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Cks1 mediates the effects of mutant p53 proteins on the mitotic spindle cell cycle checkpoint

Review Article

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Summary

The p53 tumor suppressor is the most frequently mutated gene in human cancer. Alteration of the p53 locus predisposes human cells to chromosomal instability. This is due in part to interference of mutant p53 proteins with the activity of the mitotic spindle and postmitotic cell cycle checkpoints. Recent data indicates that mutant p53 proteins affects the control of the metaphase-to-anaphase transition by up-regulating the expression of Cks1, a protein that mediates the activatory phosphorylation of the anaphase promoting complex (APC) by Cdc2. Cells carrying mutant p53 proteins overexpress Cks1 and are unable to sustain APC inactivation and mitotic arrest. These data implicate Cks1 in the onset of chromosomal instability in cells carrying mutant p53 proteins.

I. Introduction

It has been hypothesized that the genesis of cancer is a multistep process that requires a normal cell to undergo a series of changes in order to progress to a tumorigenic state (Bishop 1987). It has been also hypothesized that both, the generation of new cellular variants and their progression to neoplasia, are originated by the acquisition of genomic instability (Nowell 1976). The hypothesis that loss of genomic integrity fuels tumor progression is largely supported by karyotypic analyses of human tumors (Nowell 1986; Wolman 1983), studies of the loss of tumor suppressor function in cell culture (Livingstone et al., 1992; Yin et al., 1992; White et al., 1994; Xiong et al., 1996) and by numerous studies conducted using transgenic animal models to disrupt genes identified as playing key roles in tumor initiation and/or progression (Donehower et al., 1992; Harvey et al., 1992; Jacks et al., 1992; Moser et al.,1993; Moser et al., 1990; Nakayama et al., 1996; Jacks et al., 1994; Williams et al., 1994; Barlow et al., 1996; Elson A et al., 1996; Kinzler and Vogelstein 1996; Baross-Francis et al., 1998; Wang et al., 1998; Kamijo et al.,1999; Toft et al., 1999).

However, despite intense investigation, the molecular mechanisms that determine why normal cells go awry remain to be elucidated. Research has led to the hypothesis that in preneoplastic cells, loss of fidelity in processes which replicate, repair, and segregate the genome allow for the accumulation of the genetic alterations that eventually lead to a malignant phenotype (Hartwell and Kastan 1994). Research addressing the relationship between cell cycle control and the maintenance of genomic integrity in human cells is still in its infancy. Experimental evidence in both yeast and mammals demonstrates that the transition from one phase of the cell cycle to the next depends critically on completion of the previous phase. Superimposed upon these transitions are cell cycle checkpoint pathways (Elledge 1996). These checkpoints oversee cell cycle transitions thus playing a crucial role in the maintenance of genomic integrity by integrating the cell cycle regulatory machinery with DNA repair and cell death pathways (Tlsty et al., 1995). The first set of checkpoints occurs at the G_1/S transition and results in a delay in the progression from G_1 into S phase preventing the replication of damaged DNA (Weinert 1998). A second set of checkpoints associated with DNA replication and repair activities may occur during S phase (Foiani et al., 1998; Boddy et al., 1998; Lindsay et al., 1998). Importantly, the dependence of mitosis on the completion of DNA replication in the cell cycle ensures that chromosome segregation takes place only after the genome has been fully replicated (Agarwal et al., 1998;

Michael and Newport 1998). A third set of checkpoints is located at M phase and the G_2/M transition and results in a delay in the progression into and out of mitosis (Minshull et al., 1994; Murray 1995).

At mitosis the proper segregation of chromosomes requires the execution of a number of processes: assembly of a bipolar spindle; attachment of chromosomes to the spindle through the kinetochore; and alignment of chromosomes at the metaphase plate. The mitotic spindle checkpoint exerts its function by preventing the onset of anaphase, the actual segregation of chromosomes, until all these events have been properly completed (DeWald et al., 1994). In addition, cells with anomalous mitoses that escape the control of the mitotic checkpoint may be growth arrested (Stewart et al. 1999) or destroyed (Lanni and Jacks 1998) at a tetraploid G_1 phase by the postmitotic checkpoint, a DNA damage-responsive pathway which is illustrated in Figure 1. Therefore, abrogation of the mitotic spindle cell cycle checkpoint results in cell death and/or aneuploidy, a hallmark of tumor progression (Cahill et al., 1998).

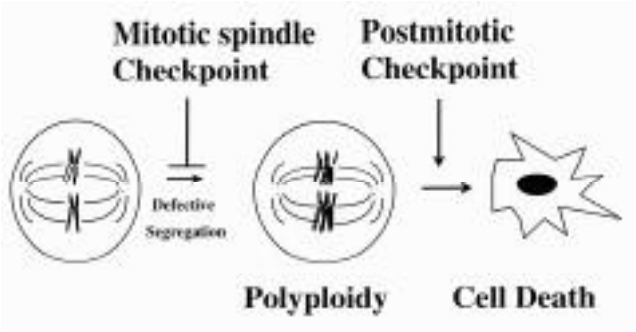

Fig. 1: Cells are protected from polyploidy by the activity of the mitotic spindle and postmitotic cell cycle checkpoints.

Commonly referred to as the "cellular gatekeeper" (Levine 1997), the p53 gene is the most frequently mutated gene in human cancer (Hollstein et al., 1994) and has been extensively studied as a key cell cycle checkpoint gene at the G_1/S transition of the cell cycle (Levine 1997). Recent reports have also suggested a role for the p53 tumor suppressor gene product at the mitotic spindle cell cycle checkpoint. For example, primary fibroblasts isolated from Li-Fraumeni syndrome (LFS) individuals, who are born with heterozygous mutations in the p53 tumor suppressor gene (Malkin et al., 1990; Srivastava et al., 1990), have a marked tendency to become heteroploid in culture (Bischoff et al., 1990). Heteroploidy is also commonly observed in p53 null mouse cells *in vitro* (Harper et al., 1993), and in p53 knockout mice (Cross et al., 1995). Likewise the expression of mutant p53 proteins in human colon carcinoma cells and murine cell lines causes chromosomal abnormalities including increased ploidy levels during growth in culture (Agapova et al., 1996). Intriguingly, the overexpression of a mutant p53 protein on a p53 null background accelerated the polyploidy in a myelomonocytic cell line (Peled et al., 1996). Moreover, LFS fibroblasts that carry structural dominant p53 mutant proteins progress quickly to polyploidy when incubated in the presence of mitotic spindle inhibitors such as colcemid (Gualberto et al., 1998).

II. Discussion

The altered mitotic spindle cell cycle checkpoint status generated by structural p53 mutant proteins is a gain of function property that can not be explained by the loss of wild type p53 function. As stated, we have shown that LFS fibroblasts that carry structural dominant p53 mutant protein progress quickly to polyploidy when incubated in the presence of mitotic spindle inhibitors such as colcemid (Gualberto et al. 1998). In contrast, normal human fibroblasts, p53 null LFS fibroblasts, or NHFs carrying the human papilloma virus 16E6 (HPV16 E6) that binds to and promotes the degradation of wild type p53 exhibit growth arrest in response to mitotic inhibitors (Gualberto et al. 1998). Furthermore, Jacks and coworkers recently reported that p53 null mouse fibroblasts have a normal mitotic checkpoint (Lanni and Jacks, 1998). Contrary to these results, polyploidy has been reported by others in HPV16 E6-expressing NHFs and in p53 null mouse embryo fibroblasts (Cross et al., 199s; Di Leonardo et al., 1997). The reason for these apparently contradictory results may be in the inherent differences in the mechanisms that underlie the onset of aneuploidy in p53 null and mutant p53-expressing cell types. Importantly, we and others have demonstrated that the mitotic cell cycle spindle checkpoint is transient in p53 null cells (Hixon et al., 1998; Lanni and Jacks et al., 1998). Therefore, even cells with active mitotic checkpoint status may become polyploid due to inactivation of a p53-dependent postmitotic checkpoint (Minn et al., 1996; Di Leonardo et al., 1997; and Lanni and Jacks 1998). This is illustrated in **Figure 2**. In response to an anomalous chromosomal segregation, normal cells (p53 +/+) delay transiently the exit from mitosis (mitotic spindle checkpoint). P53 +/+ cells with non-segregated chromosomes may eventually re-enter the cell cycle but are destroyed at the subsequent G1 phase (postmitotic checkpoint-dependent apoptosis). Alternatively, normal cells may be growth arrested at a tetraploid G1 stage (Stewart et al., 1999). Loss of p53 (p53 -/-) abrogates the postmitotic cell cycle checkpoint whereas gain of a dominant p53 mutant (p53 +/mut) abrogates both the mitotic and postmitotic checkpoints. Loss of p53 function, either by loss of p53 (p53 -/-) or mutant p53 dominance (p53 +/mut), may then originate aneuploidy due to centrosome amplification (Fukasawa et al. 1996) and multipolar division. In summary, loss of wt p53 may result in aneuploidy due to lack of a postmitotic checkpoint activity; whereas, a structural dominant mutant of the p53 protein may abrogate both the mitotic checkpoint (gain-of-function) and the postmitotic checkpoint (dominant negative).

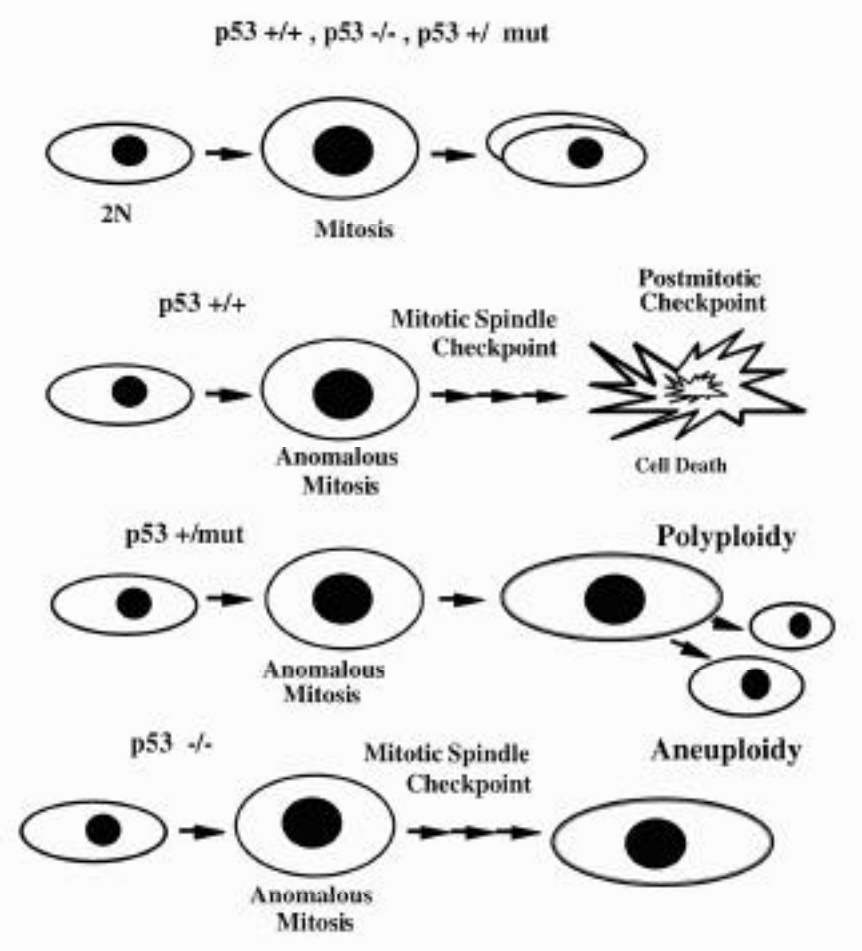

Fig. 2: In response to an anomalous chromosomal segregation, normal cells (p53 +/+) delay transiently the exit from mitosis (mitotic spindle checkpoint). P53 +/+ cells with non-segregated chromosomes may eventually re-enter the cell cycle but are destroyed at the subsequent G1 phase (postmitotic checkpoint, an apoptotic pathway). Loss of p53 (p53 -/-) abrogates the postmitotic cell cycle checkpoint whereas gain of a dominant p53 mutant (p53 +/mut) abrogates both the mitotic and postmitotic checkpoints. Loss of p53 function, either by loss of p53 (p53 -/-) or mutant p53 dominance (p53 +/mut), may then originate aneuploidy due to centrosome amplification (Fukasawa et al. 1996) and multipolar division.

In spite these results, questions still remain as to how p53 mutant proteins affect the control of mitosis. Entry into M phase requires MPF activation, a process that depends upon an increase in cyclin B expression and the dephosphorylation of cdc2. Progression through mitosis and cytokinesis requires the subsequent inactivation of MPF, which depends in part on cyclin B degradation. Experiments in yeast indicate that the mitotic spindle cell cycle checkpoint feeds into the cell cycle regulatory machinery at mitosis by a pathway that delays the degradation of cyclin B and maintains cdc2 kinase activity (Basi and Draetta 1995). Thus cyclin B is degraded and MPF inactivated only after certain aspects of mitosis related to spindle assembly and disassembly have been properly completed. Evidence indicates that mutant p53 proteins render human cells unable to control cyclin B metabolism in response to mitotic spindle depolymerizing agents (Hixon et al., 1998). Important, loss of wild type p53 does not duplicate this effect (Hixon et al., 1998). An experiment illustrating the differences in the control of cyclin B metabolism in mutant p53 cells versus cells with inactivated wild type p53 is shown in **Figure 3**. Using the retroviral vectors LXSN or pBabe we expressed the structural p53 mutant 143A or the human papilloma virus protein 16 E6 (HPV16 E6) in primary human fibroblasts. As a control, cell were also coinfected with empty LXSN or pBabe. HPV16 E6 promoted the selective degradation of wild type p53 but not mutant p53. Therefore, NHF pBabe p53 143A/LXSN-E6 showed a dramatic decrease in wild type p53 levels but expressed similar levels of mutant p53 protein to those found in the NHF pBabe p53 143A/LXSN population (**Fig. 3A,B**). An 80-hour delay in cyclin B metabolism was observed in NHFs exposed to colcemid (**Fig. 3C**). Similar to the NHFs,

the NHF16 E6-expressing cell population also delayed cyclin B metabolism in response to colcemid (**Figure 3C** and **D**). In contrast, the cell population expressing E6 and p53 143A showed a short mitotic pause, approximately 16 hours, in response to colcemid (**Figure 3E**). These results demonstrate that expression of a mutant p53 protein, but not wild type p53 inactivation, abrogates the ability of normal cells to regulate the metabolism of cyclin B protein in response to an anomalous chromosomal segregation.

Unfortunately, little is known about the molecular events that constitute the mitotic spindle cell cycle checkpoint pathway in normal human cells. However, biochemical and genetic evidence demonstrates that mitotic checkpoint signals, originated in part by the association of MAD2 proteins to unattached kinetochores (Chen et al., 1996; Li and Benezra 1996; Pangilinan and Spencer 1996), are transduced to modulate cyclin B metabolism and MPF activity (Murray et al., 1996). MAD2 has been shown to be associated with the cdc27 component of the anaphase promoting complex (APC), a protease that targets Cyclin B and other mitotic targets, inhibiting its proteolytic activity (Fang et al., 1998; Hwang et al., 1998; Wasserman and Benezra 1998). More exactly, cdc27-APC is activated by MPF in a reaction that requires its association with the Cdc2-Cks1 complex (Patra and Dunphy 1998) (**Figure 4**).

Numerous studies indicate that the level of Cks1 or homologous proteins is rate limiting in Cyclin B degradation (Moreno et al., 1989; Basi and Draetta 1995b; Patra and Dunphy 1996; Hixon et al., 1998; Patra and Dunphy 1998). Human Cks1, also called CksHs1 for Cdc2/Cdc28 kinase subunit Homo sapiens 1 (Richardson et al.,1990), was initially identified as a homologue of the Cdc28/Cdc2- associated proteins of *S. cerevisiae*, Cks1, and S. *pombe*, Suc 1 (Richardson et al., 1990). Importantly, in S. pombe, inactivation of the suc 1 gene causes mitotic arrest with high levels of cdc13 (Cyclin B homologue) and high MPF kinase activity (Moreno et al., 1989; Basi and Draetta 1995b). To further elucidate the effect(s) of mutant p53 protein on mitosis we investigated how the presence of mutant p53 proteins could affect Cks1 functions (Hixon et al., 1998). We found that normal human fibroblasts downregulate CksHs1 expression in response to mitotic inhibition (**Figure 5A**). In contrast, human fibroblasts carrying mutant p53 proteins expressed higher levels of CKsHs1 and failed to downregulate CksHs1 expression in response to mitotic spindle depolymerization (**Figure 5B**). More importantly, ectopic expression of CKsHs1 originated altered cell cycle checkpoint status (**Figure 5C**). These results identified CksHs1 as a mutant p53 gene target and provide a molecular mechanism to understand the origin of heteroploidy in mutant p53 expressing cells. However, many details remain to be elucidated. It appears that CksHs1 induces aneuploidy by interfering with the control of cyclin B metabolism by the mitotic spindle checkpoint (Hixon et al., 1998). However, whether mutant p53 directly regulates Cks1 or affects its control through interaction with other p53-related proteins warrants further study. In addition, CKsHs1 could be one of many mutant p53 targets at mitosis.

How could mutant p53 protein affect the expression of mitotic regulators that are not wild type p53 targets? Mutant p53 proteins may directly activate gene expression. Several reports have indicated gain of function transcriptional properties for p53 mutants (Suber et al., 1992; Dittmer et al., 1993; Gualberto et al., 1995; Lin et al., 1995; Frazier et al., 1998). Also, it has been postulated that mutant p53 proteins may mimic the biological function of a "proliferative" conformational stage of wild-type p53 (Milner and Watson 1990; Ullrich et al., 1992). This proliferative p53 may work in association with other transcription factors, such as Sp1 (Gualberto and Baldwin 1995). Alternatively, mutant p53 could affect the expression of CKsHs1 and other mitotic proteins indirectly, affecting the levels of its regulators. Finally, recent data (Di Como et al., 1999 Ruiz-Lozano et al., 1999) suggest an additional molecular mechanism that could explain some of the gain of function properties of mutant p53 proteins. These authors demonstrate that the dominant negative activity of mutant p53 can be exerted not only over wild type p53, but also over other p53-related proteins, such as p51 and p73. Thus, certain differences between p53 mutant and p53 null phenotypes could be due to the ability of p53 related proteins to compensate for the lack of p53 function in the latter.

In conclusion, loss of fidelity in the processes that replicate, repair, and segregate the genome may allow for the accumulation of the genetic alterations that eventually lead to a malignant phenotype. These processes are integrated with the cell cycle regulatory machinery by cell cycle checkpoint pathways. Loss or inactivation of cell cycle checkpoint genes, such as the p53, results in genomic instability and tumor progression. In addition, mutant p53 proteins may contribute to genomic instability by mechanisms that do not necessarily imply the inactivation of wild type p53. At mitosis, structural p53 mutants, but not loss of wild type p53 function, cause altered mitotic spindle cell cycle checkpoint status. This altered checkpoint status is originated, at least in part, by the upregulation of Cks1, a cofactor of MPF that targets cdc2 to the APC. In normal cells, mitotic checkpoint signals delay the progression of mitosis by inhibiting the activity of the APC through a dual mechanism, MAD2 association to APC components and dowregulation of Cks1. Overexpression of Cks1 abrogates the mitotic spindle checkpoint reproducing the effects of mutant p53 proteins on cyclin B metabolism and the progression through mitosis. In addition, p53 dominant mutants may facilitate the survival of aneuploid cells due to inactivation of a postmitotic p53-dependent checkpoint. These data contribute to our understanding of the origin of aneuploidy in mutant p53 cells. Future experiments should focus on the upregulation of CksHs1 and other members of this protein family in human tumor initiation and progression.

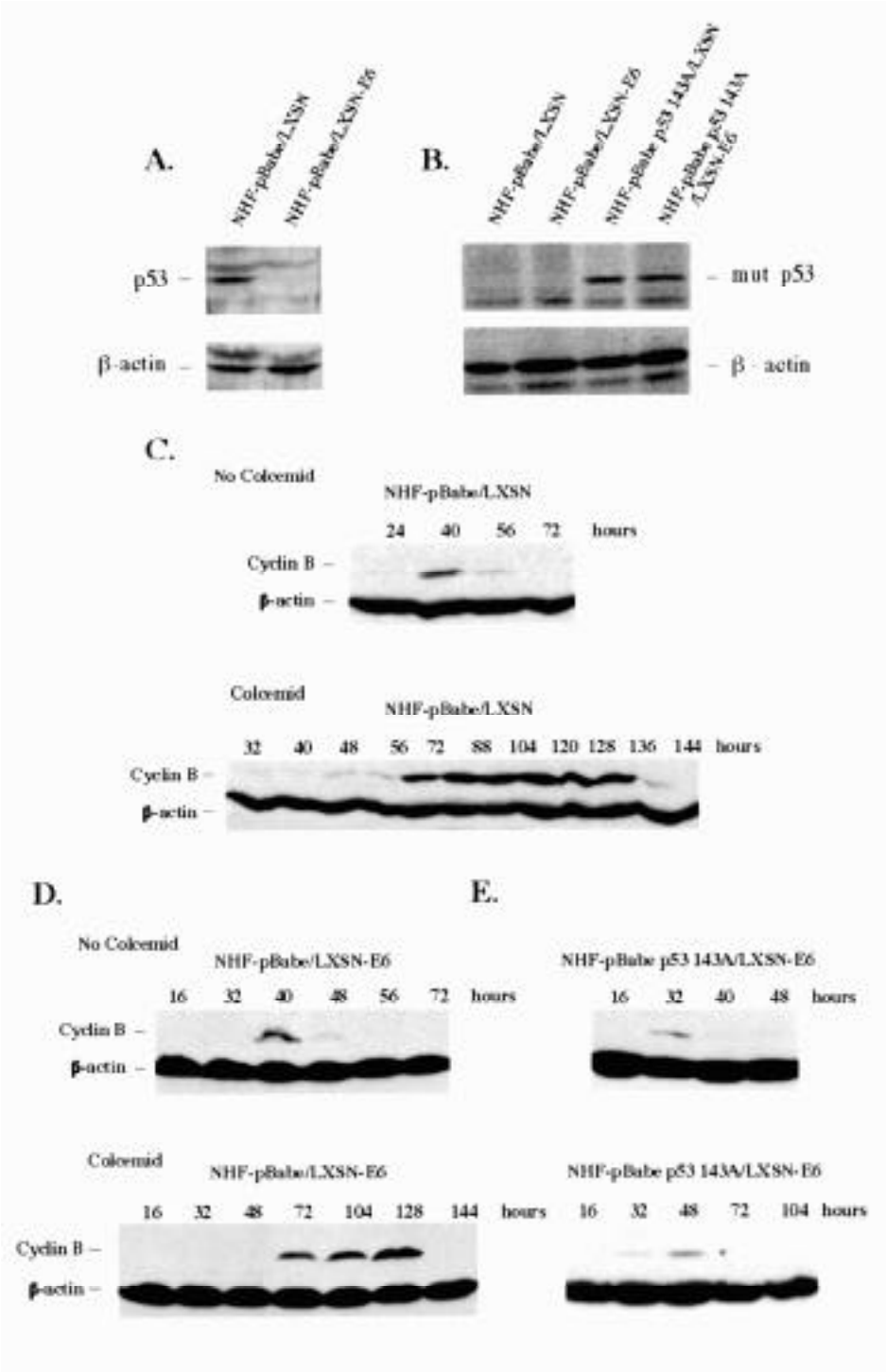

Fig. 3: (**A**) Western analysis of p53 and β-actin in NHF carrying the retroviral vectors pBabe (puromycin resistance, no insert) and LXSN (neomycin resistance, no insert) or LXSN-E6. For the induction of p53, the cells were incubated for 2 days in 10 μM mycophenolic acid (a GMP biosynthesis inhibitor). (**B**) Immunoprecipitation of mutant p53 and β-actin in primary NHF following double infection with the retroviral vector pBabe and LXSN or LXSN-E6 or with the vector pBabe p53 143A and LXSN or LXSN-E6. The cells were metabolically labeled and the proteins were analyzed (**C to E**) Western analysis of cyclin B and β-actin in NHF carrying the retroviral vectors pBabe and LXSN (**C**), pBabe and LXSN-E6 (**D**), or pBabe p53 143A and LXSN-E6 (**E**). The cells were incubated and western blotting was carried out. Data are representative of three independent experiments.

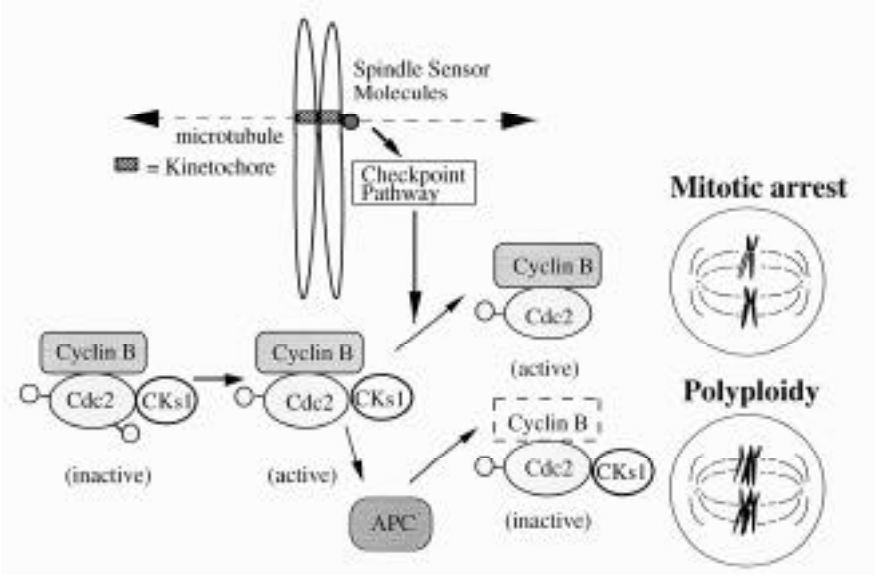

Fig. 4: Model of the mitotic spindle cell cycle checkpoint pathway. Cdc2 phosphorylates and activates the protease complex APC. Cks1 allows docking of Cdc27 onto Cdc2. Dowregulation of Cks1 expression by the mitotic spindle checkpoint pathway block APC activation and degradation of its mitotic targets such as Cyclin B.

Acknowledgments

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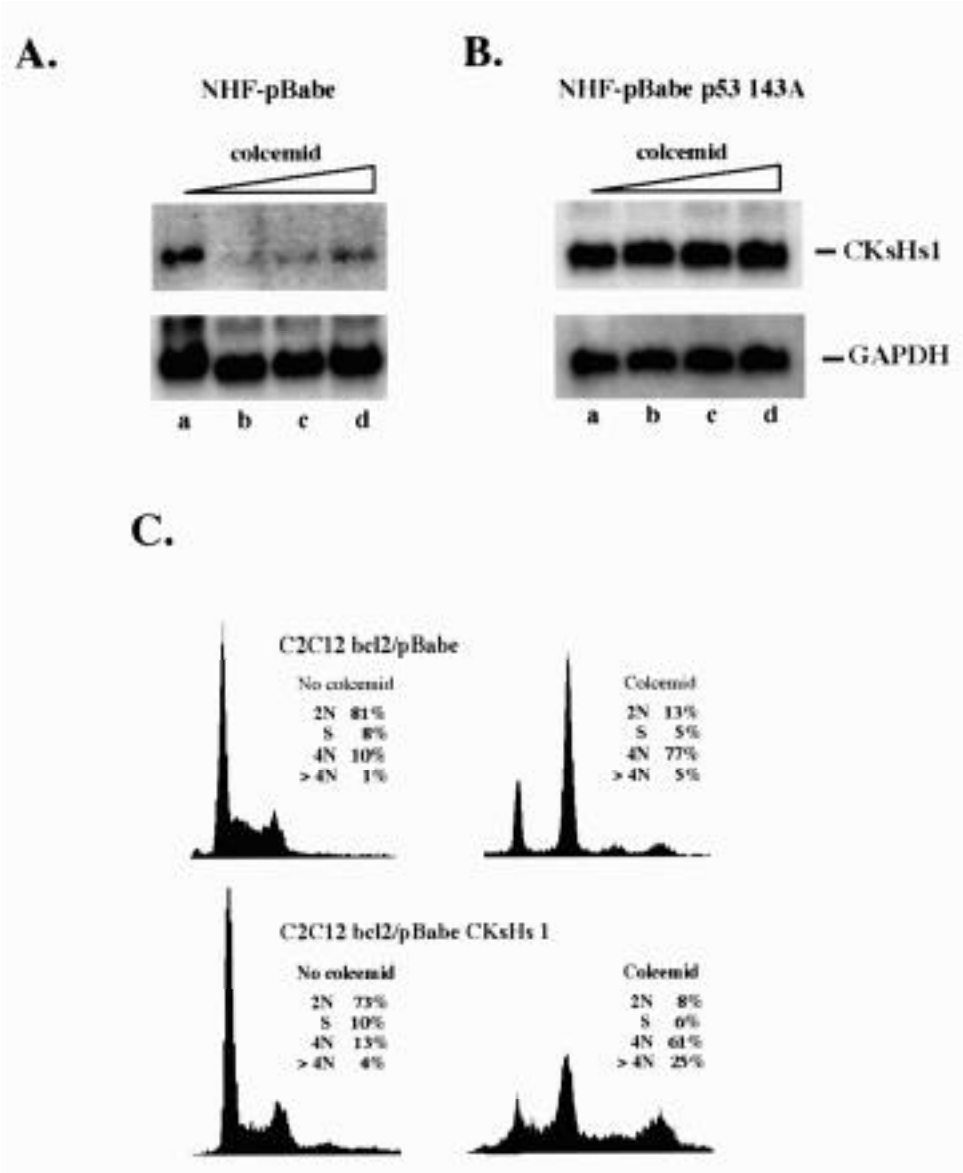

Fig. 5: Northern analysis of CKsHs1 expression in NHF-pBabe (**A**) and NHF-pBabe p53 143A (**B**) cells incubated in the presence of increasing concentrations of colcemid. Cells were synchronized at G0 by incubation in low serum media and then incubated for 40 h in 10% FBS in the presence of 0 (a), 100 (b), 200 (c), or 1000 (d) ng/ml of colcemid. Colcemid was added at 12 h after cell passage.

(**C**) Flow cytometry analysis of cell cycle distribution of DNA content in C2C12 cells ectopically expressing CKsHs1 and/or bcl-2. Cells were incubated in the absence or presence of 200 ng/ml of colcemid for 2 PDL incubation times. PDLs for C2C12 bcl2/pBabe and C2C12 bcl2/pBabe CKsHs1 cells were 38 and 32 h, respectively. Following incubations, cells were harvested and processed for flow cytometry of DNA content.

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