk2 (in *Cdk2*$^{-/-}$*Cdk4*$^{-/-}$ MEFs), ectopic expression of Cdk2, or ablation of *p53* do not allow the accumulation of cells with one centrosome. What causes the accumulation of two centrosomes in cells knocked down for Cdk2, and in cells ablated for *Cdk2* and *Cdk4*? Our experiments following the cell and centrosome cycles in *Cdk2*$^{-/-}$ and in *Cdk2*$^{-/-}$*Cdk4*$^{-/-}$ MEFs revealed a premature separation and duplication of centrosomes. The defect was present in early cell cycle stages of *Cdk2*$^{-/-}$ MEFs, but restored at subsequent phases of the cell cycle. Perhaps Cdk2 and Cdk2&Cdk4 are imposing negative regulation of centriole separases, or other centrosome kinases. For example, Cdk2 targets other than NPM may prevent centriole separases to bind to the centriole pair. When *Cdk2* and *Cdk2&Cdk4* are ablated,

those centriole separases and/or centriole duplication kinases may associate with the centrosomes prematurely to signal early centriole separation and duplication. Other targets of Cdk2 that regulate various steps within the centrosome duplication cycle include CP110, and mMps-1 (162, 163). Plk-4 has not been reported to be directly regulated by Cdk2, but requires its activity for its maximum activity (164). Our future experiments will identify the centriole separases, centrosome cycle licensers, or centriole duplication kinases deregulated in *Cdk2*$^{-/-}$, *Cdk4*$^{-/-}$, and in *Cdk2*$^{-/-}$*Cdk4*$^{-/-}$ MEFs at the transcriptional or post-translational levels.

The second major finding in this manuscript is that either Cdk2 or Cdk4 signal CA in *p53*-null MEFs. Ablation of *p53* was proposed to lead to CA in part by preventing p21^{Waf1} to control Cdk2 (107-109). We demonstrated using a genetic approach that, indeed, Cdk2 is a major mediator of CA in *p53*$^{-/-}$ MEFs. A unique finding was that ablated *Cdk4* was equally effective in suppressing CA. This was unexpected, as no one has reported elevated Cdk4 activity in *p53*$^{-/-}$ MEFs. Western blots that assessed the relative levels of cyclins or CKIs that promote or inhibit Cdk2 or Cdk4 activities failed to identify any deregulated cell cycle regulatory molecule that would promote Cdk4 activity. For example, levels of cyclin D1 were similar between all groups. On the other hand, p16 was up-regulated in *p53*$^{-/-}$ MEFs, which would be consistent with decreased Cdk4 activity. How does Cdk4 mediate CA in *p53*$^{-/-}$ MEFs? At low concentrations, p21^{Waf1} is a specific inhibitor of Cdk2 and promotes assembly of cyclin D/Cdk4 (170); while at higher concentrations it equally inhibits Cdk2, Cdk3, Cdk4, and

Cdk6 (105, 170). In fact, there is precedent for the involvement of $p21^{Waf1}$ in inhibiting Cdk2 and Cdk4 activities triggered by the Ras oncogene, as mice lacking $p21^{Waf1}$ and overexpressing MMTV-H-Ras^{G12V} display higher Cdk2 and Cdk4 activities relative to those expressing H-Ras^{G12V} alone (194). Perhaps a similar scenario exists within the centrosome, as $p21^{Waf1}$ may inhibit both Cdk2 and Cdk4 activities to regulate centrosome duplication; thus, ablation of *p53* and the concomitant absence of endogenous $p21^{Waf1}$ may hyperactivate Cdk2 and Cdk4. Another possibility is that ablation of *Cdk2* or *Cdk4* changes the CKI/Cdk ratios within the cell, allowing more $p21^{Waf1}$ to bind and inhibit the Cdk that is still present in the cell; this compensatory mechanism has been observed in *Cdk4*$^{-/-}$ MEFs, in which more $p27^{Kip1}$ is available to bind and inhibit Cdk2 activity (159). However, observations that *p53*$^{-/-}$ cells are devoid of detectable endogenous $p21^{Waf1}$ render unlikely the possibility that more $p21^{Waf1}$ is available to inhibit Cdk2 activity in *p53*$^{-/-}$*Cdk4*$^{-/-}$ MEFs to prevent CA. Other Cdks that may impose that kind of compensatory regulation are p16 and p57, overexpressed in *p53*$^{-/-}$ MEFs. Nevertheless, since p16 and p57 did not accumulate upon transient knockdown of p53, and because silenced Cdk2 or Cdk4 were able to suppress CA in the same cells, that level of indirect compensatory inhibition of Cdk2 or Cdk4 by the CKIs is unlikely. Yet, because our experiments have not ruled out that type of compensatory mechanisms, we will develop composite p53, Cdk and CKI knockdowns and knockouts to directly address the individual roles of those CKIs in preventing the hyperactivity of the remaining Cdk.

The following evidence supports a direct role for Cdk2 and Cdk4 in mediating CA in p53-null cells: Firstly, concomitant loss of *Cdk2* and *Cdk4* in *p53*$^{-/-}$ MEFs did not reduce frequencies of CA lower than the ablation of each individual kinase, rendering unlikely the possibility that the compensatory mechanism imposed by CKIs on Cdk2 or Cdk4 kinase activities are responsible for suppression of CA when either kinase is ablated. Secondly, concomitant ablation of *Cdk2* & *Cdk4* in p53-deficient MEFs cultured in HU reduced NPM^{T199} hyper-phosphorylation to the same extent as ablated *Cdk2* or *Cdk4*. In addition, rendering the NPM^{T199} site non-phosphorylatable by mutating the Cdk2/Cdk4 phosphorylation site (NPM^{T199A}) suppressed CA to the same extent as ablated Cdk2 or Cdk4, demonstrating that ablated Cdks are preventing CA to the maximum. Therefore, we conclude that both Cdk2 and Cdk4 are individually required to signal CA in *p53*-deleted cells.

How do Cdk2 and Cdk4 deregulate centrosome duplication resulting in CA? The model depicted in Figure 8, based on published observations (28, 30, 128, 129), and the observations reported in this manuscript shows two ways by which the G_1 Cdks could affect CA: firstly, by triggering unregulated phosphorylation of Rb and the release of E2Fs; secondly, by the direct phosphorylation of centrosomal targets. Although we did not perform a wide screen for all the E2F targets that may result in CA in p53$^{-/-}$ MEFs, those E2F targets that we analyzed by Westerns were unchanged (cyclins A, E and D). Thus, in this manuscript, we tested the second scenario.

In this manuscript, we resolved how Cdk2 and Cdk4 directly impinge on CA through NPM. Our experiments indicated that ablation of *Cdk2* or *Cdk4* decreased NPM^{T199} hyper-phosphorylation in $p53^{-/-}$ MEFs arrested in G_0 (serum-arrested), or in late G_1 (under an HU-block). Those experiments suggested that restoration of the normal phosphorylation of NPM^{T199} normalized the licensing of the centrosome cycle, as well as the excessive centriole reduplication in $p53^{-/-}$ MEFs to prevent CA. Our experiments showing that introduction of a mutant NPM lacking Cdk2 and Cdk4 phosphorylation sites (NPM^{T199A}) abrogated CA in $p53^{-/-}$ MEFs to the same extent as ablated Cdk2 or Cdk4 demonstrated that, indeed, the Cdk2, Cdk4→NPM^{T199} pathway is central to prevent CA. Based on our observations that Cdk2 or Cdk4 crosstalk to the centrosome through the same phosphorylation site in NPM, Thr199, we propose a novel paradigm: that full-fledged phosphorylation of NPM by both Cdk2 and Cdk4 in $p53^{-/-}$ MEFs is critical to the induction of CA. How do Cdk2 and Cdk4 prevent centriole reduplication? Our experiments showing that ablation of *Cdk2* or *Cdk4* prevented centriole re-duplication clearly identified an important step within the centrosome cycle that impinges on CA. Western blots showing that in $p53^{-/-}Cdk2^{-/-}$, $p53^{-/-}Cdk4^{-/-}$, and in $p53^{-/-}Cdk2^{-/-}Cdk4^{-/-}$ the hyper-phosphorylation of NPM^{T199} is abrogated indicated that restoration of normal centrosome cycle licensing is key to prevention of centriole reduplication. Another putative mechanism is that the Cdks control the expression or activity of centriole duplication kinases, including Plk4, Plk2, or mMps-1. So far, Western blots and real time PCR have not detected increased levels of Plk4 in $p53^{-/-}$ MEFs; we have

yet to explore whether it influences its kinase activity. Thus, future experiments will address how Cdk2 and Cdk4 modulate the activities of centriole duplication kinases.

A third major finding was that ablation of *Cdk2* or *Cdk4* in *p53*$^{-/-}$ MEFs inhibits two precursors to the two major types of chromosome instability: micronuclei formation (precursors to aneuploidy) and double-strand DNA breaks (precursors to chromosome rearrangements). We conclude that both G_1 Cdks are critical to CA and chromosome instability in *p53*$^{-/-}$ MEFs. Our data has important implications for cancer therapy, as the 50% human cancers that harbor p53 mutations can be treated with small molecule inhibitors against Cdk2 or Cdk4, currently under development or undergoing clinical trials (195). By using inhibitors specific to either Cdk we can take away the ability of ablated *p53* to generate chromosome instability, an abnormal phenotype strongly associated with poor prognosis and resistance to chemotherapy (196, 197), stopping cancer progression in its tracks. One could accomplish this inhibition without the proliferative toxicity predicted to occur when Cdk2 and Cdk4 are concomitantly inhibited or the mitotic and developmental defects triggered by Cdk1 inhibition (156, 158, 195).

Materials and Methods

Generation of mouse embryonic fibroblasts

Mice were crossed as *Cdk2*$^{+/-}$ x *Cdk2*$^{+/-}$, *Cdk4*$^{+/-}$ x *Cdk4*$^{+/-}$ and *Cdk2*$^{+/-}$*Cdk4*$^{+/-}$ x *Cdk2*$^{+/-}$*Cdk4*$^{+/-}$ (meiotically recombined, (156), *p53*$^{+/-}$*Cdk2*$^{+/-}$ x *p53*$^{+/-}$*Cdk2*$^{+/-}$, *p53*$^{+/-}$

Cdk4$^{+/-}$ x *p53*$^{+/-}$*Cdk4*$^{+/-}$ and *p53*$^{+/-}$*Cdk2*$^{+/-}$*Cdk4*$^{+/-}$ x *p53*$^{+/-}$*Cdk2*$^{+/-}$*Cdk4*$^{+/-}$. After mating, embryos were isolated from females 13.5 days after detection of seminal plugs. Embryos were collected under sterile conditions and their livers extirpated for extraction of DNA and PCR genotyping. MEFs were generated using established methods (30). Assessments of the individual genotypes were done by PCR genotyping with primers specific for the wild-type and KO*p53*, *Cdk2* and *Cdk4* alleles (156). All experiments were performed on passage 2 (p2) MEFs.

Cell culture.

MEFs were maintained under proliferating conditions, with 10% FBS/DMEM. For serum arrest experiments, cells were cultured in 0.2% FBS/DMEM for 60 hrs.

Centriole re-duplication assay

Three independent, proliferating MEFs of the indicated genotypes plated in a two-well chamber slides, were untreated or treated with 2 mM HU for 48 hrs. For co-immunostaining of α- and γ- tubulins to examine centrioles, cells were first incubated on ice for 30 min to destabilize microtubules nucleated at the centrosomes, followed by brief extraction (~ 1 min) with cold extraction buffer [0.75 % Triton X-100, 5 mM PIPES, 2 mM EGTA (pH 6.7)]. Cells were then briefly washed in cold PBS, and fixed as previously described. Cells were immunostained with anti-α-tubulin monoclonal (Sigma DMA1) and anti-γ-tubulin polyclonal antibodies (ABCAM ab11317). The antibody-antigen complexes were

detected with the appropriate ALEXA-conjugated antibodies (Molecular Probes) and the frequencies of CA were calculated by counting 200 cells per group.

Serum starvation and BrdU incorporation assay

MEFs of the indicated genotypes plated in 60 mm dishes were grown to confluence in 10% FBS/DMEM medium and then split into three groups. In group one and two the cells were plated into a 2-well tissue culture chamber slides and those for the third group in 60 mm Petri dishes. MEFs of different groups were first plated at high densities, starved for 60 hours by culturing in medium supplemented with 0.1% FBS, and then released by the addition of 10% FBS for various time-points. The cells in group one were immediately fixed for centrosome staining at the indicated time points. To measure the S phase entry of the indicated genotypes, MEFs in group two were pulse-labeled with 20 μM BrdU (BD Pharmingen 51-7581KZ) and incubated for 30 minutes as described (198, 199). BrdU-positive cells were detected using primary antibodies against BrdU (Calbiochem NA61) and an Alexa Fluor 555 secondary antibody (Molecular Probes). We counted 200 cells per group for the BrdU and centrosome assays. Lysates from cells in group three were obtained for Western blots at the indicated time points.

Immunofluorescence

Immunofluorescence was performed following our published protocols (30). MEFs were plated at 4x 10^4 cells per well into 2-well tissue culture chamber slides and grown for 2-3 days. Cells were fixed in paraformaldehyde, washed in

PBS, permeabilized in 1% NP40/PBS solution, and blocked in 5% BSA in PBS. Centrosomes were stained overnight at 4 ^{o}C with monoclonal antibodies against pericentrin (BD Biosciences 611814), and/or γ-tubulin (ABCAM ab11317). Chromosome breaks were detected using gamma-H2AX (UPSTATE 07-164) and detected with the appropriate ALEXA-conjugated antibodies (Molecular Probes). Cells were also counterstained with 4, 6-diamidino-2-phenylindole (DAPI). For each experiment involving calculations of the frequencies of centrosomes, at least 200 cells from each chamber were counted per group.

Western blots

Western blots were performed according to published protocols (30). Protein lysates were obtained by incubating cells in lysis buffer (50 mM HEPES; pH 7.9; 250mM KCl; 0.1 mM EDTA; 0.1 mM EGTA; 0.1% NP40 and 10% glycerol; 0.5 mM NaF; 0.1 mM NaVO4; 0.1 mM PMSF; 10 mM β-glycerophosphate; 0.1 mM DTT; 0.1 mg/ml aprotinin; 0.1 mg/ml leupeptin) for 30 min at 4 ^{o}C. Samples were denatured at 95 ^{o}C for 5 min in SDS sample buffer, resolved by SDS-PAGE, and transferred to PVDF membranes (BIO-RAD, Hercules, CA). The blots were incubated in blocking buffer (5%) [w/v] nonfat dry milk or BSA in Tris-buffered saline + 0.1% Tween 20 [TBS-T] for 1 hr. and then probed overnight at 4 ^{o}C with the primary antibodies. The blots were then rinsed in TBS-T and incubated with the appropriate horseradish peroxidase-conjugated secondary antibodies at room temperature. The blots were then rinsed in TBS-T, and the antibody-antigen complex was visualized by chemiluminescent HRP Antibody Detection

Reagent (Denville Scientific Inc., Metuchen, NJ). Western blots for the detection of phosphorylated NPM were done similarly, except that the serine/threonine phosphatase inhibitor Calyculin A (UPSTATE, CA) was included in the culture medium 10 min prior to harvest at a concentration of 100 nM. The antibodies used in the various Western blots were as follow: Cdk2 (Santa Cruz sc-163), Cdk4 (Cell Signaling 2906), p53 (Santa Cruz sc-6243), $p57^{Kip2}$ (Santa Cruz sc-8298), $p16^{INK4A}$ (Santa Cruz sc-1207), $p21^{Waf-1}$ (Santa Cruz sc-397), $p27^{Kip1}$ (Santa Cruz sc-528), □-actin (Cell Signaling 4970), Cyclin A (ABCAM ab38), Cyclin D1 (Cell Signaling 2922), and Cyclin E (Santa Cruz sc-481).

Micronucleus assay

The micronucleus assay was done as described previously (30). Briefly, 4×10^4 cells were plated into each well of a 2-well chamber slide (Nalge Nunc International, 177380). After 2 to 3 days in culture, cells were fixed in 4 % paraformaldehyde and stained with DAPI. Micronuclei appear as spherical structures with a similar morphology to the nucleus except that their sizes range from $1/10^{th}$ to $1/100^{th}$ the size of a nucleus; 1000 cells were counted for each genotype analyzed.

Transfections

For transient transfection of wild-type and mutant *NPM/B23*, three independent *p53*$^{-/-}$ MEFs were co-transfected with plasmid encoding either a FLAG-tagged wild-type or substitution mutant (Thr^{199} → Ala) *NPM/B23* with a neomycin

resistant gene (pcDNA3.1) using lipofectamine (Invitrogen, Carlsbad, CA). As a negative control, the empty vector was transfected. After transfection at 37 ^{o}C overnight in a 5% CO_2 incubator, cells were fed with fresh complete medium for 24 hrs. The cells were then treated with complete medium containing 2.5 mg/ml neomycin (Sigma, St Louis, MO) for 7 days. The G418 resistant cells were maintained in complete medium containing neomycin (1mg/ml) for an additional 2 days, and replated for further culture in a fresh complete medium for an additional 24 hrs.

Three independent *Cdk4*$^{-/-}$ MEFs were also transfected with plasmids encoding empty vectors (pBABE-*hygro*) and Cdk2 (pBABE-*Hygro*-Cdk2) using lipofectamine (Invitrogen) according to the manufacturer's protocol. After transfection (37 ^{o}C overnight), cells were fed with fresh complete medium for 24 hrs., and switched to selective medium (150 µg/ml hygromycin) for 4 days. Selected cells were directly plated into a two chamber slides, fixed as previously described and stained for centrosome analysis.

RNA interference

Cdk2 and Cdk4 siRNAs (Catalogue # sc-29260 and sc-29262) were purchased from Santa Cruz Biotechnology, Inc. Three independent MEFs of each genotype were grown in a six well plate until they become 60-80% confluent. The cells were then transfected with each siRNA according to the manufacturer's protocol and 72 hrs. after the transfection, the cells were used to prepare cell lysate and plated into a two chamber slide for centrosome analysis.

We also knocked down p53, with two synthesized siRNA duplex-sequences by transfecting three independent wild-type MEFs using lipofectamine 2000 (Invitrogen) according to the manufacturer's protocol. The two siRNA duplex-sequences targeting the p53 mRNA are:

ACCAF161020_1:

(sense, 5'-rCrArCrArUrGrUrArCrUrUrGrUrArGrUrGrGrArUrGrGrUrGrGrUrA-3')

(antisense, 5'-rCrCrArCrCrArUrCrCrArCrUrArCrArArGrUrArCrArUrGTG-3')

ACCAF161020_2:

(sense, 5'-rGrGrUrGrArArArUrArCrUrCrUrCrCrArUrCrArArGrUrGrGrUrUrU-3')

(antisense, 5'-rArCrCrArCrUrUrGrArUrGr GrArGrArGrUrArUrUrUCrACC-3').

All the siRNA were prepared using a transcription-based method with the silencer siRNA construction kit (Integrated DNA Technologies) according to the manufacturer's instructions. Three days after the addition of complete medium, cell lysates were prepared for appropriate assays.

Cyclin-dependent kinase 4 activity assay

Cyclin-dependent kinase 4 assay was performed according to published methods (190). Briefly, the NPM peptide is phosphorylated because of Cdk4 activity, which is measured using luminometric estimation of ATP depletion. Immuno-precipitated (Ip) Cdk4 obtained form 150 µg of total protein extract from Wild-type and $Cdk2^{-/-}$ MEFs at different time points was washed three times in cell lysis buffer, twice in kinase buffer and resuspended in 30 µl of kinase buffer, optimized to preserve the Cdk4 activity. Kinase reaction was performed adding 20 µl of a

mixture containing 0.5 µM ATP and 2µg of NPM peptide (Abcam cat# ab39518) as substrate in kinase buffer. The reaction was incubated for 30 min at 30 ^{o}C and then an equal volume of kinase-GLO reagent was added. As a reaction control, kinase reaction was performed with samples in the absence of the peptide substrate (No-NPM), with no Ip-Cdk4 control and with Ip-Cdk4 obtained from total protein extract from Cdk4$^{-/-}$ MEFs. Samples were then incubated for 20 min at room temperature and the developed luminescence was recorded using the SPECTRAMAX GEMIN^{R}XS luminometer and expressed as relative light units.

Image acquisition and manipulation

Slides were analyzed using a Zeiss Axioplan II with Plan-Apochromat X 100/1.4 NA oil immersion objective. Images were taken using a color digital camera (Axiocam HRC) and Zeiss Axiovision software. Confocal images were acquired with a Zeiss LSM 510 META point scanning laser confocal microscope mounted on a Zeiss Axioplan II upright microscope equipped with Plan-Apochromat X 63/1.4 NA oil immersion objective. Images were captured by Zeiss Image Browser. All the samples were mounted in Fluoromount-G mounting medium (Southern Biotech) and analyzed at room temperature.

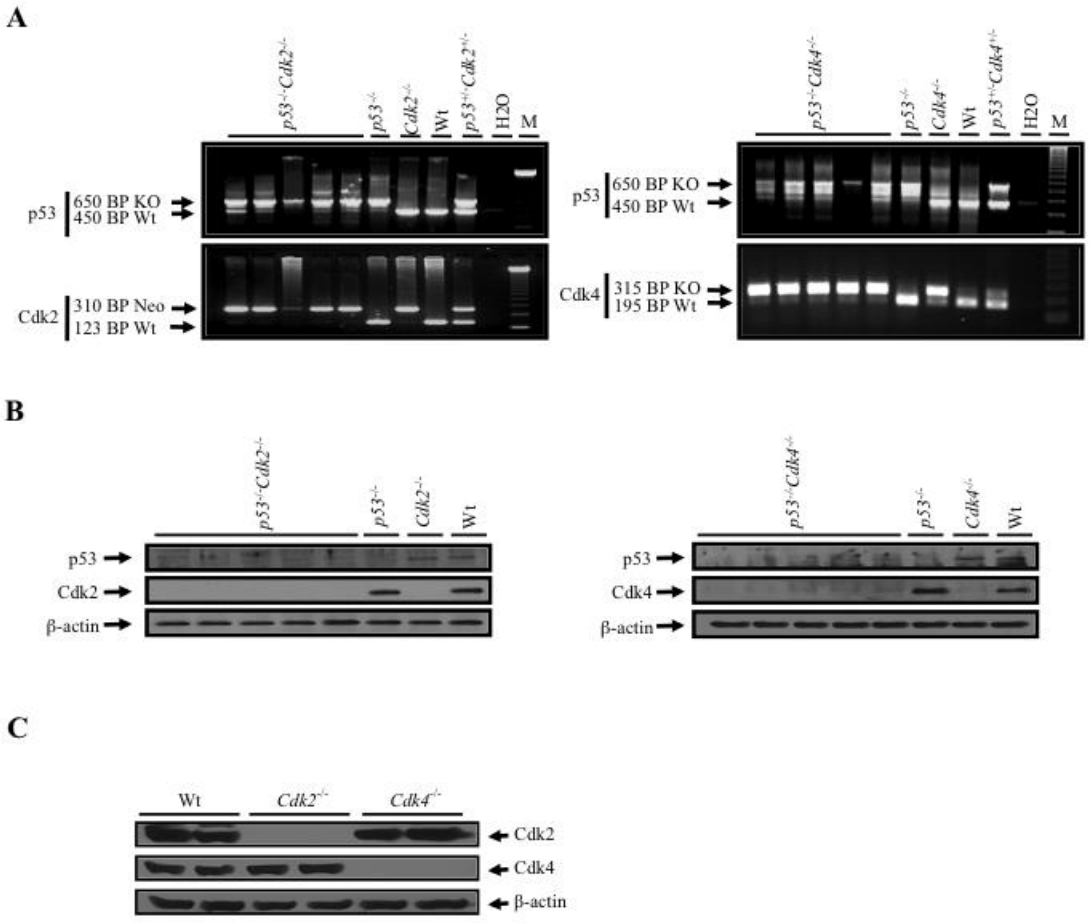

A
p53+/-Cdk2-/-
p53-/-
Cdk2-/-
Wt
p53+/-Cdk2+/-
H2O
M
p53
650 BP KO
450 BP Wt
Cdk2
310 BP Neo
123 BP Wt
p53+/-Cdk4-/-
p53-/-
Cdk4-/-
Wt
p53+/-Cdk4+/-
H2O
M
p53
650 BP KO
450 BP Wt
Cdk4
315 BP KO
195 BP Wt
B
p53+/-Cdk2-/-
p53-/-
Cdk2-/-
Wt
p53
Cdk2
β-actin
p53+/-Cdk4-/-
p53-/-
Cdk4-/-
Wt
p53
Cdk4
β-actin
C
Wt
Cdk2-/-
Cdk4-/-
Cdk2
Cdk4
β-actin

Figure 1. Cells lacking *p53*, *Cdk2* and *Cdk4* display defective CKI profiles. (A) PCR-based genotyping. PCR analysis of genomic liver DNA from E.13.5 embryos generated by crossing $p53^{+/-}Cdk2^{+/-}$ or $p53^{+/-}Cdk4^{+/-}$ mice. These gels included five double mutants ($p53^{-/-}Cdk2^{-/-}$, left panel or $p53^{-/-}Cdk4^{-/-}$, right panel), one $Cdk2^{-/-}$, one $Cdk4^{-/-}$ mutant, wild-type embryos and a control lacking DNA (H_2O). **(B)** Western blots were performed to confirm the genotyping data generated in **(A)**, using antibodies specific to p53, Cdk2 and Cdk4; the bottom panel shows the β-actin levels used as loading controls. **(C)** Western blots were conducted to determine the expression levels of Cdk4 in $Cdk2^{-/-}$ MEFs and Cdk2 in $Cdk4^{-/-}$ MEFs. To ensure that equal among of proteins was loaded, β-actin was used to probe the same membrane.

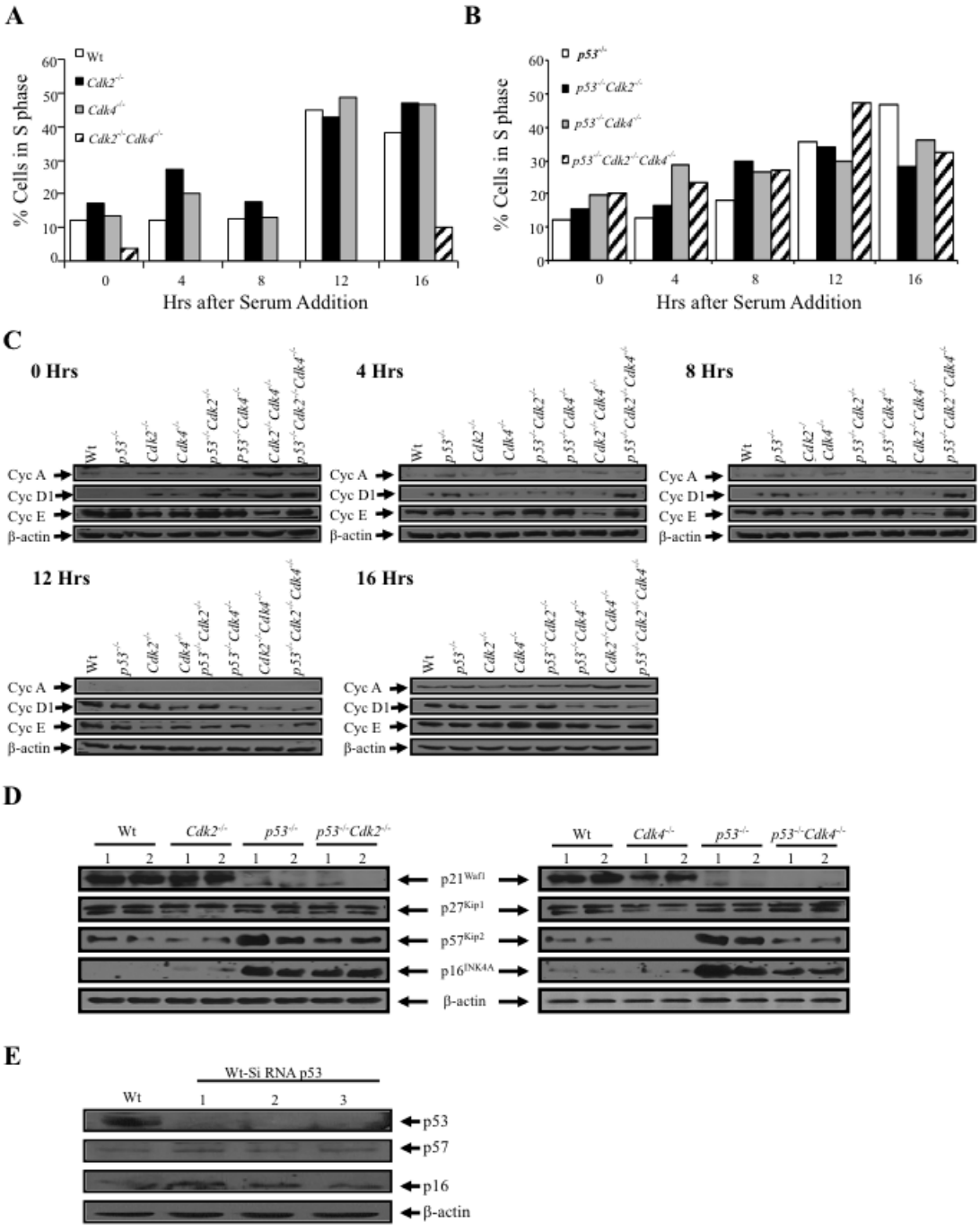

A
Wt
Cdk2-/-
Cdk4-/-
Cdk2-/-Cdk4-/-
% Cells in S phase
Hrs after Serum Addition
B
p53-/-
p53-/-Cdk2-/-
p53-/-Cdk4-/-
p53-/-Cdk2-/-Cdk4-/-
% Cells in S phase
Hrs after Serum Addition
C
0 Hrs
4 Hrs
8 Hrs
12 Hrs
16 Hrs
Cyc A
Cyc D1
Cyc E
β-actin
D
Wt
Cdk2-/-
p53-/-
p53-/-Cdk2-/-
Cdk4-/-
p53-/-Cdk4-/-
p21Waf1
p27Kip1
p57Kip2
p16INK4A
β-actin
E
Wt-Si RNA p53
Wt
p53
p57
p16
β-actin

Figure 2. Ablation of Cdk2 or Cdk4 does not significantly alter the cell cycle. (A,B) Cells of the indicated genotypes were arrested in G_0 and subsequently stimulated by addition of serum. Cells were pulse labeled with BrdU 30 minutes prior to harvest, and harvested at the indicated time-points after serum stimulation. Cells were stained with anti-BrdU antibodies and the appropriate secondary antibodies and visualized using confocal microscopy and a 63X objective. Nuclei were counterstained with DAPI. This experiment was repeated twice; a representative experiment is presented. Frequencies represent BrdU positive cells in a population of at least 200 cells per group. **(C)** Whole-cell extracts were prepared from MEFs collected at the indicated time-points following serum addition, and analyzed by Western blotting using antibodies against cyclin A, cyclin D1, cyclin E and β-actin as control. **(D)** Western blots were performed in MEFs cultured in DMEM containing 0.2% FBS for 48 hrs. One and two represent the loading of the protein lysates of two independent MEFs of the indicated genotypes. Western blots were probed with antibodies against $p21^{Waf1}$, $p27^{Kip1}$, $p57^{Kip2}$, and $p16^{INK4A}$. β-actin served as loading control. **(E)** Western blots of proteins extracted from controls (wild-type MEFs), or wild-type MEFs transfected with siRNAs specific to p53 were probed with p53, p57, p16 and β-actin (control).

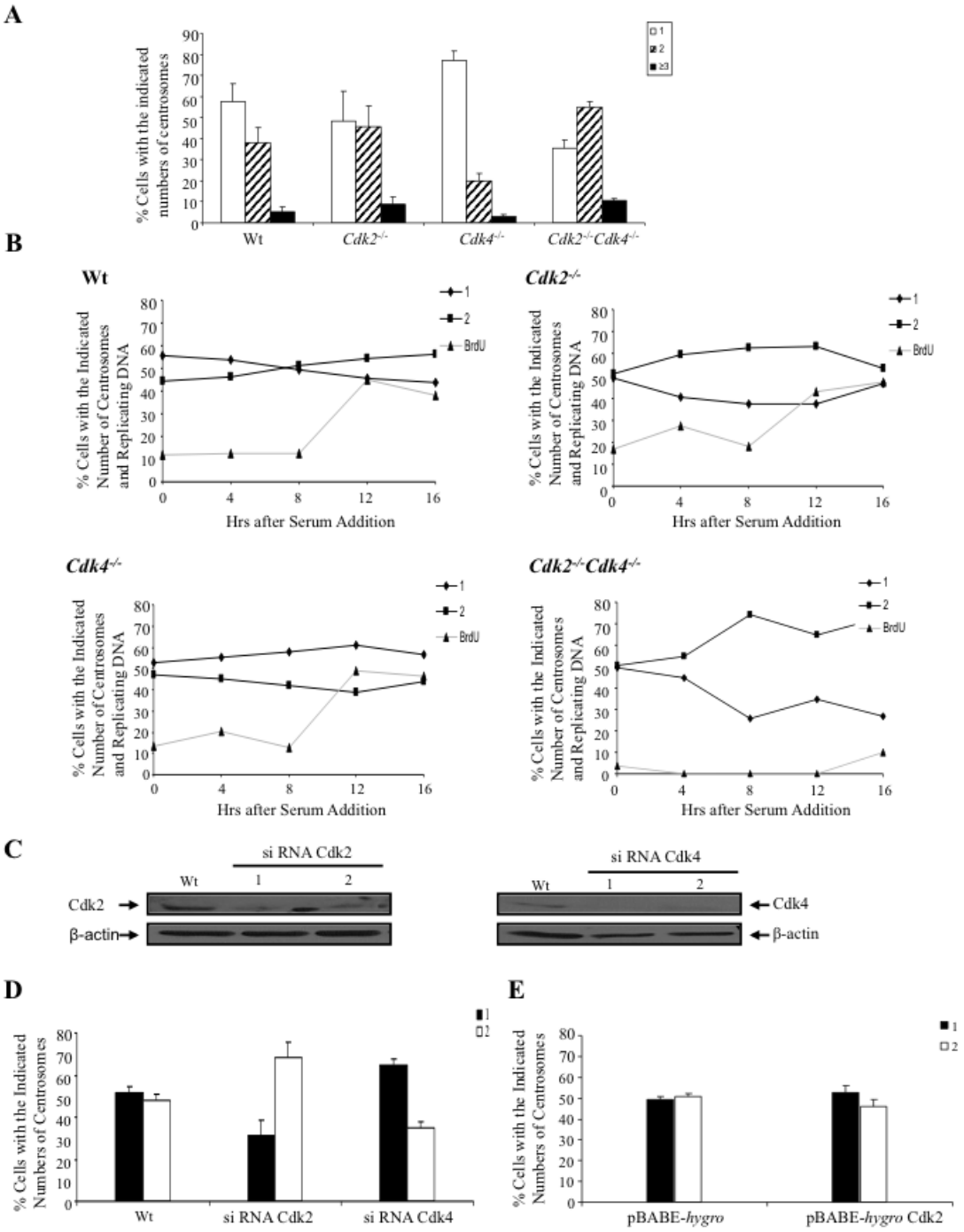

A
% Cells with the indicated numbers of centrosomes
Wt
Cdk2-/-
Cdk4-/-
Cdk2-/-Cdk4-/-
B
Wt
Cdk2-/-
Cdk4-/-
Cdk2-/-Cdk4-/-
% Cells with the Indicated Number of Centrosomes and Replicating DNA
Hrs after Serum Addition
BrdU
C
si RNA Cdk2
si RNA Cdk4
Cdk2
Cdk4
β-actin
D
% Cells with the Indicated Numbers of Centrosomes
Wt
si RNA Cdk2
si RNA Cdk4
E
pBABE-hygro
pBABE-hygro Cdk2

Figure 3. Ablation of *Cdk2* and *Cdk4,* or siRNA-mediated silencing of Cdk2 and Cdk4 lead to distinct centrosome cycle defects. (A) Proliferating E13.5 MEFs from the indicated genotypes were fixed, processed, and co-immunostained with anti-pericentrin, anti-gamma-tubulin and the appropriate secondary antibodies. Averages +/- standard deviation of the percentage of cells with one, two and three centrosomes. The exact number of embryos analyzed is as follows: Wild-type (8), *Cdk2*$^{-/-}$ (3), *Cdk4*$^{-/-}$ (4), and *Cdk2*$^{-/-}$*Cdk4*$^{-/-}$ (3). Statistical significance of the averages ($p \leq 0.05$), was established by an unequal variance T-test. T-test values of the percentage of cells in the population containing one centrosome (relative to Wild-type): *Cdk2*$^{-/-}$ (0.159885), *Cdk4*$^{-/-}$ (0.000518), *Cdk2*$^{-/-}$ *Cdk4*$^{-/-}$ (0.000544). *P* values of cells in the population containing two centrosomes relative to wild-type MEFs: *Cdk2*$^{-/-}$ (0.122182), *Cdk4*$^{-/-}$ (0.000172), *Cdk2*$^{-/-}$*Cdk4*$^{-/-}$ (0.000528). *P* values of cells with ≥3 centrosomes relative to wild-type MEFs: *Cdk2*$^{-/-}$ (0.091487), *Cdk4*$^{-/-}$ (0.06122), *Cdk2*$^{-/-}$*Cdk4*$^{-/-}$. **(B)** MEFs of the indicated genoypes were grown in duplicate to confluence followed by serum starvation for 60 hours. Quiescent cells were stimulated with serum, and at every 4 hrs for a period of 16 Hrs, the number one and two centrosomes were scored. This experiment was repeated twice; a representative experiment is presented. The BrdU data is the same presented in Figs 2A and B; the data is presented here for clarity purposes. **(C)** Western blots of protein extracted from non-transfected wild-type cells, or transfected with siRNAs against Cdk2 or Cdk4, and probed with antibodies against Cdk2 or Cdk4; the same membrane was probed with β-actin as a control. **(D)** Wild-type MEFs untransfected or transfected with siRNAs against Cdk2 or Cdk4 were immuno-stained with anti-gamma-tubulin antibodies and frequencies were established by counting cells with one and two centrosomes in a population of at least 200 cells per group**.** Three independent MEFs were used. Statistical significance of the averages ($p \leq 0.05$) was established by an unequal variance T-test. T-test values of the percentage of cells in the population containing one centrosome relative to two centrosomes per group: Wild-type (0.215535), siRNA Cdk2 (0.003271), siRNA Cdk4 (0.008772). **(E)** Three independent *Cdk4*$^{-/-}$ MEFs transfected with plasmids encoding control vector (pBABE-*hygro*) and pBABE-*hygro*-*Cdk2* and plated after selection using hygromycin were immuno-stained using anti-gamma-tubulin antibodies and the appropriate secondary. Frequencies were established by counting cells with one and two centrosomes in a population of at least 200 cells per group**.** T-test values of the percentage of cells in the population containing one centrosome relative to two centrosomes per group: pBABE-*hygro* (0.345271), pBABE-*hygro*-*Cdk2* (0.136406).

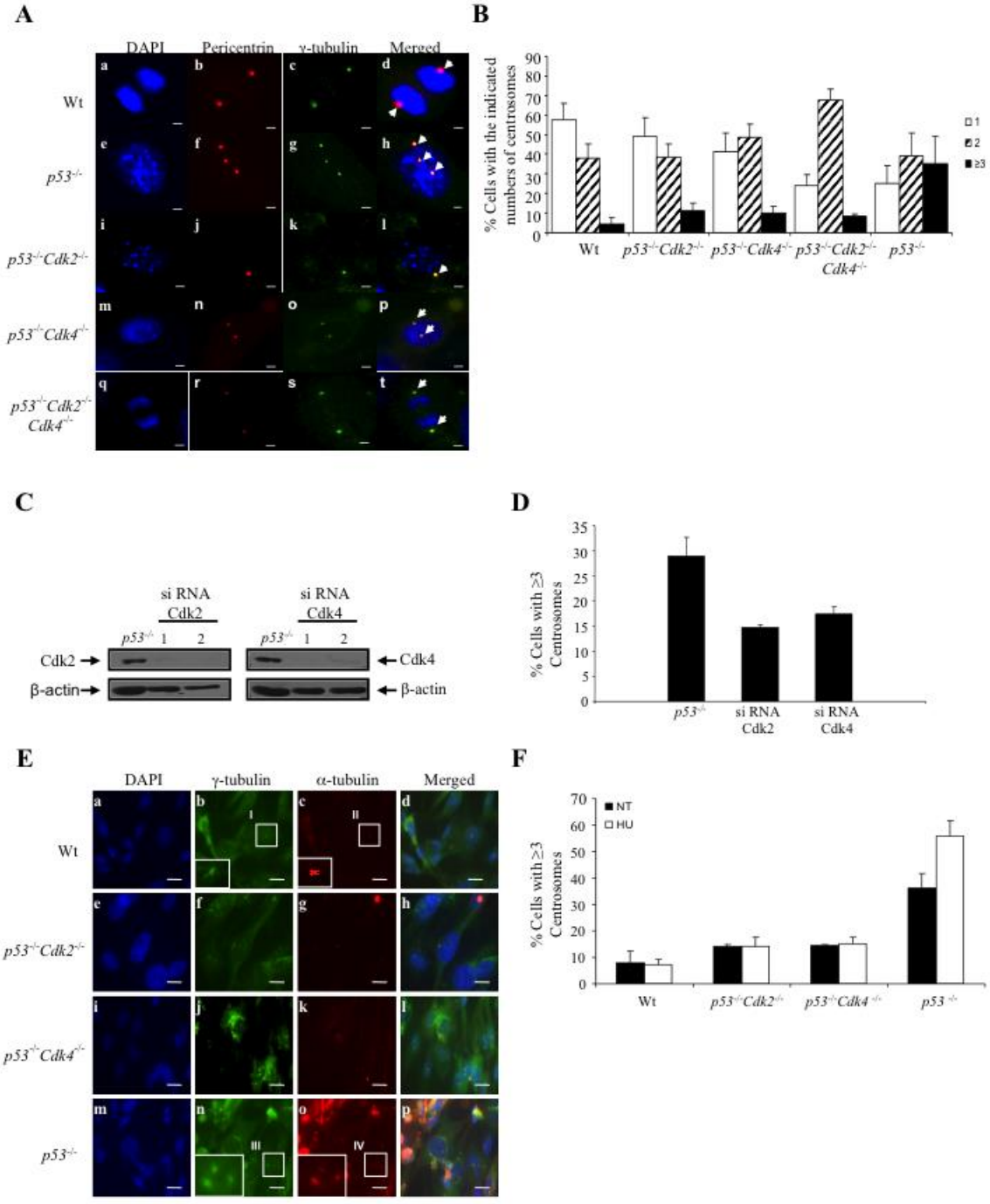

A
DAPI
Pericentrin
γ-tubulin
Merged
Wt
p53-/-
p53-/-Cdk2-/-
p53-/-Cdk4-/-
p53-/-Cdk2-/-Cdk4-/-
B
% Cells with the indicated numbers of centrosomes
1
2
≥3
Wt
p53-/-Cdk2-/-
p53-/-Cdk4-/-
p53-/-Cdk2-/-Cdk4-/-
p53-/-
C
si RNA Cdk2
si RNA Cdk4
p53-/-
1
2
Cdk2
Cdk4
β-actin
D
% Cells with ≥3 Centrosomes
p53-/-
si RNA Cdk2
si RNA Cdk4
E
DAPI
γ-tubulin
α-tubulin
Merged
Wt
p53-/-Cdk2-/-
p53-/-Cdk4-/-
p53-/-
F
NT
HU
% Cells with ≥3 Centrosomes
Wt
p53-/-Cdk2-/-
p53-/-Cdk4-/-
p53-/-

Figure. 4 Ablation or siRNA-mediated silencing of Cdk2 and Cdk4 prevents centriole re-duplication and CA in *p53*$^{-/-}$ MEFs. (A) MEFs of the indicated genotypes were co-immunostained with antibodies recognizing pericentrin (red, b, f, j n, r) and gamma-tubulin (green, c, g, k, o, s). Nuclei were stained with DAPI (blue, a, e, I, m, q). Panels d, h, l, p, t show the overlay images of the pericentrin and gamma-tubulin immuno-stainings. **(B)** Proliferating E13.5 MEFs from the indicated genotypes were fixed, processed, and co-immunostained with anti-pericentrin, anti-gamma-tubulin and the appropriate secondary antibodies. The graph presents averages +/- standard deviations of the percentage of cells with one, two and three or more centrosomes. The exact number of embryos analyzed is as follows: Wild-type (8), *p53*$^{-/-}$ (8), *p53*$^{-/-}$*Cdk2*$^{-/-}$ (5), *p53*$^{-/-}$*Cdk4*$^{-/-}$ (5), *p53*$^{-/-}$*Cdk2*$^{-/-}$*Cdk4*$^{-/-}$ (4). T-test values of the percentage of cells in the population containing one centrosome (relative to wild-type): *p53*$^{-/-}$ (0.017174), *p53*$^{-/-}$*Cdk2*$^{-/-}$ (0.137854), *p53*$^{-/-}$*Cdk4*$^{-/-}$ (0.358121), *p53*$^{-/-}$*Cdk2*$^{-/-}$*Cdk4*$^{-/-}$ (3.95E-05). *P* values of cells in the population containing two centrosomes relative to wild-type MEFs: *p53*$^{-/-}$ (0.860687), *p53*$^{-/-}$*Cdk2*$^{-/-}$ (0.9713), *p53*$^{-/-}$*Cdk4*$^{-/-}$ (0.024679), *p53*$^{-/-}$*Cdk2*$^{-/-}$ *Cdk4*$^{-/-}$ (2.69E-05). *P* values of cells with ≥3 centrosomes relative to wild-type MEFs: *p53*$^{-/-}$ (0.006967), *p53*$^{-/-}$*Cdk2*$^{-/-}$ (0.232114), *p53*$^{-/-}$*Cdk4*$^{-/-}$ (0.706722), *p53*$^{-/-}$ *Cdk2*$^{-/-}$*Cdk4*$^{-/-}$ (0.051209). **(C)** Western blots of extracts from untransfected *p53*$^{-/-}$ MEFs, or *p53*$^{-/-}$ MEFs transfected with Cdk2- or Cdk4-specific siRNAs were probed with the indicated primary antibodies. **(D)** Frequencies of CA in control *p53*$^{-/-}$ MEFs, and in *p53*$^{-/-}$ MEFs MEFs knocked down for Cdk2 or Cdk4. Three independent MEFs were used. Centrosomes were detected as in (A and B). Statistical significance of the averages ($p \leq 0.05$) was established by an unequal variance T-test. *P* values of cells with ≥3 centrosomes relative to control *p53*$^{-/-}$ MEFs: siRNA Cdk2 (0.002445), siRNA Cdk4 (0.006696). **(E)** Proliferating MEFs from the indicated genotypes (3 per group) were untreated (NT) or treated with 2mM HU for 48 hrs. To determine the presence of centrioles, the cells were subjected to cold-treatment and brief extraction prior to fixation. This treatment destabilizes microtubules nucleated at centrosomes; hence centrioles can be microscopically visualized by immunostaining for α-tubulin (a major component of centrioles), at a high magnification. Cells were co-immunostained with anti-gamma-tubulin polyclonal (green, b, f, j, n) and anti-α-tubulin monoclonal (red, c, g, k, o) antibodies, and counterstained with DAPI (blue, a, e, i m). Panels d, h, l, and p show the overlaid images of gamma-tubulin and α-tubulin immunostaining. Insets are the magnified images of the area indicated. **(F)** Frequencies of centriole reduplication were established by counting cells with ≥ 3 separated centrioles in a population of at least 200 cells per group**.** P values (HU-treated compared to non-treated, NT: Wild-type (0.791492), *p53*$^{-/-}$*Cdk2*$^{-/-}$ (1), *p53*$^{-/-}$*Cdk4*$^{-/-}$ (0.507158), *p53*$^{-/-}$ (0.012161).

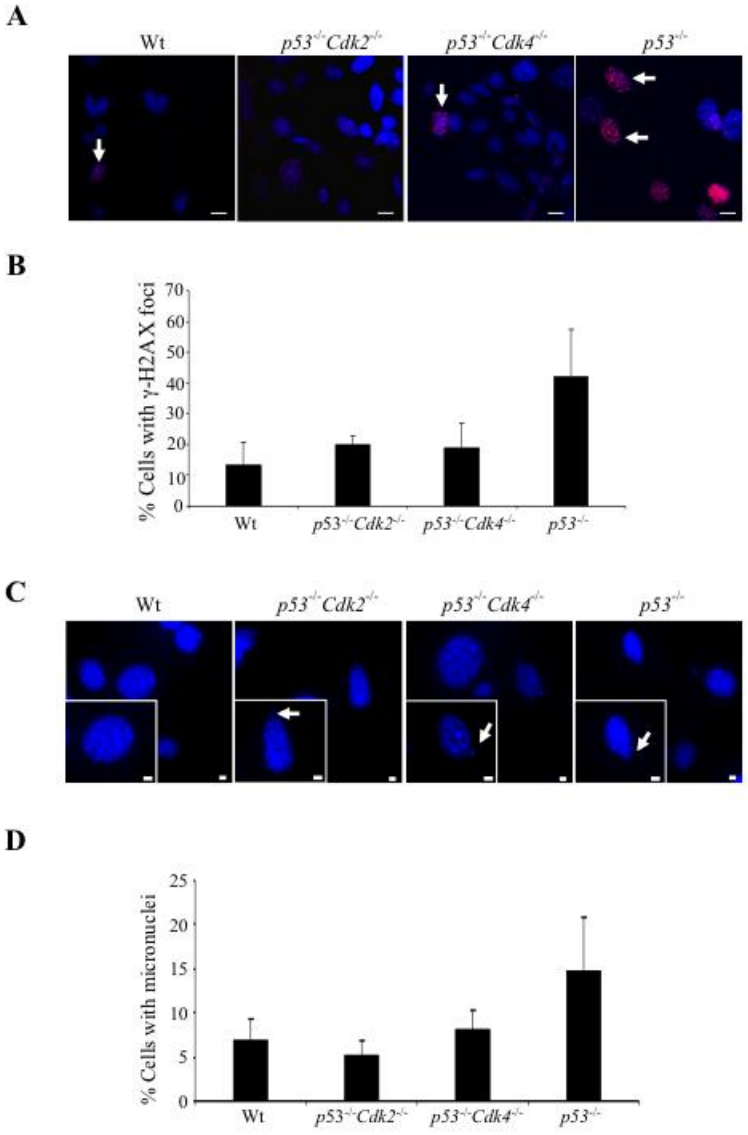

A
Wt
p53-/-Cdk2-/-
p53-/-Cdk4-/-
p53-/-
B
% Cells with γ-H2AX foci
70
60
50
40
30
20
10
0
Wt
p53-/-Cdk2-/-
p53-/-Cdk4-/-
p53-/-
C
Wt
p53-/-Cdk2-/-
p53-/-Cdk4-/-
p53-/-
D
% Cells with micronuclei
25
20
15
10
5
0
Wt
p53-/-Cdk2-/-
p53-/-Cdk4-/-
p53-/-

Figure 5. Ablation of *Cdk2* and *Cdk4* inhibits chromosome instability in cells lacking p53. (A,B) Frequencies of gamma-H2AX foci (arrows) were calculated in cells with the indicated genotypes. The scale bars represent 10 μm. The graph presented in **(B)** represent that averages +/- SD of the percentage of gamma-H2AX foci in a population of at least 200 cells. Each group included 4 different MEFs. P values (relative to Wild-type control): *p53*$^{-/-}$ (0.015218), *p53*$^{-/-}$ *Cdk2*$^{-/-}$ (0.126173), *p53*$^{-/-}$*Cdk4*$^{-/-}$ (0.346771). **(C,D)** Proliferating E13.5 MEFs from the indicated genotypes were fixed and nuclei were visualized with DAPI. Frequencies of micronuclei (inset and arrows) formation were calculated in at least 500 cells for the indicated genotypes. Each group included four different MEFs. P values (relative to wild-type Control): *p53*$^{-/-}$ (0.016122), *p53*$^{-/-}$*Cdk2*$^{-/-}$ (0.137054), *p53*$^{-/-}$*Cdk4*$^{-/-}$ (0.370282).

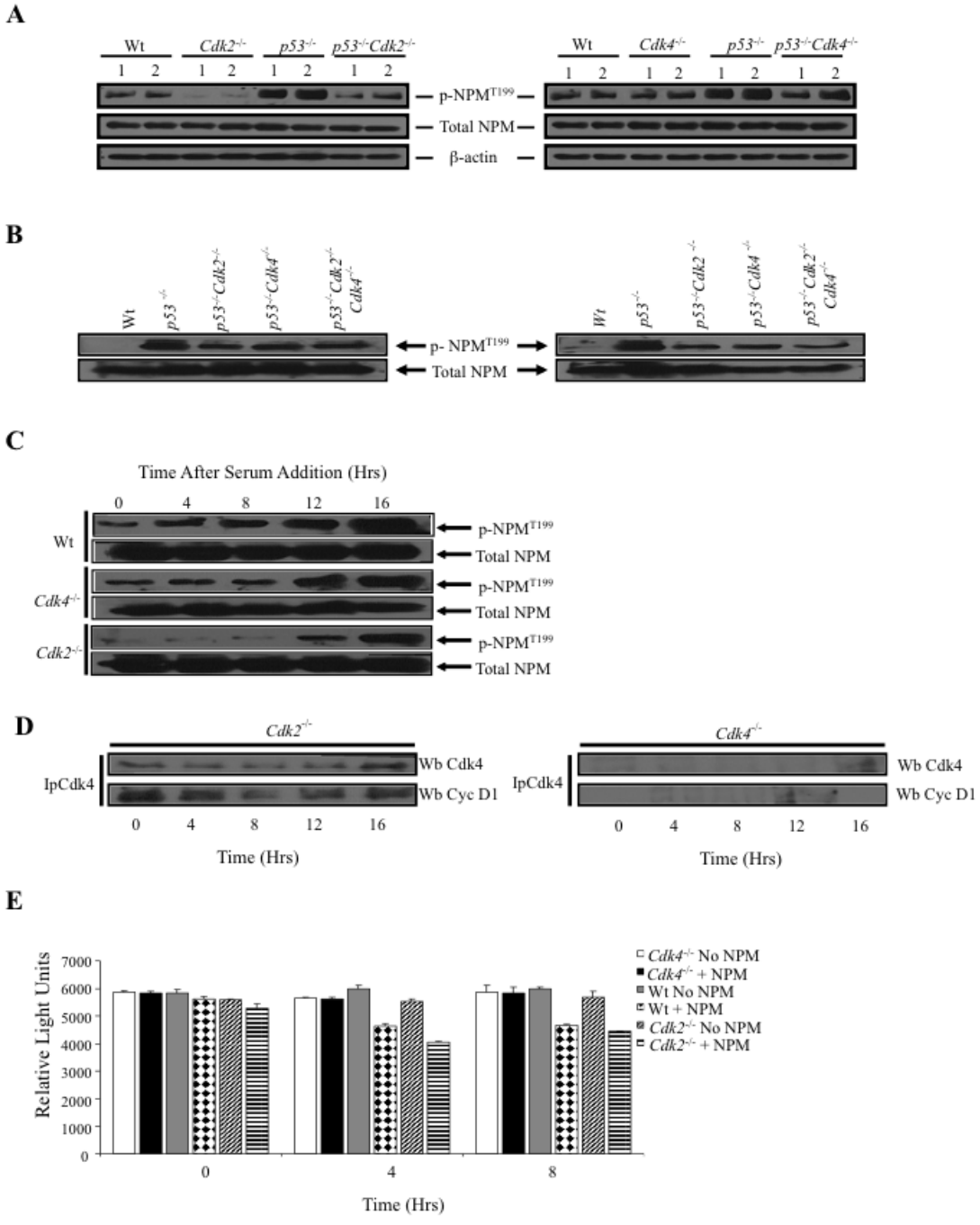
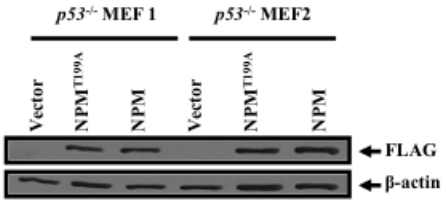
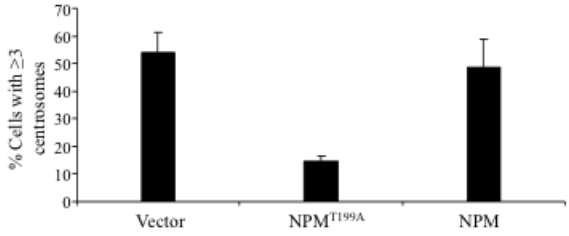

A
Wt
Cdk2-/-
p53-/-
p53-/-Cdk2-/-
Wt
Cdk4-/-
p53-/-
p53-/-Cdk4-/-
1 2 1 2 1 2 1 2
1 2 1 2 1 2 1 2
p-NPMT199
Total NPM
β-actin
B
Wt
p53-/-
p53-/-Cdk2-/-
p53-/-Cdk4-/-
p53-/-Cdk2-/- Cdk4-/-
p- NPMT199
Total NPM
C
Time After Serum Addition (Hrs)
0 4 8 12 16
Wt
Cdk4-/-
Cdk2-/-
p-NPMT199
Total NPM
D
Cdk2-/-
Cdk4-/-
IpCdk4
Wb Cdk4
Wb Cyc D1
0 4 8 12 16
Time (Hrs)
E
Relative Light Units
7000
6000
5000
4000
3000
2000
1000
0
Cdk4-/- No NPM
Cdk4-/- + NPM
Wt No NPM
Wt + NPM
Cdk2-/- No NPM
Cdk2-/- + NPM
0 4 8
Time (Hrs)

Figure 6. Cdk2 and Cdk4 affect the centrosome cycle and centrosome amplification through NPM. (A) E13.5 MEFs of the indicated genotypes were serum-starved for 60 hrs. Cells were pre-incubated with Calyculin A, a serine/threonine phosphatase inhibitor. Phospho-NPM^{T199}-blotted Western blot analysis of protein fractions of G_0-arrested MEFs. The bottom panels were probed with antibodies against total NPM and β-actin to show equal loading**. (B)** MEFs of the indicated genotypes were treated with 2 mM HU for 48 Hrs and then pre-incubated with Calyculin A, a serine/threonine phosphatase inhibitor before protein extraction. Western blots of the protein extracts were probed with antibodies against NPM^{T199}, or against total NPM (control). The MEFs on the left panel are independent of the ones in the right. **(C)** Western blot analyses of MEFs of the indicated genotypes that were serum-arrested and released into the cell cycle for various time-points. Western blots of the protein extracts were probed with antibodies against NPM^{T199}, or against total NPM (control). **(D)** Western blots of Cdk4 immuno-precipitated (Ip-Cdk4) from extracts of wild-type, *Cdk2*$^{-/-}$ and *Cdk4*$^{-/-}$ MEFs were probed with Cdk4 and Cyclin D1 antibodies**. (E)** Cdk4 kinase assays of protein lysates from wild-type, *Cdk2*$^{-/-}$ and *Cdk4*$^{-/-}$ MEFs were carried out at various time-points following serum addition; shown are the 0, 4 and 8 hr. time-points. The results are from three independent MEFs. The experiment was repeated at least twice, and a representative experiment is presented. The reactions contained no NPM peptide (No NPM), or NPM peptide (+ NPM). Luminescence was recorded by the SPECTRAMAX GEMINI^{R}XS using the SoftMax program. P values of kinase assays comparing NPM relative to No NPM at each indicated time point: Wild-type 0 hrs (0.107203829), *Cdk2*$^{-/-}$ 0hrs (0.037437678), Wild-type 4hrs (0.000111335), *Cdk2*$^{-/-}$ 4hrs(0.002861355), Wild-type 8hrs (0.000761355), *Cdk2*$^{-/-}$ 8hrs (0.000449084). The p values from the kinase assays done in *Cdk4*$^{-/-}$ MEFs were more than 0.05 at any given time-point.

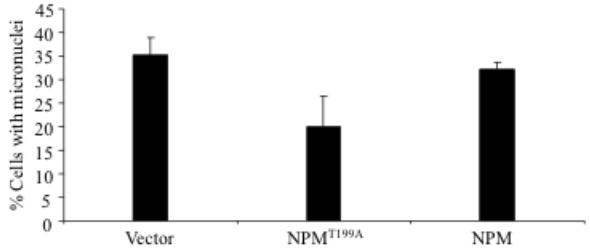
A


p53-/- MEF 1
p53-/- MEF2
Vector
NPMT199A
NPM
Vector
NPMT199A
NPM
FLAG
β-actin

B


% Cells with ≥3 centrosomes
70
60
50
40
30
20
10
0
Vector
NPMT199A
NPM

C


% Cells with micronuclei
45
40
35
30
25
20
15
10
5
0
Vector
NPMT199A
NPM

D

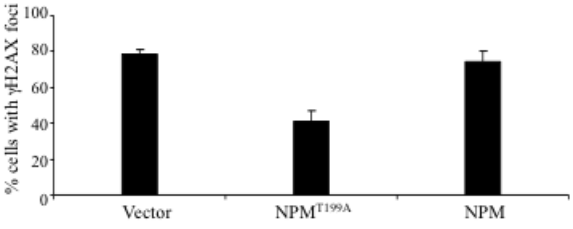
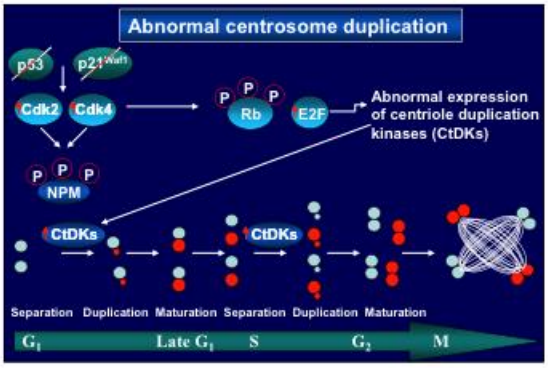

% cells with γH2AX foci
100
80
60
40
20
0
Vector
NPMT199A
NPM

Figure 7. NPM^{T199A} suppresses centrosome amplification and chromosome instability. (A) Passage 2 *$p53^{-/-}$* MEFs were transiently transfected with plasmids encoding FLAG epitope-tagged NPM and the NPM/B23 mutant (NPM^{T99A}). As control, empty vector was transfected. After Neomycin selection, cell lysates were obtained and then probed with anti-FLAG antibodies. **(B)** The transfectants described in **(A)** were fixed and immunostained with anti-γ-tubulin polyclonal antibodies and detected with Alexa 488 antibodies. Cells were counterstained with DAPI. The number of cells with ≥ 3 centrosomes in a population of at least 200 cells was statistically analyzed by fluorescence microscopy. Each group included three transfected MEFs. P values (relative to the transfected vector control): NPM^{T199A} (0.006963), wild-type NPM (0.560677). **(C)** Proliferating E13.5 MEFs from the indicated genotypes were fixed and nuclei were visualized with DAPI. Frequencies of micronuclei formation in a population of 500 cells were calculated for the indicated genotypes. P values (relative to the transfected vector control): NPM^{T199A} (0.011338), wild-type NPM (0.353737). **(D)** Frequencies of H2AX foci were calculated in cells with the indicated genotypes. P values (relative to the transfected vector control): NPM^{T199A} (0.002591), wild-type NPM (0.476327).

A


Abnormal centrosome duplication
p53
p21Waf1
Cdk2
Cdk4
P
P
P
Rb
E2F
Abnormal expression of centriole duplication kinases (CtDKs)
P
P
P
NPM
CtDKs
CtDKs
Separation
Duplication
Maturation
Separation
Duplication
Maturation
G1
Late G1
S
G2
M

B

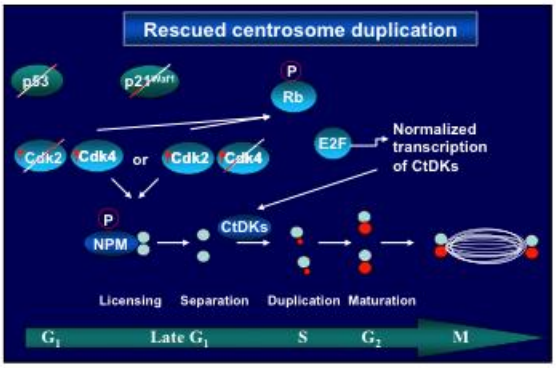

Rescued centrosome duplication
p53
p21Waf1
P
Rb
Cdk2
Cdk4
or
Cdk2
Cdk4
E2F
Normalized transcription of CtDKs
P
NPM
CtDKs
Licensing
Separation
Duplication
Maturation
G1
Late G1
S
G2
M

Figure 8. A model explaining how ablated Cdks prevent centrosome amplification. (A) Ablation of *p53* results in undetectable levels of $p21^{Waf1}$, leading to hyperactive Cdk2 and Cdk4. Hyperactive Cdks crosstalk to the centrosome via two modes: by hyper-phosphorylating Rb in the nucleus, leading to uncontrolled E2F-dependent transcription of molecules that influence various steps in the centrosome duplication cycle: those involved in centriole splitting, as well as centriole duplication kinases (CtDKs). In addition, hyperactive Cdks constitutively phosphorylate NPM^{T199}, resulting in excessive licensing of centrosome duplication. Uncontrolled expression of CtDKs and the inability of NPM to suppress normal centrosome duplication results in faster centrosome duplication cycles within a single cell cycle, resulting in the formation of multiple centrosomes. **(B)** When Cdk2 or Cdk4 are deleted in $p53^{-/-}$ MEFs, Rb is under-phosphorylated, and the E2F-dependent transcription of CtDKs is restored. In addition, under-phosphorylated NPM restores normal centrosome licensing and prevents excessive centriole duplication. This restricts the centrosome duplication cycle to one per cell cycle, thus resulting in normal centrosome numbers.

Chapter 3

The Ras oncogene signals centrosome amplification in mammary epithelial cells through cyclin D1/Cdk4 and Nek2

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*These authors contributed equally to this work. Harrison MK contributed to data shown in Figure 4.

Introduction

Overexpression of the Ras and Myc proto-oncogenes in breast cancers is associated with poor prognosis (200, 201). Ras is constitutively active in breast cancers through deregulated Her2, Erb4 and EGFR tyrosine kinase receptors (202, 203), overexpression of H-Ras, K-Ras, and N-Ras in 69% breast cancers, or mutational activation of K-Ras in 6.5% breast cancers (204, 205). Amplification of c-Myc results in its overexpression in 15-70% breast cancers (206-208). Direct evidence that Ras and c-Myc are involved in mammary cancers was obtained by the co-expression of c-Myc, H-Ras^{G12D}, telomerase and SV40 T antigens in primary human mammary epithelial cells, resulting in transformation (137). Additionally, MMTV transgenic mice expressing c-Myc (209, 210), H-Ras^{G12V} (210, 211), N-Ras (212), and K-Ras^{G12D} (213, 214), developed mammary tumors.

Concurrently, apoptosis and cell cycle arrest are transient barriers to Myc and Ras mammary carcinogenesis (209, 211). For example, overexpression of H-Ras^{G12V} in mouse mammary epithelial cells results in transient cell cycle arrest between 14 days and 32 days after induction (211). Indeed, an active p53 pathway is a major obstacle to H-Ras^{G12V}-initiated mammary tumors (211, 215), and c-Myc triggers activating K-Ras mutations to induce non-regressing mammary tumors (209). Chromosome instability (CIN) is a potential mechanism used by oncogenes to abrogate transient barriers to mammary cancers; consistent with this, mammary tumors expressing H-Ras^{G12V} and c-Myc are

genomically unstable (215, 216). However, the source, timing, and relevance of oncogene-dependent CIN to mammary tumorigenesis are unknown.

CA, the acquisition of three or more centrosomes within a cell, is one of the major contributors to CIN in human cancers (152). CA is frequently observed in human cancers - including prostate, colon, breast, and cervical cancer - which suggests an involvement in tumorigenesis (82, 84, 217, 218). CA is a potential initiator of mammary tumorigenesis, since most benign breast lesions (94, 149) and breast cancers display CA (94, 98, 218). Centrosomes ensure equal segregation of chromosomes by directing the bi-polarity of the mitotic spindle (21). Thus, CA generates multi-polar spindles, merotelic attachments (attachment of single kinetochores to microtubules emanating from different poles), chromosomal lagging and aneuploidy, a major type of CIN (136). Because CA and multipolar mitoses are potentially transforming, they are suppressed by various mechanisms, including mitotic catastrophe, centrosomal clustering during mitosis and genomic convergence (136, 219, 220)

The most direct evidence showing the involvement of CA in tumorigenesis is that ectopic expression of centrosome regulatory proteins in transplanted Drosophila neuronal stem cells resulted in tumors (23, 24). In mammalian cancers, aneuploidy is ubiquitous (188). In contrast to mammalian cells, in which low level aneuploidy initiates and sustains various mouse tumors (133, 134), and in contrast to mammalian tumors, which are aneuploid, tumorigenesis in Drosophila was not accompanied by CIN. In fact, of the five independent genetic alterations required to transform primary human mammary epithelial cells (137),

three (H-Ras^{G12V}, inactive Rb and p53) trigger CA and CIN (102, 115, 125), while c-Myc triggers aneuploidy and chromosome recombinations (216); this suggests a close relationship between CA, CIN, and mammary tumor initiation.

Demonstrating that CA is involved in mammary tumor initiation requires establishing that oncogene-driven CA occurs during pre-malignancy and identifying single or cooperating oncogenes responsible for CA. The identification of the centrosome regulatory proteins deregulated by oncogenes would allow future therapeutic interventions to abrogate the CA and aneuploidy that drive breast tumors. We demonstrate the ability of Ras to signal CA in pre-malignant mouse mammary lesions and human mammary epithelial cells through cyclin D1/Cdk4 and Nek2.

Results

Oncogene expression results in dysplasia, ectopic proliferation, and apoptosis during pre-malignancy and tumorigenesis

Doxycycline-inducible MMTV transgenic mice expressing K-Ras^{G12D} and/or c-Myc for five days, or until mammary tumors developed, were used to address various abnormal phenotypes involved in mammary tumor initiation (209). Real-time PCR using transgene-specific primers revealed that K-Ras^{G12D} and/or c-Myc expressed robustly in the corresponding transgenic groups, which was undetectable in controls (data not shown). Western blots detecting endogenous and transgenic Ras and c-Myc showed that levels of K-Ras^{G12D} (7-fold over the control) are within the average Ras expression in human breast

tumors, which are 2-10 fold relative to non-affected mammary epithelium (data not shown) (204, 205). In contrast, c-Myc levels are much higher (50-70 fold over controls) than the average c-Myc levels in human breast tumors, which are 1.8-4 fold relative to non-affected mammary epithelium (206-208).

As reported previously for H-Ras^{G12V} and K-Ras^{G12D} (210, 214), mammary tumors initiated by K-Ras^{G12D} occurred much faster relative to c-Myc (data not shown), and co-expression of K-Ras^{G12D} and c-Myc induced mammary tumors faster than either transgene did separately. These results allowed us to select a time-point of five days to investigate events associated with early pre-malignancy, since it precedes tumorigenesis by a few weeks.

We assessed various abnormal phenotypes associated with the expression of K-Ras^{G12D} and c-Myc that have been thoroughly studied in tumors, but are poorly understood in early pre-malignancy; those include histological changes, ectopic proliferation, and apoptosis (194, 209, 214, 221, 222). Mammary glands expressing oncogenes displayed distinct histopathological changes at pre-malignancy: c-Myc led to mild hyperplasia of ducts and lobules, with single-layered acini adjacent to each other. In contrast, K-Ras^{G12D}, or K-Ras^{G12D} and c-Myc severely altered the normal structure of the mammary gland; specifically, ducts and lobules were hyperplastic, the epithelial cells occupied the lumen of the acini, and had invaded into the stroma (Figure 1a). Such distinctions were obscured in the tumors caused by K-Ras^{G12D} or c-Myc, since both harbored numerous malignant epithelial cells and scanty stroma (Figure 1a).

Ki-67 immunostaining showed that all oncogenes enhanced proliferation of mammary epithelial cells, and that K-Ras^{G12D} and c-Myc cooperated to increase those frequencies in the pre-malignant lesions (Figure 1a, 1b). Cleaved caspase-3 revealed that while K-Ras^{G12D} and c-Myc increased cellular apoptosis during pre-malignancy, only c-Myc signaled apoptosis in tumors. K-Ras^{G12D} suppressed c-Myc-signaled apoptosis at both stages (Figure 1a, 1c).

Thus, K-Ras^{G12D} and c-Myc are triggering malignant phenotypes during pre-malignancy, and their synergistic nature is obvious as soon as pre-malignancy because they cooperate to modulate frequencies of proliferation and apoptosis and to accelerate mammary tumor formation.

Centrosome amplification during pre-malignancy is specific to K-Ras^{G12D}

Frequencies of CA were assessed in mammary pre-malignant lesions and tumors initiated by K-Ras^{G12D} and/or c-Myc using immunostaining against gamma tubulin and pericentrin, proteins within the pericentriolar material of centrosomes essential to the nucleation of microtubules (Figure 2a). In spite of the universal CA found in tumors (Figure 2c), only mammary glands expressing K-Ras^{G12D} or K-Ras^{G12D}and c-Myc displayed elevated frequencies of CA at premalignancy (Figure 2b). However, K-Ras^{G12D} and c-Myc did not significantly increase frequencies of CA compared to K-Ras^{G12D} alone. Thus, CA occurs during tumor initiation and it is specific to the K-Ras^{G12D} pathway.

K-Ras^{G12D} and c-Myc result in different expression levels of gene products governing the cell and centrosome duplication cycles

One of the major mechanisms generating CA is the deregulation of the centrosome duplication cycle in late G_1/S phase. That deregulation may arise as a consequence of the down-regulation of negative regulators of the cell and centrosome cycles, including p53 (102), NPM (131), p21^{Waf-1} (108), p16^{INK4A} (149, 150), Brca1 (223), Brca2 (224), and E2F3 (30). Various checkpoint controls activated in response to overexpressed H-Ras^{G12V} are also involved in the negative regulation of the centrosome cycle; for example, protein expression of p21^{Waf-1}, p16^{INK4A} and p19^{ARF} plateau at 8 days post-induction, while p53 is activated at 4 days post-induction (211). A second major mechanism leading to the deregulation of the centrosome cycle is the overexpression of cell and centrosome regulatory molecules; those include E2F2 and E2F3 (28), cyclin D1 (146), cyclins E and A in p53-null cells (122), Plk4 (22), Mps-1 (162), or Nek-2 (15).

Taking these mechanisms into account, we screened the steady-state transcriptional levels of various molecules involved in the cell and centrosome cycles using quantitative real-time PCR (Table 1). None of the CKIs were significantly down-regulated; rather, we observed significant overexpression of some of those transcripts, including p16^{INK4A}, p19^{ARF}, and p27^{Kip1}. Likewise, Nek2, E2F2, E2F3a, Cyclin D1, Cyclin B2, and Plk4 were up-regulated.

We selected a subset of the differentially expressed gene products from Table 1 and assessed their steady-state protein levels with Western blots (Figure

3). In general, K-Ras^{G12D} and c-Myc led to a more robust deregulation of most target genes relative to either single oncogene. The results from real-time PCR were not always consistent with Western blots, perhaps because real-time PCR can detect minuscule amounts of mRNAs. For example, Western blots did not detect up-regulated p19^{ARF} or cyclin B2. In addition, even though p16^{INK4A} mRNA was up-regulated by K-Ras^{G12D}, the p16^{INK4A} protein was only robustly up-regulated in mammary glands co-expressing K-Ras^{G12D} and c-Myc. In other instances, up-regulated mRNA corresponded to up-regulated proteins; for example, p27^{Kip1} was up-regulated by all oncogenes, and p21^{Waf-1} was up-regulated by K-Ras^{G12D} and c-Myc. Another important checkpoint, p53, was hyper-phosphorylated in mammary glands expressing K-Ras^{G12D}, or K-Ras-^{G12D}and c-Myc.

Various gene products associated with CA were up-regulated; for example, Nek2 was equally up-regulated by all combinations of oncogenes, and cyclin E1 was only up-regulated by K-Ras^{G12D} and c-Myc. More importantly, cyclin D1 was up-regulated at the mRNA and protein levels in mammary epithelial cells expressing K-Ras^{G12D}, or K-Ras-^{G12D}and c-Myc. Consistent with up-regulated cyclins D1 or E1 was the increased phosphorylation of Rb in mammary glands expressing K-Ras^{G12D}, or K-Ras^{G12D} and c-Myc.

Taken together, the data indicated that rather than down-modulating CKIs or p53, K-Ras^{G12D} or K-Ras^{G12D} and c-Myc-dependent CA may arise from their ability to up-regulate targets that are critical in regulating both the centrosome cycle (such as Nek2) and the cell cycle (such as cyclin s D1 and E1).

Cyclin D1/Cdk4 and Nek2 contribute to Ras- or Ras and Myc-triggered centrosome amplification

We have described the ability of K-Ras^{G12D}, either alone or co-expressed with c-Myc, to deregulate various key regulators of cell and centrosome duplication cycles. Of those, Nek2 is a centrosome separase normally active at mitosis (17). Nek2 is overexpressed in breast cancers and exhibits centriole-splitting activity when expressed in interphase (15). Deregulated cyclin E/Cdk2 signals CA (30, 107, 122). Likewise, cyclinD1/Cdk4, is associated with CA (146) and is a key regulator of centrosome duplication (79). Cyclin B is localized in the centrosome (225). Thus, it is reasonable to assume that the up-regulation of Nek2, cyclin D1 cyclin E1, or B2 may mediate Ras and Ras and c-Myc-dependent CA. Hence, MCF10a cell lines stably expressing H-Ras^{G12V}, or H-Ras^{G12V} and c-Myc were generated. MCF10a is a non-transformed human mammary epithelial cell line with intact p53 (226). The MCF10a system showed minor differences relative to the transgenic mice; for example, while H-Ras^{G12V}, or H-Ras^{G12V} and c-Myc caused up-regulation of Nek2 and cyclin D1 as observed in vivo, the expression of cyclins E1 or B2 was unchanged, but nevertheless highly expressed (Figure 4a). Because ectopic expression of H-Ras^{G12V}, or H-Ras^{G12V} and c-Myc results in CA (Figure 4c), Nek2, cyclinD1, Cdk4, cyclin E1 and cyclin B2 were silenced (Figure 4b) using siRNAs. Silencing Cdk4, cyclin D1 or Nek2 abrogated oncogene-triggered CA (Figure 4b). This data demonstrated that Nek2 and cyclin D1/Cdk4 are critical to oncogene-triggered CA.

An explanation for the ability of silenced cyclin D1, Cdk4, and Nek2 to suppress CA is that their down-regulation causes cell cycle arrest. Cell cycle analysis by flow cytometry (Table 2) showed that silencing cyclin E1, cyclin B2, and Nek2 in MCF10a cells expressing H-Ras^{G12V} significantly increased the cell population in G_1 phase and correspondingly decreased the cell population in G2/M phases, while inhibition of Cdk4 or cyclin D1 did not. In MCF10a cells expressing vector control, or co-expressing H-Ras^{G12D} and c-Myc, silencing Nek2, cyclins D1, B2, or E1 significantly elevated cells accumulating in G_1. BrdU incorporation assays (Figure 4d) showed that knockdowns of cyclin D1 and Nek2 significantly inhibited cell proliferation induced by ectopically expressing H-Ras^{G12V}, or H-Ras^{G12V} and c-Myc. Thus, deregulation of the cell cycle is not the only cause of CA.

Discussion

This study addresses some important questions regarding the relationship between CA and mammary tumorigenesis. First, we show that CA precedes tumorigenesis and is oncogene-specific, since K-Ras^{G12D} initiated CA in mammary precursor lesions. In contrast, c-Myc was unable to induce CA in early pre-malignancy but induced CA in tumors. These findings place K-Ras^{G12D} among a group of oncogenes, including Aurora A and Pin-1, causing CA in pre-malignant mammary lesions (145, 227). In contrast, c-Myc falls in the category of genetic alterations, including ablated p53, that do not display CA during pre-malignancy (228, 229). Because some oncogenic stimuli lead to CA at pre-

malignancy whereas others do so later suggests that some oncogenes directly trigger CA to rapidly initiate tumors, while others require additional genetic or epigenetic changes to induce CA. The capacity of K-Ras^{G12D} to induce CA and lower apoptotic frequencies may contribute to faster times-to-tumors relative to c-Myc, as both oncogenes are similarly efficient in triggering ectopic proliferation in pre-malignant lesions and tumors. Another major finding was the identification of a subset of K-Ras^{G12D}-specific centrosome regulatory targets mediating CA in pre-malignant mammary lesions, including Nek2 and cyclin D1/Cdk4, and that their silencing abrogated CA in human mammary epithelial cells. Nevertheless, the question remains as to whether they mediate CA in vivo.

Interestingly, c-Myc resulted in Nek2 up-regulation without causing CA. An explanation for this is that Nek2 is necessary, but not sufficient to trigger CA without cooperating with other altered centrosome regulatory molecules. There is precedent for Nek2's cooperative nature: For example, ectopic expression of Nek2 cannot induce CA unless mammary epithelial cells are pre-immortalized with the SV40 T-antigen (15). Additionally, we showed that c-Myc enhanced proliferation without causing CA during pre-malignancy, demonstrating that CA and ectopic proliferation arise independently. In contrast c-Myc mammary tumors harbored CA. This suggests that c-Myc cooperates with secondary alterations to cause CA; one of which might be K-Ras, as it is a common hotspot in non-regressing mouse mammary tumors initiated by c-Myc (209).

Evidence suggests that ablated E2F3 and p53 up-regulate Cdk2 to trigger CA (30, 102). In fact, our recent work showed that Cdk2 and Cdk4 are key

mediators of CA in p53-null cells (79). In contrast, ectopic expression of cyclin E (Cdk2's catalytic partner) in hepatocytes only results in mild CA, whereas overexpression of cyclin D1 (Cdk4's catalytic partner) results in more severe CA (146), suggesting that in some cell/tissue-types, cyclin D1/Cdk4 is more potent than cyclin E/Cdk2 in signaling CA. This is also evidenced by our observations that silencing cyclin D1/Cdk4 significantly inhibits H-Ras^{G12V}, or H-Ras^{G12V} and c-Myc dependent CA. In contrast, inhibition of cyclin E1 or cyclin B2 severely alters cell cycle profiles without affecting CA.

Even though our studies clearly showed that Cdk4 is involved in Ras-induced CA, it is unknown how it leads to CA. One explanation is that Cdk4 phosphorylates targets required for regulating the centrosome cycle. For example, there is a strong correlation between hyperactive Cdk2, hyper-phosphorylation, and inactivation of NPM — a major negative regulator of the centrosome cycle (30). Our recent work showed that NPM is phosphorylated by Cdk4 during G_1, and that expressing NPM^{T199A}, a mutant lacking the Cdk2/Cdk4 phosphorylation site prevented CA in p53-null cells (79). Likewise, deregulated Cdk4 may also use canonical Cdk2 phosphorylation sites in molecules involved in other steps in the centrosome cycle, including CP110, Mps-1, and Plk4 — regulators of centriole duplication that are direct Cdk2 targets or that require Cdk2 for their optimal activity (22, 163, 164, 230). Another explanation is that hyperactive Cyclin D/Cdk4 hyper-phosphorylates Rb, leading to increased E2F activity and the deregulation of centrosome regulatory targets. We will identify transcriptional and post-transcriptional targets of cyclin D1/Cdk4 in the future.

Because ablation of cyclin D1 or Cdk4 abrogates mammary tumorigenesis in MMTV-Ras or MMTV-Neu (Her2) mice (8, 9), Cdk4/Cdk6-specific inhibitors reduce ectopic proliferation in human Her2+ breast cancer cells (231), and inhibitors of Nek2 decrease the tumorigenic potential of breast cancer cells (14, 232), we propose that centrosomal regulatory targets downstream of Ras would represent important future targets for intervening with breast tumorigenesis.

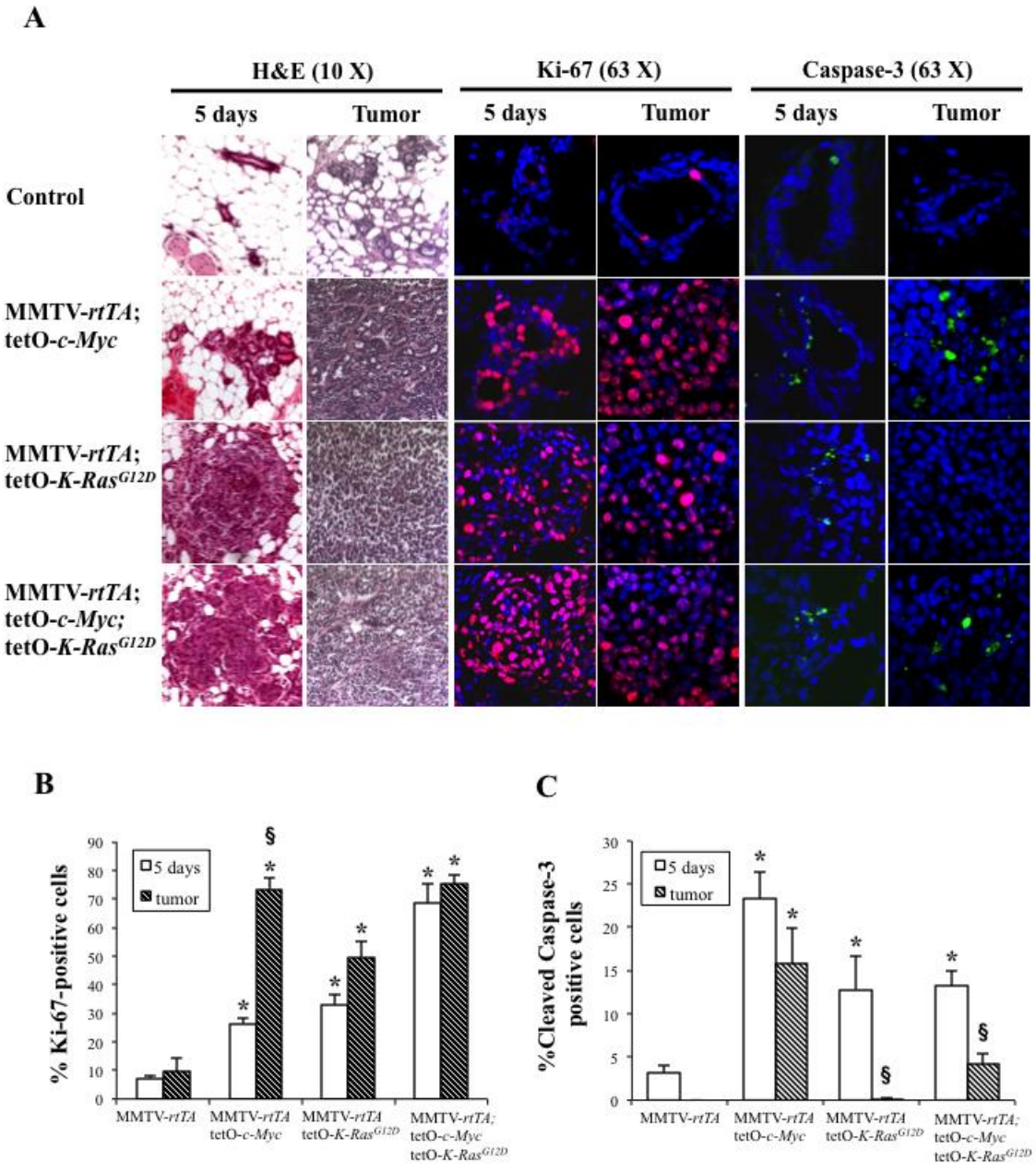
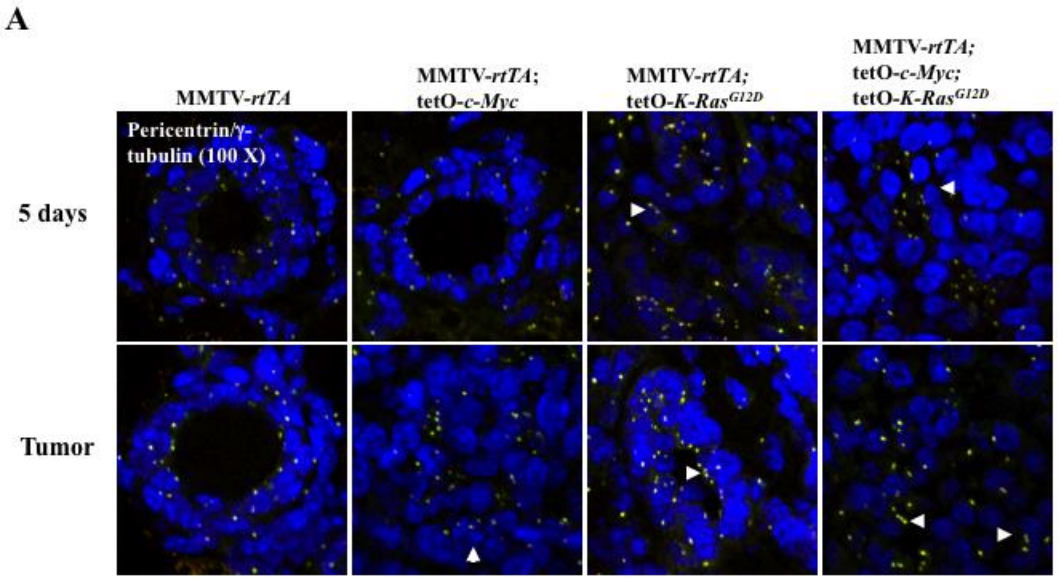
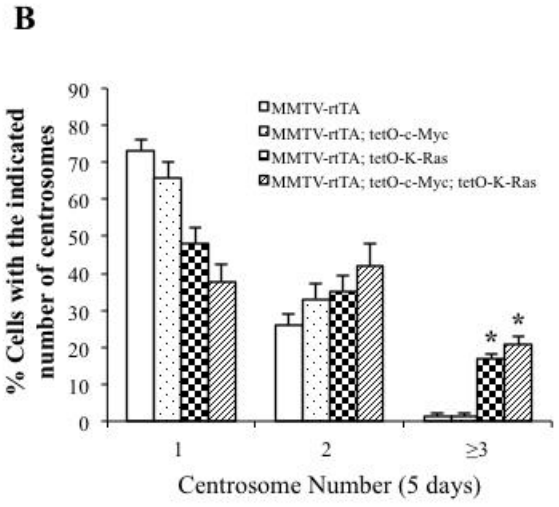

A
H&E (10 X)
Ki-67 (63 X)
Caspase-3 (63 X)
5 days
Tumor
5 days
Tumor
5 days
Tumor
Control
MMTV-rtTA; tetO-c-Myc
MMTV-rtTA; tetO-K-RasG12D
MMTV-rtTA; tetO-c-Myc; tetO-K-RasG12D
B
% Ki-67-positive cells
5 days
tumor
MMTV-rtTA
MMTV-rtTA tetO-c-Myc
MMTV-rtTA tetO-K-RasG12D
MMTV-rtTA; tetO-c-Myc tetO-K-RasG12D
C
%Cleaved Caspase-3 positive cells
5 days
tumor
MMTV-rtTA
MMTV-rtTA tetO-c-Myc
MMTV-rtTA tetO-K-RasG12D
MMTV-rtTA; tetO-c-Myc tetO-K-RasG12D

Figure 1. Inducible expression of K-Ras^{G12D} and c-Myc in mouse mammary glands results in distinct histopathology, ectopic proliferation, and apoptosis. (A) Hematoxylin–eosin (H&E) staining of mammary gland cross-sections. The first two columns show that expressions of K-Ras^{G12D} or K-Ras^{G12D} and c-Myc result in dysplasia in mice treated with doxycycline for 5 days or until tumors developed (chronic means that controls were treated chronically for 10 months but did not develop tumors). The first column was from H&E performed in paraffin-embedded 10 mm sections; the second was performed in 10 μm frozen sections. All immunostaining (columns 3–6) were performed in 7–10 μm frozen mammary cross-sections. The second pair of columns show mammary epithelial cells immunostained with antibodies against Ki-67 in red (Abcam, Cambridge, MA, USA, ab15580; the secondary antibody is conjugated with Alexa Fluor 555). The third pair of columns show mammary epithelial cells immunostained with antibodies against cleaved-caspase-3 in green (Cell Signaling, Danvers, MA, USA, 9661; the secondary antibody is conjugated with Alexa Fluor 488). The nuclei were stained with DAPI in blue. **(B,C)** The percentages of proliferating cells (with positive Ki-67 staining) and apoptotic cells (with positive caspase-3 staining) were calculated at 5 days or in chronically induced mice. Each group included three independent mice. Averages and SD were calculated as 200 epithelial cells per mouse. Significance was assessed using an unequal variance t-test, calculated in Excel (Microsoft, Redmond, WA, USA) (significance: *P<0.05 as compared with MMTV-rtTA controls, §P<0.05 when comparing the same transgenic group 5 days with long-term treatment).


A
MMTV-rtTA
MMTV-rtTA; tetO-c-Myc
MMTV-rtTA; tetO-K-Ras^G12D
MMTV-rtTA; tetO-c-Myc; tetO-K-Ras^G12D
Pericentrin/γ-tubulin (100 X)
5 days
Tumor


B
% Cells with the indicated number of centrosomes
MMTV-rtTA
MMTV-rtTA; tetO-c-Myc
MMTV-rtTA; tetO-K-Ras
MMTV-rtTA; tetO-c-Myc; tetO-K-Ras
Centrosome Number (5 days)

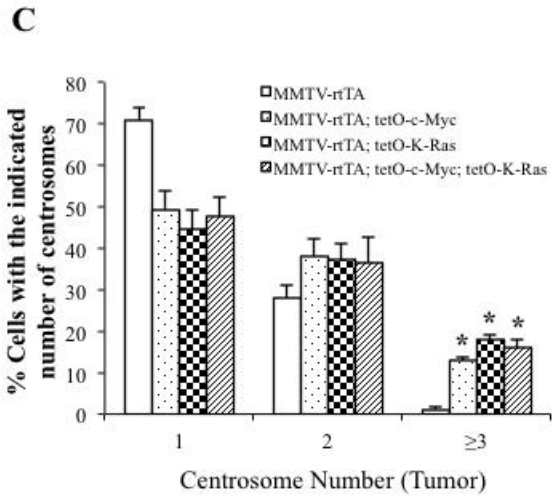

C
% Cells with the indicated number of centrosomes
MMTV-rtTA
MMTV-rtTA; tetO-c-Myc
MMTV-rtTA; tetO-K-Ras
MMTV-rtTA; tetO-c-Myc; tetO-K-Ras
Centrosome Number (Tumor)

Figure 2. Expression of K-Ras^{G12D} results in centrosome amplification in premalignant mammary lesions, whereas c-Myc-induced centrosome amplification is only detected in tumors. (A) Co-immunostaining with antibodies against γ-tubulin (Abcam, ab11317; the secondary antibody is conjugated with Alexa Fluor 555) and pericentrin (BD Biosciences, San Jose, CA, USA, 611814; the secondary antibody is conjugated with Alexa Fluor 488). Pictures presented as merged from the green and red channels, resulting in yellow signals. Arrows indicate cells with CA. Insets of the indicated area are presented for easier visualization. Chronic means that controls were treated chronically for 10 months but did not develop tumors. **(B, C)** Frequencies of mammary epithelial cells with 1, 2, or ≥3 centrosomes. Each group included three independent mice. Averages and SD were calculated as 200 epithelial cells per mouse. Significance was assessed using an unequal variance t-test, calculated in Excel (significance: *P<0.05 as compared with MMTV-rtTA controls).

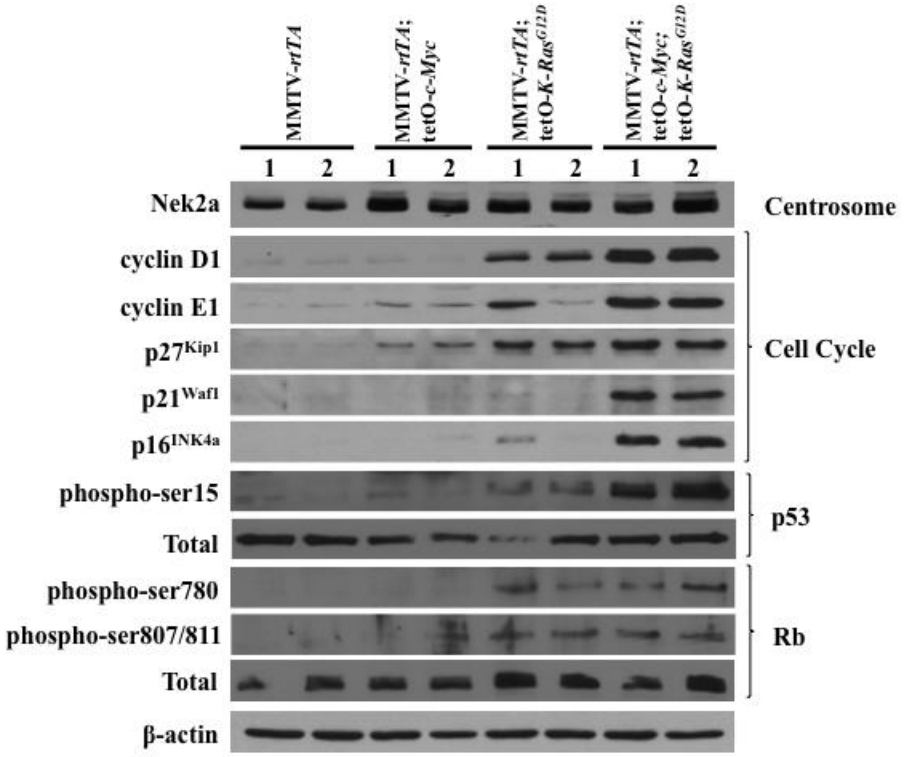

MMTV-rtTA
MMTV-rtTA; tetO-c-Myc
MMTV-rtTA; tetO-K-Ras^{G12D}
MMTV-rtTA; tetO-c-Myc; tetO-K-Ras^{G12D}
1 2 1 2 1 2 1 2
Nek2a
Centrosome
cyclin D1
cyclin E1
p27^{Kip1}
p21^{Waf1}
p16^{INK4a}
Cell Cycle
phospho-ser15
Total
p53
phospho-ser780
phospho-ser807/811
Total
Rb
β-actin

Figure 3. Expression of K-Ras^{G12D} and c-Myc during premalignancy results in differential expression of proteins governing the cell and centrosome cycles. Lysates were extracted from the mammary glands of 3-month-old mice treated with doxycycline for 5 days. Proteins were detected by western blots. Antibodies from Cell Signaling were cyclin D1 (#2922), p16Ink4a (#4824), phospho-Ser15 p53 (#9284), phospho-Rb (Ser780, #9307; Ser807/811, #9308), Rb (#9313) and β-actin (#4970). Antibodies from BD Biosciences included Nek2 (#610593); those from Santa Cruz Biotechnology (Santa Cruz, CA, USA) included cyclin E1 (sc-481), p27Kip1 (sc-528), p21Waf1 (sc-397) and p53 (sc-6243). Duplicates (1, 2) represent two independent mice from each group.

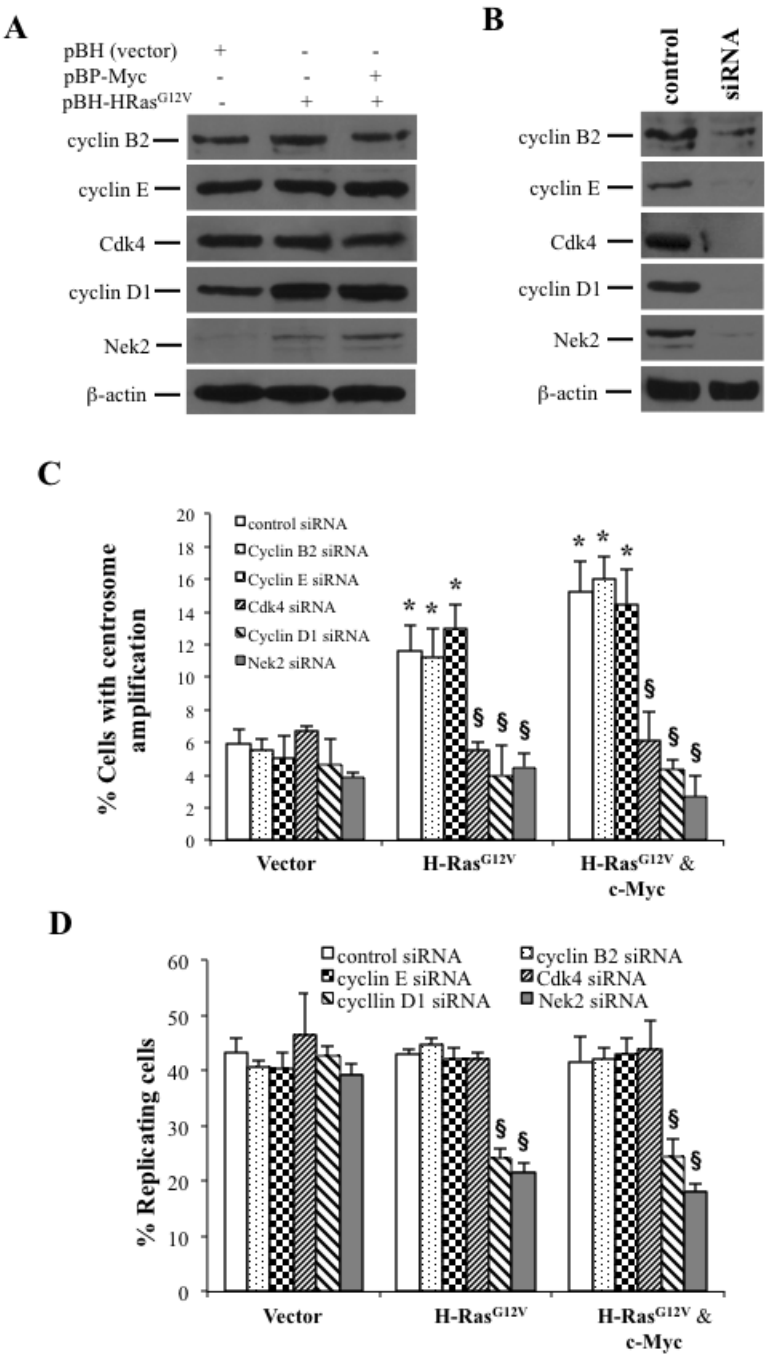

A
pBH (vector) + - -
pBP-Myc - - +
pBH-HRas^{G12V} - + +
cyclin B2
cyclin E
Cdk4
cyclin D1
Nek2
β-actin
B
control
siRNA
cyclin B2
cyclin E
Cdk4
cyclin D1
Nek2
β-actin
C
% Cells with centrosome amplification
control siRNA
Cyclin B2 siRNA
Cyclin E siRNA
Cdk4 siRNA
Cyclin D1 siRNA
Nek2 siRNA
Vector
H-Ras^{G12V}
H-Ras^{G12V} & c-Myc
D
% Replicating cells
control siRNA
cyclin B2 siRNA
cyclin E siRNA
Cdk4 siRNA
cycllin D1 siRNA
Nek2 siRNA
Vector
H-Ras^{G12V}
H-Ras^{G12V} & c-Myc

Figure 4. Oncogene-induced centrosome amplification is suppressed by siRNA-mediated silencing of Cdk4, cyclin D1 or Nek2. MCF10A cells were stably transfected with plasmids encoding empty vector (pBABE-hygro or pBABE-puro), H-Ras^{G12V} (pBABE-hygro-H-Ras^{G12V}), c-Myc (pBABE-puro-c-Myc) and H-Ras^{G12V} and c-Myc (pBABE-hygro-H-Ras^{G12V} and pBABE-puro-c-Myc). These established cell lines were then transfected with control siRNAs duplexes (Ambion, Austin, TX, USA, #4611) or against cyclins B2, D1, E1, Cdk4 and Nek2. **(A)** Western blots of cyclin B2 (Abcam, ab82287), cyclin E (Santa Cruz Biotechnology, sc-480), Cdk4 (Abcam, ab7955), cyclin D1 (Cell Signaling, 2922) and Nek2 (BD Biosciences, 610593) protein levels in MCF10A cells stably expressing vector control, H-Ras^{G12V} or H-Ras^{G12V} and c-Myc, which were starved in 0.2% FBS for 60 h. **(B)** Western blots of parental MCF10A transfected with control siRNAs, or siRNAs targeting cyclin B2, cyclin E1, Cdk4, cyclin D1 or Nek2, showing knockdown efficiencies. **(C, D)** Frequencies of CA (double immunostaining with γ-tubulin and pericentrin) and proliferation (immunostaining with BrdU, BD Pharmingen, San Jose, CA, USA, NA61) in MCF10A cells ectopically expressing empty vector, H-Ras^{G12V}, or H-Ras^{G12V} and c-Myc in the presence of control or targeted siRNAs. The average and SD were calculated from triplicate experiments (*$P<0.05$ as compared with MCF10A cells transfected with empty vector and control siRNA; §$P<0.05$ as compared with MCF10A cells transfected with H-Ras^{G12V}, or H-Ras^{G12V} and c-Myc vectors together with control siRNA). For each experiment, we counted at least 200 cells per group.

Table 1. Five-day induction of K-Ras^{G12D} and c-Myc results in the differential expression of various genes

Gene Name	MMTV-*rtTA*; tetO-*c-Myc*	MMTV-*rtTA*; tetO-*K-Ras^{G12D}*	MMTV-*rtTA*; tetO-*c-Myc*; tetO-*K-Ras^{G12D}*
Aurora kinase A	0.97 (0.25)	0.90 (0.25)	2.08 (0.13)
Brca1	3.17 (0.28)*	3.55 (1.95)	18.90 (0.43) *
Brca2	1.11 (0.19)	0.74 (0.42)	0.86 (0.24)
Cdc2	1.35 (0.10)	1.31 (0.14)	3.25 (0.30) *
Cdk2	0.60 (0.34)	0.48 (0.11)	0.51 (0.27)
Cdk4	0.86 (0.15)	0.64 (0.14)	1.41 (0.07)
c-Nap-1	0.81 (0.23)	0.54 (0.38)	0.34 (0.18)
Cyclin A2 (CCNA2)	2.12 (0.17) *	1.74 (0.14)	3.53 (0.31) *
Cyclin B1 (CCNB1)	2.12 (0.27)	1.48 (0.24)	5.71 (0.46) *
Cyclin B2 (CCNB2)	6.51 (0.08) *	4.45 (0.16) *	29.79 (0.26) *
Cyclin D1 (CCND1)	1.11 (0.27)	4.91 (0.28) *	4.03 (0.46) *
Cyclin E1	0.69 (0.23)	0.47 (0.45)	0.51 (0.48)
Cyclin E2	0.77 (0.22)	0.57 (0.57)	1.10 (0.31)
E2f1	0.99 (0.33)	0.56 (0.27)	1.14 (0.10)
E2f2	2.60 (0.62) *	4.52 (0.33) *	14.06 (0.31) *
E2f3a	2.41 (0.05) *	1.53 (0.35)	3.35 (0.35) *
E2f3b	0.52 (0.41)	0.47 (0.42)	0.70 (0.40)
E2f4	1.10 (0.42)	1.08 (0.19)	1.86 (0.35)
E2f5	0.70 (0.54)	0.53 (0.27)	1.18 (0.44)
Mps-1	1.21 (0.55)	1.00 (0.63)	1.62 (0.24)
Nek2	0.69 (1.20)	3.88 (2.87)	9.80 (0.61) *
NPM	1.94 (0.12)	0.75 (0.11)	1.39 (0.28)
$p15^{INK4B}$ (CDKN2B)	0.63 (0.07)	1.73 (0.31)	2.54 (0.14) *
$p16^{INK4A}$ (CDKN2A)	0.47 (0.69)	19.34 (0.33) *	48.84 (0.17) *
$p18^{INK4C}$ (CDKN2C)	0.61 (0.11)	0.02 (0.32) *	0.01 (0.11) *
$p19^{INK4D}$ (CDKN2D)	1.30 (0.50)	1.19 (0.14)	3.98 (0.33) *
$p19^{ARF}$	0.48 (0.03)	7.84 (0.63) *	76.64 (0.67) *
$p21^{Waf1}$ (CDKN1A)	0.54 (0.16)	1.48 (1.45)	4.13 (0.30) *
$p27^{Kip1}$ (CDKN1B)	58.76 (0.22) *	29.79 (0.47) *	50.10 (0.72) *
Plk4	3.05 (0.69)	2.33 (1.03)	11.18 (0.40) *

* Difference is significant by an unequal variance T-Test ($p < 0.05$) as compared to the control group (MMTV-*rtTA*).

† Data is presented as fold change and standard deviation (in parentheses) stemming from triplicate real-time PCR; data is from three independent mice from each genetic group.

Table 2. Cell cycle distribution of MCF10a cells transfected with empty vector, H-Ras^{G12V} and/or c-myc, and the indicated siRNAs

Vector	siRNA	G1 (%)	S (%)	G2/M (%)
Control	Control	60.2 ± 0.02	13.8 ± 0.55	25.9 ± 0.53
	Cdk4	50.3 ± 1.32 *	18.1 ± 1.15 *	31.6 ± 2.46
	Cyclin D1	63.9 ± 0.71 *	11.8 ± 0.18 *	24.3 ± 0.89
	Cyclin E1	78.8 ± 0.10 *	6.0 ± 0.06 *	15.2 ± 0.16 *
	Cyclin B2	68.3 ± 0.55 *	13.2 ± 1.99	18.5 ± 1.44 *
	Nek2	71.1 ± 1.46 *	9.4 ± 0.26 *	19.5 ± 1.21 *
H-Ras^{G12V}	Control	63.2 ± 0.03	11.3 ± 0.17	25.5 ± 0.14
	Cdk4	51.2 ± 0.46 *	17.0 ± 2.26	31.8 ± 1.80 *
	Cyclin D1	65.9 ± 1.95	11.8 ± 0.23	22.2 ± 1.72
	Cyclin E1	76.1 ± 2.76 *	6.8 ± 1.25 *	17.1 ± 1.51 *
	Cyclin B2	75.7 ± 0.28 *	8.2 ± 1.02	16.2 ± 1.30 *
	Nek2	74.6 ± 0.05 *	7.5 ± 0.10 *	17.9 ± 0.05 *
H-Ras^{G12V} and c-Myc	Control	54.9 ± 0.01	10.4 ± 1.23	34.7 ± 1.22
	Cdk4	47.6 ± 0.38 *	15.0 ± 1.76	37.4 ± 1.38
	Cyclin D1	58.8 ± 0.95 *	13.7 ± 0.78	27.5 ± 1.73 *
	Cyclin E1	64.4 ± 0.30 *	7.6 ± 0.04	28.0 ± 0.26 *
	Cyclin B2	62.8 ± 1.96 *	14.0 ± 1.38	23.3 ± 0.58 *
	Nek2	65.4 ± 2.01 *	8.2 ± 1.42	26.4 ± 0.58 *

* Differences are significant ($p < 0.05$) as compared to control siRNA. 10,000 cells were collected per experiment using flow cytometry. The columns represent the percentage of cells in G1, S, or G2/M, gated using FLOJO, and presented as Mean ± SD. The results stem from triplicates.

Chapter 4

Cdk4 and Nek2 signal binucleation and centrosome amplification in a Her2+ breast cancer model

Pitner MKH completed all work presented in this chapter.

Introduction

Theodor Boveri's work published in 1914 was the first to hypothesize a correlation between abnormal centrosome numbers, aneuploidy, and tumorigenesis (233). Almost 100 years later, the questions surrounding this correlation are still being pursued. Centrosomes play a crucial role in maintaining euploidy; the two mitotic centrosomes direct the formation of a bipolar spindle and allow equal segregation of chromosomes into daughter cells (234). CA, the acquisition of three or more centrosomes within a cell, is often observed in human cancers and has been shown to contribute to multipolar mitoses, aneuploidy, and chromosomal instability (82, 84, 152, 218). There is a growing body of evidence showing that a majority of solid tumors and some hematopoietic cancers harbor cells with centrosome abnormalities, either numerical or structural (81). Observations in breast tumors show that adenocarcinoma cells have a much higher occurrence of centrosome defects, including amplification of number, increased volume, and supernumerary centrioles, when compared to normal breast tissue (94, 95). Similar phenotypes can also be found in premalignant lesions and pre-invasive *in situ* ductal carcinoma, suggesting that these aberrations influence early breast carcinogenesis (83, 94, 96). Although the role played by CA in mammalian tumorigenesis remains a mystery, major discoveries have been made. Among these is the discovery that ectopic expression of centrosome and mitotic regulatory kinases results in CA and tumorigenesis in *Drosophila* (23, 24). Another finding is that low-level aneuploidy caused by interference with the

spindle assembly checkpoint initiates mouse tumors (133, 134), and that CA is capable of generating low levels of aneuploidy (136). CA is also known to generate more severe forms of aneuploidy, including tetraploidy, through generating multipolar spindles (101). Although tetraploidy is selected against in checkpoint-proficient cells (101, 136, 235), it contributes to carcinogenesis in p53-deficient mammary epithelial cells (236). It has been reported that the absence of p53 allows transient tetraploidy in a small subset of cell lines (235).

The centrosome duplication cycle is coordinated with the cell cycle, such that it occurs only once per mitosis (21). The biology of cell cycle regulation has been well studied (237, 238), and it is known that the faithful regulation of its phases, G_1, S, and G_2/M, is important to cancer prevention (239). More recent work has shown that there are many cell cycle regulatory proteins (including the cyclins, Cdks, CKIs, and E2Fs) that associate with the centrosome cycle and seem to play a role in centrosome homeostasis (101, 240, 241). A large number of these proteins have also been reported as deregulated in cancer. For example, Cdk2 and Cdk4 are two proteins central to the coordination of the cell and centrosome duplication cycles. It has been previously shown that Cdk4 is a regulator of centrosome duplication (79, 146), that the cyclin D1/Cdk4 complex contributes to p53-null- and Ras-driven CA (79, 242) and is important in Her2 mitogenic signaling (5, 6, 11). Many studies implicate Cdk2 as a key regulator in several centrosomal functions including: centrosome duplication (27, 70, 101, 128, 243), CA in p53-negative breast cancer cells (244) and p53-null MEFs (79, 107, 122), and cells expressing the E7 viral oncoprotein (75). Because ablation

of Cdk2 or Cdk4 suppresses Her2-driven mammary tumors (8, 11, 245) and signals CA, the two Cdks may represent important links between CA and tumorigenesis.

Her2/Neu, also known as ErbB2, a receptor tyrosine kinase, induces a complex signaling network upon binding its co-receptors, among these activated signals is the well-studied Ras-activated mitogen activated protein kinase (MAPK) pathway (5). While rarely mutated in human cancers, wild-type Her2 is often found amplified at the gene level or overexpressed at the protein level. The oncoprotein is overexpressed in approximately 30% of breast tumors, and hyper-activates and deregulates its downstream signaling networks, including the G_1/S cell cycle phase via high levels of cyclin D and active cyclin D/Cdk4/6 complexes (6). Cyclin D1 and its catalytic partners Cdk4/Cdk6 have been shown to be required for Her2-induced transformation (7-10), but the mechanism driving this phenotype remains unknown. There are studies suggesting association between Her2 overexpression and CA in breast tumors (94, 98), and one showing that mammary tumors in MMTV-*Neu* mice display CA (114), but the molecular contribution of Cdk2 and Cdk4 to Her2/Neu-mediated CA has yet to be elucidated.

It has long been thought that CA is a mechanism that leads to chromosomal instability (101, 246), a distinguishing feature of cancer cells, through abnormal mitoses. A recent study provided a direct link between CA and chromosomal instability, showing that extra centrosomes are sufficient to promote chromosome gains and losses during a pseudobipolar mitosis through a

multipolar spindle intermediate (136). Increased centrosome defects are directly proportional to chromosome aberrations in breast tumors, suggesting that CA is a driver of aneuploidy (218, 247). Because aneuploidy is transforming, and correlates with chemoresistance in tumors (248), finding agents that can prevent or suppress CA and the active generation of chromosomal instability in tumors is essential to cancer control. Direct evidence showing that CA transforms primary mammary epithelial cells is lacking, and necessitates the identification of oncogene-driven centrosomal regulatory molecules signaling CA. This study elucidates mechanisms responsible for CA in a Her2+ breast cancer model. Due to extensive evidence that Cdk2 and Cdk4 are important genetic links between CA, mitotic errors, and transformation, we explored their role as major regulators of CA in Her2+ breast cancer cells. Our results illustrate that the presence of CA, binucleation and defective cytokinesis requires Cdk4 but not Cdk2. In addition, we found that Nek2 may be a downstream target of Cdk4 that regulates its expression and mediates its role in binucleation and CA.

Materials and Methods

Cell culture

SKBr3 (ATCC, Manassas, VA, USA, HTB-30) and HCC1954 (ATCC, CRL-2338) cells were maintained under proliferating conditions in RPMI media (Sigma, St. Louis, MO, USA, R8758) supplemented with 10% fetal bovine serum (FBS) and 1% Penicillin/Streptomycin antibiotics (Gibco, Carlsbad, CA, USA, 15140). MCF10A (ATCC, CRL-10317) cells were maintained in DMEM/F-12 media

(Gibco, 12500-096) supplemented with 10% FBS, 1% Penicillin/Streptomycin, $NaHCO_3$, HEPES, 10µg/ml Insulin, 20ng/ml EGF, 0.5µg/ml hydrocortisone, and 100ng/ml cholera toxin. For serum arrest/release experiments, cells were cultured in 0.2% FBS for 72 hours under serum arrest conditions, and then released through the addition of serum. All cell lines screened, but not used for further investigation in this manuscript originated from the ATCC.

Lentiviral infections

Lentiviral infections were done to create stable cell lines. The Expression Arrest lentiviral shRNA pLKO.1 vector system was used from Open Biosystems (Thermo Scientific, Waltham, MA, USA). 293T cells were co-transfected with 1.8µg target shRNA construct, 1.8µg pHRCMV8.2ΔR, and 0.18µg pCMV-VSVG helper plasmids. Viral supernatant from 293T cell culture media was collected three times in 8-hour increments beginning 48 hours after transfection. Target cell lines were infected with viral supernatant and 10mg/ml polybrene. Forty-eight hours after the final infection, selection was begun in complete media containing 2ug/ml puromycin (Sigma, p9620). Resistant cells were assayed for knockdown of the target gene by Western blot.

Transfections

Transient transfection of siRNAs was done using Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA, 11668). siRNAs against Cdk2, Cdk4, and Nek2 were designed and purchased from IDT (IDT, Coralville, IA, USA). As a negative

control, Silencer Negative Control #1 RNA (Ambion, Carlsbad, CA, USA, 4611) was transfected. Transfections were performed as per the manufacturer's protocol; 72 hours after transfection the cells were used to prepare cell lysates for western blots or fixed in preparation of immunofluorescent staining. Nek2 was subcloned into the pMONO-Hygro-GFP plasmid (Invivogen, San Diego, CA, USA, pmonoh-gfp) by the Emory DNA Custom Cloning Core Facility. Transfection of the pMONO-Hygro-GFP-Nek2 plasmid was done using TransIT-2020 Transfection Reagent (Mirus, Madison, WI, USA, MIR5404) according to the manufacturer's instructions. HCC1954 shpLKO.1; GFP-Nek2 and HCC1954 shCdk4-4; GFP-Nek2 cells were maintained in RPMI media (Sigma, R8758) supplemented with 10% fetal bovine serum (FBS), 1% Penicillin/Streptomycin antibiotics (Gibco, 15140), 2ug/ml puromycin and 25ug/ul hygromycin (Sigma, h0654).

Immunofluorescence

Immunofluorescence was performed following our published protocols (79, 242). Proliferating cells plated in four chamber slides (Thermo Scientific, Waltham, MA, USA 154526) were fixed in cold 4% paraformaldehyde, washed in PBS, permeabilized in a 0.1% NP40-PBS solution, and blocked in 10% normal goat serum (Invitrogen, 50-062Z). Centrosomes and cytoskeletal structures were stained overnight at 4°C with antibodies against pericentrin (abcam, Cambridge, UK, ab4448) or α-tubulin (Santa Cruz, Santa Cruz, CA, USA, sc-32292), respectively. Alexa Fluor 488 goat anti-rabbit (Invitrogen, A11008) and Alexa

Fluor 488 goat anti-mouse (Invitrogen, A11001) conjugated secondary antibodies were used, respectively. Cells were counterstained with 4′,6-diamidino-2-phenylindole (DAPI).

Western blotting

Western blotting was performed according to our published protocols (30, 79). Antibodies used in western blotting experiments are as follows: Her2 (Cell Signaling, Beverly, MA, USA, 2165), Cyclin D1 (Cell Signaling, 2922), Nek2 (BD Biosciences, San Jose, CA, USA, 610593), phospho-NPM (Thr199) (Cell Signaling, 3541), NPM (Invitrogen, 32-5200), GFP (abcam, ab290), β-actin (Cell Signaling, 4970), Cdk2 (Santa Cruz, sc-163), and Cdk4 (Cell Signaling, 2906).

Image acquisition

Slides were analyzed using a Zeiss Axioplan II (Zeiss, Oberkochen, Germany) microscope with a Plan-Apochromat 63x oil immersion objective. Images were taken using the Axiocam HRC and Zeiss Axiovision software. Confocal images were acquired with a Zeiss LSM 510 META point scanning laser confocal microscope mounted on a Zeiss Axioplan II upright microscope equipped with a Plan-Apochromat 20x objective. Images were captured on the Zeiss Image Browser. All fixed samples were mounted in Fluoromount-G mounting medium (Southern Biotech, Birmingham, AL, USA) and were analyzed at room temperature.

Live microscopy

Proliferating cells were plated on an eight-chambered #1.5 German coverglass system (LabTek II, 155409). Live cells were imaged at 20x on the PerkinElmer Ultra View Spinning Disk (PerkinElmer, Waltham, MA, USA) microscope at 37°C and 5% CO_2, with a differential interference contrast (DIC) filter. Images were captured every five minutes for at least twenty-four hours, and compiled into movies for analysis. All image capture and analysis was done using the Volocity 3D Image Analysis Software (PerkinElmer).

BrdU Analysis

BrdU incorporation analysis was performed according to our published protocols (79). Pulsed cells were fixed and incubated overnight at 4°C with anti-BrdU antibody (Calbiochem, Billerica, MA, USA, NA61), then for 1 hour at room temperature with Alexa Fluor 555 anti-mouse secondary antibody (Invitrogen, A21422) and counterstained with DAPI.

Flow cytometry

Cells were dissociated from culture plates using Accutase (Sigma, A6964) and collected by spinning down in 15ml conical tubes. Cells were washed in cold 1X PBS and fixed in cold 70% Ethanol. After fixation, cells were treated with 500μl RNase (Sigma, R5125) and stained with 500μl propidium iodide (Sigma, P4170) for 45 minutes. Cells were transferred to meshed cap Falcon tubes for FACS analysis. FACS analysis was performed on a Benton-Dickinson LSRII.

Results

Establishing a model for the study of centrosome amplification in breast cancer

In order to establish a breast cancer cell model of CA, we screened several established breast cell lines of varying molecular subtypes for the presence of CA. We observed that SKBr3 and HCC1954 Her2+ER-PR- breast cancer cell lines harbor significantly higher percentages of CA in comparison to MCF10A control cells (Figure 1a). BT474 showed elevation in CA approaching statistically significance ($p<0.07$); because these cells grow in multiple layers precise calculation of CA was difficult. Analysis from CCLE and COSMIC databases, as well as results from the literature show that there are no mutations detected in HRAS, KRAS, or NRAS in MCF10A or cells displaying CA. Whereas previous reports demonstrate correlation between Her2 overexpression and CA using biopsied patient tissue, our study focuses on a Her2+ cell line experimental model. Following our initial screen, we determined that SKBr3 and/or HCC1954 would be used for further modeling of CA in breast cancer.

Her2+ breast cancer cells harbor centrosome amplification that is dependent upon Her2

The Her2+ cells used in this study have many and various genetic alterations in addition to harboring Her2 amplification (226), thus it was important to ensure the observed CA phenotype was dependent upon Her2 signaling. We generated stable knockdowns using a lentiviral short hairpin (sh) PLKO.1 vector system. We infected HCC1954 cells with shPLKO.1 control and shHer2 vectors.

After confirming down-regulation of Her2 via Western blot (Figure 1b), we pulsed proliferating cells with BrdU to test whether the reduction in Her2 protein expression would cause cell cycle arrest. There was no significant difference in the number of cells that stained positive for BrdU, thus knockdown of Her2 did not cause any detectable change in the percentage of cells undergoing DNA replication and that loss of Her2 is not cytostatic (Figure 1b). Confident that these cells stably down-regulated Her2 and were proliferating normally, we assayed them for CA. Cells expressing shHer2 showed a significant reduction in CA compared to shPLKO.1 vector control cells (Figure 1c). Taken together, these results indicate that elevated expression of Her2 protein in Her2+ breast cancer cells is necessary but not sufficient to trigger CA in this model.

Centrosome amplification in Her2+ cells is abrogated with silencing of Cdk4

It has been shown that amplification of the Her2 gene is significantly correlated with centrosome abnormalities in breast tumors (94, 98, 114), which could be indicative of a role for CA in the formation and/or progression of Her2+ breast cancer. Based on previous work (116), we sought to understand the role of the G_1 Cdks in a Her2-mediated CA model. First, we found overexpression of cyclin D1 in BT474, SKBr3, and HCC1954 compared to MCF10A control cells (Figure 1d).

Next, we targeted both Cdk2 and Cdk4 in non-tumorigenic and Her2+ breast cancer cells using independent siRNA duplexes. We confirmed knockdown of each gene by Western blot (Figure 2a). CA analysis was done on

proliferating cell populations with validated siRNA knockdown. In MCF10A cells, no difference was seen in the percentage of CA between scrambled control and siCdk2 or siCdk4 transfected cells. Both SKBr3 and HCC1954 cell lines showed little to no significant difference in the percentage of CA upon knockdown of Cdk2. However, knockdown of Cdk4 induced a dramatic decrease in CA in both Her2+ cell lines (Figure 2a). As siRNA knockdown is transient, we endeavored to establish stable cell lines expressing shCdk4 (Figure 2b). Mirroring the observations seen using siRNA, stable knockdown of Cdk4 resulted in a significant reduction in the percentage of CA in Her2+ cell lines (Figure 2b). In conclusion, we showed that inhibition of Cdk2 has a nominal effect on the CA phenotype in a Her2+ model of CA and that Cdk4 is a more influential mediator of the phenotype.

To ensure that knocking down Cdk4 did not induce cell cycle arrest, and as a byproduct, a reduction in CA due to lack of cell proliferation, we performed several cell cycle analysis experiments. To make certain shCdk4 cells were progressing through the cell cycle, HCC1954 shPLKO.1 and shCdk4-4 cells were serum arrested for 72 hours. Upon the addition of serum, starting at time zero hours, we harvested cells for cell cycle analysis every 6 hours for 24 hours. Flow cytometry results indicate that shCdk4 cells follow a very similar cell cycle pattern to control cells. A modest difference was seen in the S phase fraction at 18 hours post serum addition, but by 24 hours there is no significant difference (Table 1). This data suggests that loss of Cdk4 affects neither cell cycle entry after serum starvation nor proliferation.

To further investigate the S phase fraction of these cells, BrdU incorporation assays were used as described in the Materials and Methods section of this manuscript. We confirmed that stable shCdk4 cell lines were not deficient in S phase cells; our results showed control and shCdk4 cells had similar percentages of cells that stained positive for BrdU (Table 2). These results demonstrate that silencing Cdk4 does not affect the cell cycle, but rather, selectively affects the CA phenotype in this Her2+ breast cancer model.

Her2+ cells show a high percentage of binucleation, which is reduced upon silencing of Cdk4

There are several different mechanisms that may generate CA, including, but not limited to de novo centriole assembly, centriole reduplication, and cytokinesis failure (249). Interestingly, we observed a phenotype of binucleation in HCC1954 and SKBr3 cells compared to MCF10A control cells using antibodies against α-tubulin and DAPI, to image the cytoskeleton and nucleus, respectively (Figure 3a). This phenotype correlates with CA; cells that were binucleated were also overwhelmingly positive for CA. As shown in Figure 3a and 3b, proliferating SKBr3 cells displayed 8.0% binucleation and 75.1% of these cells also harbored CA; 12.2% of proliferating HCC1954 cells were binucleated, and 91.9% of the binucleated population had CA. There is a reasonable amount of data in the literature suggesting a mechanistic link between binucleation and centrosome abnormalities (235). The source of a potential cytokinetic defect causing binucleation and CA could span the entirety of the cell cycle. Deregulation could

lie at the level of molecules directly involved in cytokinesis or could lie upstream in molecules that regulate the cell cycle and its progression.

To ascertain the role of Cdk4 in generating binucleated Her2+ cells, we compared control and shCdk4 HCC1954 cells via microscopy. Results showed a significant decrease in binucleation in all three independent populations compared to vector control and parental cells (Figure 3c), suggesting a correlation between CA and binucleation.

For higher resolution and to reveal additional cellular mechanisms, we employed live cell imaging techniques. Using proliferating HCC1954 shCdk4-1 cells and their respective control, images were captured every 5 minutes over the course of 24 hours, and then pooled to create movies of a field of cells dividing over time. The results were rather striking; we observed cells attempting to undergo mitosis, failing, and resulting in binucleation (Figure 3d). While these events were rare, we noted them in both control and shCdk4-1 cells; however, there is a clear and significant difference in the percentage of these events between the two cell populations. Control cells present with 2.4% of attempted mitoses ending in observed novel binucleation, while in shCdk4-1 cells, only 0.4% of attempted mitoses ended in this way (Table 3). This data provides cytokinesis failure as a discernible mechanism for Cdk4 mediation of binucleation and CA in a Her2+ model.

Loss of Nek2 mimics the loss of Cdk4 and correlates with reduced centrosome amplification and binucleation in Her2+ breast cancer cells

A previous study published by our laboratory screened a broad panel of cell and centrosome cycle regulators in MCF10A cells stably expressing H-Ras^{G12V} or H-Ras^{G12V} and c-Myc (242). While this study identified genes that influenced a CA phenotype, no mechanism was revealed. The screen pulled down several interesting targets, one of which, Nek2, seemed particularly significant in light of the observed binucleation phenotype in Her2+ cells. Nek2, a NIMA-related cell cycle dependent protein kinase, is normally involved in centrosome separation at the onset of mitosis through phosphorylation of centrosomal proteins (17, 250), its activity peaking in S and G_2 phases (251). Nek2 levels have been found to be elevated in human breast cancer (15). Other proteins reported to be involved in the formation of CA were also found to be deregulated in Her2+ cell lines. NPM is a negative suppressor of centrosome licensing; it is a target of Cdk2 and Cdk4 phosphorylation during duplication initiation and a known suppressor of CA (129, 241). Deregulated NPM has been shown to mediate CA in other systems, including p53$^{-/-}$ MEFs through Cdk2 and Cdk4 (79).

We followed up with Nek2 as an important target gene in our model, based on the abnormal binucleation phenotype, and found that Nek2 protein was overexpressed in the Her2+ cell lines investigated in comparison to MCF10A cells (Figure 4a). We transiently transfected siCdk4 constructs into two Her2+ cell lines to assay the level of Nek2 upon loss of Cdk4 under proliferating conditions; interestingly we found that knockdown of Cdk4 lead to reduction of Nek2 levels (Figure 4b). Upon probing control and shCdk4 expressing HCC1954

cells with antibodies against Nek2 we discovered that knockdown of Cdk4 resulted in a decrease of Nek2 protein expression in serum-arrested cells (Figure 4c). We also detected a decrease in the level of phosphorylated NPM in cells expressing shRNAs against Cdk4 (Figure 4c). This target provides an interesting avenue for further investigation.

These novel findings suggest a functional correlation between Cdk4 and the potential CA regulator, Nek2. Next, we performed qRT-PCR experiments under proliferating and serum starvation conditions to address the role of Cdk4 in regulating Nek2 at the transcriptional level. We found no significant difference in the amount of Nek2 mRNA in any of the cell lines investigated at either proliferation or quiescence, suggesting that the silencing of Cdk4 does not impact Nek2 at the transcriptional level (data not shown). To further pursue Nek2 as a mediator of CA, we transfected siRNA constructs into MCF10A, SKBr3, and HCC1954 cells and assessed the percentage of CA. The reduction of Nek2 by siRNA phenocopied loss of Cdk4 and reduced the percentage of CA found in Her2+ cells (Figure 4d).

Overexpression of recombinant active Nek2 in human cancer cells induces premature centriole splitting at G_1/S, while still allowing cells to enter mitosis (17). Deregulated Nek2 has also been associated with abnormalities in cytokinesis in mammary epithelial cells immortalized with SV40 large T antigen (15). To elucidate a role for Nek2 in the observed binucleation phenotype of the Her2+ breast cancer model, we stained SKBr3 and HCC1954 shNek2 cells with antibodies against α-tubulin and DAPI in order to image the cytoskeleton and

nucleus, respectively. This assay revealed that knocking down Nek2 reduced the percentage of binucleation in proliferating cells, as control shPLKO.1 cells maintained high levels of binucleation, while shNek2 cells showed significantly lower percentages (Figure 4e). This data shows that Nek2 mediates CA and binucleation in Her2+ breast cancer cells.

This data suggests that Nek2 is possibly downstream of Cdk4 and important in inducing CA. To further address this possibility we attempted a rescue experiment by introducing an overexpression plasmid, GFP-Nek2, into HCC1954 cells expressing either shPLKO.1 or shCdk4. We were unable to obtain Nek2-overexpressing shCdk4-1 and shCdk4-3 cell populations, as these transfectants stopped proliferating. Nevertheless, we were able to establish stable populations of HCC1954 shPLKO.1 and shCdk4-4 cells and confirm overexpression of Nek2 via Western blot by probing for both Nek2 protein as well as GFP (Figure 5a). Interestingly, expression of Cdk4 protein was restored in cells overexpressing Nek2. The presence of GFP-Nek2 increased the percentage of CA in both control and HCC1954 shCdk4-4 cells compared to their relative controls (Figure 5a). To better understand a potential signaling pathway, we transfected siNek2 constructs into three Her2+ cell lines, HC1954, SKBr3, and JIMT1, and examined the levels of Cdk4 protein expression. We found HCC1954 and SKBr3 cells with confirmed Nek2 knockdown showed a marked reduction in Cdk4 expression. JIMT1 showed a slight reduction in Cdk4 upon knockdown of Nek2 (Figure 5b). We found no significant difference in the level of Cdk4 mRNA, suggesting that the silencing of Nek2 does not affect Cdk4 at the

transcriptional level (data not shown). In this report we show that Nek2 plays a key role in identifying the mechanism behind CA and binucleation in a Her2+ breast cancer model.

Discussion

A major proportion of human tumor cells harbor centrosome abnormalities. These aberrant phenotypes have been hypothesized to generate multipolar mitoses, microtubule nucleation errors, aneuploidy, chromosome instability, and even tumorigenesis. Understanding whether CA plays a role in breast tumorigenesis requires identifying the pathways and molecules that directly signal CA. Once such pathways and molecules are identified, their inhibition and/or overexpression will lead to a better understanding of their role in transformation. Our early work demonstrated that the Ras oncogene signals CA through the MAPK pathway, while other Ras-dependent pathways do not act on CA (141, 144). We also showed that Ras is able to trigger CA in premalignant mammary epithelial lesions, whereas c-Myc is unable to do so (242). These experiments indicate that CA is specific to certain oncogenic and signaling pathways and that CA may drive early mammary tumorigenesis.

Adding to our body of work detailing oncogene and tumor suppressor specific contributions to CA (79, 242), we studied CA in a Her2-positive breast cancer model. Previous studies addressing the involvement of cell cycle regulators in the centrosome cycle demonstrate that the loss of E2F3 and p53 deregulated Cdk2 activity, resulting in CA (30, 102). Recent work by our lab

showed Cdk2 and Cdk4 are key regulators of CA in p53-null cells (79), and that silencing of cyclin D1/Cdk4 inhibits CA driven by H-Ras^{G12V}, or H-Ras^{G12V} and c-Myc (242). The results presented in this report clearly show that Cdk4 is more influential than Cdk2 in mediating CA in Her2+ breast cancer cells. Importantly, Cdk4 inhibition abrogates CA without significantly interfering with the cell cycle, suggesting that a unique function of Cdk4 is to signal CA in a subset of Her2+ breast cancer cells. This observation is not specific to Her2 overexpression, as inhibition of Cdk4 suppressed CA in p53-null cells and in MCF10A expressing Ras, or Ras and Myc, without significantly affecting the cell cycle (79, 116). The implications of this result are potentially important to the treatment of breast cancer patients. We speculate that inhibiting Cdk4 in Her2+ breast tumors can suppress some malignant characteristics of tumor cells such as CA and the active generation of aneuploidy.

Our past work showed that Cdk4 is essential for centriole reduplication, an important intermediate to CA (79). The novelty of the studies described here revolves around Cdk4 signaling cytokinesis defects, binucleation, and CA. While the Cdks are typically related to CA through the deregulation of the centrosome cycle (30, 79, 100), so far, no one has shown that Cdk4 can influence aspects of cytokinesis. Interestingly, we found that knockdown of Cdk4 leads to a reduction in the level of Nek2 protein expression, which leads to a reduction in the percentage of binucleation and CA in Her2+ cells. This observation suggests a novel molecular mechanism where Nek2 can mediate some of the oncogenic functions of Cdk4. While cleavage failure is not sufficient to establish CA in

untransformed cells, it has been shown that in transformed CHO $p53^{-/-}$ cells several rounds of cleavage failure caused a small increase in CA that does not persist at later passages (235).

Compelling data indicates high levels of the centrosomal kinase Nek2 protein in cell lines derived from breast, cervical, and prostate carcinomas. Overexpression of Nek2 in immortalized breast cancer cells induces aneuploidy through multinucleated cells, and these cells are typically associated with CA (15). Additionally, transient overexpression of kinase-active Nek2 induces premature centrosome splitting (17). Nek2 protein can be regulated both temporally and spatially in various ways, including through transcription, post-translational modifications, and protein-protein interactions. The abundance of Nek2 is also managed by cell cycle-dependent protein degradation; it is normally targeted for proteasomal degradation following ubiquitylation facilitated by the anaphase promoting complex/cyclosome (APC/C). Failure to appropriately degrade Nek2 could increase stability and abundance within the cell (13). It is plausible, based on the role of Nek2 in centrosome separation and microtubule organization, that overexpression of Nek2 could lead to CA via binucleation, potentially through a defect in cytokinesis. Perhaps the most direct evidence for Nek2's role in cytokinesis comes from the *Drosophila* model system. At wild-type levels DmNek2 localizes to the midbody, and overexpression of DmNek2 causes a decrease in normal cytokinesis and an increase in tetraploid cells (252). There is sufficient evidence of centrosomal aberrations leading to mitotic defects, and there is a growing body of work suggesting that Nek2 is one of the molecules that

maintains mitotic events. In light of these findings, we propose a model where the overabundance of Nek2 in Her2+ cells is caused by deregulated cyclin D1/Cdk4 activity and that in turn, Nek2 is required to facilitate some of the abnormal mitotic functions triggered by cyclin D1/Cdk4 (Figure 6). Experiments attempting to rescue CA in cells stably silenced for Cdk4 were inconclusive. The only shCdk4 cells overexpressing Nek2 that proliferated were those that re-expressed Cdk4. This result could be interpreted as evidence of an interesting signaling loop, wherein high levels of Nek2 can positively regulate expression of Cdk4. This is suggested by the reduction in Cdk4 levels upon knockdown of Nek2. Alternatively, Nek2 overexpression in cells lacking Cdk4 might impose cell cycle blocks and impair cell proliferation.

Discovering that inhibition of Nek2 or Cdk4 diminishes CA in breast cancer cells, and showing that silencing of Cdk4 leads to reduced Nek2 overexpression is important, as both molecules have been shown to mediate mammary epithelial transformation (8, 14). As demonstrated in this manuscript, the inhibition of Cdk4 or Nek2 prevents CA in Her2+ cells, which is indicative of the important role of CA in mammary transformation. There are several molecules involved in centriole separation that are promising chemotherapeutic targets, including Nek2 and some of its phosphorylation substrates. There are currently no Nek2 inhibitors in clinical trials; however, interference in centrosome separation could cause mitotic abortion and cell death. There is some concern of issues with redundancy within centriole separation with the motor kinesin Eg5, as Eg5 compensates for Nek2 when Nek2 activity is reduced (253). Work done in the

studies presented here provide further evidence that Nek2 inhibitors could be of clinical relevance. Given the novel signaling feedback loop described in Chapter 4, inhibitors against Nek2 could also have an effect on Cdk4.

This manuscript furthers the understanding of the role that CA plays in breast cancers by identifying Cdk4 and Nek2 as mediators of CA in Her2+ breast cancer cells, and by identifying binucleation as a major mechanism generating CA in breast cancers. This has potential translational relevance because CA may be a driver of breast cancer biogenesis, exemplified by the presence of CA in pre-malignant mammary epithelial lesions in humans and in mice expressing various oncogenes. On the other hand, aneuploidy generated by CA can also drive resistance to chemotherapeutic agents. Thus, further studies are needed to establish whether inhibition of CA via the Cdk4-Nek2 pathway will improve the clinical outcome of breast cancer patients.

Table 1. Knockdown of Cdk4 does not affect cell cycle profiles.

Cell Line	Hours	Percent of G_1 cells (SD)*	Percent of S cells (SD)*	Percent of G_2 cells (SD)*
HCC1954 shPLKO.1	0	73.4 (3.1)	9.9 (1.2)	16.4 (4.1)
	6	68.9 (3.0)	12.2 (1.2)	18.2 (3.3)
	12	71.3 (5.4)	4.7 (1.9)	23.9 (7.5)
	18	59.2 (2.8)	24.4 (2.5)	16 (2.9)
	24	35.3 (0.6)	35.3 (9.6)	28.9 (10.1)
HCC1954 shCdk4-4	0	76 (0.1)	6.8 (3.8)	17 (4.1)
	6	72.7 (2.8)	10.1 (0.5)	16.8 (2.4)
	12	74.8 (2.5)	4.7 (0.5)	20.2 (3.1)
	18	68 (1.7)	13.7 (1.4)	17.9 (2.6)
	24	25.7 (7.5)	29.5 (5.3)	44.3 (1.5)

Results are pooled from two independent experiments.
*: p-value is calculated by T-test.

Table 2. Knockdown of Cdk4 does not affect the fraction of cells in S phase.

Cell Line	Percent of BrdU+ cells	SD	p-value*
HCC1954 shPLKO.1	34.1	12.03	
HCC1954 shCdk4-1	34.9	4.35	0.91
HCC1954 shCdk4-3	31.7	12.34	0.83
HCC1954 shCdk4-4	29.8	7.14	0.63

Results are from three independent experiments.
*: p-value is calculated by T-test.

Table 3. Knockdown of Cdk4 reduces percentage of failed mitosis ending in binucleation.

Cell Line Group	N	Observed Phenotype (%)		p-value*
		Binucleated	Non-binucleated	
HCC1954 shPLKO.1	645	15 (2.3)	630 (97.7)	0.0089
HCC1954 shCdk4-1	485	2 (0.4)	483 (99.6)	

Results are pooled from three independent experiments.
*: p-value is calculated by Chi-square test.

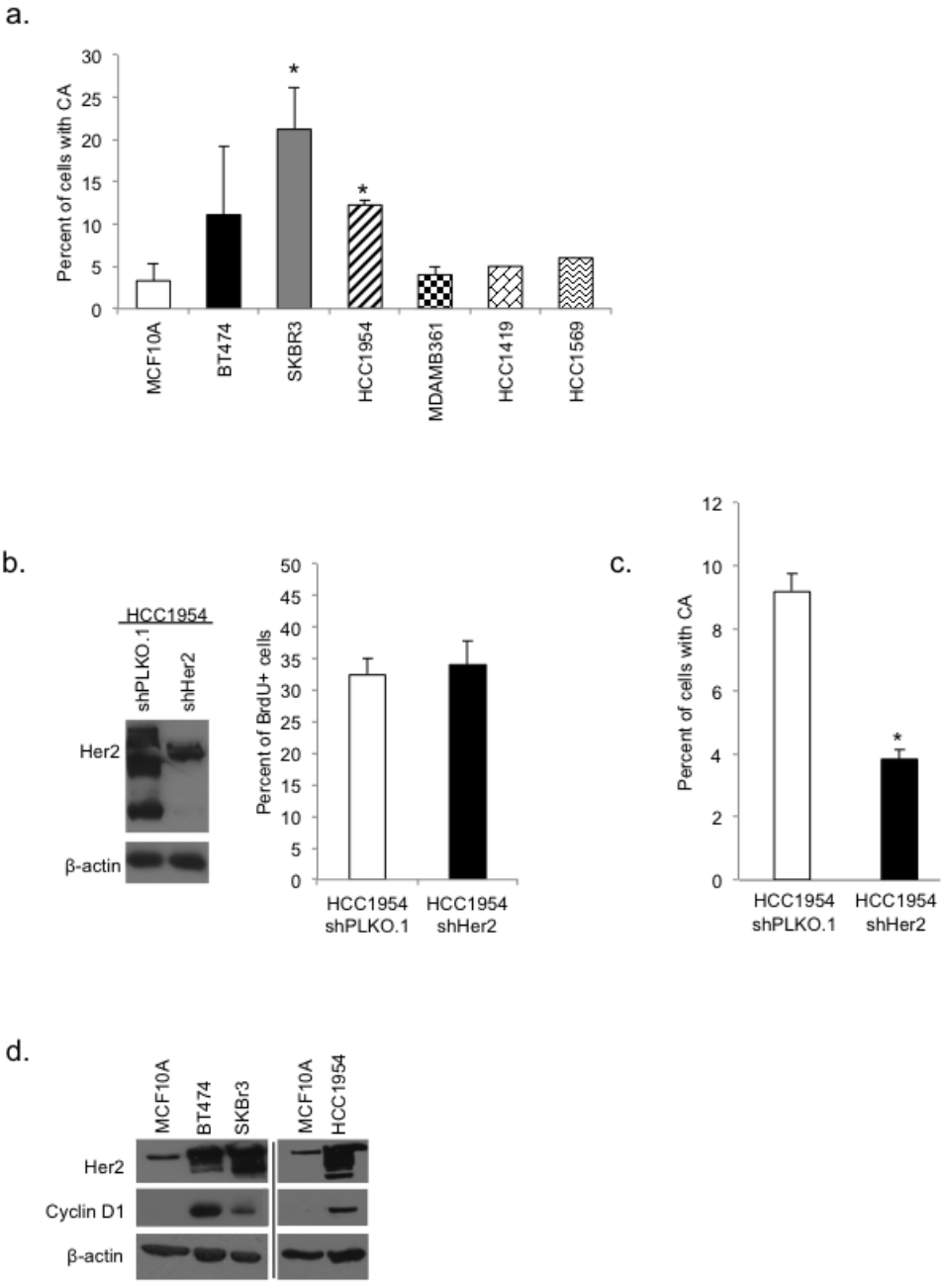
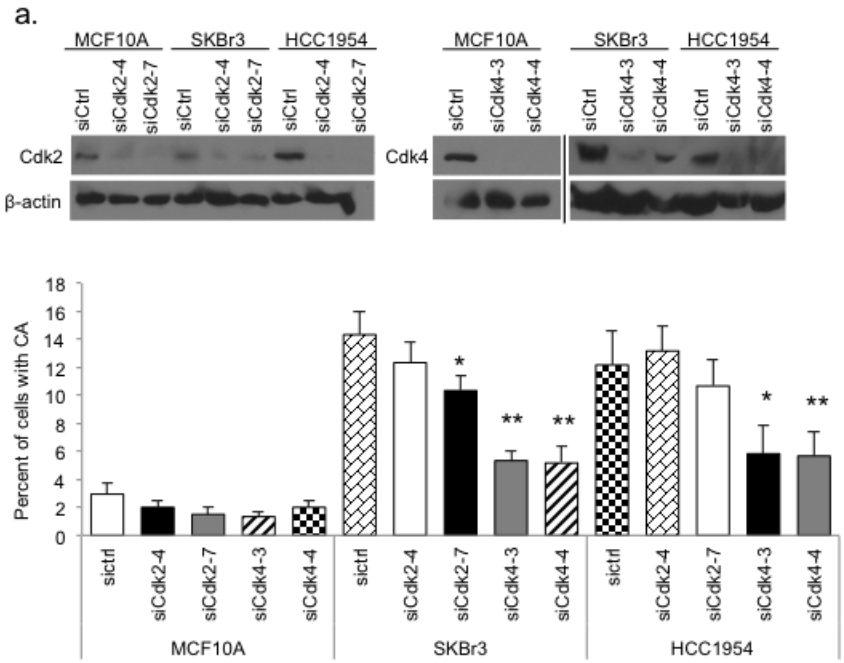

a.
Percent of cells with CA
30
25
20
15
10
5
0
*
*
MCF10A
BT474
SKBR3
HCC1954
MDAMB361
HCC1419
HCC1569
b.
HCC1954
shPLKO.1
shHer2
Her2
β-actin
Percent of BrdU+ cells
50
45
40
35
30
25
20
15
10
5
0
HCC1954 shPLKO.1
HCC1954 shHer2
c.
Percent of cells with CA
12
10
8
6
4
2
0
*
HCC1954 shPLKO.1
HCC1954 shHer2
d.
MCF10A
BT474
SKBr3
MCF10A
HCC1954
Her2
Cyclin D1
β-actin

Figure 1. Her2+ cells display centrosome amplification. (A) Centrosome amplification (CA) was measured by staining proliferating cells plated in four-chambered microscopy slides with an antibody against pericentrin and counterstaining with DAPI. Independent experiments were done three times using 200 cells per experiment. Graphs show the percent of cells with CA. Statistical significance was addressed using a T-test (*=p≤0.05). **(B)** Lentiviral shPLKO.1 control and shHer2 vectors were used to infect HCC1954, creating stable cell lines via puromycin selection. Knockdown was confirmed by western blot using an antibody against Her2; β-actin was used as a loading control. Upon confirmation of knockdown, CA was measured as described in **(A)**. **(C)** BrdU incorporation shows the percentage of cells in a proliferating population stained positively in HCC1954 shRNA cells. **(D)** Protein lysate was collected under starvation conditions. MCF10A and Her2+ breast cancer cell lines were probed with antibodies against Her2, and cyclin D1; β-actin was used as a loading control. Western blot results show two separate gels; different exposures are commensurate with protein abundance.


a.
MCF10A
SKBr3
HCC1954
siCtrl
siCdk2-4
siCdk2-7
Cdk2
β-actin
siCdk4-3
siCdk4-4
Cdk4
Percent of cells with CA
sictrl
MCF10A
SKBr3
HCC1954

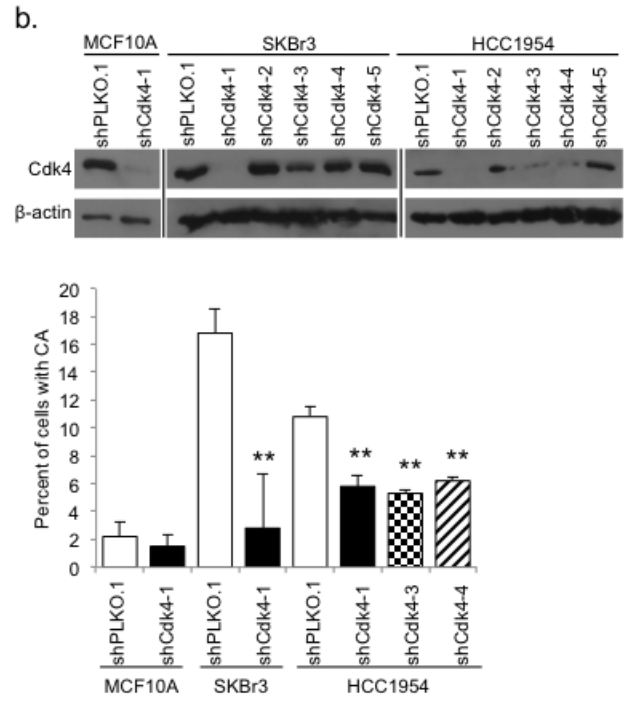
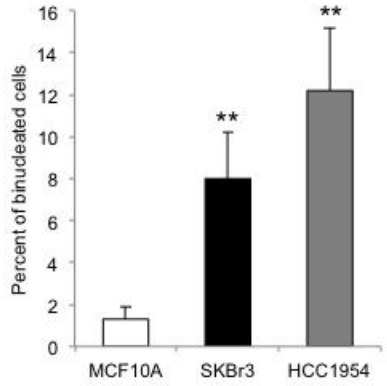
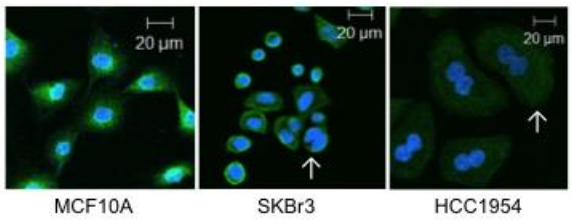

b.
shPLKO.1
shCdk4-1
shCdk4-2
shCdk4-3
shCdk4-4
shCdk4-5
Cdk4
β-actin
Percent of cells with CA
MCF10A
SKBr3
HCC1954

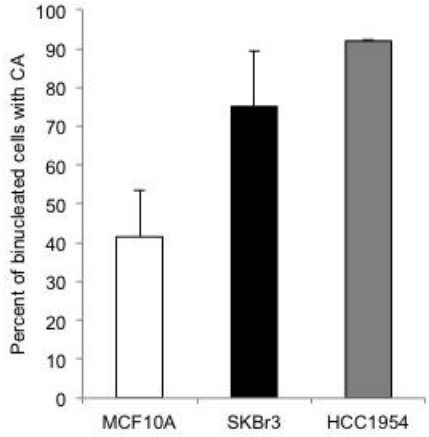
Figure 2. Centrosome amplification in Her2+ cells is mediated by Cdk4. **(A)** siRNAs against Cdk2 and Cdk4 were transfected into target cell lines; scrambled siRNA was used as a control. siRNA knockdown was confirmed by western blot using antibodies against Cdk2 and Cdk4; β-actin was used as a loading control. Western blot results show three separate gels; different exposures are commensurate with protein abundance. The number of centrosomes in proliferating cells was measured as described in Figure 1a. Statistical significance was addressed using a T-test (*=p≤0.05; **= p≤0.01). **(B)** Lentiviral shPLKO.1 control and shCdk4 vectors were used to infect MCF10A, SKBr3, and HCC1954 and create stable cell lines via puromycin selection. Independent lentiviral clones were screened in each cell line; knockdown was confirmed by western blot using an antibody against Cdk4; β-actin was used as a loading control. Western blot results show three separate gels; different exposures are commensurate with protein abundance. CA was measured in cell lines where knockdown was successful as described in Figure 1a. Statistical significance was addressed using a T-test (**= p≤0.01).

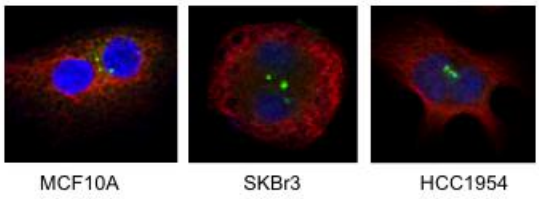
a.


Percent of binucleated cells
16
14
12
10
8
6
4
2
0
**
**
MCF10A
SKBr3
HCC1954

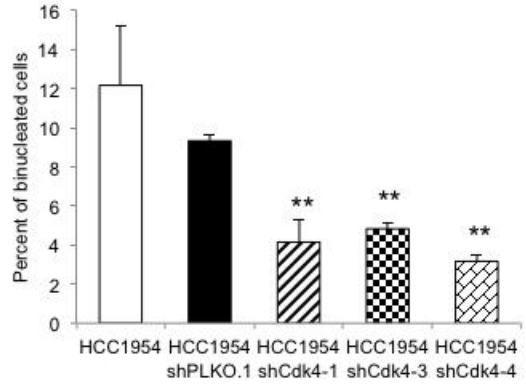

20 µm
20 µm
20 µm
MCF10A
SKBr3
HCC1954

b.


Percent of binucleated cells with CA
100
90
80
70
60
50
40
30
20
10
0
MCF10A
SKBr3
HCC1954


MCF10A
SKBr3
HCC1954

c.


Percent of binucleated cells
16
14
12
10
8
6
4
2
0
**
**
**
HCC1954
HCC1954 shPLKO.1
HCC1954 shCdk4-1
HCC1954 shCdk4-3
HCC1954 shCdk4-4

d.

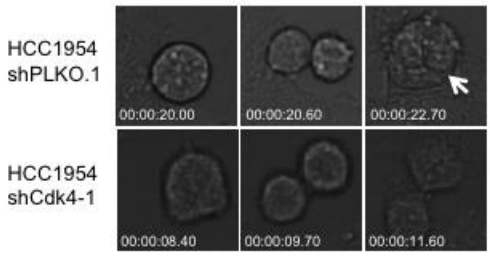

HCC1954 shPLKO.1
00:00:20.00
00:00:20.60
00:00:22.70
HCC1954 shCdk4-1
00:00:08.40
00:00:09.70
00:00:11.60

Figure 3. Her2+ breast cancer cells display elevated percentages of binucleation and cytokinesis defects. (A) Binucleation was measured in MCF10A, SKBr3, and HCC1954 parental cell lines by fixing, processing, and staining proliferating cells with an antibody against α-tubulin and counterstaining with DAPI. Arrows indicate binucleated cells. Independent experiments were done three times using 200 cells per experiment. Graphs show the percent of binucleated cells. Statistical significance was addressed using a T-test (*=p≤0.05; **= p≤0.01). **(B)** The percentage of CA in binucleated cells was measured by fixing, processing, and staining proliferating cells with antibodies against pericentrin and α-tubulin and counterstaining with DAPI. Independent experiments were done two times using 200 cells per experiment. The percentage of binucleation was measured as described in **(A)**. **(C)** Binucleation was measured in HCC1954 parental, HCC1954 shPLKO.1 control, and HCC1954 shCdk4 cells as described in **(A)**. **(D)** Still panels were captured from live cell imaging video of HCC1954 shPLKO.1 and shCdk4-1 to analyze the formation of binucleates in a proliferating population. Arrow indicates a binucleate resulting from failed cytokinesis.

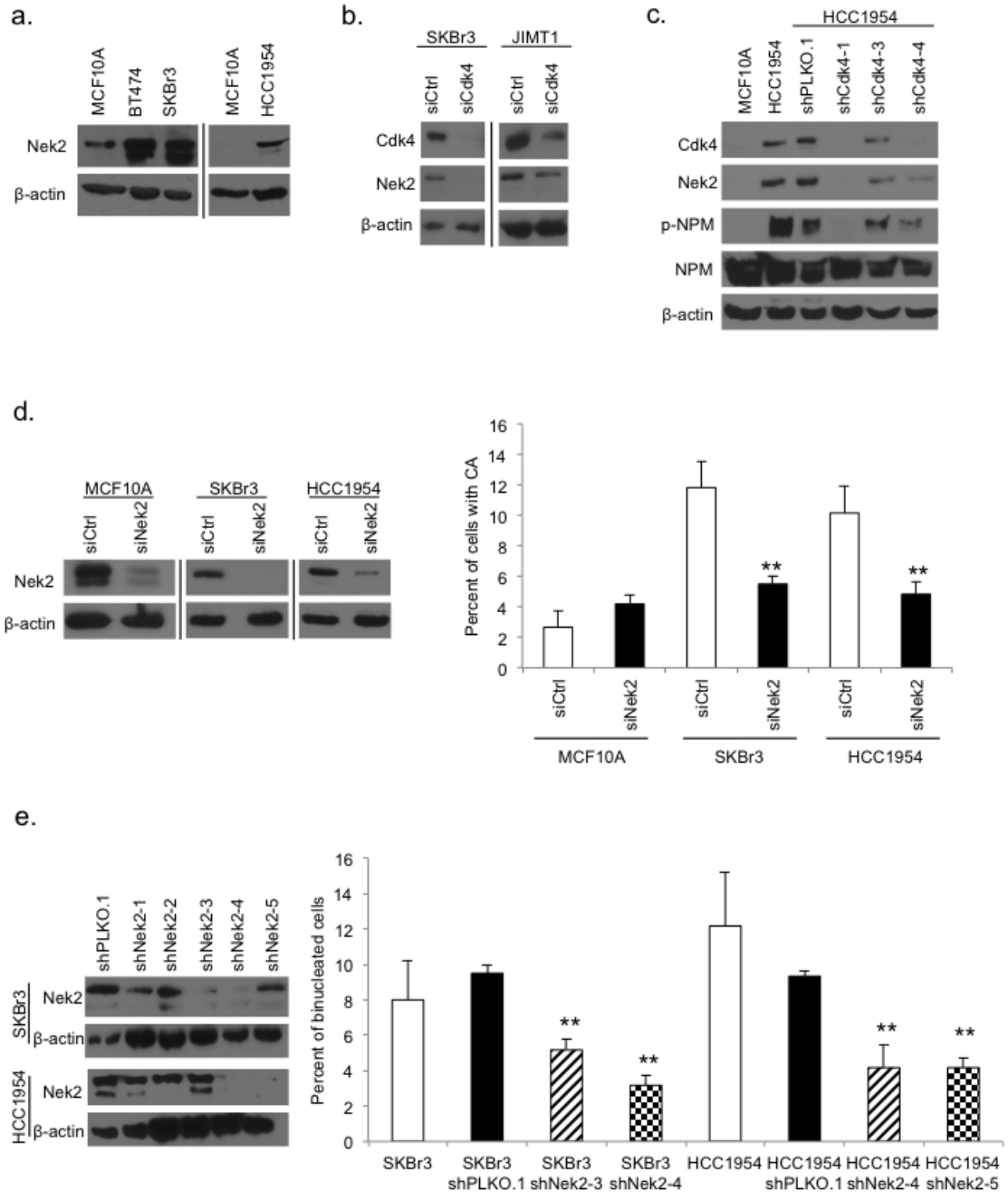
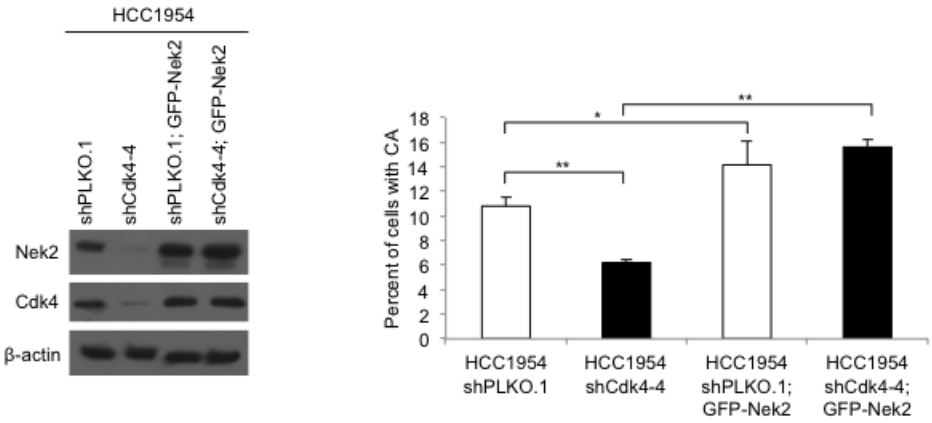

a.
MCF10A
BT474
SKBr3
MCF10A
HCC1954
Nek2
β-actin
b.
SKBr3
JIMT1
siCtrl
siCdk4
siCtrl
siCdk4
Cdk4
Nek2
β-actin
c.
HCC1954
MCF10A
HCC1954
shPLKO.1
shCdk4-1
shCdk4-3
shCdk4-4
Cdk4
Nek2
p-NPM
NPM
β-actin
d.
MCF10A
SKBr3
HCC1954
siCtrl
siNek2
siCtrl
siNek2
siCtrl
siNek2
Nek2
β-actin
Percent of cells with CA
16
14
12
10
8
6
4
2
0
**
**
siCtrl
siNek2
siCtrl
siNek2
siCtrl
siNek2
MCF10A
SKBr3
HCC1954
e.
shPLKO.1
shNek2-1
shNek2-2
shNek2-3
shNek2-4
shNek2-5
SKBr3
Nek2
β-actin
HCC1954
Nek2
β-actin
Percent of binucleated cells
16
14
12
10
8
6
4
2
0
**
**
**
**
SKBr3
SKBr3 shPLKO.1
SKBr3 shNek2-3
SKBr3 shNek2-4
HCC1954
HCC1954 shPLKO.1
HCC1954 shNek2-4
HCC1954 shNek2-5

Figure 4. Binucleation and centrosome amplification in Her2+ cells are mediated by Nek2. (A) Protein lysates from Figure 2a were used in western blots to detect levels of Nek2 in MCF10A and Her2+ breast cancer cell lines; β-actin was used as a loading control. Western blot results show two separate gels; different exposures are commensurate with protein abundance. **(B)** Western blotting was done in lysates collected from proliferating SKBr3 and JIMT1 cells transfected with siCdk4 constructs. Antibodies against Cdk4 and Nek2 were used; β-actin was used as a loading control. Western blot results show two separate gels; different exposures are commensurate with protein abundance. **(C)** Western blotting was done in lysates collected from serum arrested HCC1954 expressing shRNAs against Cdk4. Antibodies against Cdk4, Nek2, phospho-NPM, and NPM protein were used; β-actin was used as a loading control. **(D)** Transient transfection of siNek2 was performed in target cell lines; scrambled siRNA was used as a control. Knockdown was determined by western blotting using an antibody against Nek2; β-actin was used as a loading control. CA was measured as described in Figure 1a. Western blot results show two separate gels; different exposures are commensurate with protein abundance. **(E)** Lentiviral shPLKO.1 control and shNek2 vectors were used to infect SKBr3 and HCC1954 cells and create stable cell lines via puromycin selection. Independent lentiviral clones were screened in each cell line; knockdown was confirmed by western blot using an antibody against Nek2; β-actin was used as a loading control. The percentage of binucleation was compared in SKBr3 parental, shPLKO.1, and two independent shNek2 cell lines, and HCC1954 parental, shPLKO.1 control, and two independent shNek2 cell lines as described in Figure 3a. Statistical significance was addressed using a T-test (*=p≤0.05; **= p≤0.01).

a.


HCC1954
shPLKO.1
shCdk4-4
shPLKO.1; GFP-Nek2
shCdk4-4; GFP-Nek2
Nek2
Cdk4
β-actin
Percent of cells with CA
18
16
14
12
10
8
6
4
2
0
*
**
**
HCC1954 shPLKO.1
HCC1954 shCdk4-4
HCC1954 shPLKO.1; GFP-Nek2
HCC1954 shCdk4-4; GFP-Nek2

b.

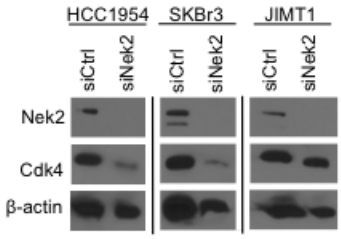

HCC1954
SKBr3
JIMT1
siCtrl
siNek2
siCtrl
siNek2
siCtrl
siNek2
Nek2
Cdk4
β-actin

Figure 5. A potential signaling loop for Cdk4 and Nek2. (A) Cells were serum starved for 72 hours, and overexpression of GFP-Nek2 plasmid was confirmed by western blotting using antibodies against Nek2 and GFP; protein lysates were also probed with an antibody against Cdk4. The number of centrosomes was assayed as in Figure 1a. Statistical significance was addressed using a T-test (*=p≤0.05; **= p≤0.01). **(B)** Western blotting was done on protein lysates collected from proliferating cells transfected with siNek2 constructs. Knockdown was confirmed using an antibody against Nek2; membranes were then probed with an antibody against; β-actin was used as a loading control. Western blot results show three separate gels; different exposures are commensurate with protein abundance.

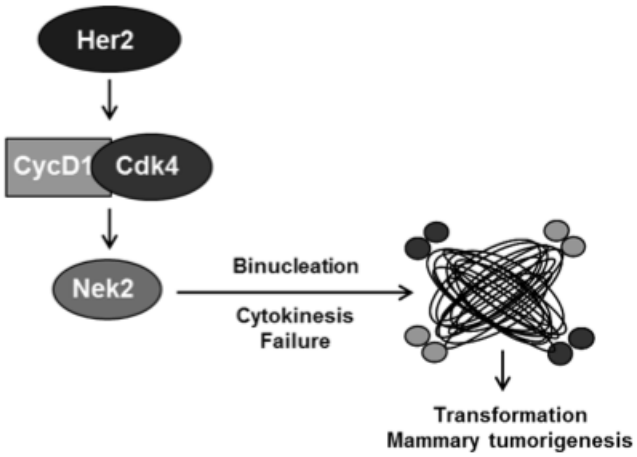

Her2
CycD1
Cdk4
Nek2
Binucleation
Cytokinesis
Failure
Transformation
Mammary tumorigenesis

Figure 6. Working model. Our working model proposes that in a Her2+ breast cancer model overexpression of cyclin D1/Cdk4 leads to an abundance of Nek2. Based on our results and the results of others, overexpression of Nek2 could drive binucleation through failed cytokinesis, leading to centrosome amplification and potentially transformation and mammary tumorigenesis.

Chapter 5

Discussion

Discussion

This dissertation is focused on the molecular mechanism of CA in a Her2 positive breast cancer model. Overall, our data indicate two kinases, Cdk4 and Nek2, working in concert, mediate CA through cytokinetic failure. We find oncogenic drivers, such as Ras (116) and Her2, are able to induce overexpression of cyclinD1/Cdk4, which in turn upregulate Nek2 levels, ultimately leading to binucleation and CA (Figure 1). In addition, we show silencing Cdk4 or Nek2 abrogates the abnormal centrosome and binucleated phenotypes. Furthermore, we identify cytokinesis failure as the mechanism effecting CA in a Her2 positive breast cancer model.

Role of Cdk4 in centrosome amplification

While the impact of cell cycle regulators on the centrosome is well understood, the role of Cdk4 as a specific effector of centrosome biology is a relatively recent one. Previously, Cdk2 was thought to be the canonical cyclin-dependent kinase involved in regulating the centrosome duplication cycle. The relative contributions of the G_1 Cdks - Cdk2 and Cdk4 - to CA in $p53^{-/-}$ MEFs, and in regulating the centrosome cycle was established in 2009 (79). While redundancy in Cdk function is well founded, this study uncovered distinct centrosome cycle defects, suggesting that the G_1 Cdk functions are potentially specific in regards to the centrosome. While Cdk2 deficiency promotes early separation and duplication of centrosomes, absence of Cdk4 promotes the accumulation of cells with one centrosome that failed to separate and duplicate.

The accumulation of cells with one centrosome in $Cdk4^{-/-}$ MEFs was not compensated with passage, as the accumulation of cells with one centrosome was also observed in MEFs silenced with siRNA directed against Cdk4.

One major consequence of deregulated oncogenes and tumor suppressor genes is the activation of G_1 cyclin-dependent kinases. Most tumors harbor centrosome abnormalities. Because of this, identifying specificity in oncogene driven CA is an important question in the field. Showing that CA precedes tumorigenesis and is oncogene-specific, we found that K-Ras^{G12D} initiated CA in mammary precursor lesions while c-Myc was unable to do so. Based on this finding, we pursued an upstream regulator of Ras, Her2, an EGFR family receptor tyrosine kinase that is overexpressed in approximately 20-30% of breast tumors. After establishing a model of CA in a panel of Her2+ breast cancer cells, we further investigated the impact of G_1 Cdks on this aberrant phenotype. By systematically knocking down Cdk2 and Cdk4 we found that Cdk4 is the strongest driver of CA in this working model. In addition, knockdown of Cdk4 does not significantly affect the cell cycle, suggesting redundancy in Cdk4's cell cycle activities, but specificity in its mediation of CA.

Each phase of the centrosome cycle is a potential target for cancer therapy. Upon inhibition of Cdk2, normal centriole duplication and cell cycle progression are maintained, while centriole overduplication is blocked. The idea that Cdk2 kinase inhibition could be used as an approach to abrogate centriole overduplication is reinforced by the study showing the CDK/GSK inhibitor, indirubin-3'-oxime (IO), was able to inhibit centriole overduplication. Perhaps

even more interestingly, use of IO caused a reduction in the steady-state population of aneuploidy cells in culture, suggesting that targeting the centrosome could reduce genomic instability (253). With this as proof-of-concept, it is also feasible to imagine a similar role for Cdk4 inhibitors. Based on the results discussed here, including the maintenance of a normal cell cycle upon knockdown of Cdk4, it is possible that the use of Cdk4 inhibitors would be able to markedly reduce CA while still allowing normal cells to proliferate as usual.

Preliminary results from in vivo experiments revealed CA in the pre-malignant breast tissue of MMTV-Neu female mice compared to control litter-mates. Crossing the MMTV-Neu mouse to the Cdk4 heterozygous knockout mouse, creating MMTV-Neu;Cdk4(-/-) mice, allowed us to analyze the effect of Cdk4 knockdown on Her2/Neu driven CA. Results from these mammary glands suggested that loss of Cdk4 abrogates CA in MMTV-Neu mouse mammary epithelial cells. As these experiments are preliminary in nature, further experiments are necessary to investigate the role of Her2/Neu in CA in vivo.

In summary, our results suggest specificity of G_1 phase Cdk usage by distinct oncogenic signaling, with p53 requiring Cdk2 and Cdk4, and Her2 and Ras requiring cyclin D1/Cdk4/Cdk6. This led us to investigate downstream regulators of CA in breast cancer cells and identify the mechanisms driving abnormal centrosome phenotypes.

Effect of Cdk4 on centrosomal regulatory kinase, Nek2

The NEK family of protein kinases is a large group of eleven serine-threonine kinases that play an important role in cell cycle control (254). In recent years it has been established that several of the NEKs participate in the structural changes that are necessary for a cell to move from interphase into mitosis. Among these, and perhaps the best studied, is Nek2. Nek2 is cell cycle regulated, and its normal function is to be expressed and peak in S and G_2 phase. It is a critical part of the human centrosome and is known to regulate centrosome disjunction and establish the mitotic spindle (14, 17). Beyond centrosome separation, Nek2 might be involved in additional aspects of mitotic progression. The support for a role of Nek2 in chromatin condensation under the control of the MAPK pathway (255) and evidence that some vertebrate NEKs could be involved in the final stages of cell division (16) were of particular interest to us as we investigated how Cdk4 mediated CA in Her2+ breast cancer cells.

The identification of Nek2, in addition to cyclinD1/Cdk4, as K-Ras^{G12D} specific centrosome regulatory targets mediating CA in pre-malignant mammary lesions (116) led to the hypothesis that deregulated Cdk4 could potentially be influencing the high levels of Nek2 seen in Her2+ cell lines. We propose a model wherein the overabundance of Nek2 in Her2+ cells is caused by deregulated cyclin D1/Cdk4 activity and that in turn, Nek2 is required to facilitate some of the abnormal mitotic functions triggered by cyclin D1/Cdk4. While Cdk4 and Nek2 are not normally present during the same phases of the cell cycle, disruption of normal levels and timing of expression by oncogene activity could put these two

kinases in an interesting signaling loop driving aberrant cellular phenotypes. Nothing is known about the signaling feedback between Nek2 and Cdk4, this represents one of the most novel findings of this work. Considering that siRNA knockdown of each molecule has no effect on the mRNA transcript of the other, it is plausible that this feedback is based in post-translational modifications. As both Cdk4 and Nek2 are kinases, their effect on each other and other regulatory molecules is potentially due to phosphorylation. It would be interesting to see if these kinases are physically interacting with each other or through an intermediate such as E2F. Deregulated protein stability could also be a factor allowing the two kinases, active in different phases of the cell cycle, to be available for interaction with each other. Further experiments, including co-immunoprecipitation and kinase assays, would provide a better indication of how this feedback loop operates.

Binucleation and centrosome amplification, the cause and effect

Several different mechanisms have been reported to generate CA. These include, but are not limited to de novo centriole assembly, centriole reduplication, and cytokinesis failure (249). Prior to this dissertation no work had been done to identify a mechanism driving Cdk4 mediated CA. We noted a high frequency of binucleates in Her2+ cell lines and discovered that this aberrant phenotype was abrogated by the knockdown of Cdk4 or Nek2. Based on this result, we pursued cytokinesis failure as a potential mechanism for CA in this model system. Using fixed and live cell imaging we established that a fraction of proliferating Her2+

cells attempt mitosis but fail to complete cytokinesis. The Nek family of protein kinases has been previously implicated in various aspects of spindle assembly, the spindle assembly checkpoint and other facets of cytokinesis, further suggesting that a Cdk4-Nek2 signaling paradigm could signal binucleation and CA through failed cytokinesis.

Future Directions

The ultimate goal of our research is to identify the role of CA in breast tumors by addressing how oncogenic signals engage the centrosome machinery. Our initial efforts to identify centrosomal proteins that were up-regulated in a Her2-driven model of CA uncovered several interesting avenues to pursue. We identified other pathways and molecules downstream of Her2 and Ras that are involved in CA, including Plk4, phospho-NPM (a phosphorylation target of Cdk2 and Cdk4), and the E2F transcriptional activators (Figure 2). This suggests that besides the regulation of Nek2 by Cdk4, presented in this dissertation, there are alternative pathways to CA. Pathways dependent on the phosphorylation of NPM and the overexpression of E2Fs (which lie downstream of Cdk4) pose interesting scientific questions and are being investigated.

E2F mediated centrosome amplification

Cdk4 drives a well-studied and extensive transcriptional program including, but not limited to a complicated schedule of E2F activators. In addition to their canonical roles regulating the cell cycle and apoptosis, the E2Fs may

influence the centrosome cycle. While targets upstream of these transcriptional activators have been established as mediators of centrosome biology, the role of E2Fs is unclear. As established downstream targets of cyclin-dependent kinases it is feasible for E2Fs to be responsible for mediating the effects of Cdks on the centrosome (Figure 2). Preliminary data shows that E2F expression is deregulated in Her2+ breast cancer cells that harbor CA, and that E2F knockdowns decrease the frequency of CA without affecting cell proliferation. Knockdown of E2F1 and E2F3 suppress CA in Her2+ cells, while their overexpression elevate CA. Consistent with my results, silencing E2Fs decrease Nek2 protein levels, while their overexpression increases those levels. This suggests that one mode of centrosomal regulation by Cdk4 is through influencing E2F-dependent expression of Nek2. Better understanding of the targets of E2F transcription factors as they pertain to the centrosome duplication cycle would add valuable information about the signaling cascade driving CA.

A role for Plk4 and Nek2 in centrosome amplification, aneuploidy, and resistance to radiotherapy

In addition to the work presented here establishing Nek2 as a major player in CA, preliminary work has identified elevated basal protein levels of the centrosome regulatory kinase Plk4 in Her2+ER-PR-breast cancer cells displaying CA. Additional studies in this subtype of human breast cancer cells showed that Cdk4 and Nek2 mediate CA and tetraploidy, with Nek2 signaling downstream of Cdk4. Also, Cdk4 can mediate radioresistance in breast cancer cells. This is of

clinical relevance because Nek2 and Plk4 are part of a signature comprising sixteen mitotic and centrosomal kinases that distinguish low prognosis basal from luminal breast cancers.

Since Nek2 and Plk4 regulate two crucial steps in the centrosome cycle: centrosome/centriole separation and duplication, respectively, it is conceivable that they may cooperate in affecting CA. In turn, CA may generate the aneuploidy that drives transformation and radioresistance. In addition, preliminary data and reports from the literature indicate the overexpression of Nek2 and Plk4 in breast cancer cells with CA and in basal breast cancers. Based on that evidence, it could be suggested that Nek2 and Plk4 contribute to breast carcinogenesis and radiation resistance by generating CA and aneuploidy.

Centrosome amplification, Her2, and Herceptin Resistance

With the development of Herceptin as a treatment of Her2+ breast cancers, patient prognosis has significantly improved. However, almost half of Her2+ patients do not respond to Herceptin treatment and many responders regress. This failure in efficacy of Herceptin has driven a search for signaling cascades and molecules associated with the receptor to prevent inherent or acquired Herceptin resistance.

As presented in this dissertation, a major consequence of Her2 deregulation is the activation of the G_1 phase cyclin dependent kinases Ckd4/Cdk6 and Cdk2, which has been shown to subsequently drive CA. However, a major limitation exists here and persists in the CA field is the

question of cause or consequence. Is the presence of CA a cause or driver of carcinogenesis or does CA arise as a consequence of the accumulation of genetic alterations present in cancer? While not within the scope of the studies presented here, future work needs to address drivers of CA in a clean inducible system in vitro and in vivo.

It is imperative to first investigate CA's dependency upon Her2 using inducible systems in vitro. Next, in order to address the role of CA in Herceptin resistance, Nek2 and Cdk4, both drivers of CA in Her2+ER-PR- cells, should be investigated. In fact, our recent work shows that Her2+ER-PR- breast cancer cells that are inherently resistant to Herceptin, specifically HCC1954 and JIMT1, display CA, and Herceptin-resistant SKBr3 cells have higher frequencies of CA relative to parental Herceptin-sensitive SKBr3 cells. This latest result suggests a close relationship between CA and Herceptin resistance. Pursuing these targets within the framework of Herceptin resistance could lead to the discovery of a predictive marker of resistance and a therapeutic target.

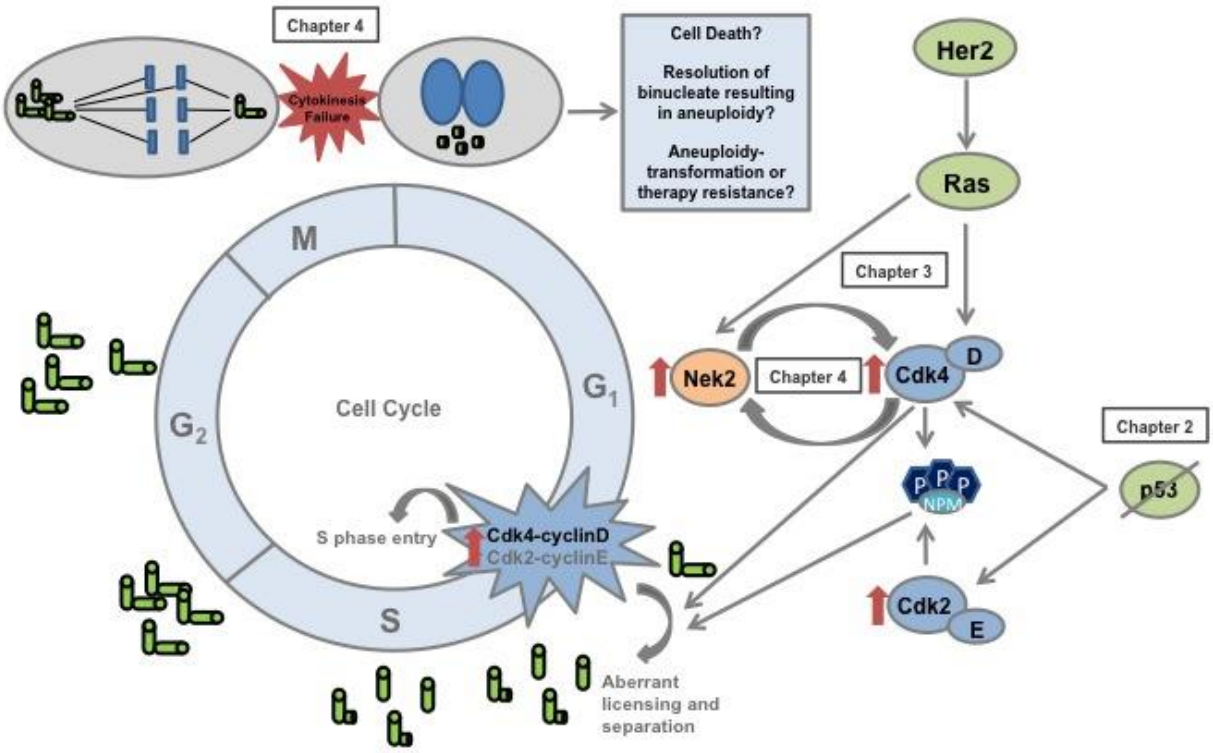


Figure 1. A model detailing the conclusions of this dissertation. Our model depicts the conclusions drawn in Chapters 2, 3, and 4 of this dissertation. Based on our studies and the work of others we find that deregulated tumor suppressors (p53) and oncogenes (Her, Ras) affect the centrosome cycle through Cdk2/Cdk4 and Cyclin D1/Cdk4, respectively. We also propose that the mechanism behind Her2 driven centrosome amplification is binucleation caused, in part, by overexpression of Nek2, a centrosomal kinase.

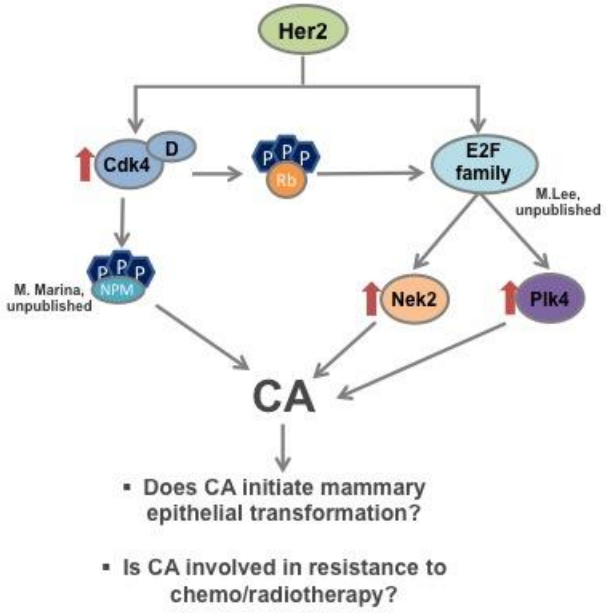


Figure 2. A schematic of future directions. Future directions based on the findings presented in this dissertation include understanding the role of hyper-phosphorylated NPM and the role of the E2F family in regulating important centrosome regulatory molecules.

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