

**IDENTIFICATION OF THE CAUSES OF CYTOKINESIS FAILURE
IN CANCER CELLS**

by

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Tetraploidy and chromosomal instability are common phenotypes of malignant cells and cytokinesis failure is a known source of tetraploidy. However, the causes of cytokinesis failure are not yet identified. An essential step in the process of cytokinesis is phosphorylation of myosin regulatory light chain (MLC), required for actin–myosin interaction and the formation of the cleavage furrow. Our data indicate that cancer cells are deficient in MLC phosphorylation and this deficiency is the cause of cytokinesis failure. Myosin light chain kinase (MLCK) is a key enzyme that phosphorylates MLC during cytokinesis and is inhibited in cancer cells. Aurora B kinase is an essential regulator of cytokinesis that is commonly over-expressed in cancer cells and can phosphorylate MLCK *in vitro*. Therefore we hypothesize that Aurora B over-expression is the cause of MLCK inhibition in cancer cells. Consistent with our hypothesis, we demonstrate that Aurora B kinase indeed is an MLCK inhibitor *in vitro* and in cultured mammalian cells. Cytokinesis failure resulting from Aurora B over-expression can largely be suppressed by constitutively active MLCK or phosphomimetic MLC and reducing protein levels of Aurora B in cancer cells increases MLCK activity and decreases cytokinesis failure. These data thus describe a novel pathway that drives Aurora B induced cytokinesis failure.

Cytokinesis failure is often observed in only a subset of cancer cells but the trigger for this divisional failure in that subset is unknown. One strong possibility is the presence of lingering chromatin at the cleavage site that has been previously proposed to block cytokinesis

completion. However, the mechanism linking lingering chromatin and cytokinesis failure is still a mystery. In this study we demonstrate that lingering chromatin causes cytokinesis failure by inducing over-expression of Aurora B and the resultant inhibition of MLCK and phosphorylated MLC. Together, my results define a novel pathway for Aurora B mediated regulation of cytokinesis and contribute to our understanding of the genomic destabilizing events of tumorigenesis.

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PREFACE

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1.0 INTRODUCTION

Every time a cell divides, it separates its duplicated genome equally amongst the two daughter cells. Aberrant cell division can result in unequal distribution of the genome, forming daughter cells with a deviation from the diploid number of chromosomes and any changes in the ploidy of the cells can have serious consequences such as development of cancer. Cancer has been interpreted as a complex Darwinian adaptive system that slowly evolves through natural selection of cellular clones (Greaves and Maley, 2012). The classical 'clonal evolution' model of cancer suggests that a tumor evolves by stepwise accumulation of somatic mutations and clonal expansion of the selective mutations (Nowell, 1976). This model describes that a progenitor cell that has acquired mutations provides selective growth advantages and initiates cancer. The resultant neoplastic clones expand either immediately or after a prolonged latent period. Genetic instability in the tumor population gives rise to additional clones with even more favorable mutations that expand further. The tumor thus evolves gradually over a prolonged period of time ranging over many years. A recent evidence for the presence of large-scale punctuated changes in cancer cells, driven by sudden catastrophic mitotic events that results in multiple genomic rearrangements, have argued that 'punctuated equilibrium' can also contribute to cancer evolution along with gradualism (Stephens et al., 2011). Changes in the ploidy of the cells, specifically whole genome duplication, has played an important role during speciation (Semon and Wolfe, 2007). A recent high throughput analysis of cancer genomes indicates that genome

doubling events occur commonly during the evolution of cancer genome as well (Carter et al., 2012). Change in the ploidy of a cell can be a source for both these mutational changes, ones that are acquired gradually over a prolonged period of time or the ones that are acquired in a shorter time frame via sudden punctuated changes, over the course of cancer evolution. Therefore, understanding the causes and consequences of changes in ploidy is an active and important area of cancer research.

1.1 TETRAPLOIDY

Polyploidy refers to a state of having greater than diploid ($2N$) number of chromosomes. Tetraploidy is a polyloid condition characterized by double ($4N$) the normal complement of chromosomes. Polyloid cells are frequently observed in plants (Galbraith et al., 1991). Surprisingly, even in mammals, occurrence of physiologically normal polyploidy, most commonly tetraploidy, is not a rare phenomenon. Megakaryocytes, the precursors of platelets, or cells of the mammalian placenta called trophoblasts, become polyloid as a part of their developmental program (Ravid et al., 2002; Zybina and Zybina, 2005). In adult rats, around 70% of hepatocytes are tetraploid, whereas in humans around 20-30 % of hepatocytes are polyploidy or tetraploid when they terminally differentiate (Guidotti et al., 2003). Other than liver, polyloid cells are also observed in many other tissues such as brain, urothelium, mesothelium, normal and lactating breast, to name a few. The presence of polyloid cells in such a variety of tissues under normal conditions has thought to be a part of differentiation or functional adaptation (Biesterfeld et al., 1994).

Polyploidy can be advantageous under certain circumstances. For example, polyploidy has played an important role during the evolution of eukaryotes (Otto, 2007). Tetraploidy is also observed during some types of cellular stress responses. For example, liver cells or hepatocytes regenerated after exposure to toxic drugs or after partial hepatectomy are predominantly polyploid (Fausto and Campbell, 2003). Heart muscle and vascular smooth muscle cells that are diploid under normal conditions become polyploidy during hypertension. Polyploid fibroblast have also been observed during wound healing and finally polyploidy is often correlated with senescence and aging (Storchova and Pellman, 2004). The presence of tetraploids during a cellular stress response can be advantageous as it can help tissues to switch to an energy saving mode by regulating organ size and increasing the production of ATP to confer metabolic benefits (Anatskaya and Vinogradov, 2010). Polyploidy can also protect against genotoxic stress by increasing the gene copy number allowing enhanced expression of key genes important for the stress response (Storchova and Pellman, 2004). This can be specifically important for tissues such as liver that primarily function in metabolizing toxic products.

1.1.1 Tetraploidy induced tumorigenesis

Although polyploidy can be advantageous under certain circumstances, unscheduled polyploidy comes with a cost. Germline polyploidy is often lethal and accounts for 5 to 10% of spontaneous abortions (Vitale et al., 2010). Perhaps one of the major disadvantages of somatic polyploidy or tetraploidy from a human health perspective is that it acts as a trigger for cell transformation and tumorigenesis (Ganem et al., 2007; Storchova and Kuffer, 2008; Storchova and Pellman, 2004; Vitale et al., 2010). Several lines of evidence suggest that tetraploids are transient intermediates

during tumorigenesis. In a variety of rodent cell lines cultured *in vitro*, development of tetraploidy or hypertetraploidy has been observed to accompany spontaneous neoplastic transformation (Shackney et al., 1989). Tetraploid cells have been observed in early stages of diverse tumor types. In a premalignant esophageal cancer, called Barrett's esophagus, occurrence of tetraploid cells precedes appearance of aneuploid tumors and correlates with the mutational inactivation of the p53 tumor suppressor gene (Galipeau et al., 1996). Tetraploids are often observed during early stages of cervical cancer and they predispose such cells to aneuploid cancer formation, frequently involving loss of chromosome 17 (Olaharski et al., 2006). Early stages of malignant gliomas, colonic adenocarcinomas and advanced stages of bladder cancers are other examples of tumors with tetraploid cell populations (Jakobsen et al., 1983; Park et al., 1995; Takanishi et al., 1996).

While these descriptive studies suggest a role for ploidy changes in cancer, they are only a correlation and many other cellular changes accompany transformation. However more recently direct experimental studies in animal models have shown that tetraploidy is sufficient for tumor formation in mice. *p53^{-/-}* mouse mammary epithelial cells made tetraploid after treatment with the actin inhibitor dihydrocytochalasin B (DTB) form tumors roughly 25% of the time upon subcutaneous injection into nude mice. However, diploid cells injected using the same procedure do not form tumors (Fujiwara et al., 2005). This seminal work shows that tetraploidy of an otherwise normal genome is sufficient to form tumor in the mouse xenograft model.

To follow up on this observation, similar studies using mechanisms of tetraploidy that may be relevant to cancer confirmed this conclusion. For example, injection of sorted tetraploid cells formed as a result of over-expression of a downstream target of c-Myc, called as Gp1b α , commonly over-expressed in cancer cells, also form larger, faster and more aggressive tumors

upon injection into nude mice, as compared to smaller and slower growing tumors formed upon injection of diploid cells over-expressing Gp1b α (Li et al., 2008). Thus the selection of tetraploids among identical cells enhances tumorigenesis in mice. In both of these examples, the cancer cells isolated after tumor formation are aneuploid and genetically unstable, similar to human tumor cells. These experimental observations strongly demonstrate causality between tetraploidy and tumorigenesis. Thus, both the correlative studies in human patients and the experimental evidence support the hypothesis that tetraploid cells act at an early stage leading to formation of aneuploid and genetically unstable tumors.

1.1.2 Mechanism of tetraploidy induced tumorigenesis

One of the major changes that accompany polyploidy is loss of genetic stability. Tetraploid cells demonstrate scaling effects, i.e. they have double the content of DNA and double the number of centrosomes, as compared to diploid cells. Two major consequences of this scaling effect that are proposed to contribute towards tetraploidy induced tumorigenesis are (a) generation of aneuploid progeny as a result of increased frequency of chromosome mis-segregation and (b) increased frequency of DNA damage. Both outcomes are inter-related.

1.1.3 Generation of aneuploid progeny from tetraploid cells and their role in tumor development

One of the most striking features of tetraploidy induced tumors is the formation of aneuploid and chromosomally unstable tumor cells (Fujiwara et al., 2005; Li et al., 2008). Chromosomal

instability (CIN) is defined as persistently high rates of gain or loss of whole or a fraction of a chromosome during cell division. An abnormal numbers of chromosomes, either more or less than the diploid number, is defined as aneuploidy (Geigl et al., 2008; Gordon et al., 2012). Whole chromosome aneuploidy refers to the changes in the number of whole chromosome and segmental aneuploidy refers to the chromosomal rearrangement like deletion, amplifications or translocations (Geigl et al., 2008). Both of these changes are present in cancer cells obtained from tetraploid induced tumors and appear to be a consequence of tetraploidy in these cells.

CIN and aneuploidy is perhaps the most distinguishing genomic characteristic of most of the solid tumors as well as leukemias and lymphomas (Thompson and Compton, 2011). According to the Mitelman Database, a large repository of cytological data from over 60,000 human cancer cases, aneuploidy is observed in a majority of cancers. A typical solid tumor karyotype can range from 40 to 60 chromosomes, sometimes even exceeding 70 (Mitelman et al., 2012). A recent comprehensive study using high-resolution mapping of somatic copy number alterations (SCNA) in cancer cells has revealed that 25% of the cancer cell genome is affected by whole chromosome or chromosome arm level SCNA and 10% is affected by focal SCNA (Beroukhi et al., 2010). CIN is also present in the majority of the tumor cells (Lengauer et al., 1997; Thompson and Compton, 2011). Moreover, CIN and aneuploidy are interdependent and aneuploidy often arises as a result of underlying CIN (Holland and Cleveland, 2009). Conversely, aneuploidy can also drive genomic instability under certain circumstances (Sheltzer et al., 2011). In spite of the presence of a widespread aneuploidy, its precise role in tumorigenesis is still under investigation.

More than a century ago, a German scientist Theodor Boveri proposed that aneuploidy is the cause of cancer (Boveri, 1914; Manchester, 1995). Boveri observed that sea urchin embryos

developed from multipolar divisions form cancerous growths. Based on these data and the observations of German pathologist David Hanseemann of asymmetrical anaphases and telophases in epithelial tumors, Boveri proposed that tumors arise from a progenitor cell that possesses an incorrectly combined set of chromosomes (known in today's world as aneuploidy).

Since the time of Boveri, contribution of aneuploidy to tumorigenesis has been strongly debated. As four to six successive mutations are required for cancer formation (Kinzler and Vogelstein, 1996), skeptics of the aneuploidy hypothesis have argued that aneuploidy is merely a benign side effect of the process of transformation and does not play a role in driving tumor development (Zimonjic et al., 2001).

However, many yeast and mice models have provided ample of evidence for fitness benefits of aneuploidy and its causative role in tumor development. Comparison of 38 aneuploid yeast strains with their euploid counterparts demonstrates that many aneuploid strains grow significantly better under various stress conditions and drug exposure and do not acquire additional mutations (Pavelka et al., 2011). Thus, it appears that aneuploidy itself provides the enhanced growth potential and is not just a way of acquiring additional mutations. Aneuploid mouse models developed from altered expression of spindle assembly checkpoint proteins such as *Mad1*, *Mad2* and *Bub1* develop a variety of tumors ranging from benign lung tumors to lethal lymphomas, lung, liver and spleen cancers and some are prone to develop carcinogen induced tumors (Baker et al., 2009; Holland and Cleveland, 2009; Iwanaga et al., 2007; Michel et al., 2001).

Aneuploidy is observed in many premalignant conditions in humans and precedes the process of transformation (Weaver and Cleveland, 2006). The presence of aneuploidy positively correlates with more aggressive tumors and worse prognosis in the general population (Shackney

et al., 1989). Moreover, aneuploidy generated by CIN is responsible for tumor relapse and recurrence (Sotillo et al., 2010). A recessive genetic condition known as mosaic variegated aneuploidy (MVA) is characterized by mosaic aneuploidy involving different chromosomes and tissues (Tolmie et al., 1988). Children suffering from this condition develop cancer at higher frequencies (Jacquemont et al., 2002; Kajii et al., 2001). Patients of Down's syndrome, characterized by trisomy 21, have significantly increases risk of hematological malignancies (Hitzler and Zipursky, 2005). All these data provide a strong line of evidence supporting the causal role of aneuploidy in tumorigenesis.

However, aneuploidy does not always result in tumor development. In many instances aneuploidy is not compatible with whole organism viability. Children born with trisomy 13 or 18 do not survive beyond few days of life (Rasmussen et al., 2003). Mouse embryonic cell lines engineered to be trisomic for specific chromosomes display embryonic lethality (Williams et al., 2008). Surprisingly, aneuploidy also acts as a tumor suppressor under certain conditions and in certain tissues (Weaver et al., 2007).

Whole chromosome or segmental aneuploidy involves altered expression of multiple different genes and pathways, depending upon the chromosomes involved. Therefore, these paradoxical roles of aneuploidy as a tumor inducer and tumor suppressor can be explained by the differences in the chromosomes involved and the differences in the extra-cellular environment that different aneuploid cells are exposed to. Thus, although aneuploidy can be fatal in certain circumstances, it might improve fitness and provide survival benefits under specific conditions. Tetraploid cells have an additional set of the entire genome that can be specifically advantageous for the survival of aneuploid progeny as the additional chromosomes can buffer the effect of gain or loss of chromosomes that would otherwise be detrimental in the diploid genome. Consistent

with this hypothesis, tetraploid cells with CIN show near normal growth rates compared to isogenic diploid strains and can tolerate nearly 1000 fold increase in the rate of gain or loss of chromosome without major impairment of cell cycle progression (Ganem et al., 2009; Storchova et al., 2006).

Although outcomes of aneuploidy can differ under different circumstances, all aneuploid cells show similar signs of energy and proteotoxic stress (Tang et al., 2011). These common downstream effects of aneuploidy are attractive targets for developing new anti-cancer therapies. Therefore, continued efforts are warranted for the study of the causes and consequences of aneuploidy for the better understanding of the process of tumorigenesis and also for the development of new therapeutic strategies.

1.1.3.1 Chromosome segregation defects as an underlying cause of aneuploid progeny

Recent work performed using diploid and tetraploid yeast cells have demonstrated that certain alleles are required for the survival of polyploid (tetraploid) cells but not for the survival of diploid cells. This phenomenon is referred to as ploidy specific lethality (PSL) (Lin et al., 2001; Storchova et al., 2006). Interestingly all of the PLS gene products are required for maintaining genomic stability such as the genes required for proper chromosome segregation, sister chromatid cohesion or homologous recombination (Lin et al., 2001; Storchova et al., 2006). Consistent with this analysis, chromosome segregation errors occur at significantly higher rates in tetraploid cells and appear to be the primary cause of tetraploidy induced aneuploidy.

Tetraploid cells demonstrate chromosome segregation errors because of the higher frequencies of incorrect kinetochore-microtubule attachments such as merotely and syntely. Merotely is a condition characterized by the attachment of a single sister chromatid to both the

spindle poles whereas syntely is defined as the attachment of both sister chromatids to one spindle pole (Kapoor, 2004).

Tetraploidy leads to the doubling of the centrosome number as well as doubling of the genome. In mitosis, each centrosome is located at the poles of a bipolar mitotic spindle. Therefore, the presence of more than two centrosomes can result in the formation of multipolar spindles, as was observed by Boveri. Formation of multipolar spindles can then lead to an unequal distribution of chromosomes and aneuploidy. However, the progeny of multipolar divisions are frequently inviable *in vitro* (Ganem et al., 2009) and the cytoplasmic motor dynein can cluster extra centrosomes into two poles (Quintyne et al., 2005). Interestingly, it was recently demonstrated that cells with extra centrosomes often undergo bipolar division and only transiently pass through a multipolar stage (Ganem et al., 2009). But, the presence of this transient multipolar stage leads to an increased incidence of merotelic kinetochore-microtubule attachments. Segregation of merotelically attached chromosomes can lead to a tug-of-war between the microtubules emanating from the opposite spindle poles and can cause chromosomes to lag behind and mis-segregate giving rise to aneuploid daughter cells. Consistently, an increase in the centrosome numbers or centrosome amplification and formation of multipolar spindles is frequently observed in cancers (Nigg, 2006; Saunders et al., 2000) and is positively correlated with genomic instability and cancer progression (Pihan et al., 2001; Sato et al., 2001).

Another source of defective chromosome segregation often observed in tetraploid cells is syntelic kinetochore-microtubule attachment. In tetraploid cells, the cell volume increases with the genome size but the size of pre-anaphase spindle does not increase and the spindle geometry is altered (Storchova et al., 2006). This disparity in the scaling of the genome versus spindle size

leads to an increased incidence of syntelically-attached chromatids that can also lag behind and mis-segregate. In summary, tetraploid cells produce aneuploid progeny because they mis-segregate chromosomes and the resultant aneuploid progeny acts as the progenitor of tetraploidy induced tumors.

1.1.4 Increased DNA damage associated with tetraploidy and aneuploidy

Another consequence of tetraploidy generation is an increase in the DNA damage. Although the kinetics of DNA replication are unaltered in tetraploid cells, they accumulate twice the DNA damage during S-phase, probably as a consequence of genome doubling (Storchova et al., 2006). Primary tetraploid human fibroblasts display an increase in γ -H2X foci compared to their diploid counterparts, a marker for DNA damage, (Hau et al., 2006). Tetraploid yeast also shows increased sensitivity to γ -radiation and other DNA damaging agents (Mayer et al., 1992; Mortimer, 1958).

1.1.4.1 Chromosome segregation errors as a source of DNA damage

Chromosome segregation errors also act as a source of DNA damage in tetraploid cells. Mis-segregating chromosomes often lag behind other chromosomes during anaphase and become damaged if cytokinesis proceeds in the presence of uncleared chromatin at the cleavage plane. The damaged DNA then elicits DNA double strand break repair and unbalanced translocations leading to aneuploidy (Janssen et al., 2011).

Mis-segregating chromosomes such as lagging chromosomes often form micronuclei adjacent to the parent nucleus. Chromosomes present in the micronuclei undergo asynchronous

and defective DNA replication. If cells enter mitosis while micronuclear DNA is still undergoing replication, chromosome pulverization can occur causing extensive DNA damage and fragmentation of chromosomes that can integrate back into the genome (Crasta et al., 2012). Pulverization of micronuclear DNA can be a source of a recently reported DNA damaging phenomenon of cancer cells termed as ‘chromothripsis’, whereby hundreds of genomic rearrangements occur during a single catastrophic event. In contrast to a serial accumulation of tumorigenic mutations over a long period of time, events like chromothripsis lead to massive tumorigenic mutational changes in a very short period of time (Stephens et al., 2011).

Long term imaging of tetraploid cells in real time indicate that the majority of tetraploid cells do not survive beyond four to five cell divisions, independent of their p53 status (Krzywicka-Racka and Sluder, 2011). Such a short survival of tetraploid cells seriously questions their tumorigenic potential. However, as tetraploid cells frequently mis-segregate chromosomes, they appear to be more prone to undergo sudden mutational changes such as chromothripsis. If this is true, even a life span of a few cell cycles may be sufficient to accumulate tumorigenic genomic changes.

Gene expression imbalances associated with aneuploidy can further increase the rate of spontaneous DNA damage by altering the cellular processes that usually cause or repair normally lower levels of DNA damage. Additionally, the extra set of chromosomes present in tetraploid cells may buffer the effect of deleterious mutations by providing an extra copy of normal allele. This masking effect might allow cells with DNA damage to survive longer until they accumulate an optimized mutational load. This is demonstrated using a haploid and a diploid yeast strain in a long-term evolution experiments where diploid yeast with defective mismatch repair show significant survival advantage over haploid yeast (Thompson et al., 2006). In summary,

tetraploid cells play a causal role in tumorigenesis by increasing the DNA damage and producing aneuploid progeny.

1.1.5 Tetraploidy checkpoints

Considering all the deleterious effects caused by tetraploidy, it is not surprising that cells have evolved checkpoint mechanisms to eliminate tetraploid cells. Non-transformed rat fibroblasts made tetraploid with a cytokinesis inhibitor DCB, arrest in G1 stage of cell cycle in a p53 dependent manner (Andreassen et al., 2001). In contrast, DCB treated cells can proliferate upon transfection with the SV-40 virus that inactivates p53 (Wright and Hayflick, 1972). Tetraploid cells formed by virus induced cell - cell fusion can propagate in the culture only in the absence of p53 (Duelli et al., 2005). All these data suggest the existence of a ‘tetraploidy checkpoint’ mediated by p53 that blocks the proliferation of tetraploid cells. However, the mechanism of p53 activation in tetraploid cells is unclear.

Tetraploid cells can also be eliminated by p53 driven apoptosis. Inhibition of apoptosis in a variety of cell types leads to increased prevalence of tetraploid populations (Nelson et al., 2004). Tetraploid cells can activate a Bax-dependent apoptosis and depletion of Bax enhances survival of tetraploid cells (Castedo *et al.*, 2010). Therefore, it is likely that mutations in p53, as frequently observed in a variety of tumor types, are important for the survival of tetraploid cells during tumorigenesis.

1.1.6 Origins of tetraploid cells

Given the role of tetraploid cells in tumor development, it is crucial to understand the causes of tetraploid cell formation in order to understand the disease better and also to develop therapies that may target tetraploids or interfere with the process of tetraploidization. The following are the mechanisms that can hypothetically result in the formation of tetraploid cells.

1.1.6.1 Cell-cell fusion

Myocytes and osteoclasts undergo cell-cell fusion as a part of their normal developmental program (Storchova and Pellman, 2004). Cell-cell fusion also happens spontaneously in cell culture (Duelli et al., 2005) or following infection with certain viruses and the resultant tetraploidy may enhance transformation and tumorigenesis (Duelli et al., 2005).

1.1.6.2 Endoreplication

Endoreplication is defined as a phenomenon in which cells undergo repeated rounds of DNA replication without undergoing mitosis. Cells undergoing endoreplication thus become polyploidy by uncoupling DNA replication with mitosis. Endoreplication is most commonly observed during normal developmental processes. Cells of *Drosophila* salivary glands can completely skip mitosis and become polyploidy via endoreplication (Edgar and Orr-Weaver, 2001). Human megakaryocytes, and placental trophoblasts are common examples of cells that become polyploid by endoreplication (Ravid et al., 2002; Zybina and Zybina, 2005). Cells that undergo endoreplication do not typically have amplified centrosomes and would not have the mitotic defects and chromosomal instability associated with extra centrosomes in the cell.

1.1.6.3 Mitotic slippage

Prolonged arrest of cells in mitosis can result in premature exit from mitosis and reentry into G1 without completion of chromosome segregation and cytokinesis, a phenomenon termed as mitotic slippage. Mitotic slippage is frequently observed in cells treated with microtubule inhibitors or other conditions that cause incorrect kinetochore-microtubule attachment activating spindle assembly checkpoint (SAC) and delay in mitosis for a prolonged period of time (Rieder and Maiato, 2004; Xu et al., 2010).

1.1.6.4 Cytokinesis failure

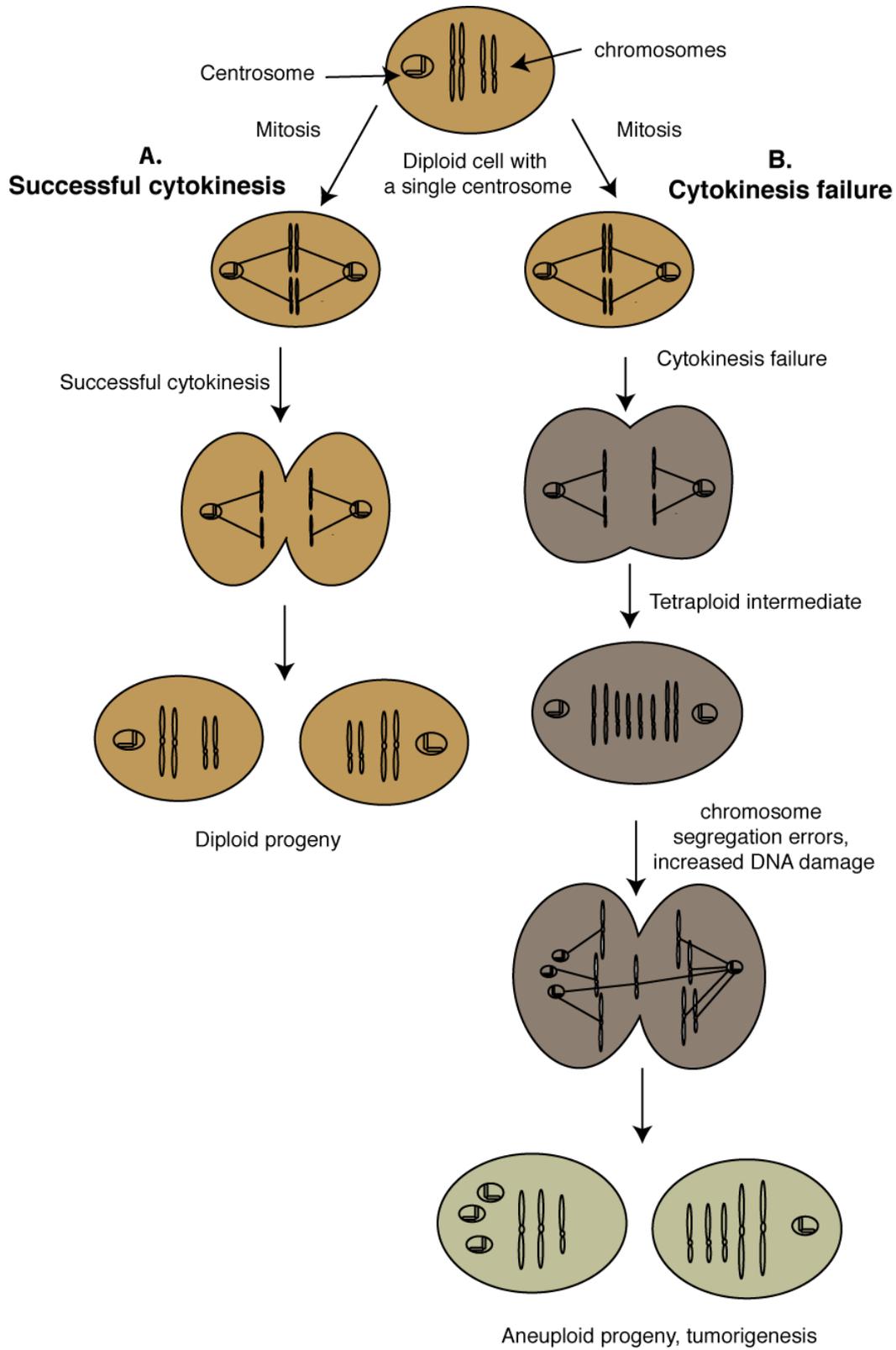
Cells that replicate DNA and enter mitosis but fail to complete cytokinesis become tetraploid. Cytokinesis failure is usually observed in pathological conditions. For example, liver cells become tetraploid under stress, predominantly by incomplete cytokinesis (Guidotti et al., 2003).

Most importantly, cancer cells appear to become tetraploid by failure of cytokinesis (Steigemann et al., 2009; Wu et al., 2010a).

Cytokinesis failure and cancer

Live cell-imaging analysis of cancer cells done in our laboratory has demonstrated that approximately 10% of cancer cells fail at cytokinesis depending upon the cancer cell type. These cells begin to ingress the cleavage furrow but later it regresses back and binucleated, tetraploid cells are formed. Cell-cell fusion is rarely observed in the tested cancer cell populations (Wu et al., 2010a). Furthermore, all cells with multipolar spindles fail cytokinesis in the previous division. Thus, cytokinesis failure appears to be the primary source of tetraploidy and multipolarity in cancer cells. As demonstrated in (Figure 1), tetraploid cells formed as a result of

cytokinesis failure pass through a pseudobipolar mitotic stage because of the presence of an extra set of chromosomes and extra centrosomes. This leads to chromosome segregation errors resulting in the formation of aneuploid progeny that can promote tumorigenesis.



Adapted from Gordon DJ *et al. Nature Rev. Genet.* 2012

Figure 1. Role of cytokinesis failure in tumorigenesis.

(A) Normal cell division produces two diploid daughter cells with a single centrosome (B) Cells that fail in cytokinesis produce a single tetraploid cell with double the number of chromosomes and centrosomes. As a result of this doubling, subsequent divisions of tetraploid cells occur with an increased frequency of chromosome segregation errors and DNA damage. The resultant aneuploid progeny can then stimulate tumorigenesis.

Many known regulators of cytokinesis are mis-regulated in cancer cells and can contribute to tetraploidy (Sagona and Stenmark, 2010). Cells over-expressing oncogene Gp1b α become tetraploid as a result of cytokinesis failure (Wu et al., 2010b). Loss of Aurora B kinase can also trigger regression of the cleavage furrow (Steigemann et al., 2009). But the mechanisms leading to tetraploidy in cancer cells are just beginning to be investigated. Given the disastrous consequences of cytokinesis failure, it is necessary to understand its origins. This is the primary goal of this dissertation. In order to discuss cytokinesis failure, it is first important to clarify the normal process of cytokinesis.

1.2 CYTOKINESIS AND ITS REGULATION

Cytokinesis is the final step of mitosis when the cytoplasm is divided into two daughter cells. It is a remarkably complicated process and needs coordinated action and regulation of more than 100 proteins (Pollard, 2010). Although the goal of cytokinesis is conserved in all the organisms i.e. to separate a mother cell into two daughter cells, different organisms carry out cytokinesis a little differently. Metazoans primarily divide by the formation of an actin-myosin contractile ring, whereas plant cells divide by building a cell plate between the two dividing daughter cells.

As the goal of this dissertation is to understand cytokinesis failure in human cancer cells, the following section will primarily discuss the mechanism of cytokinesis in animal cells.

The process of cytokinesis can be divided into four different stages as follows, specification of the cleavage plane, formation and ingression of an actin-myosin contractile ring, midbody formation and abscission (Normand and King) (Figure 2).

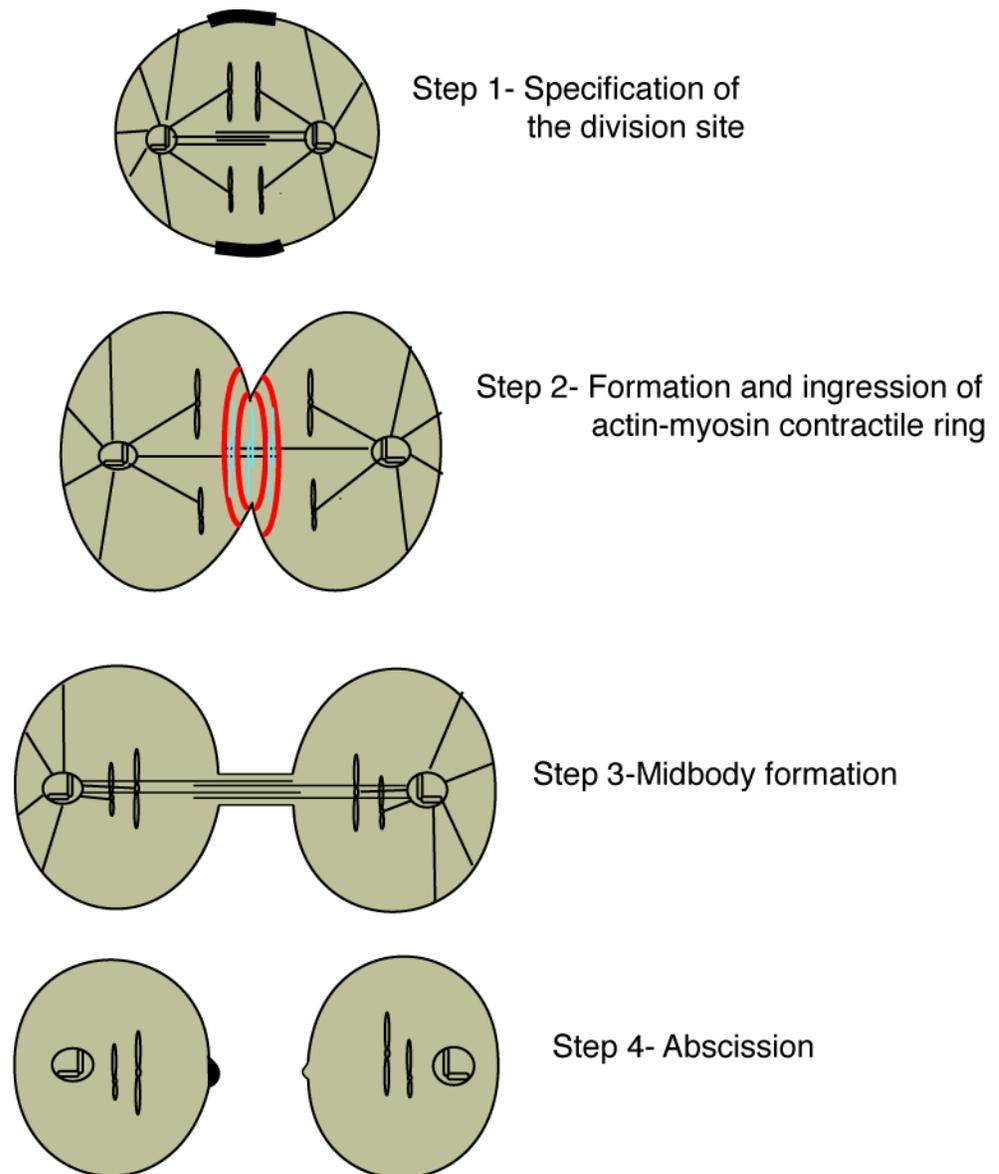


Figure 2. Stages of cytokinesis.

Process of animal cell cytokinesis can be divided into four different steps. Step-1 determines the site of division plane. Step-2 occurs with the formation and ingression of an actin-myosin based contractile ring. Step-3 results in the formation of the midbody and Step-4, known as abscission, is responsible for a physical separation of the dividing cell into two daughter cells.

1.2.1 Step 1-Specification of the division site

Accurate spatial and temporal regulation of cytokinesis depends upon positioning of the cleavage site between the segregating chromosomes, only after chromosomes have sufficiently separated. Furthermore, the divisional plane must be near the center of the cell, to allow equal partitioning of the cytoplasm, or positionally biased in the case of asymmetrical division. Therefore, the cell uses multiple cues to achieve correct positioning of the cleavage furrow. Classical experiments performed using sand dollar eggs demonstrate that if the mitotic spindle is displaced by physical manipulation, the existing cleavage furrow regresses and a new one forms at the site of the new spindle midplane (Rappaport, 1997). These and many other studies establish a central role of the mitotic spindle apparatus in providing spatial cues for positioning of the cleavage furrow. Specifically two components of the mitotic spindle, spindle asters and the spindle midzone, are most important for positioning the division site (Bringmann and Hyman, 2005). Spindle asters consist of radial arrays of microtubules nucleated by the centrosomes. Astral microtubules that grow towards the cell pole are called polar astral microtubules and astral microtubules that reach up to the equatorial region of the cell are called equatorial astral microtubules (Burgess and Chang, 2005). The polar relaxation and equatorial stimulation model of furrow positioning suggests that polar astral microtubules relay signals that inhibit contractility at the polar cortex

whereas, equatorial astral microtubules deliver positive signals that stimulate the formation and the contractility of the cleavage furrow at the equatorial cortex (Burgess and Chang, 2005).

Another component of the mitotic spindle that provides spatial cues for the site of cytokinesis is the spindle midzone. The spindle midzone or central spindle consists of bundles of antiparallel, non-kinetochore microtubules situated between the two spindle poles during cytokinesis and the proteins associated with these microtubules. As cytokinesis proceeds, the central spindle becomes compacted into a structure called the midbody. A heterotetrameric centralspindlin complex is necessary for the bundling of anti-parallel microtubules in the central spindle. This complex consists of the kinesin-6 motor protein MKLP-1 and an activator of Rho GTPase activity called RhoGAP CYK-4 (Mishima et al., 2002). The centralspindlin complex, specifically CYK-4, recruits and activates guanine nucleotide exchange factor RhoGEF ECT2 to the site of the cleavage furrow and this interaction is promoted by polo-like kinase 1 or Plk-1 (Werner and Glotzer, 2008). This localized activation of ECT2 further leads to activation of RhoA at the cleavage furrow site, which is required for the actin-myosin contractile ring assembly. Thus, central spindle appears to control cytokinesis by localizing cytokinesis regulatory proteins to the midzone. Role of RhoA in the assembly of actin-myosin contractile ring will be discussed in detail below. Centralspindlin is not required for furrow initiation but is required for completion (von Dassow, 2009). Disruption of the spindle midzone can cause various types of cytokinesis defects depending upon the organism and the cell type (Glotzer, 2004).

1.2.2 Step 2- Formation and ingression of an actin-myosin contractile ring

After the site of the cleavage plane is determined, the next step of cytokinesis is the formation of an actin-myosin contractile ring. In eukaryotes, assembly and ingression of the contractile ring provides the mechanical force required for the separation of mother cell into two daughter cells during cytokinesis.

Early identification of filamentous structures at the cytokinetic furrow (Schroeder, 1968) were later shown to contain F-actin and the molecular motor myosin (Fujiwara et al., 1978; Schroeder, 1973). Actin and myosin are targeted to the cleavage furrow in a semi-independent manner (Pollard, 2010). Myosin is assembled at the cleavage furrow in the form of membrane bound nodes (Vavylonis et al., 2008). Actin filament assembly at the cleavage furrow is promoted by the actin polymerizing protein formin (Chang et al., 1997) and the nucleating protein Arp2/3 (Pelham and Chang, 2002). Formin is also present in the discrete nodes that nucleate actin filaments. Once F-actin appears at the cleavage furrow, myosin captures and pulls the actin filaments intermittently from the adjacent nodes by a search and capture mechanism (Pollard, 2008). Thus, pulling of actin filaments by motor myosin condenses the nodes into a contractile ring. Once the ring is formed, it remains a dynamic structure with continuous assembly and disassembly of its components (Pelham and Chang, 2002).

1.2.3 Step 3-Midbody formation

Towards the end of cytokinesis, after the cleavage furrow ingression is completed, two dividing daughter cells remain connected by a thin cytoplasmic bridge. The core of the cytoplasmic bridge contains a phase-dense structure called a Fleming body or midbody. As the furrow

ingresses, it compacts the antiparallel bundles of midzone microtubules into a single large bundle that comprises the core of midbody. In addition to microtubules, the midbody contains several other proteins including centralspindlin components RhoA and anillin, proteins involved in vesicle trafficking and lipid rafts as well as chromosome passenger proteins and polo like kinase or Plk1 (Hu et al., 2012; Skop et al., 2004). The primary function of the midbody appears to be to localize the site of abscission.

1.2.4 Step 4- Abscission

The last step of cytokinesis is called abscission. This process is responsible for severing the residual thin cytoplasmic bridge formed after contraction, leading to complete separation of two daughter cells. This is probably the least well-understood step in cytokinesis and requires a cross talk between cytoskeletal, membrane trafficking and membrane remodeling processes. Abscission requires a series of complex events including vesicle transport and targeting, disassembly of midbody microtubules and plasma membrane fission/fusion. Three pathways of membrane trafficking: the secretory pathway, endocytic pathway and the components of the ESCRT (endosomal sorting complex required for transport) machinery are implicated in the process of abscission and blocking either of these pathways blocks cytokinesis (Gromley et al., 2005; Schiel and Prekeris, 2010; Schweitzer et al., 2005). Golgi-derived secretory vesicles accumulate at the intercellular bridge during late stages of cytokinesis and the protein centriolin plays an important role in recruiting these vesicles (Gromley et al., 2005). Recycling endosomes and other components of the endocytic pathway might be required during abscission to remodel the furrow plasma membrane as the furrow ingresses (Schweitzer et al., 2005) and endocytosis at the other parts of the cells such as polar region, might serve as a source of vesicles to be

delivered to the cleavage furrow. Recent studies have demonstrated that subunits of ESCRT machinery, conventionally known for their role in multivesicular body (MVB) formation, also play a role during late stage of cytokinesis. Components of ESCRT localize to the midbody and inhibition of some of the components blocks abscission (Dukes et al., 2008; Morita et al., 2007). Although precise role of these proteins in abscission is still elusive, as abscission requires a lot of membrane remodeling, understanding the role of ESCRT proteins in abscission would be interesting. After vesicle delivery at the midbody, membrane fusion is required to complete cytokinesis. SNARE proteins are critical for this process. Several SNARE proteins including Syntaxin 2 and VAMP - 8 are implicated in the process of abscission (Gromley et al., 2005; Low et al., 2003). Thus, the combined action of all the above-mentioned processes results in the resolution of intercellular cytoplasmic bridge and completion of cytokinesis. However, the precise spatial and temporal regulation of the process of abscission is just beginning to be understood.

1.2.5 Regulation of cytokinesis

All the stages of cytokinesis mentioned above are highly coordinated and tightly regulated to complete cytokinesis successfully. Being a central process, actin-myosin ring assembly is regulated at many different levels and regulation of myosin activity is probably one of the most important mechanisms of regulation of actin-myosin based contractile processes.

1.2.6 Regulation of myosin activity by myosin light chain phosphorylation

Myosin is the motor protein that drives actin-based contractile processes and these processes are primarily regulated by the contractile activity of myosin. Myosins comprise of a super family of motor proteins that share the properties of ATP hydrolysis and actin binding (Yamashita et al., 2000). They use the conformational changes driven by ATP hydrolysis to walk towards the positive or barbed ends of actin filaments. This walking slides the oppositely oriented actin filaments against each other, producing contraction. This process is central to all the contractile processes including skeletal, cardiac and smooth muscle contraction as well as the contractile processes in non-muscle tissues. More than 15 groups form the myosin superfamily labeled myosin I, myosin II, so on. Amongst these, myosin II, a conventional myosin, is the oldest member of the family, first discovered in skeletal muscle tissue (Yamashita et al., 2000). Since the discovery of the skeletal muscle isoform, myosin II has been shown to be ubiquitously expressed in skeletal, cardiac and smooth muscle as well as non-muscle cells and tissues. Non-muscle myosin II (NM II) is expressed in almost all non-muscle cells and is essential for diverse contractile processes including cell adhesion, polarity, migration and division (Conti and Adelstein, 2008; De Lozanne and Spudich, 1987; Vicente-Manzanares et al., 2009). Interestingly, NM II is also expressed in muscle tissue where it regulates muscle tissue development and differentiation (Swales et al., 2006).

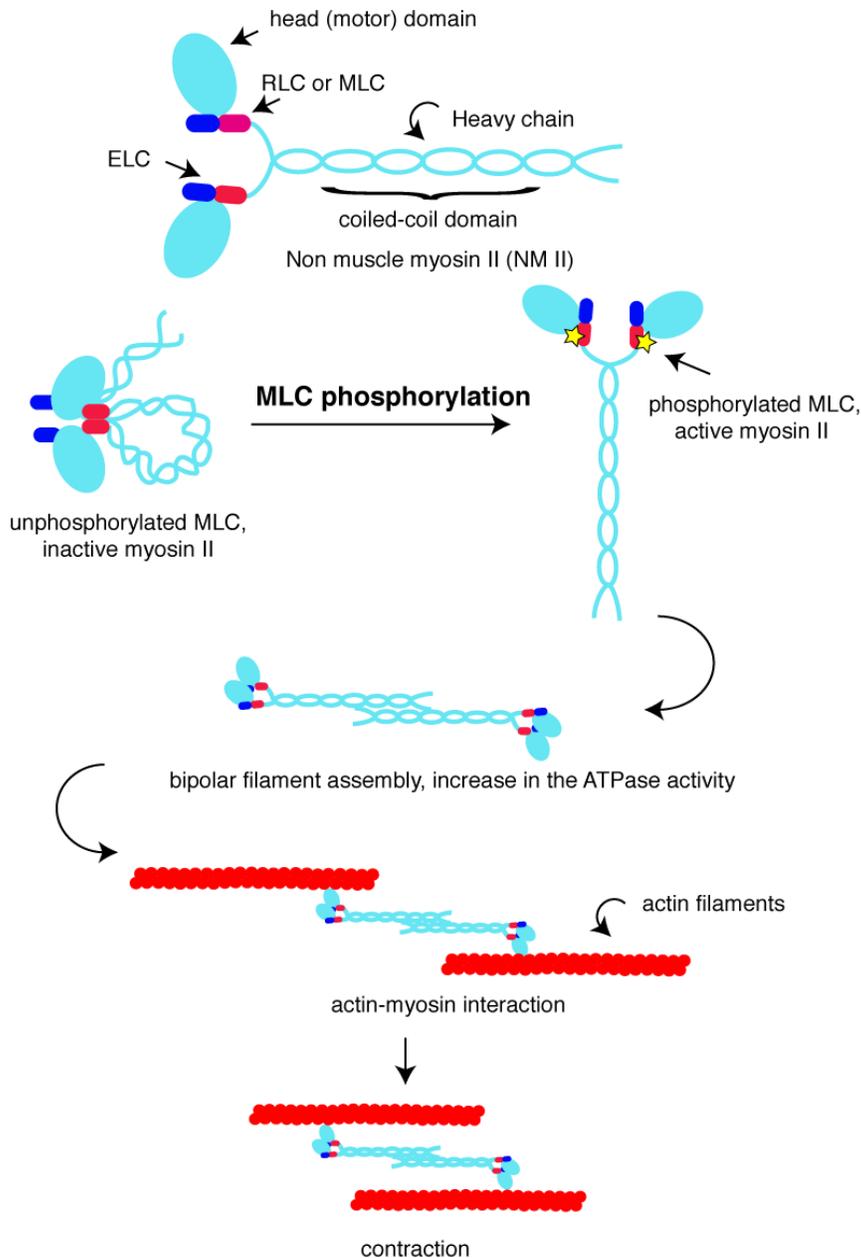
Myosin II, including muscle and non-muscle isoforms, are hexameric enzymes that comprise of a pair of heavy chains of 230 kDa, a pair of regulatory light chain (MLC) and a pair of essential light chains (ELC) of 20kDa each. The myosin heavy chain consists of head, neck, rod and tail domains (Weiss and Leinwand, 1996). The amino terminal portion of myosin heavy chain (MHC) contains the globular head or motor domain that possesses the ATPase activity and

the actin-binding site. A neck domain provides the attachment to regulatory and essential light chains and acts as a lever to amplify the head rotation during the conversion of chemical energy of ATP hydrolysis to mechanical energy. An elongated alpha-helical coiled coil rod domain and a short non-helical tail domain ajoin the neck domain. Coiled-coil rod domains can homodimerise and form tail-to-tail bipolar filaments with motor heads at each end, unique to myosin II molecules.

Three different genes encode non-muscle myosin heavy chain proteins - NMHC IIA, IIB and IIC respectively. Depending upon the heavy chain isoform, three different isoforms of NM II labeled as NM IIA, NM IIB or NM IIC are found in the mammalian tissues (Golomb et al., 2004; Simons et al., 1991). Depending upon the cell type, different NM II isoforms show differences in the localization, however they are thought to have redundant functions.

Regulation of myosin activity plays a central role in controlling actin-myosin interaction and reversible phosphorylation of myosin regulatory light chain or MLC is the major mechanism by which myosin activity is regulated (Moussavi et al., 1993; Sellers, 1991). MLC phosphorylation activates NM II in two ways. It increases Mg^{2+} ATPase activity of the motor domain by changing conformation (Sellers et al., 1981; Wendt et al., 2001), and enhances filament-forming ability of coiled coil tail domain of the heavy chain (Scholey et al., 1980).

(Figure 3)



Adapted from Vicente-Manzanares *et al. Nature Rev Mol Cell Biol* 2009

Figure 3. Regulation of myosin II activity by MLC phosphorylation.

Myosin II consists of a pair of heavy chains, a pair of essential light chains (ECL) and a pair of regulatory light chains (MLC). When MLC is unphosphorylated, myosin II is folded into a compact structure. Phosphorylation of MLC on active sites results in the unfolding of the tail domain that can dimerize to form the bipolar filaments. Phosphorylation of MLC also increases the ATPase activity of the head domain. Activated myosin then can interact with actin, leading to contraction.

In the unphosphorylated state, NM II is folded into a compact structure as the tail of unphosphorylated NM II is folded at two points and physically interacts with the head domain to make it compacted. Further, under unphosphorylated conditions, one head blocks another head of the same molecule from binding to actin. Phosphorylation of MLC disrupts head-head and head-tail interaction enabling the unfolded tail domain to dimerize and form bipolar filaments. Phosphorylation of MLC at Ser19 is the predominant activating phosphorylation as it enhances the ATPase activity and promotes the filament assembly of myosin (Ikebe and Hartshorne, 1985; Pearson et al., 1984). Ser19 phosphorylation is sometimes followed by another activating phosphorylation at Thr18 residue, which further increases the filament forming ability of myosin (Ikebe, 1989; Ikebe et al., 1986). MLC can also be phosphorylated on Thr9 and Ser1 and Ser2 (Bengur et al., 1987). However, these phosphorylation events are inhibitory and decrease the Mg^{2+} ATPase activity of myosin (Nishikawa et al., 1984).

It is well established that MLC phosphorylation is cell cycle regulated. In metaphase, MLC is predominantly phosphorylated on inhibitory sites Ser1/2. However, as the cell progresses through anaphase, MLC is de-phosphorylated on these sites and phosphorylated on Ser19, thus activating myosin during anaphase (Yamakita et al., 1994). Interestingly Ser19 phosphorylation immediately precedes the formation of the cleavage furrow and antibody staining demonstrates that MLC phosphorylated on Ser19 localizes to the cleavage furrow (Matsumura et al., 1998). All these data indicate that MLC phosphorylation of Ser19 is crucial for the process of cytokinesis.

Referring its importance in myosin regulation, MLC can be phosphorylated by a variety of kinases and a single known phosphatase (Matsumura, 2005; Matsumura et al., 2011). The

following section describes the role of these enzymes in regulating MLC phosphorylation, specifically during cytokinesis.

1.2.7 Myosin light chain kinase

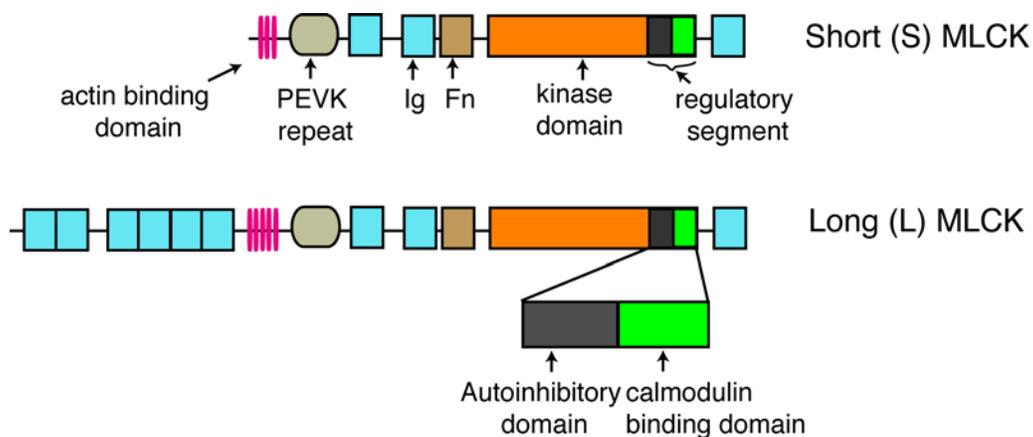
As the name suggests, myosin light chain kinase or MLCK is a dedicated MLC kinase with MLC as its only known substrate (Kamm and Stull, 2001). MLCK phosphorylates MLC primarily on Ser19 and to a lesser extent on Thr18, thus it activates both the ATPase activity of the motor domain and promotes the biopolar filament assembly of myosin II (Ikebe and Hartshorne, 1985; Pearson et al., 1984).

MLCK is a ubiquitously expressed enzyme encoded by three genes in humans: *mylk1*, *mylk2* and *mylk3*. *Mylk2* encodes a skeletal muscle specific isoform whereas *mylk3* encodes a cardiac specific isoform (Chan et al., 2008; Zhi et al., 2005). The *mylk1* gene encodes smooth muscle and non-muscle isoforms (Gallagher et al., 1991). Non-muscle isoform is widely expressed, and is detectable in most tissue and cell types. It regulates MLC phosphorylation during a variety of non-muscle contractile processes such as cell spreading, cell migration, focal adhesion, stress fiber formation, neurite growth cone advancement, apoptotic blebbing, secretion and cytokinesis, to name a few (Kamm and Stull, 2001). Using an alternative promoter, the *mylk1* gene encodes three different MLCK isoforms (Birukov et al., 1998). The long isoform (MW~220KDa, also named non-muscle or long MLCK) is ubiquitously expressed during development, but in adults it is predominantly expressed in non-muscle tissues (Blue et al., 2002). The short isoform (MW~108-130KDa, also called smooth muscle or short MLCK) is predominantly expressed in smooth muscles during development and maturity. A third transcript (MW~24 KDa, called telokin or kinase related protein- KRP) is a non-catalytic isoform and

lacks the kinase domain (Ito et al., 1989). In a non-muscle tissue, the long and the short isoform demonstrate distinct localization patterns. The short isoform is diffusely distributed throughout the cytoplasm whereas the long isoform specifically localizes to stress fibers and also to the cleavage furrow during cell division. Thus, the long isoform plays a distinct role during both, the assembly and the ingression of the actin-myosin contractile ring (Poperechnaya et al., 2000).

1.2.7.1 Structure function relationship of MLCK

MLCK enzymes are the members of the immunoglobulin super family of proteins. All the isoforms of MLCK are calcium and calmodulin dependent enzymes (Yagi et al., 1978) and there is no evidence for MLCK activity independent of Ca^{2+} /calmodulin. MLCK contains a conserved kinase domain and a regulatory segment located to the C-terminal of the kinase domain (Figure 4).



Adapted from Kamm *et al.* *JBC* 2001

Figure 4. A schematic of MLCK structure.

Both the long and the short isoforms of MLCK consist of a conserved kinase domain and a C-terminal regulatory domain. The regulatory domain contains an autoinhibitory domain that connects the

calmodulin-binding domain to the kinase domain. The long isoform is identical to the short isoform except an 922-934 amino acid extension at the N-terminus that contains two additional actin binding domains and six IgG modules.

The regulatory domain consists of an autoinhibitory domain that connects the calmodulin-binding domain to the kinase domain (Padre and Stull, 2000). In the absence of Ca^{2+} /calmodulin, the autoinhibitory domain folds back on the catalytic core and prevents the binding of MLC, but not of ATP, to the catalytic cleft. Calmodulin binding results in the displacement of the autoinhibitory domain from the catalytic cleft, with subsequent binding of the N-terminal of MLC to the catalytic cleft and transfer of the terminal phosphate of ATP to MLC (Kamm and Stull, 2001). The telokin domain is present on the C-terminus of the regulatory segment and facilitates binding of unphosphorylated MLC (Shirinsky et al., 1993). Other than the conserved kinase domain and a regulatory segment, short isoform of MLCK consists of one Fn module, three IgG modules, a PEVK repeat rich region and an actin binding domain consisting of three DFRXXL repeat motifs at the N-terminal of the kinase domain (Hong et al., 2011; Kamm and Stull, 2001). Thus, MLCK appears to be anchored to actin at its N-terminus and the catalytic core at the C-terminus is exposed to myosin filaments for MLC phosphorylation. The long isoform of MLCK is identical to the short isoform except an 922-934 amino acid extension at the N-terminal that contains two additional actin binding domains and six IgG modules (Garcia et al., 1997). The additional IgG modules in the long isoform contribute to its targeting to stress fibers and the cleavage furrow (Dulyaninova et al., 2004; Poperechnaya et al., 2000).

1.2.8 Regulation of MLCK activity by phosphorylation by Aurora B kinase

In addition to Ca^{2+} /calmodulin, non-muscle MLCK activity can be modulated by a variety of signaling pathways that regulate cytoskeletal morphology during cell motility, migration and division. Upstream kinases that phosphorylate non-muscle MLCK during different times include growth factors such as mitogen activated kinases (MAPK) ERK1 and ERK2 (Klemke et al., 1997), Src kinase (Garcia et al., 1999), cAMP dependent protein kinase - PKA (Horman et al., 2008), Rho family GTPases p21-activated kinase PAK2 and PAK1 (Goekeler et al., 2000) and Aurora B kinase (Dulyaninova and Bresnick, 2004). Phosphorylation of MLCK by different kinases is required for the regulation of different processes such as cell spreading, stress fiber formation, cell migration, cell division and cytokinesis.

Perhaps the most relevant kinase that controls MLCK activity during cytokinesis is a known regulator of cytokinesis - Aurora B kinase (Dulyaninova and Bresnick, 2004). MLCK activity varies during the cell cycle. It is lower during early mitosis but increases almost two-fold during cytokinesis (Poperechnaya et al., 2000). This variation in MLCK activity correlates very well with the phosphorylation status of MLC during cytokinesis, suggesting that MLCK is the key kinase that phosphorylates MLC during cytokinesis. However, the upstream signals that regulate MLCK phosphorylation and activity temporally and spatially are not completely understood at this point. Consistent with the changes in its activity, the long isoform of MLCK is differentially phosphorylated, predominantly on serine residues during the cell cycle (Dulyaninova and Bresnick, 2004). Interestingly, Aurora B binds to long MLCK *in vitro* and phosphorylates serine residues in the IgG modules. Considering the specific localization of long MLCK to the cleavage furrow and the crucial role of Aurora B in regulating cytokinesis, it can be proposed that phosphorylation of MLCK by Aurora B is a key regulatory event during

cytokinesis. However the significance of this phosphorylation and its effect on MLCK activity are not yet clear and need to be investigated further to study the normal process of cytokinesis and also to understand the causes of its failure.

1.2.9 MLC phosphorylation by other kinases and a phosphatase

Another important regulator of actin-myosin assembly and contraction is the small GTPase RhoA. RhoA is activated by RhoGEF-ECT2 (Yuce et al., 2005). During anaphase, ECT2 is localized to the central spindle and interacts with the centralspindlin component CYK-4/MgcRacGAP. This interaction stabilizes ECT2 in an active conformation allowing it to activate RhoA (Yuce et al., 2005). Restriction of the centralspindlin component to the central spindle thus limits localization of ECT2 and the activation of RhoA to a narrow zone during furrow formation. Cyk-4/MgcRacGAP is a GTPase activating protein (GAP) for RhoA. In addition to activating ECT2, Cyk-4/MgcRacGAP is also thought to inactivate RhoA late in cytokinesis, along with another GAP protein p190RhoGAP (Su et al., 2003).

Activated RhoA regulates myosin assembly by activating its downstream targets Rho-dependent kinase (ROCK) and Citron kinase (Citron K). ROCK can directly activate myosin by phosphorylating MLC on Ser19 (Amano et al., 1996). ROCK also indirectly activates myosin by inhibiting myosin binding subunit (MBS) of myosin phosphatase that takes off the activating phosphorylation of myosin (Kimura et al., 1996). Citron Kinase, another effector protein of RhoA, can phosphorylate MLC on Thr18 and Ser19 (Yamashiro et al., 2003). ROCK is required for the ingression of the cleavage furrow whereas Citron kinase is required for the maintenance of the cleavage furrow and completion of cytokinesis. Death associated protein kinase (DAPK) and DAPK-like kinase, known as DAPK3 also appear to regulate MLC phosphorylation during

cytokinesis. However, the detailed role of these kinases in cytokinesis is not yet known (Preuss et al., 2003).

In contrast to the redundant kinases, there is a single known phosphatase that dephosphorylates MLC (Matsumura et al., 2011). Myosin phosphatase consists of a large targeting subunit known as MYPT1, a catalytic subunit known as PP1c and a small subunit (Hartshorne et al., 2004). It is interesting to note that many kinases such as ROCK which phosphorylate MLC, can also phosphorylate and inhibit myosin phosphatase (Hartshorne et al., 2004).

1.2.10 Regulation of cytokinesis by Aurora B kinase

All the stages of cytokinesis are regulated by Aurora B kinase, a member of a conserved serine/threonine kinase family. Aurora kinases were first identified in *Drosophila melanogaster* during the search for the genes that regulate structure and function of mitotic spindle (Glover et al., 1995) and are homologous to the *Saccharomyces cerevisiae* Ipl-1 (Francisco et al., 1994). So far three members have been identified in the human Aurora family namely, Aurora A, Aurora B and Aurora C (Adams et al., 2001). Aurora A primarily functions in centrosome maturation and separation. Aurora B kinase regulates kinetochore-microtubule attachments, spindle assembly checkpoint and cytokinesis. Aurora C appears to function redundantly with Aurora B, however its function is not yet well understood.

1.2.10.1 Aurora kinases and cancer

Interestingly, human Aurora kinase family members Aurora A and B were first identified as homologs of fly aurora kinases in a PCR screen performed for kinases over-expressed in colorectal cancer (Bischoff et al., 1998). Since then, both Aurora A and Aurora B are found to be

over-expressed in a majority of tumor tissues. Unlike Aurora A, Aurora B gene amplification has not been clearly demonstrated and it is usually not classified as a classical oncogene. Nonetheless, a variety of tumor tissues over-express Aurora B including colon, prostate, thyroid, liver, breast, and lung cancer to name a few (Chieffi et al., 2006; Katayama et al., 2003; Lin et al., 2010; Mountzios et al., 2008; Qi et al., 2007; Sorrentino et al., 2005). Although Aurora B cannot transform cells on its own, it potentiates Ras-mediated transformation (Kanda et al., 2005). Elevated levels of Aurora B are a predictor of poor cancer prognosis and positively correlate with advanced stages of cancer progression (Kurai et al., 2005) and increased genomic instability (Smith et al., 2005). Many Aurora B inhibitors are currently in phase I and phase II clinical trials as chemotherapeutic agents and are showing promising results (Kollareddy et al., 2012).

1.2.10.2 Mitotic functions of Aurora B

Aurora B regulates mitosis as a component of the chromosomal passenger complex (CPC). Core CPC components include Aurora B kinase and non-enzymatic subunits include inner centromere protein –INCENP, survivin and borealin (Ruchaud et al., 2007). Aurora B binds to a region near the C-terminus of INCENP called the IN box. This binding leads to auto-phosphorylation of Aurora B and is necessary for its activity. Activated Aurora B then phosphorylates INCENP leading to a further increase in its kinase activity in a positive feed-back loop (Bishop and Schumacher, 2002; Yasui et al., 2004). Survivin and borealin are also substrates of Aurora B and appear to function in targeting of CPC to different locations during mitosis (Gassmann et al., 2004; Wheatley et al., 2004).

As the name suggests, being a part of CPC, Aurora B demonstrates very dynamic localization during cell division (Murata-Hori et al., 2002). It associates with chromosome arms

very briefly but concentrates at the inner centromeric region through prometaphase and metaphase. At the onset of anaphase, Aurora B redistributes from chromatin to the spindle midzone and is concentrated at the equator along with the midzone microtubules, whereas during late anaphase it concentrates at the midbody (Murata-Hori et al., 2002; Terada et al., 1998).

Known Aurora B functions correlate well with this dynamic localization of Aurora B during mitosis. During early mitosis, prior to the onset of anaphase, Aurora B localizes to the chromosome arms and the centromeric region and regulates chromosome condensation, sister chromatid cohesion, kinetochore-microtubule attachment error correction and spindle assembly checkpoint. Aurora B regulates chromosome condensation by phosphorylating Histone H3 on serine 10 (Hsu et al., 2000). It is required for the recruitment of condensin on the chromosomes in some organisms (Giet and Glover, 2001) and for confining the localization of the Shugoshin family of proteins that are required for maintaining sister-chromatid cohesion (Resnick et al., 2006).

For accurate chromosome segregation, all sister chromatids need to attach to the microtubules emanating from the opposite spindle poles. Aurora B plays an important role in establishing kinetochore-microtubule attachments by phosphorylating kinetochore-associated proteins such as Ncd80/Hec-1 and Dam1 complexes in yeast (Cheeseman et al., 2002). In addition to establishing normal bipolar attachments, Aurora B plays a pivotal role in correcting erroneous merotelic or syntelic kinetochore-microtubule attachments. Aurora B is enriched at the site of merotelic attachments and destabilizes them by activating depolymerase MCAK that leads to depolymerization of incorrectly attached microtubules (Knowlton et al., 2006). Even a single unattached or incorrectly attached kinetochore activates spindle assembly checkpoint (SAC). SAC delays the onset of anaphase by inhibiting the activation of anaphase promoting complex

(APC) by formation of inhibitory complexes of MAD and BUB proteins, until correct bivalent orientation of chromosomes is achieved (Musacchio and Salmon, 2007) (Rieder et al., 1995; Wassmann and Benezra, 2001). SAC is activated either due to the absence of tension between the sister chromatids or the presence of unattached chromatids. Aurora B appears to activate SAC by sensing the absence of tension in the presence of incorrect kinetochore-microtubule attachments (Musacchio, 2011).

1.2.10.3 Aurora B as a regulator of cytokinesis

During late mitosis, Aurora B localizes to the spindle midzone and midbody and primarily functions to regulate cytokinesis. Interestingly, the predominant phenotype of Aurora B mis-regulation is cytokinesis failure and induction of multinucleation and polyploidy. Exogenous over-expression of Aurora B in rat cells, human cells or the Chinese hamster ovary cells results in defective cleavage furrow formation, cytokinesis failure and polyploidy (Tatsuka et al., 1998; Terada et al., 1998). Conversely, complete depletion of Aurora B by siRNA or by chemical inhibition also results in cytokinesis failure and multinucleation (Ditchfield et al., 2003; Tsuno et al., 2007). Thus, optimal levels of Aurora B appear to be essential for successful cytokinesis. Aurora B over-expressing cells form tumors upon injection into nude mice (Nguyen et al., 2009; Ota et al., 2002). Interestingly, tumors formed upon injection of Aurora B over-expressing tetraploid cells are larger than those formed upon injection of their diploid counterparts (Nguyen et al., 2009). Moreover, these tumors are metastatic and the tumor cells are multinuclear, aneuploid and show various degrees of chromosome amplifications and deletions (Nguyen et al., 2009; Ota et al., 2002). Thus, cytokinesis failure and tetraploidy induction appears to be the underlying mechanism of Aurora B-induced tumorigenesis.

Although Aurora B mis-regulation phenotype clearly demonstrates its necessity during cytokinesis, the precise molecular mechanism by which Aurora B regulates cytokinesis is not yet clearly understood. Considering the tumorigenic potential of Aurora B over-expressing tetraploid cells and promising results of Aurora B inhibitors as therapeutic targets, it is necessary to understand in detail the role of Aurora B in cytokinesis regulation.

A substrate profile of Aurora B kinase indicates that Aurora B spatially and temporally regulates cytokinesis by phosphorylating different substrates at different stages of the process. At the onset of anaphase a phosphorylation gradient of Aurora B substrates is established at the spindle midzone through interaction of Aurora B with midzone microtubules in a positive feedback loop (Fuller et al., 2008). At the midzone, Aurora B phosphorylates the centralspindlin component MgcRacGAP, thus regulating RhoA activity during establishment of the cleavage site and the initiation of the cleavage furrow ingression (Minoshima et al., 2003). Interestingly, Aurora B dependent NoCut pathway negatively regulates abscission by delaying it in the presence of un-segregated chromatin (Norden et al., 2006; Steigemann et al., 2009). So far, MKLP-1 and ESCRT-III component CHMP4C are identified as Aurora B substrates that are involved in abscission inhibition (Carlton et al., 2012). However, future work is required to uncover additional Aurora B targets that might be involved in cytokinesis regulation. Other substrates of Aurora B that localize to the cleavage furrow and might be important targets during cytokinesis include vimentin, desmin, glial fibrillary acidic protein and MLCK (Dulyaninova and Bresnick, 2004; Goto et al., 2003; Minoshima et al., 2003).

We are specifically interested in understanding the significance and consequences of phosphorylation of MLCK by Aurora B. Aurora B phosphorylates MLCK on serine residues, predominantly located within its IgG modules (Dulyaninova and Bresnick, 2004). This

phosphorylation matches well the changes in MLCK activity and MLC phosphorylation status during cell cycle. As described later, taking into account the necessity of MLCK for MLC phosphorylation during cytokinesis, we aim to study in detail the effect of Aurora B kinase on MLCK activity. This dissertation project provides specific insights into the role of MLCK as an Aurora B substrate and the effect of Aurora B mis-regulation on MLCK activity and on cytokinesis outcomes in cancer cells.

1.3 DISSERTATION OVERVIEW

Research performed over the past few decades makes it clear that tetraploidy plays a causal role in genomic instability, transformation and neoplasia. However, further investigations are required to address the causes of tetraploidy and to uncover the mis-regulated pathways that under normal circumstances maintain the diploid status of the cells. These studies will also provide novel drug targets for efficient chemotherapy.

Previous studies in our laboratory, performed to identify the causes of tetraploidy in cancer cells, reveal that cytokinesis failure is the primary source of tetraploidy in cancer cells. These cells specifically fail to maintain actin-myosin based contraction at the cleavage furrow, resulting in cleavage furrow regression and cytokinesis failure. Actin-myosin contraction is tightly regulated by the phosphorylation of MLC and our studies identify deficient MLC phosphorylation as the cause of cytokinesis failure in cancer cells. The first goal of this dissertation project is to dissect the upstream pathways that lead to deficient MLC phosphorylation in cancer cells. Given the oncogenic potential and frequently observed mis-regulation of Aurora B kinase in cancer cells, we have explored the role of Aurora B mis-

regulation in cytokinesis failure. For this study, the most relevant substrate of Aurora B is MLCK, a kinase that regulates MLC phosphorylation during cytokinesis and is inhibited in cancer cells. Therefore, we hypothesize that Aurora B mis-regulation causes cytokinesis failure by inhibition of MLCK. Chapter 2 of this dissertation explains in details the results that led us to this hypothesis and the data that support it.

Our previous study reported abortive cytokinesis in approximately 10% of the cancer cell population, depending on the cancer cell type (Wu *et al.*, 2010a). These observations are intriguing, as the cytokinesis failure appears to occur only in a certain fraction of the population. Whether this fraction fails in cytokinesis randomly or there is an underlying anomaly that predisposes only a subset of cells to fail is not known at this stage. Therefore, the second goal of this dissertation project is to understand the reasons as to why cytokinesis fails only in a subset of the cells. Chromosome segregation errors are frequently observed in cancer cells and are proposed to cause cytokinesis failure. Therefore we hypothesize that chromosome segregation errors act as a trigger for mis-regulation of Aurora B, inhibition of MLCK and cytokinesis failure in cancer cells. Chapter 3 of this dissertation explains in detail the rationale behind this hypothesis and the results supporting it.

2.0 INHIBITION OF MLCK BY AURORA B KINASE CAUSES CYTOKINESIS FAILURE IN CANCER CELLS

2.1 INTRODUCTION

As described in the Introduction (Chapter 1, section 1.1), tetraploidy in some cell types poses a serious threat in terms of the cellular transformation and tumorigenesis. Studies undertaken to identify the causes of tetraploidy point to cytokinesis failure as the primary cause of tetraploidy generation (Steigemann et al., 2009; Wu et al., 2010a). Cytokinesis failure doubles the number of chromosomes and centrosomes. Both of these events lead to an increase in the incidence of chromosome mis-segregation, aneuploidy and tumorigenesis. However the causes of cytokinesis failure are still not completely understood. Investigation into identifying the pathways that culminate in failed cytokinesis will give us further insights into the course of tetraploidy induced tumorigenesis.

Cytokinesis in animal cells is accomplished by the formation and ingression of the cleavage furrow composed of an actin-myosin contractile apparatus. The contractile ability of this apparatus is highly dependent on the phosphorylation of myosin regulatory light chain or MLC. MLC phosphorylation is necessary to induce bipolar filament assembly and increase in the ATPase activity of motor myosin. Thus, MLC phosphorylation controls both the assembly and contractility of the actin-myosin apparatus.

Not surprisingly, spatial and temporal regulation of cytokinesis depends on the phosphorylation status of MLC. During cytokinesis, MLC is phosphorylated at Thr18 and Ser19 residues and phosphorylation at these sites is indispensable for the process. Expression of an unphosphorylatable mutant of MLC (T18A/S19A) in mammalian cells results in distorted cleavage furrow formation, cytokinesis failure and a significant increase in the formation of multinuclear cells (Komatsu et al., 2000). Similarly, *Drosophila* clones expressing unphosphorylatable MLC (T20A/S21A, corresponding MLC phosphorylation sites in *Drosophila*) cannot perform cytokinesis and have a phenotype almost identical to MLC null flies (Jordan and Karess, 1997). Consistently, live cell-imaging analysis of cancer cells report cytokinesis failure at an early stage, as a result of abnormal contractility of actin-myosin at the cleavage furrow (Wu et al., 2010a). Based on these data, we hypothesize that cancer cells fail in cytokinesis because of deficiencies in MLC phosphorylation.

2.2 DEFICIENT MLC PHOSPHORYLATION IS THE CAUSE OF CYTOKINESIS FAILURE IN CANCER CELLS

2.2.1 MLC phosphorylation is reduced in cancer cells and correlates with the multinucleation frequency

To test the hypothesis of MLC phosphorylation deficiency as the cause of cytokinesis failure in cancer cells, we compared MLC phosphorylation status between a panel of non-cancer control cell lines and cancer cell lines. As described previously, the unphosphorylated form of MLC (un-pMLC) was separated from monophosphorylated (mono-pMLC) and diphosphorylated (di-

pMLC) forms using urea glycerol gel electrophoresis (Word et al., 1991). After measuring the band intensities, MLC phosphorylation ratios were calculated as a ratio of (mono-pMLC+di-pMLC) / (un-pMLC+mono-pMLC+di-pMLC). Consistent with our hypothesis, non-cancer cells showed significantly higher phosphorylation ratios compared to cancer cells ($p < 0.001$) (Figure 5A) (Wu *et al.*, 2010a). Lower phosphorylation ratios were relevant to cytokinesis, as MLC phosphorylation was induced at similar times in mitosis in non-cancer and cancer cells but remained lower in cancer cells even after normalizing the percentage of cells in anaphase (Wu et al., 2010a). Cytokinesis failure is the origin of multinucleated tumor cells. Therefore we tested if there was a correlation between MLC phosphorylation ratio and the frequency of multinucleated cells. As expected, non-cancer cells that possessed high MLC phosphorylation ratio had a low frequency of multinucleated cells. In contrast, cancer cells that demonstrated a low MLC phosphorylation ratio had a higher frequency of multinucleation and the correlation was statistically valid (Figure 5B) (Wu *et al.*, 2010a). These data suggest that cancer cells are deficient in MCL phosphorylation and this deficiency correlates with the observed cytokinesis failure.

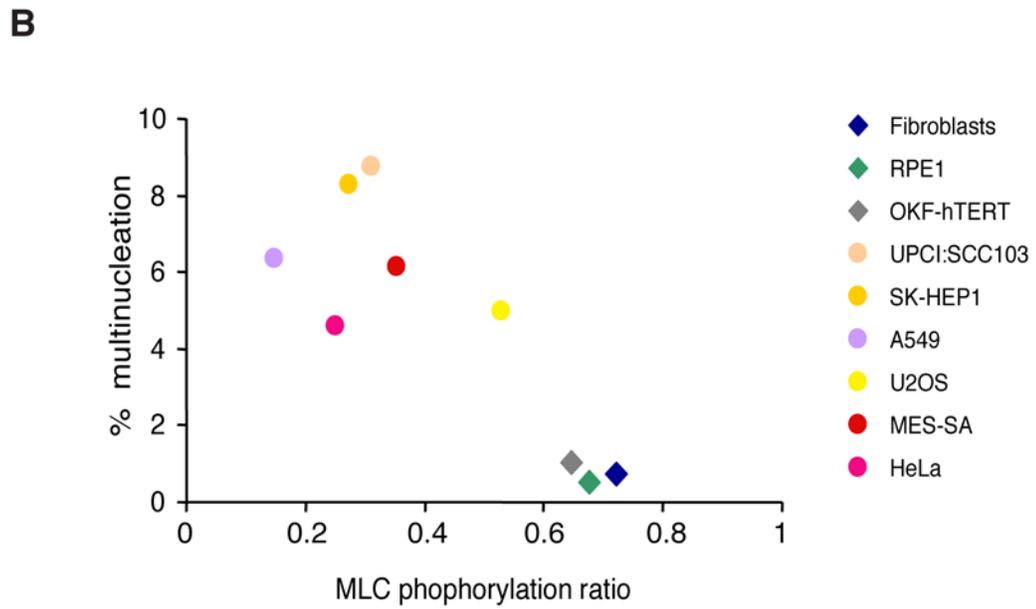
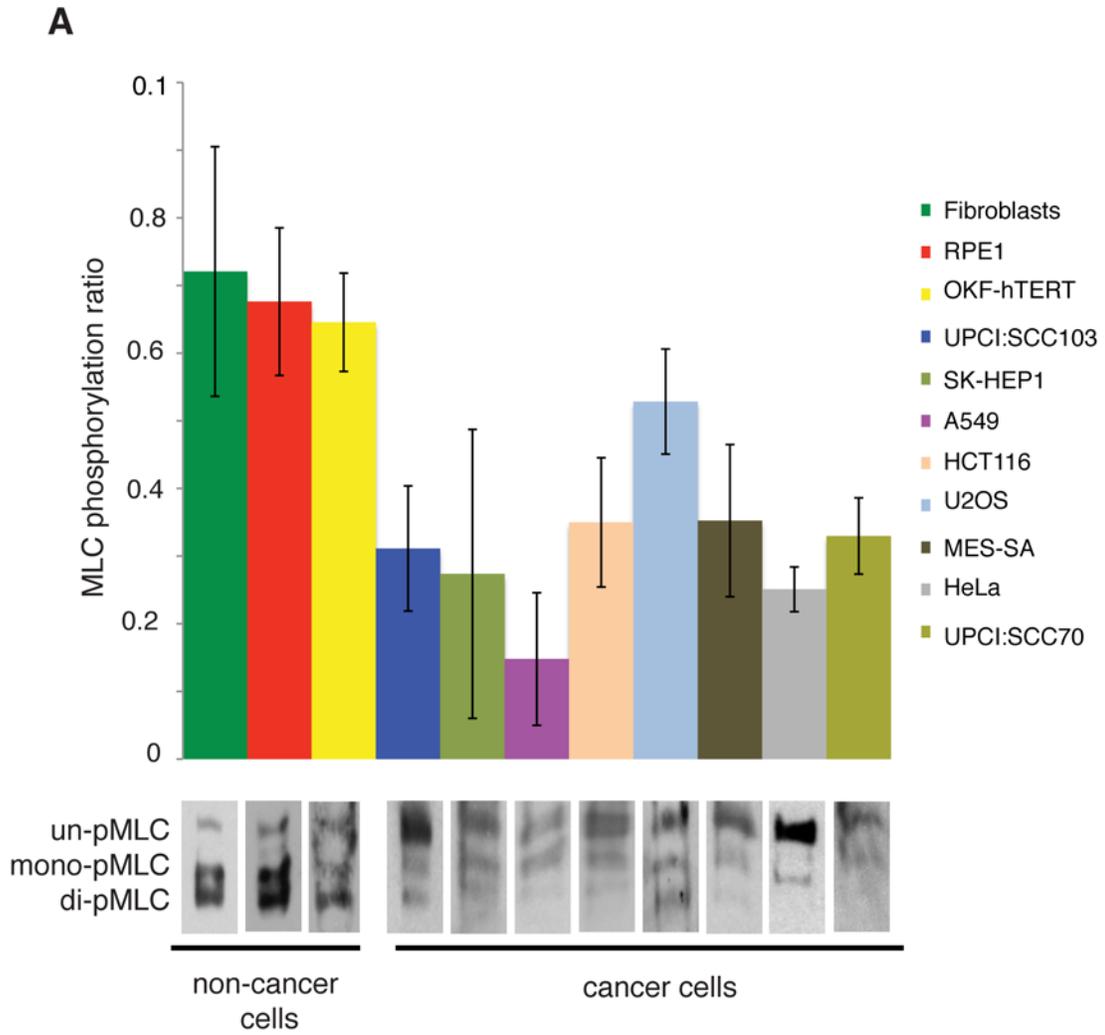


Figure 5. MLC phosphorylation is reduced in cancer cells and correlates with the multinucleation frequency.

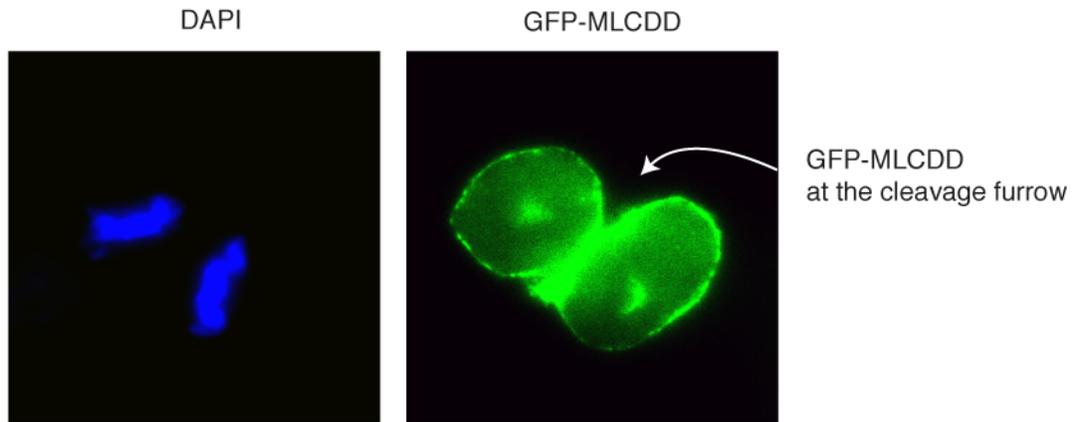
(A) MLC phosphorylation levels were compared between a panel of asynchronous non-cancer and cancer cells by urea glycerol gel electrophoresis. MLC Phosphorylation ratio = $(\text{mono-pMLC} + \text{dipMLC}) / (\text{un-MLC} + \text{mono-pMLC} + \text{dipMLC})$. Un-pMLC= unphosphorylated MLC, mono-pMLC= MLC phosphorylated at a single residue and di-pMLC= MLC phosphorylated at both the residues. Error bars represent mean \pm S.D from more than three experiments, $p < 0.001$. (B) The ratio of MLC phosphorylation was correlated with the frequency of multinucleation in the indicated cell lines. Diamonds represent non-cancer cells and circles represent cancer cells (Wu *et al.*, 2010a).

2.2.2 Deficient MLC phosphorylation is the cause of cytokinesis failure in cancer cells

To test if MLC phosphorylation deficiency played a causal role in cytokinesis failure, we examined cytokinesis failure after transfection of a phosphomimetic MLC construct tagged with GFP (Ward *et al.*, 2002). This construct consists of an aspartic acid residue at the position 18 and 19 (T18D/S19D) instead of WT threonine and serine and will hereafter referred to as GFP-MLCDD. As MLC is predominantly phosphorylated on T18/S19 during cytokinesis, this construct mimics phosphorylated state of MLC during cell division, bypassing the need for its upstream regulators. The mutant protein expressed abundantly and localized correctly at the cleavage furrow (Figure 6A). In this and all the subsequent experiments, multinucleation or binucleation frequency was counted by staining the cells with DAPI to mark the nuclei and actin to denote the cell boundary. 48 hours after transfection of the phosphomimetic plasmid, moderate but statistically significant decrease in the frequency of multinucleation was observed in all the four cancer cell lines tested (Figure 6B) (Wu *et al.*, 2010a). These observations were also confirmed by imaging the oral cancer cell line UPCI:SCC103 in real time with both DIC

and fluorescent markers (Wu et al., 2010a). MLC is de-phosphorylated by a single known phosphatase. Increasing MLC phosphorylation by knock-down of myosin targeting subunit of myosin phosphatase, known as MYPT1 also resulted in a reduction in the multinucleation and thus rescued cytokinesis failure in cancer cells (Wu et al., 2010a). Taken together, all these data support our hypothesis that deficient MLC phosphorylation is the cause of cytokinesis failure in all the tested cancer cell lines.

A



B

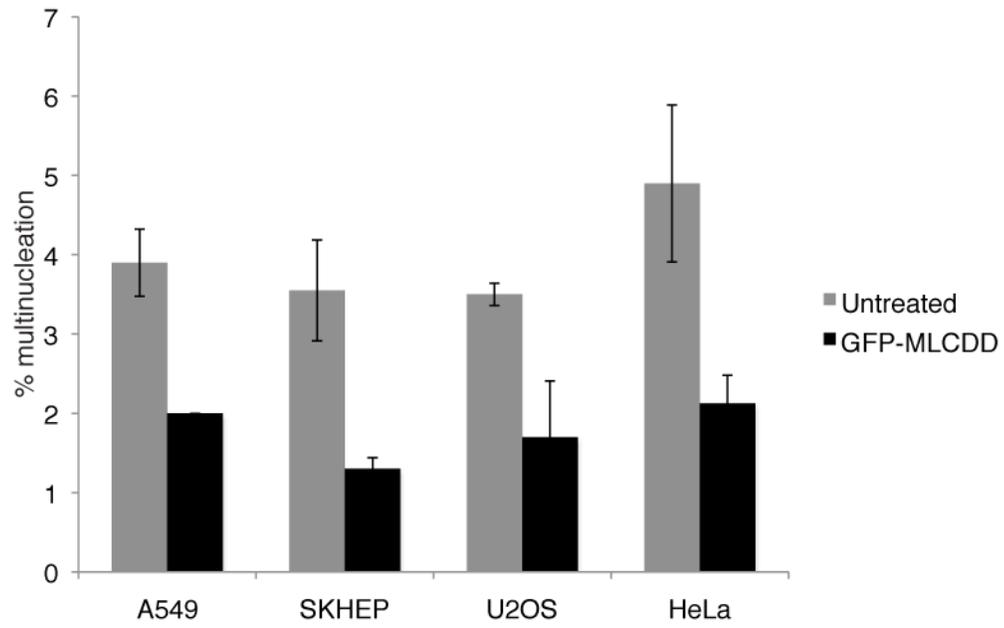


Figure 6. Phosphomimetic MLC reduces cytokinesis failure in cancer cells.

(A) A representative image of the correct localization of phosphomimetic MLC (GFP-MLCDD) at the cleavage furrow of the dividing cell. DAPI stain indicates nuclei. (B) Multinucleation frequency was measured after 48 hours of transient transfection of phosphomimetic MLC in the indicated cancer cell lines. Data includes mean \pm SD from three independent experiments and n > 500 cells for each experiment (Wu *et al.*, 2010a).

2.3 MLCK IS INHIBITED IN CANCER CELLS AND THIS INHIBITION IS THE CAUSE OF CYTOKINESIS FAILURE

2.3.1 MLCK is expressed at low levels in cancer cells

Establishing deficient MLC phosphorylation as the cause of cytokinesis failure led us to our next task of identification of the sources of deficient MLC phosphorylation in cancer cells. Reflecting the robustness of the process of cytokinesis, MLC phosphorylation is controlled redundantly by many kinases and a single phosphatase (Matsumura, 2005; Matsumura et al., 2011), described in detail in the Introduction.

Out of these kinases, MLCK is the key kinase that phosphorylates MLC. During cytokinesis, MLC is phosphorylated by MLCK predominantly on Ser19 and to a lesser extent on Thr18 (Ikebe and Hartshorne, 1985). This phosphorylation appears to be essential for the completion of cytokinesis as demonstrated by studies in different model systems. In sea urchin embryos, MLCK dependent MLC activity precedes cleavage furrow formation and is maintained throughout cytokinesis (Lucero et al., 2006). During fly spermatogenesis, MLCK is required for contractile ring contraction at all the stages of cytokinesis and MLCK inhibition causes cleavage furrow regression and cytokinesis failure (Silverman-Gavrila and Forer, 2001; Wong et al., 2007). MLCK inhibition in non-cancer cells also results in reduced MLC phosphorylation and a significant increase in multinucleation (Wu et al., 2010a). All of these results suggest that MLCK is indispensable for MLC phosphorylation during cytokinesis. Consistent with the role of MLCK during cytokinesis and the observed cytokinesis failure in cancer cells, MLCK is down-regulated in some cancer cell types such as prostate and colon cancer (Lee et al., 2008; Leveille et al., 2009).

Therefore, to test if MLCK down-regulation was the cause of cytokinesis failure in cancer cells, we compared MLCK protein levels by immunoblotting across the panel of different cancer and non-cancer cell lines and expectedly found them to be lower in cancer cells as compared to non-cancer cells (Figure 7A). However, cancer cells have higher mitotic indices compared to non-cancer cells. To rule out the possibility of cell cycle differences between cancer and non-cancer cells as the underlying cause of the observed MLCK down-regulation, MLCK levels were examined in cell cycle synchronized non-cancer cells RPE1 and oral squamous cell carcinoma cells UPCI:SCC103. This pair of non-cancer and cancer cell line was chosen for their compatibility with cell cycle synchronization and their common epithelial origin. Nocodazole was used to arrest cells in mitosis, as it is a drug that depolymerizes microtubules, thus inhibiting the formation of mitotic spindle and arresting cells in mitosis. MLCK expression was then compared in mitotically arrested cells as well as cells released from the arrest for four hours. Even at the comparable mitotic indices, MLCK expression remained lower in cancer cells as compared to non-cancer cells (Figure 7B), confirming low abundance of MLCK in cancer cells independent of the cell cycle differences.

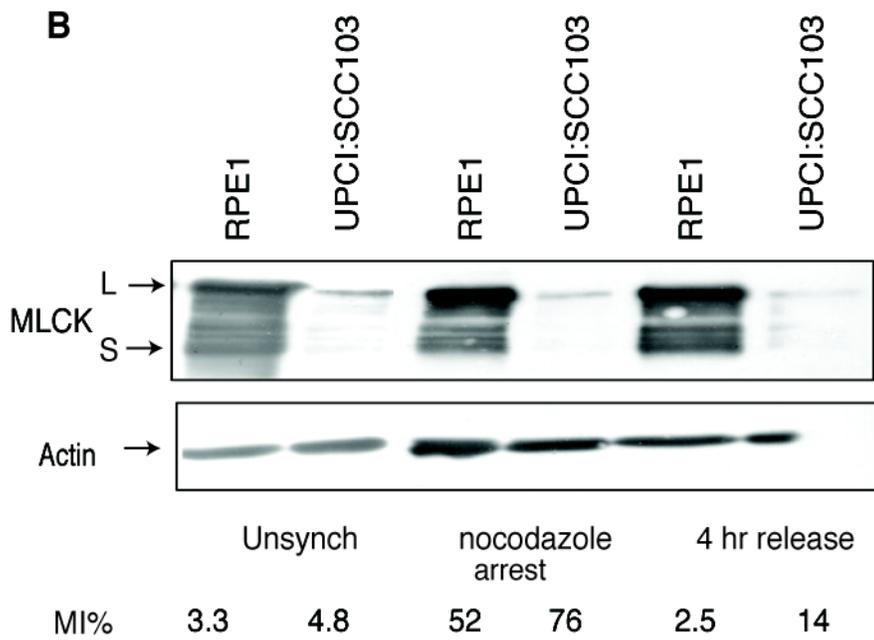
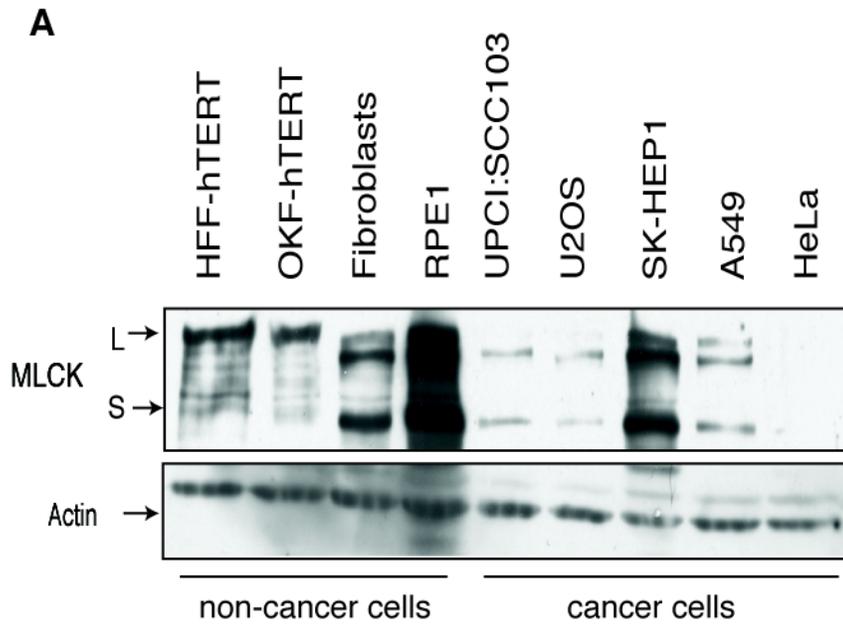


Figure 7. MLCK is expressed at lower levels in cancer cells.

(A) MLCK protein levels in the indicated asynchronous non-cancer and cancer cells were determined by immunoblotting. L and S represent the long and the short isoform of MLCK respectively. Actin was used as a loading control. (B) MLCK expression was compared between cell cycle synchronized RPE1 (non-cancer) and UPCI:SCC103 (cancer) cells. Cells were arrested in mitosis with nocodazole and released for four hours. Different concentrations of nocodazole (400ng/ml for RPE1 and 300ng/ml for UPCI:SCC103 were used to achieve comparable mitotic indices. Bottom row indicates mitotic indices (MI) as percentage of cells in mitosis judged by DAPI staining and epifluorescence microscopy (n > 500).

2.3.2 MLCK activity is inhibited in cancer cells

It is possible that the low abundance of MLCK, as described above, is the cause of deficient MLC phosphorylation and cytokinesis failure in cancer cells. If this is true, exogenous expression of MLCK should increase MLC phosphorylation and rescue cytokinesis defects. To test this possibility we over-expressed a WT MLCK-GFP construct in cancer cell line UPCI:SCC103. The construct was shown to be active and exogenous protein expressed at abundant levels. However surprisingly, in spite of increasing the protein abundance, MLC phosphorylation did not increase and we did not observe a reduction in cytokinesis defects (Wu et al., 2010a). This suggested that in addition to its lower abundance, MLCK kinase activity is inhibited in cancer cells.

To test this hypothesis, I examined MLCK activity by an *in vitro* kinase assay. The assay was performed as described previously using a synthetic peptide substrate and the activity was measured as pMoles of ATP transferred to the substrate (Y axes) over a period of time (X axis) (Poperechnaya et al., 2000). First of all, the specificity of the assay was confirmed. As described in the Introduction, MLCK is a calmodulin-dependent enzyme. When MLCK activity was

measured in the presence and absence of calmodulin, no activity was detected in the absence of calmodulin, indicating the measurement of a calmodulin-dependent enzyme activity (Figure 8A). Kinase activity was also undetectable in the presence of MLCK inhibitor ML-7, which specifically inhibits the catalytic activity of MLCK (Saitoh et al., 1987)(Figure 8B). MLCK is known to autophosphorylate (Foyt and Means, 1985). However, no phosphorylation was detected in the absence of a substrate, ruling out the possibility of MLCK autophosphorylation as a source of the measured counts (Figure 8C). Taken together, all these controls confirm that the *in vitro* kinase assay was specifically measuring MLCK activity and was only detecting peptide substrate phosphorylation.

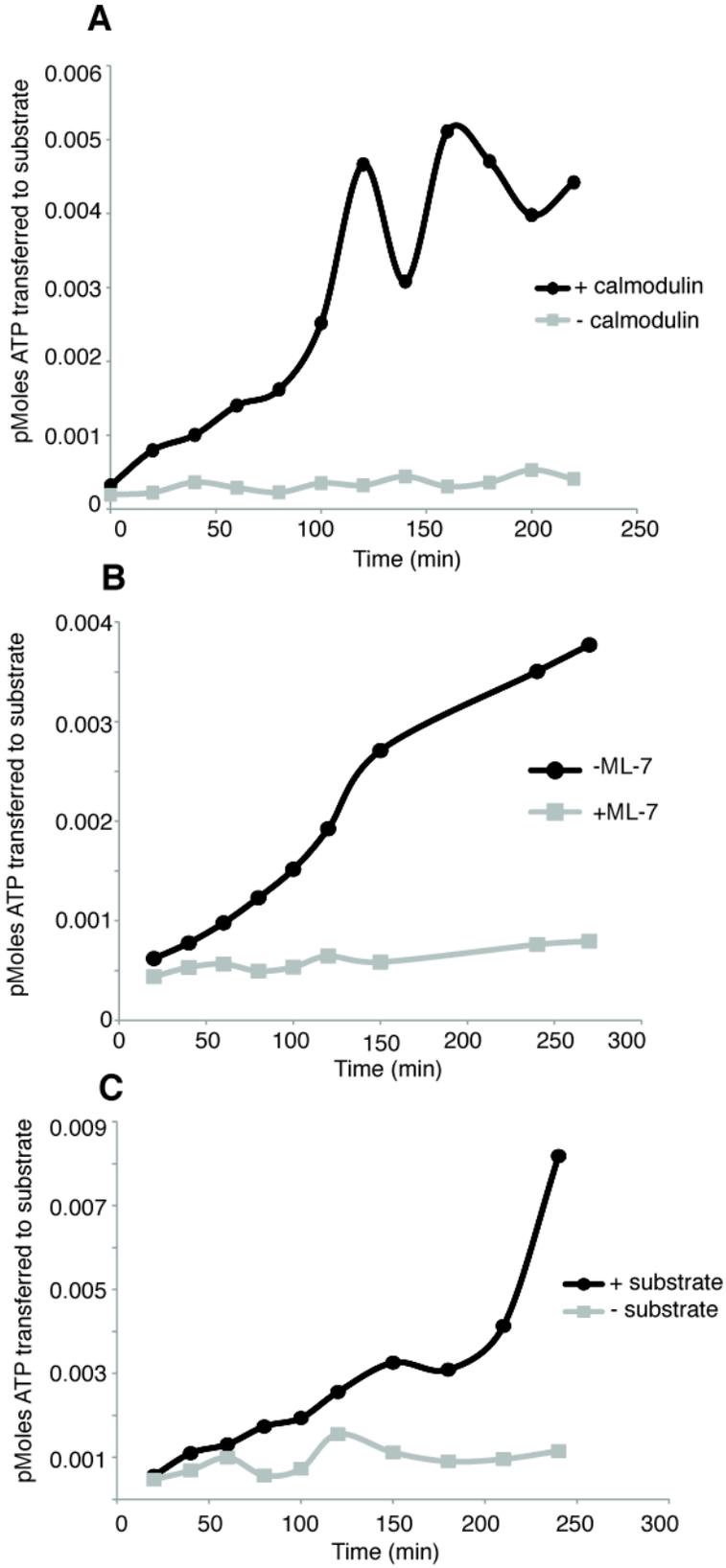


Figure 8. Specificity of the *in vitro* kinase assay.

Specificity of the *in vitro* kinase assay, used to measure MLCK activity, was ensured by three different controls. (A) Kinase assay values were determined in the presence and the absence of calmodulin to ensure the measurement of the activity of a calmodulin dependent enzyme. (B) Kinase assay was performed after the addition of MLCK specific inhibitor ML-7. (C) Kinase assay values were measured in the presence and the absence of a synthetic peptide substrate to rule out the possibility of MLCK autophosphorylation being detected as the phosphorylation counts. In this and all the subsequent kinase assays, Y-axis indicates transfer of a radiolabeled phosphate from ATP to a peptide MLCK substrate and X-axis indicates a time period after the start of the reaction. Irregularities in some data points indicate performer's error as these experiments were performed during the optimization of the kinase assay.

After confirming the specificity of the kinase assay, I measured the kinase activity of MLCK purified from non-cancer and cancer cells. Equal amounts of MLCK were immunoprecipitated from RPE1 and UPCI:SCC103 cells. In this and all the subsequent kinase assay experiments, half of the immunoprecipitates were used for immunoblotting to confirm MLCK immunoprecipitation and the other half was used for the kinase assay. Immunoblots were also quantified using an image-processing program 'Image J' and minor adjustments (<10%) were given to the kinase assay values to account for any differences in the immunoprecipitation efficiencies. Results of the *in vitro* kinase assay indicated that MLCK purified from cancer cells UPCI:SCC103 was less active as compared to MLCK purified from non-cancer cells RPE1 (Figure 9A). Similar results were also obtained for another pair of cancer (U2OS) and non-cancer cells (Fibroblasts) (Figure 9B). These data suggest that MLCK is less active in cancer cells compared to non-cancer cells.

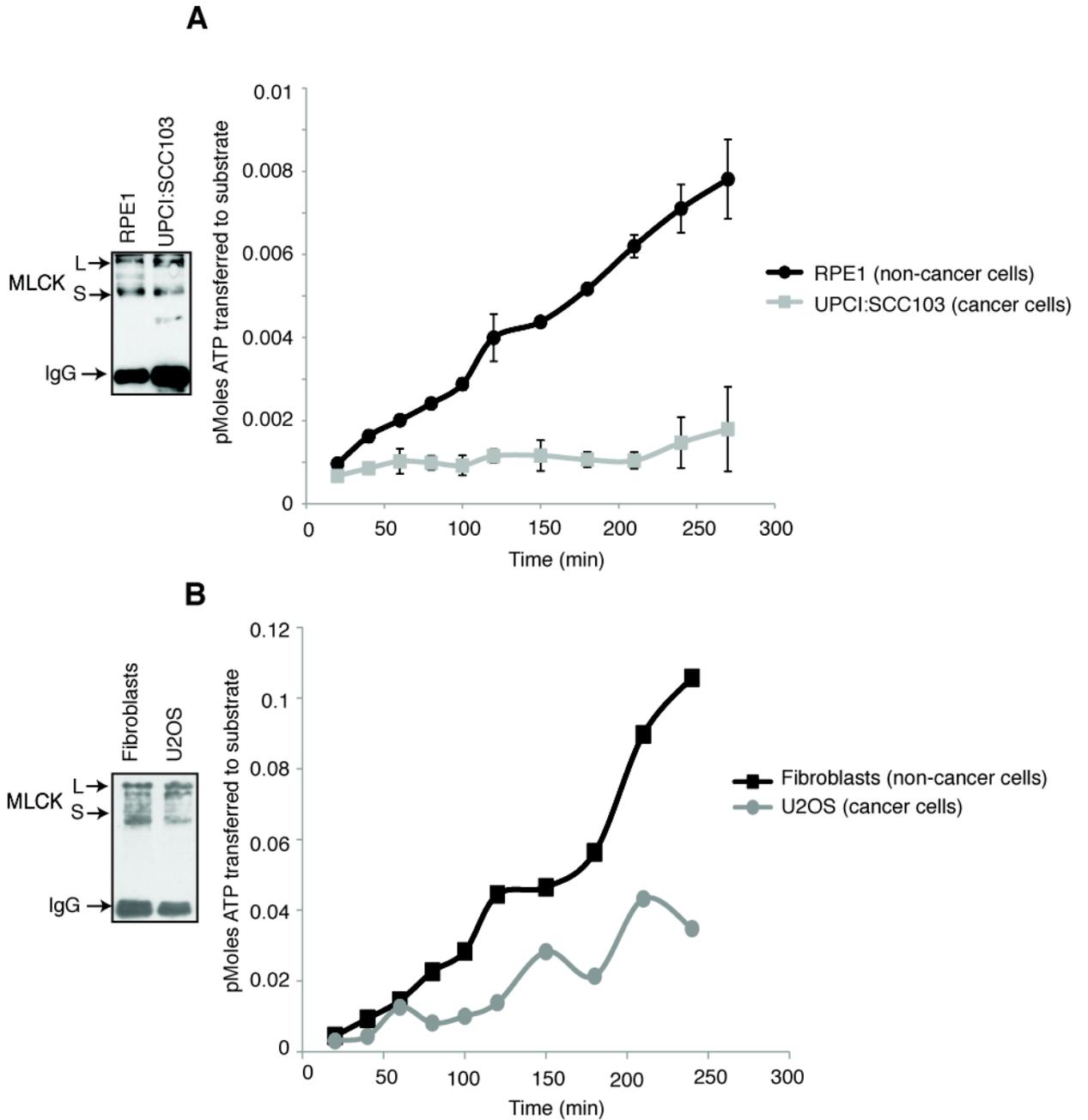


Figure 9. MLCK activity is inhibited in cancer cells.

(A) MLCK activity was compared between asynchronous non-cancer cells (RPE1) and cancer cells (UPCI:SCC103) by the *in vitro* kinase assay. Immunoblot on the left indicates the amount of immunoprecipitated MLCK used in the kinase assay. Immunoblots in this and all the subsequent experiments were also quantified using image-processing program Image J and minor adjustments were

given to the kinase assay values to account for any differences in the immunoprecipitation efficiencies. (B) MLCK activity was compared between another pair of non-cancer cells (Fibroblasts) and cancer cells (U2OS).

However, MLCK activity also differs during the cell cycle. It is lower during early mitosis but increases almost two-fold during anaphase and cytokinesis. The above-mentioned inhibition of MLCK in cancer cells was observed in unsynchronized cell populations. As cancer cells typically have higher mitotic indices, we wanted to rule out the possibility of the observed MLCK activity difference being a result of cell cycle differences. Therefore, I compared MLCK activity in cell cycle synchronized non-cancer and cancer cells. Again, RPE1 and UPCI:SCC103 were chosen as a representative non-cancer and cancer cell lines respectively, for the reasons described previously. Cells were synchronized in mitosis by nocodazole and in S-phase by a double thymidine block. In both instances, MLCK activity remained lower in cancer cells compared to non-cancer cells (Figure 10A and Figure 10B). Taken together, these data support our hypothesis that MLCK is inhibited for its kinase activity in cancer cells. In summary, MLCK is inhibited at two levels in cancer cells; it is expressed at lower levels and is also enzymatically less active.

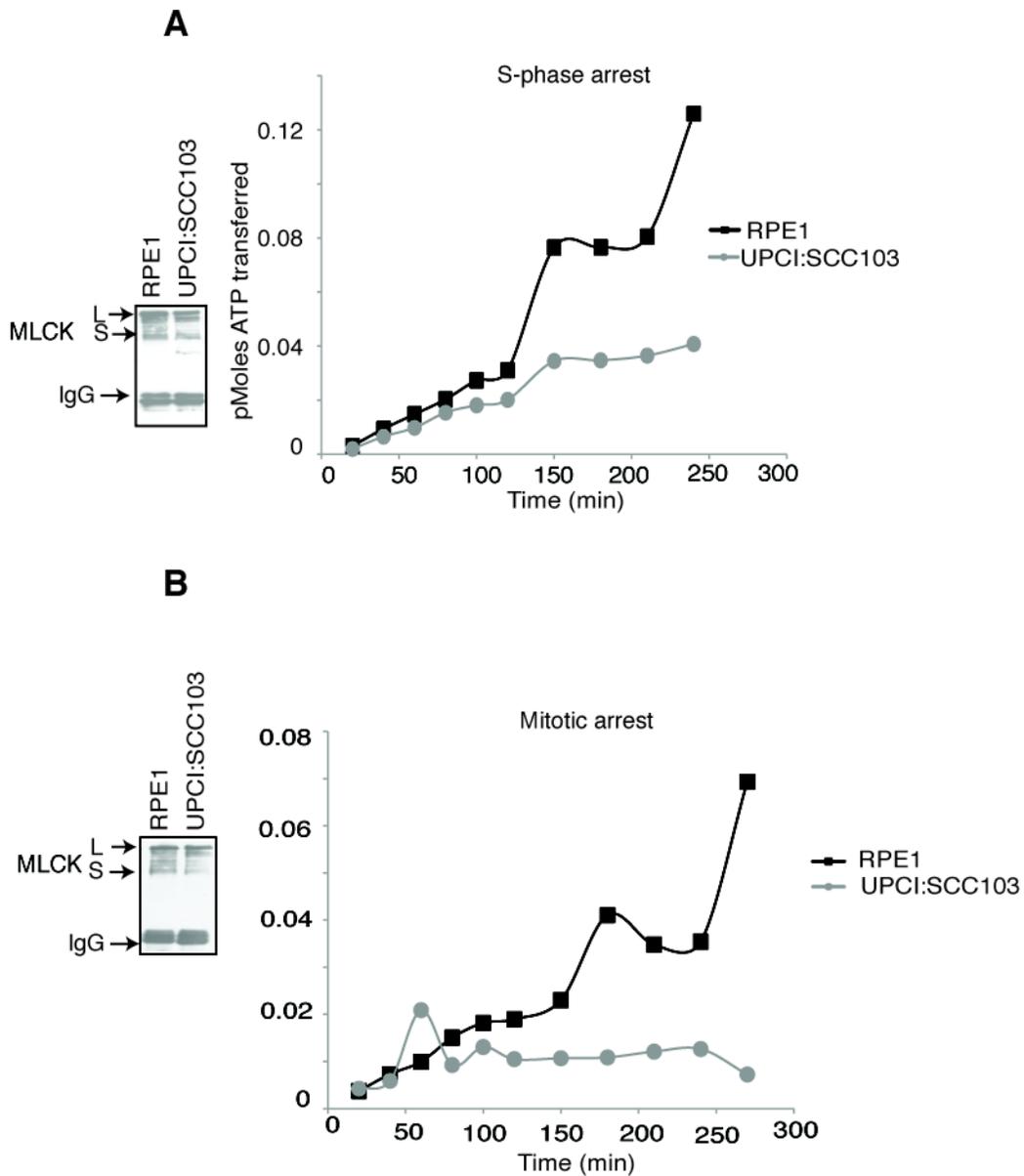


Figure 10. MLCK activity is inhibited in cancer cells independent of the cell cycle differences between non-cancer and cancer cells.

(A) MLCK activity was compared *in vitro* between RPE1 and UPCI:SCC103 cells after arrest in S-phase by a double thymidine block. Left panel shows immunoprecipitation of MLCK from the indicated cell lines used in this assay. (B) MLCK activity was compared *in vitro* between RPE1 and UPCI:SCC103 cells after arrest in mitosis by nocodazole. Left panel indicates immunoprecipitation of MLCK from the indicated cell lines.

2.3.3 MLCK inhibition is the cause of cytokinesis failure in cancer cells

To test if the observed MLCK inhibition was indeed the cause of cytokinesis failure, I examined the frequency of binucleation as an indicator of cytokinesis failure, after expression of a constitutively active MLCK construct in cancer cells. Constitutively active MLCK lacks C-terminal amino acids 1745-1914 that encode an autoinhibitory domain, making the mutant MLCK constitutively active (Wadgaonkar et al., 2003). A kinase dead MLCK mutant that contains a deletion of an ATP binding site was used as a control (Wadgaonkar et al., 2003). Expression of both the mutant constructs was confirmed by immunoblotting (Figure 11A and Figure 11B).

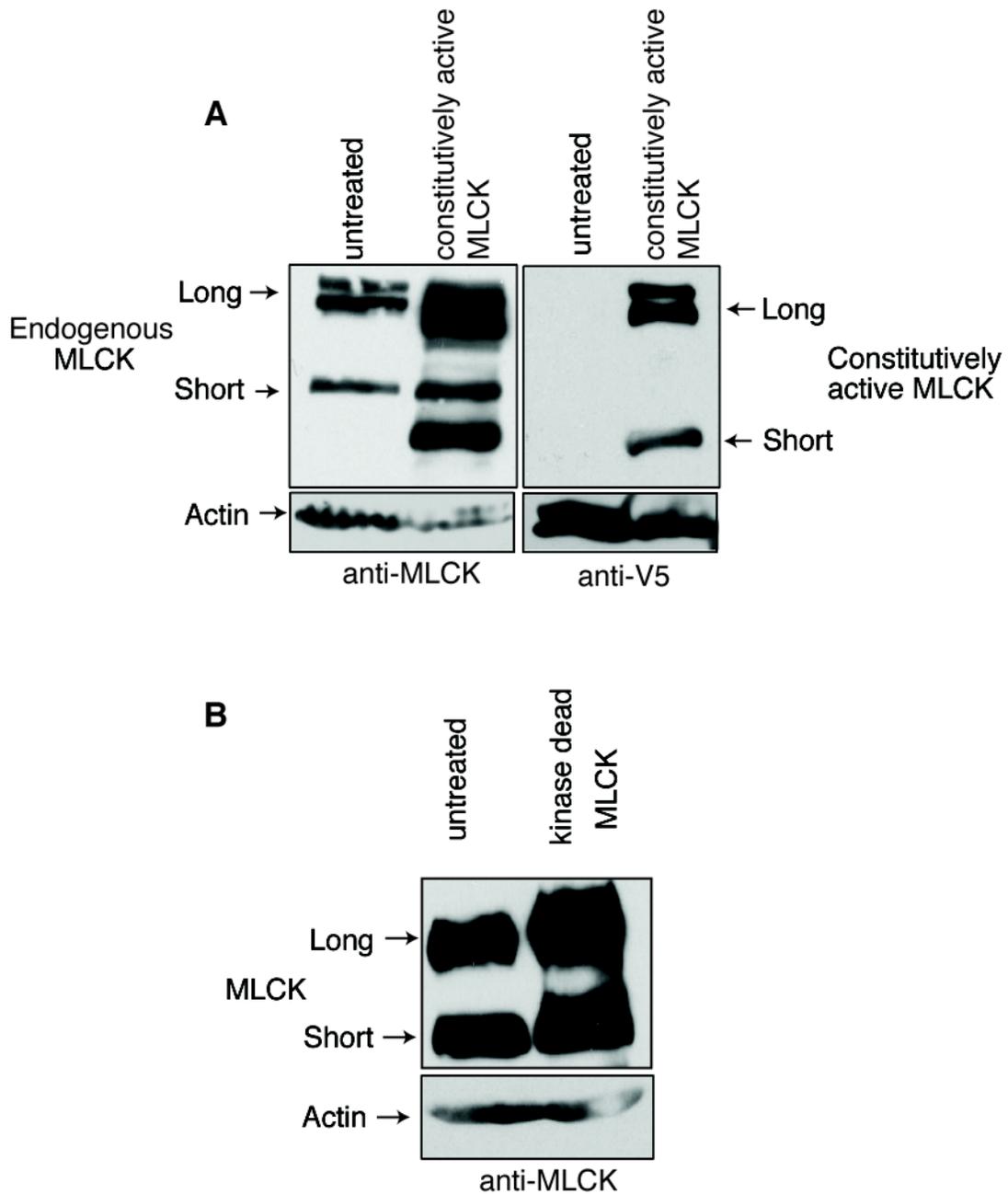


Figure 11. Expression of a constitutively active and a kinase dead MLCK construct is confirmed by immunoblotting.

(A) UPCI:SCC103 cells were transiently transfected with a V5 tagged constitutively active MLCK construct that contains a deletion of an autoinhibitory domain consisting of amino acids 1745-1914.

Immunoblots were probed using anti-MLCK antibody (left panel) and anti-V5 antibody (right panel). Only endogenous MLCK is seen in untreated cells, whereas along with endogenous MLCK, shorter fragments of the long and the short isoform, corresponding to the above-mentioned deletion are detected in cells transfected with a constitutively active MLCK. Both the shorter fragments are also detected with anti-V5 antibody confirming their expression from the mutant plasmid. (B) UPCI:SCC103 cells were transiently transfected with a kinase dead MLCK construct that contains a 27 amino acid deletion of the ATP binding site. It is difficult to detect a change in 27 amino acids as a shift in the molecular weight of the long and the short isoforms. However, an increase in the protein levels of both the isoforms suggests successful expression of the mutant construct.

Expression of a constitutively active MLCK construct, but not a kinase dead construct resulted in a significant decrease in the frequency of cytokinesis failure after two rounds of transfection in all the cancer cell lines tested (Figure 12) ($p < 0.05$ for all the cancer cell lines tested). These results suggest that inhibition of MLCK is a cause of cytokinesis failure in the tested cancer cell lines.

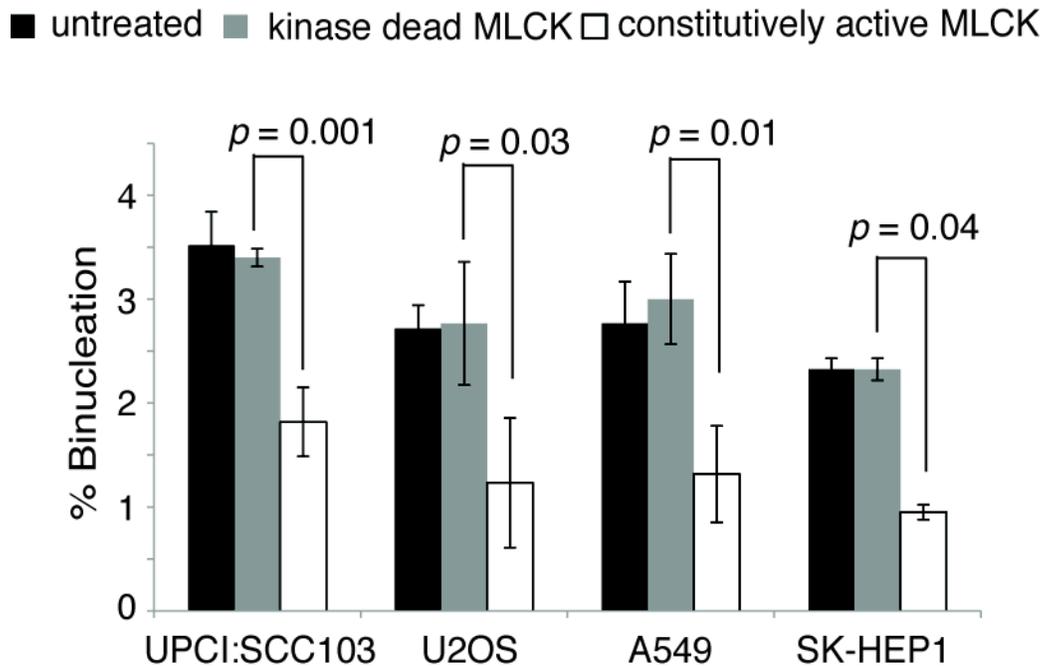


Figure 12. MLCK inhibition is a cause of cytokinesis failure in cancer cells.

Binucleation frequency was measured in the indicated cancer cell lines that were either untreated or were transfected with a kinase dead or a constitutively active MLCK constructs. In this and all the

subsequent experiments, binucleation frequency was determined by DAPI staining and epifluorescence microscopy (n=500-1000 cells for each experiment). Standard deviation between three independent experiments is shown as error bars. *p* value is calculated using a Student's-t test and $p < 0.05$ indicates a statistically significant difference.

2.4 OVER-EXPRESSION OF AURORA B KINASE IS THE CAUSE OF MLCK INHIBITION AND CYTOKINESIS FAILURE

After identifying MLCK inhibition as a cause of cytokinesis failure in cancer cells, our next goal was to identify the cause of MLCK inhibition. Differentially phosphorylated serine residues in MLCK are the consensus sites for Aurora B kinase phosphorylation. Furthermore, Aurora B can phosphorylate MLCK *in vitro*, but the consequences of this phosphorylation are not known. Considering the essential role of Aurora B during cytokinesis, well documented over-expression of Aurora B in a variety of tumor tissues and the tumorigenic potential of Aurora B over-expression, I hypothesized that Aurora B kinase is an inhibitor of MLCK and over-expression of Aurora B is the cause of cytokinesis failure in cancer cells. I tested this hypothesis by three different approaches. Firstly, MLCK activity was measured *in vitro* in the absence and the presence of purified Aurora B. Secondly, Aurora B was over-expressed in non-cancer cells to test if it was sufficient to inhibit MLCK and cause cytokinesis failure. And thirdly, Aurora B protein levels were reduced in cancer cells to test if it could increase MLCK activity and reverse cytokinesis failure of cancer cells.

2.4.1 Aurora B can inhibit MLCK *in vitro*

To test if Aurora B kinase was indeed an inhibitor of MLCK, I examined MLCK activity after addition of purified Aurora B kinase to an *in vitro* MLCK assay. Consistent with our hypothesis, addition of purified Aurora B resulted in a significant decrease in MLCK activity and suggested that MLCK could be inhibited directly by Aurora B (Figure 13).

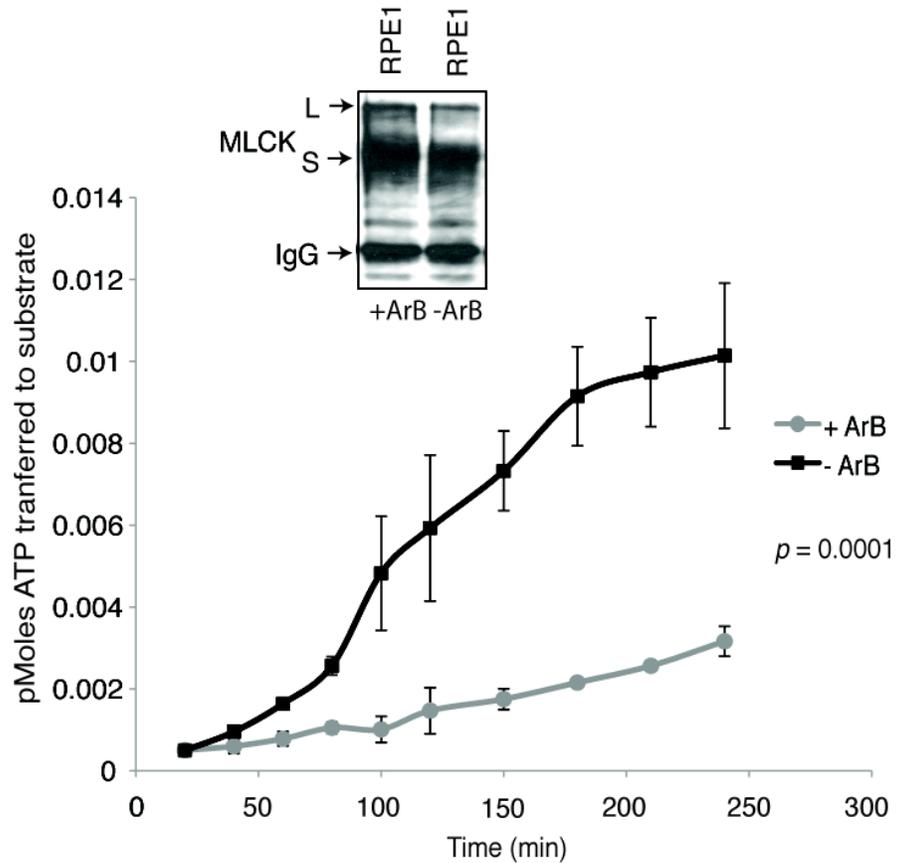


Figure 13. Aurora B inhibits MLCK *in vitro*.

MLCK was immunoprecipitated from RPE1 cells and *in vitro* MLCK activity was determined in the presence (+ArB) and the absence (-ArB) of added Aurora B. Immunoblot indicates MLCK immunoprecipitation used for two reactions. Standard deviation is shown as error bars that are present for all the data points. *p* value was calculated using a t-test for independent samples.

2.4.2 Aurora B over-expression in non-cancer cells recapitulates MLCK inhibition in cancer cells

To validate *in vitro* inhibition of MLCK by Aurora B in cultured cells, Aurora B kinase was over-expressed in a non-cancer cell line Human foreskin fibroblasts immortalized by expression of human telomerase reverse transcriptase (hTERT) known as HFF-hTERT. Considering the elimination of tetraploid cells in a p53 dependent manner, Aurora B was stably over-expressed on a WT and a p53 null background. Hereafter these cells will be named as follows: HFF+vector refers to HFF-hTERT expressing empty vector alone. HFF+ArB refers to HFF-hTERT stably over-expressing Aurora B on WT p53 background. HFFshp53 refers to HFF-hTERT with a stable shRNA mediated knock-down of p53 and HFFshp53+ArB refers to HFF-hTERT with a stable knock-down of p53 and stable over-expression of Aurora B. Aurora B over-expression was confirmed in the indicated cell lines by immunoblotting (Figure 14).

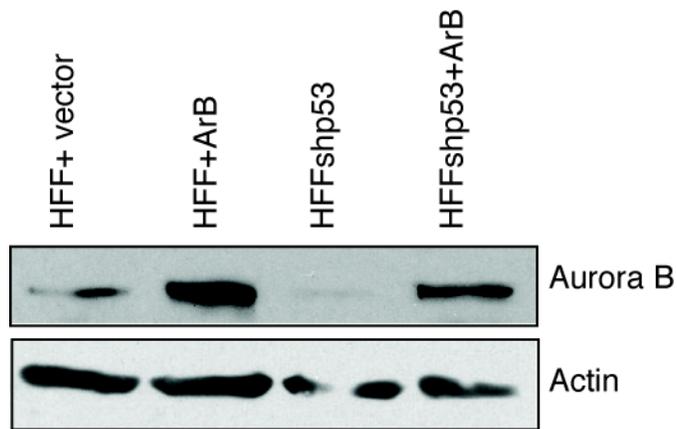


Figure 14. Aurora B over-expression in non-cancer cells is confirmed by immunoblotting.

Aurora B protein levels in non-cancer cell line HFF-hTERT, stably transfected with the indicated plasmids were determined by immunoblotting. Actin was used a loading control.

I first examined the protein levels of MLCK in all the above-mentioned cell lines. Interestingly, MLCK was expressed at lower levels only in HFFshp53+ArB cell line, indicating that down-regulation of MLCK expression can be recapitulated by over-expression of Aurora B in non-cancer cells (Figure 15A).

To determine if Aurora B could inhibit MLCK activity, I compared the *in vitro* activity of MLCK immunopurified from HFFshp53 control and HFFshp53+ArB cells. Consistent with the MLCK enzymatic inhibition observed in cancer cells, MLCK immunopurified from Aurora B over-expressing HFF cells was less active compared to control cells (Figure 15B). Together, these data suggests that Aurora B over-expression in non-cancer cells is sufficient to down-regulate MLCK expression and activity.

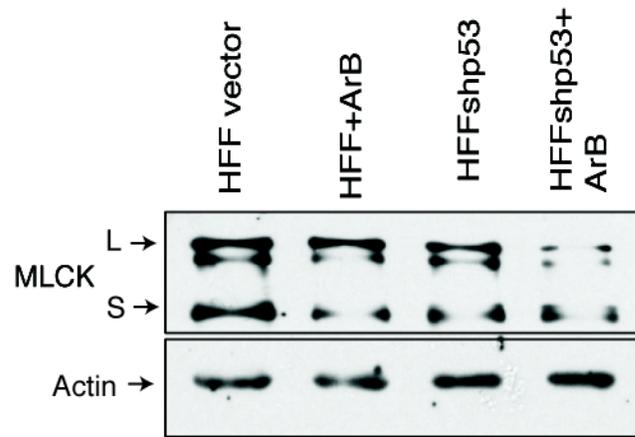
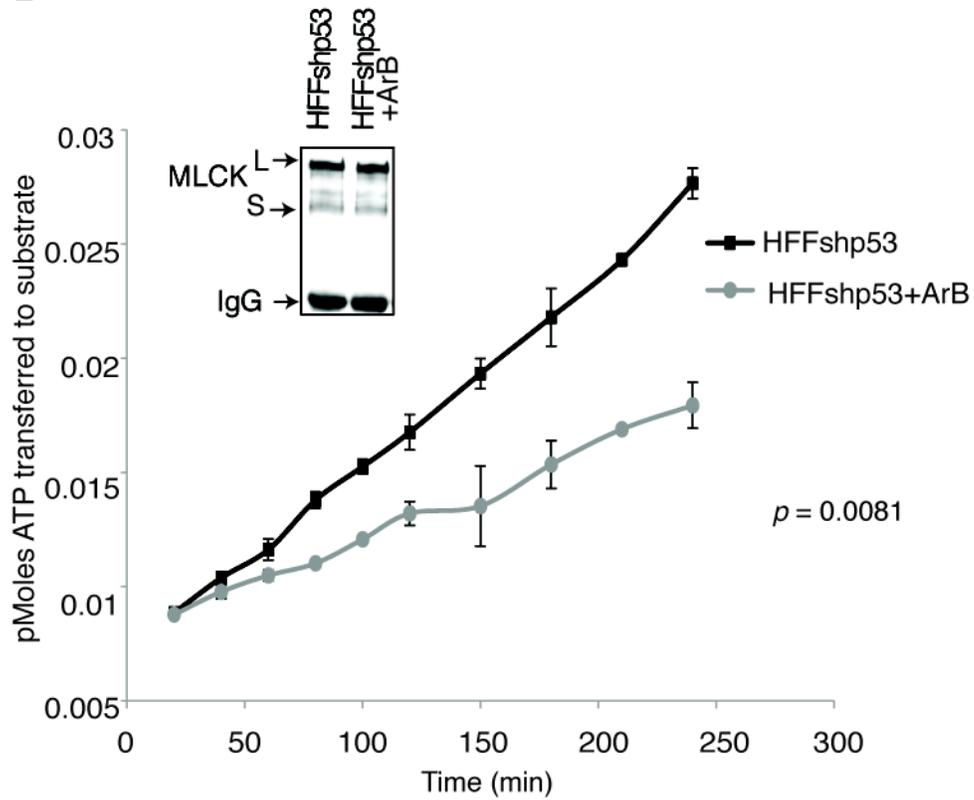
A**B**

Figure 15. Over-expression of Aurora B in non-cancer cells recapitulates MLCK defects of cancer cells.

(A) MLCK expression was compared between HFF-hTERT cells with a stable over-expression of empty vector control (HFF+vector), over-expression of Aurora B (HFF+ArB), stable knock-down of p53 (HFFshp53) and knock-down of p53 plus over-expression of Aurora B (HFFshp53+ArB). L and S represent the long and the short isoforms of MLCK respectively. Actin was used as a loading control. (B) MLCK activity is compared between HFFshp53 and HFFshp53+ArB cell line by *in vitro* kinase assay. Immunoblot in (B) indicates immunoprecipitation of MLCK from the indicated cell lines.

2.4.3 Aurora B over-expressing cells fail in cytokinesis as a result of MLCK inhibition and deficient MLC phosphorylation

Over-expression of Aurora B is a known cause of tetraploidy (Tatsuka et al., 1998; Terada et al., 1998). The above-mentioned data suggest that Aurora B over-expression in non-cancer cells results in MLCK inhibition. MLC is the only known substrate of MLCK and we have demonstrated that MLCK inhibition and deficient MLC phosphorylation are the causes of cytokinesis failure. Based on these data, I hypothesized that Aurora B over-expressing cells fail at cytokinesis as a result of MLCK inhibition and the resultant deficient MLC phosphorylation. In order to test this hypothesis, I firstly confirmed if HFF-hTERT cells over-expressing Aurora B fail in cytokinesis, as has been observed for other cell lines used in previously published studies. In agreement with the published literature, I observed an increase in the frequency of binucleation or cytokinesis failure in Aurora B over-expressing HFF cells. Consistent with the role of p53 in limiting the survival of tetraploid cells and observed MLCK down-regulation, an increase in the binucleation frequency was observed only in the cell line over-expressing Aurora B in a p53 null background (Figure 16A). Next, to test if the observed cytokinesis failure was a result of MLCK inhibition and deficient MLC phosphorylation, I examined binucleation frequency in HFFshp53+ArB cell line after transient transfection of a phosphomimetic MLC (GFP-MLCDD) and a constitutively active MLCK construct. In contrast to empty GFP vector or kinase dead MLCK controls, expression of phosphomimetic MLC or constitutively active MLCK resulted in a significant decrease in the binucleation frequency in HFFshp53+Aurora B cell line (Figure 16B).

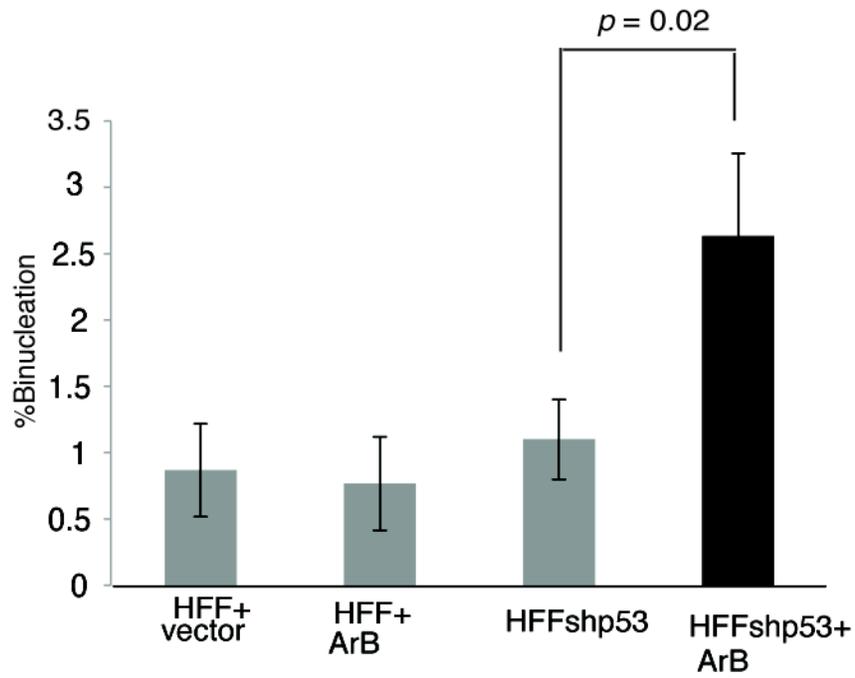
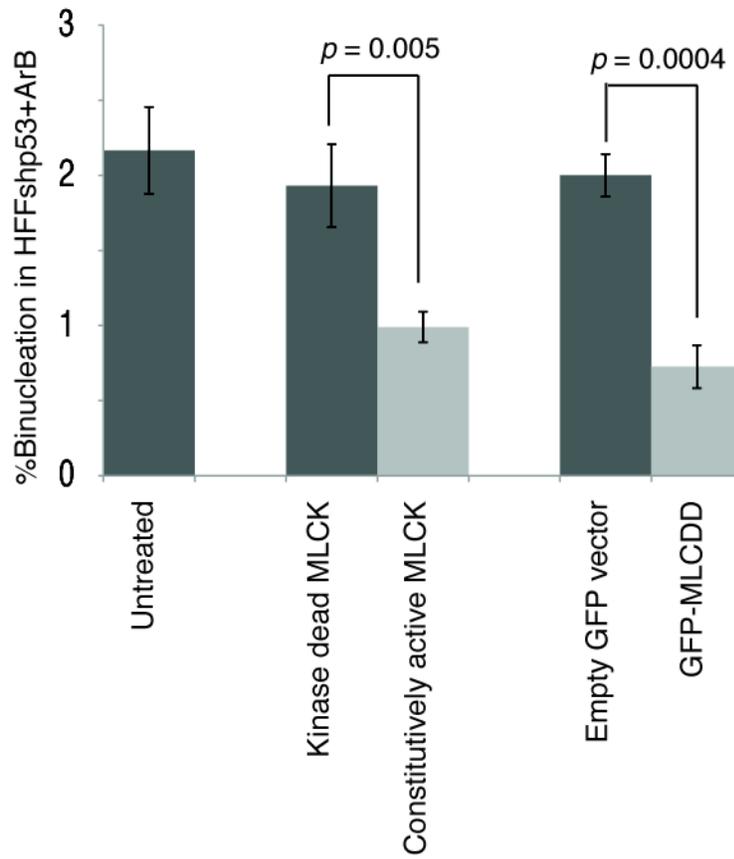
A**B**

Figure 16. Aurora-B induced cytokinesis failure is mediated through MLCK inhibition and a reduction in MLC phosphorylation.

(A) Binucleation frequency was determined in the indicated HFF cell lines. (B) Binucleation frequency was measured in HFFhsp53+ArB cell line after a transient transfection of a kinase dead MLCK or a constitutively active MLCK construct and an empty GFP vector control or a phosphomimetic GFP (GFP-MLCDD) construct.

In summary so far, we have demonstrated that Aurora B can inhibit MLCK directly. In cultured non-cancer cells, over-expression of Aurora B is sufficient to down-regulate protein levels and kinase activity of MLCK and Aurora B over-expressing non-cancer cells fail in cytokinesis because of MLCK inhibition and deficient MLC phosphorylation.

2.5 AURORA B OVER-EXPRESSION IS THE CAUSE OF CYTOKINESIS FAILURE IN CANCER CELLS

2.5.1 Cancer cells over-express Aurora B

My next goal was to investigate if Aurora B over-expression was indeed the cause of observed MLCK inhibition and cytokinesis failure in cancer cells. As demonstrated by many studies, Aurora B over-expression is one of the common characteristics of a variety of tumor tissues (Chieffi et al., 2006; Lin et al., 2006; Qi et al., 2007; Sorrentino et al., 2005). Therefore I wanted to confirm if it was true for the cancer cell lines used in this study as well. I firstly compared Aurora B protein levels between unsynchronized non-cancer and cancer cell lines by immunoblotting. Consistent with the previous published literature, Aurora B was expressed at higher levels in all the cancer cell lines tested (Figure 17A). However, Aurora B protein levels vary during cell cycle. Considering its multi-dimensional role during mitosis, Aurora B levels peak at G2-M transition (Bischoff et al., 1998). As cancer cells have higher mitotic indices, it is often debated whether elevated Aurora B levels seen in unsynchronized cancer cells are merely a reflection of increased mitotic indices and not a real pathological feature of cancer cell populations (Nikiforov, 2005). To address this concern, I compared Aurora B protein levels in three different pairs of non-cancer and cancer cells after synchronizing them in mitosis with nocodazole. It was essential to treat different cell lines with different concentrations of nocodazole as described in the Methods section (Chapter 4, section 4.7.2), to achieve comparable mitotic indices. Comparison of matched pair of non-cancer and cancer cell lines demonstrated that cancer cells had markedly higher Aurora B protein levels even at similar mitotic frequencies. Induction of higher frequency of mitotic indices such as in RPE1 cells, minimized the difference

in Aurora B levels as expected, but it is important to note that unsynchronized RPE1 cells still expressed Aurora B at lower levels compared to other cancer cells (Figure 17B).

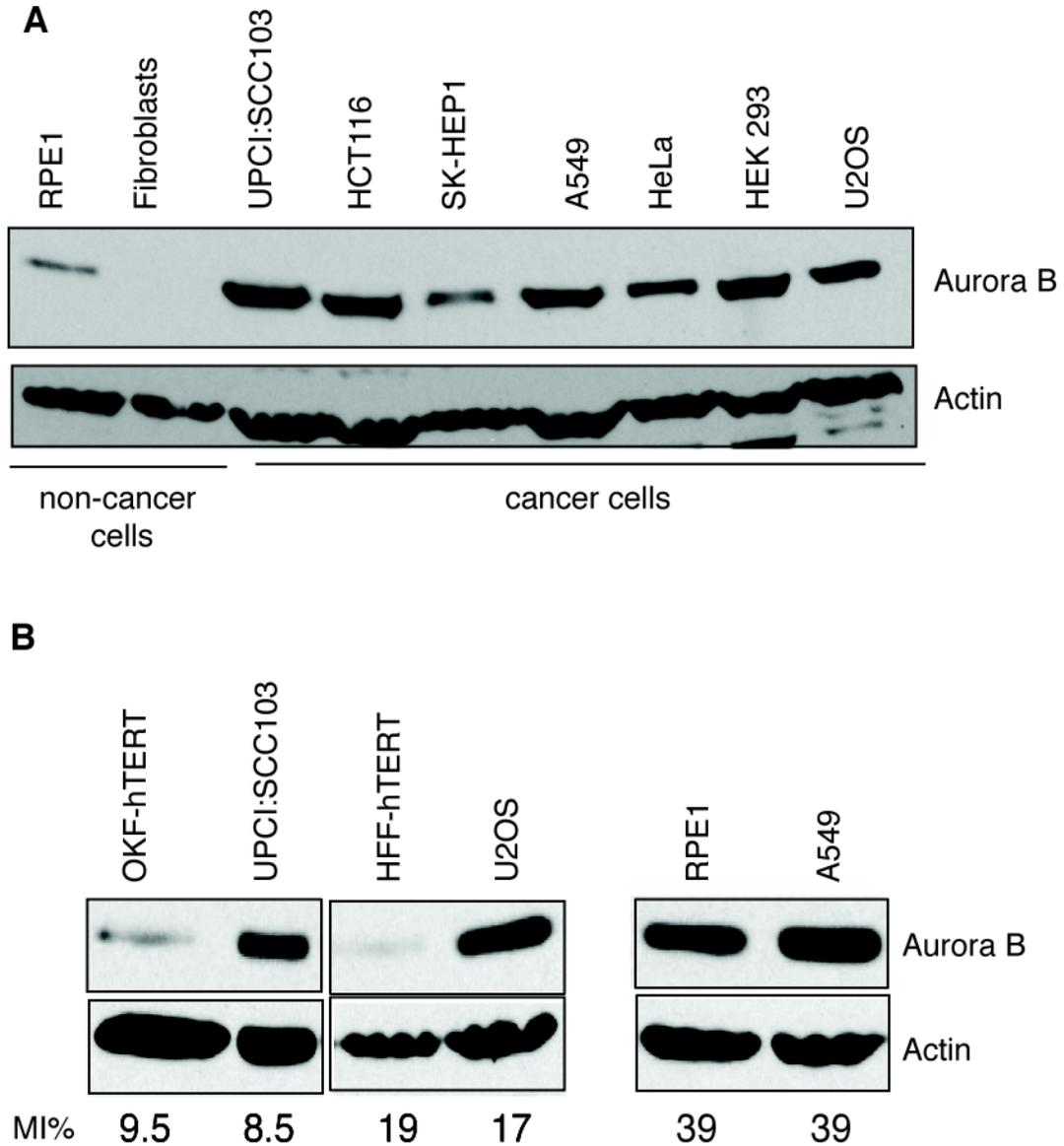


Figure 17. Aurora B is over-expressed in cancer cells independent of the differences in mitotic indices.

(A) Aurora B protein levels were compared by immunoblotting between asynchronous non-cancer and cancer cells. Actin was used as a loading control. (B) Aurora B expression was compared between three different pairs of non-cancer and cancer cells after achieving comparable mitotic indices from mitotic arrest

with nocodazole. OKF-hTERT, HFF-hTERT and RPE1 are non-cancer cells whereas UPCI:SCC103, U2OS and A549 are cancer cells. MI represents mitotic indices.

2.5.2 Over-expression of Aurora B kinase is the cause of cytokinesis failure in cancer cells

Aurora B over-expression results in cytokinesis failure and tetraploidization (Tatsuka et al., 1998; Terada et al., 1998). In contrast, complete depletion of Aurora B levels either by siRNA or inhibition of its kinase activity by chemical inhibitors also results in the formation of tetraploid or polyploidy cells (Ditchfield et al., 2003; Tsuno et al., 2007). Therefore it appears that ‘optimal’ levels of Aurora B are required for successful cytokinesis such that imbalance in Aurora B levels on either the higher or lower side of the ‘optimal’ level causes cytokinesis failure. To test this possibility, I counted the frequency of binucleation after complete or partial knock-down of Aurora B using siRNA. To rule out the possibility of any off-target effects of RNAi mediated silencing, two different siRNAs were used to knock-down Aurora B. siRNA # 1 targeted the N-terminal region of Aurora B mRNA and siRNA # 2 targeted the C-terminal region. Aurora B has a very high sequence similarity with Aurora A. Aurora A is a known oncogene and mis-regulation of Aurora A can also cause cytokinesis defects (Marumoto et al., 2003). Considering the high sequence homology between Aurora B and Aurora A, non-specific targeting of Aurora A mRNA by Aurora B siRNA is a common concern. Therefore, we examined Aurora A protein levels after Aurora B knock-down and found them to be unaltered, thus ruling out the possibility of non-specific knock-down of Aurora A by Aurora B siRNA (Figure 18).

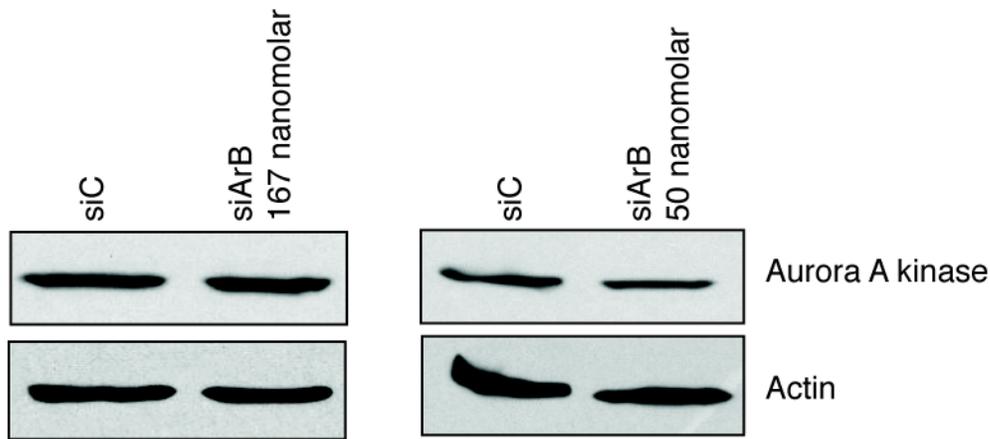


Figure 18. Aurora B siRNA does not have an off-target affect on Aurora A mRNA.

Aurora A kinase protein levels were measured after transfection of U2OS cells with 167 and 50 nanomolar concentration of Aurora B siRNA for 24 hours respectively. Actin was used as a loading control.

After confirming the specificity of Aurora B siRNAs, I completely knocked-down Aurora B protein levels in cancer cell line U2OS using both siRNAs. To achieve complete knock-down of Aurora B, cells were treated cells with 167 nanomolar of siRNA for 48 and 24 hours. Consistent with the published literature, a complete knock-down of Aurora B resulted in a significant increase in the binucleation frequency (Figure 19A leftmost and middle panels). As expected, 48 hours of knock-down resulted in a greater increase in the binucleation compared to 24 hours of complete knock-down. To test our hypothesis that optimal levels of Aurora B are required for cytokinesis, I then partially knocked-down Aurora B protein levels in U2OS cells by treating them with 50 nanomolar of siRNA for 24 hours. Consistent with our prediction, a partial knock-down of Aurora B resulted in a significant decrease in the binucleation frequency (Figure 19A, rightmost panel). Similar results were obtained with the use of siRNA # 2 as well (Figure 19B)

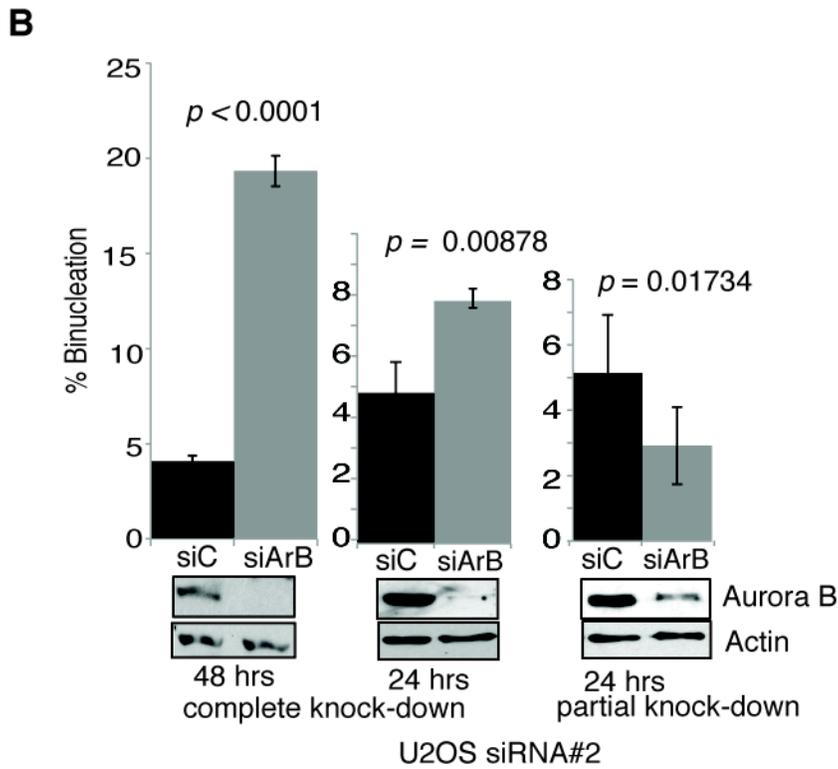
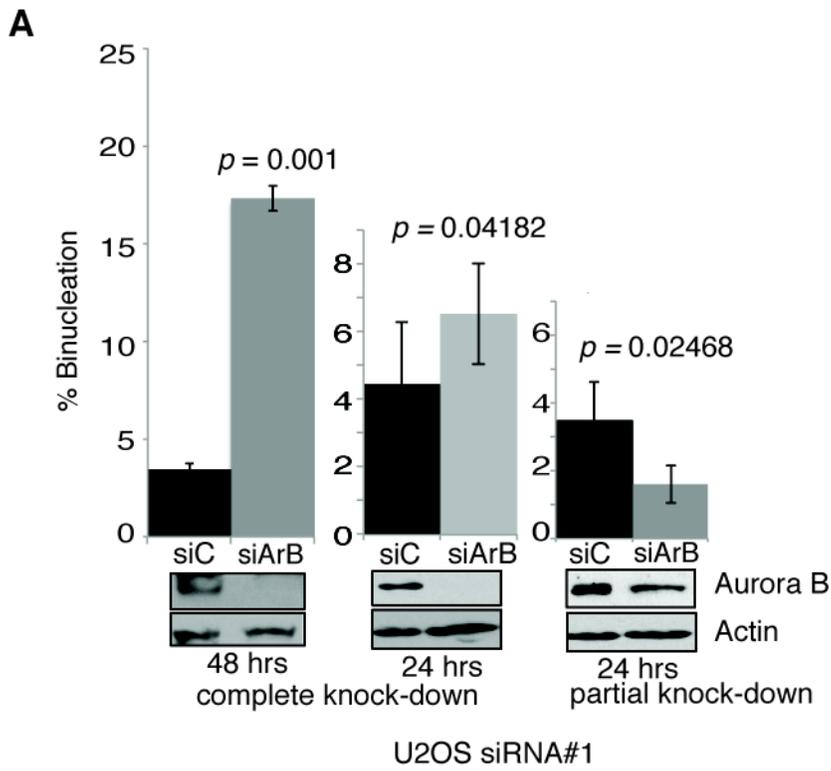


Figure 19. A partial knock down of Aurora B reduces cytokinesis failure in cancer cells U2OS.

(A) Binucleation frequency was compared between control siRNA (siC) and Aurora B siRNA #1 (siArB) treated U2OS cells. Leftmost and middle panel show the data obtained from 48 and 24 hours of complete knock-down of Aurora B respectively, achieved with the use of 167 nanomolar of siRNA. Rightmost panel shows the data obtained from 24 hours of a partial knock-down of Aurora B achieved with 50 nanomolar of Aurora B siRNA. Bottom panels demonstrate immunoblots of Aurora B protein levels after respective knock-downs. (B) Similar analysis as in (A) was performed in U2OS cells using a second non-overlapping siRNA against Aurora B (siRNA # 2)

This hypothesis was also confirmed using oral squamous cell carcinoma cell line UPCI:SCC70 (Figure 20A and Figure 20B).

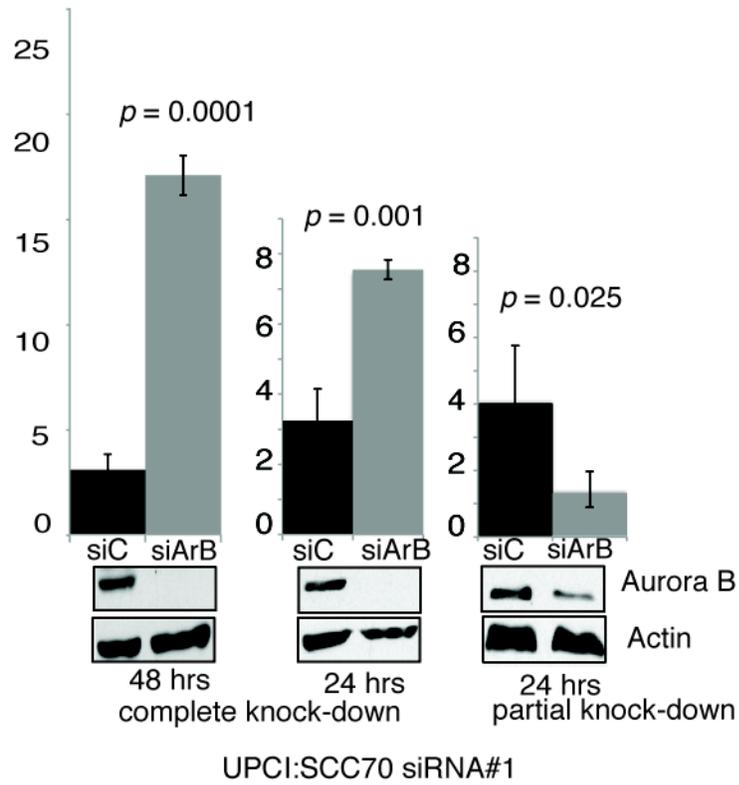
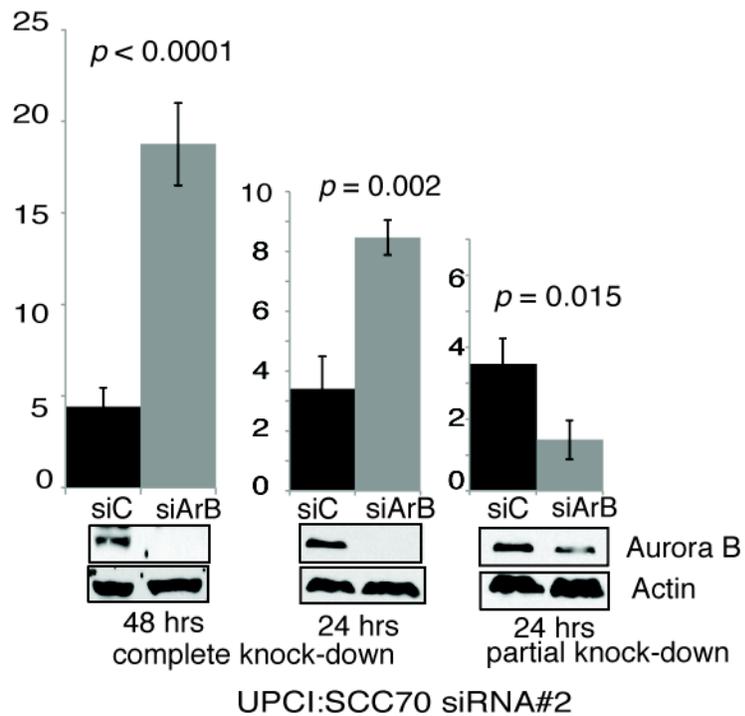
A**B**

Figure 20. Cytokinesis failure is reduced after a partial knock-down of Aurora B protein levels in cancer cells UPCI:SCC70.

(A) Binucleation frequency was compared between control siRNA (siC) and Aurora B siRNA # 1 (siArB) treated oral squamous cell carcinoma cells UPCI:SCC70. Data obtained after 48 and 24 hours of complete knock-down of Aurora B is indicated in the leftmost and the middle panel respectively. Data obtained after 24 hours of a partial knock-down of Aurora B protein levels is demonstrated in the rightmost panel. Bottom panels are the immunoblots of Aurora B protein levels in the indicated knock-downs. (B) Similar analysis as in (A) was performed using siRNA # 2 in UPCI:SCC70 cells.

Taken together, these data tells us that Aurora B over-expression is a cause of cytokinesis failure in cancer cells and a partial knock-down of Aurora B protein levels reduces the frequency of cytokinesis failure, supporting the idea that optimal levels of Aurora B are required for successful cytokinesis.

2.5.3 A partial knock-down of Aurora B in cancer cells increases MLCK activity and expression

The data presented so far suggests that Aurora B over-expression is the cause of cytokinesis failure in cancer cells. We hypothesize that the observed cytokinesis failure in Aurora B over-expressing cancer cells occurs due to inhibition of MLCK. To validate this hypothesis, we examined MLCK activity in U2OS cancer cells after knock-down of Aurora B. As we observed a reduction in cytokinesis failure in cancer cells after knocking-down Aurora B to a partial levels, we measured MLCK activity at the similar level of Aurora B knock-down, achieved by treating cells with 50 nanomolar of siRNA for 24 hours. Corresponding to a decrease in the binucleation frequency, an increase in MLCK activity was observed after a partial knock-down of Aurora B

(Figure 21A). Similarly, MLCK expression also increased after a partial knock-down of Aurora B (Figure 21B). Thus both the parameters of MLCK inhibition in cancer cells i.e. decreased expression and reduced kinase activity were reversed upon decreasing Aurora B protein levels. These data provided further evidence that Aurora B over-expression results in cytokinesis failure because of the inhibition of MLCK.

U2OS siRNA#1 MLCK activity

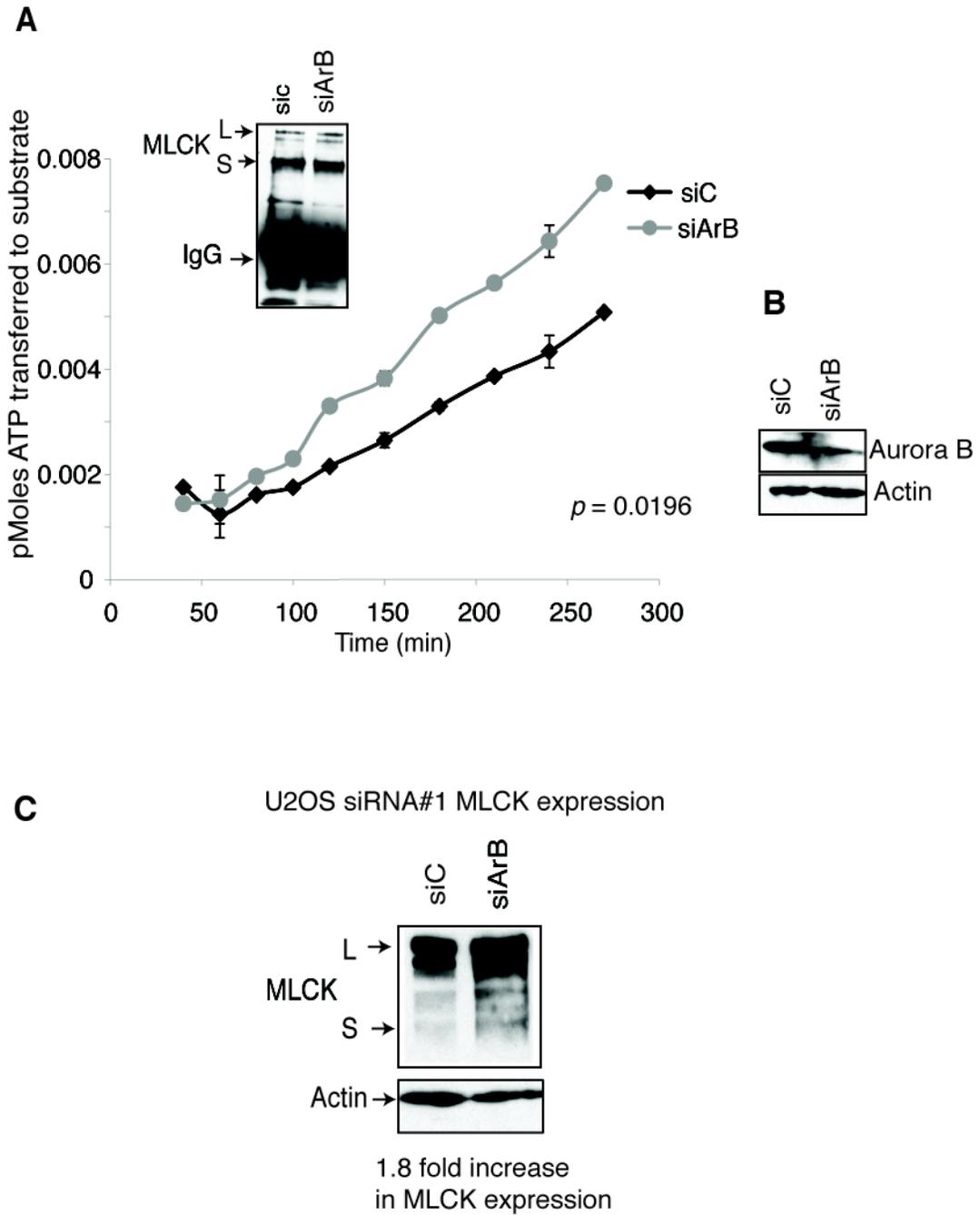


Figure 21. A partial knock-down of Aurora B with siRNA # 1 increases MLCK activity and protein levels in cancer cells U2OS.

MLCK *in vitro* activity (A) and MLCK expression (C) was compared between control siRNA (siC) and Aurora B siRNA #1 (siArB) treated U2OS cells. Left panel of immunoblot in (A) demonstrates

immunoprecipitation of MLCK from siC and siArB treated U2OS cells that were used in the kinase assay. Immunoblot in (B) demonstrates a partial knock-down of Aurora B observed in this experiment. Actin was used as a loading control. For some data points errors bars are very small and therefore not visible.

Similar results were obtained using a second siRNA against Aurora B in U2OS cells (Figure 22 A and Figure 22B) and a different cancer cell line UPCI:SCC70 (Figure 23A). In summary, these data confirms that Aurora B over-expression is a cause of MLCK inhibition and resultant cytokinesis failure in cancer cells

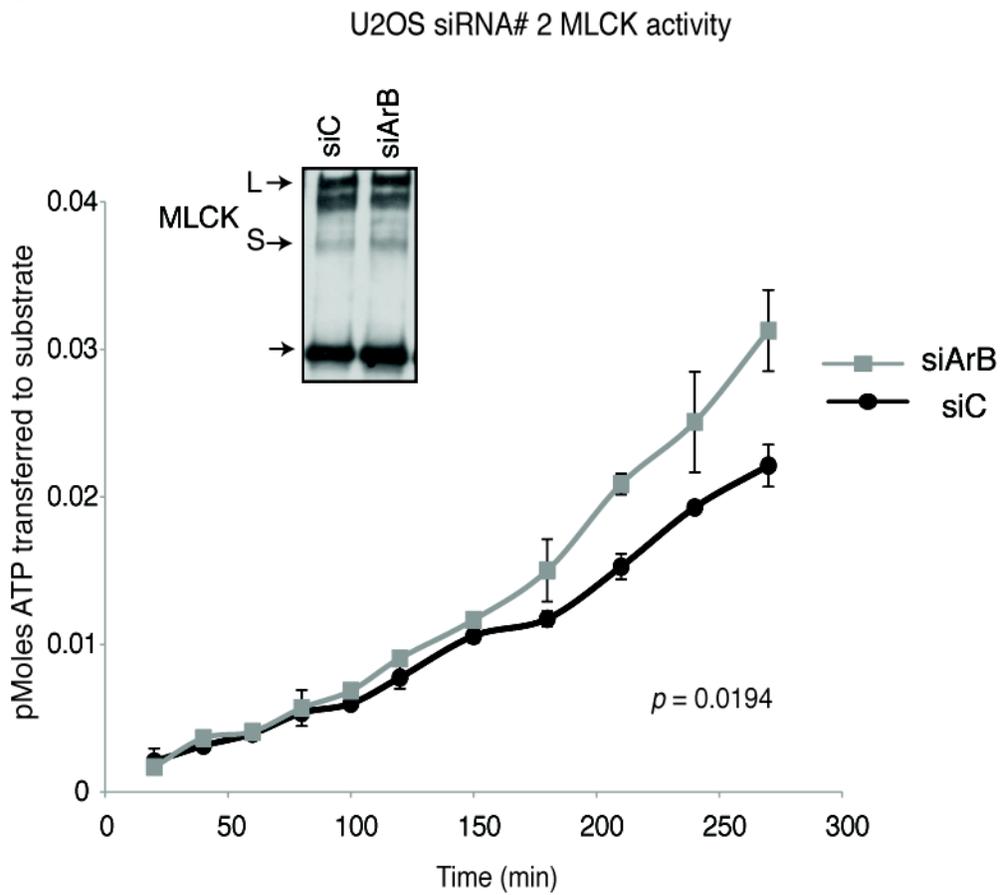
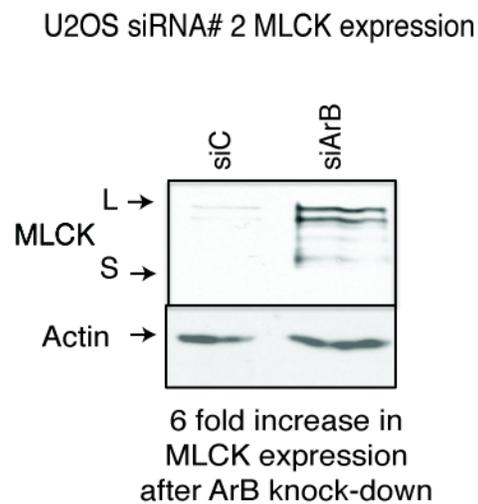
A**B**

Figure 22. A partial knock-down of Aurora B with siRNA # 2 also increases MLCK activity and expression in U2OS cells.

MLCK *in vitro* activity (A) and MLCK protein levels (B) were measured in control siRNA (siC) and Aurora B siRNA # 2 (siArB) treated U2OS cells. Immunoblot in (A) demonstrates MLCK immunoprecipitation from the indicated cells.

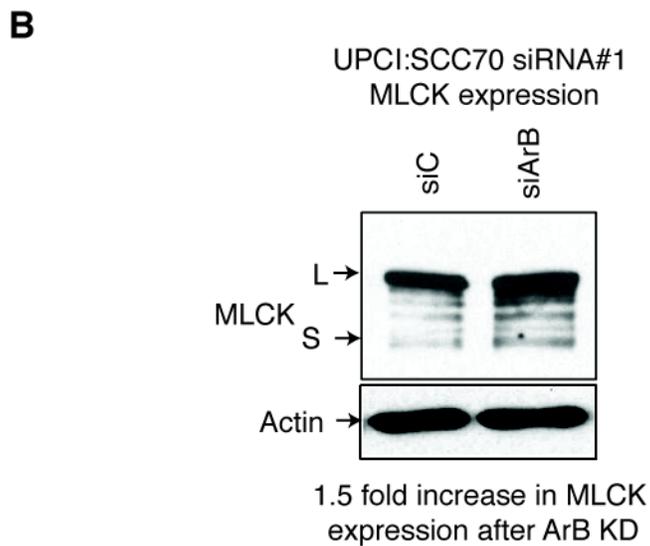
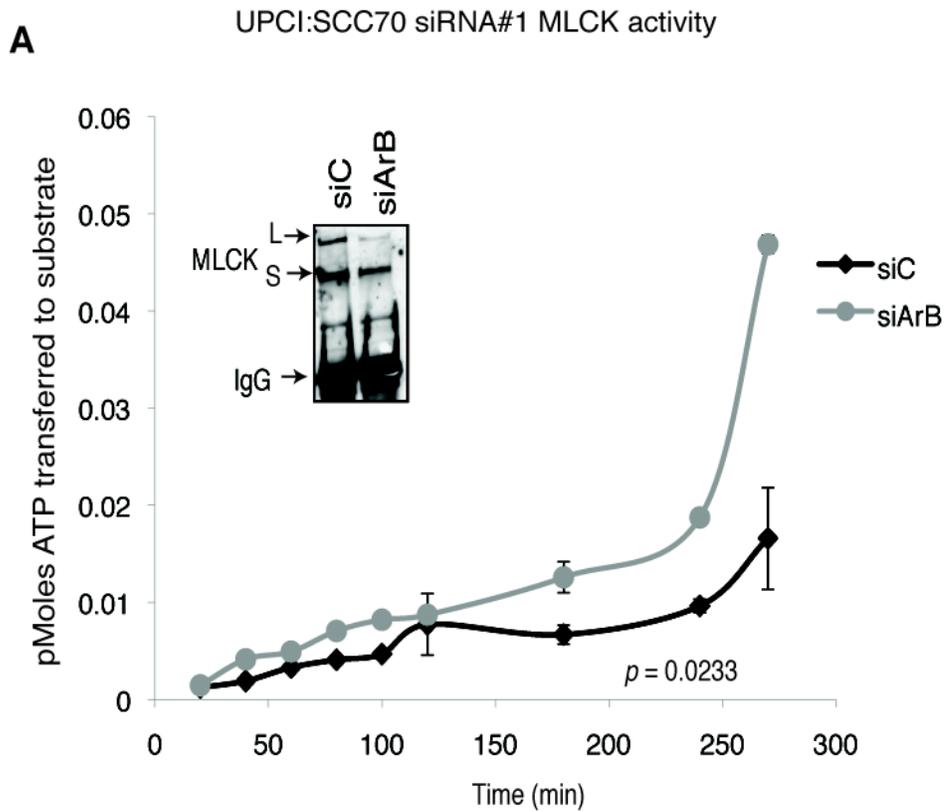


Figure 23. A partial knock-down of Aurora B increases MLCK activity and expression in cancer cells UPCI:SCC70.

MLCK *in vitro* activity (A) and MLCK expression (B) was measured in a second cancer cell line UPCI:SCC70 after transfection of a control siRNA (siC) or Aurora B siRNA (siArB). Immunoblots in (A) show MLCK immunoprecipitation used in the respective reactions.

2.5.4 Aurora C kinase also phosphorylates MLCK and increases MLCK activity

Aurora kinase family has three members, Aurora A, Aurora B and Aurora C kinase. Out of the three members, functions of Aurora C are probably the least well understood. Aurora C was indentified by a homology search for kinases homologous with Aurora B kinase. It was shown to be more closely related to Aurora B kinase with 83% of sequence homology between Aurora B and Aurora C kinase domains (Kimura et al., 1999). It is predominantly expressed in testis and is required for spermatogenesis (Kimmins et al., 2007). Adults also express Aurora C in other tissues such as placenta, ovary and liver but at very low levels (Yan et al., 2005). Interestingly, Aurora C gene *AIK3* is mapped on chromosome 19q13.43, a region that is commonly rearranged or deleted in cancer cells (Kimura et al., 1999), demanding further investigations into functions of Aurora C. Consistent with the sequence homology between Aurora B and Aurora C, Aurora C also demonstrates functional redundancy with Aurora B. Similar to Aurora B, Aurora C interacts with CPC components INCENP and survivin and localizes to the spindle midzone during cytokinesis (Sasai et al., 2004; Yan et al., 2005). Kinase dead mutant of Aurora C as well as siRNA mediated knock-down of Aurora C results in cytokinesis failure and multinucleation, further demonstrating its functional similarity with Aurora B (Sasai et al., 2004; Yan et al., 2005; Yang et al., 2010). Aurora C appears to replace Aurora B for normal CPC functioning in the pre-implantation stage of embryogenesis (Fernandez-Miranda et al., 2011). Interestingly, in cultured cells, Aurora C can complement Aurora B function and can rescue multinucleation phenotype of

Aurora B inhibition (Sasai et al., 2004). These data demonstrate that Aurora C functions redundantly with Aurora B. Therefore I decided to investigate if Aurora C is also a regulator of MLCK activity.

Firstly, we examined if MLCK was a substrate of Aurora C kinase. To test this possibility, I performed *in vitro* phosphorylation assay. Purified Aurora C was added to the kinase assay containing radiolabeled ATP and immunoprecipitated MLCK as a substrate. After 30 minutes of incubation, the reaction was run on a gel and imaged using phosphorimager to detect any possible phosphorylation of MLCK. As MLCK autophosphorylates (Tokui et al., 1995), a phosphorylation band corresponding to the long isoform of MLCK was detected even in the absence of added Aurora C and was absent after the addition of MLCK specific inhibitor ML-7. Phosphorylation of the long isoform was detected even in the presence of ML-7 after addition of purified Aurora C indicating that MCLK is an Aurora B substrate. A phosphorylation band corresponding to the autophosphorylation of Aurora C was detected at the correct molecular weight ensuring that added Aurora C was active. These results suggest that MLCK is indeed a substrate of Aurora C kinase *in vitro* and Aurora C specifically phosphorylates the long isoform of MLCK (Figure 24A). To further investigate the effects of Aurora C phosphorylation on MLCK activity, MLCK activity assay was performed in the presence of purified Aurora C kinase. Surprisingly, in contrast to Aurora B kinase, addition of Aurora C kinase resulted in an increase in MLCK activity (Figure 24B).

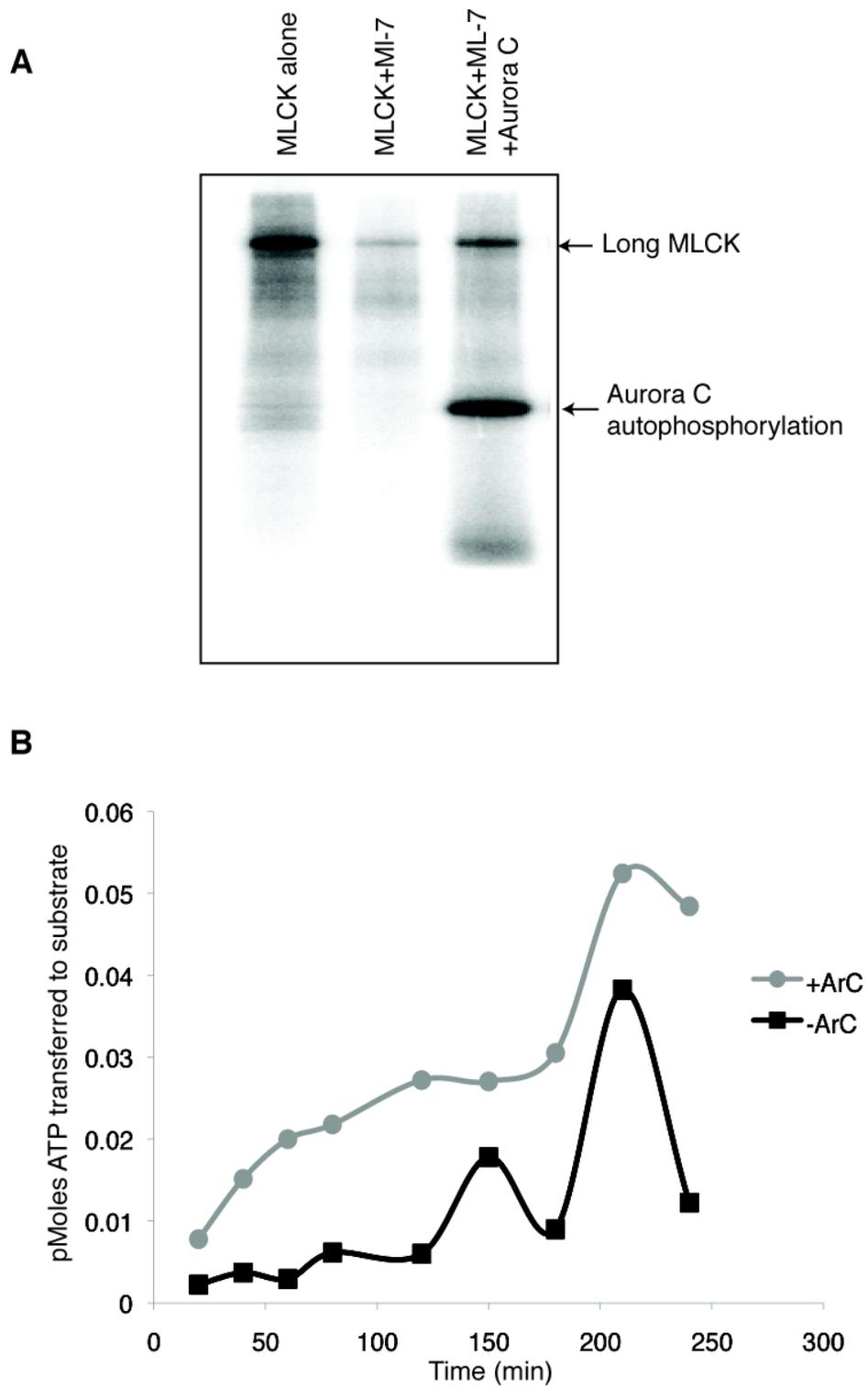


Figure 24. Aurora C kinase phosphorylates MLCK and increases its activity.

(A) MLCK phosphorylation assay was performed in the presence of MLCK alone, in the presence of MLCK specific inhibitor ML-7 (used to inhibit MLCK autophosphorylation) and after addition of purified Aurora C plus ML-7. (B) MLCK activity was measured *in vitro* in the presence (+ArC) and the absence (-ArC) of purified Aurora C kinase. Immunoblot in (B) indicates immunoprecipitation of MLCK used in the reaction.

Thus, even though MLCK is a substrate of Aurora C similar to Aurora B, Aurora C appears to activate MLCK in contrast to inhibitory effect of Aurora B. Consistent with the inhibition of MLCK in cancer cells and activation of MLCK by Aurora C kinase, I observed lower expression of Aurora C in UPCI:SCC103 cancer cells compared to RPE1 non-cancer cells (Figure 25). Similar results of the lower levels of Aurora C transcripts have been reported in a few other cancer cell lines (Lin et al., 2006). However, the consequences and significance of MLCK activation by Aurora C is not clearly understood at this point and needs further investigation.

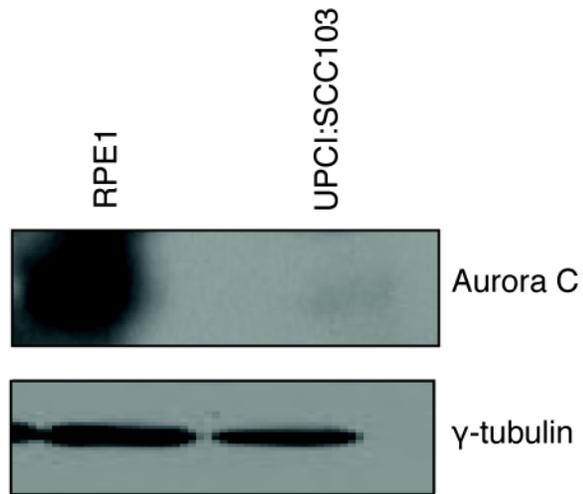


Figure 25. Aurora C is expressed at lower levels in cancer cells UPCI:SCC103.

Aurora C protein levels were compared between non-cancer cells RPE1 and oral squamous cell carcinoma cells UPCI:SCC103. Actin was used as a loading control.

2.6 CONCLUSIONS AND DISCUSSION

My results uncover for the first time, a molecular pathway that leads to cytokinesis failure in cancer cells. The pathway enumerated by our data is shown below:

Aurora B over-expression → MLCK inhibition → deficient MLC phosphorylation → cytokinesis failure

We have taken a bottom up approach to uncover the causes of cytokinesis failure. We have demonstrated that cancer cells are deficient in MLC phosphorylation and deficient MLC phosphorylation is the cause of cytokinesis failure. MLCK, an upstream regulator of MLC phosphorylation, is expressed at lower levels in cancer cells and shows reduced activity and this inhibition plays a causal role in cytokinesis failure.

Further, I have demonstrated that Aurora B over-expression is the cause of MLCK inhibition and cytokinesis failure. MLCK activity is inhibited directly *in vitro* by addition of purified Aurora B kinase. MLCK inhibition observed in cancer cells can be reproduced in non-cancer cells through over-expression of Aurora B. And lastly, cancer cell inhibition of MLCK expression and activity and the resultant cytokinesis failure can be reduced by partially knocking-down Aurora B. Taken together, these data establish a clear mechanistic link between Aurora B over-expression and cytokinesis defects, both commonly observed characteristics of tumor cells.

I believe these results are relevant for the process of tumorigenesis *in vivo*. Isolation of tetraploid mouse mammary epithelial cells, made tetraploid by cytokinesis failure alone is sufficient to stimulate tumor formation (Fujiwara et al., 2005). Interestingly, injection of Aurora B over-expressing tetraploid murine epithelial cells form nearly two-fold larger tumors compared to diploid Aurora B over-expressing cells, suggesting that cytokinesis failure is critical for the

tumor forming ability of Aurora B over-expression (Nguyen et al., 2009). Our data suggest that MLCK inhibition and deficient MLC phosphorylation play a central role in mediating Aurora B induced tumorigenesis, a model that can be explored further in animal studies.

MLCK is phosphorylated differently during interphase and mitosis (Poperechnaya et al., 2000). In metaphase MLCK is phosphorylated primarily within the IgG domain, at additional unique sites than those observed in interphase. Considering the lower activity of MLCK during metaphase, these phosphorylation sites appear to be predominantly inhibitory. Our results and the previous work from the Bresnick lab identify Aurora B as the upstream kinase responsible for inhibition of MLCK. During anaphase, MLCK activity increases back allowing cytokinesis to proceed. However, as cancer cells over-express Aurora B, metaphase inhibition of MLCK appears to continue during anaphase resulting in persistent MLCK inhibition and cytokinesis failure.

[R/K] [X] [T/S] [I/L/V] has been identified as the consensus Aurora B phosphorylation site (Cheeseman et al., 2002). Sequence analysis of long MLCK reveals 12 potential Aurora B phosphorylation sites, eight of which are located within the IgG domain (Dulyaninova and Bresnick, 2004). In the future, phosphopeptide mapping and mutagenesis studies can identify the actual sites that are phosphorylated and the effect of these phosphorylation events on MLCK activity. It would also be interesting to investigate potential differences if any, in the phosphorylation of these putative sites in cancer and non-cancer cells.

Lower expression of MLCK has been reported previously in some of the tumor types (Lee et al., 2008; Leveille et al., 2009). However, the mechanism of MLCK down-regulation has never been shown. Consistent with other reports, our results confirm lower abundance of MLCK

protein in a variety of cancer cells. As both the expression and the activity of MLCK appear to be regulated by Aurora B, it is an interesting possibility that they are inter-dependent.

Aurora B phosphorylates the long isoform of MLCK *in vitro* but not the short isoform. (Dulyaninova and Bresnick, 2004). Preferential localization of the long isoform to the cleavage furrow makes it a more relevant Aurora B target during cytokinesis. However, MLCK antibody used in this study binds both the isoforms and the kinase assays measure the combined activity of both the long and the short isoforms and do not distinguish between the effects of Aurora B on the two isoforms separately. It is more likely that the observed changes are entirely due to the phosphorylation of the long isoform. However, the possible effects of Aurora B mis-regulation on the activity of the short isoform cannot be ruled out at this stage.

While I emphasize the role of MLCK during cytokinesis, *in vitro* kinase assay performed with S-phase arrested cells demonstrates inhibition of MLCK even in the interphase of cancer cells. Cells can divide by the traction forces acting at the periphery, independent of the cleavage furrow (Kanada et al., 2005). As MLCK regulates contractile processes associated with cell adhesion and motility (Katoh et al., 2001), changes associated with the cell motility outside of the cleavage furrow can also influence cytokinesis outcome in tumor cells. Additionally, interphase inhibition of MLCK can influence cell motility relevant to the metastatic potential of the cancer cells. So far, all the known functions of Aurora B involve mitotic processes. But in one example of T lymphocytes, Aurora B is found to be present in all the stages of cell cycle and is required for the G1 to S transition (Song et al., 2007). Thus it is possible that interphase inhibition of MLCK is also mediated by Aurora B kinase.

My data also identify Aurora C as an additional regulator of MLCK activity. Aurora C binds to CPC components similar to Aurora B and rescues Aurora B depletion phenotypes. Thus,

Aurora C appears to function redundantly with Aurora B. Interestingly, my data demonstrates that unlike Aurora B, Aurora C activates MLCK. Both Aurora B and Aurora C mRNA levels are elevated at G2/M stage of cell cycle. However, Aurora B levels peak and drop ahead of Aurora C during mitosis. Aurora B levels taper at the end of M phase but Aurora C persists even after exit of M phase (Sasai et al., 2004). This cell cycle distribution of Aurora B and C levels and their opposing effect on MLCK activity correlate very well with the observed inhibition of MLCK during metaphase and activation during anaphase and these apparently antagonistic effects of Aurora B and C appear to be important for the temporal regulation of MLCK during cytokinesis. There are some conflicting results about Aurora C levels in tumor tissues. Some studies report elevated levels whereas others report decreased levels of Aurora C transcripts in tumor tissues (Lin et al., 2006; Sasai et al., 2004). Although I observed reduced Aurora C protein levels and reduced MCLK activity in oral cancer cells, this observation needs to be validated in other cancer cell lines.

In summary, my work identifies a novel mechanism of MLCK regulation by Aurora B and Aurora C kinase and provides insights into the causes of cytokinesis failure in cancer cells.

3.0 LINGERING CHROMATIN TRIGGERS CYTOKINESIS FAILURE

3.1 INTRODUCTION

Data presented in chapter 2 describes a novel pathway of cytokinesis failure mediated by Aurora B over-expression and the downstream MLCK and pMLC inhibition. As described in the introduction, cytokinesis failure is typically observed in only in a subset of cancer cells. As the conclusions presented in chapter 2 were based on population based studies, it was not possible to distinguish if the activation of this pathway and abortive cytokinesis occurred stochastically in some of the cells or if there was an underlying factor that specifically triggered this pathway in a certain population of the cells.

Faithful cytokinesis requires tight coordination of chromosome segregation and cleavage furrow ingression. Under normal circumstances, two sets of chromosomes segregate away from the cleavage plane shortly after the onset of anaphases and the progression of cytokinesis in the presence of uncleared chromatin results in extensive DNA damage (Janssen et al., 2011). Therefore, cytokinesis completion needs to await complete clearance of the chromatin from the cleavage plane. Consistent with this idea, it has been demonstrated that lagging chromatin at the cleavage furrow results in abortive cytokinesis (Mullins and Biesele, 1977; Shi and King, 2005). However, the mechanism of lingering chromatin induced cytokinesis failure remains unknown. Based on our data, we hypothesize that the cells with lingering chromatin fail in cytokinesis by triggering MLCK and MLC phosphorylation inhibition pathway. In other words, we believe that

cytokinesis failure is not a stochastic process but occurs specifically in cells with lingering chromatin at the cleavage plane.

More than a century ago, David Hansemann reported frequent occurrence of asymmetrical anaphases in epithelial cancers characterized by the presence of lagging chromosomes or the bridges formed by the sticky chromosomes (Hansemann, 1890; Hardy and Zacharias, 2005). Hansemann's work was forgotten for many decades until the advances in cytogenetics established chromosome segregation defects as a very common characteristic of cancer cells. They occur in about one percent of the dividing somatic cells but at a much higher frequency in cancer cells, a possible explanation for the higher frequency of cytokinesis failure in cancer cells (Cimini et al., 2003; Gisselsson, 2008; Gisselsson et al., 2000; Saunders et al., 2000). Chromosome segregation errors that can lead to the presence of lingering chromatin at the cleavage plane are primarily anaphase bridges and lagging chromosomes. Anaphase bridges and lagging chromosomes will be described individually in the following sections but will be referred together as lingering chromatin for the purposes of this study.

3.1.1 Anaphase bridges

Anaphase bridge can be defined as a continuous string of DNA connecting two main segregating masses of chromosomes at two anaphase poles. They can be further classified into chromatid or chromosome bridges (McClintock, 1941). Chromatid bridge is formed by the fusion of two sister chromatids before the separation of centromeres. This leads to the stretching of the intercentromeric DNA during anaphase resulting in the breakage of a chromatid segment at one or multiple locations. Similarly, if two chromosomes fuse together, they face a tug of war during anaphase resulting in DNA double strand breaks. Once the DNA ends are broken, they can be

ligated back or can fuse illegitimately with other chromosomes forming novel dicentric chromosomal structures. The resultant dicentric chromosome again undergoes a tug of war in the following mitosis resulting in the breakage-fusion-breakage cycle of anaphase bridges formation (McClintock, 1938, 1941).

Any DNA damaging event that triggers DNA double strand breaks (DSB) can initiate the formation of anaphase bridges. Cells have evolved a variety of DNA damage repair pathways such as homologous recombination or non-homologous end joining to restore the original chromosomal structure after DSB (Kass and Jasin, 2010). However an inaccurate repair process can fuse non-identical chromosome ends together resulting in the formation of anaphase bridges (Pfeiffer et al., 2000). DSB and the resultant anaphase bridges can occur spontaneously during cellular processes or can be induced by exogenous agents such as ionizing radiation or IR (Hagen, 1994; Humphrey and Brinkley, 1969; Pfeiffer et al., 2000). Another common source of anaphase bridges is the shortening of telomeric TTAGGG repeats (Artandi et al., 2000; Gisselsson et al., 2001). Shortened telomeric ends, as observed in aging cells or in tumors, can be inappropriately treated as DSB leading to terminal fusion of two chromosomes or sister chromatids and initiation of breakage-fusion-breakage cycle (Gisselsson, 2005). Inactivation of p53 plays an important role in promoting breakage-fusion-breakage cycle after telomere shortening (Artandi et al., 2000).

3.1.2 Lagging chromosomes

Lagging chromosomes is a rather poorly defined term used to describe chromosomes that are left behind at the spindle equator during anaphase when the majority of the sister chromatids have moved away from each other towards the opposite spindle poles (Cimini et al., 2001; Cimini et

al., 1999). Lagging chromosomes are frequently observed in cancer cells and are recognized as an important source of aneuploidy and chromosomal instability (Reing et al., 2004; Saunders et al., 2000; Thompson and Compton, 2008).

Lagging chromosomes arise primarily from errors in kinetochore–microtubules attachments, specifically merotelic kinetochore-microtubule attachment (Cimini et al., 2001). When a single kinetochore attaches to microtubules emanating from both the poles, it faces the pulling forces in opposite direction causing it to lag behind. Merotelic attachment are commonly observed in early mitosis even in normal tissues (Salmon et al., 2005). However, there are cellular mechanisms that prevent and correct merotelic attachments before the onset of anaphase and involve proteins that primarily regulate chromosome and kinetochore structures and the dynamics of microtubules at the kinetochore (Gregan et al., 2011). Centromerically located Aurora B kinase is at the centre of these corrective mechanisms. Aurora B is enriched at the merotelically attached chromosomes and corrects these erroneous attachments by altering the activity of kinetochore proteins such as the KMN network and microtubule depolymerizing proteins such as MCAK (Knowlton et al., 2006; Welburn et al., 2010). However, mechanisms correcting merotelic attachments can get overwhelmed if misaligned chromosomes occur at a very high frequency. For example, cells recovering from nocodazole treatment experience a very high frequency of merotelic that is beyond the correction capacity of cells and hence they experience a very high frequency of lagging chromosomes (Cimini et al., 1999). Similarly, cancer cells experience a higher frequency of merotelic attachments, most commonly during the coalescence of extra centrosomes, and hence demonstrate higher frequency of lagging chromosomes (Ganem et al., 2009; Silkworth et al., 2009). Also, deficiencies in the corrective

mechanisms can further promote merotely and the occurrence of lagging chromosomes in cancer cells.

3.1.3 Role of Aurora B kinase in coordinating cytokinesis with chromatin clearance from the cleavage plane

How cytokinesis is coordinated with chromatin clearance to avoid DNA damage was a completely unanswered question until a recent discovery of the NoCut pathway, described for the first time in yeast *Saccharomyces cerevisiae* (Norden et al., 2006). The NoCut pathway ensures that cytokinesis proceeds only after all the chromosomes are moved away from the cleavage plane. Aurora B kinase Ipl1 is at the center of this pathway. In the presence of midzone defects, Ipl1 delays cytokinesis completion by recruiting anillin related protein Boi1 and Boi2 at the cleavage site to inhibit abscission. Later, similar pathway was also identified in mammalian cells with a conserved role for Aurora B (Steigemann et al., 2009). In the presence of unsegregated chromatin, mammalian Aurora B delays abscission by phosphorylating and stabilizing Mklp1 at the intracellular canals.

As Aurora B plays an essential role in coordinating chromatin clearance with cytokinesis timing, we hypothesize that Aurora B dependent MLCK and MLC inhibition pathway is activated in a response to lingering chromatin to prevent cytokinesis completion.

3.2 RESULTS

3.2.1 Lingering chromatin interferes with cytokinesis

To test the hypothesis of lingering chromatin interfering with cytokinesis by inhibition of MLCK and MLC phosphorylation, I first tested if cells indeed fail at cytokinesis in the presence of lingering chromatin by measuring the binucleation frequency after induction of chromosome segregation defects in HFFshp53 cells and the cancer cells U2OS with a treatment of 2.8-3 Gy of ionizing γ -radiation (IR). IR primarily causes DNA double strand breaks and results in the formation of anaphase bridges most likely due to delayed or incomplete repair of these breaks (Fenech et al., 2011; Hagen, 1994; Pfeiffer et al., 2000). Cells with WT p53 senesce in the presence of chromosome bridges. Therefore it was important to use p53 null HFF cell line to promote the formation of anaphase bridges (Artandi et al., 2000). As expected, cells treated with IR demonstrated an increase in the frequency of lingering chromatin (Figure 26A) and consistent with other studies, it resulted in cytokinesis failure as demonstrated by an increase in the frequency of binucleation (Figure 26B). It is important to note that even untreated cancer cells U2OS had a higher frequency of lingering chromatin and binucleation compared to untreated HFFshp53 cells. This observation was expected as cancer cells have higher frequency of segregation errors and cytokinesis failure.

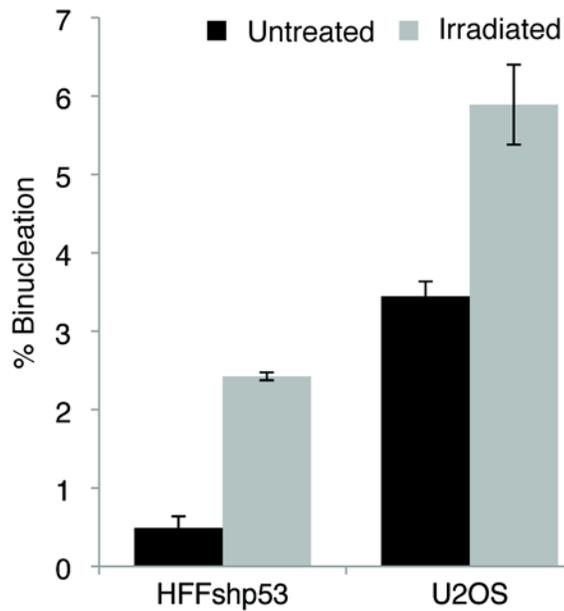
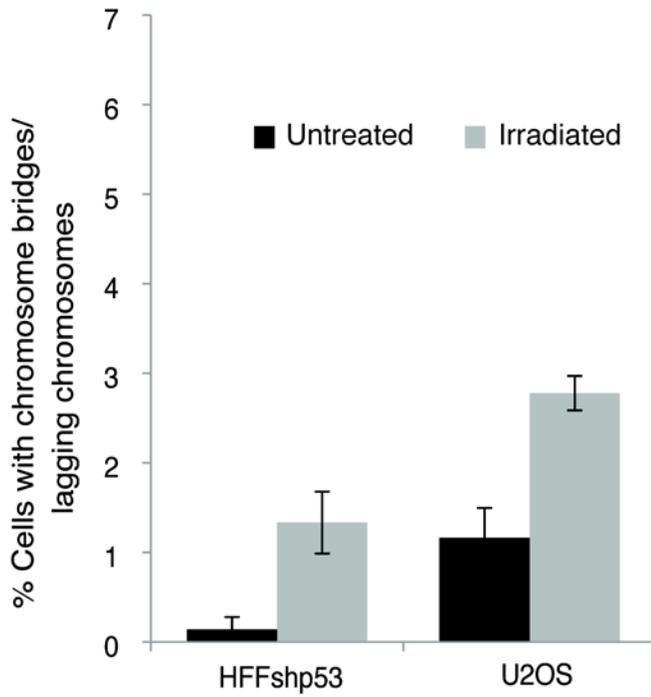


Figure 26. Induction of lingering chromatin increases cytokinesis failure.

(A) Percentage of cells with chromosome bridges and lagging chromosomes were measured in untreated cells (black bars) and cells treated with 2.8-3 Gy of ionizing γ -radiation (grey bars). **(B)**

Binucleation frequency was measured in untreated (black bars) or irradiated (grey bars) cells. Two bars on the left of each figure show the data from HFFshp53 cells while two bars on the right show the data from U2OS cells.

3.2.2 Cells with lingering chromatin are deficient in phosphorylated MLC at the cleavage furrow

My hypothesis predicts that lingering chromatin will interfere with actin-myosin contractile ability as a result of MLCK inhibition and deficiencies in MLC phosphorylation. MLCK activates MLC by predominantly phosphorylating Ser19 residue and to a lesser extent Thr18 residue (Ikebe and Hartshorne, 1985). Therefore to test our hypothesis, I induced lingering chromatin in HFFshp53 and U2OS cells by treating them with IR and then examined the localization of Ser19 phosphorylated MLC (hereafter referred to as pMLC) at the cleavage furrow in the presence and absence of lingering chromatin. As shown in the representative examples, in the absence of lingering chromatin pMLC was robustly enriched at the cleavage furrow in approximately 80% of the anaphases of HFFshp53 cells (Figure 27A). However, in the presence of lingering chromatin pMLC enrichment was greatly diminished in almost 80% of the anaphases. Similar results were also observed for cancer cells U2OS (Figure 27B). These data suggest that lingering chromatin could interfere with actin-myosin contraction during cytokinesis by interfering with activating phosphorylation of MLC at the cleavage furrow.

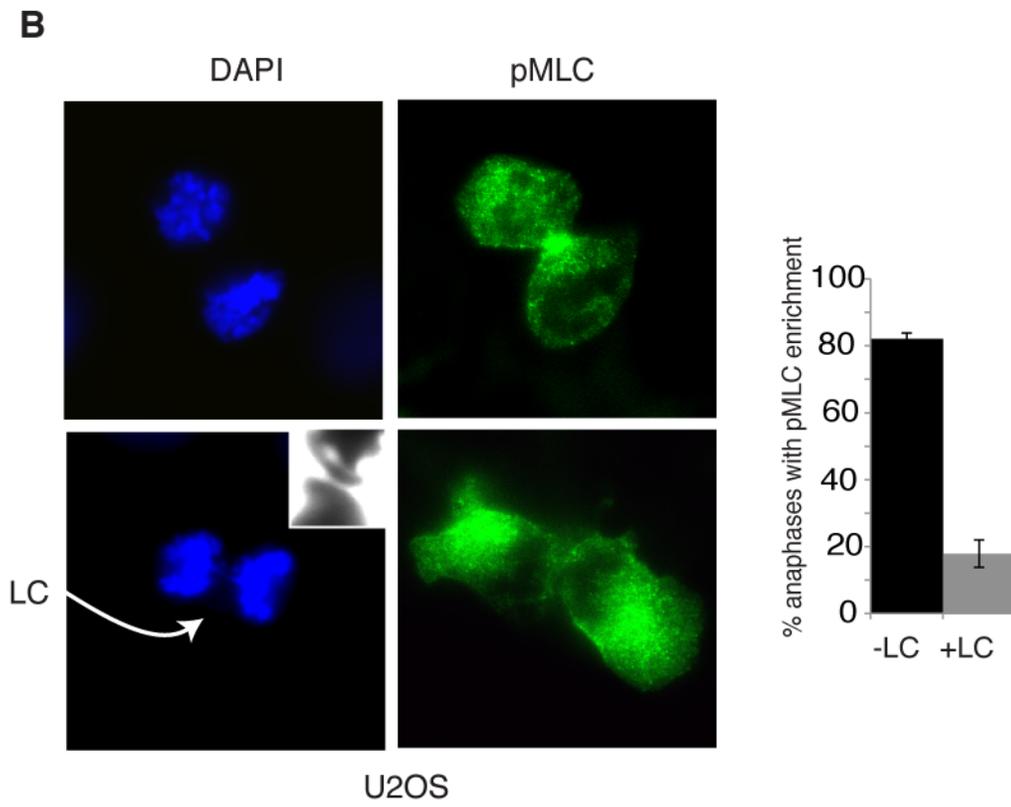
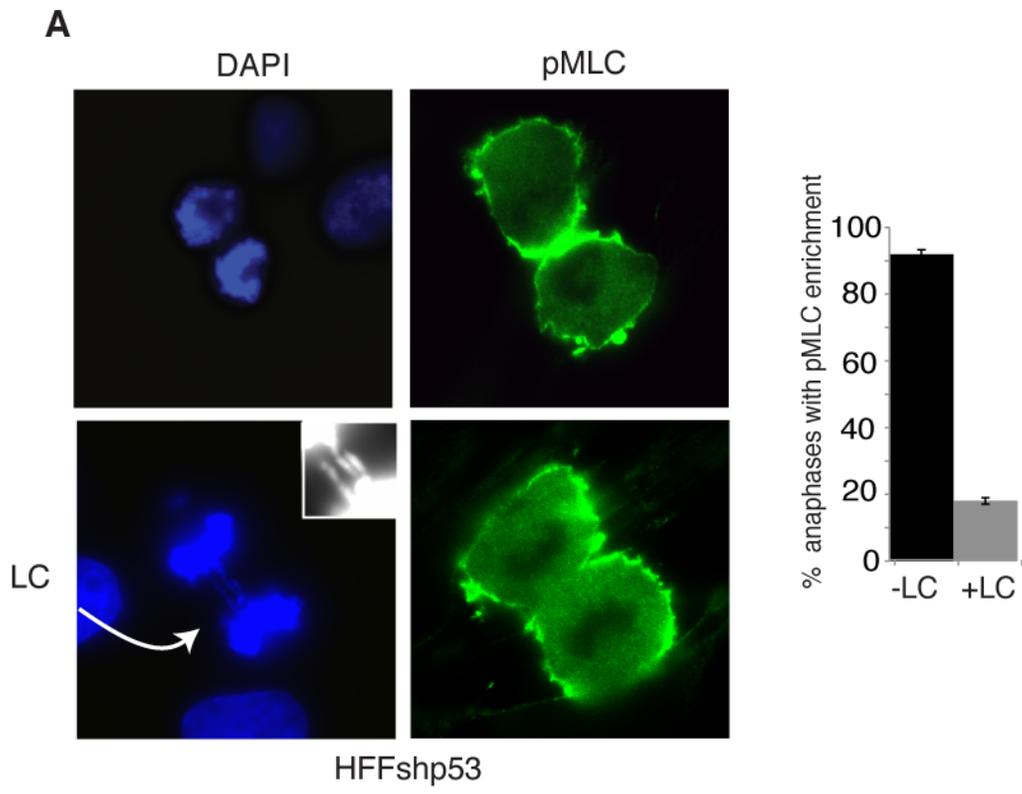
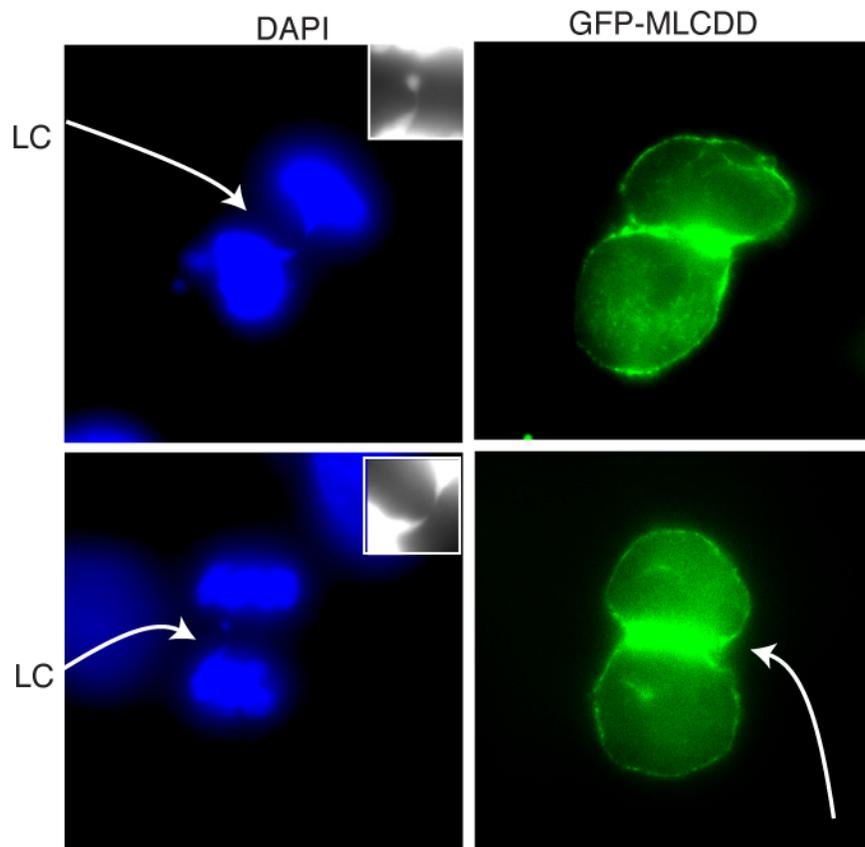


Figure 27. MLC phosphorylation is deficient in the presence of lingering chromatin.

(A) Representative images of the localization of MLC phosphorylated on Ser19 (pMLC) at the cleavage furrow in HFFshp53 cells in the presence (bottom panel) and the absence (top panel) of lingering chromatin (LC) are shown. Nuclei were stained with DAPI. Right panel indicates quantitation of pMLC localization in the presence (+LC) and the absence (-LC) of lingering chromatin (n=40 in two independent experiments each). (B) Representative images of pMLC localization at the cleavage furrow in the presence and the absence of lingering chromatin in U2OS cells are shown. Right panel indicates quantitation of pMLC localization (n=40 in two independent experiments). Insets in (A) and (B) are zoomed in images demonstrating the presence of lingering chromatin.

To further confirm these observations, I transfected a GFP tagged phosphomimetic MLC construct in cells prior to induction of bridges and examined its localization at the cleavage furrow. As demonstrated in (Figure 28), phosphomimetic MLC localized correctly at the cleavage furrow in the presence and absence of lingering chromatin. These data indicated that lingering chromatin specifically induced defects in MLC phosphorylation at Ser19 and Thr18. Taken together, these data suggests that lingering chromatin could interfere with actin-myosin contraction during cytokinesis by interfering with the activating phosphorylation of MLC at the cleavage furrow.



Localization
of phosphomimetic MLC
at the cleavage furrow in the
presence of lingering chromatin

Figure 28 Phosphomimetic MLC localizes at the cleavage furrow even in the presence of lingering chromatin.

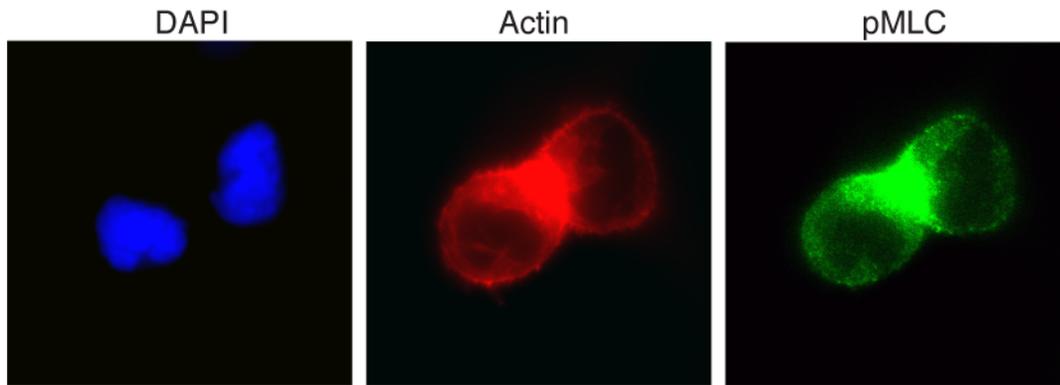
Two representative images of the localization of phosphomimetic MLC (GFP-MLCDD) at the cleavage furrow in the presence of lingering chromatin (LC) in U2OS cells are shown. Nuclei were stained with DAPI. Insets indicate zoomed in images of lingering chromatin.

3.2.3 Cells with lingering chromatin exhibit excessive blebbing during cytokinesis

While examining anaphases with lingering chromatin, I observed abnormal membrane protrusions indicative of blebbing. Blebs are protrusions of the cell membrane that result from actin-myosin contraction of the cortex (Charras, 2008). Even during normal cytokinesis, smaller blebs are often observed at the polar cortex. However, abnormal actin-myosin contraction can manifest in excessive blebbing activity that is often indicative of cytokinesis failure (Birkenfeld et al., 2007; Wu et al., 2010b). Interestingly, cells with dys-regulated MLCK activity are known to exhibit striking blebbing activity (Fishkind et al., 1991). Therefore, to further investigate the observed membrane protrusions, I co-immunostained cells for actin and pMLC. In the absence of lingering chromatin, I did not observe blebbing activity at the cell cortex (Figure 29A). However, in the presence of lingering chromatin, distinct membrane blebs were observed most commonly at the polar cortex (Figure 29B). These blebs were strongly positive for pMLC staining indicating abnormal MLC contractility at the polar cortex. These data suggest that anaphases with lingering chromatin demonstrate abnormal actin-myosin contractility in the form of blebbing, most likely preceding abortive cytokinesis. I would like to emphasize the detection of strong actin enrichment at the cleavage furrow even in the presence of lingering chromatin. Actin and myosin are recruited to the cleavage furrow independently. Therefore this actin enrichment indicates that cells with lingering chromatin are specifically deficient in MLC phosphorylation at the cleavage furrow and are not manifesting a general deficiency in contractile proteins. Note that deficient MLC phosphorylation alone should still lead to defects in actin-myosin contraction and cytokinesis failure.

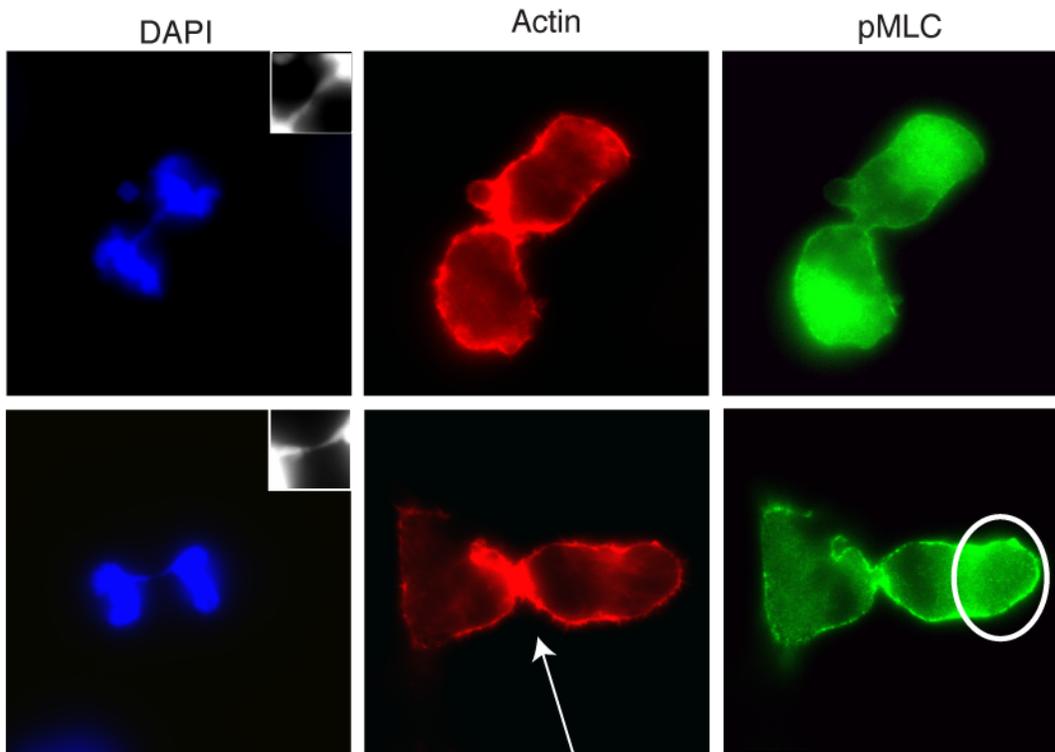
A

Anaphase without lingering chromatin



B

Anaphases with lingering chromatin at the cleavage plane



Actin is enriched at the cleavage furrow even in the presence of lingering chromatin

Blebbing with pMLC enrichment

Figure 29. Anaphases with lingering chromatin exhibit excessive blebbing.

(A) A representative image of a normal anaphase with localization of actin and pMLC at the cleavage furrow in U2OS cells. (B) Two examples of Anaphases with lingering chromatin in U2OS cells, stained for actin and pMLC, are shown. Blebbing is observed as an abnormal protrusion of the cell cortex indicated by a white circle. Insets in (A) and (B) demonstrate zoomed in images of lingering chromatin.

3.2.4 MLCK is deficient at the cleavage furrow in the presence of lingering chromatin in HFFshp53 cells

MLCK is the key kinase that phosphorylates MLC at the cleavage furrow. My results presented in Chapter 2 demonstrate that MLCK inhibition is the primary cause of deficient MLC phosphorylation and cytokinesis failure. Therefore I wanted to test if cells with lingering chromatin are also deficient in MLCK localization at the cleavage furrow. To test this possibility, cells were immunostained for MLCK after treatment with IR. As expected, in HFFshp53 cells MLCK was typically enriched at the cleavage furrow in the absence of lingering chromatin but was enriched at the cleavage furrow in only ~ 60% of anaphases with lingering chromatin at the cleavage site (Figure 30).

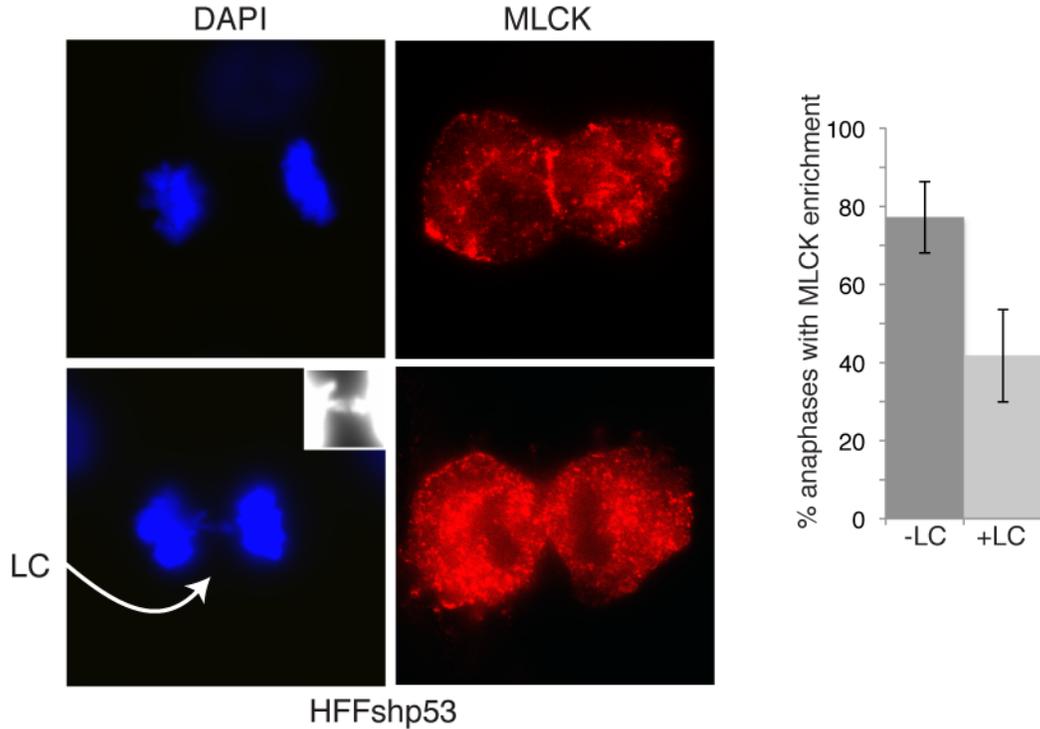


Figure 30. MLCK is mis-localized from the cleavage furrow of HFFshp53 cells in the presence of lingering chromatin.

MLCK localization at the cleavage furrow in the presence and the absence of lingering chromatin (LC) is shown. Inset is a zoomed in image demonstrating lingering chromatin. Right panel indicates quantitation of MLCK localization during anaphase in the presence (+LC) and the absence (-LC) of lingering chromatin.

3.2.5 MLCK is not enriched at the cleavage furrow of cancer cells

I performed similar analysis for MLCK enrichment at the cleavage furrow in the presence of lingering chromatin in cancer cells U2OS. Surprisingly, I failed to detect MLCK at the cleavage furrow in any dividing U2OS cells irrespective of the presence or absence of lingering chromatin

(Figure 31). To rule out the possibility of immunostaining artifacts, we modified the fixation and staining protocols, but consistently failed to detect MLCK at the cleavage furrow. To examine if these observations were true in other cancer cells lines, I compared MLCK localization in a panel of non-cancer and cancer cell lines. Consistent with the above observations, MLCK was enriched at the cleavage furrow of the tested non-cancer cells but was not detected at the cleavage furrow of the tested cancer cells (Figure 32).

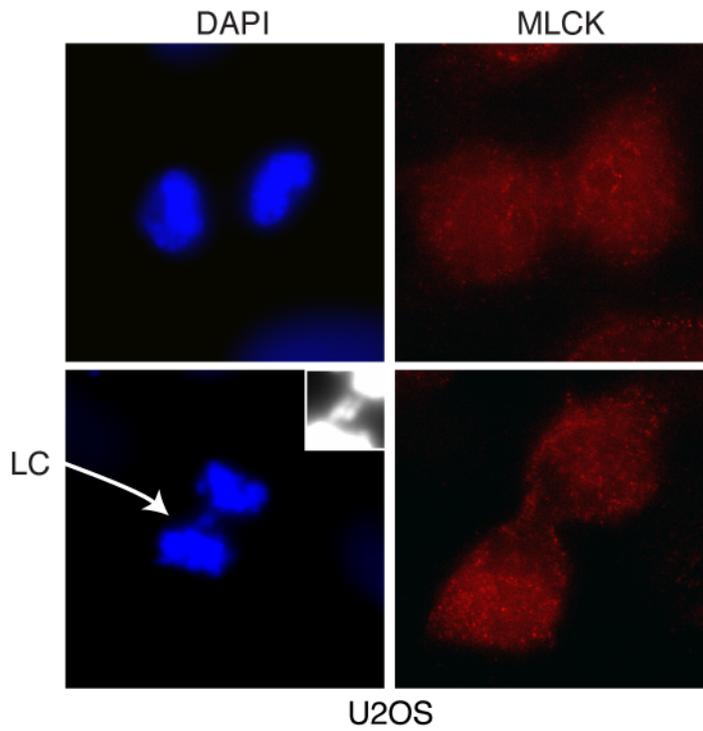
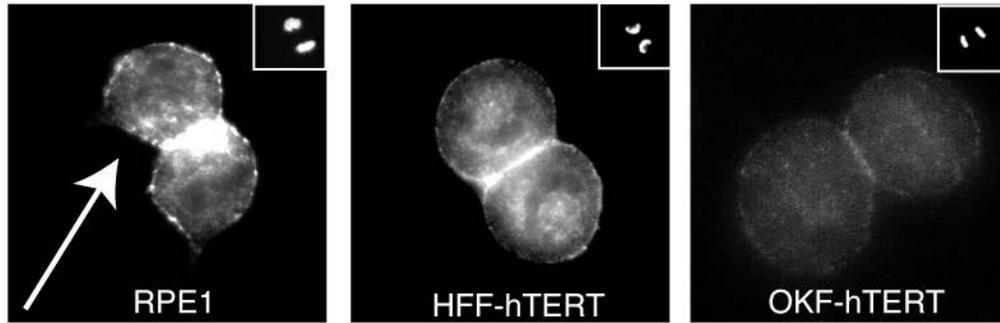


Figure 31 MLCK is mis-localized from the cleavage furrow of all the dividing U2OS cells.

Representative images of MLCK localization at the cleavage furrow in cancer cells U2OS in the absence (top panel) and the presence (bottom panel) of lingering chromatin at the cleavage plane are shown. Nuclei were stained with DAPI.

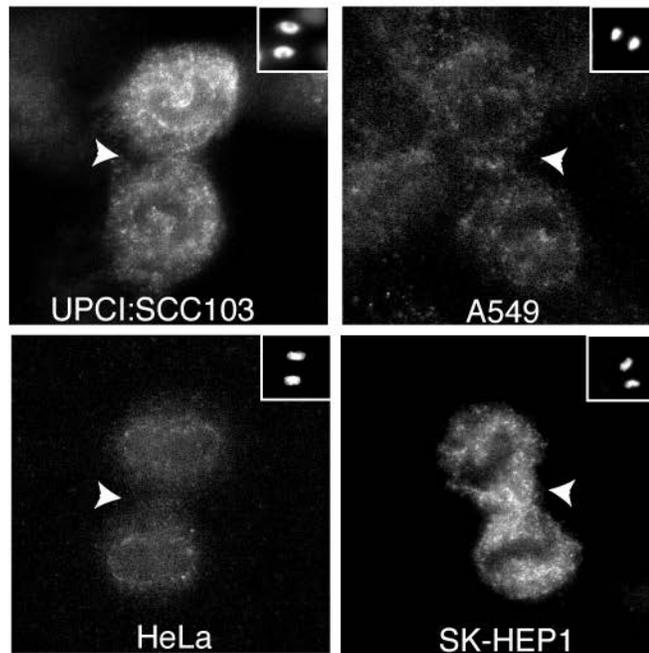
A



Non-cancer cells

MLCK
at the cleavage furrow

B

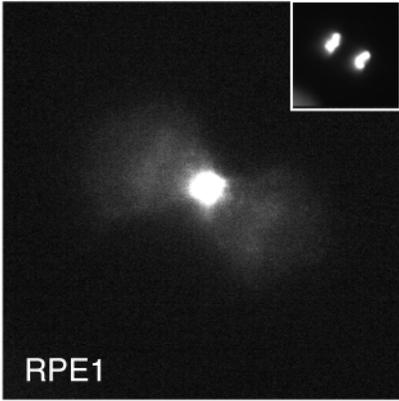


Cancer cells

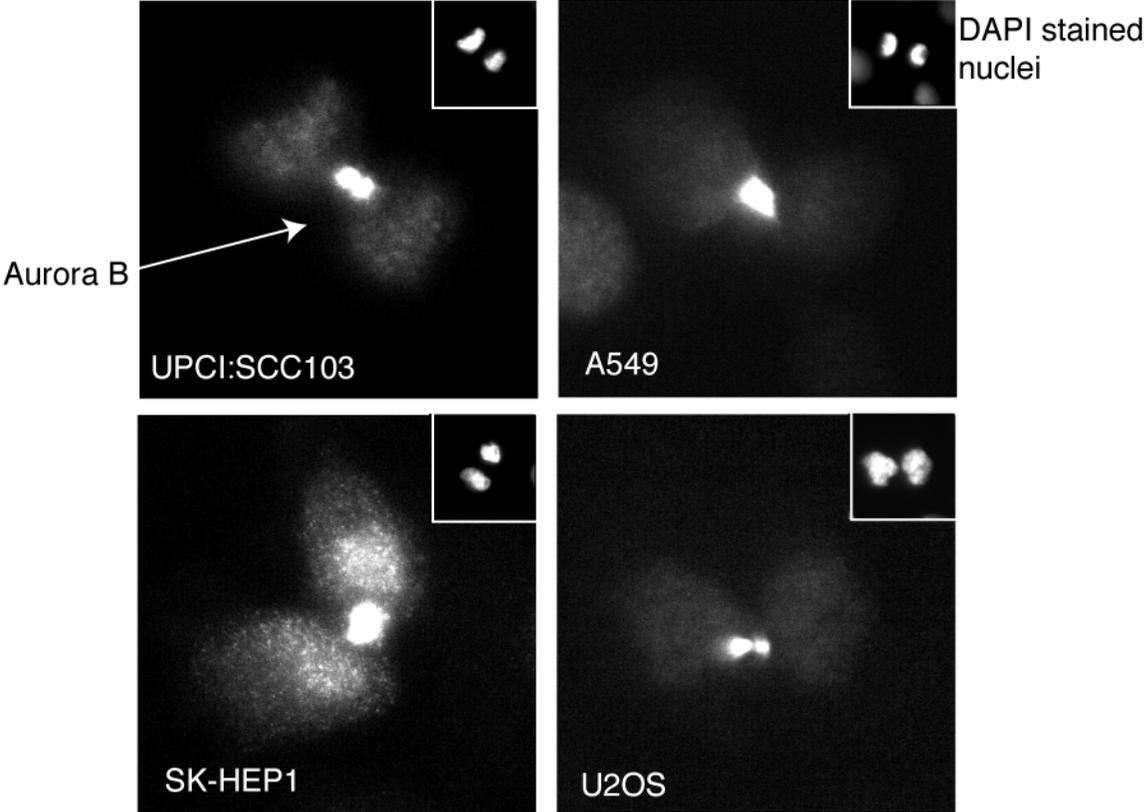
Figure 32. MLCK is mis-localized from the cleavage furrow in all the tested cancer cells.

(A) MLCK localization at the cleavage furrow of non-cancer cells RPE1, HFF-hTERT and OKF-hTERT is shown. (B) MLCK staining in the anaphases of cancer cells UPCI:SCC103, HeLa, A549 and SK-HEP1 is shown. Arrowheads denote mis-localization of MLCK from the cleavage furrow of cancer cells. Insets demonstrate corresponding DAPI stained nuclei.

MLCK mis-localization from the cleavage furrow of cancer cells is not a result of a general defect of cytokinetic furrow formation as other protein like Aurora B localized correctly at the cleavage furrow of cancer cells during division (Figure 33).



Non-cancer cell



Cancer cells

Figure 33. Aurora B is correctly localized at the cleavage furrow in cancer cells.

Localization of Aurora B in non-cancer cells RPE1 and cancer cells UPCI:SCC103, A549, SK-HEP1 and U2OS cells is shown. Arrow indicates the cleavage furrow. Insets demonstrate DAPI stained nuclei.

These results suggest that MLCK is mis-localized from the cleavage furrow of all the cancer cells. There can be several explanations for these observations. Firstly, I have demonstrated that MLCK is expressed at very low levels in cancer cells. Therefore low abundance of protein might prevent its detection at the cleavage furrow. However, MLCK was not detected at the cleavage furrow in cancer cells SK-HEP1 that expresses MLCK at relatively higher levels compared to other cancer cells. Therefore, low abundance of MLCK protein may not completely explain its absence from the cleavage furrow, but this possibility cannot be ruled out at this stage. Secondly, MLCK might be mis-localized as a result of more transient and often undetectable aberrant chromosome segregation events such as metrotelic or syntelic kinetochore-microtubule attachments. And finally, as cancer cells over-express Aurora B, it might interfere with MLCK localization even in the absence of lingering chromatin.

3.2.6 MLCK inhibition and deficient MLC phosphorylation is a cause of cytokinesis failure in cells with lingering chromatin

Data presented in the above two sections suggest that cells with lingering chromatin are deficient in MLCK and activating MLC phosphorylation at the cleavage furrow. To test if these deficiencies were the sources of cytokinesis failure associated with lingering chromatin, I tested if phosphomimetic MLC or constitutively active MLCK could block lingering chromatin induced cytokinesis failure. Cells were transiently transfected with these constructs and the

binucleation frequency was determined after IR treatment. Compared to empty GFP vector or kinase dead MLCK controls, phosphomimetic MLC and constitutively active MLCK significantly reduced the frequency of binucleation after treatment with IR. These results were true in both HFFshp53 and U2OS cells (Figure 34A and Figure 34B respectively). Taken together, these data suggest that lingering chromatin interferes with MLCK activity and activating phosphorylation of MLC and restoring MLCK and pMLC largely prevents cytokinesis failure associated with lingering chromatin.

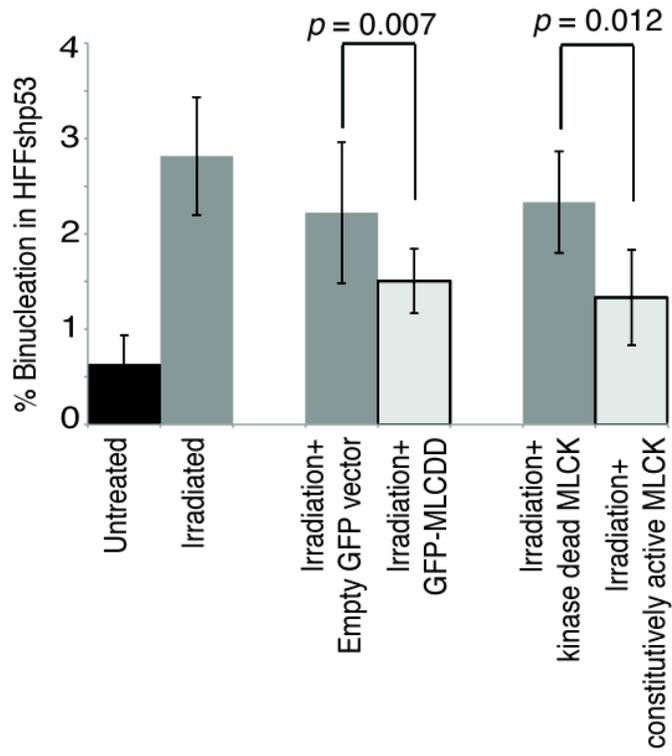
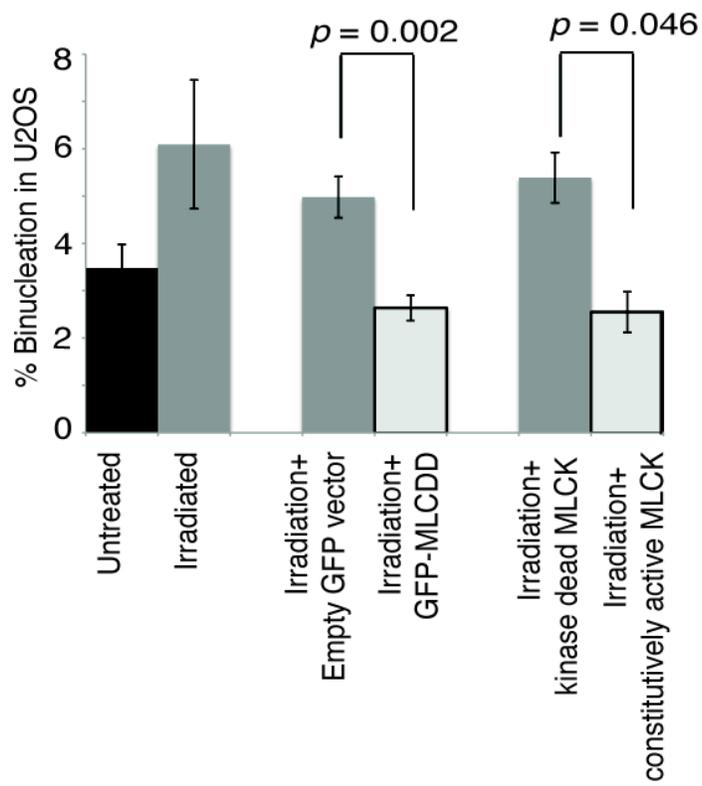
A**B**

Figure 34. MLCK inhibition and deficient MLC phosphorylation is a cause of cytokinesis failure in cells with lingering chromatin.

(A) Binucleation frequency was measured in untreated or irradiated HFFshp53 cells and cells irradiated and transfected with an empty GFP vector control or phosphomimetic MLC and a kinase dead or a constitutively active MLCK construct. (B) Binucleation frequency was measured in untreated or irradiated U2OS cells or cells irradiated and transfected with the indicated constructs. (n=500-100 in each of the three independent experiments)

3.2.7 Aurora B is up-regulated in the presence of lingering chromatin

Results from the above two sections indicate that MLCK inhibition and deficient MLC phosphorylation occur in the presence of lingering chromatin. I have also demonstrated that Aurora B over-expression is the upstream cause of MLCK inhibition. These two observations give rise to two possibilities. Either, lingering chromatin and Aurora B independently inhibit MLCK or the two events are linked together and indeed Aurora B over-expression is induced by lingering chromatin to cause downstream inhibition of MLCK. Interestingly, Aurora B is often over-expressed in cancer cells without changes in the gene copy number (Smith et al., 2005). It is required for correcting chromosome segregation errors such as merotely and also for coordinating cytokinesis with chromosome segregation. Taken together, these data make me favor the later possibility and we hypothesize that lingering chromatin is the underlying cause of Aurora B up-regulation and the resultant MLCK and pMLC inhibition. To test this hypothesis, we examined Aurora B protein levels after induction of lingering chromatin in HFFshp53 cells. We employed two different methods to induce lingering chromatin. Cells were treated with IR to cause DSB and induce anaphase bridges. Alternatively, cells were treated with monastrol to induce lagging chromosomes from spindle defects, as described previously (Janssen et al., 2011).

Monastrol is an inhibitor of molecular motor Eg5, a motor required for bipolar spindle assembly (Mayer et al., 1999). Hence, cells treated with Eg5 inhibitor monastrol arrest in mitosis as a result of monopolar spindle formation. During the recovery from monastrol block, there is a significant increase in the frequency of lagging chromosomes probably as a result of increased incidence of merotely (Thompson and Compton, 2008). As per my prediction, induction of lingering chromatin, both by IR and by monastrol treatment led to an increase in Aurora B protein levels (Figure 35A). Exposure of cells to another DNA damaging agent UV-C that did not induce anaphase bridges or lagging chromosomes and did not increase Aurora B protein levels indicating that the increase in Aurora B levels was not a result of generalized DNA damage but a specific effect of lingering chromatin. Changes in Aurora B levels were not a result of changes in mitotic indices either as mitotic indices remained comparable after all the treatments (Figure 35B). These data supports our hypothesis that lingering chromatin indeed elevates Aurora B levels.

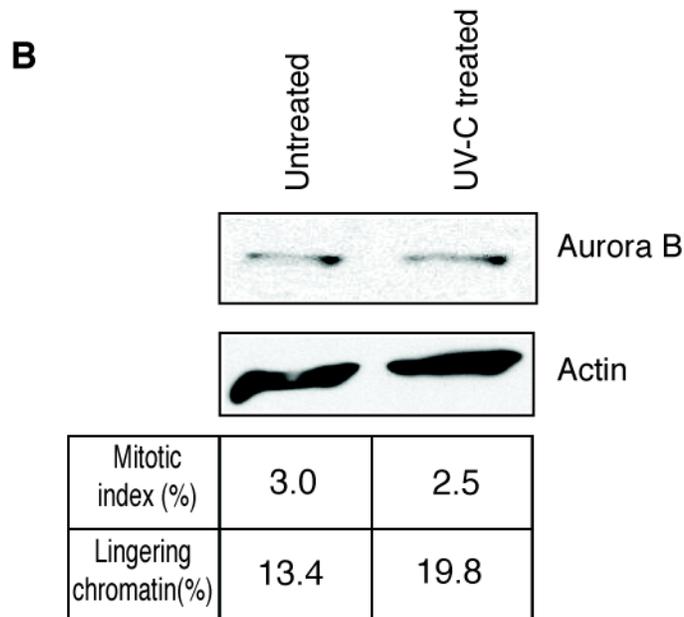
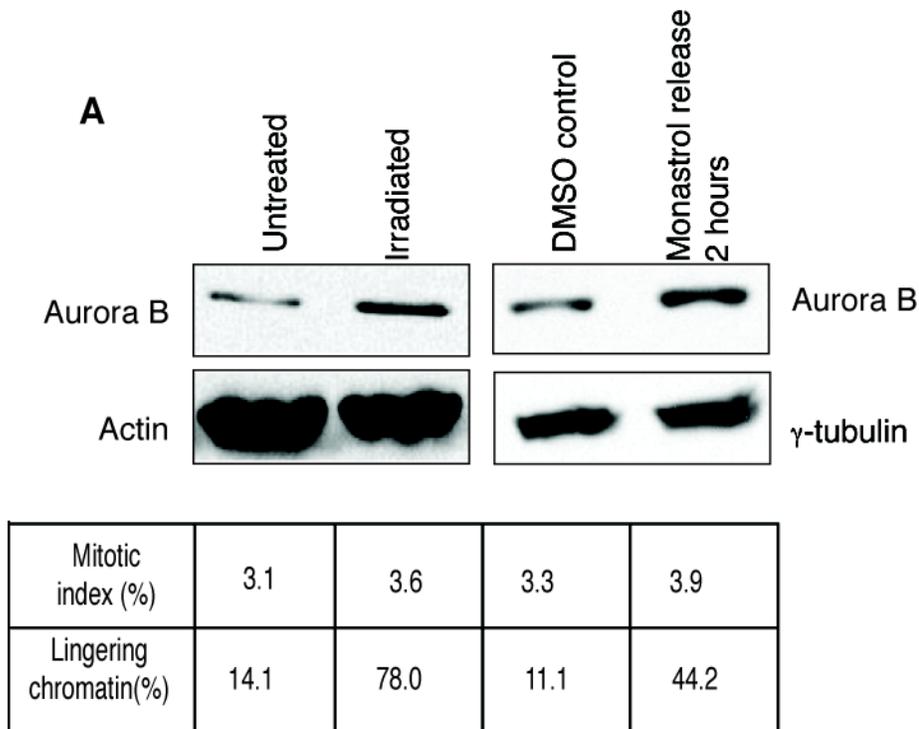


Figure 35. Aurora B protein levels are increased upon induction of lingering chromatin.

(A) Aurora B protein levels were measured by immunoblotting after induction of lingering chromatin either by γ -radiation (right panel) or by release from monastrol block (left panel). (B) Aurora B

protein levels were measured after exposure to DNA damaging agent UV-V. Bottom table in (A) and (B) show quantitation of mitotic indices and the frequency of lingering chromatin with respective treatments. Lingering chromatin percentage is calculated as the fraction of total anaphases with any chromatin not segregating with the main chromosome group. Actin and γ -tubulin were used as a loading control.

3.2.8 Aurora B over-expression and MLCK inhibition is the cause of mis-localization of phosphorylated MLC in the presence of lingering chromatin

As lingering chromatin induced Aurora B over-expression, we further wanted to test if this up-regulation of Aurora B was responsible for MLC phosphorylation deficiencies in the presence of lingering chromatin. To test this possibility, we examined localization of pMLC at the cleavage furrow in the presence and absence of lingering chromatin after chemical inhibition of Aurora B kinase. A chemical inhibitor of Aurora B kinase named ZM447439 (ZM) was used for these purposes. ZM is a selective ATP competitive inhibitor of Aurora B kinase and was used at 2 μ M concentration for 45 minutes (Ditchfield et al., 2003). Aurora B inhibition was confirmed by diminished staining of a known Aurora B substrate, histone H3 phosphorylated on Ser10 (pH3) (Hsu et al., 2000). In untreated cells, pMLC was not enriched at the cleavage furrow in the presence of lingering chromatin (Figure 36). However, consistent with our hypothesis, pMLC enrichment returned to the cleavage furrow after inhibition of Aurora B. 68% of ZM treated cells showed enrichment of pMLC at the cleavage furrow in the presence of lingering chromatin in contrast to 16% of untreated cells. These results indicate that Aurora B activity is required for the inhibition of MLC phosphorylation in the presence of lingering chromatin.

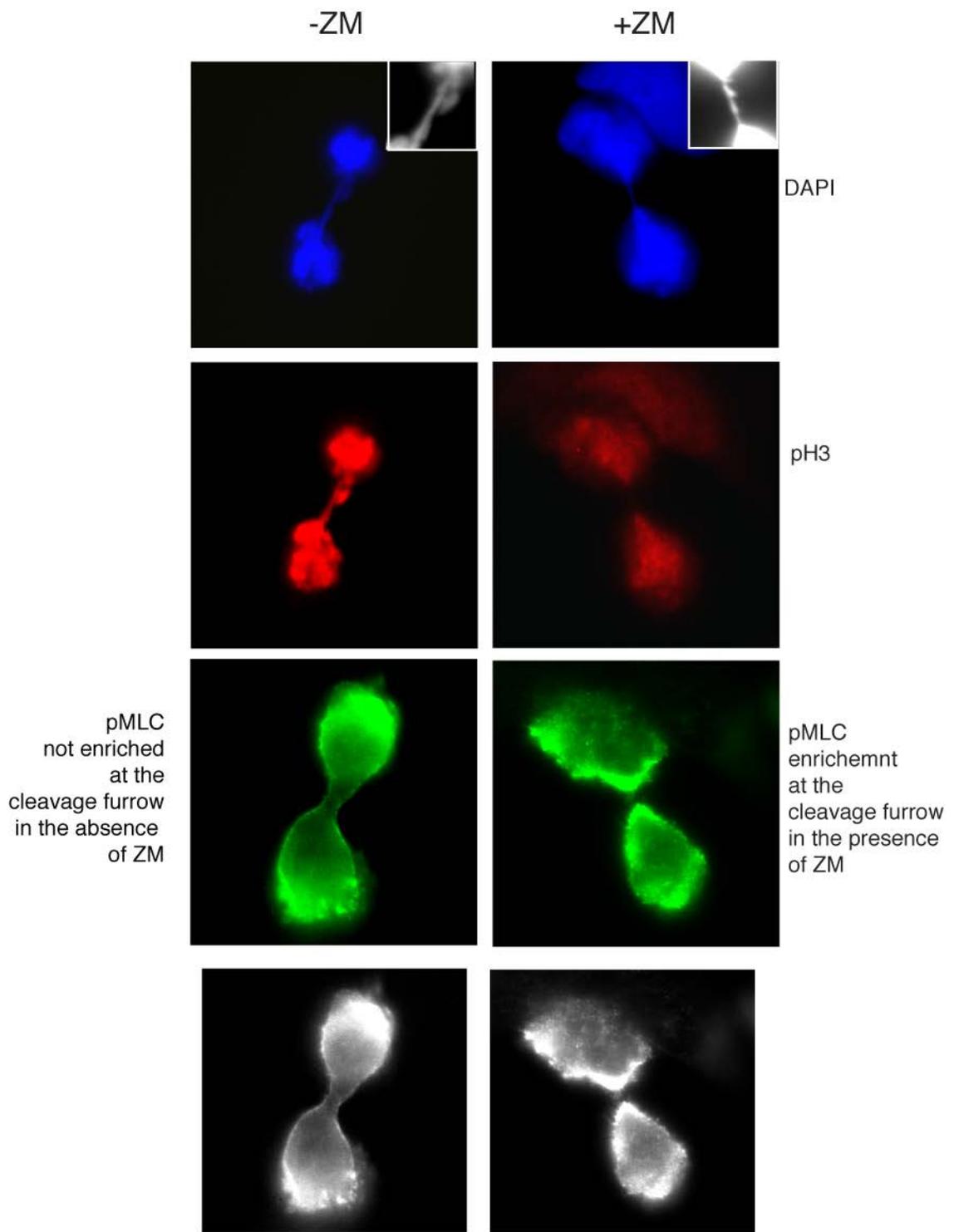


Figure 36. MLC phosphorylation is restored in cells with lingering chromatin after inhibition of Aurora B.

Localization of pMLC in the presence of lingering chromatin (LC) in U2OS cells was examined in cells treated with Aurora B kinase inhibitor ZM447439 (+ZM) for 45 minutes at 2 μ M concentration and cells treated with DMSO control (-ZM). Phospho-histone H3 (pH3) staining was used to indicate Aurora B inhibition. Insets indicate zoomed in images of lingering chromatin. Nuclei were stained with DAPI.

As inhibition of MLCK is the primary downstream effect of Aurora B over-expression, I further analyzed if MLCK inhibition was the cause of non-enrichment of pMLC at the cleavage furrow. To test this possibility, I examined the localization of pMLC after induction of lingering chromatin in cells that were transiently transfected with a constitutively active or a kinase dead MLCK construct. In untreated cells, I consistently observed reduced enrichment of pMLC in anaphases with lingering chromatin (Figure 37). As per our prediction, cells treated with constitutively active MLCK showed enrichment of pMLC at the cleavage furrow even in the presence of lingering chromatin. 75% of cells transfected with a constitutively active MLCK demonstrated pMLC enrichment at the cleavage furrow compared to 20% of cells transfected with a kinase dead MLCK construct. These data indicate that MLCK inhibition is responsible for lingering chromatin induced MLC phosphorylation defects. Restoration of pMLC staining with Aurora B inhibition or MLCK activation suggest that these procedures restore rather than bypass pMLC inhibition pathway and support the hypothesis that deficient MLC phosphorylation caused by lingering chromatin at the cleavage furrow is due to increased Aurora B activation and resultant MLCK inhibition.

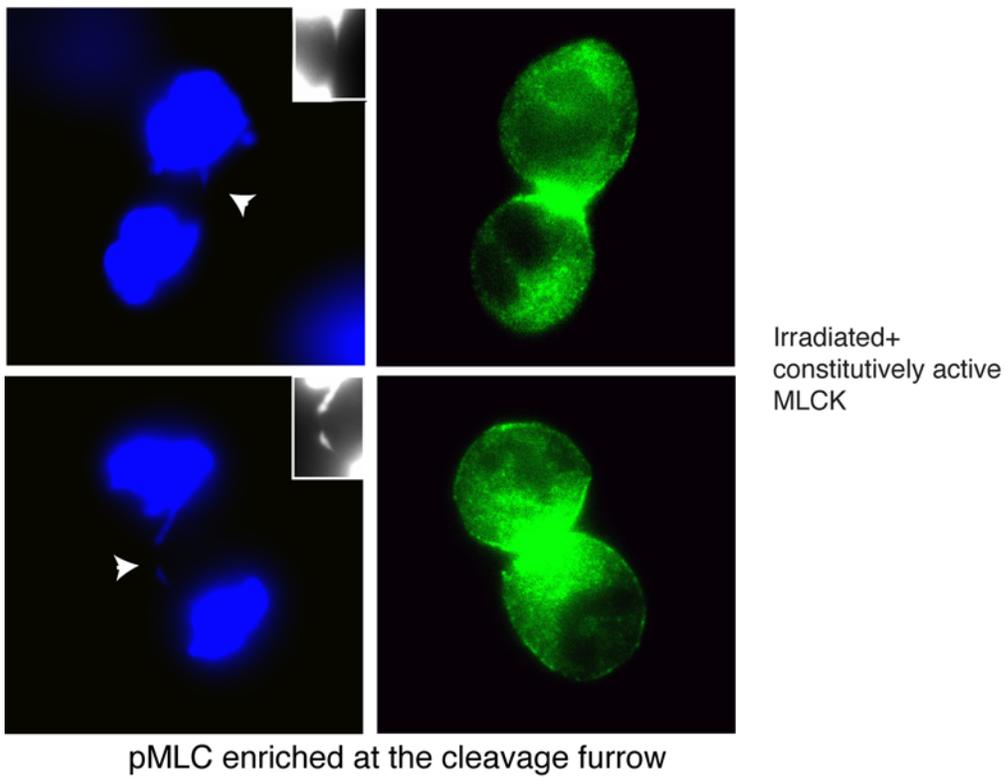
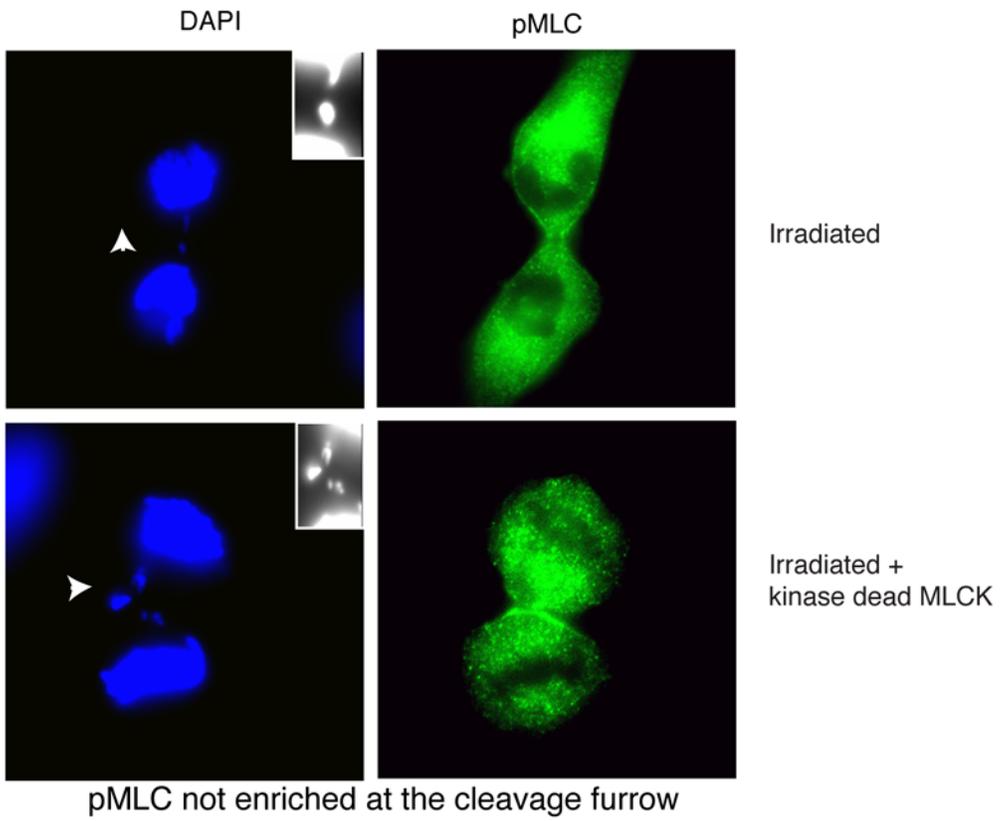


Figure 37. Phosphorylated MLC localizes to the cleavage furrow in the presence of lingering chromatin after activation of MLCK.

Representative images in the top two panels demonstrate localization of pMLC at the cleavage furrow in the presence of lingering chromatin in untreated U2OS cells or cells transfected with a kinase dead MLCK construct. Representative images in the bottom two panels indicate pMLC localization at the cleavage furrow in the presence of lingering chromatin after transfection with a constitutively active MLCK construct. Insets indicate zoomed in images of lingering chromatin.

3.2.9 Inactivation of the pMLC inhibition pathway causes DNA damage

Cytokinesis progression in the presence of lingering chromatin results in DNA damage (Janssen et al., 2011). Therefore, cytokinesis inhibition caused by reduced MLC phosphorylation appears to be a protective mechanism, activated to avoid DNA damage that could be caused by cutting of the lingering chromatin by the ingressing cleavage furrow. If this were true, reversing pMLC inhibition should lead to an increase in the damage of lingering DNA. To test this hypothesis, I transfected U2OS cancer cells with a phosphomimetic MLC construct and examined the levels of phosphorylated γ -H2AX by immunoblotting, an indicator of DNA damage (Rogakou et al., 1998). Compared to empty GFP control, transfection with a phosphomimetic MLC led to an increase in the levels of phosphorylated γ -H2AX, suggesting an increase in DNA damage. U2OS being a cancer cell line has higher frequency of lingering chromatin at the cleavage furrow. Therefore it appears that increased DNA damage after transfection of phosphomimetic MLC could be resulting from cleavage furrow cutting through the lingering chromatin. However, these results need to be verified in other cancer cell lines and also need to be compared with non-cancer cells that typically do not have lingering chromatin at the cleavage furrow.

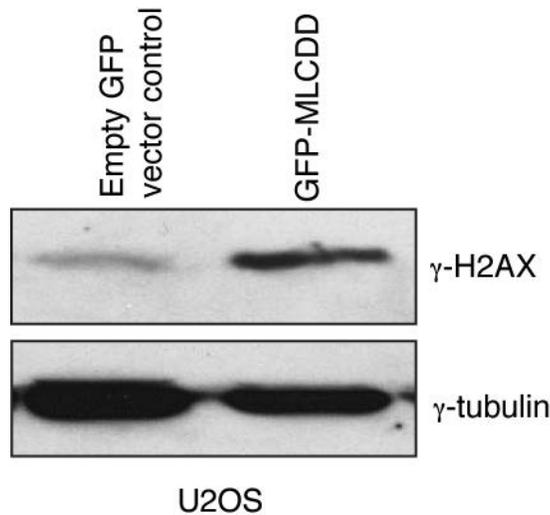


Figure 38. DNA damage is increased upon transfection of a phosphomimetic MLC construct in cancer cells U2OS.

γ -H2AX protein levels were measured as an indicator of DNA damage after transfection of an empty GFP vector control or a phosphomimetic MLC (GFP-MLCDD) construct in cancer cells U2OS. γ -tubulin were used as loading controls.

3.3 CONCLUSIONS AND DISCUSSION

Results presented in the above two sections identify lingering chromatin as the most upstream event in the cytokinesis failure pathway that can now be further extended as follows:

Lingering chromatin at the cleavage furrow → Aurora B over-expression → MLCK inhibition → deficient MLC phosphorylation → cytokinesis failure

I have demonstrated that MLCK and phosphorylated MLC are inhibited in the presence of lingering chromatin and these deficiencies are the cause of lingering chromatin associated cytokinesis failure. Further, I have demonstrated that lingering chromatin and Aurora B over-expression are not independent sources of MLCK inhibition but rather lingering chromatin

induces Aurora B over-expression to cause MLCK inhibition. I have provided the first evidence for lingering chromatin induced over-expression of Aurora B and have dissected the downstream pathway responsible for cytokinesis failure in the presence of lingering chromatin.

Summarizing all the data presented in this dissertation, I would like to propose a model for the regulation of cytokinesis (Figure 39). At the onset of anaphase, when chromosomes begin to segregate away from the cleavage plane, MLCK becomes active leading to MLC phosphorylation, cleavage furrow ingression and cytokinesis completion. However in the presence of lingering chromatin such as anaphase bridges or lagging chromosomes, Aurora B is up-regulated to cause MLCK inhibition, deficient MLC phosphorylation and cytokinesis failure.

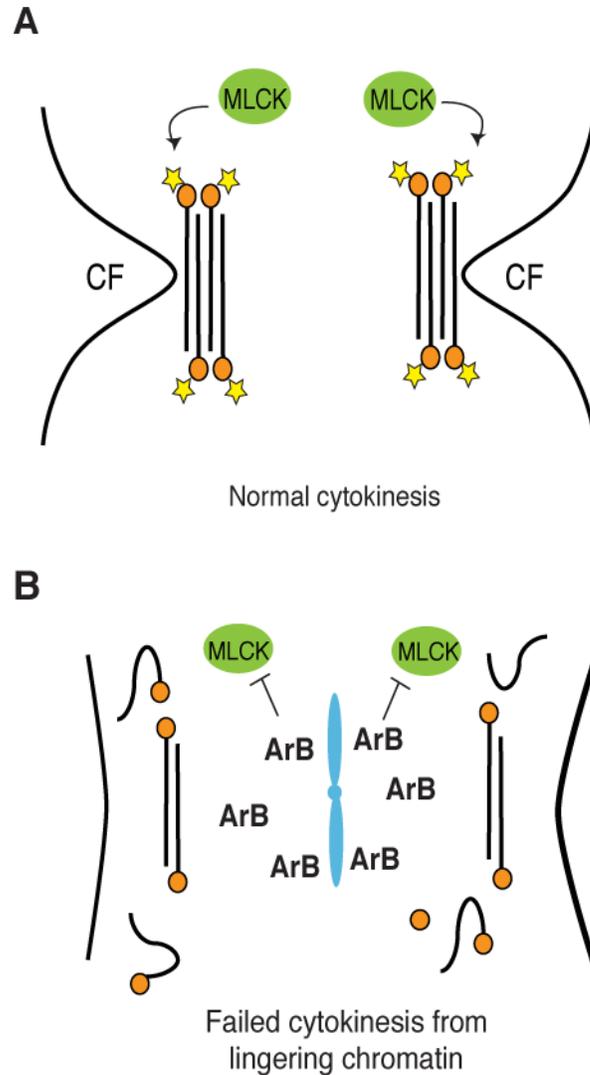


Figure 39. Proposed model for the regulation of cytokinesis.

(A) During normal cytokinesis, MLCK phosphorylates MLC. Phosphorylated MLC (yellow stars) activates motor protein myosin II by promoting bipolar filament assembly and increasing its ATPase activity. The activated myosin then can interact with actin providing the contractile force necessary for the ingression of the cleavage furrow during cytokinesis. (B) Lingering chromatin at the cleavage furrow up-regulates Aurora B kinase (ArB). Up-regulated Aurora B kinase then leads to inhibition of MLCK, deficient phosphorylation of MLC and cytokinesis failure.

Blue fill represents lingering chromatin. CF indicates cleavage furrow.

Role of Aurora B kinase in coordinating cytokinesis with chromatin clearance from the cleavage plane has been described before in the NoCut pathway (Norden et al., 2006; Steigemann et al., 2009). Being a component of the NoCut pathway, Aurora B delays abscission in the presence of chromatin bridges by stabilizing MKLP1 to anchor the intracellular canal between the dividing cells and inactivation of Aurora B leads to furrow regression. The pathway described in our study identifies a similar function for Aurora B in regulating cytokinesis with chromatin clearance, but proposed to function at a different stage of cytokinesis. Our study identifies MLCK inhibition as the primary downstream effect of Aurora B activity in the presence of lingering chromatin and demonstrates that Aurora B over-expression is required for furrow regression. As MLCK and MLC phosphorylation are required for cleavage furrow ingression, which is an earlier stage of cytokinesis than abscission, it is possible that our pathway is different than the NoCut and acts early on to cause furrow regression before cytokinesis is progressed through abscission.

The NoCut pathway described so far suggests that abscission is delayed until chromatin bridges are resolved. However, consequences of unresolved bridges are still unknown. Therefore, alternative possibility is that the delay in abscission in fact signals furrow regression and both MLCK inhibition and MKLP1 stabilization are a part of a single NoCut pathway. If this is true, it is possible that delay in abscission acts as a signal for MLCK inhibition and deficient MLC phosphorylation.

In the yeast NoCut pathway, anillin-related proteins Boi1 and Boi2 localize at the site of the cleavage in an Aurora B-Ipl1 dependent manner to act as abscission inhibitors. Human anillin is also a known regulator of midbody formation, abscission and cleavage furrow ingression (Gai et al., 2011; Kechad et al., 2012; Piekny and Glotzer, 2008). Interestingly, anillin binds pMLC,

specifically phosphorylated by MLCK and this binding is required for maintenance of the furrow ingression (Straight et al., 2005). These data make anillin an attractive candidate that could potentially link the delay in abscission to furrow regression. Future work would be required to test this hypothesis.

I have demonstrated that Aurora B protein levels are increased in the presence of lingering chromatin. However, the mechanism of this up-regulation is still not known. It is likely that lingering chromatin stabilizes Aurora B protein levels, a hypothesis that can be tested in the future. It was recently shown that ADA histone acetyltransferase is required for the activation of NoCut and forcing Aurora B on chromatin activates NoCut independent of midzone defects (Mendoza *et al.*, 2009). I would like to test if a similar signaling mechanism is responsible for the up-regulation of Aurora B and the downstream inhibition of MLCK in the presence of lingering chromatin.

3.3.1 Relevance of MLCK inhibition pathway in non-cancer cells

At its inception, the main focus of this project was to identify the causes of cytokinesis failure in cancer cells. In doing so, I uncovered a pathway that appears to be an aberrancy of Aurora B over-expression phenotype of cancer cells. However, our results of lingering chromatin acting as an inducer of Aurora B over-expression and the observation of increased DNA damage after reversal of pMLC inhibition suggest that this pathway must be relevant during the regulation of normal cytokinesis as well. Even non-cancer cells experience chromosome segregation defects, albeit at lower frequency. In such rare cases, if the cleavage furrow cuts through the lingering chromatin, it can have disastrous consequences such as formation of grossly aneuploid progeny that might be unviable or could be tumorigenic if key tumor suppressors are inactivated by the

cutting of DNA. Therefore it is crucial to prevent DNA damage caused by the ingressing cleavage furrow by making it regress. It might be more beneficial to form a tetraploid cell that can be eliminated in a p53 dependent manner rather than forming a grossly aneuploid or unviable progeny. This idea can be supported by the fact that p53 loss often precedes an appearance of tetraploid cells during early stages of tumorigenesis and only tetraploids that are null for p53 possess the ability to form tumors in mice.

Cancer cells mis-segregate chromosomes at a much higher frequency causing an increase in Aurora B protein levels, probably without any changes in its gene copy number. Incidental loss of p53 along with increased Aurora B levels can then lead to illicit survival of the resultant tetraploid progeny making this pathway an aberrancy of tumor tissues.

I would further like to suggest that MLCK inhibition by Aurora B kinase is required even at earlier stages of cytokinesis. In early mitosis, Aurora B is chromatin associated but later dissociates from chromatin during cytokinesis (Kelly et al., 2007; Vader et al., 2006). Similarly, MLCK activity is inhibited in early mitosis but increases during anaphase. This raises the possibility that chromatin associated Aurora B might inhibit MLCK in early mitosis to avoid premature ingression of the cleavage furrow when chromosomes are still at the metaphase plate. These ideas are still at a speculative stage and further work is required to validate them experimentally.

Even in 21st century, cancer is a major cause of morbidity and mortality in human population (Siegel et al., 2012). Basic research in cancer biology is at the frontier of our war on cancer. By dissecting the cytokinesis failure pathway in cancer cells, we have contributed our small share in understanding the genomic destabilizing events of tumor development as well as have gained insights in the regulation of the cytokinesis in non-cancer cells. This research

improves our understanding of the pathology of the disease and may also open avenues for new therapeutic approaches.

4.0 MATERIALS AND METHODS

4.1 CELL CULTURE

Table 1 shows a list of non-cancer and cancer cell lines used in this study. The cells were propagated in the growth medium recommended by the manufacturer. Oral cancer cells lines UPCI:SCC103 and UPCI:SCC70 were kindly provided by Dr Susanne Gollin (University of Pittsburgh). HFF-hTERT cell lines were kindly provided by Dr. Edward Prochownik (University of Pittsburgh).

Table 1. List of cell lined used in this study

<u><i>Non-cancer cell lines</i></u>	<u><i>Abbreviation</i></u>	<u><i>Tissue of origin</i></u>	<u><i>Growth medium</i></u>
Retinal pigment epithelial cells immortalized with human telomerase reverse transcriptase or hTERT	RPE1	Retina	DMEM-F12 + 10% FBS
Primary fibroblasts	Fibroblasts	Skin	DMEM + 10% FBS
Oral keratinocytes immortalized with hTERT	OKF-hTERT	Oral mucosa	Defined keratinocyte medium

Human foreskin fibroblasts immortalized with hTERT	HFF-hTERT	Foreskin	DMEM + 10% FBS +1% Penicillin-streptomycin + 1% L-glutamine
<u>Cancer cell lines</u>	<u>Abbreviation</u>	<u>Tissue of origin</u>	<u>Growth medium</u>
Oral squamous cell carcinoma	UPCI:SCC103	Tongue	MEM + 10% FBS + 1% non-essential amino acids + 1% L-glutamine
Oral squamous cell carcinoma	UPCI:SCC70	Retromolar trigone or RMT	MEM + 10% FBS + 1% non-essential amino acids + 1% L-glutamine
Osteosarcoma	U2OS	Bone	McCoy's 5A medium + 10% FBS
Lung carcinoma	A549	Lung	F-12K or MEM +10% FBS+1% non-essential amino acids 1% L-glutamine
Cervical adenocarcinoma	HeLa	Cervix	DMEM + 10% FBS
Liver adenocarcinoma	SK-HEP1	Liver	MEM+10% FBS +1% non-essential amino acids+1% l-glutamine

Colorectal carcinoma	HCT116	Colon	McCoy's 5a medium+ 10% FBS
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4.2 MLCK IMMUNOPRECIPITATION

Cells were lysed using modified radioimmunoprecipitation (RIPA) buffer (50mM Tris-HCL pH7.4, NaCl 150mM, NP-40%) with freshly added protease inhibitors (1mM PMSF, 100mM leupeptin and pepstatin each) and phosphatase inhibitors (100mM NaF and 1mM Na₃VO₄). Lysates were clarified by centrifugation at 10,000g for 15 minutes. Supernatants were incubated with MLCK antibody (Sigma Aldrich) at 4⁰C for 2 hours. A slurry of Protein A sepharose beads (Amersham Biosciences) was added to the reaction and the reaction was further incubated at 4⁰C for 2 hours. Immunoprecipitates were washed with RIPA buffer three times and were divided into two halves. One half was subjected to electrophoresis and immunoblotting to confirm MLCK immunoprecipitation and the other half was subjected to an *in vitro* kinase assay to measure MLCK activity. Immunoblots were also quantified using Image J program and the kinase assay values were given minor adjustments (<10%) by multiplying the substrate phosphorylation data with the ratio of the intensities of the scanned immunoblots.

4.3 *IN VITRO* KINASE ASSAY

MLCK activity was determined using an *in vitro* kinase assay, performed as described previously (Poperechnaya et al., 2000). Half of the immunoprecipitates were incubated with a reaction mixture containing 10mM MOPS pH 7.0, 1mM DTT, 4mM MgCl₂, 0.1mM CaCl₂, 1μM calmodulin (Sigma Aldrich), 0.1mM [γ ³²P] ATP (Perkin-Elmer) and 15μM MLCK substrate (Biomol). The reaction was kept agitated using a low speed vortexer. At the indicated time points 10μl of the reaction supernatant was spotted onto P81 phosphocellulose squares (Millipore). Squares were washed 10 times each using 2mls of 75mM phosphoric acid. Transfer of radio-labeled ATP to the substrate was measured using scintillation counter.

4.3.1 MLCK activity assay in the presence of purified Aurora B kinase and Aurora C kinase

To study the effect of Aurora B on MLCK activity, assay was performed in the presence of purified Aurora B kinase (Upstate Cell Signaling Solutions) at the concentration of 1μg/100μl of reaction buffer (500mM Tris-HCL, pH 7.5, 1.0mM EGTA, 150mM DTT and 1X Magnesium ATP cocktail (Upstate cell signaling solutions).

To study the effect of Aurora C on MLCK activity, the assay was performed in the presence of purified Aurora C kinase (Cell signaling solutions) at the concentration of 1 μg/100μl of reaction buffer containing 25mM Tris-HCl pH 7.5, 10mM MgCl₂, 5mM β-glycerophosphate, 0.1mM Na₃VO₄ and 2mM DTT.

4.4 IMMUNOBLOTTING

Cells were lysed using RIPA buffer or hot SDS buffer. Protein concentration was measured by Lowry assay (Bio-Rad). Lysates were subjected to SDS - polyacrylamide gel electrophoresis and were transferred to a PVDF or a nitrocellulose membrane. Membranes were blocked with 5% milk in TBSN and were probed with the primary antibodies diluted in 5% milk in TBSN. After overnight incubation with primary antibody, membranes were incubated with HRP-conjugated secondary antibodies (1:5000, GE healthcare). Proteins were detected using chemiluminescent substrate (Thermo Fisher) and M35 A X-OMAT Processor (Kodak). Primary antibodies and the required concentrations are given in Table 2.

4.4.1 Urea/glycerol gel electrophoresis

Unphosphorylated and phosphorylated forms of MLC were detected by urea/glycerol gel electrophoresis and immunoblotting as described previously (Word et al., 1991). Cells plated in 6 well plates were harvested using ice cold TCA containing 10mM DTT. After centrifugation, cell pellets were washed three times with diethyl ether and were resuspended in urea sample buffer containing 8M urea, 20mM Tris-HCl, 23mM glycine, 10% glycerol, 10mM EGTA, and 0.2% bromophenol blue, pH 8.6. Lysates were subjected to electrophoresis, were transferred onto PVDF membrane, and were probed with anti-MLC antibody (A kind gift from Dr. James Stull, University of Texas Southwestern Medical Centre).

4.5 IMMUNOFLUORESCENCE MICROSCOPY

Cells were seeded on sterile 22X22 mm coverslips placed in 35mm tissue culture dishes. Immunofluorescence staining for MLCK was performed as described previously using gradient methanol fixation (Dudnakova et al., 2006). Cells were fixed with 100% ice cold methanol at -20°C for 10 minutes followed by fixation with 75%, 50% and 25% methanol at 4°C for 3 minutes each. For the immunofluorescence staining of pMLC, Aurora B kinase and pH3, cells were fixed with 4% paraformaldehyde (Sigma-Aldrich) in PBS for 20 minutes and cell membranes were permeabilized using 0.1% Triton (Fisher) for 5 minutes.

Cells were blocked with 1% BSA in PBS for 1 hour and were stained with the indicated primary antibodies for 1 hour at the concentration given in Table 2. Alexa Fluor 488 or Alexa Fluor 568 conjugated secondary antibodies (Invitrogen) were used at 1:250 dilutions for 1 hour. Nuclei were stained with 4,6-diamidino-2-phenylindole (DAPI) at $1\mu\text{g/ml}$ concentration for 5 minutes. Actin was stained using rhodamine-conjugated phalloidin (Cytoskeleton). Cells were visualized using an Olympus BX60 epifluorescence microscope and images were captured using Hamamatsu Argus-20 CCD digital camera.

Table 2. List of antibodies used in this study

Primary Antibody	Manufacturer	Dilution for immunoblotting	Dilution for immunofluorescence microscopy
MLCK	Sigma	1:5000	1:500
Aurora B kinase	BD transduction	1:500	1:150

Aurora A kinase	BD transduction	1:500	NA
Aurora C kinase	Abcam	1:500	NA
MLC phosphorylated on Ser19	Cell Signaling Technology	1:500	1:200
Actin	Cytoskeleton	1:3000	1:200
γ -Tubulin	Sigma	1:5000	NA
Phosph-histone H3	Millipore	1:5000	1:1000
Phospho- γ -H2A.X	Cell Signaling Technology	1:5000	

4.6 TRANSFECTIONS

4.6.1 Recombinant DNA

Phosphomimetic MLC construct was kindly provided by Dr. Kathleen Kelly (National Cancer Institute, Bethesda) (Ward *et al.*, 2002). As described in this paper, pEGFP-MLC construct was made using T7-7-MLC. Site-directed mutagenesis (Stratagene) was performed using pEGFP-MLC as a template to generate pEGFP-MLC (18D, 19D) mutant. Constitutively active and kinase dead MLCK constructs were kindly provided by Dr. Raj Wadgaonkar (SUNY downstate medical center) (Wadgaonkar *et al.*, 2003). As described in this paper, constitutively active MLCK lacks C-terminus amino acid 1745- 1914. Endothelial MLCK was used as a template to obtain the deleted inserts by PCR analysis. PCR product was cloned into TOPO expression vector (Invitrogen) in frame with V5 and His tags. Kinase dead MLCK construct lacks the ATP

binding site consisting of amino acids 1580-1607. To generate kinase dead construct, PCR product of amino acids 1-1580 was cloned into TOPO expression vector followed by the addition of C-terminal portion. Both the constructs are expressed under CMV promoter.

4.6.2 DNA transfection

Cells were seeded on 22X22 mm sterile coverslips. After 6-8 hours, cells were transfected with 1 μ g of the indicated DNA using transfection reagent FuGENE6 (Roche diagnostics) as per manufacturer's guidelines. Following transfection, cells were grown in starvation medium OPTI-MEM for 24 hours followed by growth in full medium for 12 hours. Cells transfected with phosphomimetic MLC were used for further analysis 36 hours after transfection. For constitutively active and kinase dead MLCK constructs, the transfection procedure was repeated after 36 hours and cells were used for further analysis after 72 hours of total transfection.

4.6.3 RNA transfection

Aurora B knock-down was performed using two different siRNAs. siRNA # 1 targets the N-terminal region of the Aurora B mRNA and was commercially available from Qiagen (Hs_AURKB_5, catalog number S102622032). siRNA # 2 was designed as published previously (Klein et al., 2006) using the sequence 5' GGAAAGAAGGGAUCCCUAA 3' (Dharmacon) that targets the C-terminal region of the mRNA. Cells were transfected using a reverse transfection protocol using transfection reagent HiPerFect as per manufacturer's guidelines. To achieve complete knock-down of Aurora B, siRNAs were used at 167 nanomolar

concentration for 48 hours or 24 hours. To achieve partial knock-down of Aurora B, siRNAs were used at 50 nanomolar concentration for 24 hours.

4.7 CELL CYCLE SYNCHRONIZATION

4.7.1 S-phase arrest

Cells were arrested in S phase with a double thymidine block. Cells were treated with thymidine (5mM for non-cancer cells and 2mM for cancer cells) for 18 hours. They were released in the fresh medium for 8 hours followed by second treatment with thymidine for 18 hours.

4.7.2 Mitotic arrest

To compare MLCK activity and expression, cells were arrested in mitosis with 18 hours of nocodazole treatment at the concentration of 400 ng/ml for RPE1 and 300 ng/ml for UPCI:SCC103 cells. To achieve comparable mitotic indices for Aurora B expression analysis, it was necessary to treat cells with nocodazole as follows: OKF-hTERT- 600 ng/ml, HFF-hTERT – 500 ng/ml both treated for 24 hours. RPE1- 400 ng/ml, A549 and U2OS – 300 ng/ml and UPCI:SCC103 – 25 ng/ml, each treated for 18 hours.

4.8 IRRADIATION AND UV- C TREATMENT

After plating the cells for 18-24 hours, they were exposed to 2.8-3 Gy of γ -radiation using ^{137}Cs as a source. Cells were used for further analysis after allowing them to recover for 24 hours. Cells were exposed to 10J of UV-C using UV-C lamp model X-15 (Spectroline) and were used for further analysis 24 hours later.

4.9 DRUG TREATMENTS

Aurora B kinase was inhibited using Aurora B inhibitor ZM447439 (Tocris) at 2 μM concentration for 45 minutes.

To induce lagging chromatin, cells were treated with Eg5 inhibitor monastrol (Tocris) at 100 μM concentration for 16 hours. Cells were allowed to proceed through anaphase by releasing them from the block for 2 hours.

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