# The Regulation of Keratin 8 During The Cell Cycle

**by**

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***This thesis is dedicated to my dearest family;***

***Recep, Mümine and my brother Sinan,***

***Who are always there to support me morally and physically.***

## ABSTRACT

Cell division is essential for living organisms to accomplish their most fundamental tasks such as transferring their genetic information to next generations. During cell division, a cells' morphology and biochemistry undergo dramatic changes. These complex processes are tightly regulated by various cell cycle dependent kinases. Chromosomes, cell membrane and cytoskeletal elements are highly dynamic during mitosis and cytokinesis. Many aspects of cell division are yet to be discovered including the function of master regulators and their dynamic targets at the molecular level. For instance, actin and microtubule filaments, two major members of the cytoskeleton, play critical roles in cell division and their role and regulations are relatively well characterized. However, intermediate filaments, another member of the cytoskeleton, are the less studied aspect in cell division. Therefore, we aimed to study the regulation of keratins, the major member of intermediate filaments, and their regulation by the master mitotic kinases during both mitosis and cytokinesis. We took a mass spectrometry-based phosphoproteomic approach to identify specific phosphorylation sites of Keratin 8 during cell division. Further, we analyzed cytokinesis specific phosphorylation sites of Keratin 8 by using small molecule inhibitors. Our study revealed the phosphorylation dependent regulation of Keratin 8 and its cellular function during cytokinesis

# ÖZET

Hücre bölünmesi, canlı organizmaların genetik bilgilerini gelecek kuşaklara aktarmak gibi en temel görevlerini yerine getirmesi için gereklidir. Hücre bölünmesi sırasında hücrelerin morfolojisinde ve biyokimyasında çarpıcı değişiklikler gerçekleşir. Bu karmaşık süreç çeşitli hücre döngüsüne bağlı kinazlarla sıkı bir şekilde düzenlenir. Kromozomlar, hücre zarı ve sitoskeletal elementler mitoz ve sitokinez sırasında en çok değişkenlik gösteren yapılardır. Hücre bölünmesi pek çok yönden araştırılmış ve birçok moleküler düzeyde mekanizma keşfedilmiştir. Örneğin, hücre iskeletinin başlıca üyeleri olan aktin ve mikrotübül filamentleri, hücre bölünmesinde kritik rol oynar ve onların özgül rolleri ve regülasyonları literatürde iyi tanımlanmıştır. Bununla birlikte, hücre iskeletinin diğer bir üyesi olan ara fılamentler, hücre bölünmesindeki rolleri ve regülasyonları açısından daha az çalışılmıştır. Bu nedenle, bu çalışmada ara filamentlerin başlıca üyesi olan Keratin'lerin regülasyonunun mitoz ve sitokinez sırasında ana mitotik kinazlar tarafından düzenlenmesini incelemeyi amaçladık. Hücre bölünmesi sırasında Keratin 8'in özgül fosforilasyon bölgelerini tanımlamak için kütle spektrometresine fosfopeptitlerin kalitatif tayini yapılmıştır. Ayrıca, küçük molekül inhibitörleri kullanarak Keratin 8'in sitokineze özgü fosforilasyon bölgelerini analiz ettik. Çalışmamız, Keratin 8'in fosforilasyona bağlı regülasyonunu ve bunların sitokinez sırasında hücresel fonksiyonlarını ortaya koymuştur.

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## NOMENCLATURE

| | |
|---|---|
| ALIX | ALG-2-interacting protein X |
| APC | Anaphase-Promoting Complex |
| APC/C | Anaphase-Promoting Complex/Cyclosome |
| ATP | Adenosine 5’-Triphosphate |
| BSA | Bovine Serum Albumin |
| CDK | Cyclin-Dependent Kinase |
| CEP55 | Centrosomal protein of 55 kDa |
| CPC | Chromosomal Passanger Complex |
| DAPI | 4',6-diamidino-2-phenylindole |
| DMEM | Dulbecco's Modified Eagle Medium |
| DTT | Dithiothreitol |
| ECL | Enhanced chemiluminescence |
| ECT2 | Epithelial Cell Transforming 2 |
| EDTA | Ethylenediaminetetraacetic acid |
| EMT | Epithelial-to-mesenchymal transition |
| ESCRT | Endosomal Sorting Complex Required For Transport |
| FBS | Fetal Bovine Serum |
| G0 | Gap Zero |
| G1 | Gap One |

| | |
|---|---|
| G2 | Gap Two |
| GFP | Green Fluorescent protein |
| GST | Glutathione S-transferase |
| GTPase | Guanosine Triphsphophatase |
| HeLa | Human cervical cancer cell line |
| HRP | Horseradish peroxidase |
| IAA | Iodoacetamide |
| IF | Intermediate Filaments |
| INCENP | Inner Centromere Protein |
| IPTG | Isopropyl β-D-1-thiogalactopyranoside |
| kDa | Kilodalton |
| K18 | Keratin18 |
| K8 | Keratin 8 |
| M | Mitosis |
| MPF | Mitosis Promoting Factor |
| PBS | Phosphate buffered saline |
| P/S | Penicillin-Streptomycin |
| PLK1 | Polo-like Kinase 1 |
| PRC1 | Protein Regulator of Cytokinesis 1 |
| RhoA | Ras Homolog Family Member A |

| | |
|---|---|
| ROCK | Rho-associated protein kinase |
| S | Synthesis |
| SDS-PAGE | Sodium dodecyl sulfate polyacrylamide gel electrophoresis |
| STC | S-Trityl-L-cysteine |
| TBS | Tris buffered saline |
| TEMED | Tetramethylethylenediamine |
| WCL | Whole cell lysate |
| WT | Wild Type |

# Chapter 1

## INTRODUCTION

Cell division is an essential process for the reproduction of a cell. The cell undergoes an ordered series of events, which is named ‘the cell cycle’, to complete the process of duplication. The cell cycle consists of sequenced events of DNA duplication of the cell and its division into two. At the end of the cell cycle, one complete new organism is reproduced from a parental cell. The cell cycle consists of two major phases: The S phase, which is required for chromosome duplication, and the M phase, which is required for chromosome segregation and cytoplasmic division. Cell division is tightly controlled and coordinated at specific cell cycle points. Mistakes in cell cycle check points may result in abnormalities in the cell and may even lead to death.

In typical mammalian cells, the S phase takes 10-12 hours to complete. In contrast, the M phase, which requires less than an hour, is much shorter compared to the S phase. Contrarily, the division of the cytoplasm occupies only a few minutes during the completion of the M phase. Although the M phase takes a short period of time, the cell undergoes dramatic morphological and biochemical changes, which take place mostly at chromosome, membrane and cytoskeleton components. Cell cycle regulators provide spatial organization of these components. Aurora kinases are among the major cell cycle regulators. Aurora A

and Aurora B play a crucial role in chromosome orientation and segregation in mitosis [1, 2, 3, 4]. However, Aurora B is fundamental for the completion of cytokinesis. The cell does not undergo cytokinesis, when Aurora B is catalytically inactive [5]. Moreover, its overexpression is associated with a large number of cancer types by causing aneuploidy and chromosomal instability [6].

While Aurora B is active in both mitosis and cytokinesis, its targets change throughout the cell division. Cytoskeleton proteins are one of the major targets of Aurora B in both mitosis and cytokinesis. It has been well established, that microtubule and microfilament regulation during mitosis and cytokinesis through Aurora B activation. However, there are huge gaps in terms of intermediate filament regulation during cell division. Vimentin, which is a type III intermediate filament, is a well-known target of Aurora B during cytokinesis [7]. However, there is insufficient investigation of other intermediate filament proteins, especially keratin proteins.

The main function of keratin proteins is to provide mechanical strength to the cell. For instance, the strongest parts of the body are highly enriched with keratin proteins like hair, nails and horn. Although they are enriched in the strongest structures, they are also significantly expressed in epithelial cells. However, it has not been studied well, how these solid and robust structures do not prevent the cell division and how they are regulated during very dynamic process like cytokinesis. Since, Aurora B is one of the major kinases during

both mitosis and cytokinesis, it is possible that keratin proteins may be one of the targets of Aurora during cell division. In this thesis, I aimed to investigate the re-organization of Keratin 8 in cell division upon Aurora B regulation by using biochemical analysis and phospho-proteomic approaches.

Chapter 2 includes a detailed literature review related to the study in this thesis. It begins with a short description of the cell cycle, and continues with the specific role of Aurora B and the importance of keratin proteins. It ends with recent findings of the relationship between Aurora B and Keratin 8. In chapter 3, the used materials and the applied methods are given. The results of the experiments are described in chapter 4. A detailed interpretation and discussion of the results and future directions of the study are provided in chapter 5.

# Chapter 2

# LITERATURE REVIEW

## 2.1 Cell Cycle

The cell cycle consists of serial events of cell growth and division into two identical cells. The cell cycle is necessary for cells to reproduce by accomplishing its most fundamental task which is transferring its genetic information to the next generation. In this way, more complex organisms can maintain their existence with the development of the organism and constant repair and renewal of tissues. In eukaryotic cells, the cell cycle consists of four main stages: The G1-, S-, G2- and M-phase. If the environment is not favorable in the early stage of cell cycle (G1), the cell decides to enter the so-called G0 stage, which is a resting phase. Required proteins and mRNA are synthesized in the G1 phase to prepare the cell for the following steps. The S phase is critical for cells to duplicate DNA correctly, otherwise cells may encounter genetic abnormalities, which may end in the formation of diseases or even death. Rapid cell growth and protein synthesis occur in the G2 phase just before mitosis. G1, S and G2 phases are called interphase, which usually takes 23 hours in mammalian cells. During this time, the cell makes sure that internal and external environments are suitable for cell division. In the M phase, the two main processes mitosis and cytokinesis lead to

chromosome segregation and cytoplasmic division. Mitosis consists of 5 different stages: prophase, prometaphase, metaphase, anaphase and telophase. A schema of the cell cycle is given in Figure 2.1.

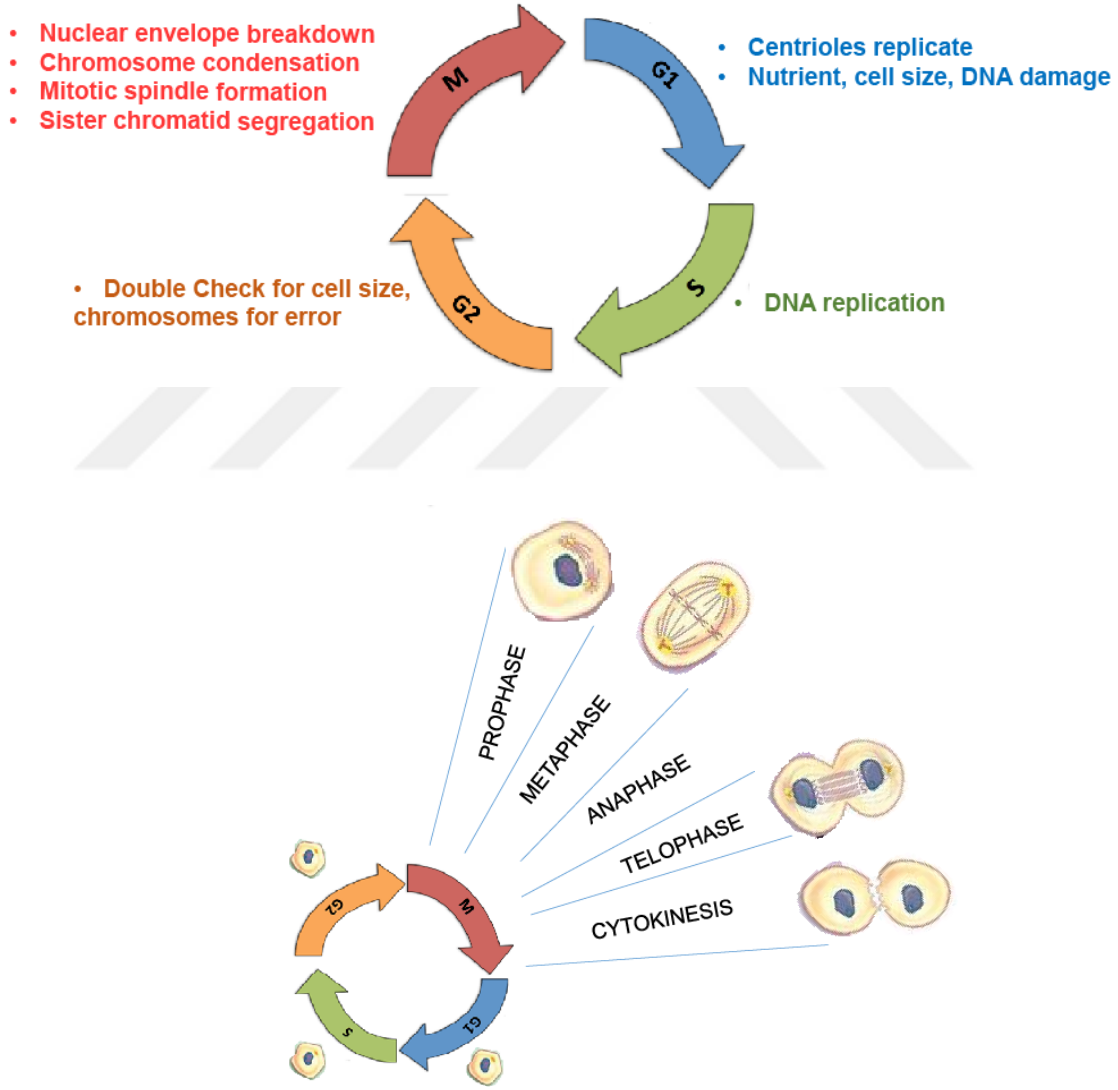



**Figure 2.1 The Cell Cycle Stages of Eukaryotic Cell.** The major steps for cell division

## 2.2. Cell Cycle Regulation

The cell controls the cell cycle precisely at specific check points to maintain proper cell division. There are three major cell cycle checkpoints: The G1 checkpoint, the G2/M checkpoint and the spindle checkpoint. Each checkpoint ensures the favorable cell cycle process and is controlled by cyclin proteins and cyclin-dependent kinases (CDKs) [8,9]. Otherwise, this can cause cells to lose their functionalities or result in cell death.. For instance, bypassing the checkpoints may result in cancer, which leads to uncontrollable division of the cell.

There is a robust and well organized control mechanism for cell cycle checkpoints. The expression of cyclins is cell cycle stage specific and oscillates accordingly. There are four main classes of cyclins, which are named respectively to their active phases: G1-cyclins, G1/S-cyclins, S-cyclins and M-cyclins. Briefly, G1-cyclins coordinate cell growth. G1/S-cyclins initiate entering a new cell cycle. S-cyclins trigger chromosome duplication. The cell enters mitosis by initiation of M-cyclins. Figure 2.2 shows the oscillation of cyclins during the cell cycle. Cyclins assemble with CDKs to form partially active cyclin-CDK complexes. Table 2.1shows specific CDK partners of cyclins. Phosphorylation on activating sites result in fully active cyclin-CDK complexes. Similarly, phosphorylation on inhibitory sites of cyclin-CDK complexes cause inactivation. If a cell decides that internal and external factors are favorable to progress to the next cell cycle stage, related cyclin proteins are ubiquitylated

to be degraded by proteases. Positive and negative control mechanisms provide an error-correctionmechanism. Arresting the cell cycle grants the necessary time for this process.

The cell cycle progresses through phosphorylation and dephosphorylation events, which cause the activation of downstream pathways. For instance, CDK1 phosphorylation on nuclear envelope proteins results in their disassembly and the accession to the chromosome [10, 11, 12]. Spindle assembly and sister chromatin alignment is also provided through CDK1 phosphorylation on its targets. Metaphase to anaphase transition is initiated by phosphorylation of the anaphase-promoting complex (APC), which is a member of the ubiquitin ligase family of enzymes, by CDK1 in case of proper chromosome alignment and kinetochore attachment [13]. APC targets two main types of proteins, securin, which holds sister-chromatid pairs together, and S- and M-cyclins. Securin degradation causes induction of chromosome segregation and eventually cytokinesis.

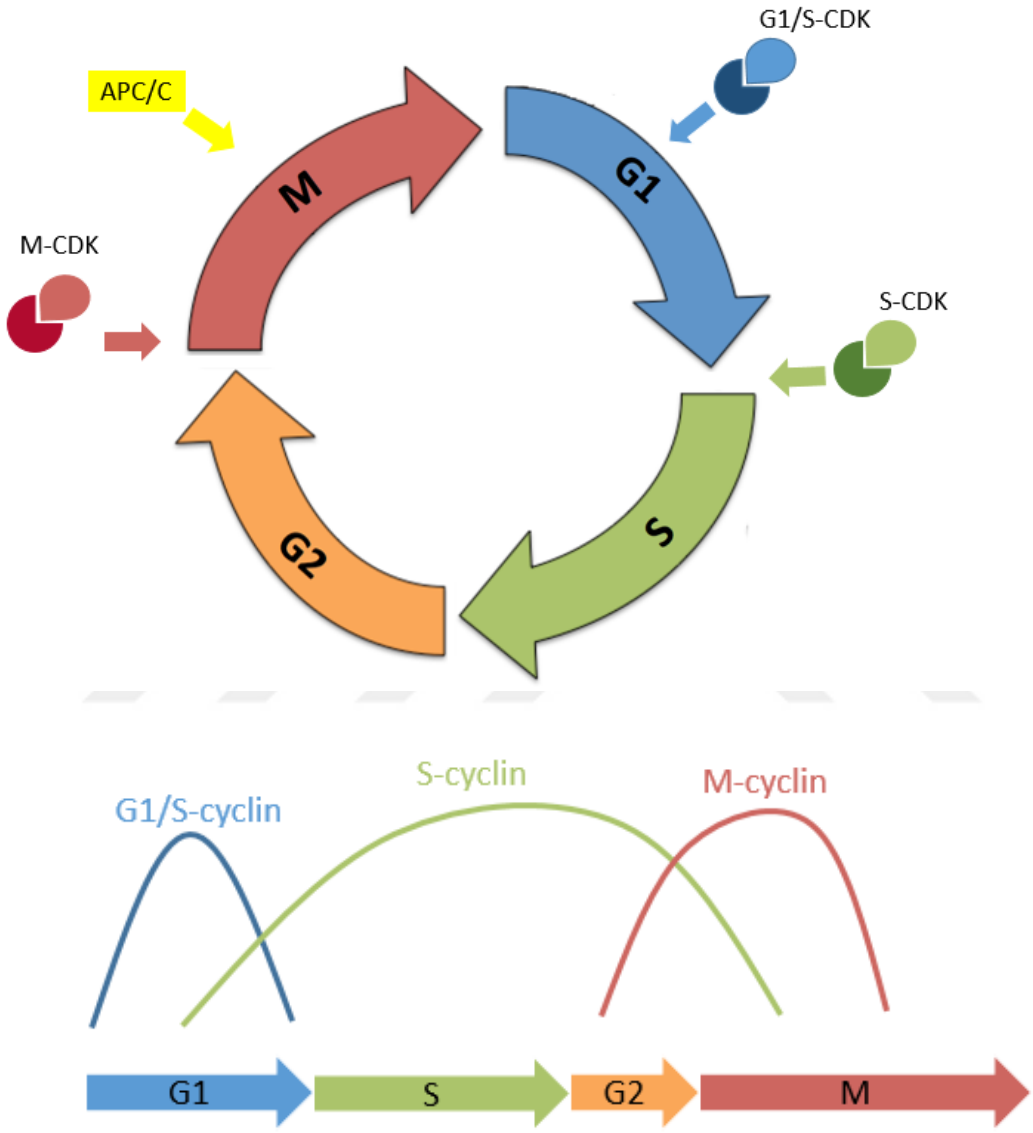



**Figure 2.2 The Cell Cycle Regulation by Cyclins and CDK.** The overview of different cyclins and cyclin/CDK complexes. Cyclins fluctuate in specific pattern during the cell cycle. Inspired by [38]

**Table 2.1 Cyclins and Their CDK partners.** Each cyclins interact with their specific partners depending on the cell cycle stage.

| PHASE | CYCLIN | CDK |
|---|---|---|
| G0 | Cyclin C | Cdk3 |
| G1 | Cyclin D,E | Cdk2, Cdk4, Cdk6 |
| S | Cyclin A, E | Cdk2 |
| G2 | Cyclin A | Cdk1, Cdk2 |
| M | Cyclin B | Cdk1 |

### 2.3 Cytokinesis

The last step of cell division is cytokinesis, in which two daughter cells separate from each other by cytoplasmic division. Cytokinesis proceeds through four different stages: cytokinetic entry, early cytokinesis, late cytokinesis and abscission (Figure 2.3) [14]. Cytokinetic entry starts with the formation of a contractile ring and the appearance of anti-parallel midzone microtubules between segregated chromosomes. The contractile ring is composed of actin filaments, myosin II filaments and other regulatory and structural proteins [15]. In early cytokinesis, gradually contraction of the contractile ring causes the formation of a cleavage furrow and narrows it respectively. Compacted microtubules and other

electron-dense material create an intercellular bridge, named the midbody, between two daughter cells in late cytokinesis. In the last stage of cytokinesis, the midbody formation leads to a favorable stage for abscission by recruiting and orchestrating abscission proteins.

In mammalian cells, RhoA, a small GTPase of the Ras family, is one of the key regulators of cytokinesis [16]. RhoA promotes the formation of a contractile ring at the cell cortex and orchestrates the contraction of the ring. It has two major functions. Firstly, it activates formins, which initiate the nucleation of unbranched actin filaments. Secondly, it stimulates ROCK kinase that directly phosphorylates the regulatory light chain of myosin II to activate it [17].

Although RhoA plays a crucial role in the contractile ring formation, other key regulatory proteins orchestrate cytoplasmic division because the cytoplasmic division is a complex mechanism which includes the cooperation of different pathways, for instance, Golgi apparatus-derived endosomal vesicles. ESCRT-III, ECT2, CEP55, ALIX, PLK1, PRC1 and Aurora B are crucial players of cytokinesis. Although there is no cell cycle checkpoint in contrast to other cell cycle stages, harmony between those proteins operates the separation of the plasma membrane between two daughter cells.

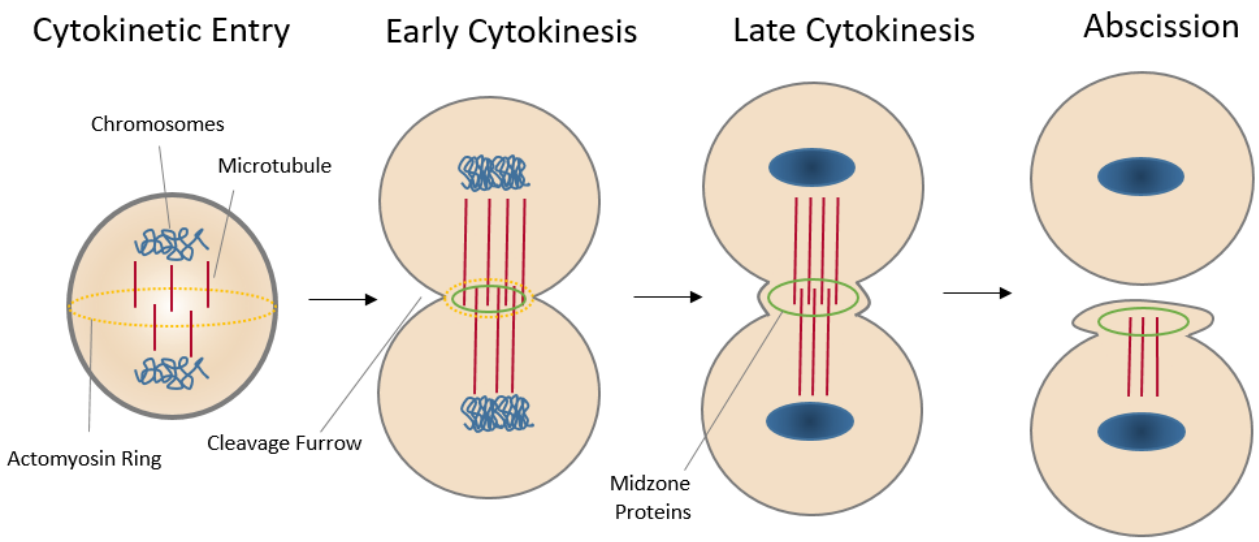



**Figure 2.3 Stages of Cytokinesis.** Four stages of Cytokinesis: Cytokinetic entry, early cytokinesis, late cytokinesis and abscission. Adapted from [14]

### 2.3.1 Aurora Kinases

The members of the Aurora family are serine/threonine kinases. The family is composed of three groups of kinases: Aurora A, Aurora B and Aurora C. These enzymes are essential for cell division. Besides their critical role in cell cycle, their overexpression was also identified in different types of cancer [18]. This property of Aurora kinases made it interesting targets for cancer therapeutics [19].

Since Aurora A/B/C are in the same family, they share common homologies in their kinase domains. However, they have different functions and cellular localizations during the cell

cycle [20]. Aurora A activity is essential in mitosis and peaks during G2/M transition. It enriches at microtubule spindles and centrosomes to regulate centrosome maturation, spindle assembly and stability. In contrast, Aurora B is essential for both mitosis and cytokinesis. Aurora B is a member of the Chromosomal Passenger Complex (CPC) that is one of the key regulators of mitosis. A detailed description of Aurora B will be given in the next section. Lastly, there is little knowledge regarding Aurora C, except that it is also member of CPC like Aurora B [21].

### 2.3.2 Aurora B Kinase

Aurora B activity continues during both mitosis and cytokinesis in contrast to other aurora kinase groups. Its activity peaks in the G2/M phase during the cell cycle. In mitosis, Aurora B functions with its interaction partners such as the Inner Centromere Protein (INCENP), Survivin and Borealin. Assembly of these four proteins form CPC, which organizes various processes including chromosome alignment and histone modification. CPC secures chromosome alignment and attachments between chromosomes and microtubules of the mitotic spindle [22]. The enzymatic core of the protein complex is Aurora B, whereas INCENP is functions as a scaffold protein. All subunits of CPC are required for localization to the centromere, microtubule spindle and cleavage furrow. Malfunctioning of CPC causes chromosome congression and segregation defects which eventually result in multinucleated

cells, tumorigenesis or cell death. Since Aurora B is the catalytic domain that plays a crucial role in both CPC localization and phosphorylation of targets, inactive Aurora B inhibits the cell cycle progress. For this reason, Aurora B has become a more popular target for cancer treatments rather than other Aurora kinases.

Aurora B diffuses in the cytoplasm in interphase and its localization shifts to the nucleus, specifically to chromosome arms and centromeres, through prophase to metaphase. When metaphase to anaphase transition starts, it accumulates at the central spindle after leaving the centromere. In the telophase, it localizes to the cleavage furrow to promote cytokinesis and eventually appears at the midbody until cytokinesis is completed. Aurora B targets a wide variety of substrates with the help of its dynamic localization during the cell cycle. Histone H3 protein is one of the substrates of Aurora B during mitosis. It phosphorylates Histone H3 at Ser10 [23]. Histone H3 phosphorylation on Ser10 appears specifically in mitosis. Therefore, it is defined as a mitotic marker. Furthermore, the kinesin-like proteins MKLP and ZEN4 are two of the major substrates of Aurora B for cleavage furrow formation during cytokinesis [24]. Interestingly, the most studies revealed that vimentin, which is a cytoskeleton protein, is phosphorylated at S56 by Aurora B in cytokinesis [25].

As described above, Aurora B is one of the major regulators of the cell cycle by orchestrating multiple cell cycle phases and targeting multiple substrates. Its substrates are of many different protein types including kinases, kinesins, histones, membrane and cytoskeleton

proteins. However, there are more substrates of Aurora B and their phosphorylation sites to be discovered to understand underlying mechanisms of mitosis and cytokinesis.

## 2.4 Cytoskeleton

Cytoskeleton proteins are the major players to maintain various functions in the cell, which include mechanical strength and spatial organization of the cell. Three cytoskeleton filament families, which differ in their role in the aspect of cell mechanics and biological role , contribute to these functions: Microfilaments (actin filaments), microtubules and intermediate filaments (IF). Cytoskeleton filaments differ from each other by their intracellular distribution, characteristic structures and distinct types of protein subunits polymerizing to specific filaments. Actin polymers are 7 nm in diameter, whereas microtubules are polymers of tubulin and 25 nm in diameter. In contrast, intermediate filaments are polymerized by various proteins depending on the type of IF and their size changes from 8 to 12 nm in diameter. Actin filaments play a critical role in the cell motility and the pinching of two daughter cells during cell division, whereas microtubules provide intercellular organization of organelles and chromosome segregation during mitosis. We have a more distinctive understanding of actin filaments and microtubules in terms of their roles and associations. However, intermediate filaments are not well characterized compared to the other two family members.

### 2.4.1 Intermediate filaments

IF consist of various types of proteins, whereas actin and microtubule filaments consist of a single type of polymer. Therefore, they can form both homodimers and heterodimers. More than 60 genes contribute to subtypes of IF. Six subtypes of IF are categorized according to their amino acid sequence similarities. They have similar secondary structures, which consist of three main domains: The head, the alpha helical rod and the tail domains. Figure 2.4 shows a schematic diagram of IF secondary structures. The greatest variety of IF comes from Type I (acidic) and Type II (basic/neutral) keratin classes. Type III are composed of desmin, GFAP, Peripherin and vimentin. Type IV are composed of α-internexin, neurofilaments, synemin and syncoilin. Type V are lamins which contribute to the structure of the cell nucleus. Type VI are nestins which are present mostly in nerve cells. Figure 2.5 illustrates the distribution of intermediate filaments in different mammalian cell types.

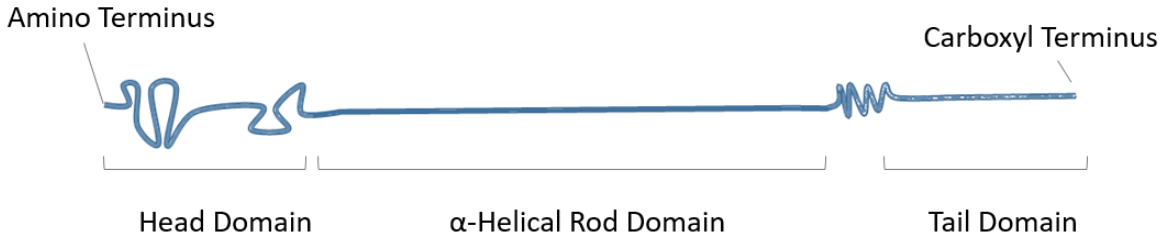



**Figure 2.4 Secondary Structure of Intermediate Filaments**. Similarity in secondary structure of subclasses of intermediate filaments. Adapted from [39].

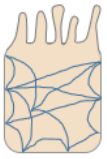
| Class | Protein | Distribution | Function | |
|---|---|---|---|---|
| I | Acidic Keratins | Epithelial Cells | Tissue Strength |  Epithelial cell |
| II | Basic Keratins | Epithelial Cells | Tissue Strength | |
| III | Desmin, Vimentin, GFAP | Muscle, Mesenchymal cells, Glial cells | Sarcomere Organization |  Smooth muscle |
| IV | Neurofilaments | Neurons | Axon Organization |  Axon |
| V | Lamins | Nucleus | Nuclear Organization |  Nuclei |

**Figure 2.5 Intermediate Filaments in Mammals**. Intermediate filaments are expressed in different cell types. Adapted from [40]

Some intermediate filaments are well characterized in terms of their cellular function and regulation mechanisms. For example, vimentin is differentially expressed in mesenchymal cells which makes it a marker for mesenchymally derived cells or for the epithelial-to-mesenchymal transition (EMT) [26]. Moreover, it is a clinically significant protein because it is used as biomarker for colon cancer [27]. Besides those properties of vimentin, the mitosis-promoting factor (MPF) and Aurora B provide phosphorylation dependent regulation of vimentin through the cell cycle. Vimentin reorganization during mitosis is mediated by p34 phosphorylation and Aurora B regulates the cleavage furrow specific vimentin phosphorylation in the cytokinesis process [7]. Apart from vimentin, lamins are also regulated in a cell cycle dependent manner by CDK1 to initiate nuclear envelope breakdown. Although cell cycle dependent regulation of some important members of IF are revealed, there is insufficient information about the cell cycle dependent regulation of keratin proteins, the major members of IF.

#### 2.4.1.1 Keratin Proteins

Keratin proteins are composed of Type I and Type II classes of IF. There are two types of keratins: Acidic (Type I) and basic/neutral (Type II). Fifty-four genes in humans contribute to keratin expression in epithelial cells. Keratins are expressed in different epithelial cells in a tissue-specific manner as shown in Figure 2.6. Their cellular function is mainly to provide

mechanical strength, stability and integrity to the cell. Moreover, some keratins have distinct functions in differentiation or intracellular signaling to protect the cell from stress, to promote wound healing and to regulate apoptosis [28].

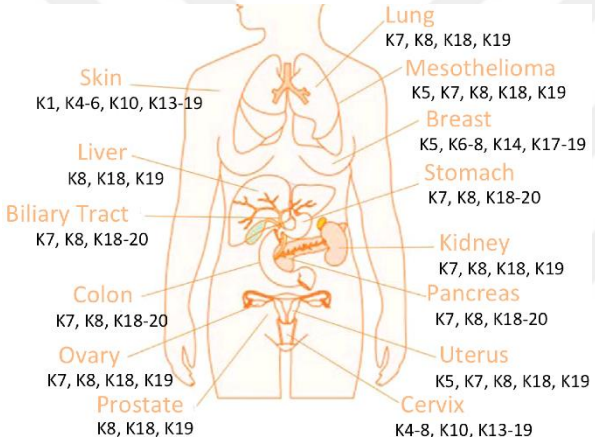



**Figure 2.6 Keratin Tissue-Specific Expression.** [43]

Type I and Type II keratins pair and form heterodimers to function properly, which requires at least one acidic and basic/neutral protein. Keratins are expressed in epithelial cells in a tissue/organ specific manner. For example, the K8/K18 pair is expressed in simple epithelial cells, whereas the K5/K14 pair is expressed in stratified epithelium. Mutation in either pair

of keratin heterodimers may result in severe conditions including weakness in mechanical stress and fragility also known as the epidermolysis bullosa simplex [29, 30].

Although malfunctioning of keratin proteins primarily causes mechanical strength related diseases, an association of keratin proteins with other diseases such as cancer has been discovered. K5, K14 and K17 are upregulated in squamous cell carcinoma (SCC) and K18 is upregulated in adenocarcinoma [31]. Another study has revealed that collective invader cells are K14+ in major breast cancer subtypes [32].

Like other IF proteins, the regulation of keratin through post translational modifications (PTM) plays a critical role for its cellular function. Phosphorylation, sumoylation, O-linked glycosylation and ubiquitination are major PTMs to regulate the reorganization of keratin filaments. Specific PTMs on keratin proteins are associated with different cellular functions and even with diseases (Figure 2.7). For example, K8/K18 hyperphosphorylation has been correlated with chronic liver disease progression, whereas dephosphorylation of K8-S73/S431 correlates with increased cell migration and tumorigenesis in oral squamous cell carcinoma (OSCC) [33, 34, 35].

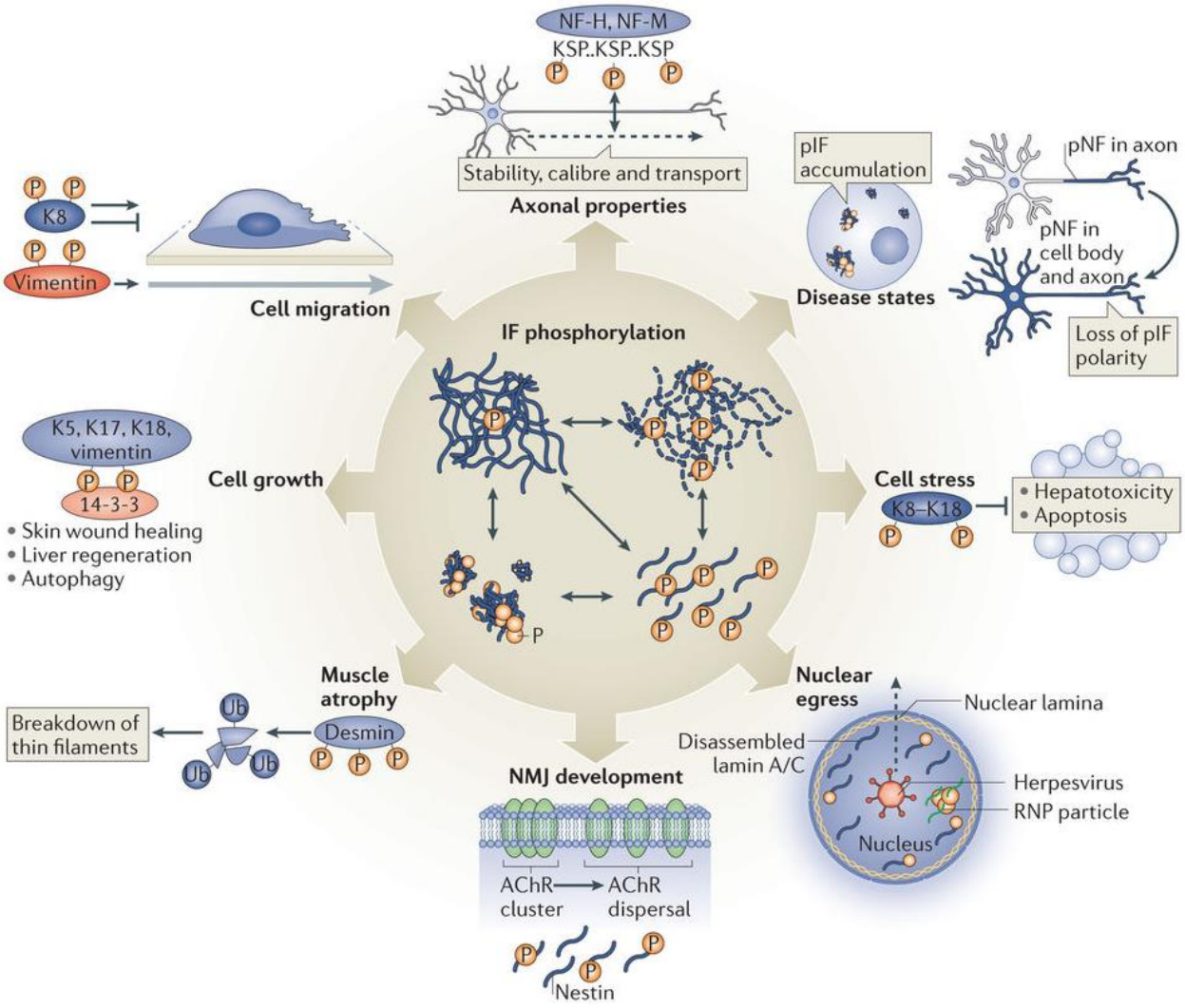



**Figure 2.7 Keratin Regulation through Post Translational Modification**. Intermediate filaments are regulated through PTMs, especially keratin proteins functions in different cellular mechanisms depending on their modifications. [41]

Although the importance of keratin proteins is well established in the literature in terms of different aspects, the underlying mechanism of the regulation of keratin proteins is not clear. One of the less studied subjects is the regulation of keratin during cell division. Interestingly, it is unknown how the mechanical property of keratins does not disrupt cell division especially during cytokinesis. For the successful completion of cell division, keratin should be cut or reorganized during cell division. Otherwise, the cell cannot complete the process of cytokinesis which will cause multinucleated cells. Therefore, there must be a regulatory mechanism for the reorganization of keratin during cell division. First evidences about the regulation of keratin during cell division has been revealed in the early 1980's. They have showed that some cells had speckles formation of Keratin 8 during prophase to telophase [36]. However, the speckle formation was not common among all the cells, but this phenotype was observed among different cell types. It shows that there could be multiple mechanisms for reorganization of Keratin 8 during cell division. It could be speculated that speckle formation of keratin during mitosis is provoked by CDK1 which is one of the master mitosis regulators and they generated a model for regulation of Keratin 8 during the cell cycle (figure 2.8). However, reorganization of Keratin 8 in cytokinesis is more crucial to provide plasma division, otherwise it will end up with multinucleation. For that reason, CDK1 may not be a regulator of Keratin 8 during cytokinesis. It is more likely that master regulators of cytokinesis including Aurora B, Plk1, PRC1 and ESCRT II may have an active role in the reorganization of Keratin 8.

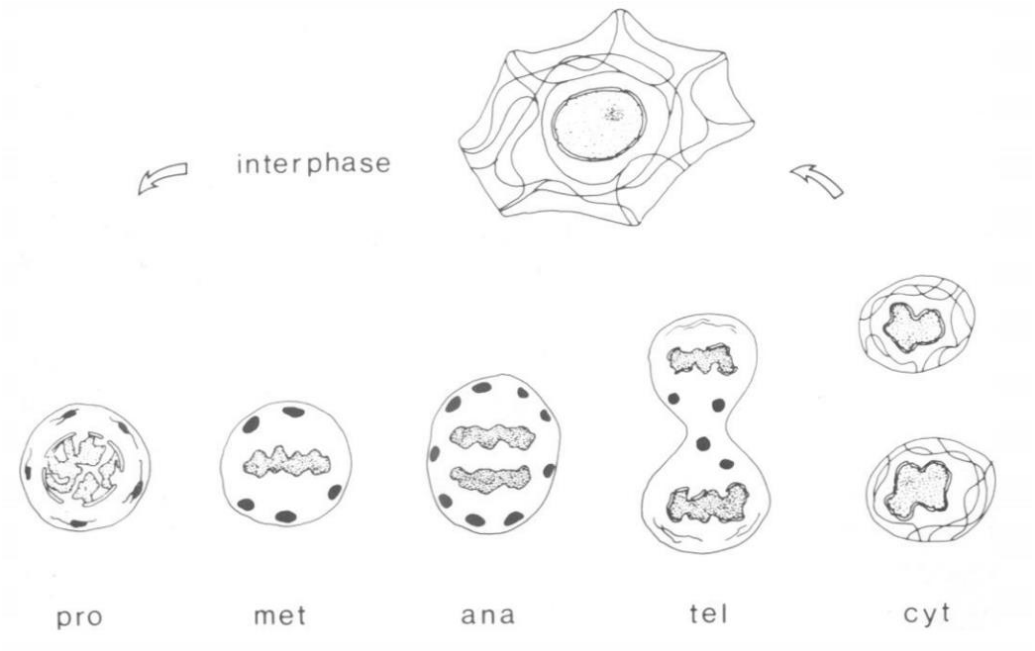



**Figure 8 Keratin Filament Disruption and Reformation During mitosis.** Suggested mechanism for Keratin 8 regulation through speckle formation. [36]

### 2.5 Keratin 8 and Aurora B

Previous genome-wide phosphoproteome analysis identified some Aurora B specific phosphosites on Keratin 8 during cytokinesis. In this study, cytokinesis specific phosphopeptides of Keratin 8 identified by quantitative mass spectrometry are described (Figure 2.9). Then, Aurora B specific phosphopeptides established by combining quantitative phosphoproteomics with chemical genetics. It has been showed that two cytokinesis specific phosphopeptide intensities decreased upon VX680-mediated Aurora B inhibition during cytokinesis [37].

| Protein Name | Sequence | Phos. Site | Mock/VX680 | Mit/Cyt |
|---|---|---|---|---|
| Keratin-8 | SYKV**S**TSGPR | S5 | 10.49 | 0.24 |
| | SYTSGPGSRI**S**SS**S**FSR | S11, S14 | 8.45 | 0.38 |

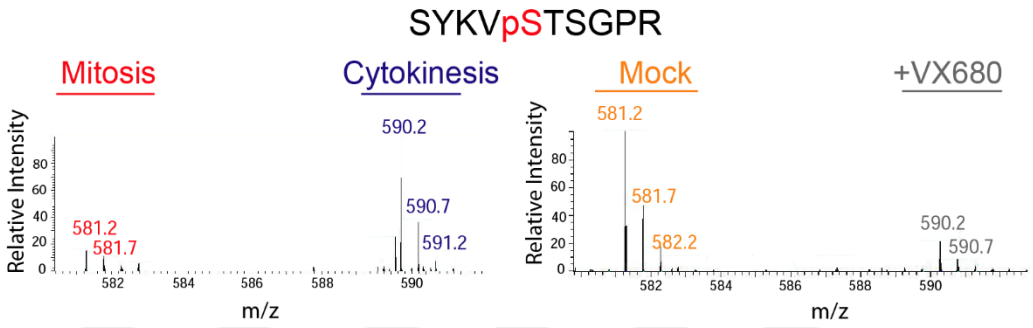



**Figure 2.9 The Cytokinesis Specific Keratin 8 Phospho Peptides.** Aurora B dependent cytokinesis phosphorylation sites of Keratin 8 are identified as S13 and S34. [37]

A current study revealed that intermediate filaments are also major substrates of Aurora B kinase besides microtubules and actin [7]. Over 20,000 phosphopeptides were quantified in this study by using a quantitative proteomic approach (SILAC). 246 of them were identified as unique phosphopeptides which are down regulated upon VX680 and AZD1152- mediated Aurora B inhibition. Cytoskeleton proteins were one of the major targets of Aurora B (Figure 2.10). In the light of these findings, we aimed to reveal the cell cycle dependent regulation of Keratin 8 through Aurora B phosphorylation.

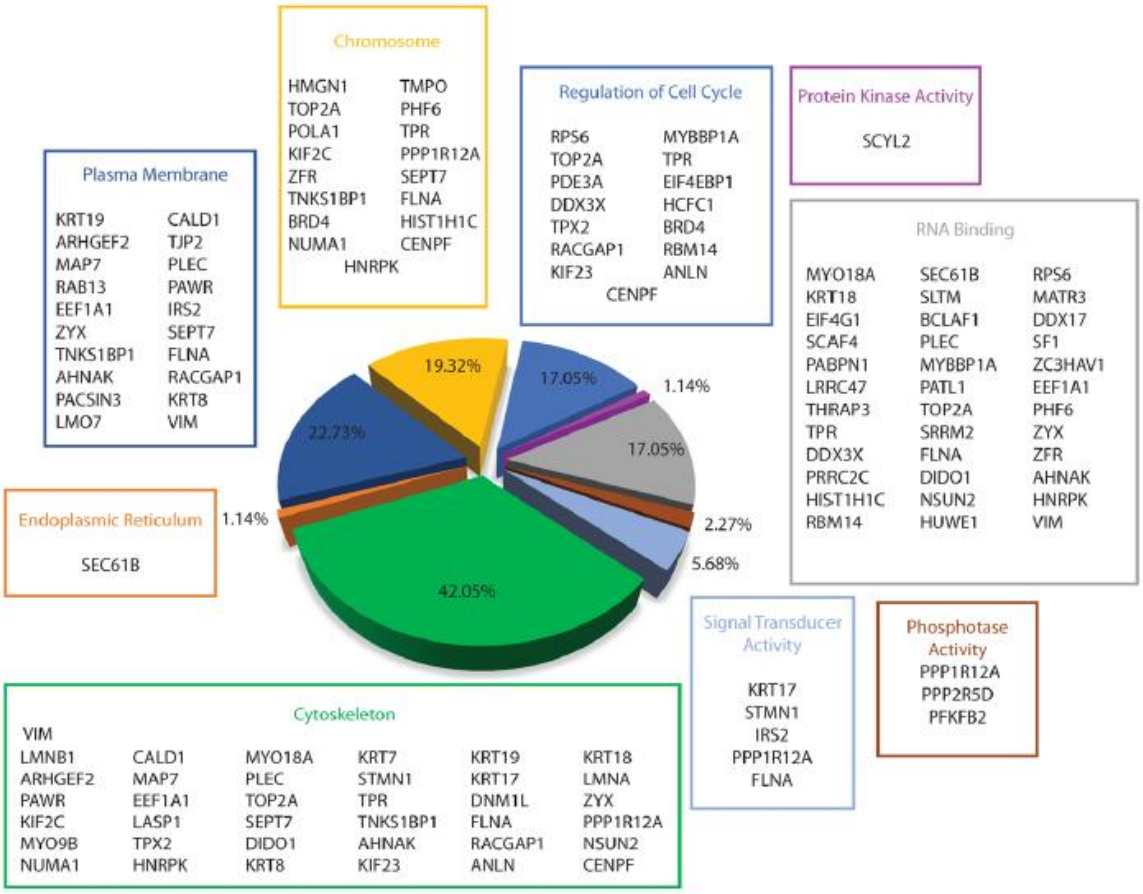



**Figure 2.10 The Major Substrates of Aurora B in Cytokinesis.** [7]

# Chapter 3

# MATERIALS AND METHODS

## 3.1 Cell Culture

HeLa S3 cells were routinely cultured and maintained in Dulbecco's modified Eagle's medium (DMEM) (Lonza, BE12-741F) containing 10 % Fetal Bovine serum (FBS), 100 unit/ml Penicillin and 100 µg/ml Streptomycin (Lonza, DE17-602E) at 37°C and 5% CO2. The cells were passaged every 3 days to prevent over confluency. Briefly, the cells were washed with 1X phosphate saline buffer (PBS) once before adding 0.25 % trypsin/EDTA (Sigma Aldrich, T4049). Cells were incubated for 5 minutes at 37°C and 5% CO2 to detach them from plate. Trypsin was deactivated by adding 4x volume of DMEM. Then, the cells were transferred to new cell culture plate. For harvesting of the cells, trypsinization procedure was followed by centrifuge of collected cells at 1,200 rpm for 4 minutes at 4°C to pellet them. Centrifuge step was repeated once with 1X PBS after sucking DMEM. Then, the pellet was frozen in liquid nitrogen and stored at -80°C. To freeze cells, $10^6$ Cells were suspended in freezing medium (70 % DMEM, 20% FBS and 10 % dimethyl sulfoxide (DMSO) and stored in liquid nitrogen or at -80°C.

HeLa Kyoto cells stably expressing Aurora B-LAP-tagged BAC transgenes were cultured in DMEM containing 10 % FBS, 100 unit/ml Penicillin, 100 µg/ml Streptomycin and 400

μg/ml Geneticin at 37°C and 5% CO2. To passage, freeze or harvest cells, previously described procedure was performed.

### 3.2 Cell Cycle Synchronization

The cells were grown at 30% confluency. If immunostaining procedure was performed further, the cells were seeded into coverslips. Double thymidine block was proceed to synchronize the cells to interphase. Briefly, cells were incubated with DMEM including 2 mM Thymidine (Santa Cruz, 296542A) for 20 hours. Cells were arrested at G1/S phase at the end of the first thymidine block. Then, they washed three times with 1X PBS and they released for 8 hours in fresh DMEM. Cells were incubated in 2 mM Thymidine for 17 hours at the end of release. Then, they washed three times with 1X PBS to remove excess thymidine and they released from second thymidine block for 7 hours in fresh DMEM. To arrest cells at prometaphase, cells were incubated in DMEM including 30 nM Nocodazole (Calbiochem, 487928), which is reversible microtubule polymerization inhibitor, for 5 hours. Nocodazole arrested cells were gently washed with 1X PBS three times. Then, they released from nocodazole arrest in fresh complete DMEM. Cells were harvested or fixed to coverslips at the end of one hour release from nocodazole to collect cells at bipolar cytokinesis. To induce Aurora B inhibition at cytokinesis, cells were treated with 1 μM AZD1152 or 0.5 μM VX680 Aurora B inhibitors at the 30 minutes of nocodazole release.

In order to arrest cells at mitosis more efficiently, double thymidine block was followed 12 hours incubation with 10 μM Kinesin 5 inhibitor S-Trityl-L-cycsteine (STC) (Sigma, 164739). At the end of STC incubation, cells were collected with 80-90% of mitotic cells.

## 3.3 Cloning and Transfection

### 3.3.1 Side Directed Mutagenesis of wild type Keratin 8:GFP vector

Wild type Keratin 8 in pEGFP-N3 (Addgene) (WT-K8-GFP) vector was gift from Dr. Milind Vaidya (ACTREC). GFP was tagged at the N terminus of Keratin 8 in the vector. Keratin 8 mutated constructs contains S13A and S34- 35A mutations were cloned into pEGFP-N3 vector by Gurkan Mollaoglu (former M.Sc. student of Cell Biology and Proteomics Laboratory, Koc University) and Goksu Ozlu (former intern student) with site directed mutagenesis procedure. Additional S36-37A mutations to Keratin 8 S13A, S34-35A were constructed with the same strategy. Briefly, exponential amplification of target template was performed by polymerase chain reaction (PCR). In PCR reaction, 50 ng/μl Keratin 8 S13A, S34-35A in pEGFP-N3 vector was used as template in 25 μl reaction supplemented with 1X Pfu DNA polymerase reaction buffer, 200 nM forward primer (5' GGTTCCCGCATCGCCGCCGCCGCCTTCTCCCGAGTGGGC 3'), 200 nM reverse primer (5' GCCCACTCGGGAGAAGGCGGCGGCGGCGATGCGGGAACC 3'), 280 μM dNTP mix (NEB, N0447S), 1.25 unit Pfu Turbo DNA polymerase (Stratagene, 600252) and

distilled water ($dH_2O$) to complete 25 µl. PCR condition was set as following: Initial denaturation at 98°C for 30 seconds, 19 cycles of ; denaturation at 95°C for 40 seconds, annealing at 58°C for 3.5 minutes, extension at 68°C for 15 minutes and final extension at 68°C for 5 minutes. PCR products were run in 1 % agarose gel electrophoresis to confirm template amplification by comparing band intensity with control without primers. Then, PCR products were treated with 5 Unit DpnI enzyme (NEB, R0176) to digest methylated parental template at 37°C for 1 hour. After DpnI digest, templates were transformed into Escherichia coli (E. coli) DH5α competent cells by following transformation protocol. In summary, all PCR products were transformed into 100 µl E. coli DH5α bacteria. Mixture was incubated on ice for 30 minutes. To induce heat shock, mixture was incubated at 42°C for 45 seconds. Then, cells were transferred on ice immediately. Transformed cells were incubated at 37 °C for 1 hour and 30 minutes in Lysogeny broth (LB) without any selective antibiotic. Concentrated bacteria were inoculated into 50 µg/ml Kanamycin agar plate. Plates were incubated at 37 °C for 17 hours. Single colonies were growth in 50 µg/ml Kanamycin LB at 37 °C for 17 hours. Plasmid DNA purification was performed with Macherey-Nagel Nucleospin Plasmid mini-prep kit by following manufacturer's instructions. Purified plasmids were send to Macrogen (Europe) to be sequenced with universal CMV forward primer. Confirmed sequenced were used in further experiments.

### 3.3.2 Cloning of Wild Type Keratin 8 into pGEX-6P-1 vector

Wild type Keratin 8 was cloned into pGEX-6p-1 (Addgene) (WT-K8-GST) for Keratin 8 purification experiments. Keratin 8 was amplified from Wild type Keratin 8-GFP-pEGFP-N3 with PCR. ). In PCR reaction, 20 ng/µl Keratin 8 S13A, S34-35A in pEGFP-N3 vector was used as template in 20 µl reaction supplemented with 1X Phusion High Fidelity (HF) DNA polymerase reaction buffer, 200 nM forward primer including EcoRI restriction site (5' *GCGCGCGAATTCATGTCCATCAGGGTGACCCAG* 3'), 200 nM reverse primer including SalI restriction site (5' *GCGCGCGTCGACCTTGGGCAGGACGTCAGAGGAC* 3'), 200 µM dNTP mix (NEB, N0447S), 0.6 unit Phusion High Fidelity DNA polymerase (NEB, M0530S) and $dH_2O$ to complete 20 µl. PCR condition was set as following: Initial denaturation at 98°C for 1 minute, 35 cycles of ; denaturation at 95°C for 10 seconds, annealing at 70°C for 30 seconds, extension at 72°C for 3 minutes and final extension at 72°C for 5 minutes. PCR products were run in 1 % agarose gel electrophoresis to confirm template amplification by comparing DNA ladder. PCR products were purified with Macherey-Nagel Nucleospin Gel and PCR Clean-up Kit by following manufacturer's instructions. Purified PCR fragment and acceptor vector (p-GEX-6p-1) was digested with EcoRI-HF and SalI-HF restriction enzymes. Digestion reaction was performed 1X Cut Smart buffer, one unit of each restriction enzymes, 1 µg of PCR fragment or acceptor vector and $dH_2O$ to complete 50 µl at 37°C for 2 hours. Acceptor vector was treated with 1 unit Antarctic Phosphotase (NEB, M0289S) with addition of Antarctic phosphatase reaction buffer (1X

final concentration). Digested PCR fragments were incubated at 65°C for 10 minutes to heat inactivate restriction enzymes. Digested acceptor vector were incubated at 70°C for 10 minutes to heat inactivate both restriction enzymes and Antarctic phosphatase. Digested products were purified with Macherey-Nagel Nucleospin Gel and PCR Clean-up Kit by following manufacturer's instructions. Then, 20 µl ligation reaction were performed by using 1X T4 DNA ligase buffer, 50 ng acceptor vector (digested p-GEX-6p-1), 45 ng digested PCR fragment (K8 insert), 1 µl T4 DNA ligase (NEB, M0202S) and $dH_2O$ to complete 20 µl at 16 °C for overnight. After ligation, templates were transformed into Escherichia coli (E. coli) Stbl3 competent cells by following transformation protocol. In summary, all ligation products were transformed into 100 µl E. coli DH5α bacteria. Mixture was incubated on ice for 30 minutes. To induce heat shock, mixture was incubated at 42°C for 45 seconds. Then, cells were transferred on ice immediately. Transformed cells were incubated at 37 °C for 1 hour and 30 minutes in (LB) without any selective antibiotic. Concentrated bacteria were inoculated into 100 µg/ml Ampicilin agar plate. Plates were incubated at 37 °C for 17 hours. Single colonies were growth in 100 µg/ml Kanamycin LB at 37 °C for 17 hours. Plasmid DNA purification was performed with Macherey-Nagel Nucleospin Plasmid mini-prep kit by following manufacturer's instructions. Purified plasmids were send to Macrogen (Europe) to be sequenced with universal M13-pUC-reverse primer. Confirmed sequenced were used in further experiments.

### 3.3.3 Transfection

WT-K8-GFP and Mutated-K8-GFP vectors were transfected to HeLa S3 cells with Lipofectamine 2000 by following manufacturer's instructions for immunostaining and live cell imaging experiments. If transfected HeLa S3 cells was used for synchronization, they were transfected with vectors 8-24 hours before thymidine block. In summary, cells were grown in 12-well plate on coverslips until they reach 50 % confluency. Then, 300-1500 ng DNA was incubated with 1-5 µl Lipofectamine 2000 in 150 µl Optimem (Thermo Fisher, 31985047) for 10 minutes at room temperature. DNA was given to cells drop by drop. Cells were washed with 1X PBS and DMEM was changed after 8-10 hours transfection.

## 3.4 Keratin 8:GST Purification from Bacteria

### 3.4.1 Keratin 8:GST Isolation from Inclusion Body of Bacteria

WT-K8-GST was expressed in bacteria to purify Keratin 8 as unmodified in terms of post translation modifications. Basically, WT-K8-GST vector was transformed into Escherichia coli (E. coli) BL21 competent cells by following standard transformation protocol as described previously. Single colony was chosen and grown in 50 ml 100 µg/ml Ampicilin LB at 37 °C for 17 hours. On the second day, 10 ml of bacteria was transferred in fresh 250 ml 100 µg/ml Ampicilin LB and it was left to grow at 37 °C until its OD600 reached to 0.5.

Bacteria was induced with 1 mM Isopropyl β-D-1-thiogalactopyranoside (IPTG) at OD600≈0.5. It was left at 37°C for overnight. On the third day, Bacteria was pelleted at 5000rpm for 10 min at 4 °C. Bacteria pellet (g) was weighted and 4 fold volume (ml) of lysis buffer (100 mM TrisCl pH 8, 5mM EDTA, 5mM DTT and 1X Protease Inhibitor (Roche, *11836170001*)) was used. The pellet was re-suspended in lysis buffer with tissue grid homogenizer. Then, it was sonicated with sonicator at power 45, 8 % for 10 seconds for 4 cycles by 30 seconds break on ice between cycles. Suspension was centrifuged at 13,500 rpm at 4°C for 1 hour. Pellet was re-suspended in wash buffer (50 mM TrisCl pH 8, 10 mM EDTA, 5 mM DTT, 1 M NaCl, 1% NP40) equal volume to lysis buffer at the beginning. The pellet was homogenized with tissue grid homogenizer. Then, it was sonicated with sonicator at power 45, 8 % for 10 seconds for 4 cycles by 30 seconds break on ice between cycles. Suspension was centrifuged at 13,500 rpm at 4°C for 30 minutes. Pellet was washed two more times without sonication step. To remove excess NaCl, washing step was repeated with lysis buffer once. After washing, pellet was extracted with extraction buffer (8 M Urea, 5 mM DTT, 2 mM EDTA, 10 mM TrisCl pH 8) and homogenized with tissue grid homogenizer. Suspension was rotated at room temperature for 3 hours. It was centrifuged at 100,000 xg for 1 hour at 4°C. Supernatant was taken as isolated recombinant protein from inclusion body.

### 3.4.2 Refolding of Keratin 8:GST by Dialysis

Isolated recombinant protein was dialyzed with dialysis buffer (25 mM Hepes pH 7.4, 100 mM KCl, 5mM $MgCl_2$, 0.5 mM EGTA) to discard urea from solution. High concentration of urea prevents nature folding of proteins. Therefore, keratin 8 folding was provided by incubating in dialysis bag at 4°C for overnight by stirring.

### 3.4.3 Batch Purification of Keratin 8:GST

Glutathione beads were prepared for binding to isolated recombinant WT-K8-GST. 75 µl glutathione bead were washed in 10 times volume of 1X PBS. Suspension was pelleted at 500 xg at 4°C for 4 minutes. Dialysis buffer was used as last wash buffer. One volume of dialysis buffer was added to bead to make it 50% slurry. Then, 10 times volume of isolated recombinant WT-K8-GST was added and incubated at 4°C for overnight by gentle agitation. Next day, bead was centrifuged at 500 xg at 4°C for 4 minutes to discard unbound proteins. Centrifuge condition was kept the same for following steps. Bead was washed with wash buffer (25 mM Hepes, 150 mM KCl, 5mM $MgCl_2$, 0.5 mM EGTA, 1 mM DTT, 0.01% NP40) at 4°C for 10 minutes by gentle agitation for twice. Harsh wash buffer (25 mM Hepes, 600 mM KCl, 5mM $MgCl_2$, 0.5 mM EGTA, 1 mM DTT, 0.01% NP40) was used for third wash. To remove excess KCl, last wash was repeated with wash buffer. At the end of procedure, recombinant WT-K8-GST was cleaned from bacteria protein. Cleaned purified protein was used in further experiments.

### 3.5 In Vitro Kinase Assay with HeLa S3 Whole Cell Lysate

For phosphorylation of WT-K8-GST, isolated recombinant protein was incubated with HeLa S3 whole cell lysate (WCL) which is synchronized to mitosis or cytokinesis by double thymidine block followed with nocodazole treatment. AZD1152 and VX680-mediated Aurora B inhibition of cytokinesis cells were used as negative control for phosphorylation assay. Harvested cells were lysed with three times pellet volume of lysis buffer (25 mM Hepes pH 7.4, 150 mM KCl, 5mM $MgCl_2$, 0.5 mM EGTA, 1 mM DTT, 0.1% NP40) on ice 15 minutes. Cell lysates were centrifuged at 10,000 rpm 10 min at 4°C. Supernatant were taken as WCL. To simulate phosphorylation event, WCL and purified recombinant protein was incubated in Kinase Assay Buffer supplemented with 25 mM Hepes pH 7.4, 150 mM KCl, 5mM $MgCl_2$, 0.5 mM EGTA, 1 mM DTT, 0.01% NP40 and 0.05 mM ATP at at 25 °C for 1 hr 20 min by gentle agitation. AZD1152 and VX680-mediated Aurora B inhibition were maintained during preparation of lysates and phosphorylation assay. For this purpose, 1 μM AZD1152 and 0.5 μM VX680 were used. To isolate WT-K8-GST from lysate, washing steps which was described previously were followed. At the end of washing steps, WT-K8-GST bound to gluthahione bead was eluted with 3X SDS Blue Loading Buffer (NEB, B7703S) supplemented with 100 mM Dithiothreitol (DTT) by boiling it at 85°C for 10 minutes.

### 3.6 SDS-PAGE and Western Blotting

SDS-PAGE gel was prepared following the indicated amounts for separating and stacking gel. 10% separating gel contained 5ml 30% acrylamide/ 0.8% bisacrylamide, 3.75ml 4X Tris.Cl/SDS pH 8.8, 6.25ml ddH2O, 0.05ml 10% (w/v) APS, 0.01ml TEMED and stacking gel contained 0.65ml 30% acrylamide/ 0.8% bisacrylamide, 1.25ml 4X Tris.Cl/SDS pH 8.8, 3.05 ml ddH2O, 0.025ml 10% (w/v) APS, 0.005ml TEMED. Equal amounts of protein samples were loaded and run on the gel at 160 V for 1.5 hours.

Separated proteins were transferred to nitrocellulose membrane (Whatman Protran BA85) overnight at 40V at 4°C. Transfer was verified by staining with Ponceau dye. After 45 minutes blocking with 4% nonfat milk in TBS/0.1 % Tween20 (TBST), the membrane was incubated with primary antibodies which were prepared in 2%BSA-TBST for 3hours at RT to overnight at 4°C. Then the membrane was rinsed 3 times with TBST for 10min each. Secondary antibody incubation was done for 1.5 hours at RT with donkey anti-rabbit or anti-mouse secondary antibodies prepared in 4% nonfat milk-TBST. Then the membrane was rinsed 3 times with TBST Buffer for 10min each. For the final wash, only TBS was used to avoid interfering of the detergent with horse radish peroxidase detection system. Proteins were detected with ECL (Pierce ECL western blotting substrate 32106).

### 3.7 Immunostaining, Microscopy and Quantification

Cells were grown on coverslips in 12-well plates, washed with PBS and fixed with 3% PFA for 15 min at 37°C. Fixed coverslips were washed with PBS-0.1% Triton-X (PBS-Tx) 3 times for 5 minutes. They incubated in blocking solution (2% BSA in PBS-Tx) for overnight at 4°C or 30 minutes at room temperature. Incubation of primary antibody in 2% BSA in PBS-Tx was done at 4°C for overnight or room temperature for 2 hours. Secondary antibody in 2% BSA in PBS-Tx was incubated room temperature for 1 hours after washing coverslips in PBS-Tx 3 times for 5 minutes. Cell nuclei staining was done 1 μg/ml DAPI in 2% BSA in PBS-Tx for 10 minutes at room temperature. After washing 3 times for 5 minutes in PBS-Tx, coverslips were mounted on homemade mounting medium and sealed with nail polish. They stored at 20°C.

Immunostained coverslips were imaged with Nikon 90i Confocal Microscope using NIS-Element Imaging software. At least 5 different images for each coverslips were captured using Nikon Eclipse APO λ 100X/1.40 Oil objective lens.

Keratin 8 and INCENP immunofluorescence intensities were quantified using ImageJ software (Wayne Rasband, NIH). Basically, RGB values of three different spots on cleavage furrow were measure by keeping area constant and average of these values were named as fluorescence intensity on cleavage furrow. Then, the same process applied to random three spots on background of cell and named fluorescence intensity of the cell background. Finally,

the fluorescence intensity on the cleavage furrow were normalized to the fluorescence intensity of the cell background.

### 3.8 Live Cell Imaging

Live cell imaging was performed on an inverted fluorescence microscope (Olympus Xcellence, Hamburg, Germany) equipped with ANDOR iXon3 EMCCD camera and LUCPLNFLN 40X NA 0.6 dry objective (Olympus). Time-lapse GFP images were collected under GFP turret at different time interval depending on the experiment. Hela S3 cells were maintained at 37 °C and 5% CO2 in a humidified chamber during imaging.

### 3.9 Protein Identification with Mass Spectrometry

Keratin 8:GST eluted from glutathione beads in 3X Blue Loading Buffer (NEB, B7703S) supplemented with 100 mM Dithiothreitol (DTT) by boiling it at 85°C for 10 minutes. Dissociated proteins were alkylated by 100 mM IA (I6125, Sigma Aldrich). Then samples were loaded into 12% Tris-Glycine Precast Gels (Pierce, 25247) for separation according to their kDa. The corresponding band to Keratin 8:GST (66-81 kDa) was stained with Page Blue Protein Staining (Thermo Fisher Scientific, 24620) and cut for further processes. Gel were cut into small cubic pieces and collected in one eppendorf tube. Gel pieces were washed

to remove staining solution by following ABC, ABC:ACN and ACN cycling. First, 100 mM ABC solution was added to gel pieces until it covers gel plugs and it incubated at room temperature for 10 minutes. Then it was removed and 1:1 ABC:ACN solution was incubated at room temperature for 10 minutes. At the end of cycle, AB:ACN mixture was discarded and 100 % ACN was incubated at room temperature for 10 minutes. This cycle was repeated until gel plugs become white in ACN. Washed gel plugs were dried in speed vacuum to remove excess ACN. Gel plugs were digested by using 1:50 (Trypsin: Protein amount ratio) Sequencing Grade Modified Trypsin (Promega) at 37 °C overnight. Digests were desalted by Stage Tipping using Empore C18 47mm disks, then dried in Speed-Vacuum. Further, they were resuspended in 5% FA and 5% ACN for LC-MS/MS analysis.

### 3.10 Mass Spectrometry Data Acquisitions and Processing

The peptides were subjected to a reversed phase Nano LC-MS/MS (EASY-nLC, Thermo) connected to a Q Exactive quadrupole Orbitrap mass spectrometer (Thermo Fisher Scientific, Bremen). The peptides in the fractions were directly loaded onto an in-house packed 100 μm i.d. × 17 cm C18 column (Reprosil-Gold C18, 5 μm, 200Å, Dr. Maisch). Survey spectra were acquired on the Orbitrap with the resolution of 70,000. MS2 analysis consisted of collision-induced dissociation (Higher-energy collisional dissociation (HCD)) with the parameters: resolution 17,500. Raw data files were processed with Protein Discoverer (version 1.4,

Thermo Scientific) and MaxQuant (version 1.5.2.8) for protein identification. The raw data was searched against database including Keratin 8:GST sequence.

# Chapter 4

# RESULTS

## 4.1 Subcellular Localization of Keratin 8 During Cell Cycle

In this project, we aimed to reveal the regulation mechanism of Keratin 8 during the cell cycle by Aurora B that is one of the master regulator in both mitosis and cytokinesis. Keratin 8 dynamics during the cell cycle was investigated. For this purpose, Keratin 8 subcellular localization was analyzed in different cell cycle stages by performing immunohistochemistry experiments. Endogenous Keratin 8 and INCENP proteins were stained with the fluorescence labeled antibodies. Keratin 8 subcellular localization in the cell cycle stages is given in Figure 4.1. In interphase, Keratin 8 diffused in the cytoplasm by forming interlinking filaments. Its localization synchronously changed with mitosis. It started to accumulate in the periphery of the rounded cell through prophase. In metaphase, its interlinking formation decreases and it diffused to the cell periphery. Keratin 8 evenly localized in the cytoplasm without any preferences. When the cell progressed through anaphase to telophase, it displayed the preference not to localize on the cleavage furrow and it accumulated on the opposite poles of two daughter cells. However, it evenly diffused through the cytoplasm during abscission which is late cytokinesis. Keratin 8 undergoes dynamic changes during the cell cycle and it disappears on the cleavage furrow until cytokinesis is completed.

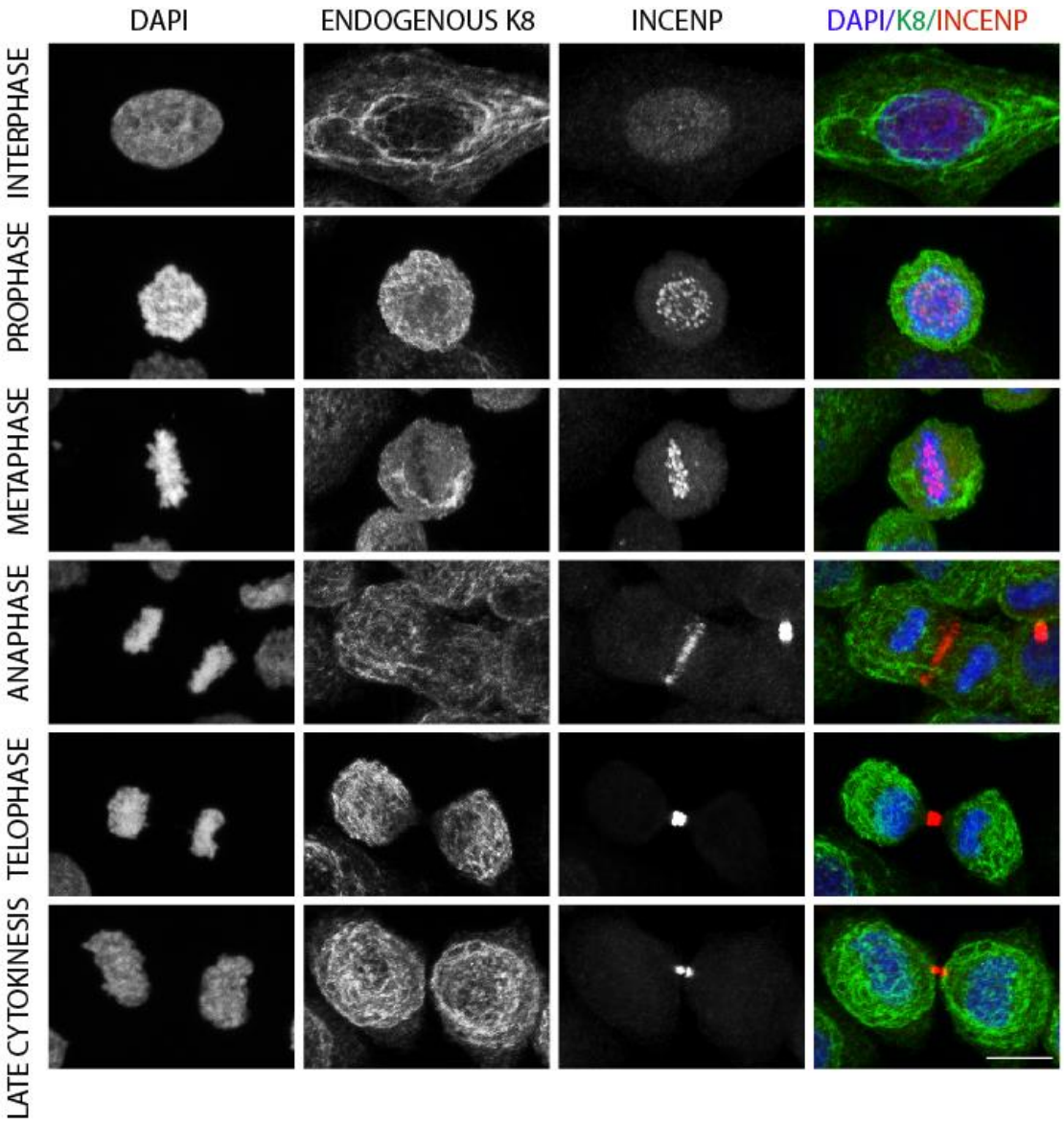



**Figure 4.1 Subcellular Localization of Keratin 8 During Cell Cycle.** Immunostaining of HeLa S3 cells was probed in different cell cycle stages for Keratin 8 (green), INCENP (red) and DAPI (blue). Scale bar, 10 μm.

### 4.2 Keratin 8 Dynamics During Cell Cycle

Live cell imaging was performed to understand better Keratin 8 dynamics during the cell cycle. For this purpose, Hela S3 cells were transfected with the wild type Keratin 8:GFP plasmid. Cells were imaged under an inverted microscope for 24 hours. Figure 4.2 displays the cell cycle progress of two different cells. Hela S3 cells spent the most of their time in interphase during the cell cycle and Keratin 8 showed similar localization through cytoplasm, from nucleus to the plasma membrane, with interphase cell in Figure 4.1. When cells started to round up, Keratin 8 accumulated around the periphery of the cell nucleus. Although cells spent some time in mitosis, cytokinesis was completed quite fast around 20 minutes. Keratin 8 disappeared on the cleavage furrow until the plasma membrane division completed between two daughter cells (figure 4.1).

Live cell imaging results show that Keratin 8 shows dynamic subcellular localization depending on the cell cycle. It disappears from the cleavage furrow when cytoplasmic division starts with anaphase. It reconstructs its distribution to the cytoplasm of the cell immediately after the abscission. Keratin 8 displays the most dramatic and dynamic changes during the cytokinesis. It shows an unique re-organization that is specific to the cytokinesis.

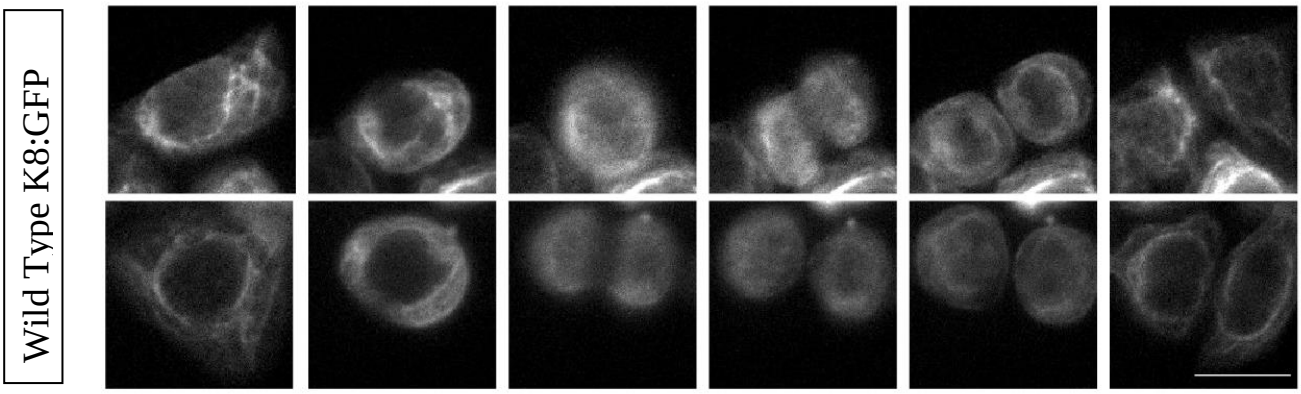



**Figure 4.2 Keratin 8 Dynamics During Cell Cycle.** HeLa S3 cells transfected wild type Keratin 8:GFP were imaged for 24 hours. Scale bar, 10 μm.

### 4.3 Aurora B Inhibition Effect on Keratin 8 Localization During Cytokinesis

Further, the underlying mechanism of the cytokinesis specific re-organization of Keratin 8 was questioned. As it is described in the literature review, previous studies showed that cytoskeletal elements are one of the major substrates of Aurora B that is one of the master regulators during cytokinesis. The cytokinesis specific re-organization of Keratin 8 was analyzed by using small molecule Aurora B inhibitors.

HeLa S3 cells were seeded on the coverslips and grown until it reaches 30-40% confluency. Cells were synchronized to interphase by double thymidine block. Cells were further synchronized to prophase with 10 ng/ml Nocodazole treatment for 5 hours after thymidine

release. Then, cells were released into cytokines with refreshing media. After 30 min release of nocodazole, cells were treated with 1 μM AZD1152 and 0.5 μM VX680 Aurora B inhibitors. Hela S3 cells were specifically treated with drugs after 30 min release of nocodazole to stimulate the cytokinesis specific inhibition of Aurora B. Cells were fixed with PFA after 1 hour release from nocodazole. The experimental workflow for the synchronization and drug treatments is given in figure 4.3.

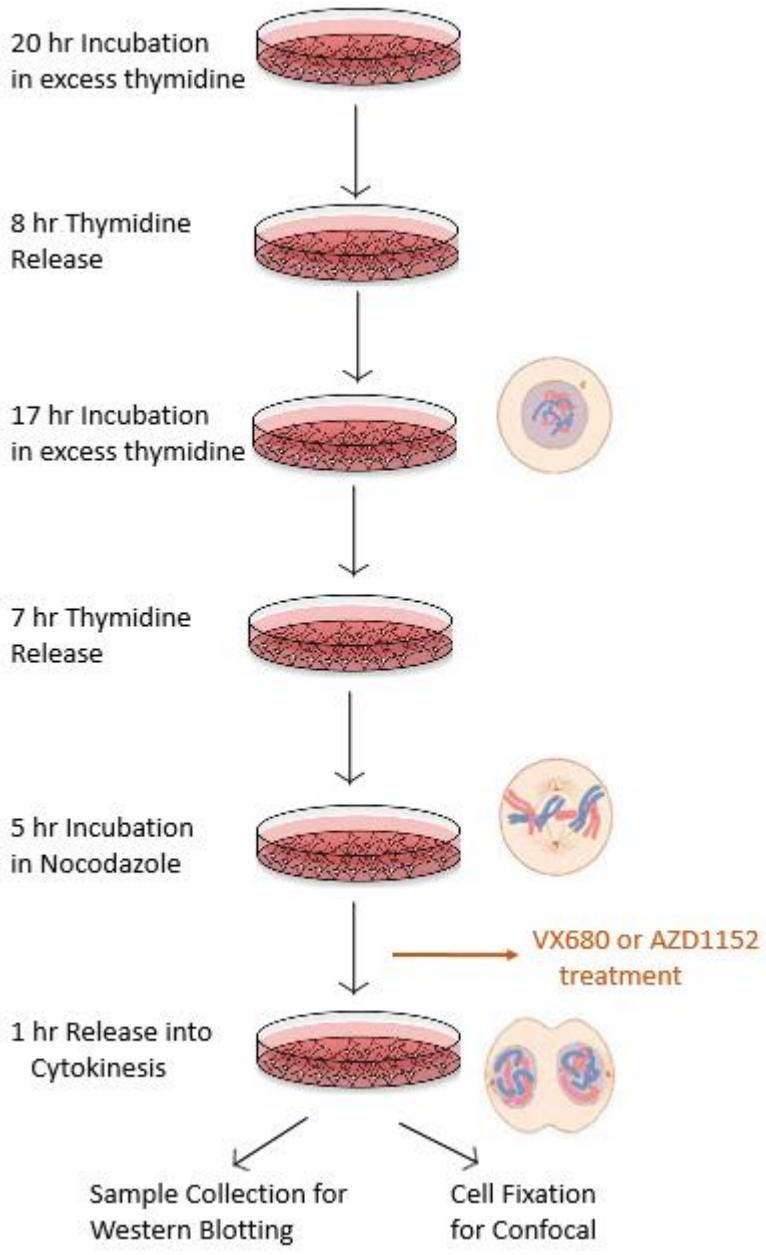



**Figure 4.3 The Cell Cycle Synchronization Experimental Workflow.**

Figure 4.4 shows the effect of AZD1152 and VX680-mediated Aurora B inhibition on Keratin 8 localization in the cytokinesis. Control HeLa S3 cells show predicted Keratin 8 localization on the cleavage furrow. However, when Aurora B is specifically inhibited in the cytokinesis, Keratin 8 persists on the cleavage furrow. It does not disappear from the cleavage furrow. Keratin 8 bridge and fibers appears between two daughter cells. Additionally, Aurora B inhibition disrupts the chromosome segregation and INCENP localization which are expected phenotype.

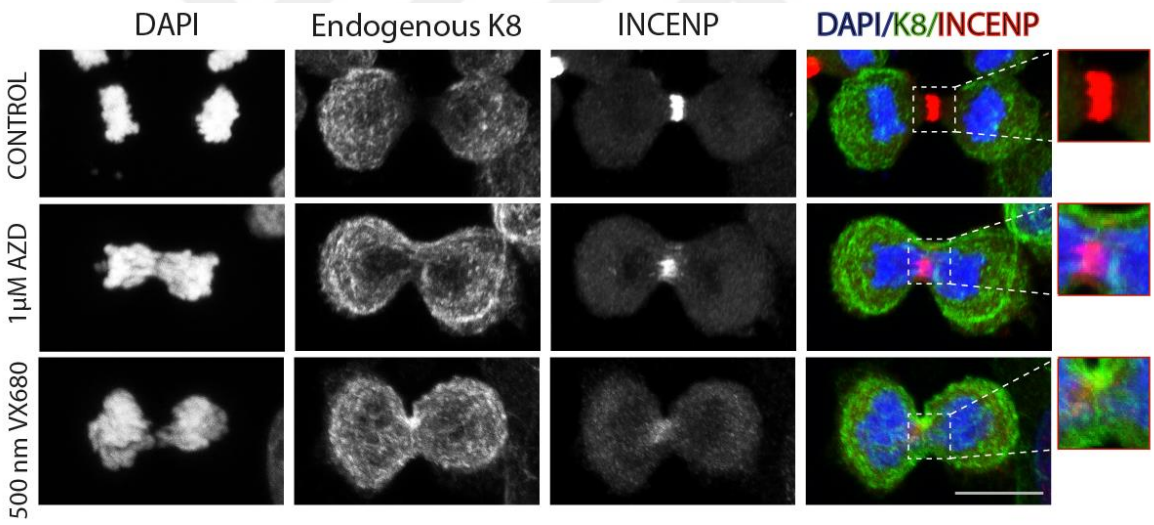
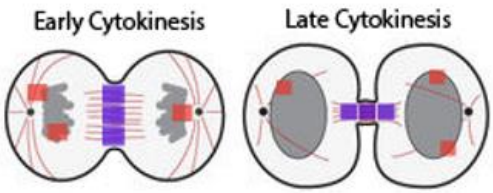



**Figure 4.4 Aurora B Inhibition Effect on Keratin 8 Localization in the Cytokinesis.** Hela S3 cells were synchronized to mitosis with nocodazole arrest and then released to cytokinesis. Cells were treated with AZD1152 and VX680 at 30$^{th}$ minute of nocodazole release. Cells were immunostained for endogenous Keratin 8 (green), INCENP (red) and DAPI (blue). Scale bar, 10 μm.

Keratin 8 localization on the cleavage furrow was quantified in the control and drug treated cells. At least 40 cells for each conditions were analyzed for quantification. The Early and late cytokinesis cells were chosen for fluorescence intensity quantification on the cleavage furrow. 3 different spots on the cleavage furrow with the constant area and 3 different spots on a random place of the rest of cell, named as background of cell, were selected and Keratin 8 and INCENP fluorescence intensities are measured. The illustration of process is shown in figure 4.5. The average intensity of each three spots were calculated. The average fluorescence intensity of Keratin 8 or INCENP in the cleavage furrow were normalized to the average fluorescence intensity of Keratin 8 or INCENP in the cell background.




$$\text{Normalized Intensity of Flouresence} = \frac{\text{Average Intensity of Three Spots in Cleavage Furrow of Cell}}{\text{Average Intensity of Three Spots in Background of Cell}}$$

**Figure 4.5. Quantification Method for the Fluorescence Intensity in the Cleavage Furrow.**

Figure 4.6 represents normalized Keratin 8 and INCENP fluorescence intensity on the cleavage furrow. AZD1152 treated cells shows 2-fold increase in Keratin 8 intensity on the cleavage furrow and VX680 treated cells shows almost 2.5-fold increase in Keratin 8 intensity on the cleavage furrow. A reverse situation is observed in INCENP localization in the cleavage furrow. AZD1152 treatment causes 2.5-fold decrease INCENP intensity whereas VX680 treatment causes 1.5-fold decrease.

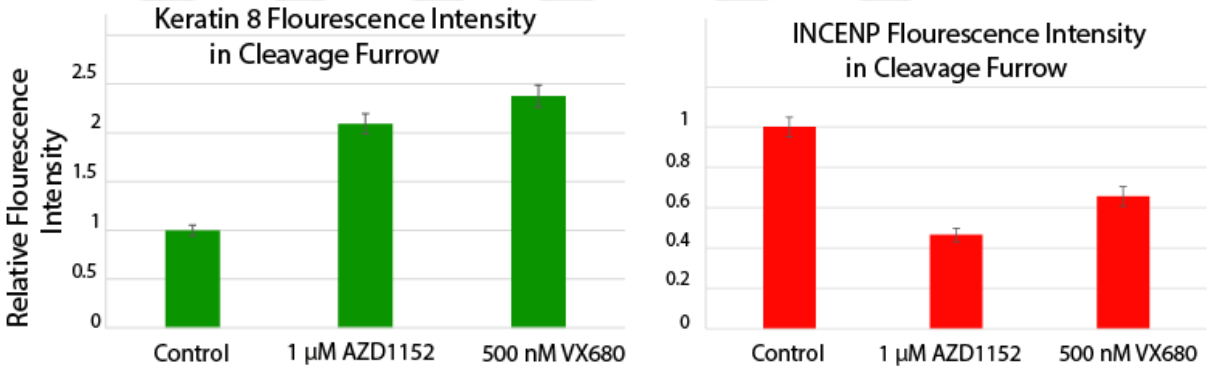



**Figure 4.6. Quantification of Aurora B Inhibition Effect on Keratin 8 Localization on the Cleavage Furrow.** Keratin 8 and INCENP fluorescence intensities of cytokinesis synchronized HeLa S3 cells were quantified with ImageJ software. Control (n=65), 1 µM AZD1152 (n=48), 500 nM VX680 (n=57)

### 4.4 Keratin 8 Dynamics upon Aurora B Inhibition During Cell Cycle

The live cell imaging was performed to analyze Keratin 8 dynamics upon AZD1152 and VX680-mediated Aurora B inhibition during the cytokinesis. HeLa S3 cells were transfected with wild type Keratin 8:GFP. Transfected cells were synchronized and treated as described in the previous part (figure 4.3). The cell imaging was started immediately after drug treatments. Cells were captured each 2 minutes for 2 hours.

The time lapse image of Keratin 8:GFP expressing HeLa S3 cells is shown in figure 4.7. After 30 minutes cells were released to cytokinesis from nocodazole block, cells were treated with AZD1152 and VX680 to initiate Aurora B inhibition. At that point, cell imaging was started. In control cell, the cytoplasm division started at $22^{th}$ minute and it is competed at $28^{th}$ minute. As expected, Keratin 8 disappeared from cleavage furrow during that time and reconstructed again at $60^{th}$ minute. Although Keratin 8 localization is normal in mitosis in drug treated cell (AZD1152 and VX680), it persists on the cleavage furrow during the cytokinesis. Keratin 8 accumulation on cleavage furrow was occurred between $36^{th}$- $42^{th}$ minute of cell division in 500 nM AZD1152 treated cells. Similarly, maximum Keratin 8 intensity was observed on cleavage furrow between $22^{th}$ and $28^{th}$ minute of the cell division. It is observed distinguishable fiber bridges between daughter cells and its intensity peaks on the cleavage furrow upon AZD1152 and VX680-mediated Aurora B inhibition.

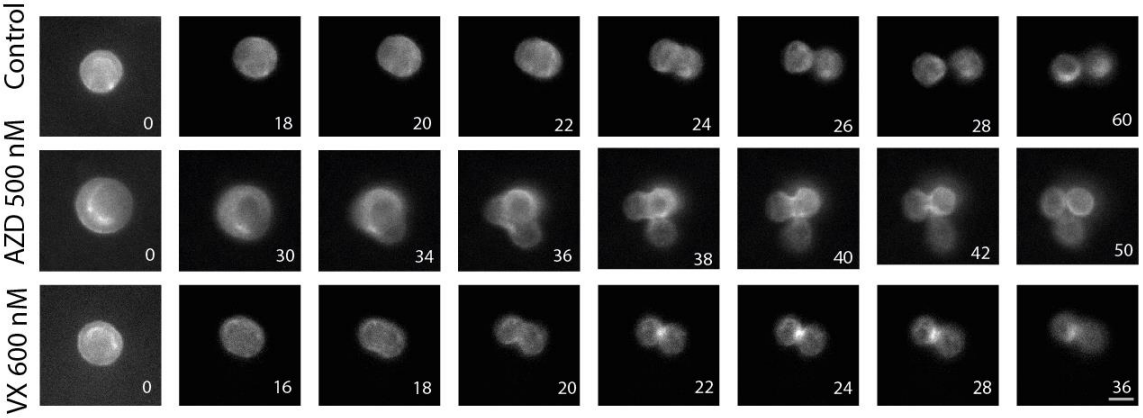



**Figure 4.7 Keratin 8 Dynamics upon Aurora B Inhibition during Cell Cycle.** HeLa S3 cells transfected with wild type Keratin 8:GFP were synchronized to mitosis with nocodazole arrest. Cells were treated with AZD1152 and VX680 after 30$^{th}$ minutes of release from nocodazole arrest that is starting point of cell imaging. Scale bar, 10 μm.

## 4.5 The Effect of Mutation in Aurora B Phosphorylation Site of Keratin 8 in the Cytokinesis

In part 4.4, it was clearly observed that Aurora B inhibition causes dramatic changes in Keratin 8 dynamics during the cytokinesis. Somehow, Aurora B promotes disappearance of Keratin 8 on the cleavage furrow. To understand the underlying mechanism of Keratin 8 regulation by Aurora B, I analyzed Aurora B specific phosphorylation site of Keratin 8. Aurora B specific phosphorylation site of Keratin 8 is determined based on previous genome-

wide phosphoproteome analysis in response to Aurora B kinase inhibitors on the proteome. In the study, two peptides of Keratin 8 showed cytokinesis specific phosphorylation whereas the phosphorylation of these peptides decreased upon Aurora B inhibition in the cytokinesis. Except of the study, there is no information regarding Keratin 8 regulation in the cytokinesis. Therefore, the previously identified Aurora B specific phosphorylation sites of Keratin 8 was analyzed. Phosphorylation sites of these two peptides were shown in figure 1.9. These peptides were SYKVSTSGPR (underlined amino acid is S13) and SYTSGPGSRISSSSFSR (underlined amino acids are S34, S35,36, 37). In the study, the phosphorylation of S13, S34 and S35 was diminished upon VX680-mediated Aurora B inhibition.

First, it is aimed to construct Keratin 8 plasmid including mutations on candidate phosphorylation sites. For this purpose, the site directed mutagenesis was performed on the wild type Keratin 8:GFP construct to generate S13,34,35,36,37A mutations. Keratin 8 Mutation sites were shown in figure 4.8. These serines were located at the head domain of Keratin 8 which is highly phosphorylated during its regulation.

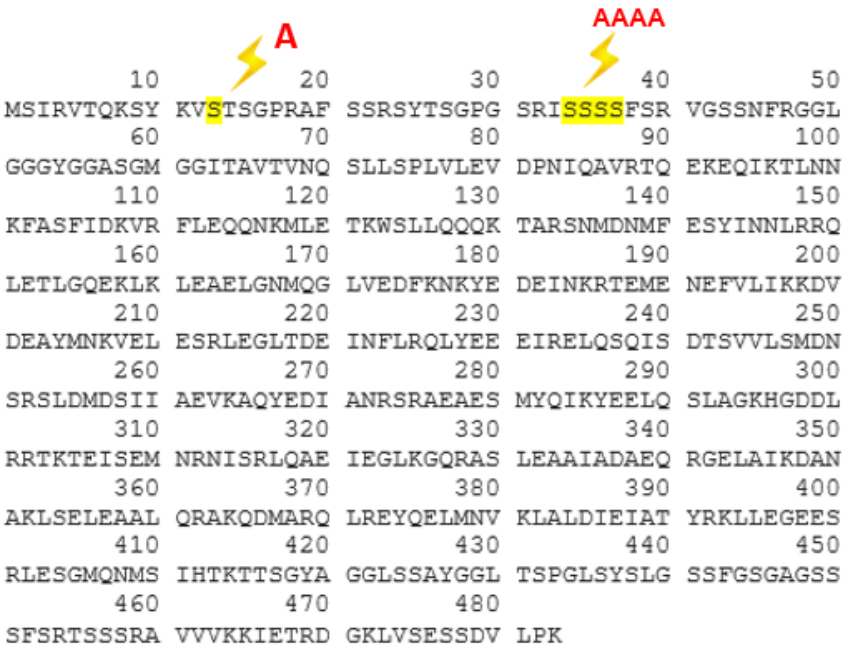



**Figure 4.8 Mutated Aurora B Phosphorylation Site of Keratin 8.** S13, S34, S35, S36 and S37 were converted to alanine in Keratin 8:GFP construct by site directed mutagenesis method.

Further, the effect of mutation on Aurora B specific phosphorylation site of Keratin 8 was analyzed. For this purpose, HeLa S3 cells were transfected with wild type and mutated K8:GFP constructs. Transfected cells were synchronized to the cytokinesis and fixed with PFA for the microscopy imaging. Figure 4.9 shows the wild type Keratin 8:GFP and mutated K8:GFP expressing HeLa S3 cytokinesis cells. The wild type Keratin 8:GFP shows the consistent localization with endogenous Keratin 8 (figure 4.4, control cell). It disappears on the cleavage furrow during the cytokinesis. However, the mutated Keratin 8:GFP persists on

the cleavage furrow. Filament fibers and bridge formation are also observed in mutated Keratin 8:GFP constructs.

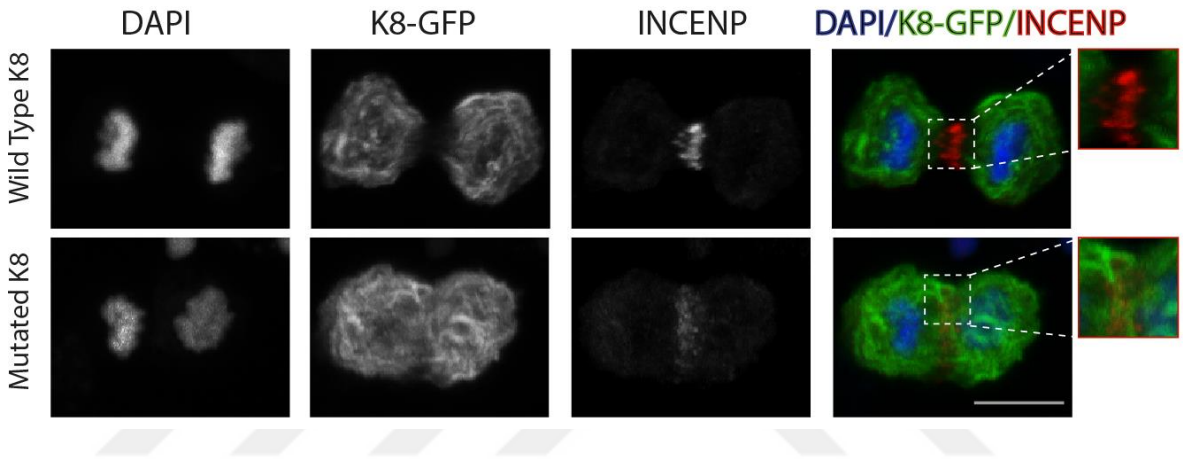



**Figure 4.9 Effect of Mutation in Aurora B Phosphorylation Site of Keratin 8 in Cytokinesis.** HeLa S3 cells were transfected with the wild type Keratin 8:GFP and S13A, S34-37A mutated Keratin8:GFP constructs. They were synchronized to mitosis with nocodazole arrest and then released to cytokinesis. Cells were immunostained for INCENP (red) and DAPI (blue). Scale bar, 10 μm.

When quantification of Keratin 8 and INCENP fluorescence intensity on the cleavage furrow for at least 10 cells was applied as described previously, an identical pattern with Aurora B inhibition was observed changes in fluorescence intensities between wild type and mutated Keratin 8. Keratin 8:GFP intensity was increased 2.5-fold on the cleavage furrow and INCENP intensity is decreased 1.5-fold (figure 4.10) upon the mutation on the phosphorylation sites of Keratin 8.

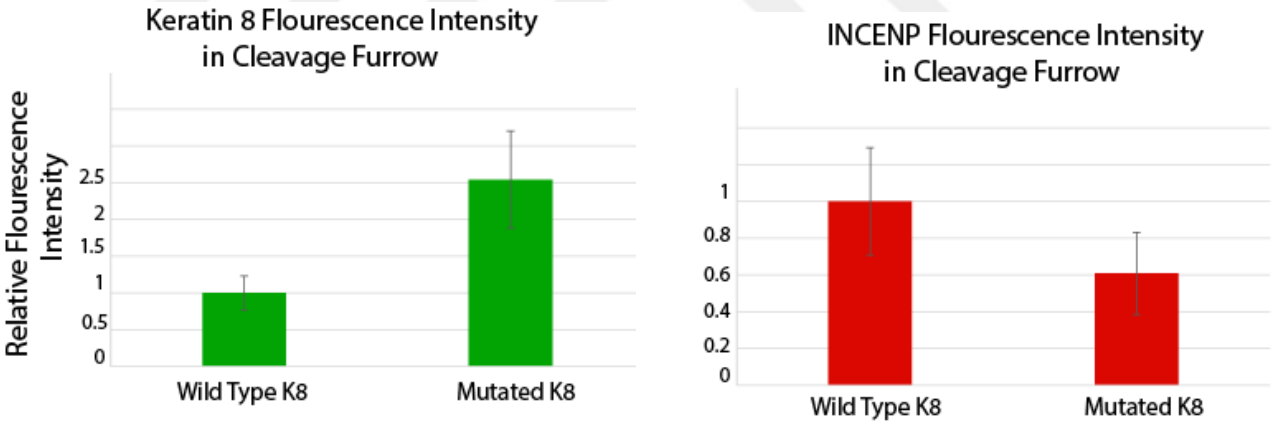



**Figure 4.10 Quantification of Aurora B phosphorylation site mutated Keratin 8 Localization on Cleavage Furrow.** Exogenous Keratin 8 and endogenous INCENP fluorescence intensities of cytokinesis synchronized HeLa S3 cells were quantified with ImageJ software. Wild Type K8 (n=14), Mutated K8 (n=13).

## 4.6 Phosphoproteome Analysis of Keratin 8 upon AZD1152 and VX680-mediated Aurora B Inhibition

In previous parts, it is established that Aurora B activity in the cytokinesis is critical for Keratin 8 subcellular localization. Additionally, serines mutations to alanine on Aurora B specific phosphorylation site of Keratin 8 has a negative effect on the localization of Keratin 8 in the cytokinesis. These two finding show a fundamental relationship between Aurora B and Keratin 8 during the cytokinesis. However, these findings are not a direct evidences to show Aurora B phosphorylation on Keratin 8 during the cytokinesis. In vitro kinase assay is one of the most valid methods to confirm the kinase-substrate interaction. For this purpose, in vitro kinase experiment setup was designed to simulate in vitro phosphorylation of Keratin 8.

In the experimental setup, it is aimed to show in vitro phosphorylation of unmodified Keratin 8 protein by active Aurora B. First, the wild type Keratin 8:GST in pGEX-6P-1 plasmid was generated by cloning protocol. The construct was transformed into bacteria and Keratin 8:GST was expressed in bacteria without any post translational modification. Since Keratin 8:GST was expressed in bacteria without an interaction partner Keratin 18, it was accumulated in the inclusion body which is insoluble protein aggregation. Keratin 8 was isolated from the inclusion body of bacteria by using high concentration of NaCl and UREA as described in material and methods. Then, the isolated recombinant Keratin 8 was dialyzed

in a favorable buffer to provide the nature folding of it. To remove excess bacteria proteins, Keratin 8:GST was purified with the glutathione bead by applying batch purification protocol without an elution step. Purified Keratin 8:GST was stored as substrate source for in vitro kinase assay.

Aurora B purification is more challenging than substrate purification, because activity of Aurora B is quite important for in vitro kinase assay. Aurora B is not active without its interaction partner INCENP. Therefore, it should be purified as complex to obtain active Aurora B. For this reason, whole cell lysate of HeLa S3 cells was used as active Aurora B kinase source by keeping its interaction partners intact. To stimulate Keratin 8 phosphorylation in mitosis and cytokinesis, HeLa S3 cells were synchronized to mitosis and cytokinesis. Mitosis and cytokinesis and AZD1152/VX680 treated cytokinesis cell pellets were collected (figure 4.3).

Keratin 8:GST bound to glutathione bead were incubated in the kinase buffer including control and drug treated (AZD1152 and VX680) whole cell lysates. To initiate kinase reaction, ATP was added to the environment. On the parallel, AZD1152 and Vx680 was supplemented to maintain Aurora B inhibition. The kinase reaction was incubated for 1 hour and 20 min at 25°C. Then, Keratin 8:GST bound to glutathione bead was cleared from the whole cell lysate by applying the batch purification protocol. The experimental setup is

shown in figure 4.11. Eluted Keratin 8:GST was proceeded with in-gel digest protocol. In vitro phosphorylation of Keratin 8:GST was analyzed with mass spectrometry.

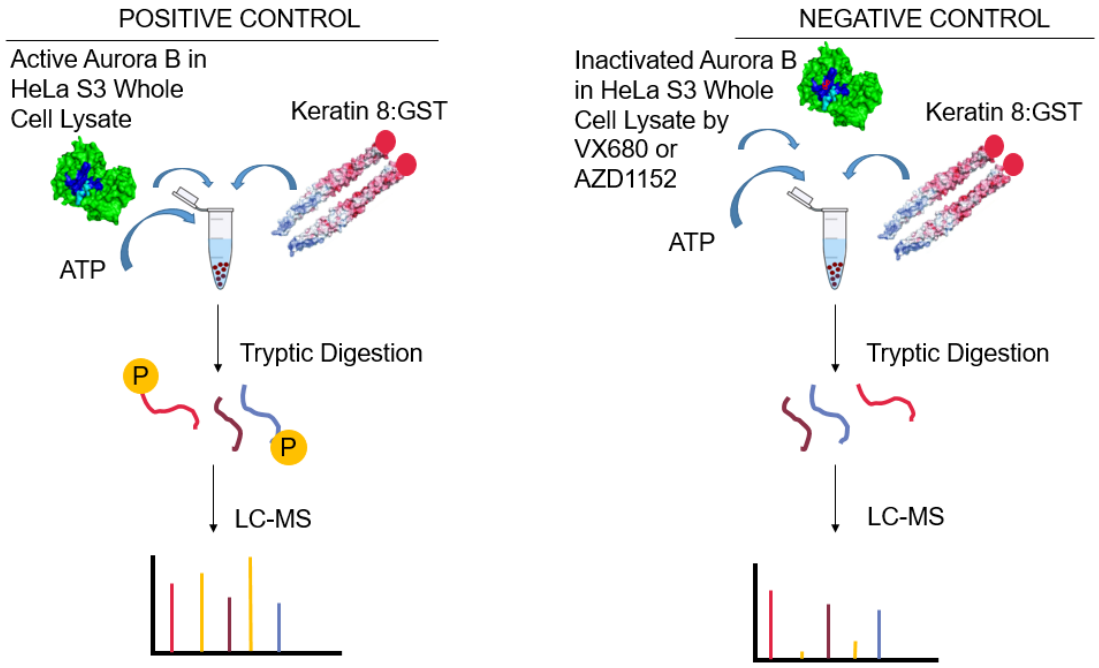



**Figure 4.11 Experimental Workflow of Phosphoproteome Analysis of Keratin 8 upon aurora B Inhibition.**

Biological and technical replicates were analyzed and quantified in MaxQuant software by applying label free quantification. First, phosphorylation of S34 Keratin 8 was compared between mitosis and cytokinesis, because phosphorylation of Keratin 8:GST may not depend on type of the cell cycle stages. Surprisingly, Keratin 8 S34 phosphorylation was higher in

cytokinesis. Phospho IpSSSSFSR peptide intensity was normalized to non-phospho ISSSSFSR peptide intensity. There was two-fold difference in phosphorylated peptide intensity (figure 4.12). Additionally, the reverse situation was identified for the phosphorylation of FASFIDK peptide. The phosphorylated FApSFIDK peptide was 3.2-fold in mitosis compared the cytokinesis (figure 4.13). These results displayed that phosphorylation of Keratin 8:GST was related with cell cycle stage of the lysate. Then, cytokinesis control and drug treated cytokinesis samples were compared for ISSSSFRS phosphorylation. It revealed that Keratin 8 S34 phosphorylation was diminished 1.5-fold upon VX680-mediated Aurora B inhibition in cytokinesis (figure 4.14). Also, the phosphorylation was totally vanished in AZD1152 treatment (figure 4.14).

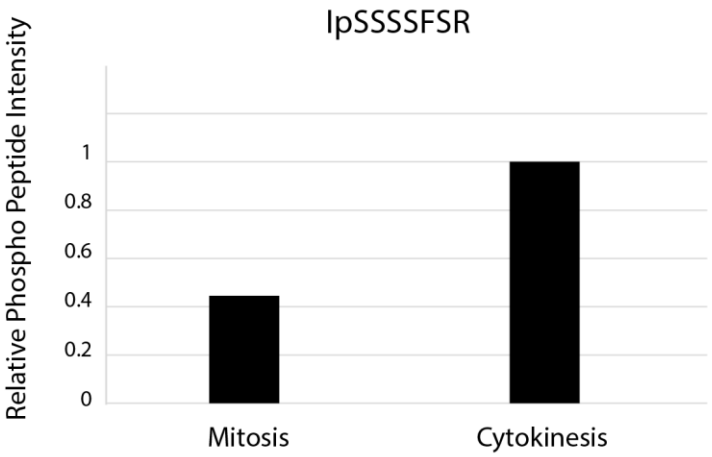



**Figure 4.12 ISSSSFSR peptide Quantification in Mitosis and Cytokinesis.** Mitosis and Cytokinesis peptides were quantified in Maxquant. To compare phospho IpSSSSFSR peptide between mitosis and cytokinesis, phosphopeptide intensity was normalized to non-phospho ISSSSFSR peptide intensity. Normalized phophopeptide intensity in mitosis and cytokinesis were scaled to 1 to reflect the fold difference.

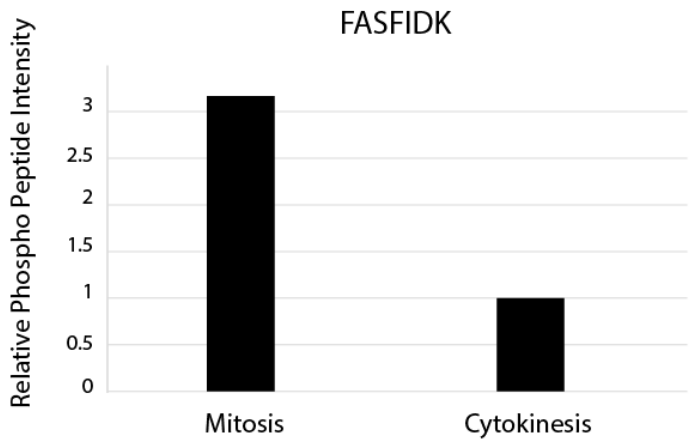
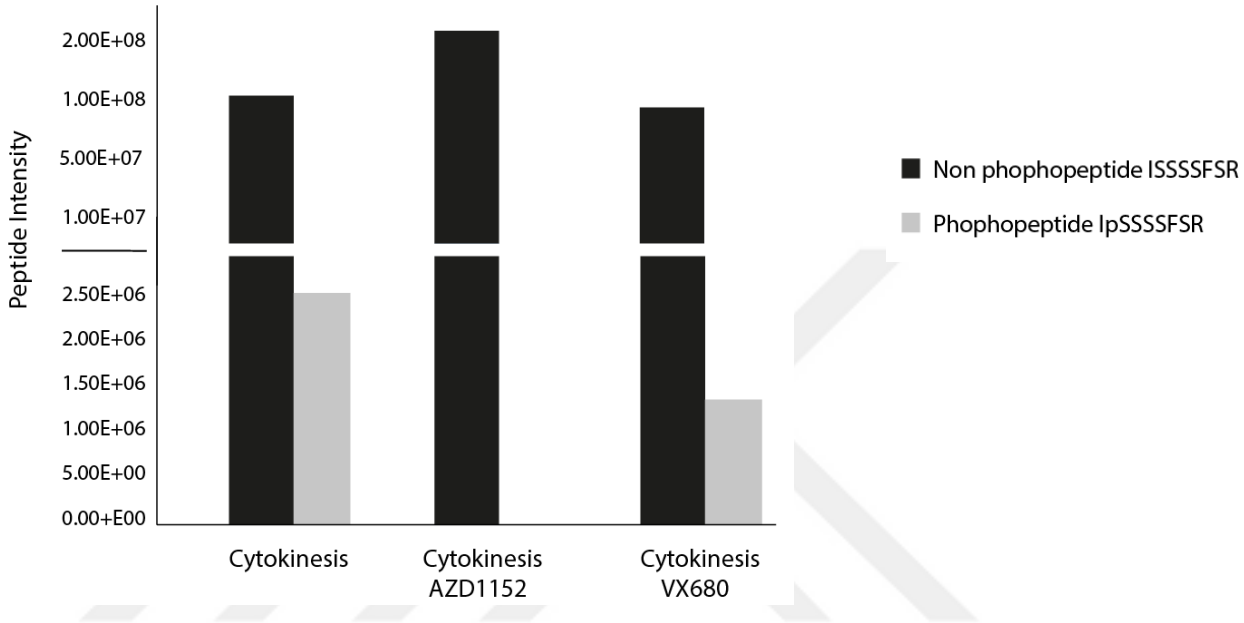



**Figure 4.13 FASFDIK peptide Quantification in Mitosis and Cytokinesis.** Mitosis and Cytokinesis peptides were quantified in Maxquant. To compare phospho FApSFIDK peptide between mitosis and cytokinesis, phosphopeptide intensity was normalized to non-phospho ISSSSFSR peptide intensity. Normalized phophopeptide intensity in mitosis and cytokinesis were scaled to 1 to reflect the fold difference.

A


Peptide Intensity
2.00E+08
1.00E+08
5.00E+07
1.00E+07
2.50E+06
2.00E+06
1.50E+06
1.00E+06
5.00E+00
0.00+E00
Cytokinesis
Cytokinesis AZD1152
Cytokinesis VX680
Non phophopeptide ISSSSFSR
Phophopeptide IpSSSSFSR

B

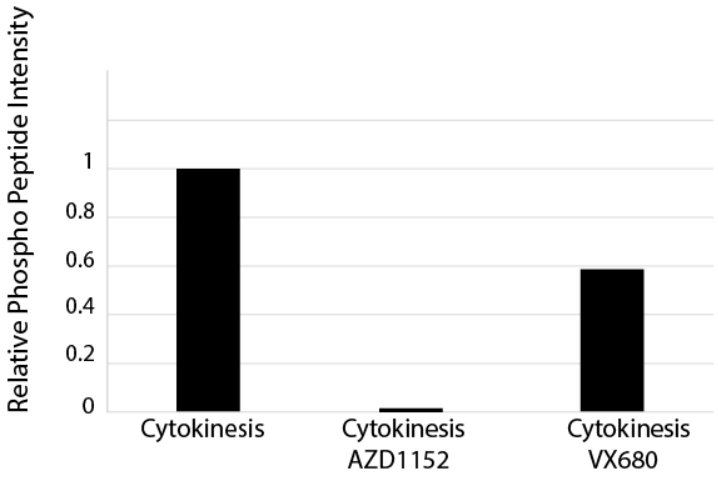

Relative Phospho Peptide Intensity
1
0.8
0.6
0.4
0.2
0
Cytokinesis
Cytokinesis AZD1152
Cytokinesis VX680

**Figure 4.14 Analysis of Keratin 8 protein upon Aurora B Inhibition during Cytokinesis**. MaxQuant was used for identification and quantification of peptides. A) nonphosphopeptide ISSSSFSR and phosphopeptide IpSSSSFSR intensities were shown. B) Quantification of ISSSSFSR phosphopeptide. The phosphopeptide intensity was normalized with the nonphosphopeptide intensity of ISSSSFSR. Then value were scaled to 1.

### 4.7 Interaction of Keratin 8 and Aurora B

Up to now, aurora B and Keratin 8 interaction has not been showed. Further, we showed Keratin 8 and Aurora B interaction with Keratin 8:GST pull down. We incubated Keratin 8:GST with cytokinesis synchronized Hela S3 whole cell lysate. On the parallel, we used the empty GST as control. Then, GST and Keratin 8:GST were pulled down by the batch purification of the glutathione bead. Interacting proteins were analyzed with the western blotting (figure 4.15). Keratin 8:GST and GST proteins were observed in the elute. Aurora B was also pulled down as interacting protein in Keratin 8:GST elute. However, Aurora B and Keratin 8 was also observed in Empty GST pulldown.

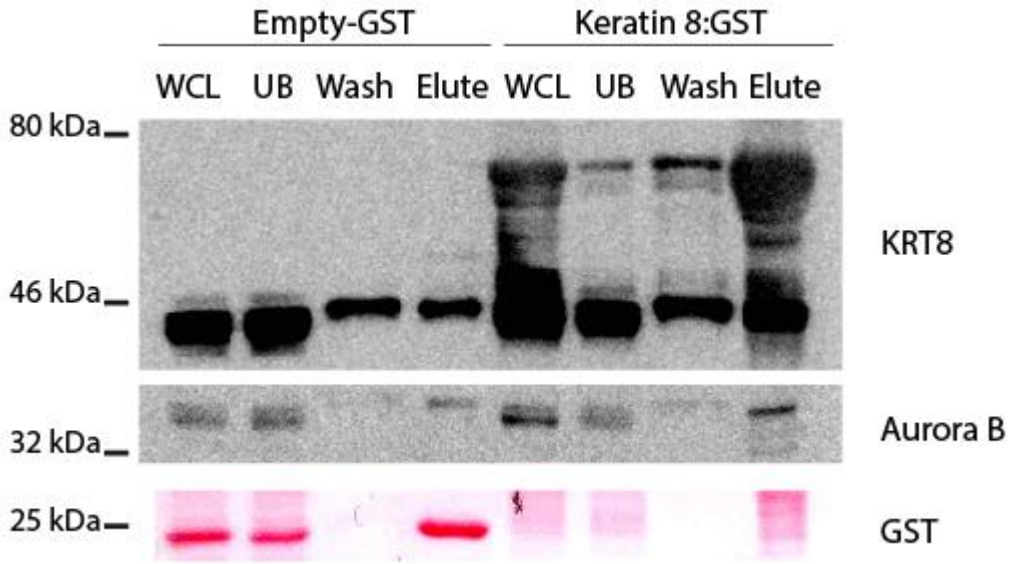



Figure 4.15 Immunoprecipitation of Keratin 8:GST with Aurora B. Cytokinesis synchronized HeLa S3 cells lysate was incubated with Keratin 8:GST. Keratin 8:GST was immunoprecipiptated with the glutathione bead. Empty GST was used as negative control.

# Chapter 5

# DISCUSSION

In this study, we aimed to show Aurora B dependent regulation of Keratin 8 in cytokinesis. Keratin 8 protein has been studied in aspects of various cellular mechanism, such as differentiation, apoptosis or migration. However, its regulation during cell cycle is one of the least studied subject. Its biochemical properties makes it more interesting in terms of its regulation during cell division. It is important question to answer how the cell organizes such a robust protein in very short time. The study tries to enlighten the question by focusing on its regulation in cytokinesis which takes around 15 minutes in the mammalian cell to complete.

The study is also important to understand the substrate interaction of Aurora B during cytokinesis. Last studies has showed that cytoskeleton proteins are one of the major substrates of Aurora B during cytokinesis. It has been revealed that Aurora B phosphorylates S55 and S56 of Vimentin, which is the member or intermediate filament, specifically in cytokinesis. Additionally, this study shows that Aurora B also regulates Keratin 8, another cytoskeleton member, during cytokinesis by S34 phosphorylation. Besides that, S103 phosphorylation of Keratin 8 may also depend on Aurora B activity specifically in mitosis.

First, Aurora B activity during cytokinesis is critical for localization of Keratin 8 (figure 4.4). Aurora B inhibition in cytokinesis causes Keratin 8 bridge-like formation and it persists on cleavage furrow during process. The outcome of Aurora B inhibition on Keratin 8 indicates that Aurora B activity may provide solubility to Keratin 8 in cytokinesis, because it is already known that phosphorylation on keratin protein increases its solubility. We hypothesizes that Aurora B phosphorylation on the certain site of Keratin 8 may facilitate its solubility and prevent its accumulation on cleavage furrow.

Then, we tried to simulate phosphorylation of Keratin 8 in mitosis and cytokinesis by Aurora B. In this way, the validation of Keratin 8 S34 phosphorylation by Aurora B was aimed. To show cell cycle dependent Keratin 8 phosphorylation, its phosphorylation sites were compared between mitosis and cytokinesis. FApSFIDK (Keratin 8 S103) was upregulated in the mitosis and IpSSSSFSR (Keratin 8 S34) was upregulated in the cytokinesis (figure 4.12, figure 4.13). These findings was a preliminary evidence to show Keratin 8 cell cycle dependent phosphorylation. Although it is not an expected result to have different phosphorylation of Keratin 8 in vitro in the same protein complexes (mitosis and cytokinesis), it shows how complex cellular events at the molecular level. To speculate the phosphorylation event difference, Aurora B may interact with additional partners to target different substrates in mitosis and cytokinesis or it is possible that even Aurora B phosphorylation dynamics are changing depending on mitosis or cytokinesis. It has been previously showed that auto phosphorylation of Aurora B Thr-232 is an essential for

regulation of cytokinesis [42]. Therefore, to understand the underlying mechanism of Aurora B substrate specificity in cytokinesis, further analysis of kinase-substrate complex is required.

Besides, the study showed the differentially Keratin 8 S34 phosphorylation by Aurora B in cytokinesis by taking advantage of in vitro kinase assay by using small molecule Aurora B inhibitors. After showing S34 phosphorylation is specific to cytokinesis, it is aimed to validate that S34 phosphorylation is related to Aurora B activity. MS Analysis of in vitro kinase assay of Keratin 8 in cytokinesis synchronized HeLa S3 cell revealed that S34 phosphorylation diminished upon AZD1152 and VX680-mediated Aurora B inhibition (figure 4.14). However, the finding does not prove that Keratin 8 is directly phosphorylated by Aurora B, because the downstream kinases of Aurora B may promote Keratin 8 phosphorylation. To show the direct kinase-substrate interaction, Keratin 8 phosphorylation should be analyzed only in existence of Aurora B kinase. For that, active Aurora B complex can be purified as kinase. Then, in vitro kinase assay of only purified Aurora B (kinase) and Keratin 8 (substrate) may provide a proof for kinase-substrate interaction. Mass spectrometry analysis of this experiment can reveal all Aurora B phosphorylation sites of Keratin 8.

Additionally, targeted and quantitative proteomics approaches can be used to further analyze the phosphorylation event. Parallel-reaction monitoring (PRM) or selected reaction

monitoring (SRM) are methods used in quantitative proteomics by mass spectrometry. These methods give more accurate quantification.

In this study, it is aimed to show Aurora B and Keratin 8 interaction in cytokinesis. For that reason, Keratin 8:GST pull down experiment was performed (figure 4.15). Our result is not conclusive, because Aurora B was observed in empty GST (negative control) pull down elution. However, the existence of Keratin 8 in empty GST (negative control) elute causes a suspicion interaction between Aurora B and empty GST. It is not clear whether Aurora B interacts with Keratin 8 or GST. Keratin 8 pull down may not be good approach to show Aurora B interaction. Since Keratin 8 is cytoskeleton protein and abundant in the cell, its pull down may be dirty with unspecific protein interaction. Aurora B pull down may be better approach to show specific interaction.

For the future direction, CRISPR mediated genome editing on Keratin 8 phosphorylation site may provide strong evidence to show its importance during cytokinesis. The effect of mutation on Keratin 8 phosphorylation site was showed with immunostaining. In the experiment, mutated Keratin 8 was exogenously expressed in the cell to observe localization defect (figure 4.9). However, the cell continues to express non mutated Keratin 8 endogenously. Although the localization dynamics was observed, it is not clear how the mutation affects the cell cycle progress. Lastly, it is speculated that Aurora B

phosphorylation may be provide solubility of Keratin 8 during the cytokinesis. To prove the hypothesis, Keratin solubility assay may be performed.

Overall, the study showed that Aurora B activity has effect on the localization of Keratin 8 during cytokinesis. The re-organization of Keratin 8 during cytokinesis may be provided by its S34 phosphorylation by Aurora B.

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