Quantitative and Network Analysis of Cytokinesis Specific Phosphoproteins

by

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This is to certify that I have examined this copy of a master's thesis by

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This thesis is dedicated to my family.

I love you unconditionally.

You have been "here" for me always, and I will be there for you always.

ABSTRACT

Nuclear division (mitosis) phosphorylation events have been characterized extensively however phosphorylation events taking place during cytoplasmic division (cytokinesis) specific phosphoproteins and responsible kinases are not well characterized. Our mass spectrometry analysis revealed ~1500 phosphorylation events that are specific to cytokinesis. We identified responsible kinases that are active during cytokinesis using a kinase-substrate prediction algorithm on our dataset. Including previously known cell cycle regulator kinases such as MAPK, CDK1/2 and Aurora, total 31 kinases were predicted to be active during cytokinesis. We also identified phosphorylation sites that are only present during cytokinesis in MKI67 protein which is previously reported as proliferation marker. MKI67 localizes to chromosomes during mitosis, acting as a surfactant preventing chromosome condensation during mitosis. These phosphorylation sites are found in N terminus successive repeat domains with unknown function. We suggest that cytokinesis specific phosphorylation of MKI67 might be important for its function in cell division during cytoplasmic division.

Lastly, we compared evolutionary conservation scores of phosphorylation sites that are specific to monopolar and bipolar cytokinesis to understand if differing phosphorylation events between two models are crucial. We observed no significant difference between two models, meaning monopolar cytokinesis specific phosphorylation events are not redundant.

ÖZETÇE

Çekirdek bölünmesi (mitoz) sırasında gerçekleşen protein fosforilasyonları geniş ölçüde tanımlanmıştır ancak çekirdek bölünmesi (sitokinez) sırasında gerçekleşen protein fosforilasyonları ve bu fosforilasyonlardan sorumlu kinazlar kapsamlı olarak tanımlanmamıştır. Kütle spektrometrisi analizimiz yaklaşık olarak 1500 adet protein fosforilasyonunun sitokinez sırasında gerçekleştiğini açığa çıkarmıştır. Bulduğumuz sitokineze özel fosforilasyonlar için kinaz – substrat tahmini algoritması kullanarak, sitokinez sırasında aktif olan 31 adet kinazı ortaya çıkardık. Bu kinazların içinde, daha önceden hücre döngüsünü düzenlediği belirlenmiş olan MAPK, CDK1/2 ve Aurora gibi kinazları da gördük. Yine bu fosforilasyonlar içinden, proliferasyon işaretçisi olan protein MKI67'nin sitokineze özel fosforilasyonunu ortaya çıkardık. MKI67 mitoz sırasında kromozomların etrafına yerleşerek, kromozomların kümelenmesini önleyen bir protein olarak bilinmektedir. Bu proteinde bulduğumuz sitokinez sırasında gerçekleşen fosforilasyonlar, proteinin N terminusuna yakın kendini tekrar eden bölümlerindedir ve bu bölümlerin işlevi bilinmemektedir. Sitokineze özel bu fosforilasyonlar MKI67 proteinin sitoplazmik bölünme sırasındaki işlevini aydınlatmak açısından önemli olabilir.

Son olarak, tek kutuplu ve çift kutuplu sitokinez modellerinde görülen fosforilasyonların türler arasındaki korunumlarını, tek kutuplu sitokinezin çift kutuplu sitokinezden farkını görebilmek için karşılaştırdık. İki model arasında kayda değer bir fark göremedik ve tek kutuplu sitokinez modelinde gördüğümüz fosforilasyonların sitokinez çalışmalarında kullanılabileceği sonucuna vardık.

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NOMENCLATURE

MS	Mass Spectrometry
SCX	Strong Cation exchange
SAX	Strong Anion Exchange
S	Synthesis (phase)
APC	Anaphase Promoting Complex
MPF	Maturation Promoting Factor
CDK	Cyclin Dependent Kinase
PLK	Polo Like Kinase
PTM	Post Translational Modification
ePK	Eukaryotic Protein Kinase
aPK	Atypical Protein Kinase
IMAC	Immobilized Metal Affinity Chromatography
MOAC	Metal-Oxide Affinity Chromatography

CHAPTER 1

INTRODUCTION

Almost every living cell is a result of cellular division. Therefore fundamental mechanisms which constitute cell cycle is similar in different species. These shared mechanisms include many checkpoint mechanisms which separate distinct phases of cell cycle. Checkpoints are crucial for cell to be able to replicate its DNA and segregate into newly formed two daughter cells correctly. Aberrations during replication and segregation or mutations in cell cycle regulator proteins might result aneuploidy and/or cell proliferation [1], [2].

During interphase, longest phase of the cell cycle, cellular functions are carried out normally. If cell is going to divide, DNA is replicated during interphase. DNA replication of HeLa cells takes about 6 hours and the whole interphase takes around 19 hours [3]. After DNA replication, cell goes into mitosis where genetic material is separated into newly formed daughter cells and finally cytoplasmic division occurs during cytokinesis. During cytokinesis cell undergoes dramatic physiological changes. Cellular cytoskeleton reorganizes itself to complete physical cellular division through plasma membrane abscission. Total time required for mitosis is around 1 hour [3]. Cytokinesis takes about 15 minutes which is very short compared to other cell cycle phases. Pace of the cytoplasmic division makes it challenging to study its biochemical changes.

Cell cycle phases are known to be regulated through reversible phosphorylation of proteins by kinases such as CDKs [4]–[7]. Due to the pace of cytokinesis, kinases which are controlling the cytokinesis are not well studied. There are identified kinases which are known to be functioning in cytokinesis initiation and progression such as Rho-kinase, MAPK and PLK1 [8]–[10].

In this thesis, first, I aim to identify proteins which are phosphorylated only in cytokinesis using quantitative mass spectrometry (MS) analysis of phosphoproteins from HeLa cells which are synchronized in interphase, mitosis and monopolar cytokinesis [11]. MS analysis performed in our lab quantified phosphopeptides relatively between Mitosis/Interphase, Mitosis/Cytokinesis and Interphase/Cytokinesis. Using these ratios I have clustered phosphopeptide abundance profiles into 6 different groups each representing different regulation trends. Clusters that are upregulated during mitosis to cytokinesis transition are defined as cytokinesis specific clusters. Further, I have performed kinase prediction via NetworKIN3.0 and motif analysis via motif-x on the cytokinesis specific clusters of phosphorylated sites to reveal kinases and regulatory motifs which are active during cytokinesis [12], [13].

Finally, I have compared monopolar and bipolar cytokinesis models, again using quantitative MS analysis previously performed in our lab. I have compared evolutionary conservation scores of phosphorylation sites shared between two models, phosphorylation sites specific to monopolar cytokinesis and phosphorylation sites specific to bipolar cytokinesis to evaluate monopolar cytokinesis' relevance to bipolar cytokinesis.

CHAPTER 2

LITERATURE REVIEW

2.1. Cell cycle

Cell cycle is a term that describes the life cycle of cells. Eukaryotic cell cycle consists of different stages, however two prominent stages in cell cycle are interphase and mitosis [7]. Interphase is the stage where cell grows in size and prepares for the mitosis, cell division. Investigation of two prominent cell cycle stages revealed that interphase and mitosis has different phases. G_0, G_1, S and G_2 phases are identified as stages of interphase and prophase, metaphase, anaphase and telophase are identified as stages of mitosis [14]. Depending on the cell type and cell environment, cells can go into the G_1 or G_0 after division. G_0 phase is seen mostly in somatic cells that are differentiated into their final cell type [15]. On the other hand, cells that are going in G_1 phase prepare for DNA replication which will take place during S phase. After the S phase, cells enter into another gap phase, G_2, until the mitosis starts. During mitosis, nucleus disappears and chromatin fibers condenses into visible chromosome pairs. At the end of the mitosis, chromosome pairs are segregated into two newly forming cells and physical separation of two daughter cells is completed in stage called cytokinesis [16].

Interphase

Interphase is the longest stage of the cell cycle, around 23 hours for human cells in culture and consists of G_1, S (synthesis) and G_2 stages for a cell that is dividing [16]. Interphase is the stage before mitosis, where cell grows in size, produce proteins and organelles. Gap phase G_1 is an important stage for cells to make decision of dividing. If the environmental and internal cues favor cell division at the end of G_1, cell goes into DNA synthesis phase, S phase [7], [15].

Entering into S phase means that cell is going to divide. This stage of the cell cycle is an important check point where cell commits for division [16].

During S phase, cell replicates the genetic material, DNA. DNA helicase, protein that unwinds DNA to make it accessible for replication, are recruited onto replication start sites early G_1 and late mitosis [16]. This allows cells to start replication immediately during S phase only, because helicase proteins are activated only in S phase via S-CDKs [16]. Histone proteins are used to pack chromatin fibers therefore cell produce histones during S phase to accommodate newly formed DNA. At the end of S phase copied DNA strands, sister chromatids, are stuck together via cohesin proteins [17]. This allows sister chromatids to stay together and be separated into daughter cells equally. During S phase, centrosome is duplicated too [4]. Centrosome is the microtubule organizing center that are functioning in cytoskeleton organization and present in mammalian cells [16]. There must be one centrosome per cell, therefore centrosome duplication has to be controlled [4].

After completion of S phase, dividing cell goes into G_2 phase, where protein synthesis and preparation for mitosis continues. Some eukaryotic cells do not go into G_2 phase, and directly go into M phase after DNA replication [18]. During late G_2, maturation promoting factor (MPF) is activated by cyclin B and cyclin A upon dephosphorylation of CDK1 subunit by CDC25. Activation of MPF allows cell to go into M phase [16], [19], [20].

If cell is not going to divide due to environmental and internal factors such as DNA damage, instead of going into S phase, it enters to G_0 stage called resting phase [15].

Mitosis

Mitosis stage consists of prophase, metaphase, anaphase and telophase. This phase of the cell cycle is shorter than the interphase. Mitosis takes around 1 hour to complete in mammalian cells [16].

Prophase

During prophase, first stage of the mitosis, nucleolus disappears and chromatin fibers are condensed into visible sister chromatid pairs. Condensation of fibers is crucial for the cell division because fibers in their natural form are not easily separable. Each sister chromatid pairs are physically parted from each other and become visible as pairs with the help of condensin protein [21], [22]. Sister chromatids stay connected on the centromeres, however chromosome arms are separated during prophase [16].

Further, nuclear envelope disappears and sister chromatids are attached to the opposite poles of the cell via mitotic spindle [23]. Spindle is formed during chromatid condensation between opposite poles of the cell and is composed of microtubules. Motor proteins such as kinesin-5 and dynein move and ensure spindle poles are separated [16]. Plus ends of the microtubules originate from the spindle poles and ends coming from the opposite poles meet on the sister chromatids. Spindle formation and chromatid attachments to spindle are the first steps towards sister chromatid separation. Although CDK family proteins are reported as the main regulators of the cell cycle, there are other kinases such as Polo like kinases and Aurora kinases are found to be active during spindle formation and activation of motor proteins [16]. Spindle poles are attached to the replicated chromosomes via kinetochore proteins on the centromeres in vertebrates and most of the plants [16], [24]. Kinetochores in nematodes are distributed along the chromatids [25].

Metaphase

Sister chromatids that are attached to the opposite poles are lined in the middle of the cell during metaphase. Kinesin 4 and 10 motor proteins move sister chromatids towards plus ends of the microtubules, away from the poles, to align them on the equatorial plane of the cell [16]. Although microtubules are highly dynamic, chromosomes are stabilized and hold on the equatorial plane of the cell until every chromosome pair is attached to the opposite spindle poles correctly. When all sister chromatids are lined up, cell becomes ready to separate them. There is spindle assembly checkpoint at the end of metaphase to make sure each chromatid pairs are oriented correctly. This mechanism checks if every kinetochore is attached to the spindle [24].

Cohesin proteins hold sister chromatids together and resist the microtubules which are ready to separate chromatid pairs [16].

Anaphase

Removal of the cohesin proteins is required to separate sister chromatids [16]. Anaphase promoting complex (APC) is activated during anaphase [26]. APC degrades securin. Securin normally blocks the activity of another protein, seperase. When securin is removed by APC, seperase is able to remove cohesin proteins between sister chromatids [16], [27]. Therefore, sister chromatids are released from each other. Microtubules which are attached to sister chromatids from opposite poles are able to pull chromosome pairs apart. Pulling force is achieved by plus end de-polymerization of microtubules [16]. After initiation of chromosome separation, further separation is accompanied by spindle poles that are moving away from each other. Movement of the poles are mediated by microtubule motor proteins [16].

Telophase

Separation of sister chromatids to opposite poles of the cell first results in reduced density of mitotic spindle [16]. Chromosomes recruit nuclear membrane fragments around them and these fragments join together to form new nuclear envelope and nucleus proteins are moved into nucleus [28]. Therefore two newly formed nucleus reappears in opposite poles of the cell.

Cytokinesis

Dividing cell has to divide its cytoplasm into two to generate two physically separated cells. Cytoplasmic division phase is named as cytokinesis and in most of the animal cells cytokinesis start during anaphase [16]. Cytokinesis is different from mitosis, because mitosis is the nuclear division whereas cytokinesis is the cytoplasmic division.

During cytokinesis, cleavage furrow around cell becomes visible. Cleavage furrow is formed via contractile ring which squeezes cell membrane for division [16]. Mitotic spindle is identified to be the deciding factor where the contractile ring is formed [29]. However how the exact positioning is achieved via molecular mechanisms are not clear [16], [30]. One of the model explaining this phenomena is named astral stimulation model because experiments showed that localization of furrow is determined via astral microtubules originating from spindle poles [31]. Another model is named central spindle stimulation. In this model, contractile ring is formed around central spindle microtubules originating from opposite spindle poles that are interacting in the equatorial plane [32]. Interacting sites are able found to be interacting with signaling proteins, such as RhoA, to point the localization of the contractile ring formation [16]. Third model is the astral relaxation model, which proposes that the cortical relaxation in the equatorial plane is higher than the poles, therefore it is easier to form contractile ring around [16],

[33]. It seems to be the combination of the three model proposed working together during cytokinesis.

Contractile ring is composed of actin and myosin 2 filaments. Formin protein is found to be nucleating actin filaments to form contractile ring. Contractile ring is dynamically reduced in size during cytokinesis [16].

When contractile ring is narrow enough to complete cytoplasmic division, remaining microtubule spindles become concentrated on the narrow connection between two newly formed cells. This dense connection is called midbody [34]. Midbody is visible at the end of the cytokinesis and separated between two newly formed cells [16]. However many proteins found in the midbody still need to be characterized [35].

2.2. Regulation of the cell cycle through phosphorylation

Cell division is required for organisms to develop and reproduce. Organisms regulate cell cycle of the cells depending on internal and external cues. Cell division progression and control depends on spatial and temporal regulation of phosphorylation of proteins. Kinases such as CDKs, Plks and Aurora kinases regulate cell division dynamically [6], [10], [36].

Cyclin dependent kinases (CDKs) are kinase protein family which are the regulator of the cell cycle phases, G_1, S, G_2 and M phases, via cyclin proteins [1], [4], [6], [25]. There are more than 20 CDKs identified [6]. During G_1 phase CDK4, CDK6 and CDK2, during S phase CDK2, during G_2 and M phase CDK1 are found to be activated via cyclin proteins [3]. Abundance of cyclin proteins changes as the cell cycle progresses, therefore their partner CDK activity changes. For example, Cyclin A activates CDK2 during S phase. While cell progresses from G_1 to S phase, Cyclin D activates CDK4 and CDK6 and Cyclin E activates CDK2. Cyclin B activates CDK1

during G_2 to M phase transition [8], [25]. During mitotic exit, previously activated CDKs are inactivated by anaphase promoting complex (APC) which degrades cyclin partners of CDKs. APC activity is controlled via CDC20 and CDH1 [10]. Gained activity of CDKs and modulating cyclin abundances during specific cell cycle phases shows that cell cycle progression is regulated tightly by CDKs (Table 1).

CDK	Cyclin	Cell cycle activity
CDK4	Cyclin D1, D2, D3	G_1
CDK6	Cyclin D1, D2, D3	G_1
CDK2	Cyclin E	G_1/S transition
CDK2	Cyclin A	S
CDK1	Cyclin A	G_2/M transition
CDK1	Cyclin B	Mitosis
CDK7	Cyclin H	CDK activating kinase

Table 1. CDKs in cell cycle: CDKs and their cyclin partners function in distinct phases of cell cycle [15]**.**

Polo like kinases (Plks) are functioning in various pathways in cell division [10]. PLK1 activates CDK1 by phosphorylation. PLK1 also activates CDC25, inhibits WEE1 activity and cause degredation of MYT1 by phosphorylating it. These activities of PLK1 promotes mitotic entry. Plks also regulate centrosome duplication, maturation, chromosome separation during division. Plks are required for cytokinesis phase too. PLK1 activates RhoGTPase which is required for contractile ring contraction [10].

Aurora kinase family is conserved among species. There are three types of Aurora kinases identified in mammals, Aurora A, Aurora B and Aurora C. Aurora A functions in spindle formation, Aurora B regulates chromosome alignment and Aurora C is chromosomal passenger protein that can bind to INCENP and surviving (Table 2).

Member	Substrates	Localization	Cell cycle phase
Aurora A	Kinesin related motor Eg5, CPEB, Histone H3, TPX2, LIM, p53, BRCA-1	Centrosome	Prophase, Metaphase and Telophase
		Spindle microtubules	
Aurora B	Histone H3, Histone H2A, MCAK, Topoisomerase II, INCENP, survivin	Kinetochores (prophase to metaphase)	Prophase, Metaphase
		Mid zone	Anaphase
		Midbody	Cytokinesis
Aurora C	INCENP, survivin	Centrosomes	Anaphase to Cytokinesis

Table 2. Aurora kinase family proteins. Localization and known substrates of the Aurora kinases [36], [37].

2.3. Phosphoproteomics

Spatial and temporal regulation of proteins are achieved through post translational modifications (PTMs). Once a protein is synthesized, its structure can be modified through PTMs. PTMs are reversible covalent modification of amino acid(s) in protein. PTMs can act as turn on/off switch for proteins and multiple PTMs of a single protein can create different functioning modes of protein through changing its structure, stability and enzymatic activity [38], [39]. There are more than 200 PTMs such as phosphorylation, acetylation and methylation [16].

Phosphorylation is one of the most common and studied among PTMs (Figure 1) [40]. Protein kinases phosphorylate proteins by catalyzing transfer of γ –phosphate group of ATP or GTP to serine, threonine or tyrosine residues. There are 518 putative kinases identified in the human genome [41]. Kinases can be grouped in eukaryotic protein kinases (ePKs) and atypical protein kinases (aPKs). ePKs share a similar catalytic domain sequence responsible for the phosphorylation. aPKs are different from ePKs in terms of their catalytic domains. aPKs do not have the catalytic domain similar to ePKs and they are discovered through biochemical assays. aPK family is relatively small compared to ePKs (Table 3). On the other hand, another group of proteins named phosphatases transfer phosphate groups from phosphorylated amino acids to water molecules. Phosphorylation is crucial for cell to be able to response external and internal stimuli. Generally kinases are activated by a signal and phosphorylation is used as an information transferring signals.

Approximately 2% of the human genome encode for kinases and at least 75% of the human proteome is phosphorylated on more than 50000 phosphorylation sites [41], [42]. Dynamic phosphorylation of proteins are crucial regulators for signal transduction pathways. For example

receptor tyrosine kinase on the plasma membrane phosphorylates downstream cytoplasmic proteins to regulate several signal transduction pathways [43].

Identification of phosphorylation events is challenging due to dynamic nature of protein phosphorylation events. There are several methods to identify phosphorylation statuses of proteins. One of the techniques utilizes SDS-page analysis which requires detection of phosphorylated protein using antibodies [44]. Another method is based on immunoprecipitation of phosphorylated proteins via radioactively labeled phosphate group which is transferred from radioactively labeled ATP [44], [45]. However, mass spectrometry (MS) based methods are capable to reveal global phosphorylation statuses and abundances of phosphoproteins[46]–[48].

Phosphoproteomic analyses via MS are usually carried out in the following order. First, cell lysate is obtained in the presence of phosphatase inhibitors to inhibit dephosphorylation. After lysing the cell, fractionation of the cell lysate is performed to purify proteins and purified proteins are broken down into peptides using proteases. Most widely used protease is the trypsin which cleaves proteins at C termini of lysine or arginine residues. Peptides are further fractioned and enriched to overcome sample complexity.

Phosphorylated peptides are not abundant and can be easily masked out during MS analysis. That is why fractionation and phosphoprotein enrichment steps must be carried out during sample preparation [47]. Enrichment methods mostly depend on increasing phosphopeptide abundance by removing non phosphorylated proteins from sample to be analyzed. Antibodies that recognize phosphorylated residues can be used for immunoprecipitation of phosphorylated proteins/peptides however there is no antibody working properly against phosphorylated serine and threonine residues. Another way of enriching phosphopeptides is achieved via chemical modification of phosphoserine and phosphothreonine residues which can be converted into thiol groups. These

thiol groups can be coupled to biotin which can be further coupled and eluted via avidin [43]. However widely used phosphopeptide enrichment methods are immobilized metal affinity chromatography (IMAC), metal-oxide affinity chromatography (MOAC) and Phos-Tag. All three methods capture negatively charged phosphorylated residues via positively charged groups such as Fe^{3+}, TiO_2 in columns [49], [50].

Another way of improving the identification of phosphoproteins by MS is the sample fractionation. Fractionation of the sample reduces the complexity in each fraction by separating peptides according to their chemical properties. Strong cation exchange (SCX) and strong anion exchange (SAX) are commonly used in fractionation of phosphopeptide. Tryptic phosphopeptides usually have net +1 charge on them whereas non-phosphorylated tryptic peptides have +2 net charge at low pH. Charge difference caused by phosphorylation makes non-phosphopeptides to retain in SCX column, because SCX colum contains negatively charged acid residues, therefore phosphorylated and non-phosphrylated peptides can be separated. However SCX does not work well for peptides which are phosphorylated in multiple sites because the total charge state of these phosphopeptides is usually negative. In SAX, stationary column has positively charged residues and phosphopeptides are fractioned separated from non-phosphopeptides using elution buffers with decreasing pH.

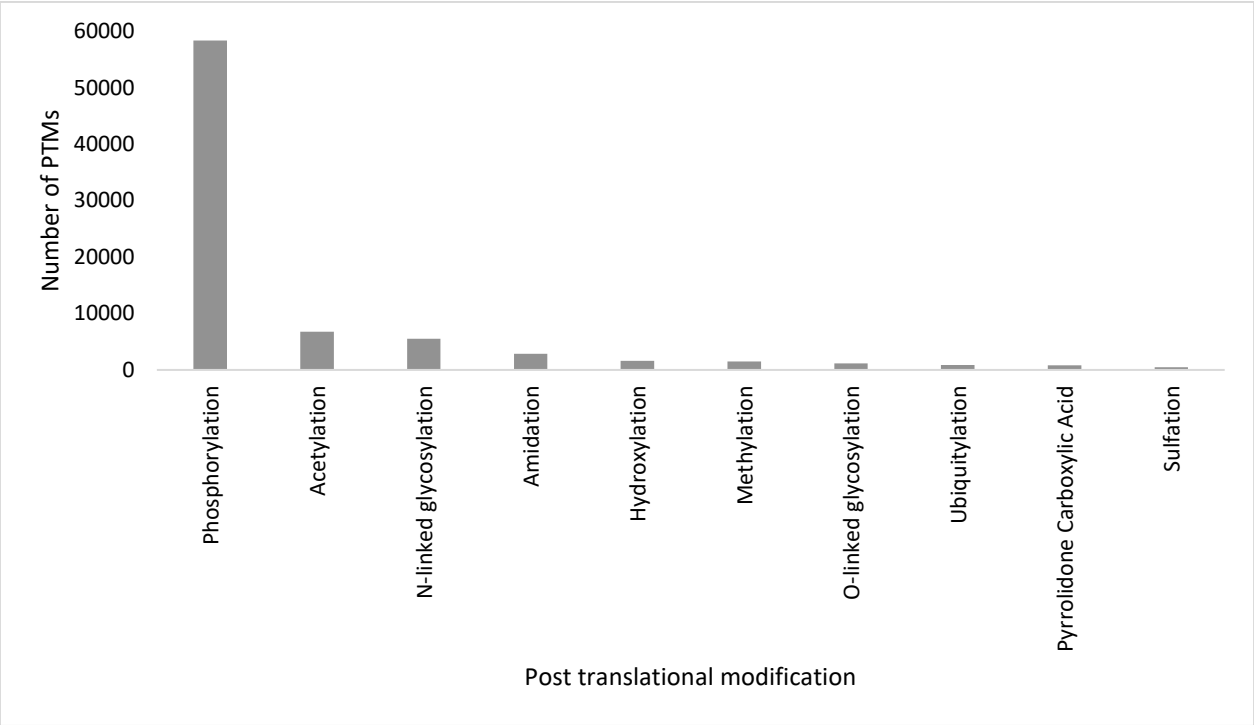

Figure 1. Number of top ten most common PTMs curated from UniProtKB/Swiss-Prot.

PTM numbers refer to number of unique PTMs which are experimentally identified in total of 85,336 proteins in UniprotKB/Swiss-Prot database (release 2015_02) [40].

Kinase Group	Human Kinases	Novel human Kinases
AGC	63	7
CAMK	74	10
CK1	12	2
CMGC	61	3
Other	83	23
STE	47	4
Tyrosine kinase	90	5
Tyrosine kinase-like	43	5
RGC	5	0
Atypical-PDHK	5	0
Atypical-Alpha	6	0
Atypical-RIO	3	2
Atypical-A6	2	0
Atypical-Other	9	4
Atypical-ABC1	5	5
Atypical-BRD	4	1
Atypical-PIKK	6	0
Total	518	71

Table 3. Number of kinases in ePK and aPK groups [41]**.**

2.4. Kinase – Substrate prediction

Mobility shift and luminescence based assays are developed to identify kinases responsible for protein phosphorylation experimentally [51], [52]. However, in vitro kinase assays are found to be decreasing kinase specificity [53]. That is why physiological phosphorylation statuses of the proteins are important to reveal kinase specificity and regulation.

MS analysis can reveal physiological phosphorylation statuses and sites of proteins. Active site of the catalytic subunit of kinases phosphorylate protein targets through recognizing phosphorylation sites [44]. Using site precise protein phosphorylation information from MS analysis and specificity of kinases to motifs around phosphorylation sites, it is possible to assign kinases to phosphorylation sites [53]. There are various computational methods predicting phosphorylation sites and matching kinase motif to phosphorylation sites, however phosphorylation motif and protein sequence is not always good enough to assign a kinase to a phosphorylation [53], [54].

Phosphorylation requires physical interaction between kinase and substrate. These interactions can be direct or mediated by intermediate proteins. Only sequence based predictions do not consider physical barriers. For example if a predicted phosphorylation site of a kinase is buried in a protein, sequence based prediction methods can match that site to a kinase however in reality that kinase cannot access to that site. Structural similarities between phosphorylation sites can be used to identify kinases can bind to those sites. Therefore, sequence motif based kinase predictions can be improved by identifying structural motifs of the phosphorylation sites [55].

Although structure of proteins possesses a great chance to reveal structural motifs of phosphorylation and identify kinases that can bind to those sites, most of the protein structures are not known. However, protein-protein interaction networks can be used to improve sequence motif based kinase substrate prediction [54]. If a phosphoprotein is interacting with a known kinase or found in close proximity to a kinase in protein-protein interaction network, it is most likely phosphorylated by that kinase [12], [56]. In this thesis we used NetworKIN3.0, which combines motif based prediction and network proximity of kinases to phosphoproteins to predict kinases [12].

CHAPTER 3

MATERIALS AND METHODS

3.1. Sample Preparation

In this thesis, we have used the quantified phosphopeptide ratios from previously performed experiments in our lab [11]. Phosphopeptides purified from HeLa S3 cells which were synchronized in interphase, mitosis and monopolar cytokinesis were quantified previously in [11]. Similarly, phosphopeptides from HeLa S3 cells which are synchronized in monopolar cytokinesis and bipolar cytokinesis were quantified previously in [11] too.

Following sections contain summary of the performed experiments.

3.1.1. Interphase, mitosis and monopolar cytokinesis synchronization

HeLa S3 cells were synchronized at G1/S phase using double thymidine block and collected as interphase cells. Remaining cells at interphase were treated with S-trityl-l-cysteine for 12 hours and collected as mitosis cells. Lastly, remaining mitosis cells were treated with Purvanol A for 15 minutes and monopolar cytokinesis synchronized cells were collected [11].

3.1.2. Monopolar cytokinesis and Bipolar cytokinesis synchronization

Monopolar cytokinesis synchronization was performed using Purvanol A as explained previously. For bipolar cytokinesis synchronization, cells, similarly, were arrested at G1/S phase by double thymidine block, incubated for 7 hours in complete media, treated with Nocadazole. When cells

are observed at the prometaphase, Nocadozole is washed away and 1 more hour of incubation of mitotic cells resulted bipolar cytokinesis synchronized cells [11].

3.2. Mass spectrometry analysis of phosphopeptides

Protein extracts from mitosis, interphase and monopolar cytokinesis samples were trypsinized and dimethyl labeled with medium, heavy and light labels (Figure 3.1). Samples are mixed, 1:1:1 ratio according to their average total peptide intensities, fractioned via SCX, phosphoenriched via TiO_2 enrichment. MS analysis is performed on Q Exactive quadrupole Orbitrap mass spectrometer and the raw files are analyzed via Proteome Discoverer 1.4 using Uniprot 2014_08 human proteome [11].

Similarly, protein extracts from bipolar cytokinesis and monopolar cytokinesis samples were trypsinized and dimethyl labeled with heavy and light labels (Figure 2). Samples are also mixed 1:1 ratio, fractioned, enriched by SCX and TiO_2, analyzed on Q Exactive quadrupole Orbitrap mass spectrometer and the raw files are analyzed via Proteome Discoverer 1.4 using Uniprot 2014_08 human proteome [11].

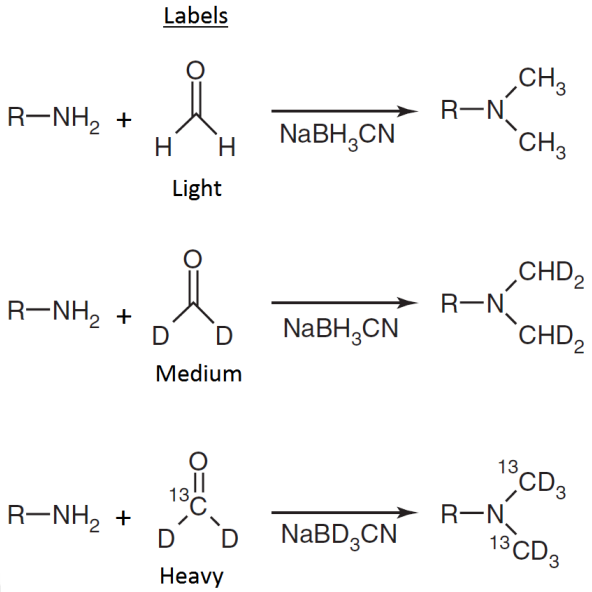

Figure 2. Labeling of peptides with stable isotope dimethyl. Modification on primary amine of peptide results +4 Da mass difference between labels. Adapted from [57].

3.3. Data processing

Numerical processing of ratios and peptide sequence editing are performed using Python 2.7.

3.3.1. Statistical analysis of phosphopeptide ratios

Median of nonphosphorylated peptide ratios are calculated to normalize each ratio group: Mitosis/Interphase, Mitosis/Cytokinesis and Interphase/Cytokinesis. Then each ratio group is normalized by their nonphosphorylated peptide ratios' median. Therefore, phosphopeptide ratios are corrected according to nonphosphorylated peptides. Nonphosphorylated peptide ratio medians are used for normalization and statistical analysis, because phosphoenrichment of the samples might be causing bias toward a subset of phosphopeptides.

After normalization, we converted ratios into $\log_{10}$ scale and expect peptide ratios to be normally distributed around 0. Therefore, median, which is equal to 0 for nonphosphopeptides, is set as the center of the normal distribution. Using corrected median absolute deviation (MAD) of nonphosphopeptides as the scaling parameter (alternative to standard deviation), a normal distribution is fit to the peptide ratios of Mitosis/Interphase, Mitosis/Cytokinesis and Interphase/Cytokinesis. MAD is corrected by the factor of 1.4826 [58].

Then we calculated outlier values for for each ratio group. Ratios which fall to the bottom 2.5% quantile and ratios which fall to the top 97.5% quantile of the normal distribution are defined as outlier ratios. For example, if mitosis/interphase ratio of a phosphopeptide belongs to the bottom 2.5% quantile, that phosphopeptide is assigned as interphase specific phosphopeptide. Therefore we assigned each outlier phosphopeptide to a specific cell cycle. Quantiles are calculated by SciPy's stats module [59].

3.3.2. Converting ratios into relative expression values

After determining significantly regulated phosphopeptides, we calculated relative expression values for each phosphopeptide by setting interphase to 1. Each phosphopeptide has Mitosis/Interphase, Mitosis/Cytokinesis and Interphase/Cytokinesis ratios. First we calculated "Mitosis expression" value from Mitosis/Interphase ratio. Similarly, we calculated "Cytokinesis expression" values too. In the end, we had expression values for each cell cycle phase for each phosphopeptide.

$$Phosphopeptide\ ratios: \frac{Mitosis}{Interphase}, \frac{Mitosis}{Cytokinesis}, \frac{Interphase}{Cytokinesis}$$

$$Interphase\ expression = 1$$

$$Mitosis\ expression = \left(\frac{Mitosis}{Cytokinesis}\right) \text{ x } \left(\frac{Cytokinesis}{Interphase}\right)$$

$$Cytokinesis\ expression = \left(\frac{Interphase}{Cytokinesis}\right)^{-1}$$

3.3.3. Clustering of relative expression values

Calculated relative expression values are unitless values that reflect original ratios of the phosphopeptides. In order to cluster phosphopeptides into cytokinesis specific and mitosis specific groups, expression values for each phosphopeptide is converted to $\log_{10}$ scale and we filtered out phosphopeptides which do not show any significant changes between interphase, mitosis and cytokinesis. Relative expression values in $\log_{10}$ are visualized using parallel plot in the order of interphase, mitosis and cytokinesis.

Using relative expression values plotted on parallel plot for each phase, interphase, mitosis and cytokinesis, we defined 6 different clusters such that each cluster representing a distinct regulation trend of phosphopeptides (Table 4).

Clusters	Phosphorylation status during:		
	Interphase to Mitosis transition	Mitosis to Cytokinesis transition	Cytokinesis
Cluster 1	Increases	Increases	Higher than interphase and mitosis
Cluster 2	Decreases	Increases	Lower than interphase, higher than mitosis
Cluster 3	Decreases	Increases	Higher than interphase and mitosis
Cluster 4	Increases	Decreases	Higher than interphase, lower than mitosis
Cluster 5	Increases	Decreases	Lower than interphase and mitosis
Cluster 6	Decreases	Decreases	Lower than interphase and mitosis

Table 4. Relative expression clusters defined according to phosphorylation regulation through interphase, mitosis and cytokinesis. First clusters which have the phosphorylation sites that upregulated during mitosis to cytokinesis transition are defined as cytokinesis specific clusters (red). From remaining phosphopeptides, similarly, clusters which have the phosphorylation sites upregulated during interphase to mitosis transition are defined as mitosis specific clusters (blue).

Clusters 1, 2 and 3 are defined as cytokinesis specific phosphopeptide clusters and clusters 4 and 5 are defined as mitosis specific clusters. We further performed biological process enrichment analysis for proteins of cytokinesis and mitosis specific clusters to compare phosphopeptides biological functions during cell cycle phases via Enrichr [60]. Adjusted p-value cut off is set to 0.05.

3.4. Kinase prediction for cytokinesis specific phosphoproteins

In order to identify kinases responsible for phosphorylation of cytokinesis specific clusters, we used NetworKIN 3.0 [56].

NetworKIN 3.0 predicts kinases that are responsible for phosphorylation of proteins in two steps. Prediction starts with matching known motifs of kinases to phosphorylation sites. Experimentally identified and trained kinase motif classifiers for each kinase group are matched to the possible phosphorylation sites of the protein and NetPhorest probability score of each match is calculated[61]. A second score is calculated from known protein-protein kinase interaction networks. Network proximity scores for kinase and proteins are calculated using STRING protein-protein interaction database by multiplication of kinase's and protein's connecting nodes' confidence scores [62]. However network proximity score is penalized by the length of edges connecting kinase and protein in the network. It means that if a protein and kinase are found close to each other in interaction network a higher proximity score is assigned. However, if the nodes connecting kinase and protein have low confidence scores, network proximity score decreases. Finally, a NetworKIN score for each predicted kinase on phosphorylation site is reported as a unified likelihood ratio which is calculated by combining NetPhorest and network proximity scores of each prediction.

FASTA sequences of proteins in cytokinesis specific cluster is compiled into a single file and kinases are predicted by NetworKIN3.0 standalone program which is available at http://networkin.info/download.shtml. We filtered out NetworKIN scores lower than 5 which are low confident predictions [63]. High confident predictions are visualized using Cytoscape [64].

3.5. Motif analysis of cytokinesis phosphopeptides

Sequence window of each phosphoprotein, 7 amino acid upstream and 7 amino acid downstream centered around phosphorylation site is acquired from Uniprot 2014_08 proteome database. Motif enrichment analysis is performed for combined cytokinesis specific clusters via motif-x web server [13]. Human proteome is set for background and lowest suggested significance threshold, 0.0005, is used in the analysis. Serine, threonine and tyrosine phosphorylations are searched in three separate runs.

3.6. Evolutionary conservation rate comparison of bipolar cytokinesis and monopolar cytokinesis phosphorylation sites

Monopolar and bipolar cytokinesis specific phosphorylation events are quantified via stable isotope dimethyl labeling as explained in [11]. Differentially regulated phosphorylation sites between bipolar and monopolar cytokinesis are identified by statistical analysis similar to explained in 3.3.1. Phosphopeptide ratios are normalized using median of nonphosphorylated peptide ratios, centered on 0 in $\log_{10}$ scale, and normal distribution is fit to using median absolute deviation as scaling factor. Outlier phosphorylation sites belonging to the bottom 2.5% and top 97.5% quantiles are identified as monopolar and bipolar cytokinesis specific phosphorylations. Phosphopeptides which are not significantly regulated between monopolar and bipolar cytokinesis are identified as shared phosphopeptides between two models.

BLASTp search for every protein identified in monopolar and bipolar cytokinesis comparison is performed against non-redundant protein sequence database with E value cut off set to 0.0001 to reveal homologous sequences [65]. Multiple sequence alignment of top 100 scoring proteins for each phosphoprotein is performed via MAFFT (version 7) [66]. Evolutionary rates of each amino acid residue for every phosphoprotein is calculated by Rate4Site 2.01 [67]. For each

protein, rates amino acids are scaled between 0, being the lowest, and 10 being the highest evolutionary conservation score.

Scaled conservation scores of individual phosphorylation sites are pooled in bipolar cytokinesis specific, monopolar cytokinesis specific and shared groups. Groups are compared via Mann–Whitney U test to asses if medians of the groups are significantly different from each other.

CHAPTER 4

RESULTS

4.1. Clusters of relative expression values in interphase, mitosis and cytokinesis

Significantly regulated phosphopeptides between two or three phases are selected for clustering because if a phosphopeptide is not significantly changed between any of the phases (interphase, mitosis and cytokinesis) it does not have a cell cycle dependent regulation. Number of phosphopeptides regulated between every phase, interphase, mitosis and cytokinesis, are significantly lower than phosphopeptides regulated between at least two phases (Figure 3). Therefore, phosphopeptides which significantly regulated between interphase, mitosis and cytokinesis are clustered into 6 different main expression trends and plotted into parallel plot. Medians of each group is represented in Figure 4 and every phosphopeptide in each cluster plotted in Figure 5.

Cytokinesis specific clusters 1,2 and 3 have the phosphoproteins which are upregulated during mitosis to cytokinesis transition. Similarly clusters 4 and 5 represent the mitosis specific clusters.

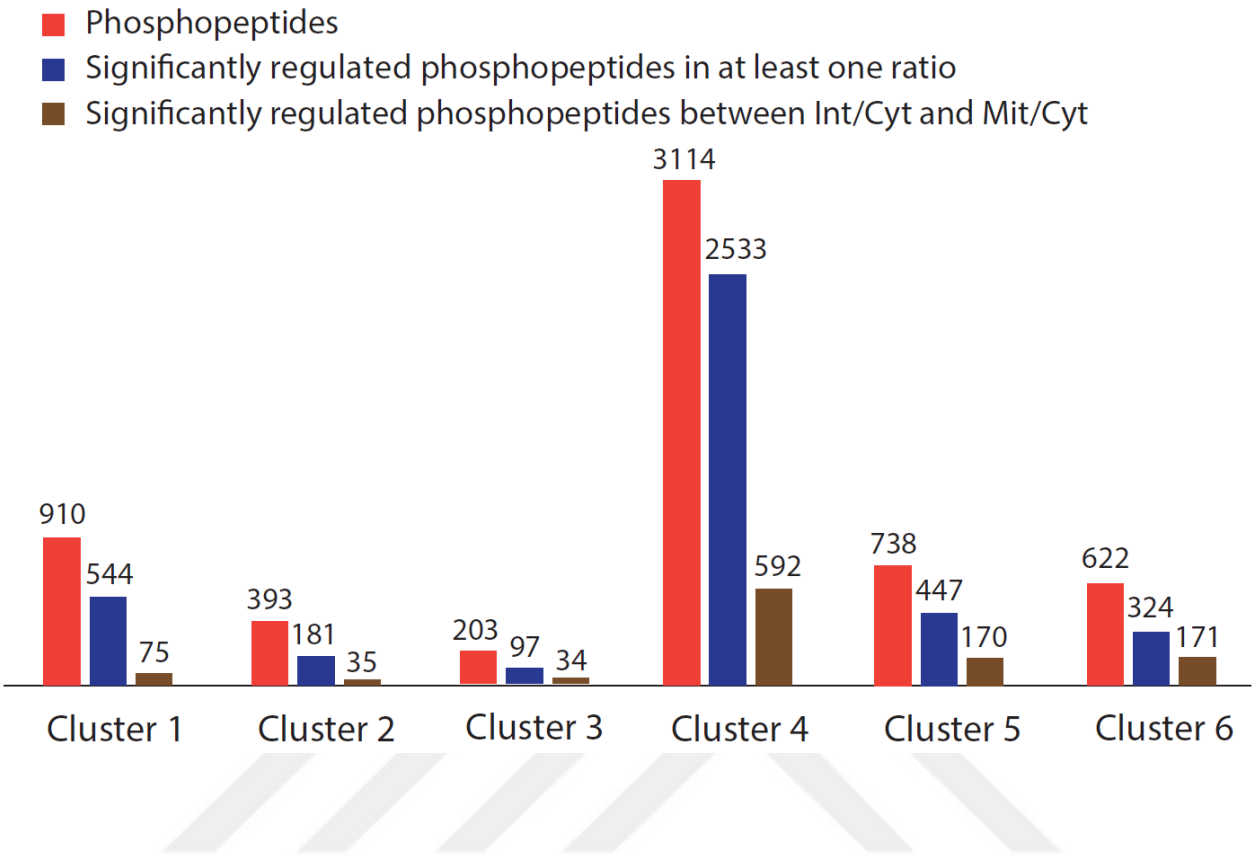

Figure 3. Phosphopeptide numbers of clusters. Total phosphopeptide numbers of each cluster, number of phosphopeptides which are regulated between at least two phases and number of phosphopeptides regulated between interphase (Int)/cytokinesis (Cyt) and mitosis(Mit)/cytokinesis(Cyt) are shown.

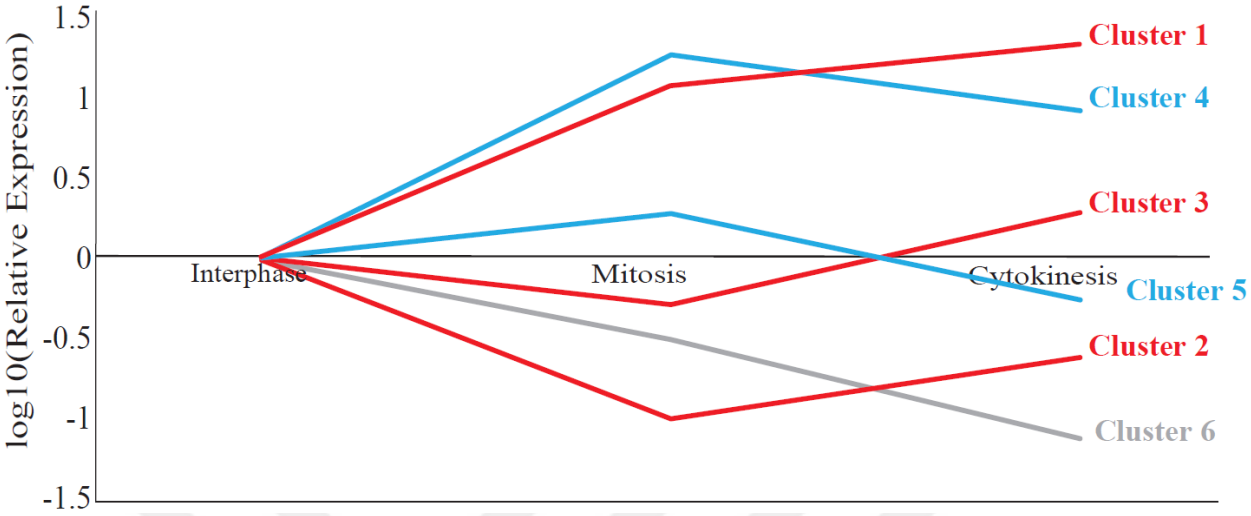

Figure 4. Medians of relative expression values of each phosphopeptide cluster. Cluster 1, 2 and 3 (red) are defined as cytokinesis specific clusters: Phosphorylation during mitosis to cytokinesis transition increases. Clusters 4 and 5 are defined as the mitosis specific clusters (blue): Phosphorylation during interphase to mitosis transition increases.

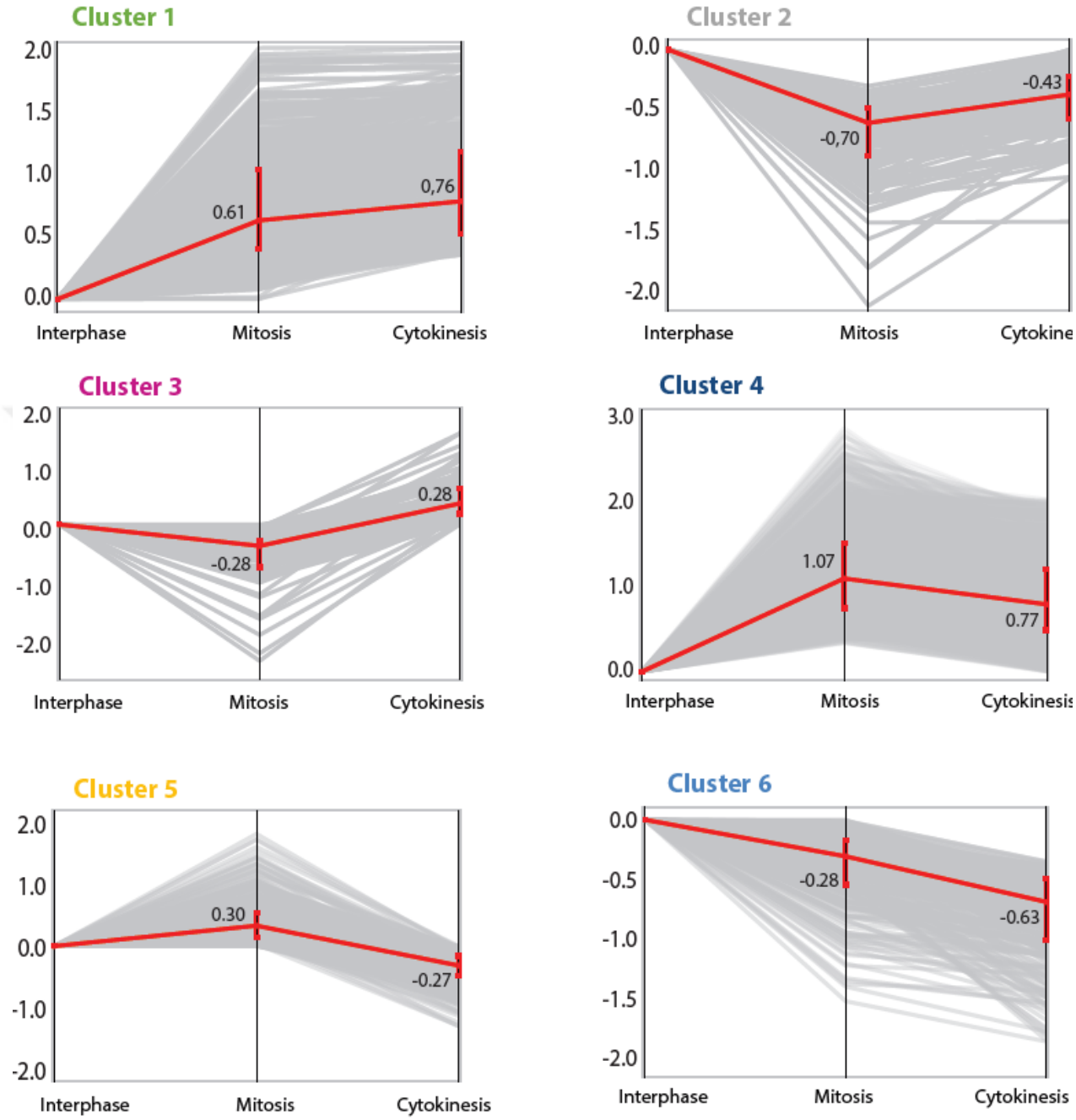

Figure 5. Relative expression values of phosphopeptide clusters in Interphase, Mitosis and Cytokinesis. Clusters 1, 2 and 3 represent cytokinesis specific phosphorylation events. Cluster 4 and 5 represent the mitosis specific phosphorylation events. Relative expression values are in $\log_{10}$. Trends in red are the median of each group.

4.2. Kinase-substrate network of cytokinesis specific clusters

Converting quantitative Proteome Discoverer ratios of phosphopeptides into relative expression values allowed us to identify cytokinesis specific phosphorylation events and total of 31 responsible kinases were predicted via NetworKIN3.0[56].

Predicted kinases for clusters 1, 2 and 3 are visualized via Cytoscape 3.4.0 in figures 6,7 and 8 respectively, and combined network of clusters 1,2 and 3 is in Figure 9.

Significantly up-regulated phosphopeptides in cytokinesis and predicted kinase network shows CDK1 is not completely shut down during cytokinesis and functioning together with Aurora and MAPKs (Figure 9).

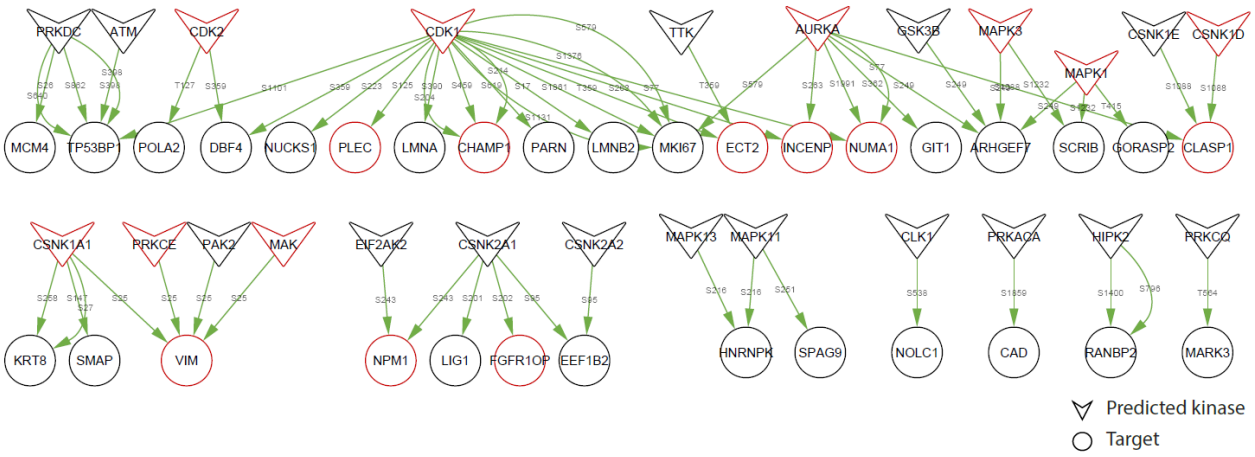

Figure 6. Predicted kinases of phosphoproteins in Cluster 1. Triangle nodes represent medium and above scoring predicted kinases via NetworKIN3.0 [56]. Edges show phosphorylation sites of proteins by kinases. Proteins and kinases function in cytoskeleton organization is highlighted with red borders. Network is visualized via Cytoscape 3.4.0[64].

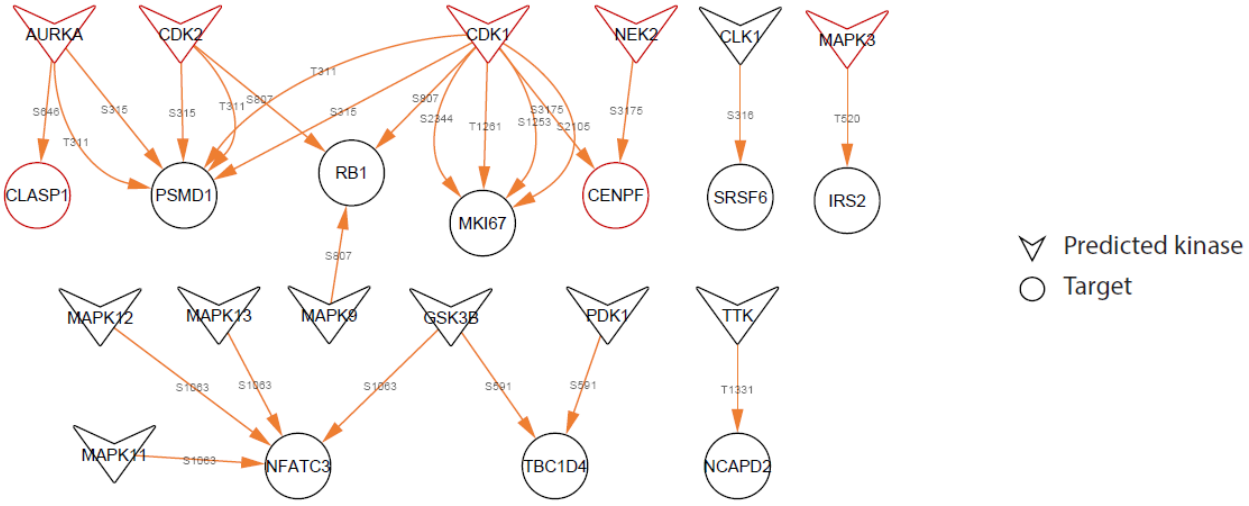

Figure 7. Predicted kinases of phosphoproteins in Cluster 2. Triangle nodes represent medium and above scoring predicted kinases via NetworKIN3.0 [56] Edges show phosphorylation sites of proteins by kinases. Proteins and kinases function in cytoskeleton organization is highlighted with red borders. Network is visualized via Cytoscape 3.4.0[64].

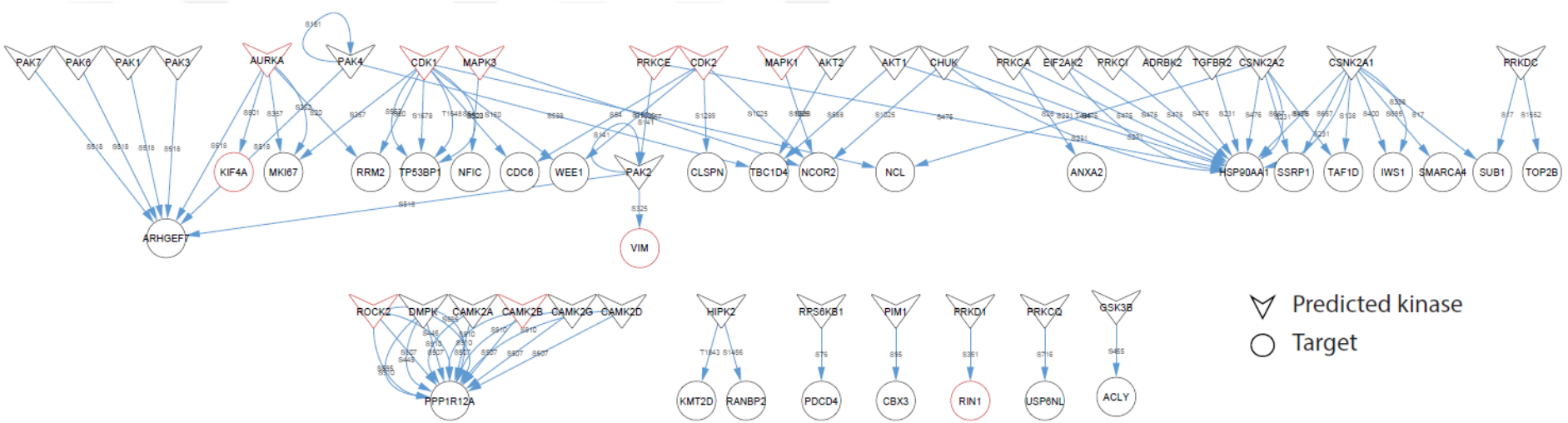

Figure 8. Predicted kinases of phosphoproteins in Cluster 3. Triangle nodes represent medium and above scoring predicted kinases via NetworKIN3.0 [56]. Edges show phosphorylation sites of proteins by kinases. Proteins and kinases function in cytoskeleton organization is highlighted with red borders. Network is visualized via Cytoscape 3.4.0[64].

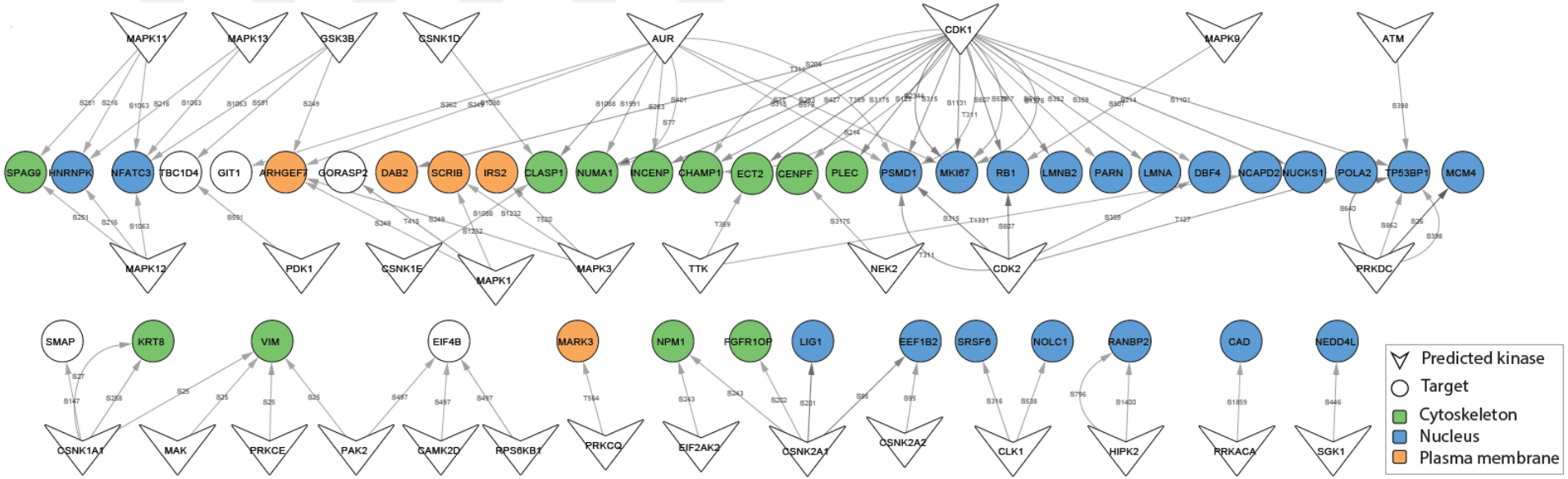

Figure 9. Predicted kinases of cytokinesis specific phosphoproteins. Triangle nodes represent medium and above scoring predicted kinases via NetworKIN3.0 [56], circle nodes represent phosphoproteins from cytokinesis specific clusters. Edges represent phosphorylation sites of phosphoproteins. Proteins are colored according to their cellular localizations. Cytoskeleton (green), nucleus (blue) and plasma membrane (orange) annotations are fetched from Uniprot [68] using BioServices python package[69]. Network is visualized via Cytoscape 3.4.0[64].

4.3. Motif analysis reveals cytokinesis specific phosphorylation of Ki-67

Motifs enriched in cytokinesis specific phosphorylation events revealed a distinct motif originating from 4 phosphopeptides which maps only to protein Ki-67 unlike other motifs enriched (Figure 10 and Figure 11). Ki-67 is a proliferation marker which is identified around mitotic chromosomes[70]. Although its function is unclear, it is thought to be acting as a surfactant around mitotic chromosomes [71]. Motif identified in the repeat regions of Ki-67 shows cytokinesis specific regulation of the protein through phosphorylation (Figure 12 and Figure 13).

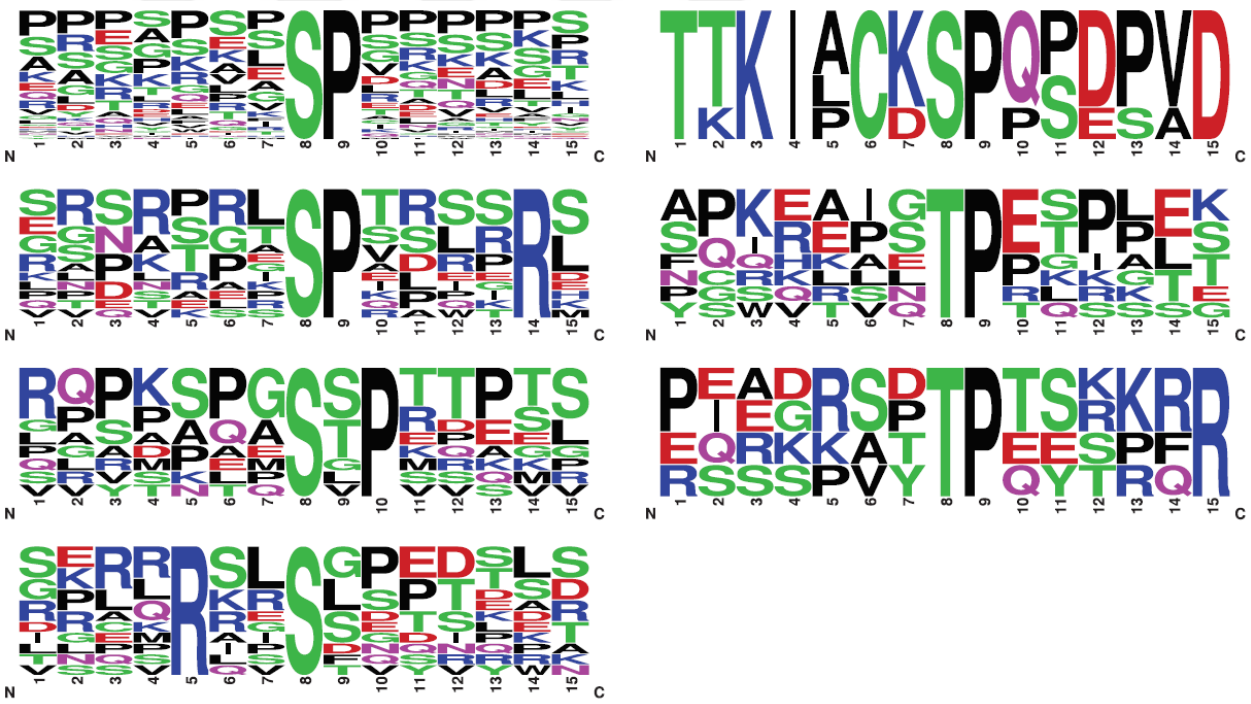

Figure 10. Motifs enriched in cytokinesis specific clusters. Motif-x webserver [13] is used to identify motifs enriched in cytokinesis specific phosphopeptides against human proteome background.

Figure 11. Phosphorylated Ki-67 motif during cytokinesis. Motif found in Ki-67 is enriched in cytokinesis specific phosphopeptides compared to the human proteome background [72].

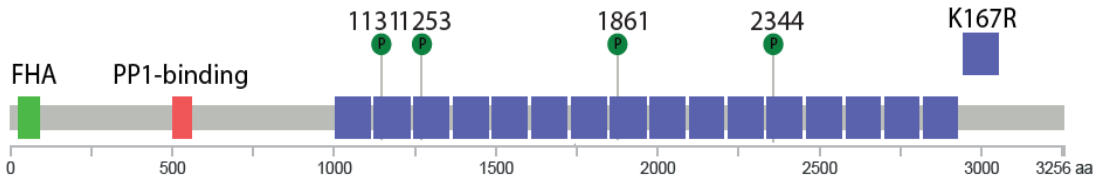

Figure 12. Cytokinesis specific phosphorylation sites bearing enriched motif of Ki-67 on K167R domains. Domain architechture of Ki-67 is visualized and cytokinesis specific phosphorylation sites are shown on K167R domains.

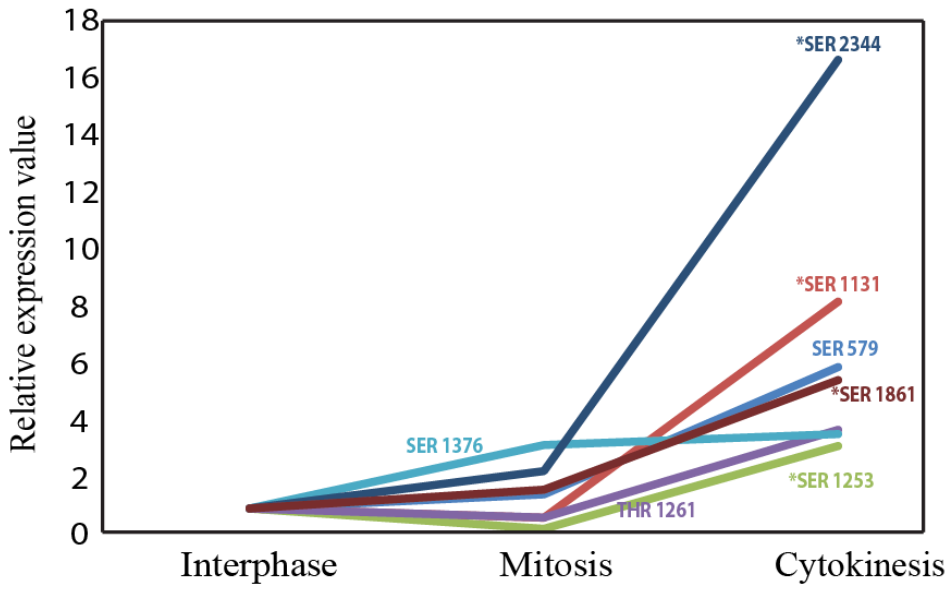

Figure 13. Phosphorylation of Ki-67 through interphase, mitosis and cytokinesis. Phosphorylation sites of Ki-67 identified in our analysis. (*) labeled sites share same phosphorylation motif in Figure 11.

4.4. Gene ontology enrichment comparison of cytokinesis and mitosis specific phosphorylations

Proteins of cytokinesis specific phosphorylations (Clusters 1,2 and 3) and mitosis specific phosphorylations (Clusters 4 and 5) compared for enriched terms of biological processes and cellular components (Figure 14 and Figure 15).

Spindle assembly and kinetochore organization terms which are expected to be related with mitosis phase are found to be enriched in cytokinesis phosphoproteins however not in mitosis phosphoproteins. That could show that phosphorylation events during cytokinesis might be still regulating past mitotic events. Similarly, “mitotic cell cycle” term is found to be enriched for cytokinesis and mitosis phosphoproteins. However, nucleus related terms such as nucleus organization, nuclear pore complex assembly and nuclear envelope are only enriched in mitosis

specific phosphoproteins. Interestingly, contractile fiber part is only enriched for cytokinesis phosphoproteins whereas actomyosin and cleavage furrow parts related terms are enriched in mitosis phosphoproteins.

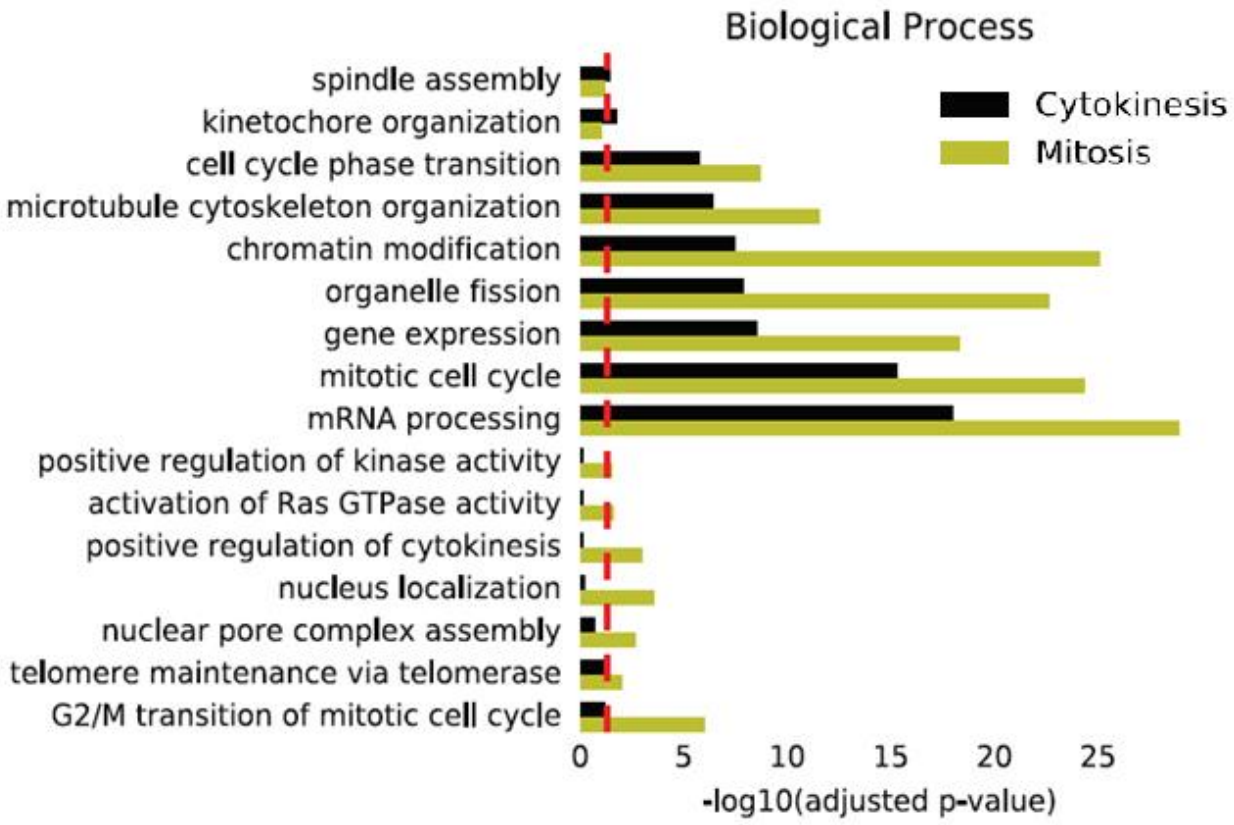

Figure 14. Comparison of enriched biological process terms of cytokinesis and mitosis specific phosphoproteins. Adjusted p-values are converted into –log10(adjusted p-values) to visualize enrichments better. Dashed red line shows significance threshold $p < -\log_{10}(0.05)$. Enrichment analysis is performed via Enrichr [60].

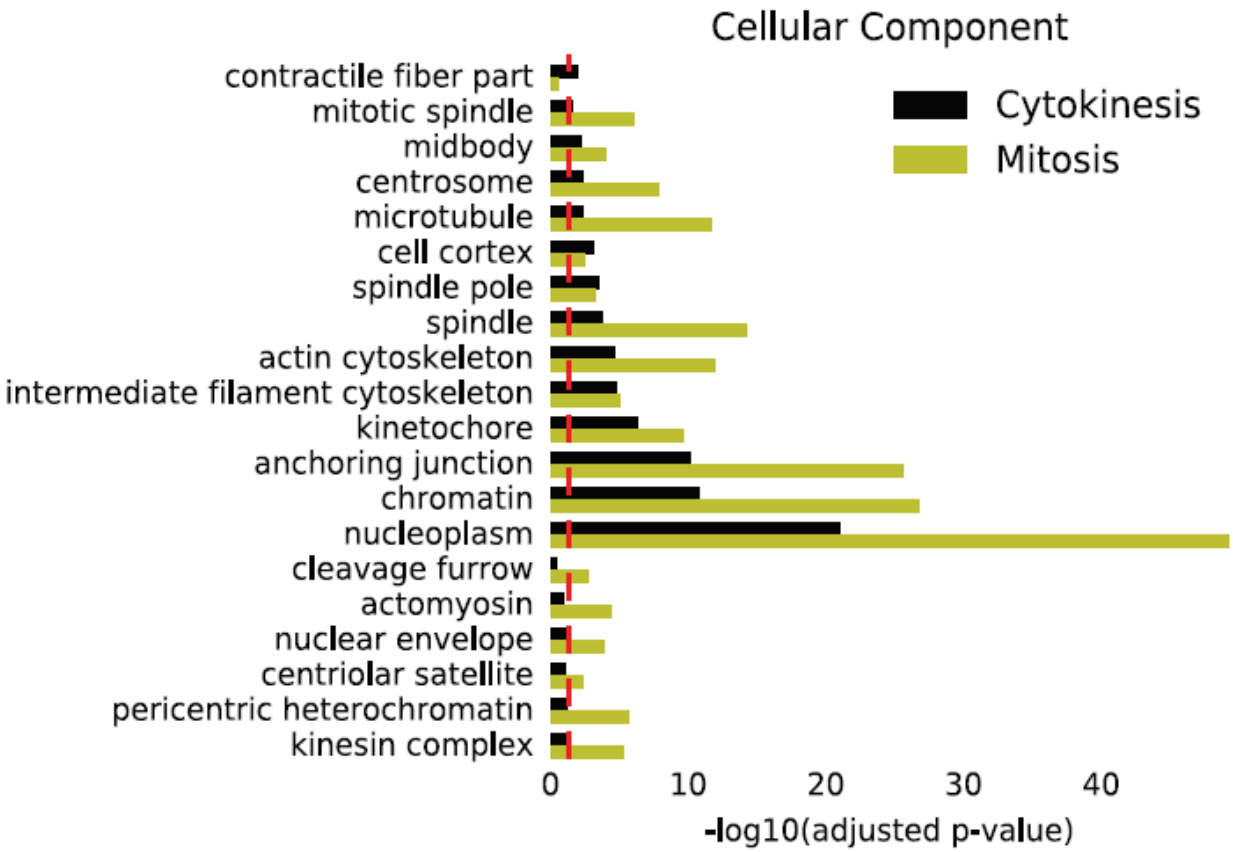

Figure 15. Comparison of enriched cellular component terms of cytokinesis and mitosis specific phosphoproteins. Adjusted p-values are converted into –log10(adjusted p-values) to visualize enrichments better. Dashed red line shows significance threshold $p < -\log_{10}(0.05)$. Enrichment analysis is performed via Enrichr [60].

4.5. Comparison of evolutionary conservation scores of phosphorylation sites between Monopolar cytokinesis and Bipolar cytokinesis

Monopolar cytokinesis is used in our analysis because of its cytokinesis synchronization efficiency [11], [73], [74]. However monopolar cytokinesis cells do not divide into two daughter cells like regular bipolar cytokinesis complete. In order to evaluate monopolar cytokinesis specific phosphorylation sites against bipolar cytokinesis, we compared phosphorylation site conservation scores of monopolar specific phosphorylation sites with bipolar cytokinesis specific phosphorylation sites.

Bipolar cytokinesis synchronization rate in our analysis was around 60-70% percent, whereas monopolar cytokinesis synchronization rate is 100% [11].

For each phosphoprotein, homologous sequences are searched using BLASTp and top 100 scoring similar protein sequences from non-human organisms are chosen. If monopolar cytokinesis is irrelevant to study cytokinesis biochemistry and dynamics, we expect the phosphorylation sites specific to monopolar cytokinesis, most likely, would not be conserved among species.

We compared medians conservation scores of phosphorylation sites of monopolar and bipolar specific phosphorylation sites and observed no difference (Mann-Whitney U Test) and concluded that the monopolar cytokinesis specific phosphorylation sites are conserved as bipolar cytokinesis specific phosphorylation sites between species (Figure 16).

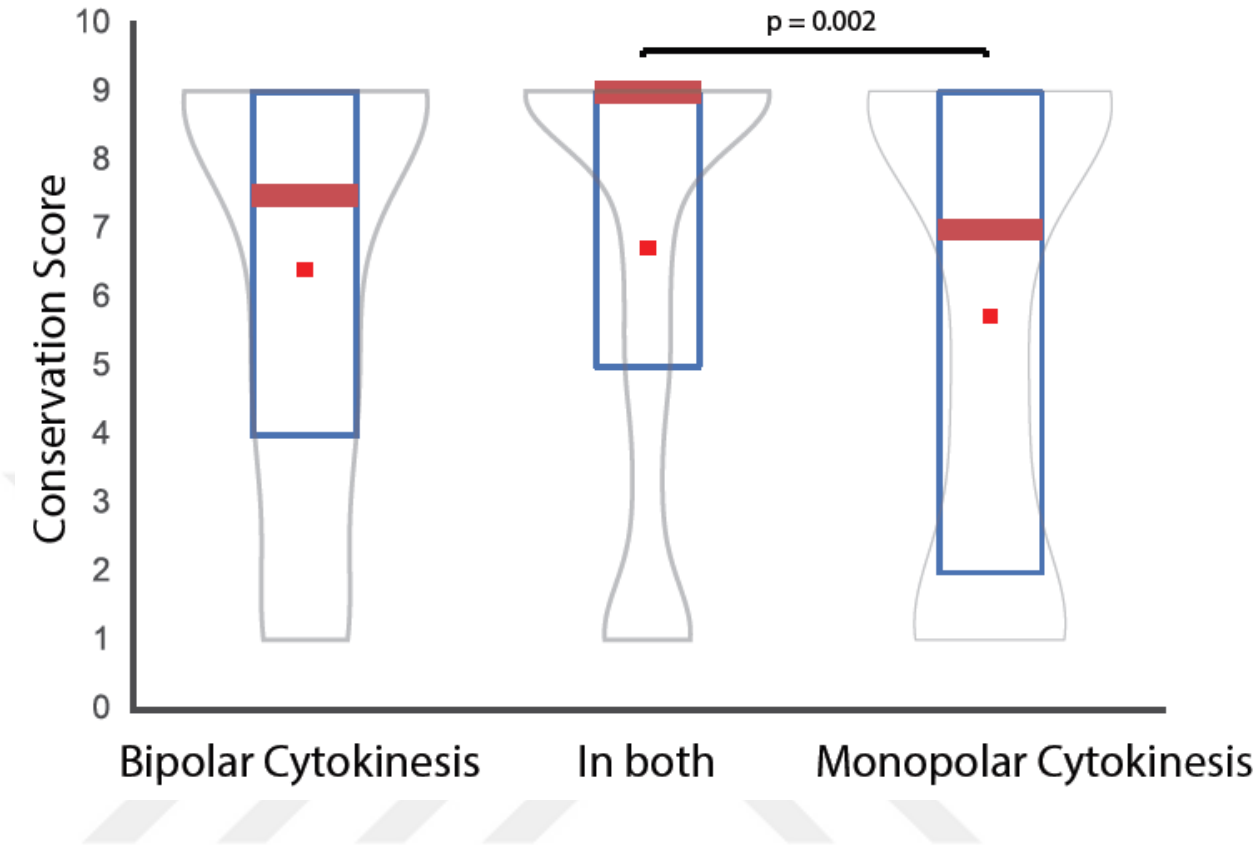

Figure 16. Conservation scores comparison of bipolar cytokinesis specific phosphosites (66 sites), monopolar cytokinesis specific phosphosites (156 sites) and phosphosites shared between monopolar and bipolar cytokinesis (1132 sites). Median of conservation scores of phosphorylation sites in both and monopolar cytokinesis are significantly different. (Mann–Whitney U test $p < 0.05$, Red lines: Median of conservation scores Red Squares: Mean of conservation scores**)**

CHAPTER 5

DISCUSSION

Cytokinesis is the fastest stage of the cell cycle and phosphorylation dynamics during cytoplasmic division change dramatically. Phosphoproteome of the cytokinesis has not been studied extensively due to its speed and challenges in synchronization of cells in cytokinesis [75]. In this study, we have identified phosphorylation events taking place during cytoplasmic division and total of 31 kinases responsible for the biochemical and physiological changes during cytokinesis. Previously reported cytokinesis related kinases such as Aurora, MAP and Casein kinases, TTK and GSKB are found to be active at the start of cytoplasmic division.

CLASP1 is a microtubule tracking protein is predicted to be phosphorylated by Aurora kinase in our analysis [76]. Phosphorylated CLASP1 localizes to microtubules and interacts with PRC1 to stabilize anti parallel microtubules on central spindle during cell division [77], [78]. ARHGEF and GIT1 are predicted to be phosphorylated during cytokinesis by Aurora kinase. ARHGEF and GIT1 are reported to be functioning together to regulate Rac1 function [79]. This might imply that this complex might be regulated by Aurora kinase during cytokinesis. Our analysis also revealed cytokinesis specific phosphorylations by MAP kinase family. MAPK is previously reported to be functioning in midbody to facilitate abscission therefore predicted phosphorylations by MAPKs might be regulating midbody function during cytokinesis [80]. Kinases predicted and their identified targets in our analysis are a collection of cytokinesis specific phosphorylation events and the kinases which are active during cytokinesis. Experimental analysis of individual kinase substrate interactions reported in our analysis can further reveal each phosphorylation's cytokinesis specific functions in detail.

We suggest that cytokinesis specific phosphorylation of Ki-67 might be used as a cytokinesis biomarker. Cytokinesis specific biomarkers are valuable to asses cytokinesis phase biochemically. Ki-67 protein is used as a proliferation marker already and cytokinesis specific phosphorylation of its K167 repeat domains can improve its use to identify proliferating cells' division progression [70]. Also we suggest that K167 repeat domain function of Ki-67 might be important for chromosomal organization during cytokinesis because Ki-67 localizes to chromosomes during mitosis. This localization of Ki-67 acts as a surfactant therefore helping chromosome segregation during mitosis [71].

In this study we also systematically showed that phosphorylations of drug induced monopolar cytokinesis synchronization and bipolar cytokinesis mostly overlap. Only about 10% of the phosphorylations differ and about 90% of the phosphorylations are shared between two models. Comparison of evolutionary conservation of phosphorylation sites showed that phosphorylations between two models are similarly conserved between species. Monopolar cytokinesis exhibits most of the physiological phases that bipolar cytokinesis have such as midzone formation and spindle bundling in the mid zone. This shows that monopolar cytokinesis specific phosphorylations events are not redundant and monopolar cytokinesis is a good alternative to study cytokinesis biochemistry.

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