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Ubiquitin receptor protein UBASH3B – a novel regulator of mitotic progression

Le récepteur à l'ubiquitine UBASH3B, un nouveau régulateur de la mitose

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To my Mother, Olga

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Résumé de these

La mitose assure la ségrégation parfaite du génome aux cellules filles et des erreurs dans la signalisation mitotique peuvent conduire à l'aneuploïdie et à la polyploïdie, caractéristiques des cancers. L'un des facteurs mitotiques majeurs contrôlant l'euploïdie des cellules eucaryotes, correspond à la kinase Aurora B. Aurora B coordonne l'alignement des chromosomes avec la ségrégation de ces derniers par la régulation de l'assemblage des fuseaux mitotiques et l'attachement des kinétochores aux microtubules. Les fonctions essentielles de la protéine Aurora B dans la ségrégation des chromosomes, sont dépendantes de sa localisation dynamique aux centromères au cours des phases de prométaphase et de métaphase et aux microtubules de la région dénommée « midzone » lors de l'anaphase. Des études précédentes effectuées par notre laboratoire ont mis en évidence le rôle critique de l'ubiquitination non-protéolytique d'Aurora B, catalysée par une E3 ligase à sous-unité de type Culin3 (CUL3), pour sa relocalisation des centromères aux microtubules de la « midzone ». Néanmoins, il reste à savoir comment et à quel moment la fraction ubiquitinée d'Aurora B est ciblée vers ces structures mitotiques.

L'attachement de molécules d'ubiquitine à des substrats protéiques régule la fidélité de la division mitotitique via des mécanismes protéolytiques et non-protéolytiques. L'action coordonnée des enzymes E1, E2 et E3 catalyse la réaction d'ubiquitination pouvant aller à l'attachement d'une seule molécule d'ubiquitine (monoubiquitination) à la synthèse de chaines de polyubiquitine de différentes topologies. Les protéines possédant des domaines d'interaction à l'ubiquitine (Ubiquitin Binding Domain ou UBD) peuvent servir en tant que récepteurs ou décodeurs pour des signaux spécifiques médiés par l'ubiquitine permettant ainsi le transfert de substrats ubiquitinés à des composants situés en aval de la voie de signalisation et/ ou à des compartiments cellulaires.

Les récepteurs à l'ubiquitine impliqués dans les processus protéolytiques, sont connus pour transférer les substrats au protéosome 26S afin de les dégrader, ou pour les cibler vers la voie de dégradation des protéines associées au réticulum endoplasmique (endoplasmic-reticulum associated

protein degradation ou ERAD) ou vers les voies de signalisation de l'autophagie. Les récepteurs à l'ubiquitine peuvent également décoder des signaux d'ubiquitination non-protéolytique tel qu'il a été montré dans la régulation des mécanismes de réparation de l'ADN ou dans la voie de signalisation NFκB. Il est intéressant de souligner qu'à ce jour, malgré le nombre élevé de protéines à UBD répertoriés dans le protéome humain, aucune n'est connu pour être associée à la régulation de la division mitotique.

Le but de mon projet de thèse consiste à identifier la (les) protéine(s) à UBDs qui agisse(nt) dans la voie de signalisation médiée par l'axe Aurora B-CUL3 au cours de la mitose permettant ainsi le contrôle de l'euploïdie des cellules humaines.

Afin d'identifier le récepteur à l'ubiquitine, impliqué dans la voie de signalisation dépendant de l'axe Aurora B-CUL 3, j'ai réalisé un criblage visuel à haut-débit (siRNA) de l'ensemble des gènes humains codant les protéines à UBD (connues et prédites) dans les cellules HeLa. La procédure de criblage a été conçue de sorte à identifier les facteurs responsables de la coordination de la ségrégation chromosomique avec la cytokinèse. L'inactivation de ces facteurs conduit à l'obtention d'un phénotype terminal majeur correspondant à une augmentation de la proportion des cellules multinuclées et des cellules à noyau multilobé. Ce phénotype est similaire à celui observé après extinction de l'expression du gène codant Aurora B. Seul un des candidats obtenus à l'issue du criblage, la protéine Ubiquitin-associated (UBA) and SH3 domain-containing protein B, UBASH3B, a été identifié comme interacteur des E3 à CUL3 au cours d'une précédente expérience d'immunoprécipitation suivi par une analyse en spectrométrie de masse.

En accord avec les résultats obtenues par le criblage, l'extinction de l'expression du gène codant UBASH3B par un pool de siRNA conduit à une augmentation significative du nombre de cellules avec un noyau multilobé de forme hétérogène par rapport à l'échantillon contrôle.

Afin de comprendre comment UBASH3B régule la progression mitotique, j'ai analysé par immunofluorescence la distribution des différentes phases mitotiques de cellules synchronisées. J'ai observé une augmentation drastique du nombre de cellules en prométaphase suite à l'extinction de l'expression du gène codant UBASH3B, suggérant un défaut dans la congression des chromosomes et/ou dans le temps nécessaire pour déclencher l'anaphase. J'ai confirmé ces résultats par la microscopie en temps réel qui a également permis de montrer que les cellules exprimant les siRNA ciblant le messager du gène codant UBASH3B, présentent une réduction de la survie cellulaire, un délai prolongé des phases de pro/ métaphase et des erreurs de nombre au cours de la ségrégation des chromosomes.

En utilisant un anticorps spécifique de la protéine UBASH3B, j'ai montré que cette protéine s'accumule au niveau des fuseaux mitotiques en prométaphase et métaphase suggérant que la protéine UBASH3B est un facteur mitotique associé aux fuseaux mitotiques et contrôle la fidélité de la ségrégation des chromosomes.

Afin d'analyser les interactions moléculaires de la protéine UBASH3B avec l'axe de signalisation Aurora B- CUL3, j'ai réalisé des tests d'immunoprécipitation. J'ai ainsi mis en évidence une forte interaction entre la protéine UBASH3B endogène et une protéine recombinante GFP-Aurora B. Il est important de préciser que cette interaction est dépendante de la présence de la protéine E3 à CUL3 suggérant que UBASH3B peut réguler directement la fraction d'Aurora B modifiée par l'E3 ligase à CUL3. Ces résultats sont corroborés par un test de pulldown utilisant une protéine recombinante qui correspond au domaine de liaison à l'ubiquitine (UBA) de la protéine UBASH3B fusionné à la GST. Cette construction (GST-UBA) interagit avec des formes modifiées, pouvant probablement être des formes mono et di-ubiquitinées de la protéine endogène Aurora B.

L'analyse de la localisation mitotique de la protéine Aurora B a révélé que l'extinction de l'expression du gène codant UBASH3B, conduit à la perte de localisation d'Aurora B au niveau des microtubules de la « midzone » lors de l'anaphase.

Afin de déterminer si UBASH3B peut réguler le recrutement de la protéine Aurora B aux microtubules, j'ai surexprimé UBASH3B dans des cellules arrêtées è un stade dit « prométaphase-like ». Cette surexpression est suffisante pour déclencher l'association de la protéine Aurora B endogène avec les microtubules.

La surexpression de la protéine UBASH3B induit également l'apparition d'un noyau multilobé, une répartition anormale et prématurée des chromosomes dans les cellules en prométaphase, une réduction du niveau protéique de la sécurine ainsi que de BubR1 suggérant une levée de l'arrêt mitotique imposée par le point de contrôle de l'assemblage des fuseaux (spindle assembly checkpoint ou SAC).

Ces résultats suggèrent fortement que la protéine UBASH3B contrôle la ploïdie des cellules par régulation de la localisation de la protéine Aurora B et par conséquence ces fonctions essentielles dans la ségrégation des chromosomes.

Les données obtenues lors de mes travaux de thèse suggèrent que la protéine UBASH3B est impliquée de manière critique dans la régulation de la ségrégation des chromosomes, agit en tant que lien moléculaire entre la fraction centromérique d'Aurora B et celle associée aux microtubules, et contrôle ainsi un mécanisme dit « switch-like » favorisant l'association d'Aurora B avec les microtubules avant le début de l'anaphase.

De plus, mes résultats suggèrent que la relocalisation d'Aurora B aux microtubules est suffisante pour inhiber la signalisation médiée par le SAC au niveau des kinétochores. . Par conséquent, la perte et le gain de fonction de la protéine UBASH3B ont des effets néfastes sur la fidélité de la ségrégation des chromosomes. C'est pourquoi je suppose que la protéine UBASH3B doit être précisément régulée au cours de la progression mitotique par un mécanisme moléculaire qui reste à identifier. Mes données démontrent également que la redistribution d'Aurora B des centromères aux microtubules contrôle l'inhibition de l'arrêt mitotique exercé par le SAC, la durée et la fidélité de l'anaphase et l'euploïdie des cellules. Curieusement, UBASH3B est

surexprimée dans les cancers très agressifs du sein, où elle participe à la promotion de la croissance tumorale, l'invasion et la métastase. Il serait intéressant de comprendre la régulation de ce récepteur au signal d'ubiquitination non-protéolytique, son profil d'expression dans d'autres cancers et de corréler son potentiel oncogénique avec son rôle dans la ségrégation des chromosomes.

De plus, mon travail met en lumière un mécanisme où la signalisation médiée par l'ubiquitine peut être décodée lors de la mitose et fournit un exemple de récepteur intracellulaire à l'ubiquitine contrôlant une voie de signalisation médiée par l'ubiquitination non-protéolytique. En effet, mes résultats suggèrent fortement que la protéine UBASH3B contrôle la localisation mitotique de la protéine kinase Aurora B de manière non-protéolytique. L'extinction de l'expression du gène codant UBASH3B ainsi que la surexpression de cette protéine n'affecte pas le niveau protéique d'Aurora B mais influence dramatiquement sa localisation cellulaire.

Par la suite, il serait important d'étudier les rôles mitotiques précis des autres protéines à domaines UBD et leurs spécificités au regard des différents substrats et modifications par l'ubiquitine.

Thesis summary

Mitosis ensures equal segregation of the genome to daughter cells and defects in mitotic pathways can lead to aneuploidy and polyploidy, frequently observed in cancers. One of the key factors controlling mitosis in eukaryotic cells is Aurora B kinase. Aurora B coordinates chromosome alignment and segregation by regulating spindle assembly and kinetochore-microtubule attachments. Essential functions of Aurora B in chromosome segregation are dependent on its dynamic localization to centromeres in prometa- and metaphase stages and to midzone microtubules during anaphase. Previous findings of our laboratory demonstrated that nonproteolytic ubiquitylation of Aurora B by CUL3-based E3 ligases is important for its relocalization from centromeres to microtubules of the spindle midzone. However, it remains unknown how and when ubiquitylated Aurora B is targeted to these mitotic structures.

Ubiquitin attachment to substrate proteins regulates fidelity of mitotic division through proteolytic and non-proteolytic mechanisms. Coordinated actions of E1, E2 and E3 enzymes catalyze substrate ubiquitylation, ranging from a single ubiquitin molecule (monoubiquitylation) to topologically different chains of interconnected ubiquitins. Ubiquitin Binding Domain (UBD) proteins can serve as receptors, or decoders, for the specific ubiquitin signals and transfer ubiquitylated substrates to the downstream signaling components and cellular compartments.

Ubiquitin receptors acting in proteolytic pathways have been shown to transfer substrates to 26S proteasome for degradation or target them to endoplasmic-reticulum-associated protein degradation (ERAD) and autophagy pathways. Ubiquitin receptors can also decode non-proteolytic ubiquitin signals in regulation of DNA repair or NF-kB signalling. Surprisingly, despite a high number of known UBDs in mammalian cells, their mitotic roles remain unexplored.

The goal of my PhD project is to identify UBDs that act in the Aurora B-CUL3 pathway during mitosis and control euploidy of dividing human cells.

To identify ubiquitin receptor, acting in the Aurora B-CUL3 pathway, the laboratory performed a high content visual siRNA screen in HeLa cells for known and predicted human UBDs. The screening procedure was designed to identify factors that coordinate chromosome segregation with cytokinesis, inactivation of which leads to strong terminal phenotypes of multilobed nuclei and multinucleated cells observed upon downregulation of Aurora B. Only one of the identified hits, Ubiquitin-associated (UBA) and SH3 domain-containing protein B, UBASH3B, was also found to interact with CUL3.

In agreement with the results obtained by unbiased screening, downregulation of UBASH3B by a pool of siRNAs led to a significant increase in a number of cells with multilobed nuclei of heterogenous forms as compared to the control siRNAs.

To understand how UBASH3B regulates mitotic progression, I first used immunofluorescence microscopy and analyzed distribution of different mitotic stages in cells synchronized by double thymidine block and release. I observed a drastic increase in a number of cells in prometaphase upon downregulation of UBASH3B as compared to control cells, suggesting defects in chromosome congression and/or timely onset of anaphase. I confirmed these results using live video microscopy, which showed that downregulation of UBASH3B reduced survival of mitotic HeLa cells, caused a prolonged delay in pro/metaphase-like states of surviving cells and lead to numerous errors during chromosome segregation.

Moreover, using a specific UBASH3B antibody, I showed that UBASH3B is localized to the mitotic spindles in prometa- and metaphase, suggesting that UBASH3B is a spindle-associated, mitotic factor controlling fidelity of chromosome segregation.

To investigate the molecular interactions of UBASH3B with Aurora B-CUL3 pathway, I performed immunoprecipitation analysis and found a strong interaction of endogenous UBASH3B with GFP-tagged Aurora B.

Importantly, this interaction was dependent on the presence of CUL3 protein, suggesting that UBASH3B may directly regulate CUL3-modified Aurora B. These results were corroborated by a pulldown assay, using a recombinant fragment of UBASH3B corresponding to the ubiquitin binding domain (UBA), which interacted with modified, presumably mono- and di-ubiquitinated forms of endogenous Aurora B, in ubiquitination-dependent manner.

Analysis of mitotic localization of Aurora B revealed that downregulation of UBASH3B abolished Aurora B localization to the mitotic spindle and led to redistribution of the centromeric Aurora B along chromosome arms. These results suggest that UBASH3B acts as a non-proteolytic ubiquitin receptor directly regulating localization of Aurora B to microtubules during mitosis.

To investigate whether UBASH3B may regulate the balance of ubiquitylated Aurora B by actively recruiting it to microtubules, I overexpressed UBASH3B protein in cells arrested in a prometaphase-like stage. Strikingly, overexpression of UBASH3B was sufficient to trigger association of endogenous Aurora B with microtubules.

Overexpression of UBASH3B also induced appearance of multilobed nuclei and premature and aberrant chromosome partitioning in prometaphase cells, reduction of Securin and BubR1 levels, suggesting the overriding of spindle assembly checkpoint (SAC).

These results strongly suggest that UBASH3B controls ploidy of cells by regulating microtubule localization of Aurora B and thereby its essential functions in chromosome segregation.

Collectively, my data suggest that UBASH3B is critically involved in the regulation of chromosome segregation, acting as a molecular link between centromeric and spindle-associated fractions of Aurora B and controls a switch-like mechanisms promoting Aurora B association with microtubules prior to anaphase.

Moreover, my findings suggest that relocalization of Aurora B to microtubules is sufficient to silence the SAC signalling at the kinetochore and

controls timing and fidelity of anaphase and thus euploidy of cells. Therefore, both loss and gain of function of UBASH3B has detrimental effects on fidelity of chromosome segregation. These data strongly suggest that UBASH3B has to be precisely regulated during mitotic progression by yet to be identified molecular mechanisms.

Intriguingly, UBASH3B is overexpressed in h ighly aggressive breast cancer, where it was shown to promote malignant growth, invasion, and metastasis. It will be of interest to understand the regulation of this non-proteolytic, intracellular ubiquitin receptor, its expression patterns in other cancers and to correlate its oncogenic potential with the role in chromosome segregation.

In addition, my work sheds some light how the versatile ubiquitin code can be read during mitosis and provides an example of an intracellular ubiquitin receptor controlling a non-proteolytic ubiquitynation pathway. Indeed, my data strongly suggest that UBASH3B controls mitotic localization of Aurora B kinase in a non-proteolytic manner. Both downregulation and overexpression of UBASH3B do not affect protein levels of Aurora B, but dramatically influence its subcellular localization.

In conclusion, for the future prospective, it will be important to study the precise mitotic roles of UBASH3B as well as other ubiquitin receptors and their specificities towards different substrates and ubiquitin modifications.

1. Introduction

1.1. Cell cycle and mitosis

The cell cycle can be defined as an organized sequence of events that ensures correct reproduction of the cell. The process of cell reproduction starts with duplication of the cell content (including the central event- duplication of genetic material) in S phase, followed by its precise distribution between 2 daughter cells during mitosis.

The process of mitosis in mammalian cells is subdivided into several parts: prophase, prometaphase, metaphase, anaphase, telophase and cytokinesis (Fig.1).

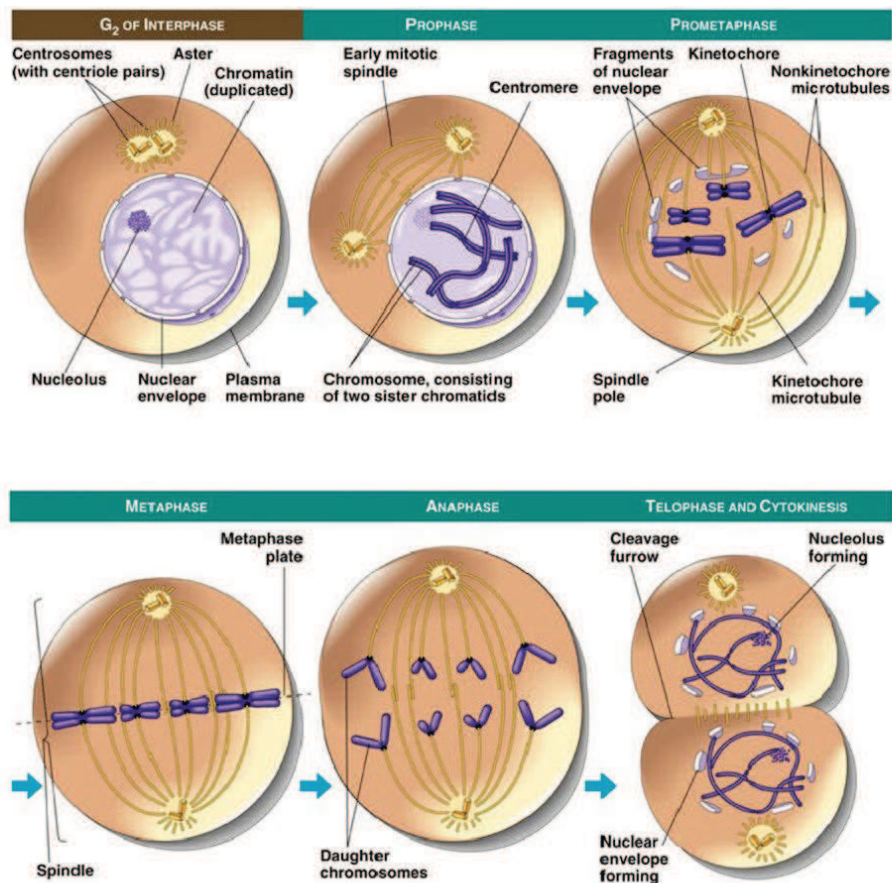

Fig.1. Graphic representations that show the different stages of mitosis(adapted from[1]) (see explanations in the text)

During prophase chromosomes begin to condense and assemble their kinetochores (protein complexes mediating attachments of microtubules to chromosomes) and centrosomes (the main microtubule organizing centers in mammalian cells) are separated to allow for spindle assembly. After the nuclear envelope breakdown, the mitotic spindle starts to access the sister chromatids and mediates sister chromatids movements towards the center of the cell in prometaphase. In metaphase chromosomes are aligned in the center of mitotic spindle and form the structure called the metaphase plate. At this stage it is absolutely necessary for kinetochores to have bipolar attachment to the microtubules of mitotic spindle, as any error in these attachments may lead to unequal distribution of genetic material between daughter cells. When all kinetochores are correctly attached to the spindle microtubules, emanating from opposite poles of the cell, chromosome segregation is licensed to start. This happens in anaphase when cohesion ring between sister chromatids is eliminated and two equal sets of chromosomes are moved to the opposite poles of the cell by microtubules' pulling forces. During telophase the spindle is disassembled, nuclear envelope is being re-formed around two segregated sets of chromosomes, which are decondensing and finally two daughter cels separate during cytokinesis by contraction of actin-myosin ring (for review, see [2]).

1.2. Building blocks of mitosis

In order to ensure faithful mitotic progression, several important mitotic structures have evolved, such as kinetochores, mitotic spindle, centrosomes, spindle midzone, midbody and others. These "building blocks" do not only constitute mitosis itself, but also pivotally regulate its progression.

1.2.1. Mitotic chromosomes

1.2.1.1. Chromosome condensation

In order to facilitate the process of DNA segregation, interphase chromatin is converted to mitotic chromosomes. This process starts in the beginning of mitosis, just before nuclear envelope breakdown, and is triggered by Cdk2-CyclinA and Cdk1-CyclinB1 phosphorylation[3,4]. Structure of mitotic chromosome is defined not only by histones, but also by non-histone proteins, that altogether form a chromosome scaffold, such as Condensin, topoisomerase II alpha and motor protein KIF4A (see below for overview)[5–7]. Eukaryotes have two Condensin complexes, I and II (Fig.2), and they both associate with chromosomes in prophase.

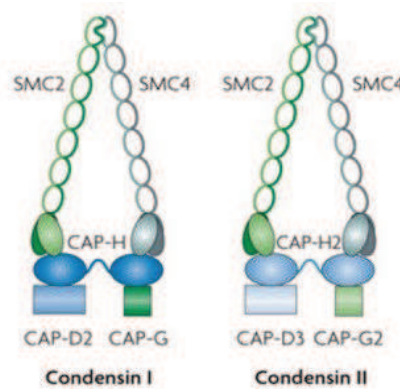

Fig.2. Molecular architecture of Condesin I and II. Condensins contain a core a heterodimer of SMC2 and SMC4, and associate with a distinct set of non-SMC regulatory subunits (CAP-H/CAP-H2, CAP-D2/CAP-D3, CAP-G/CAP-G2 for Condensin I/Condensin II) (adapted from[8])

Several models were proposed describing chromosome condensation by Condensin, although the exact mechanism is still not known. According to these models, some specific sites along chromosomes are important for binding of Condensin, and this interaction is followed either by "sequential entrapment" of DNA by Condensin, or by di- or multimerisation of Condensin with captured DNA, or both[9]. Condesin II is associated with chromatin inside the nucleus, so it is thought to initiate chromosome condensation even before the nuclear envelope breakdown, possibly triggered by Cdk2-CyclinA phosphorylation. Condensin I is a cytoplasmic

protein and it can access chromosomes only after nuclear envelope breakdown. Two subunits of Condensin I are phosphorylated by Cdk1, thus promoting the supercoiling of DNA and chromosome maturation. Topoisomerase II alpha is associated with mitotic chromosomes, alternating with Condensin[10], and its depletion leads to chromosome condensation problems[11], although the effects can be indirect. Histone H3 phosphorylation by mitotic kinase Aurora B is also considered to be important for chromosome condensation[12].

1.2.1.2. Centromere and kinetochore

Centromere is a region of the eukaryotic chromosome where kinetochores are assembled. Centromeric chromatin, as well as associated proteins (named the Constitutive Centromere-Associated Network, or CCAN), are present during the whole cell cycle[13]. In most cases, each chromosome contains only one centromeric region and such chromosomes are called monocentric. Although the centromeric region of human chromosomes is quite big (0.3-5 megabases)[14], there are no specific DNA sequences that define this region. Instead, centromeres possess a special epigenetic mark, histone H3 variant CENP-A, that is required for centromere formation[15,16]. CENP-A is a key factor defining the site for the assembly of functional kinetochores in mammalian cells[17]. Its association with the centromeric region on chromosomes is very stable[18] and its loading on centromeres is self-dependent, which means that new CENP-A binds to centromeres only in the region with already associated CENP-A. Additional DNA-binding proteins were shown to bind the centromeric region and to form a complex called CENP-T-W-S-X[19–21]. Altogether, these epigenetic marks ensure a very specific binding of the kinetochore proteins to only one chromosome region, which is crucial for correct mitotic progression.

Kinetochore is a multiprotein complex that comprises of more than 100 different components[13] (Fig.3).

Inner kinetochore proteins (16 proteins of CCAN) provide a building platform for kinetochore assembly and mediate interactions with

chromosomal DNA, as these proteins are the closest to centromeric regions. Outer kinetochore proteins, such as the KMN network (KNL1, Mis12 complex and Ndc80 complex) play a key role in kinetochore-microtubule connections. Moreover, kinetochore is a very dynamic structure, as it assembles and disassembles during the cell cycle. How does kinetochore connect DNA to microtubules? It has been shown that CENP-C directly binds CENP-A-rich centromeric regions[23,24] and at the same time the Mis12 complex (which is comprised of four subunits)[25,26]. The Mis12 complex mediates interaction with microtubules through binding to the KNL1 and Ndc80 complex[27,28].

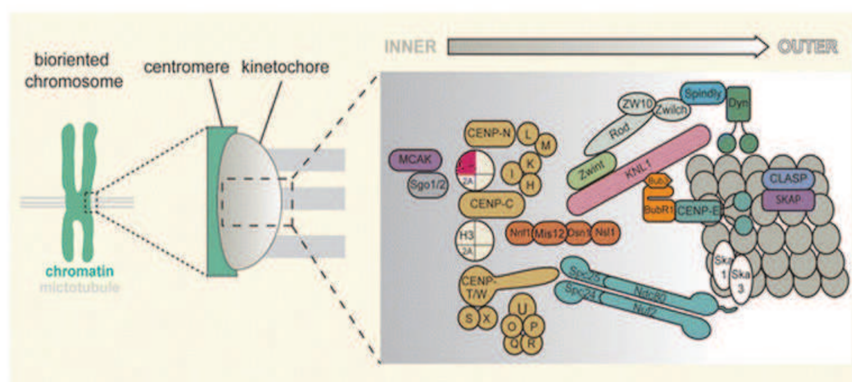

Fig.3. Schematic structure of the vertebrate kinetochore. Kinetochore components are arranged to highlight the overall geometry of the kinetochore, with more chromosome-proximal components considered inner and the microtubule-proximal components considered outer (adapted from[22])

This connection to microtubules is ensured by other interaction pathways, including additional DNA binding proteins (for example, CENP-T)[21]. Moreover, kinetochores were also shown to nucleate microtubules, which facilitate the formation of K-fibers[29]. Assembly and activity of kinetochores is controlled by Cdk1 phosphorylation and cell cycle-dependent localization of Ndc80[30].

1.2.2. Centrosomes

Centrosomes are large protein structures that serve as a microtubule organizing centers in the animal cells[31–34]. Centrosome consists of normally two centrioles (barrel-like structures built of microtubule triplets) in a

pericentriolar protein matrix (PCM), which recruits gamma-tubulin ring complexes to nucleate microtubules[35]. During S phase of the cell cycle, the centrosome is duplicated and separated upon mitotic entry, which leads to the formation of a bipolar spindle[36]. It is important to note that excessive centriole number, as well as changes in the centrosome structure, result in the multipolar spindle formation and potentially in unequal chromosome segregation and genomic instability.

1.2.3. Mitotic spindle

As the key function of mitosis is to precisely segregate the duplicated genome between two daughter cells, a special molecular machine is involved in the correct positioning of chromosomes at the metaphase plate that is able to pull the sister chromatids apart before cytokinesis. Mitotic spindle is composed of microtubules, motor proteins and numerous spindle associated factors, and was shown to be a highly dynamic structure. Spindle microtubules are arranged as two antiparallel arrays with "-" ends at the spindle poles and "+" ends in the center of the cell, marking the equator area (Fig.4)[37,38].

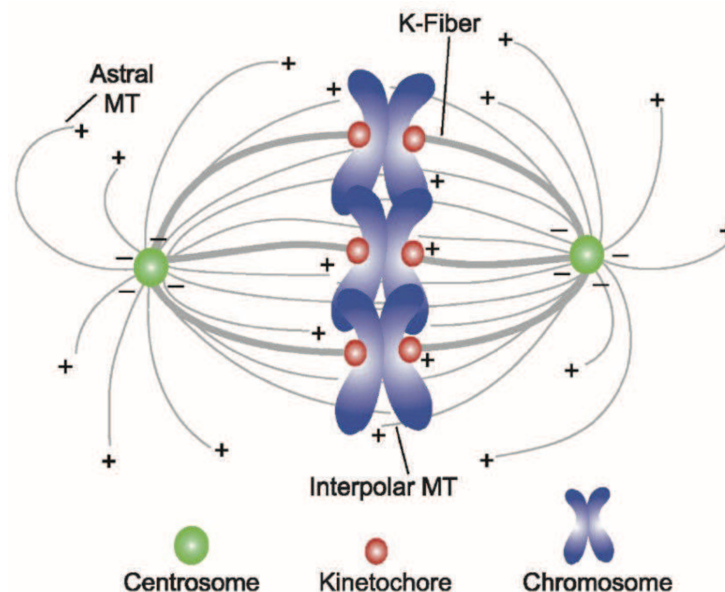

Fig.4. Mitotic Spindle Components. The scheme shows the basic components of the mitotic spindle. Microtubules are shown in gray, centrosomes in green, kinetochores in red, and chromosomes in blue. Positions of microtubule "-" ends versus "+" ends are indicated (adapted from[39])

A key feature of spindle microtubules, which allows them to segregate chromosomes, is their ability to self-organize in kinetochore fibers (K-fibers), which are microtubule bundles in the bipolar mitotic spindle[40]. These K-fibers connect spindle poles to kinetochores on each sister chromatid. Microtubules that radially emanate from centrosomes and mediate spindle positioning by interacting with cell cortex are called astral microtubules. Two spindle poles are connected by interpolar microtubules, which overlap their "+" ends in the spindle midzone (or central spindle) and coordinate anaphase and cytokinesis.

Dynamic instability of spindle microtubules is a key feature allowing for faithful mitotic progression. Microtubules are constantly switching between shrinkage and growth depending on the rate of GTP hydrolysis and availability of new tubulin dimers (Fig.5).

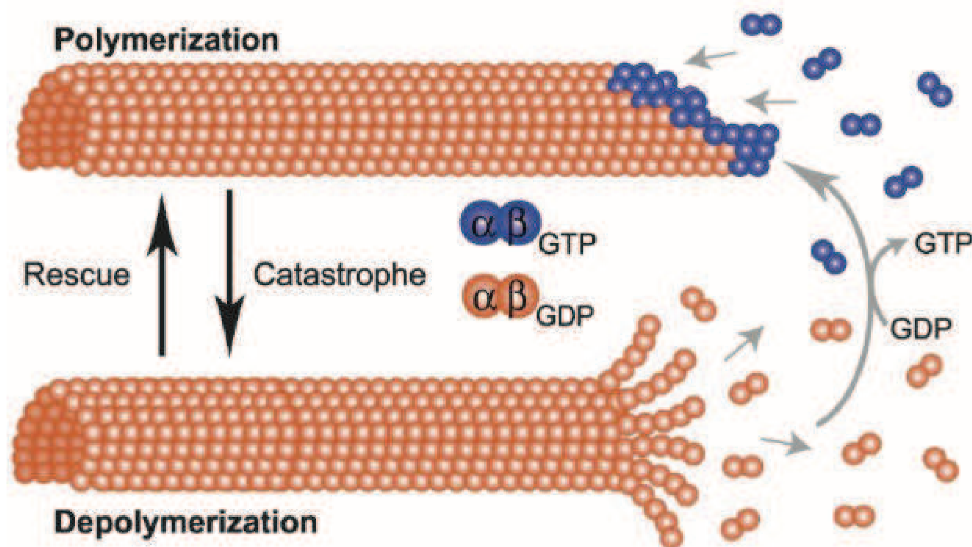

Fig.5. Dynamic instability of microtubule. Microtubules switch between stages of polymerization and depolymerization. Although dynamic instability can occur at both ends of the microtubules, only one end is shown for simplicity. GDP bound tubulin is in orange, and GTP bound tubulin, which generates the GTP cap, is in purple (adapted from[39])

Another dynamic feature of microtubules is so-called microtubule flux, which is the result of depolymerisation of kinetochore microtubules from “-“ ends together with addition of new tubulin to the ”+” ends attached to kinetochore. The resulting “flux” of tubulin subunits from kinetochores to the spindle poles generates force facing poleward and pulling kinetochores of chromosomes apart[2].

1.2.4. Motor proteins of mitotic spindle

Microtubule-associated motor proteins kinesin and dynein assist spindle assembly and chromosome segregation, by generating a force to drive chromosome segregation[41,42]. The spindle contains multiple motor proteins from both of these families, and the balanced function and coordinated actions of these proteins ensure correct spindle functioning.

1.2.4.1. Kinesins

Multiple motor proteins which are members of different kinesin’s families generally move towards “+”-ends of microtubules (with some exceptions), thus driving the motion in this direction. There are 14 different superfamilies of kinesins, they share a common catalytical core domain (conserved ATP-ase domain), but are differently localized and perform distinct functions (Fig.6). Kinesins can be processive, when they walk along microtubule as a single molecule, or non-processive (like KIFC1, member of kinesin-14 family), working in a team, but in any case kinesins use the cycle of ATP turnover, microtubule binding/release for transporting a cargo, sliding microtubules or regulating microtubule dynamics. Kinesin motors were also shown to participate in the spindle assembly. Centrosome separation upon mitotic entry is driven by KIF1/Eg5 (a member of kinesin-5 family) and KIF15/KLP2 (a member of kinesin-12 family), which mediate and coordinate sliding of antiparallel microtubules[43–47]. The correct spindle length after assembly of bipolar spindle is maintained by balanced sliding forces outward (KIF11 and/or KIF15) and inward (KIFC1). Focusing of microtubule “-“ ends in the

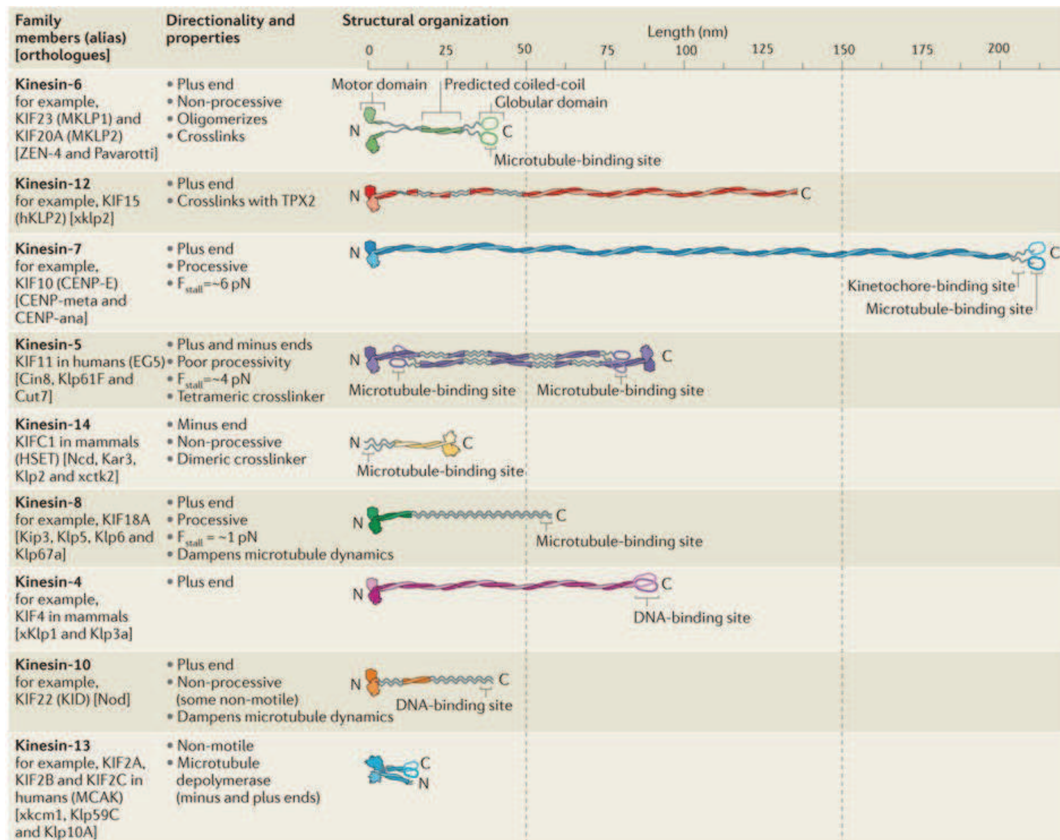

Fig.6. Topology of mitotic kinesins. Several mitotic kinesin's families are listed in the table, together with their directionality, properties and schematic structural organization (adapted from[41])

spindle poles independently of centrosomes is facilitated by KIFC1 by parallel crosslinking and sliding[48]. Moreover, chromosomal capture and congression at the metaphase plate is ensured by such kinesin motors as KIF2B and KIF2C/MCAK (depolymerisation of kinetochore microtubules from "+" and "-" ends), KIF18A (microtubule sliding and regulation of "+" end dynamics), KIF10 (lateral sliding of kinetochores to the "+" ends of microtubules) and KIF4 (regulation of microtubule lengths and chromosome compaction)[49–56].

1.2.4.2. Dyneins

Dynein belongs to another family of motor proteins involved in mitotic progression. Dynein has been shown to slide "+" ends of antiparallel

microtubules outward and focus "-" ends of microtubules in the spindle poles[57,58]. In addition, dynein is involved in the transport of other components of mitotic spindle (Nuclear Mitotic Apparatus, or NuMA)[58]. Upon establishment of correct and bipolar kinetochore-microtubule attachments, dynein has been shown to remove spindle assembly checkpoint (SAC) components from kinetochores and move them away from the place of SAC assembly[59,60]. Moreover, chromosome alignment during metaphase was reported to be controlled by cytoplasmic dynein, which moves chromosomes poleward[61,62].

1.2.5. Central spindle

During metaphase-anaphase transition, mitotic spindle is dramatically reorganized, and a special structure called central spindle, or spindle midzone, is formed between two poles of the cell, in the place of the former metaphase plate. This region includes intercalating microtubules, named midzone microtubules, which lost their connection with the spindle poles[40]. Later in anaphase and telophase, the central spindle is compacting, and finally evolves into a structure called midbody, which consists of former midzone microtubules and vesicular transport-associated proteins[63,64]. The turnover of midzone microtubules is slower in comparison to spindle microtubules[65], and microtubules themselves are more stable, although not fully inert[66–68]. The assembly of central spindle is regulated by mitotic kinases, microtubule associated proteins and motors (kinesins). The main components of central spindle formation is microtubule binding protein PRC1 (Protein Regulator of Cytokinesis 1), centralspindlin and Chromosomal Passenger Complex (CPC). PRC1 induces microtubule bundling, especially for antiparallel microtubules, and interacts with motor protein kinesin KIF4A at the central spindle[69–71]. Another key component of the midzone is protein complex centralspindlin, which consists of MKLP1 dimer (motor protein of kinesin-6 family) and CYK4 dimer (Rho family GTPase-activating protein, also called RacGAP1). Centralspindlin localizes in the center of the midzone and induces microtubule bundling and attracts proteins needed for

abscission)[72,73]. CPC also contributes to the central spindle formation by ensuring microtubule bundling and phosphorylation of some spindle components[74]. In addition, other microtubule associated and motor proteins ensure correct functioning of the spindle midzone, such as MKLP2 (kinesin-6 family member, which targets CPC component Aurora B to this region), KIF4A, Orbit (microtubule "+" end associated protein, which is important for kinetochore-microtubule attachments, but also thought to have a role in the central spindle assembly) and others[75-79]. How do all these components work together to assemble central spindle? During metaphase to anaphase transition, CPC is extracted from centromeres, other central spindle assembly factors are activated by removing mitotic phosphorylation and start to move to the region of microtubule bundles by associated "+" end directed motors (KIF4A for PRC1, SYK4 for centralspindlin and MKLP2 for CPC component Aurora B). At the same time chromosomes are moving poleward by microtubule pulling forces. Collectively, central spindle assembly factors stabilize microtubule bundles as well as their own association with spindle midzone, and provide a binding platform for regulators of cytokinesis and abscission.

1.3. Regulation of mitotic progression

A very complex process of mitosis, which involves many important players and processes, should be precisely controlled in time and in space. This regulation allows faithful mitotic progression, directing the cell to go through a set of controlling mechanisms and checkpoints, which are specific for different mitotic stages and responsive to plenty of errors that can potentially lead to abnormal chromosome segregation. In this chapter I will focus on two major posttranslational modifications, involved in control of mitosis-phosphorylation and ubiquitination, and then describe the mechanisms of regulation of kinetochore-microtubule attachments in detail.

1.3.1. Mitotic kinases

More than 600 kinases are known to control mitotic progression by phosphorylation of hundreds of substrates[80]. It is hardly possible to describe and discuss all of them in my Thesis, so I decided to focus on the main mitotic kinases and give a brief overview of balanced coordination mechanisms which they are involved in.

1.3.1.1. CDK1

CDK1 is the major kinase in mammalian cells, which controls mitosis. Early in prophase, CDK1 associates with Cyclin B1, which is a major mitotic cyclin, and CDK1-Cyclin B1 complex activation is considered to be a trigger of mitosis and a starting point for activation of many other mitotic signaling pathways[81]. For example, in prophase, CDK1-Cyclin B1 complex induces nuclear envelope breakdown[82] and chromosome condensation through phosphorylation of condensin (see above). CDK1 activates many kinases involved in the regulation of mitosis, such as PLK1, which is important for centrosome separation, bipolar spindle formation and kinetochore-microtubule attachments, Aurora A (controlling spindle formation and centrosome separation), or Greatwall kinase and NIMA kinase, ensuring full CDK1 activation through some feedback loops. CDK1-mediated phosphorylation also activates the Anaphase Promoting Complex/Cyclosome (APC/C) after all chromosomes are correctly aligned at the metaphase plate. In turn, APC/C triggers chromosome segregation and inactivation of CDK1 itself, thus ensuring mitotic exit[81].

1.3.1.2. PLK1

PLK1 (Polo-Like Kinase 1) plays a very important role in the regulation of mitosis. In late G2-early prophase it controls CDK1-Cyclin B1 activation by phosphorylation of Cyclin B1 and CDC25C[83,84]. At the same time, activation

of PLK1 itself depends on CDK1-dependent phosphorylation of PLK1 cofactor Bora[85], thus providing a feedback loop for timely and irreversible mitotic entry, and on Aurora A activity[86,87].

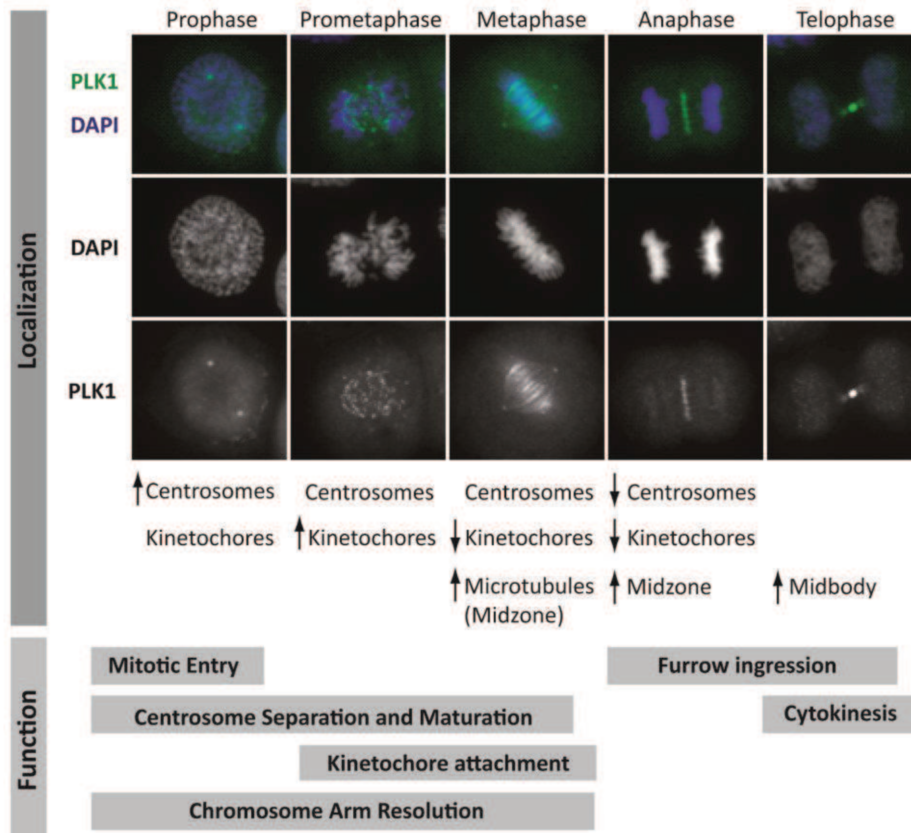

Fig.7. Localization and function of human PLK1 during mitosis. PLK1 is shown in green, DNA in blue. PLK1 localization to different mitotic structures is indicated by arrows (increased or decreased association) below immunofluorescence images. The main mitotic functions of PLK1 are highlighted below the corresponding mitotic phases (light grey bars) (adapted from[88])

Similar to Aurora B and other CPC components (see below), PLK1 regulates many mitotic processes, and this is achieved by a very specific and dynamic localization of PLK1 during different stages of mitosis (Fig.7).

PLK1 localizes to the centrosomes starting from G2 phase, where it contributes to the centrosome maturation through phosphorylation of

pericentrin and Ninein-Like Protein (Nlp)[89–91]. At the centrosomes, PLK1 is also involved in the phosphoactivation of centrosome separation regulators (Eg5, C-nap1 and Rootletin)[92,93]. After centrosome separation, PLK1 contributes to the nuclear envelope breakdown in prophase through phosphorylation of nucleoporins and p150glued dynactin subunit[94]. During prometaphase and metaphase, PLK1 localizes at the centrosome and kinetochores, where it takes part in the positioning of mitotic spindle, regulation ofkinetochore-microtubule attachments by phosphorylation of BubR1 and NUDC, and in the activation of APC/C[95–98]. Starting from anaphase, when chromosomes segregate, PLK1 relocalizes to the central spindle. Interaction of PLK1 with midzone microtubule bundles depends on Protein Regulator of Cytokinesis (PRC1), which crosslinks antiparallel microtubules[71]. PLK1 phosphorylates PRC1, thus mediating its own binding to PRC1[99]. During cytokinesis, PLK1 promotes the interaction of centralspindlin and Ect2 (Epithelial Cell Transforming sequence oncogene 2), which are important for central spindle maintenance and cytokinesis, as well as recruitment of Mklp2 and NudC (Nuclear Distribution gene C homolog) to the midzone[99–104]. After completion of mitosis, polyubiquitination of PLK1 results in its degradation by proteasome[105].

1.3.1.3. Aurora A

Aurora A is a member of Aurora kinase family, together with three other kinases (Aurora B and Aurora C). Aurora A contributes to many mitotic processes, including centrosome maturation and separation, bipolar spindle assembly, chromosome alignment and completion of cell division. After centrosome duplication in S phase, Aurora A is activated by Bora and NEDD9[106,107] and starts to localize at the centrosomes (Fig.8), where it helps to recruit centrosome maturation proteins (such as γ-tubulin, centrosomin and others)[108]. Together with centrosomal maturation, Aurora A was shown to be important for the activation and entry of CDK1-Cyclin B1 complex to the nucleus[110,111]. In complex with Bora, Aurora A activates PLK1 kinase in G2

phase, which subsequently promotes association of Aurora A with centrosome, phosphorylation of CDK activator CDC25B and BRCA1, thus resulting in mitotic entry. During mitosis, Aurora A migrates along mitotic spindle to the spindle midzone (Fig.8). Aurora A supports correct functioning of microtubule-organizing center (MTOC), formation of bipolar spindle (by phosphorylation of MCAK and CENP-E, and interactions with TPX2, Eg5 and other proteins), plays a role in kinetochore-microtubule attachment and chromosome congression (together with SAF-A and TPX2) and coordinates microtubule dynamics. Thus, coordinated actions of Aurora A with other mitotic regulators ensures normal mitotic progression[108,112].

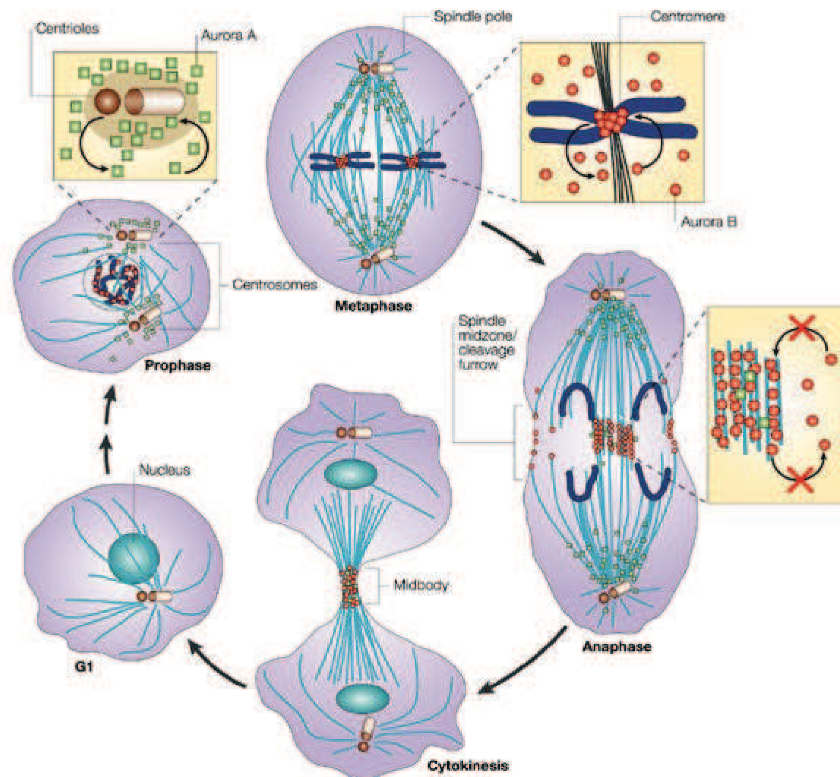

Fig.8. The relative localization of Aurora A and Aurora B in mitotic cells. Aurora A is shown as green square, Aurora B as red circles (adapted from[109])

1.3.1.4. Aurora B and Chromosomal Passenger Complex (CPC)

Aurora B kinase is one of the major mitotic regulators, orchestrating many pathways and processes during mitotic progression. Together with INner CENtromeric Protein (INCENP), Survivin and Borealin, it constitutes the Chromosomal Passenger Complex, or CPC (Fig.9)[113]. INCENP serves as a scaffold for assembly of CPC components into one complex, its N-terminus binds Borealin and Survivin, which is important for localization of the whole CPC complex and Aurora B, the enzymatic core of the complex, binds to the C-terminal IN box of INCENP[114–117]. Depletion or inhibition of any single component of CPC affects complex formation and leads to defects in microtubule-kinetochore attachments, chromosome congression and spindle

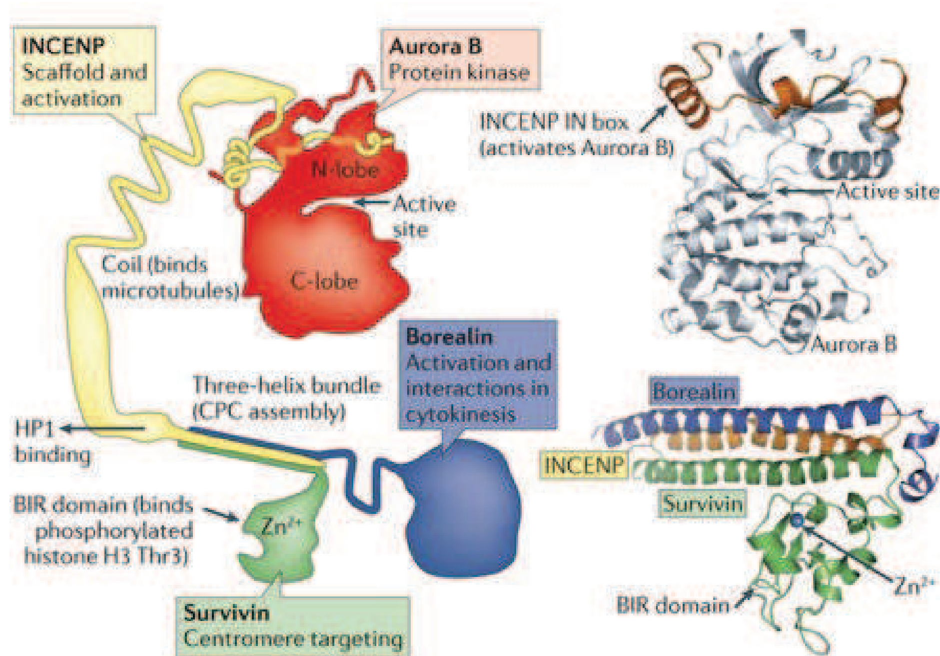

Fig.9. Schematic structure of the CPC. The domains and functions for each CPC component are indicated. The crystal structures of full length Aurora B in complex with the INCENP carboxyl terminus (amino acids 790–894) (top right) and of the three-helix bundle of INCENP (amino acids 1–58), borealin (amino acids 10–109) and full length survivin are shown (bottom right) (adapted from[113])

formation, and finally chromosome missegregation and cytokinesis defects[118]. The multifunctionality of Aurora B in complex with other CPC components is reflected by a very dynamic and specific localization during different cell cycle stages (Fig.8, Fig.10).

In the beginning of mitosis during prophase, Aurora B and other passenger proteins are localized on chromosomal arms, where they contribute together with CDK1 to the loss of cohesion induced by phosphorylation of Sororin[119,120]. Starting from prometaphase, and during metaphase, Aurora B and other CPC proteins are concentrated to the inner centromeric region. This localization, being very dynamic, is pivotal for regulation of kinetochore-microtubule attachments ("error correction") and establishing chromosome biorientation[121–125]. During metaphase-anaphase transition, CPC loses its

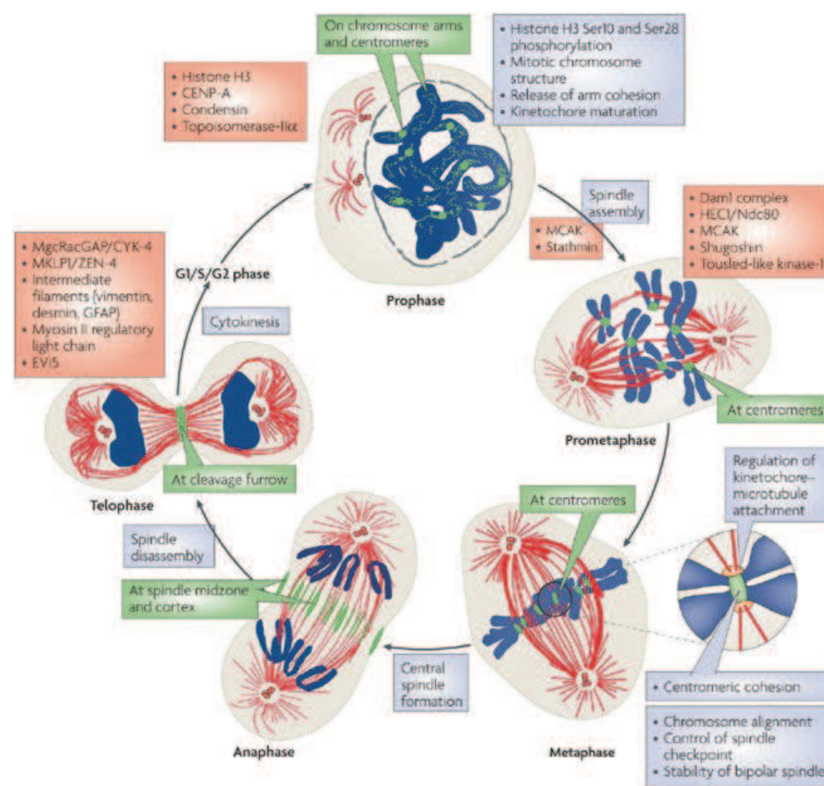

Fig.10. Schematic representation Aurora B localization. Aurora B localization (green) correlated with its multiple functions (grey boxes) and principal targets (red boxes) during the different phases of mitosis relative to tubulin and chromosome dynamics (adapted from [113])

affinity to the centromeric region, and relocalizes to the forming bundles of the central spindle microtubules, where it regulates midzone formation and cleavage furrow ingression[104,126–130]. During telophase, CPC is associated with the midbody, where it contributes to the process of abscission[131–133].Localization of the CPC components at the inner centromere depends on Borealin and Survivin. Borealin binds to the histone H2A-T120, phosphorylated by kinetochore associated kinase Bub1 and this interaction depends on Shugoshin proteins. Survivin, on the other hand, binds to the histone H3-T3, which is phosphorylated by kinase Haspin, localized in the centromeric chromatin[134–137]. These two phosphorylations overlap in the region of inner centromere, which leads to the specific recruitment of CPC components there. Aurora B itself participates in the several feedback mechanisms, which facilitate a very specific recruitment of the CPC to the inner centromere. On the one hand, Aurora B phosphorylates Haspin on multiple sites, leading to increase in Haspin-dependent phosphorylation of histone H3-T3 and thus enhanced centromeric CPC accumulation[138]. On the other hand, recruitment of Shugoshins to the centromere also depends on CPC components, as well as kinetochore localization of Bub1 and Mps1 depends on active Aurora B[135,139–144]. Finally, histone H3-S10 phosphorylation by Aurora B helps to extract CPC components from chromosome arms, thus enriching its localization on centromeres[145]. In addition, microtubules of mitotic spindle enrich centromere-associated Aurora B in prometaphase and metaphase[146]. Altogether, these feedback mechanisms ensure rapid and specific localization of Aurora B and other CPC components at centromeres, which is important for Aurora B-mediated correction of errors in kinetochore-microtubule attachments (see below).

Relocalization of the CPC from centromeres to the microtubules of central spindle takes place during metaphase to anaphase transition (Fig.10), when kinetochores are correctly attached to the spindle microtubules and CDK1 activity drops after Cyclin B1 degradation. This relocalization is a continuous and dynamic process, which requires CPC dissociation from centromeres, increasing microtubule binding affinity, active translocation to the

microtubules of the central spindle and targeting to the central spindle. Direct targeting (or holding) of CPC to the midzone microtubules depends on two main mechanisms, identified so far: MKLP2-dependent "fixing" of INCENP and Aurora B on the midzone microtubules and INCENP interaction with microtubules through its coiled-coil N-terminal domain[118,147–150]. Aurora B activity was also shown to be important for localizing of CPC in the midzone[130]. Moreover, ubiquitination of Aurora B was shown to play a pivotal role in CPC relocalization to the spindle midzone[151–154]. Cul3 E3 ligase in complex with adaptor proteins KLHL9/13 and KLHL21 monoubiquitinates Aurora B during transition to anaphase, and this modification leads to the translocation of CPC to the midzone, although the mechanism of this translocation, as well as its active participants, have remained unclear until now, and are subject of this study.

1.3.2. Regulation of kinetochore-microtubule attachments

During cell division, the genomic material duplicated in S-phase of the cell cycle should be precisely distributed between two daughter cells in mitosis, and the accuracy of this partitioning is a pivotal feature of faithful mitosis. Kinetochores and microtubules of mitotic spindle play a central role in the chromosome segregation, and interplay between these two structures should be precisely regulated. Such regulation involves controlling of kinetochore-microtubule interactions and attachments, kinetochore capture and transport, as well as establishment of chromosome biorientation.

1.3.2.1. Attachments of microtubules to kinetochores

After nuclear envelope breakdown in the late prophase/early prometaphase, microtubules of mitotic spindle gain access to kinetochores, and the cell starts to establish kinetochore-microtubule attachments (Fig.11). Dynamics of spindle microtubules allow them to capture kinetochores, which is described in the "search and capture" model (Fig.11): after nucleating at centrosome,

microtubules are elongating in different directions, shrinking when they do not encounter kinetochores, and stabilizing after attachment to kinetochores[156]. Another model of kinetochore capture depends on RanGTP gradient (Fig.11), which is high around chromosomes during mitosis. This gradient allows microtubules to grow in the direction of chromosomes, where it facilitates microtubule rescue[157] and at the same time activates release of spindle assembly factors from importin alpha and beta around chromosomes[155]. It has been shown that kinetochores themselves can also nucleate microtubules[158]. Such

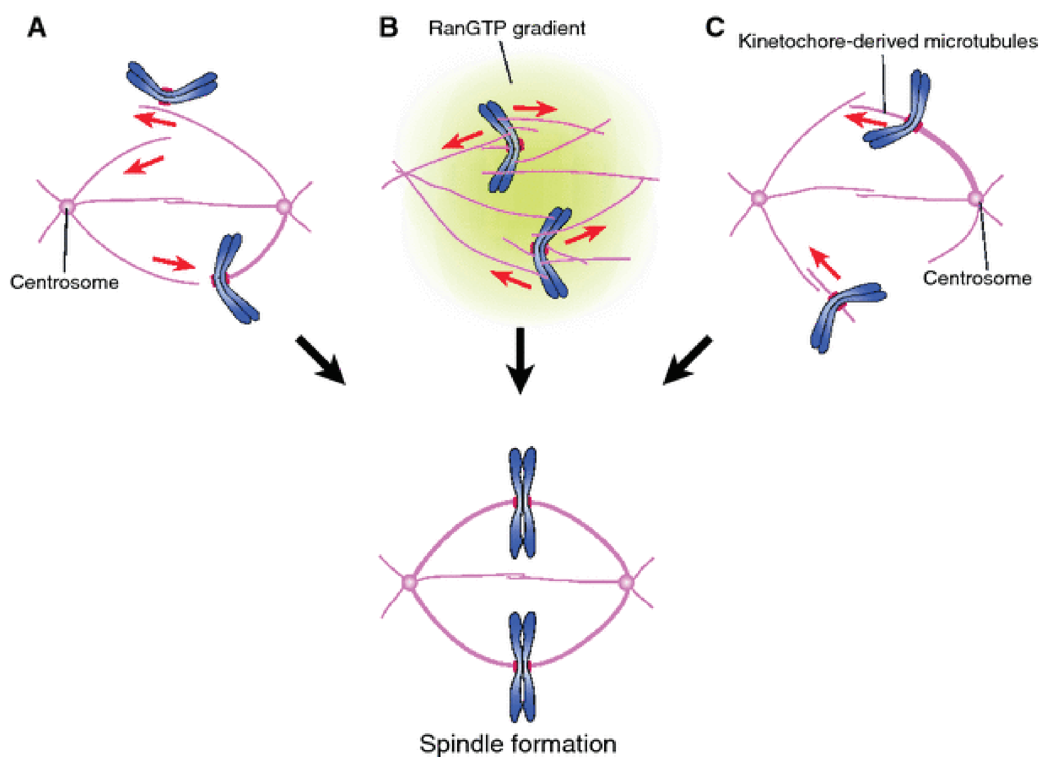

Fig.11. Mechanisms facilitating kinetochore-microtubule attachment. **A** - “search and capure” model, arrows indicate microtubule growth and shrinkage, **B** - RanGTP gradient around chromosomes promotes microtubule assembly around chromosomes, arrows indicate microtubule formation around chromosomes, **C** - kinetochore-derived microtubules facilitate efficient spindle formation, arrows indicate microtubule generation at kinetochores (adapted from[155])

kinetochore-derived microtubules form bundles with pole-derived microtubules and facilitate kinetochore-microtubule attachments (Fig.11).

Initial attachments of microtubules to kinetochores are lateral, thus providing more efficient kinetochore capture, as the contact surfaces are large in this case in comparison to microtubule ends. Some motor proteins are known to take part in such lateral attachments, such as "-" end directed motor dynein and KIFC1 and "+" end directed motor CENP-E, or KIF10[159–162]. KIF10 transports laterallyattached chromosomes to the "+" ends of microtubules, counteracting KIFC1 and dynein activities, which move chromosomes poleward. This process of lateral attachment and sliding is very dynamic, and Aurora B plays a role in the establishment of these attachments[163–165]. Unstable and dynamic lateral attachments are transformed into more stable end-on attachments, with Bub1 and Bub3 involved in this conversion[166,167]. It is important to note, that end-on attachments can be formed already initially or during K-fiber formation[155]. The KMN network, associated with kinetochores, was shown to be important for end-on attachments, as it directly binds to microtubules in Ndc80- and KNL1-dependent manner[168,169].

After kinetochore capture and establishment of end-on attachments, each chromosome is transported in a KIF10-dependent manner to the metaphase plate to establish biorientation, when both kinetochores are attached to microtubules emanating from opposite poles[160]. At this stage, errors in microtubule-kinetochore attachments can happen, leading to monotelic attachments, when only one kinetochore is attached to microtubules, or syntelic attachments, when both sister kinetochores are attached to microtubules from the same pole. Such errors in attachments should be corrected to allow proper chromosome segregation. The main player in the error correction is Aurora B[22,170–173]. Aurora B phosphorylates all three components of KMN network, KNL1, Mis12 complex and Ndc80, which leads to destabilization of incorrect kinetochore-microtubule attachments[174]. During prometaphase and metaphase, when Aurora B-mediated error correction takes place, Aurora B is localized in the centromeric region. This localization was shown to be very dynamic, with cycles of association-dissociation,

leading to a phosphorylation gradient of Aurora B, which decreases radially away from centromeres[175]. According to the tension-dependent model (which is also called "spatial separation model"), in case of mono- and syntelic attachments, the two sister kinetochores are physically not under tension, with a short distance between them (Fig.12). In this case, Aurora B is situated closer to KMN network, in comparison to amphitelic attachments (biorientation), when kinetochores are stretched and Aurora B gradient is not able to reach the substrates. As a result, kinetochore-microtubule attachments, which are not under tension, are destabilized by Aurora B through KMN network phosphorylation, kinetochores are released from attachments and re-orientation can be achieved. Protein phosphatases are known to counteract Aurora B phosphorylation, ensuring efficient dephosphorylation of KMN network. Another model suggests that upon tension, substrates of Aurora B change their conformation or become unavailable for Aurora B phosphorylation (Fig12)[176]. In this case, Aurora B is situated closer to KMN network, in comparison to amphitelic attachments (biorientation), when kinetochores are stretched and Aurora B gradient is not able to reach the substrates. As a result, kinetochore-microtubule attachments, which are not under tension, are destabilized by Aurora B through KMN network phosphorylation, kinetochores are released from attachments and re-orientation can be achieved. Protein phosphatases are known to counteract Aurora B phosphorylation, ensuring efficient dephosphorylation of KMN network. Another model suggests that upon tension, substrates of Aurora B change their conformation or become unavailable for Aurora B phosphorylation (Fig12)[176].

Dynamics of microtubules also contribute to establishment of correct kinetochore-microtubule attachments, as microtubule overstabilization will lead to maintenance of incorrect attachments. Microtubule dynamics is controlled by Kif2b in prometaphase and MCAK in metaphase. MCAK, a microtubule destabilizing protein in mammalian cells, is a substrate of Aurora B. Aurora B phosphorylation recruits MCAK to centromeres together with Sgo2[177–179], and reduces its microtubule destabilizing activity. Another

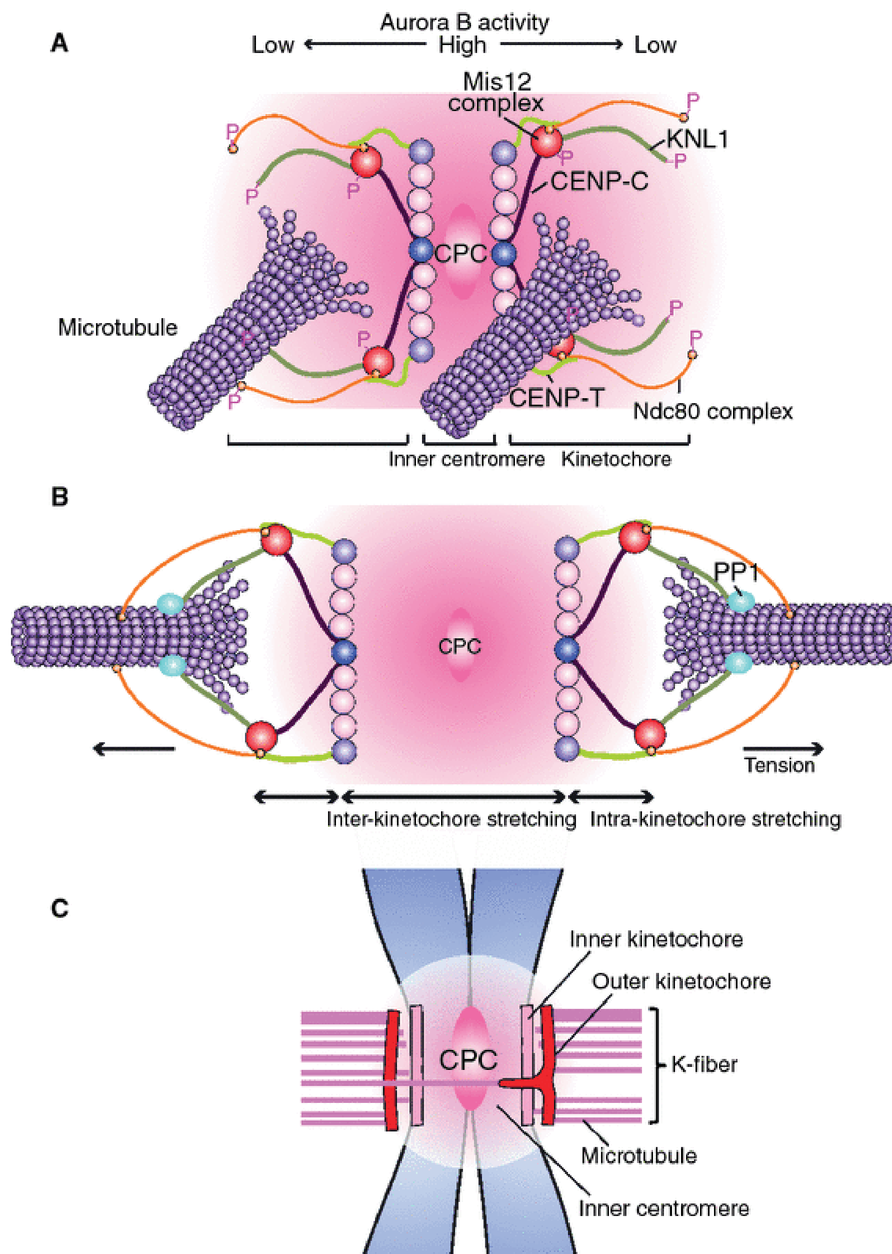

Fig.12. Schematic diagram depicting the "spatial separation model" of Aurora B-mediated correction of erroneous kinetochore-microtubule attachments. **A** –Upon syntelic attachment, sister kinetochores are not stretched due to the lack of tension. As a result, proximity of the kinetochores to the CPC makes the KMN network highly phosphorylated by Aurora B, and kinetochore-microtubule attachments are destabilized. **B** – Upon bi-orientation, kinetochores are stretched both internally and externally due to the tension exerted between them, the KMN network is under-phosphorylated and kinetochore-microtubule attachments are stabilized. Phosphatases take part in the shifting the balance to dephosphorylation of the KMN network. **C** – Merotelic attachments lead to the kinetochore stretching towards spindle poles. Therefore, a portion of the microtubule attachment sites on the kinetochore is closer to inner centromere, such attachment is destabilized by Aurora B (adapted from[155])

microtubule depolymerizing protein, Kif2b, is phosphorylated by PLK1 and becomes associated with outer kinetochore, where it destabilizes kinetochore-microtubule attachments[180]. Upon biorientation, Kif2b is displaced from kinetochore, and this stabilizes correct attachments. PLK1 phosphorylates many kinetochore substrates to regulate kinetochore-microtubule attachments, thus supporting and complementing Aurora B function[181,182]. Mps1 also supports Aurora B's function, targeting Bub1-Bub3 to KNL1, which is required for proper kinetochore-microtubule attachments[22] (see below). Another player in the establishment of chromosome biorientation is Nek2A kinase, which stabilizes attachments through phosphorylation of Ndc80[183]. Protein phosphatase PP2A-B56 provides a dynamic balance to phosphorylation by counteracting Aurora B and PLK1, and stabilizing kinetochore-microtubule attachments[184]. PP1 was also shown to have a role in biorientation, interacting with KIF10, KIF18a, KNL1 and Sds22[185–187].

1.3.2.2. Spindle Assembly Checkpoint

A special "proofreading network", called Spindle Assembly Checkpoint (SAC) ensures and controls proper chromosome segregation. SAC prevents the onset of anaphase in response to unattached kinetochores to the spindle microtubules[188]. When SAC is inactive, the Cohesin complex, which has been shown to hold sister chromatids together, is cleaved and cells can proceed to the segregation step even upon some errors in kinetochore-microtubule attachments, which in turn leads to developmental problems, aneuploidy and cancers[189–191].

SAC proteins form the Mitotic Checkpoint Complex (MCC), which inhibits ubiquitin-dependent degradation of the key cell cycle regulators such as Cyclin B and Securin by the Anaphase-Promoting Complex/Cyclosome (APC/C)[188,192]. Lack of kinetochore-microtubule attachments leads to the phosphorylation of Kinetochore Null Protein 1 (KNL1) by Ser/Thr kinase Monopolar Spindle Protein 1 (MPS1). Phosphorylated KNL1 leads to recruitment of BUB1, BUB3, BUBR1/Mad3 and RZZ complex, association of Mad1/Mad2 heterodimer with kinetochore, inhibitory binding of Mad2 to

APC/C activator Cdc20, which in turn binds Bub3, BubR1 (MCC complex assembly). Cdc20, included in the MCC, can no longer activate APC/C and this prevents chromosome segregation and mitotic exit (Fig.13)[193].

It has been shown recently that SAC generates a gradient response rather than a threshold-dependent switch-like response. For example, Collin et al[194] could show a correlation between the number of kinetochore Mad2 and SAC signalling as well as dependence of spindle-poisons induced mitotic arrest on the strength of SAC response. These findings were complemented by another recent publication[195], where the authors used a laser-based method to study

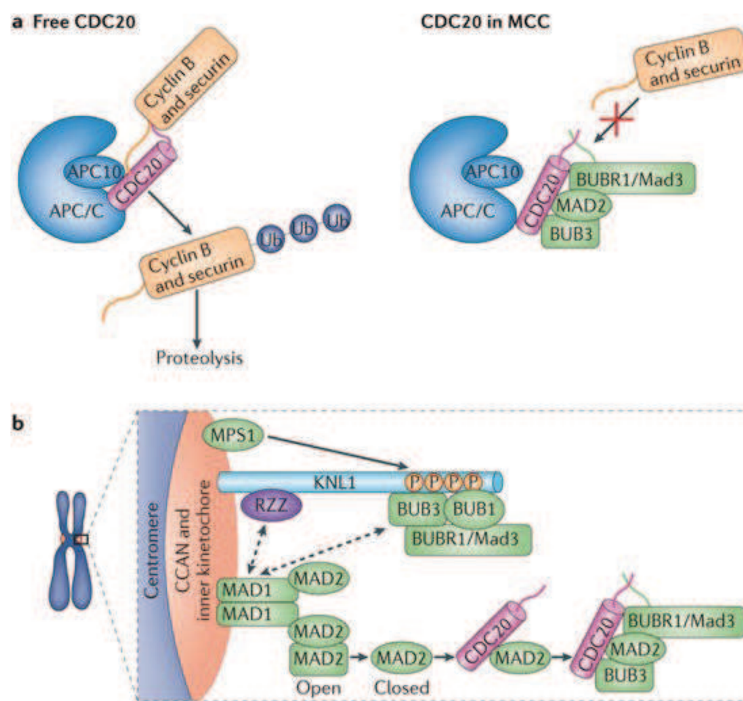

Fig.13. Chromosome segregation and exit from mitosis are triggered by the APC/C. A - CDC20, which is an activator of the APC/C, forms a docking site with APC10 that recognizes destruction box sequences (not shown) in cyclin B and securin. CDC20 promotes ubiquitylation (Ub) of these substrates and thus their degradation (left panel). SAC promotes the formation of a CDC20 inhibitory complex that is known as mitotic checkpoint complex, MCC, containing MAD2, BUB3 and BUBR1/Mad3 (right panel). In the MCC, CDC20 interaction with APC10 is disrupted, and BUBR1/Mad3 obscures degron recognition sites in CDC20. Together, these MCC-dependent processes prevent ubiquitylation of cyclin B and securin. B - Schematic of SAC activation at the kinetochore (adapted from[193])

SAC response in living cells. Together, these important findings provide a clear evidence for possibility to override the SAC during mitosis. This can be done is several ways, one of which is a detachment of chromosomes from spindle microtubules too close to metaphase-anaphase transition, when the cell is too limited in time to properly react on emerging problems with attachments[195]. Merotelic attachments (one kinetochore attached to both spindle poles) can also lead to SAC override as such attachments do not produce a checkpoint response and finally induce missegregation and lagging chromosomes[196].

1.3.3. Ubiquitination

Ubiquitination is another posttranslational modification that plays a pivotal role in the regulation of mitotic progression through controlling the degradation, localization and activity of important cell cycle regulators. Ubiquitination is a covalent attachment of a 8.5 kDa polypeptide ubiquitin to a substrate protein, which involves three enzymatic reactions: ubiquitin activation, ubiquitin conjugation and ubiquitin ligation. The first step, ubiquitin activation, includes ATP-dependent binding of ubiquitin to E1 ubiquitin activation enzyme. During the second step, ubiquitin is transferred to E2 conjugating enzyme. Finally, E3 ubiquitin ligase mediates binding of ubiquitin to the lysine residue of the substrate protein (Fig.14)[197]. Ubiquitin itself is known to serve as a substrate for binding of other ubiquitin molecules, as seven internal lysine residues can serve as docking sites (Lys6, Lys11, Lys27, Lys29, Lys33, Lys48, Lys63). As a result, a huge signaling network of different ubiquitin modifications can be built, which controls a variety of processes. For example, Lys48 and Lys11 polyubiquitination leads to proteasomal degradation of substrate proteins[199,200]. Lys63 polyubiquitination does not serve as a proteolytic signal, but defines the fate of substrate protein during stress response and DNA damage[201]. At the same time, monoubiquitination (when single ubiquitin is attached to a single site) and multiubiquitination (several single ubiquitins are attached to different

sites) are also involved in signaling pathways, regulating protein localization in endocytosis, DNA repair, transcription and mitosis[202].

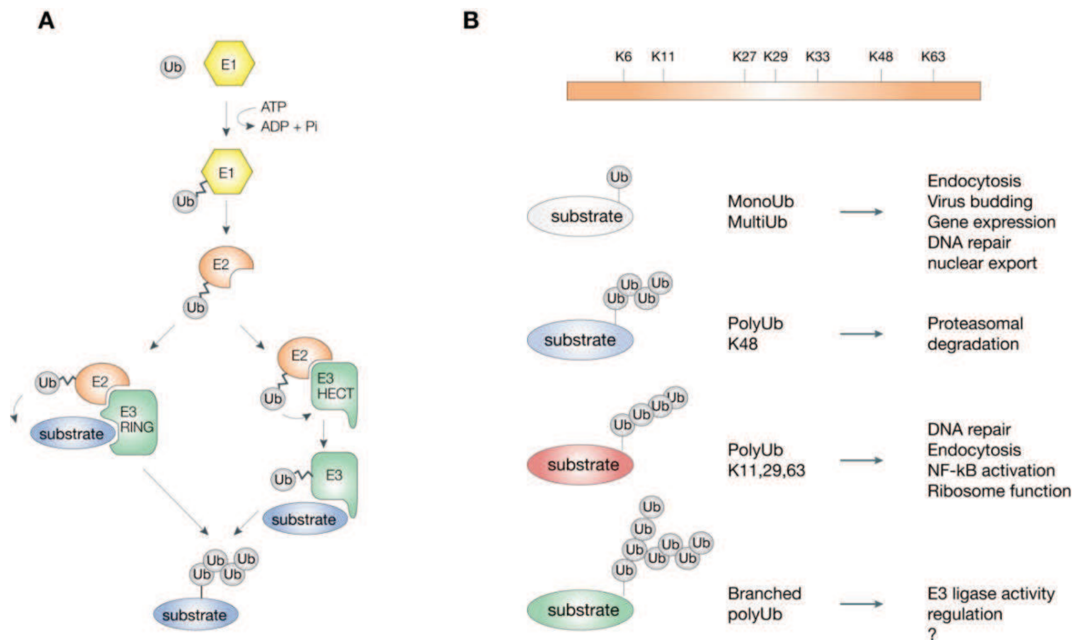

Fig.14. The ubiquitin pathway. **A** - Schematic representation of the ubiquitination process. A hierarchical set of three types of enzyme is required for substrate ubiquitination: ubiquitin-activating (E1), ubiquitin-conjugating (E2) and ubiquitin-protein ligase (E3) enzymes. The two major classes of E3 ligases are depicted. **B** - Schematic representation of the different Ub modifications with their functional roles. The question mark indicates that the functions of branched chains are largely unknown (adapted from[198])

1.3.3.1. E3 ubiquitin ligases in mitosis

More than 1000 E3 ubiquitin ligases are known to control the specificity of ubiquitin signaling in the cell[203]. I will focus on three major types of E3 ubiquitin ligases that are known to control mitotic progression: the SCF complex, APC/C and CUL3.

1.3.3.1.1. SCF complex

The SCF complex is a multisubunit E3 ligase complex, which regulates degradation of about 20% of proteins that are targeted by ubiquitin

proteasome system. It consists of a scaffold protein Cul1, the RING protein Rbx1 (which recruits E2 conjugating enzyme), adaptor protein Skp1 (S-phase kinase associated protein 1) which binds to F-box protein, the substrate binding subunit[204]. Distinct F-box proteins recognize different substrates, and more than 60 F-box proteins are encoded in the human genome, thus providing specificity for a variety of processes, controlled by SCF complexes.

During mitosis, SCF-betaTrpCP promotes ubiquitination and subsequent degradation of Emi1, which is an inhibitor of APC/C[205,206], thus promoting APC/C activation. Another target of SCFbetaTrCP, as well as SCFTome1, is a CDK1 inhibitor WEE1, ubiquitin-dependent degradation of WEE1 activates CDK1 and induces mitotic entry[207,208].

1.3.3.1.2. APC/C

Another E3 ubiquitin ligase involved in the mitotic regulation is APC/C (Anaphase Promoting Complex/Cyclosome). The APC/C is a very complex ubiquitin ligase, which consists of several blocks: catalytic module, tetrico-peptide repeats (Trps), scaffolding module and co-activator (Fig.15). The APC/C is classified as a RING-Cullin ubiquitin ligase, as its catalytic module consists of Apc2 subunit (cullin-like protein) and Apc11 (Zn+2-binding RING domain containing protein) (Fig.15)[210,211]. Interactions of APC/C with co-activators are mediated by TRP repeats proteins[212–215]. The scaffolding module optimizes the distance between catalytic block and TRP repeats protein, ensuring efficient catalysis. Coactivator proteins are important for APC/C activation, and their main function is to present substrates to the catalytic module of APC/C[216,217]. The best known coactivators of APC/C in mitosis are Cdc20 and Cdh1[218]. One of the main functions of APC/C in complex with Cdc20 is to induce metaphase to anaphase transition by ubiquitination of Cyclin B and Securin, which is an inhibitor of Separase. This ubiquitination leads to cleavage of cohesin complex on chromosomes and induces separation of sister chromatids[219–222]. After beginning of anaphase, APC/C in

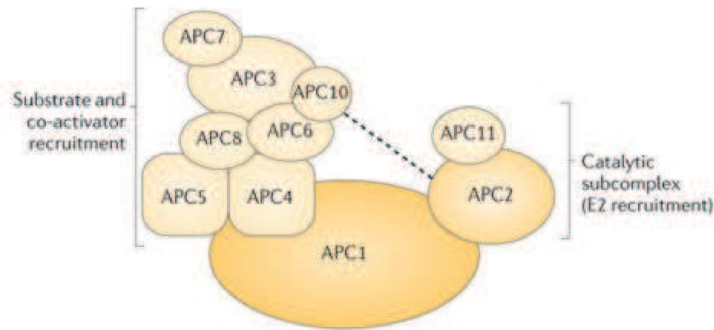

Fig.15. Scheme of the APC/C subunits. The APC/C consists of two subcomplexes that are connected by a scaffolding subunit (APC1). One subcomplex is catalytic and comprises APC2 and APC11 (which recruit the E2). The other subcomplex is composed TPR-containing proteins (APC3, APC6, APC7 and APC8) and binds substrates, APC10 and the co-activator (CDC20 or CDH1) (adapted from[209])

complex with another coactivator, Cdh1, induces ubiquitin-dependent degradation of Cdc20[223–225]. APC/C-Cdh1 promotes mitotic exit by targeting CyclinB, CyclinA, Aurora B, PLK1 and other substrates for degradation[226–233].

Localization of mammalian APC/C is very dynamic: it is associated with centrosomes and mitotic spindle, and the phosphorylated (active) form of APC/C is localized on centrosomes[234,235].

1.3.3.1.3. CUL3

CUL3 serves as a scaffold for a variety of RING-Cullin E3 ubiquitin ligases, which control many cellular processes. In these complexes CUL3 was shown to interact with substrate-binding adaptor BTB/POZ domain proteins (Bric-a-brac, Tramrack and Broad Complex/POx virus and Zinc finger)[236–238]. These substrate-specific adaptors bind Cul3 via BTB domains, and at the same time serve as a linker to mediate interaction with a substrate (for example, through a MATH domain in Cul3-Mel26 E3 ubiquitin ligase) (Fig.16).

One of the key mechanisms to modulate activity of CUL3 E3 ubiquitin ligases is the dynamic covalent modification of CUL3 by neddylation (attachment of ubiquitin-like protein Nedd8/Rub1)[241,242]. This modification activates CUL3 and facilitates recruitment and correct positioning of E2 ubiquitin conjugating

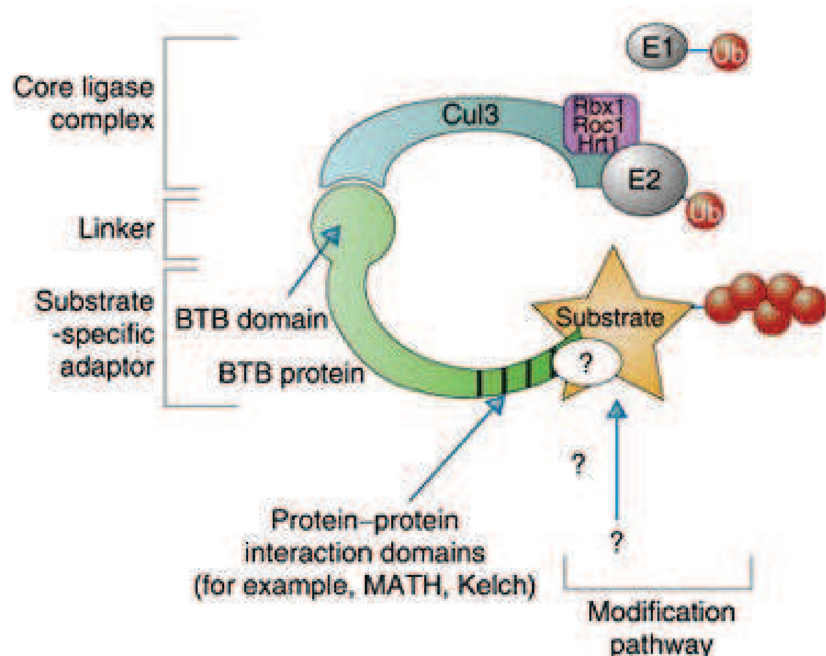

Fig.16. Structural organization of RING-Cullin E3 complexes. The catalytic core module consists of the scaffold protein CUL3 and the RING finger protein Rbx1. Single-subunit BTB domain proteins connect CUL3-Rbx1 to the substrate proteins. Substrate recognition is provided by an independent protein-protein interaction domain found in most of the CUL3-interacting BTB domain proteins (adapted from[239,240])

enzymes to the substrate protein[243,244]. The opposite pathway- deneddylation of CUL3- leads to the complex dissociation and CUL3 inhibition[245].

Another type of regulation of CUL3 activity is its dimerization and oligomerization through BTB domains of the substrate recognizing modules. Dimerization enhances substrate recruitment and facilitates the speed and processivity of ubiquitin chain formation[246,247].

CUL3 has an important role in the regulation of mitotic progression in mammalian cells. During mitosis, BTB domains of BTB-Kelch domain containing proteins interact with CUL3, at the same time the Kelch domains serve as adaptors for interaction with mitotic kinases, such Aurora B, PLK1 and Aurora A. One of the main functions of CUL3 in complex with Kelch BTB

proteins KLHL9 and KLHL13 is to ubiquitinate a key mitotic kinase Aurora B on prometaphase chromosomes, which leads to its extraction from mitotic chromosomes and further relocalization to the spindle midzone[154]. Another Kelch-BTB protein, KLHL21, was shown to monoubiquitinate Aurora B on the microtubules of the central spindle, ensuring correct progression of cytokinesis[151]. The difference in adaptor proteins, both mediating ubiquitination of Aurora B, provides an evidence for a tightly balanced ubiquitin-dependent regulation of Aurora B in a specific place during specific mitotic stages.

CUL3 in complex with KLHL22 monoubiquitinates PLK1 on kinetochores to remove it from there after bipolar kinetochore-microtubule attachments are established in metaphase[248]. Thus, PLK1 ubiquitination by CUL3 regulates chromosome alignment, satisfaction of SAC and correct chromosome segregation.

In addition, CUL3 in complex with KHLH18 was shown to be important for mitotic entry. CUL3-KHLH18 ubiquitinates and activates centrosomal Aurora A, which is required for the entry into mitosis[249].

1.3.3.2. Role of UBPs and DUBs in mitosis

Published manuscript (review): "Decoding Ubiquitin for Mitosis"

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Decoding Ubiquitin for Mitosis

Sadek Fournane, Ksenia Krupina, Charlotte Kleiss, and Izabela Sumara

Abstract

Conjugation of ubiquitin (ubiquitination) to substrate proteins is a widespread modification that ensures fidelity of many cellular processes. During mitosis, different dynamic morphological transitions have to be coordinated in a temporal and spatial manner to allow for precise partitioning of the genetic material into two daughter cells, and ubiquitination of key mitotic factors is believed to provide both directionality and fidelity to this process. While directionality can be achieved by a proteolytic type of ubiquitination signal, the fidelity is often determined by various types of ubiquitin conjugation that does not target substrates for proteolysis by the proteasome. An additional level of complexity is provided by various ubiquitin-interacting proteins that act downstream of the ubiquitinated substrate and can serve as "decoders" for the ubiquitin signal. They may, specifically reverse ubiquitin attachment (deubiquitinating enzymes, DUBs) or, act as a receptor for transfer of the ubiquitinated substrate toward downstream signaling components and/or subcellular compartments (ubiquitin-binding proteins, UBPs). In this review, we aim at summarizing the knowledge and emerging concepts about the role of ubiquitin decoders, DUBs, and UBPs that contribute to faithful regulation of mitotic division.

Keywords: mitosis, ubiquitin, DUBs, UBPs

Conjugating and "Decoding" Ubiquitin

Conjugation of ubiquitin is one of the major posttranslational modifications of proteins in eukaryotic cell. Highly dynamic and reversible, ubiquitination modulates and orchestrates a broad range of cellular processes, including protein degradation, quality control and trafficking, signal transduction, differentiation, and cell division.[1-4] In this pathway, ubiquitin is covalently attached to a substrate by coordinated cycles of 3 enzymatic reactions, ubiquitin activation (E1 enzyme), ubiquitin conjugation (E2 enzyme), and ubiquitin ligation (E3 ubiquitin ligase) (Fig. 1). One of the mostly described consequences of ubiquitination is degradation of the substrate proteins by a large protease complex, 26S proteasome. Due to its irreversible character, this process provides essential directionality toward various pathways. Importantly, ubiquitination of the targeted substrates does not always serve as a signal for the proteasomal degradation but may also regulate protein localization, binding to other proteins, or even enzymatic activities.[5] The covalent linkage of single ubiquitins (monoubiquitination or multi-ubiquitination) to the substrates or various modes of ubiquitin binding to the already attached ubiquitin moiety (polyubiquitin chain formation) determine the fate of the substrate protein (Fig. 1). A huge variation of the polyubiquitin chains can be formed, as one of the 7 internal lysine residues or the N-terminal methionine can serve as attachment sites in a single ubiquitin molecule. This creates a plethora of possible ubiquitin signals.

An additional level of complexity is provided by various ubiquitin-interacting proteins that act downstream of the ubiquitinated substrate and can serve as "decoders" for the ubiquitin signal. They may, specifically reverse ubiquitin attachment (deubiquitinating enzymes, DUBs) or, act as a receptor for transfer of the ubiquitinated substrate toward downstream signaling components and/or subcellular compartments (ubiquitin-binding proteins, UBPs) (Fig. 1). Eukaryotic genomes encode for high numbers of both ubiquitin conjugation machinery and "decoders" of ubiquitin signal, constituting a network which by analogy to the signal transduction pathways should ensure a high complexity regulation of cellular processes. It is predicted that human cells express about 600 different E3-ubiquitin ligases, 100 DUBs, and as many as 200 various UPBs. Yet, the precise roles and substrate specificity of these factors are only beginning to be understood. In this review, we aim at summarizing the recently emerging concepts and knowledge about the role of ubiquitin decoders during regulation of mitosis. Excellent existing reviews have described the roles of the E3-ubiquitin ligases known to act during mitotic division,[6-8] and likewise the role of ubiquitin coding and decoding machinery in regulation of other cell cycle stages has been extensively described.[9-13] Thanks to the high-throughput, whole genome screening efforts in mammalian cells and genetic studies in yeast, we have started to unravel novel mitotic factors that decode versatile ubiquitin signal.

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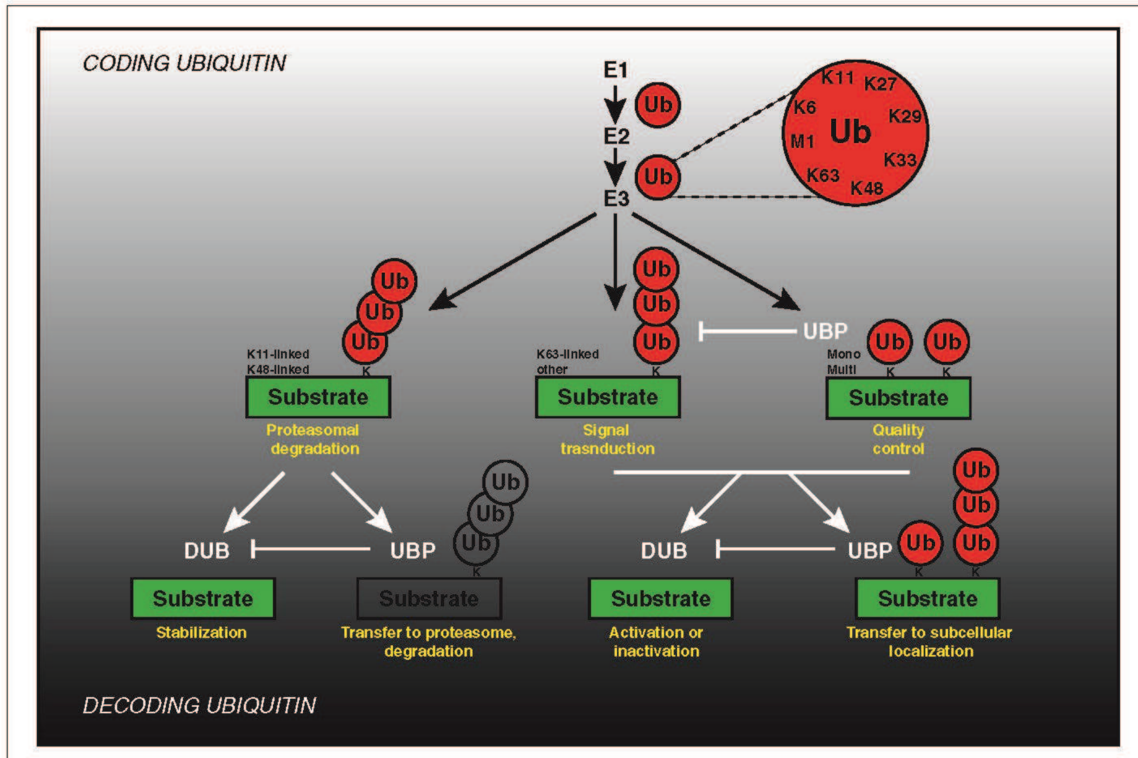

Figure 1. Coding and decoding ubiquitin. Coding: Ubiquitin (Ub) is covalently attached to the lysine residues (K) of substrate proteins by a 3-step mechanism involving the sequential actions of E1 (ubiquitin-activating enzyme), E2 (ubiquitin-conjugating enzyme), and E3 (ubiquitin ligase) enzymes, resulting in formation of both mono- and multi-ubiquitination signal as well as formation of the poly-ubiquitin chains linked via one of the internal lysine residues or N-terminal methionine residue. The possible physiological outcomes of different ubiquitination signals are indicated in yellow. Decoding: Ubiquitin modifications may be removed by specific de-ubiquitinating enzymes (DUB) and may stabilize substrates or lead to their activation or inactivation (yellow). Ubiquitin-binding proteins (UBP) interact with ubiquitinated proteins and may prevent the conversion of mono-ubiquitin into polyubiquitin chains, protect ubiquitin modifications from DUBs, target proteins to the 26S proteasome, and/or mediate downstream signaling events perhaps through new protein–protein interactions or targeting them to subcellular compartments (yellow).

Mitosis

During the mammalian cell cycle, genetic material has to be duplicated and then undergo mitosis, in which the two copies of each chromosome are segregated into two daughter cells (Fig. 2). Each of the daughter cells must receive an exact copy of the genetic material, as defects in chromosome segregation can cause genetic instability and aneuploidy, which has been linked to tumorigenesis.[14] Thus, for successful mitosis, a precise coordination of the morphological changes with time has to be accomplished. Onset of mitosis is typically marked by the nuclear envelope breakdown and condensation of the replicated DNA during prophase. The individualized chromosomes assemble their kinetochores during prometaphase and metaphase stages. The assembly of the mitotic spindle and its attachment to the sister kinetochores allows proper chromosome segregation. Accurate chromosome segregation requires that sister kinetochores attach to microtubules from opposite spindle poles (bipolar attachment) in metaphase. Kinetochore attachment is a stochastic process and as such is prone to errors, which may result in chromosome misalignment. A complex network of regulatory factors constituting so-called spindle assembly checkpoint (SAC) ensures that sister chromatid segregation in anaphase does not take place before all chromosomes are properly aligned at the metaphase plate and before their kinetochores are under sufficient occupancy and tension by spindle microtubules.[15-18] Following successful chromosome segregation, the spindle microtubules undergo a dramatic reorganization, and the anaphase central spindle or spindle midzone is formed. During this transition, the antiparallel,

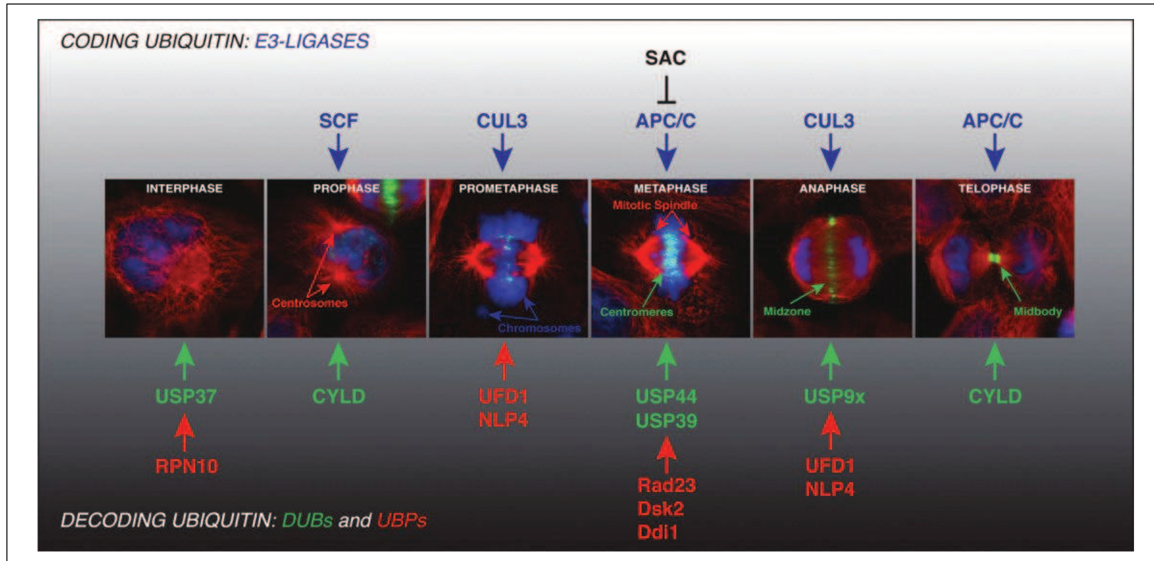

Figure 2. Regulation of mitosis by ubiquitin system. Faithful regulation of mammalian mitotic division (prophase, prometaphase, metaphase, anaphase, and telophase) requires precise coordination of structural transitions. This machinery involves many critical factors and enzymes that need to be precisely regulated in time and space. Thus, both their timely expression and correct localization to the subcellular compartments must be achieved during different morphological transitions. Blue indicates nucleus and chromosomes; green (representing the CPC component, Aurora B) marks the centromere structures during prometa- and metaphases and midzone and midbody regions during anaphase and telophase, respectively; and red marks microtubules and spindle structures. The critical and major ubiquitin E3-ligases (blue) responsible for coding ubiquitin on mitotic factors are depicted. APC/C E3-ligase is a subject of fidelity control by the spindle assembly checkpoint (SAC) network. The ubiquitin decoders, DUBs (green) and UBPs (red), contribute to fidelity and directionality of mitotic division at specific stages and transitions (for details, see text).

interdigitating microtubules and many associated proteins become organized into discrete bundles in the spindle midzone. Midzone microtubules play an important role in cytokinesis, which separates the cytoplasm of the two daughter cells.[19-21] Cytokinesis begins with the assembly of a contractile actin-myosin ring. Its contraction results in the formation of the midbody, which is composed of the remnants of the spindle midzone. In the final step, the actomyosin ring disassembles and the plasma membranes resolve in a process called abscission.[22] An accepted view is that cytokinesis cannot be completed if chromosomes are pulled apart erroneously or if the anaphase spindle midzone is not formed properly.[23] In budding yeast, these defects lead to the activation of the recently described "NoCut" checkpoint pathway.[24] In mammalian cells, abscission also fails in such cases, leading to regression of the cleavage furrow and the formation of multinucleated cells.[19-21]

Two major E3-ligases are known to control cell division: SCF-complexes regulate the G1 to S-phase transition, and the anaphase-promoting complex/cyclosome (APC/C) coordinates metaphase to anaphase transition and mitotic exit. However, recent findings hint at a more complex picture of regulation of this process, and accumulating evidence suggests a role of SCF ligases in controlling the early mitotic phases.[25] Likewise some novel cullin-based E3s, like CUL3, essentially contribute to regulation of mitosis[26,27] (Fig. 2).

Role of DUBs During Mitotic Progression

The human genome encodes for about 100 different DUBs, which belong to 5 distinct families. Four of them—USPs (ubiquitin-specific proteases), UCHs (ubiquitin C-terminal hydrolases), OTUs (ovarian tumor), and Josephin family—are cysteine proteases, whereas the fifth DUB family comprises a group of Zn^{2+} metalloproteases, which are referred to as JAB1/MPN/MOV34 (or JAMMs).[28-30] In addition to DUBs acting mostly on substrates modified by ubiquitin, specific enzymes exist that act on other ubiquitin-like molecules (UBLs), but their roles are poorly understood thus far.[31] DUBs have been implicated in many physiological and pathophysiological processes, including apoptosis, DNA repair, neuro degenerative diseases, and cancer, but their precise substrates and regulation are known only in some cases. Here, we summarize the DUBs with known functions during the cell division cycle (Table 1) and use examples of DUBs required specifically for mitosis to illustrate complexity of their regulation.

Table 1. List of DUBs Regulating Cell Cycle

Name	Family/*Domains*	Cell Cycle Stage	Function	Substrate(s)	References
USP13	USP/*ZnF-UBP, UBA, USP*	G1	Together with Ufd1, couples the endoplasmic reticulum stress response to cell cycle control	Skp2	129
BAP1	UCH/*UCH, coiled coil*	G1/S	Promotes cell cycle progression by interacting with HCF-1, a transcriptional cofactor for genes required for S phase	HCF-1	130
USP1	USP/*USP*	S	Switches off DNA damage pathways, limits the error-prone replication in absence or presence of extrinsic DNA damage	FANCD2-FANCI, PCNA	131-134
USP3	USP/*ZnF-UBP, USP*	S	Necessary for proper progression through S phase, involved in DNA damage response and prevents replicative stress	H2A (major substrate), H2B	135
BRCC36	JAMM/*JAMM, coiled coil*	DNA damage checkpoint	Allows the turnover of BRCA1-mediated repair in DNA damage response	H2A, H2AX	136, 137
OTUB1	OTU/OTU	DNA damage checkpoint	Noncanonical inhibition of DNA damage response (by blocking ubiquitin transfer)	UBC13 (interacting protein)	67, 138
USP28	USP/*UBA, UIM, USP, coiled coil*	DNA damage checkpoint	Regulates the stability of DNA-damage signaling factors, controls stability of c-Myc	Chk2, 53BP1, Claspin, c-Myc	139, 140
USP7	USP/*MATH, USP, UBL*	G1/S and G2/M	Regulates the p53-MDM2 pathway	MDM2, p53	141, 142
USP37	USP/*UIM, USP*	G1/S, G2/M	Promotes the G1/S transition by inhibiting the APC/C-dependent degradation of Cyclin A, regulation of unknown substrate necessary for the prevention of mitotic entry	Cyclin A, itself, other unknown substrate	79, 84
USP42	USP/*USP*	G1/S and G2/M	Necessary for early activation of p53 response	p53	143
USP2a	USP/*USP*	G1/S and G2/M	Regulates the p53-MDM2 pathway	MDM2	144
USP50	USP/*USP (predicted to be inactive)*	G2/M	Regulates the HSP90-dependent stability of Wee1 (unknown mechanism)	Not known	145
CYLD	USP/*CAP-Gly domains, USP with B-box*	M, G1/S	Regulates mitotic entry, assembly of the mitotic spindle, cytokinesis rate after TPA treatment, negatively regulates G1/S progression after TPA treatment	PLK1 (?), Bcl3	43, 58, 59
USP16	USP/*ZnF-UBP, USP*	M	Necessary for M phase progression, associated with reversal of DNA silencing after DNA damage response	H2A	146, 147
USP44	USP/*ZnF-UBP, USP*	M	Regulation of spindle assembly checkpoint (SAC)	CDC20	36, 37
USP4	USP/*DUSP, UBL, USP*	M	Regulation of spindle assembly checkpoint (SAC)	Splicing of α-tubulin and Bub1 mRNA, other mRNAs important for SAC (?)	70
USP39	USP/*ZnF-UBP, USP (inactive)*	M	Regulation of spindle assembly checkpoint (SAC)	Splicing of Aurora B mRNA, other mRNAs important for SAC (?)	62
USP9x	USP/*UBL, USP*	M	Regulation of chromosome alignment and segregation by controlling the dynamic dissociation of Survivin from centromeres	Survivin	125
UBPY	USP, *MIT, Rhodanese, USP*	M	Deubiquitination of proteins (including VAMP8) during cytokinesis	VAMP8, other substrates	148
AMSH	JAMM/MPN+, *MIT*	M	Deubiquitination of proteins (including VAMP8) during cytokinesis	VAMP8, other substrates	148

The table depicts DUBs known to be involved in cell cycle progression at different cell cycle stages (S [S phase], G1, G2, and M [mitosis]) and transitions. The role and regulation of mitotic DUBs are described in detail in the text. The domain composition and specific substrates of the DUBs are depicted. [?]indicates unconfirmed speculation.

Recent mass spectrometry experiments suggest that as many as 5,000 of ubiquitinated substrate proteins may exist in human cells, with 19,000 possible modification sites.[32] Thus, evolutionary pressure could exist that allowed for expansion of the DUB family, ensuring a high level of specificity of these enzymes. Indeed, inactivation of many DUBs leads to very specific phenotypes in cells and organisms, which could arise from the ability of these enzymes to recognize and bind specifically to the diverse ubiquitination signals (Fig. 1).

DUBs Reversing Nonproteolytic Ubiquitination Signal

USP44. The SAC ensures that mitosis does not go beyond metaphase until all chromosomal kinetochores are correctly attached to spindle microtubules, allowing for faithful chromosome segregation and partition of genetic information between the two daughter cells. Both occupancy of kinetochores by the spindle microtubules and tension generated by bipolar attachment between sister kinetochores are sensed by the SAC.[18] Activated SAC inhibits the APC/C E3-ubiquitin ligase by sequestering its major mitotic co-activator, CDC20 protein.[33,34] This is achieved by formation of the mitotic checkpoint complex (MCC) composed of the main effector SAC protein, MAD2, along with BubR1, Bub3, and CDC20.[35] Binding of MAD2 to CDC20 occurs at unattached kinetochores, but many other regulatory checkpoint components are required to ensure and strengthen the inhibitory effect of the SAC. Attachment of all kinetochores releases SAC inhibition, allowing APC/C^{CDC20} to target Securin (inhibitor of sister chromatid separation) and Cyclin B proteins for proteolytic ubiquitination, leading to the anaphase onset and mitotic exit. Stegmeier et al.,[36] aiming to identify novel components of SAC in human cells, screened a shRNA library targeting approximately 800 genes related to ubiquitin-signaling for their ability to bypass the spindle checkpoint arrest induced by microtubule poison paclitaxel. One of the identified hits was the DUB, USP44. Indeed, co-transfection of the USP44 siRNA and the siRNA-resistant construct of wild-type USP44 (but not catalytically inactive enzyme) results in rescue of the spindle checkpoint defect. Moreover, inactivation of USP44 in untreated cells leads to premature anaphase onset and chromosomal segregation defects, suggesting that deubiquitinating activity of USP44 is required for efficient SAC signaling and anaphase timing. Intriguingly, SAC defect observed in USP44-depleted cells was not caused by a defect in kinetochore recruitment of checkpoint components. Instead, binding of MAD2 to APC/C^{CDC20} was perturbed in these cells, suggesting that USP44 maintains the mitotic arrest by stabilizing the association between MAD2 and CDC20. Interestingly, in another parallel study, APC/C-dependent, nonproteolytic ubiquitination of CDC20 has been shown to be responsible for the disassembly of the MAD2-CDC20 complexes.[37] This CDC20 ubiquitination event is mediated by the UbcH10 E2-conjugating enzyme and is required for activation of the APC/C. Consistent with these findings, Stegmeier et *al.* have shown that in mitotic extracts, USP44 inhibits the ubiquitination activity of APC/C. Consequently, inactivation of UbcH10 in USP44-depleted cells leads to reduction of ubiquitinated CDC20, restoration of MAD2-CDC20 association, and rescue of the mitotic checkpoint arrest. These results suggest that USP44 acts as an antagonist of UbcH10 toward APC/C's own-dependent ubiquitination of CDC20. Thus, the SAC is regulated through a dynamic balance of APC/C-dependent nonproteolytic ubiquitination and USP44-dependent deubiquitination. During final stages of metaphase, when not all chromosomal kinetochores are occupied by spindle microtubules, the SAC is activated through a USP44-dependent "safeguard" mechanism, which by deubiquitinating CDC20 stabilizes MAD2-CDC20 complexes. Thus, the SAC is built on two antagonistic pathways, which can switch rapidly from one to the other, sensing the occupancy state of microtubules on kinetochores.

How is this fine balance between ubiquitination and deubiquitination of CDC20 regulated? It has been shown that USP44 protein levels peak in mitotic cells and are decreased immediately after chromosome attachment to mitotic spindles.[36] Moreover, USP44 is also phosphorylated in early mitosis and immediately dephosphorylated after SAC satisfaction but before degradation of Cyclin B. The RNA polymerase II carboxy terminal domain phosphatase, Fcp1, has been shown to mediate dephosphorylation of USP44.[38] Fcp1 is a transcription regulator, and it has been shown to be an antagonist to CDK1 in *Aspergillus nidulans*,[39] where expression of a defective Fcp1 allele together with an inhibitory phosphorylation-resistant CDK1 allele induced severe mitotic defects including impaired nuclear separation. Mitotic extracts have been used to show that the phosphorylation status of USP44 modulates its activity: Dephosphorylation by Fcp1 leads to the reduction of USP44 activity, which is associated with a dramatic decrease of the MAD2-CDC20 association and mitotic exit. Thus, the phosphorylation status of USP44 may govern the dynamic balance between ubiquitination and deubiquitination of CDC20.

The model by Stegmeier et al.[36] of the mitotic checkpoint regulation by antagonistic ubiquitination and deubiquitination has been challenged by another study.[40] According to this work, in checkpoint-activated HeLa cells, CDC20 is ubiquitinated by APC/C, leading to its proteasomal degradation. Indeed, ubiquitination-dependent degradation of yeast Cdc20 homolog during activated checkpoint has been demonstrated in *Saccharomyces cerevisae*.[41] Moreover, a CDC20 lysine-less mutant allows the inhibition of the mitotic checkpoint by promoting the mitotic exit, which is more consistent with the model in which ubiquitination-dependent degradation of CDC20 maintains the SAC.[40] This mitotic exit does not happen immediately, as it has been shown in checkpoint-arrested cells expressing the CDC20 lysine-less

mutant, as these cells have an active SAC during at least 2 hours, suggesting that additional ubiquitin-independent mechanism is required to sustain the checkpoint arrest. Reddy et *al.*[37] have also observed a partial proteolysis of CDC20 upon addition of UbcH10 and ubiquitin on checkpoint-arrested extracts; however, this proteolysis event does not regulate stability of the MCC complex. The other point is that the CDC20 lysine-less mutant binds more strongly to the checkpoint proteins MAD2 and BubR1 than the wild-type CDC20 in checkpoint-arrested cells, suggesting that CDC20 ubiquitination could modulate the binding to MAD2 and BubR1 as suggested by the model of Stegmeier et *al*. However, Nilsson et *al.*[40] argued that this strong association between the CDC20 lysine-less variant, MAD2, and BubR1 is due to an increased affinity upon the exchange of a conserved lysine into the arginine residue in the MAD2 binding site of CDC20. In principle, two mechanisms of maintaining the SAC signaling are not mutually exclusive and could work at the same time: CDC20 associated with the MCC components could be ubiquitinated by APC/C, allowing its dissociation from the MCC, and it would be degraded subsequently by the proteasome. In such a scenario, CDC20 should be progressively modified by two different types of ubiquitin signal: first a nonproteolytic and then proteolytic one. The action of USP44 would be required to "proofread" ubiquitination status of CDC20 and prevent assembly of inappropriate ubiquitin chains. Thus, future studies need to clarify the type of ubiquitin signals present on CDC20 during the precise time periods of SAC response. Interestingly, overexpression of USP44 in mouse embryonic cells has been shown to increase the association between MAD2 and CDC20 and to lead to an anaphase delay consistent with role of USP44 in the regulation of SAC.[42]

CYLD. The same shRNA-based screen that led to identification of USP44 revealed the role of another DUB, CYLD, as a regulator of early mitotic phases.[43] Mutations in the tumor suppressor gene *CYLD* cause genetic predisposition to human familial cylindromatosis,[44] and CYLD deubiquitinating enzyme was shown to negatively regulate NFκB, JNK,[45-47] and Wnt signaling pathways.[48] Some *in vitro* studies have suggested that CYLD is able to cleave specifically K63-linked and linear ubiquitin chains.[49] In the study by Stegmeier *et al.*,[43] CYLD knockdown led to accumulation of nonmitotic cells after paclitaxel treatment. These cells had a normal interphase nuclear morphology rather reflecting a delay in the mitotic entry process. This phenotype was independent of the regulatory function of CYLD in the NFκB pathway. The premitotic arrest phenotype is rescued by the expression of wild-type CYLD but not an inactive CYLD mutant, suggesting that deubiquitinating activity of CYLD is required for its early mitotic function. Consistently, CYLD-depleted cells are characterized by delayed accumulation of Serine 10-phosphorylated histone H3, delay in phosphorylation of CDC25C, and delayed degradation of Emi1. Intriguingly, downregulation of the mitotic kinase, Polo-like kinase 1 (PLK1), led to the same phenotypes and delayed both CDC25C phosphorylation and mitotic entry.[50-52] Interestingly, PLK1 has been identified as a binding protein of CYLD using proteomic approaches, suggesting a cooperative role of both proteins in the early mitotic pathway. To give a functional explanation for the delayed entry into mitosis in CYLD-downregulated cells, the authors argued that CYLD could be involved in the "prophase" checkpoint, which has been proposed to regulate entry into early mitotic phases[53] and which delays chromosome condensation in response to impaired microtubule structure. The key component of this pathway is CHFR (checkpoint with FHA and RING domains), which is an E3-ubiquitin ligase.[54-56] It has been shown that *in vitro*, CHFR is able to generate K63-linked ubiquitin chains in cooperation with the E2 conjugating heterodimer Ubc13-MMS.[56] Thus, it is tempting to propose that CYLD antagonizes the CHFR activity in order to restart the progression of the cell cycle after resolving microtubule defects. Indeed, based on experiments with *Xenopus* egg extracts, it has been proposed that PLK1 is ubiquitinated by CHFR and then degraded in order to establish a delay in the entry of mitosis upon mitotic stress.[54] Whether CHFR ubiquitinates PLK1 in cells and which kind of ubiquitin signal is used are not known. Alternatively, other, unknown components of the prophase checkpoint signaling modified by CHFR-mediated K63-linked ubiquitination could be direct potential substrates of CYLD. Thus, further studies are required to confirm the involvement of CYLD in the prophase checkpoint.

Interestingly, it has been shown that stable expression of CYLD in U2OS cells results in an increase of fragmented nuclei and multinucleated cells, reflecting impairment in chromosome segregation and/or cytokinesis.[43] Furthermore, localization analysis has shown that overexpressed CYLD is associated with microtubules in interphase and at the midbody in telophase stage, suggesting another potential mitotic function of CYLD. Consistent with these observations, in addition to its USP domain, CYLD contains 3 cytoskeletal-associated protein-glycin-conserved (CAP-Gly) repeats. CAP-Gly domain is a conserved motif found in a large number of microtubule-associated proteins and could explain microtubule localization of CYLD. Indeed, CYLD interacts with the microtubule subunit tubulin through its two first CAP-Gly independent of its deubiquitinating activity.[57,58] This interaction could allow regulation of the microtubules' dynamics by CYLD, as CYLD depletion in HeLa cells induces a slow microtubule regrowth after nocodazole treatment,[57] and its overexpression leads to accelerated microtubule regrowth in melanoma cells.[58] Furthermore, the midbody localization of CYLD has been confirmed at the level of endogenous protein in primary mouse keratinocytes.[58] Because many

proteins involved in the regulation of cytokinesis are localized to the midbody, CYLD could have a potential function in this process. It has been shown that CYLD co-localizes at the midbody with the histone deacetylase HDAC6, leading to its inhibition and high levels of acetylated, more stable microtubules, suggesting that CYLD could participate in the regulation of microtubule dynamics during the final stages of cytokinesis. However, since CYLD's role in regulation of acetylation status of microtubules does not involve its deubiquitinating activity, it is not clear how exactly CYLD exerts its function at the midbody at the mechanistic level.

However, CYLD seems also to play a role in the assembly of the mitotic spindle.[59] This function could be linked to the interaction of CYLD with the centrosomal protein CEP192, which is involved in the centrosome maturation and nucleation of microtubules.[60,61] In CEP192-depleted cells, microtubules are assembled in the vicinity of chromosomes and are unable to self-organize into bipolar spindles. Co-depletion of CEP192 and CYLD alleviates the spindle assembly defects, suggesting that CYLD function could be inhibited by CEP192. Because CYLD regulates microtubule dynamics in interphase cells, Gomez-Ferreria et *al.*[59] proposed that the potential inhibition of CYLD by CEP192 could take place at the onset of mitosis to allow for depolymerization of microtubules and/or to maintain possible K63-linked ubiquitination-mediated interaction(s) necessary for the spindle assembly.

Taken together, CYLD appears to fulfill some important functions during both early mitotic stages and mitotic exit. Further studies are needed to identify the specific and direct targets of this DUB as well as the precise modes of its regulation during mitosis.

USP39 and USP4. Using a RNAi-based screen, van Leuken *et al.*[62] identified USP39 as another regulator of the SAC. Knockdown of USP39 in synchronized U2OS cells leads to an increase in the cellular DNA content reflecting a defect in chromosome segregation and/or cytokinesis. Interestingly, the USP39-depleted cells were able to bypass the mitotic arrest induced by treatment with a microtubule stabilizing drug, paclitaxel, but not by nocodazole, which induces microtubule depolymerization. As mentioned previously, SAC is activated not only by the presence of unattached kinetochores but also by a lack of tension exerted across sister centromeres. Indeed, paclitaxel decreases the interkinetochore tension, suggesting that USP39 is specifically required to sustain the tension-dependent branch of the SAC. USP39 is also known as a 65 kDa SR-related protein of the U4/U6.U5 tri-snRNP. It associates with small ribonuclear proteins and is involved in the splicing process.[63] van Leuken *et al.*[62] found that depletion of USP39 leads to a decrease of the mRNA levels of the mitotic kinase Aurora B and consequently its protein levels. Ectopic transfection of Aurora B cDNA restored Aurora B protein levels in USP39-depleted cells but failed to rescue the mitotic arrest upon paclitaxel treatment. These observations suggested that USP39 is a critical regulator of the tension-dependent mitotic checkpoint through its function in the splicing of the Aurora B mRNA and possibly other mRNAs of essential mitotic factors. Aurora B kinase is a catalytic component of the so-called chromosomal passenger complex (CPC), containing INCENP, Survivin, Borealin/Dasra B, and Telophase-Disc-60 (TD60) proteins. Aurora B is able to correct erroneous microtubule-kinetochore attachments in particular monotelic and syntelic attachments where not enough tension is generated between sister chromatids.[64-66]

Although USP39 contains an ubiquitin-protease domain, the cysteine and histidine residues belonging to the catalytic triad of the DUB's cysteine-protease are not conserved, and consequently the authors have reported absence of the catalytic activity of USP39 *in vitro*. Despite the fact that USP39 might be catalytically inactive, it could participate in an important molecular pathway. Indeed, other studies have postulated that some DUBs do exert a function in a noncatalytic manner. For example, OTUB1 inhibits DNA double-strand breaks independently of its catalytic activity,[67] and yeast Ubp6 is involved in regulation of the proteasome-dependent degradation in a noncatalytic manner.[68] These studies highlight the possibility that DUBs that have been predicted to be inactive, such as USP50 and USP52 to 54,[69] exert noncanonical DUB functions, possibly by acting as ubiquitin-binding receptors rather than processing enzymes.

Another elegant study demonstrated the role of splicing in SAC signaling and identified DUB USP4 as a regulator of the spliceosome function during SAC response induced by treatments with paclitaxel and monastrol (a specific inhibitor of kinesin Eg5 required for formation of bipolar spindles).[70] Downregulation of USP4 leads to SAC bypass after these treatments, and mRNA levels of α-tubulin (constituent of microtubules) and Bub1 (SAC protein) are reduced in these cells. The assembly of spliceosome is initiated by binding of the U1 snRNP to the 5′ splice site followed by recognition of the branch point site and 3′ splice site by SF1/BBP and U2AF, respectively.[71] The prespliceosome is formed after recruitment of the U2 snRNP. The U4/U6 and U5 snRNPs are then added as preassembled U4/U6.U5 tri-snRNP to form an inactive complex. Upon release of U1 and U4 snRNPs, splicing reactions start. After the mRNA splicing, the spliceosome is disassembled, allowing another round of splicing. USP4 together with its substrate targeting factor, the U4/U6 recycling factor Sart3, is able to deubiquitinate the Prp3 protein,[70] which is a component of the U4 snRNP. Prp3 ubiquitination is catalyzed by the U-box-containing protein Prp19, which is a member of the Prp19 complex (Nine Teen Complex, or NTC). In yeast, this complex participates in the splicing process by influencing the biogenesis of U4/U6 snRNP and stabilizing the

binding of U5 and U6 snRNPs on the spliceosome after U4 release.[72,73] The ubiquitination signal of the Prp3 protein is nonproteolytic, and these K63-linked ubiquitin chains are then recognized by the U5 snRNP component, Prp8. Indeed, it has been previously shown that yeast Prp8 is able to bind ubiquitin *in vitro* through its variant Jab1/MPN domain.[74] This interaction is necessary for the stabilization of the U4/U6.U5 tri-snRNP, confirming previous study about the requirement of ubiquitin for U4/U6.U5 stability.[75] The working model suggests that Prp3 ubiquitination acts on U4/U6.U5 stabilization during the formation of inactive spliceosomal complex. After recruitment of the U4/U6.U5 tri-snRNP to the spliceosome, USP4-Sart3 deubiquitinates Prp3, facilitating the release of the U4 snRNP necessary for activation of the splicing machinery. To highlight the requirement of splicing for the fidelity of cell division, Song et *al.*[70] showed that depletion of several other spliceosomal factors leads to mitotic defects in HeLa cells treated with paclitaxel drug. Interestingly, knockdown of the 65 kDa SR-related protein of the U4/U6.U5 tri-snRNP, USP39, is responsible for the SAC bypass, confirming results obtained by van Leuken et *al.*[62] Thus, USP39 and USP4 regulate splicing and are indirect but critical regulators of the mitosis.

Altogether, these studies suggest that splicing is a very dynamic process and that the spliceosome is a subject of constant and profound conformational rearrangements. Throughout the splicing cycle, distinct RNAs and proteins are assembled and disassembled at precise time points. Thus, modifications of proteins by ubiquitination allow for modulation of these highly dynamic rearrangements in the spliceosome composition. Both reversibility of ubiquitin modification and binding of the specific ubiquitin receptor proteins to the nonproteolytic ubiquitin chains allow for regulation of stable protein complexes and subcomplexes during splicing. These studies also highlight the possibility that other dynamic cellular processes could be regulated by ubiquitination of the splicing machinery.

Interestingly, the *usp4* gene belongs to a chromosomal region that is frequently deleted in small-cell lung cancer (SCLC). Furthermore, SCLC is associated with reduced expression of USP4[76] and alterations of ploidy.[77] Because defects in the SAC often lead to increase in polyploidization, loss of USP4 could be linked to tumorigenesis.

DUB Reversing Proteolytic Ubiquitination Signal

USP37. Irreversible degradation by the ubiquitin-proteasome system confers directionality of the cell cycle. The APC/C E3 ligase together with its co-activators (CDC20 and CDH1) orchestrates cell cycle progression through mitosis and G1 phases[6] (Fig. 2). The CDC20 activates APC/C during mitotic phases, whereas CDH1 activates APC/C as cells exit mitosis and in G1 phase. This sequential activation manner, also referred to as "substrate ordering," allows the temporal order of degradation of APC/C substrates such as Cyclin A, Securin, Cyclin B, Geminin, PLK1, and Aurora A[78] and ensures faithful cell cycle progression. By counterbalancing the activities of E3 ubiquitin ligases, DUBs could be involved in the timing of degradation of the ubiquitination substrates. To identify potential DUBs implicated in the regulation of substrates of APC/C, Huang et *al.*[79] used a proteomic approach. Interestingly, USP37 has been found to interact specifically with the co-activator CDH1 but not with CDC20.[79] Furthermore, APC/C subunits such as CDC27, APC5, and APC7 co-immunoprecipitated with the endogenous USP37, and interaction with CDC27 is decreased upon CDH1 knockdown, suggesting that USP37 binds to APC/C through the CDH1 co-activator. Interestingly, overexpression of USP37 in synchronized U2OS cells led to an accumulation of Cyclin A protein level, which could reflect a reduced rate of its proteasomal degradation. Moreover, USP37-overexpressing cells are also characterized by an accelerated entry into S phase. Conversely, cells expressing specific USP37 shRNAs exhibited a delay in accumulation of Cyclin A after release from nocodazole-induced mitotic block and were also delayed for the entry into S phase. Thus, USP37 may regulate turnover of Cyclin A and thereby G1/S transition. Furthermore, Huang et *al.*[79] have also demonstrated that endogenous USP37 is able to bind to Cyclin A, suggesting that Cyclin A could be a direct USP37 substrate. Indeed, unlike catalytically inactive mutant, the wild-type form of USP37 is able to reduce ubiquitination of Cyclin A in cells and to counterbalance the activity of APC/C^{CDH1} *in vitro*.[79] These results strongly establish USP37 as an antagonist of APC/C^{CDH1} toward ubiquitination of Cyclin A, allowing for rescue from its proteasomal degradation and faithful progression beyond the G1/S transition. However, how is the function of USP37 regulated to ensure temporal specificity? By analyzing the USP37 protein levels, the authors observed that USP37 fluctuates in a cell cycle–dependent manner. USP37 levels peaked in the late G1 phase, decreased in late mitosis, and reappeared in G1 again, reflecting a typical profile of degradation events governed by APC/C^{CDH1} at the M/G1 transition. It is known that APC/C^{CDH1} recognizes substrates via short D- or KEN-box motives.[80,81] Indeed, USP37 contains a KEN-box motif necessary for the K11-linked ubiquitination mediated by APC/C^{CDH1} and its subsequent degradation. Interestingly, USP37 is also able to rescue itself from degradation in late G1. Thus, USP37 acts as an APC/C^{CDH1} substrate in late mitosis, and it counterbalances the APC/C^{CDH1} activity toward itself and Cyclin A in late G1. These opposing functions are modulated by CDK2-mediated phosphorylation of USP37. In their working model, the authors present evidence that Cyclin E-CDK2 phosphorylates USP37 in late G1, resulting in its activation and its rescue from degradation. USP37 acts also on ubiquitinated Cyclin A, which in turn associates with CDK2 and amplifies the

activation of USP37. This positive feedback loop promotes the G1/S transition. The USP37 activation is reinforced by phosphorylation-dependent inhibition of APC/C^{CDH1} by Cyclin A-CDK2.[82] In late mitosis, cyclin partners were degraded, preventing phosphorylation-mediated activation of USP37. At the same time, activated APC/C^{CDH1} is responsible of the proteolytic ubiquitination of USP37. The work by Huang et *al.* nicely illustrates the dynamic interplay between a DUB and E3-ubiquitin ligase, which is necessary for timely regulation of the key cell cycle regulators. In a recent proteomic survey it was found that indeed many DUBs associate with E3-ligase complexes,[83] suggesting that cross-regulation observed between USP37 and APC/C^{CDH1} could be a commonly used mechanism for spatiotemporal regulation of other important biological pathways. It will be interesting to understand whether additional DUBs exist that specifically counteract ubiquitination of some of the other numerous targets of APC/C. Likewise, the role of putative novel DUBs that may act on substrates of the SCF E3-ligases during early mitotic phases, and targets of the recently described CUL3-based E3-ligases coordinating mitotic progression, are currently unknown.

Recently, Burrows et al.[84] demonstrated that USP37 is also subjected to a second wave of degradation during the cell cycle. Indeed, a fraction of USP37 is degraded by the SCF$^{\beta TrCP}$ E3 ligase at the G2/M transition. This degradation event requires a PLK1-dependent phosphorylation at a noncanonical degron within the sequence of USP37. Interestingly, the expression of a SCF$^{\beta TrCP}$-resistant USP37 mutant leads to a delay of the mitotic entry, suggesting that one of the USP37 pools regulates unknown substrates required for the inhibition of entry into mitosis. This USP37 pool does not seem to regulate Cyclin A.[84] Thus, identification of the new USP37 substrate will be essential to understand the cell cycle functions of both USP37 pools.

UBPs in Mitosis

Recognition of ubiquitinated substrates is mediated by a variety of ubiquitin-binding proteins (UBPs) that serve as ubiquitin receptors (or decoders) and contain at least 1 ubiquitin-binding domain (UBD) within their structure.[85-87] Based on their structural features, UBDs can be divided into several subgroups, such as ubiquitin-associated (UBA) domain, ubiquitin-interacting motif (UIM), motif interacting with ubiquitin (MIU), and many others.[88] The common feature of these UBDs is the ability to noncovalently bind ubiquitin moiety. Increasing experimental evidence suggests that UBPs play a critical role in defining the fate of ubiquitinated targets in time and space, either by mediating their proteasomal degradation or by regulating their localization and/or interaction with other proteins. UBPs have recently been also shown to play a role in mitosis (Fig. 2). Rad23, Dsk2, Ddi1, and Rpn10 were identified in *Saccharomyces cerevisiae* as regulators of mitotic progression by shuttling ubiquitinated targets to the proteasome for degradation,[89-93] whereas the second group includes UBPs mediating relocalization of its targets involving the Cdc48/p97-Ufd1-Npl4 (p97) complex in mammalian cells. Although the p97 complex was proposed to play a role in chromosome segregation and spindle dynamics,[94-96] very little is known about specific UBPs controlling mitosis in mammalian cells, in particular the functional homologs of Rad23 pathway. Here, we summarize the role of UBPs controlling cell cycle (Table 2), with the main focus on known yeast UBPs and their unpredicted redundancy in controlling mitotic progression. Furthermore, we discuss the role of human p97 complex during mitosis.

UBPs Targeting Substrates for Proteasomal Degradation

Rad23, Dsk2, and Ddi1. In yeast *Saccharomyces cerevisiae*, Rad23 and Ddi1 were shown to shuttle ubiquitinated proteins to the 26S proteasome for degradation.[93] These proteins belong to UBPs that possess an UBA domain (2 UBA domains in case of Rad23) interacting with ubiquitin and ubiquitinated substrates.[97,98] In addition, Rad23 and Ddi1 have the N-terminal ubiquitin-like domain (UBL) that is able to interact with the proteasome.[99,100] RAD23 and DDI1 genes were initially identified in a screen for suppressors of the temperature sensitive mutant allele of PDS1, pds1-128.[90] Pds1 (securin in higher vertebrates) is an essential regulator of mitosis and inhibitor of anaphase onset.[101,102] Proteasomal degradation of Pds1 releases its inhibitory action on Separin, Esp1 (Separase in higher vertebrates) that mediates cleavage of cohesin complex, sister chromatid separation, and anaphase spindle elongation, ensuring successful completion of mitosis in yeast cells.[103] Pds1 is polyubiquitinated by APC/C prior to the onset of anaphase, which leads to recognition of the ubiquitin chain by 26S proteasome and degradation of Pds1 at the beginning of anaphase.[104] Interestingly, deletions of both RAD23 and DDI1 genes were able to rescue pds1-128 temperature sensitive phenotype, suggesting a possible role of these proteins in the regulation of Pds1 proteolysis during mitosis.[90] Furthermore, the mechanism of anaphase control was proposed in which Rad23 and Ddi1 bind ubiquitinated Pds1, thus inhibiting ubiquitin chain elongation and subsequent Pds1 proteolysis.[90] It is not known however, what is the mechanism of Rad23-Ddi1-Pds1 complex dissociation that finally leads to Pds1 degradation by proteasome.

The third UBA-UBL protein, Dsk2, was also shown to play a role in governing ubiquitinated substrates to proteasome.[99,105] Overexpression of Dsk2 is toxic for cells due to accumulation of ubiquitinated substrates and leads to mitotic arrest, an abnormal nuclear positioning, and affected spindle dynamics.[89,99] There are controversial data about involvement of Dsk2 in spindle pole

Table 2. List of UBPs Regulating Cell Cycle

Name	Domains	Cell Cycle Stage	Function	Substrate(s)	References
KPC2	UBL-UBA	G1	Cell proliferation control: degradation of the cyclin-dependent kinase inhibitor p27 (cooperatively with KPC1)	p27	149, 150
YEAST RPN10 (Human: PSMD4)	UIM	G1/S M/G1	Proteasome receptor for ubiquitinated substrates, regulates G1/S and M/G1 transition in budding yeast	Clb2, Sic1 (Yeast)	91, 110
Polymerase eta	UBZ	S	Translesion synthesis, tolerance of DNA damage by replicative bypass, negative self-regulation of the activity	PCNA, polymerase eta itself	151, 152
Polymerase kappa	UBZ	S	Translesion synthesis, tolerance of DNA damage by replicative bypass	PCNA	153, 154
Polymerase iota	UBM	S	Translesion synthesis, tolerance of DNA damage by replicative bypass	PCNA	155, 156
Polymerase Rev1	UBM	S	Translesion synthesis, tolerance of DNA damage by replicative bypass	PCNA	156
YEAST DDI1/VSM1	UBL (DDI1), UBA-UBL (VSM1)	G1/S G2/M M	Sister chromatid separation, *Pds1p-dependent S-phase checkpoint control*, degradation of a SCF component, the F-box protein Ufo1, involved in the G1/S transition, spindle dynamics	(?), Pds1p, Ufo1 (Yeast)	98, 90, 106, 108
YEAST: DSK2 (Human: Ubiquilin2, hPLIC1/2)	UBA, UBL	G2/M M	Transfer of the ubiquitylated proteins to the proteasome, spindle pole body duplication, spindle dynamics	(?), Pds1, Kre22 (Yeast)	89, 90, 106, 157
YEAST RAD23 (Human: hRAD23A/B)	UBA, UBL	G2/M M	Metaphase-anaphase transition, spindle pole body duplication, G2/M, sister chromatids separation, spindle dynamics	(?), Pds1p (Yeast)	89, 98, 90, 93
RAP80	IUM	G2/M	BRCA1-dependent DNA damage response and double-strand break repair	“Lys-63”-linked ubiquitinated histones H2A and H2AX at DNA lesions sites	158, 159
Ufd1	NZF	M	Nucleus reformation, chromosome alignment and segregation	Aurora B, Survivin	96, 94, 125
Npl4	NZF	M	Nucleus reformation, chromosome alignment and segregation	Aurora B, Survivin	96, 94, 125

The table depicts UBPs known to be involved in cell cycle progression at different cell cycle stages (S [S phase], G1, G2 and M [mitosis]) and transitions. The role and regulation of mitotic UBPs are described in detail in the text. The predicted human orthologs of these genes are indicated. The domain composition and specific substrates of the UBPs are depicted. [?]indicates unconfirmed speculation.

body (centrosomes in mammalian cells) duplication. Some studies have proposed a model according to which Dsk2 together with Rad23 assists the assembly of Cdc31 into the spindle pole body, which is considered to be an essential event in its duplication.[89] However, a follow-up study has shown that deletion of Rad23 and/or Dsk2, as well as triple deletion of Rad23-Ddi1-Dsk2, did not lead to the spindle pole body duplication failure.[106] Interestingly, it has been shown that Rad23, Ddi1, and Dsk2 exert partially redundant roles in cell cycle progression.[106] Double deletions of Rad23-Dsk2 and Rad23-Ddi1 showed slight accumulation of cells in G2/M; however, single deletions of these 3 genes and also double deletion of Ddi1-Dsk2 did not show any defect in mitotic progression. Furthermore, triple deletion Rad23-Ddi1-Dsk2 led to striking cell cycle arrest either in G2 or in anaphase. Additionally, spindle dynamics were shown to be affected in triple deletion strain; however, the underlying mechanisms still remain unclear.[106] According to these results, Rad23 shares a common function with Ddi1 and Dsk2 in mitotic control, but it is not clear how these proteins are regulated to coordinate the common tasks. One possible level of such regulation that could allow for specificity in binding of the ubiquitinated substrates is formation of both homo- and heterodimers between Rad23, Ddi1, and Dsk2 proteins.[105,107]

UBA and/or UBL domains were shown to be essential in Rad23 and Dsk2 homodimerization and Rad23 heterodimerization with Dsk2 and Ddi1.[93,107,108] According to one of the proposed models, dimerization of UBA/UBL proteins can potentially play a role in preventing unnecessary ubiquitin chain elongation or premature disassembly during the transit of substrates to the proteasome: Polyubiquitin chain of a substrate might be tightly covered with a complex containing multiple UBA domains, thus blocking access of E3-ligases and/or deubiquitinated enzymes to the substrate.[93] However, the biological significance of UBPs dimerization still remains to be determined experimentally.

In addition to the role in mitosis, Ddi1 was shown to be implicated in the negative regulation of the late secretory pathway by interacting with exocytic v- and t-SNAREs.[108] It is not yet clear how Ddi1 coordinates both processes, in particular, which stimuli can possibly serve as a switch between these two functions by regulating the change in localization of Ddi1 in the cell (either on a plasma membrane or in the nucleus).

Taken together, these results suggest a role of UBA-UBL proteins Rad23, Ddi1, and Dsk2 in controlling mitotic progression. These proteins were shown to inhibit degradation of ubiquitinated Pds1, thus delaying the onset of anaphase, and to influence spindle dynamics. The regulatory functions of UBPs are complicated by redundancy and cooperation between ubiquitin receptors. Thus, the exact mechanisms of mitotic control by Rad23, Ddi1 and Dsk2 remain to be elucidated. Future studies are needed to identify other yet unknown targets of Rad23-Ddi1-Dsk2 proteins that play a role during mitotic progression. Likewise, the functional orthologs of this pathway in higher vertebrates need to be characterized.

Rpn10. The Rad23-pathway components are not the only UBPs known to play a role in the regulation of mitosis in yeast. Another UBP, Rpn10, was shown to play a role in determining the fate of critical mitotic factors. Rpn10 is a stoichiometric component of 26S proteasome, and it was predicted to be one of the ubiquitin receptors governing ubiquitinated proteins for degradation.[109,110] Rpn10 contains 2 domains: N-terminal von Willebrand A domain, which is necessary for interaction with proteasome, and C-terminal ubiquitin-interacting motif (UIM) domain, which ensures Rpn10 binding to the ubiquitinated targets. Among other targets, Rpn10 controls turnover and degradation of one of the key cell cycle regulators, Sic1.[91,110] Sic1 is a specific stoichiometric inhibitor of Cdk1/Clb (CDK/Cyclin in mammalian cells) complexes that are required for S-, G2-, and M-progression and promote spindle pole body separation in yeast.[111,112] Sic1 is synthesized at the end of mitosis and degraded at the beginning of S-phase in a Rpn10-dependent manner.[110,113] During late stages of mitosis, Sic1 inhibits Cdk1/Clb2 activity and is considered to be an activator of M/G1 transition, although the precise mechanisms need to be elucidated.[114-117] At the same time, there is some evidence that other ubiquitin receptors, in particular Rad23-Ddi1-Dsk2 proteins (and other UBPs), can share the same function of shuttling targets for degradation with Rpn10, as deletion of Rpn10 in yeast is not lethal and recombinant Rad23 is able to rescue Rpn10 deletion phenotype to some extent. In contrast, deletion of Rpn10 in *Drosophila melanogaster* results in pupal lethality, and siRNA-mediated knockdown of Rpn10 in *Trypanosoma brucei* leads to G2/M cell cycle arrest.[91,118,119] Thus, the redundancy with other UBPs and the precise role of different domains of Rpn10 remain the unresolved issues that deserve extensive study.

UBPs Regulating Substrates Localization

p97 complex. Cdc48/p97-Ufd1-Npl4 is the evolutionally conserved protein complex that plays a role in the so-called ERAD (endoplasmic reticulum-associated degradation) pathway. It binds ubiquitinated proteins and is necessary for the export of the misfolded proteins from the ER to the cytoplasm, where they are degraded by the proteasome.[120] In this complex, the AAA+ ATPase Cdc48/p97 (also known as VCP in vertebrates) interacts with its adapters Ufd1-Npl4, which are able to bind ubiquitin through their NZF (Npl4 Zn-F) domains.[121-123] Studies performed in *Xenopus laevis* egg extracts revealed that apart from its function in the ER, the p97 complex regulates spindle disassembly at the end of mitosis and is necessary for formation of a closed nuclear envelope. This occurs through removal of ubiquitinated mitotic kinase Aurora B, a component of the CPC complex, from chromatin by Ufd1-Npl4 adapters during the late stages of mitosis.[96] In contrast to this observation, in HeLa cells Ufd1-Npl4 adapters of the p97 complex were shown to antagonize chromosome-associated Aurora B activity already during earlier mitotic stages, which resulted in defects in faithful chromosome segregation as cells progressed through mitotic division.[94] These observations are consistent with the model in which Aurora B and possibly other components of CPC are ubiquitinated on the mitotic chromosomes and “extracted” by the action of p97 complex. It is not clear, however, what is the fate of the ubiquitinated Aurora B bound to p97 complex or how this association is regulated. Aurora B kinase is indeed a subject of APC/C-mediated, proteolytic ubiquitination, but this event takes place during later stages of mitotic exit.[124] Interestingly, Aurora B was shown to be ubiquitinated earlier during mitosis by CUL3-based E3-ligase, but in contrast to the APC/C-mediated modification, this ubiquitination event does not seem to influence protein stability of Aurora B but rather its faithful relocalization to mitotic spindle.[26,27] It is not clear at this point whether Ufd1-Npl4 adapters localize to the mitotic spindle of whether they may change the affinity

of the ubiquitinated CPC toward microtubules. It is necessary to mention that results of another study performed in HeLa cells suggested that Ufd1 rather recruits Aurora B to chromosomes during mitosis.[125] Additionally, the authors have identified a putative DUB, USP9x, involved in regulation of dynamics of another CPC component, Survivin protein. Using siRNA, immunostaining, and FRAP techniques, investigators showed that USP9x regulates dissociation of Survivin from the centromeres and thereby chromosome alignment, segregation, and completion of cytokinesis.[125] However, the protein levels of Survivin were not affected by downregulation of USP9x. Interestingly, it has been shown that USP9x specifically hydrolyzes both Lys 29 and Lys 33 linked polyubiquitin chains,[126] which leads to regulation of activity of AMPK-related kinases and not their proteolysis. It will be interesting to uncover the precise subcellular localization of USP9x in cells during mitotic progression.

Despite evident difficulty in reconciling these observations, it is possible that different CPC components are regulated by several cooperating pathways, involving modification by different ubiquitination signals, proofreading by USP9x deubiquitinating enzyme, and finally dissociation from chromosomes by the action of p97 complex or yet to be identified microtubule-associated UBPs. Intriguingly, several other studies performed in worm *Caenorhabditis elegans* did not show the requirement of p97 complex for mitotic progression.[127,128] Overall, Cdc48/p97-Ufd1-Npl4 complex is considered to play a role in mitosis in vertebrates, but the exact function of ubiquitin-binding adapters Ufd1-Npl4 in this process requires further studies.

Concluding Remarks and Future Perspectives

Recent progress in study of the ubiquitin pathways has uncovered an enormous versatility and flexibility of this signaling network. This complexity is reflected not only by a plethora of different ubiquitination signals that can be attached to the substrates but also by the fact that their conjugation may take place at a very precise time points during the physiological processes and in a very specific subcellular localizations. This image is further complicated by so-called "decoders" of the ubiquitin signal, which may, on one hand, reverse ubiquitination like DUBs or, on the other hand, transfer the modified substrate to the downstream pathways as in the case of UBPs. For both large family of proteins, at least two functional groups can be distinguished: one that regulates proteasomal degradation (rescue or transfer, respectively) and another that can process the nonproteolytic type of ubiquitination signals and regulates substrate localization, activity, and/or stability of protein complexes (Fig. 1). Despite the recent advances in understanding the components of this network in regulation of mitosis, the current list of DUBs and UBPs involved in mitotic progression is far from complete. Thus, identification of new key players in this field will significantly improve our understanding of the principles of mitotic regulation. Likewise, major efforts are needed to understand the precise modes of regulation of these new "decoders," which could guarantee the essential specificity toward the substrates. We believe that development of imaging technologies, in particular specific "ubiquitin-fluorescent sensors" that could allow visualization of attachment of specific ubiquitin signals in a cellular context, will greatly advance our knowledge about this fascinating signaling network.

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2. Aim of this study

Mitosis ensures equal chromosome segregation between two daughter cells, and defects in mitotic pathways can lead to aneuploidy and polyploidy, frequently observed in cancers. Aurora B is one of the key mitotic kinases regulating chromosome alignment and segregation by controlling spindle assembly and kinetochore-microtubule attachments. Dynamic localization of Aurora B is pivotal for its correct function in mitosis. Previous findings of our laboratory demonstrated that nonproteolytic ubiquitination of Aurora B by CUL3 E3 ubiquitin ligase is important for correct Aurora B localization. However, the effectors of CUL3-mediated Aurora B ubiquitination still remain unknown.

Ubiquitin binding domain proteins can serve as receptors for the specific ubiquitin signals. The goal of my PhD studies is the identification and functional characterization of ubiquitin binding domain protein(s) that act in the Aurora B-CUL3 pathway during mitosis and control euploidy of dividing cells.

3. Results

3.1. Ubiquitin receptor protein UBASH3B mediates a switch-like mechanism of Aurora B localization to microtubules

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Ubiquitin receptor protein UBASH3B mediates a switch-like mechanism of Aurora B localization to microtubules

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Summary

Mitosis ensures equal segregation of the genome and is controlled by variety of ubiquitylation signals on substrate proteins. However, it remains unexplored how the versatile ubiquitin code can be read during mitotic progression. Here we identify the ubiquitin receptor protein UBASH3B that critically regulates mitosis. UBASH3B interacts with ubiquitylated Aurora B, one of the main kinases regulating chromosome segregation, and controls its subcellular localization but not protein levels. Importantly, UBASH3B is a rate-limiting factor, which is sufficient for targeting Aurora B to microtubules prior to anaphase. Indeed, super-resolution imaging reveals spindle-associated Aurora B in metaphase cells. Moreover, targeting Aurora B to microtubules by UBASH3B determines timing and fidelity of chromosome segregation and thereby euploidy of human cells. Our findings uncover an important mechanism how ubiquitin attachment to a substrate protein is be decoded during mitosis, which can be utilized by propagating cancer cells.

Introduction

Mitosis ensures equal segregation of the genome to daughter cells, and defects in mitotic pathways can lead to aneuploidy and polyploidy, frequently observed in cancers (Ganem et al., 2007; Jallepalli and Lengauer, 2001). Accurate chromosome segregation requires assembly of the mitotic spindles and their attachment to both sister kinetochores of all chromosomes, and is strictly controlled by the activity of the Spindle Assembly Checkpoint (SAC) (Musacchio and Salmon, 2007). Defects in kinetochore attachment lead to the activation of SAC, preventing chromosome segregation and often inducing apoptotic death in human cells. In contrast, cells with compromised or defective SAC response segregate their chromosomes erroneously and acquire aneuploid or polyploid state.

Ubiquitin attachment to substrate proteins is one of the essential mechanisms that regulate fidelity of mitotic division through both proteolytic and non-

proteolytic mechanisms (Bassermann et al., 2014). E3 ligases catalyze substrate ubiquitylation, ranging from a single ubiquitin molecule (monoubiquitylation) to topologically different chains of interconnected ubiquitins (Komander and Rape, 2012; Li and Ye, 2008). Ubiquitin Binding Domain (UBD) proteins can serve as receptors, or decoders, for the specific ubiquitin signals and transfer ubiquitylated substrates to downstream signaling components and compartments thereby determining their cellular functions (Husnjak and Dikic, 2012). Surprisingly, despite a high number of known UBD proteins in mammalian cells, their mitotic roles remained unexplored.

Using high-content siRNA screening and proteomic approaches, we identified UBD protein UBASH3B that critically regulates chromosome segregation, acting as ubiquitin receptor for Aurora B. Aurora B is one of the key mitotic factors controlling euploidy of eukaryotic cells. Aurora B is a component of the Chromosomal Passenger Complex (CPC) that coordinates chromosome alignment with segregation by regulating kinetochore-microtubule attachments, spindle assembly and localized activity of SAC (van der Horst and Lens, 2013). Essential functions of Aurora B in chromosome segregation are dependent on its dynamic localization to centromeres in prometa- and metaphase stages and to midzone microtubules during anaphase (Carmena et al., 2012). Centromeric recruitment of Aurora B is controlled by histone phosphorylation events enhanced by positive feedback loops (van der Horst and Lens, 2013) and spindle microtubules (Banerjee et al., 2014). Relocalization of Aurora B to midzone microtubules requires mitotic kinesin MKlp2, the CPC protein INCENP and a drop of CDK1 activity (Hummer and Mayer, 2009). Besides phosphorylation, other postranslational modifications were shown to regulate CPC localization during mitosis (van der Horst and Lens, 2013). Previous findings also identified an important role of non-proteolytic ubiquitylation of Aurora B by CUL3-based E3 ligases for its relocalization from centromeres to microtubules during mitosis (Maerki et al., 2009; Sumara and Peter, 2007, 2007). However, it remained unknown how and when ubiquitylated Aurora B is targeted to the mitotic structures.

Because we identified UBASH3B in the siRNA screen for mitotic factors and in the interactome of CUL3, we postulated that it might represent a missing molecular link that targets ubiquitylated Aurora B to microtubules and determines its mitotic functions.

Here we show that UBASH3B directly binds to ubiquitylated Aurora B and to CUL3 and its interaction with Aurora B is dependent on CUL3 and ubiquitin. Similar to CUL3, UBASH3B does not regulate protein levels of Aurora B. Instead, UBASH3B localizes to the mitotic spindles and is required for timely relocalization of Aurora B from centromeres to microtubules. Strikingly, UBASH3B interacts with MKlp2 and is sufficient to target both Aurora B and MKlp2 to microtubules, even in the presence of high CDK1 activity. Thus, by localizing to the metaphase spindles, UBASH3B is a rate-limiting factor for Aurora B targeting to microtubules prior to onset of anaphase. In line with this, super-resolution microscopy reveals a microtubule-associated pool of Aurora B in metaphase cells with fully aligned chromosomes. Moreover, our data show that redistribution of Aurora B from centromeres to microtubules silences SAC and thereby controls timing and fidelity of chromosome segregation and consequently euploidy of cells. Taken together, our findings show that UBASH3B mediates Aurora B localization in a switch-like mechanism during mitotic progression, which relies on decoding of a non-proteolytic ubiquitin signal. This important mechanism can be used to inhibit activity of elevated SAC to allow for survival of cancer cells.

Results

UBASH3B controls chromosome segregation.

To identify ubiquitin receptors that control euploidy of dividing human cells, we performed a high content visual siRNA screen in HeLa cells for known and predicted human UBD proteins (Table S1). Our screening procedure was designed to identify factors that coordinate chromosome segregation with

cytokinesis (Table S2 and S4), inactivation of which leads to strong terminal phenotypes of multilobed nuclei and multinucleated cells typically observed upon downregulation of Aurora B (Supplementary Results, Figure S1). Interestingly, one of the identified hits, Ubiquitin-associated (UBA) and SH3 domain-containing protein B, UBASH3B (also known as Suppressor of T-cell receptor signaling 1, STS1 or T-cell ubiquitin ligand 2, TULA2) was also found to interact with CUL3, which was immunoprecipitated and analyzed by mass spectrometry (Table S5). UBASH3B is ubiquitously expressed protein and was previously shown to bind monoubiquitylated proteins to regulate internalization of the receptor protein kinases (Hoeller et al., 2006; Kowanetz et al., 2004), but has been never linked to the regulation of mitosis. To confirm its potent role in mitotic progression, we downregulated UBASH3B by a pool of specific siRNAs (Figures S1B and C), distinct from the two pools used in the primary and secondary siRNA screens. Both mRNA (Figure S1B) and protein levels (Figure S1C) of UBASH3B were markedly decreased upon treatment with UBASH3B-specific siRNA pool but not by the control siRNAs. In agreement with results obtained by unbiased screening (Figure S1), downregulation of UBASH3B dramatically increased the number of cells with multilobed nuclei of heterogenous forms as compared to the controls (61.33 ± 13.86 % and 31 ± 3.46 %, respectively) (Figures 1A and S1D).

To understand how UBASH3B regulates mitotic progression, we first used immunofluorescence microscopy and analyzed distribution of different mitotic stages in cells synchronized by double thymidine block and release. We observed a drastic increase in a number of prometaphase cells upon downregulation of UBASH3B (46.66 ± 2.51 %) as compared to control cells (24.34 ± 3.78 %), suggesting defects in chromosome congression and/or timely onset of anaphase (Figure S1E). To corroborate these results, we employed live video microscopy of HeLa cells stably expressing histone marker H2B-mCherry and the probe for postmitotic nuclear reassembly, the importin-β-binding domain of importin-α, IBB–EGFP, which co-localizes with chromatin regions after reassembly of a functional nuclear envelope (Schmitz et al., 2010). This analysis showed that downregulation of UBASH3B reduced

survival of mitotic HeLa cells from 100 ± 11.59 % to 45.24 ± 6.4 % and led to death in prometaphase (Figures 1B, C and S2). Moreover, in cells treated with siRNA to UBASH3B the average time from prophase to anaphase was 98.8 ± 22.63 min in contrast to 48.03 ± 12.54 min in control siRNA-treated cells (Figures 1C, D and S2, 3). The time from prophase until metaphase (or metaphase-like stage) was also prolonged upon inactivation of UBASH3B as compared to control cells (34.9 ± 4.69 min and 22.77 ± 2.53 min, respectively). These results are consistent with the accumulation of UBASH3B-depleted cells in prometaphase and suggest that UBASH3B controls chromosome segregation (Figures S1E and 1C, D). Furthermore, 67.9 ± 15 % of UBASH3B-inactivated cells erroneously segregated their chromosomes, as compared to 12.45 ± 11.4 % in control-depleted cells, leading to unequal distribution of chromatin to daughter cells (Figure 1E). Similar observations were made using the reporter cell line expressing EGFP-Tubulin and H2B-Cherry markers (Schmitz et al., 2010) (21.3 ± 12.6 % versus 83.1 ± 15.69 % in control and UBASH3B downregulated cells, respectively) (Figure S3). These results explain how surviving UBASH3B-depleted cells acquire multilobed nuclei of very heterogenous forms (Figures S1D and S3) observed by unbiased siRNA screening (Figure S1A).

Our results strongly suggest that UBASH3B is involved in one of the essential mitotic pathways controling timing and fidelity of chromosome segregation. To confirm a direct role of UBASH3B in regulation of mitosis, we first analyzed its subcellular localization using specific UBASH3B antibody (Figure S4A). UBASH3B exhibited largely diffuse cytosolic distribution with some enrichment at the vesicle-like structures in the vicinity of the nucleus in interphase and prophase cells (Figure S4A), consistent with the previous report (Raguz et al., 2007). Upon chromosome condensation, UBASH3B started to accumulate on the mitotic spindles in prometaphase, with the strongest signals found in metaphase cells that fully aligned their chromosomes. In anaphase and telophase stages, a residual weak staining on microtubules can be found in addition to the diffuse cytoplasmic signal (Figure S4A). The direct interaction of UBASH3B with microtubules was

confirmed using microtubule pelleting assays of mitotic extracts and full-length recombinant UBASH3B protein (Figures S4B and C). Taken together, our data identify UBASH3B as a metaphase spindle-associated mitotic factor that controls chromosome segregation.

UBASH3B interacts with Aurora B-CUL3 complex in ubiquitin-dependent manner.

Interestingly, inactivation of UBASH3B leads to cellular phenotypes strongly resembling mitotic defects occurring upon misregulation of Aurora B kinase (Fu et al., 2007) (Figure S1A) and UBASH3B interacted with CUL3 in human cells (Table S5). Thus, we hypothesized that UBASH3B acts in the Aurora B-CUL3 pathway possibly as a previously postulated factor tethering Aurora B to microtubules (Maerki et al., 2010). If this is the case, UBASH3B should interact with Aurora B and CUL3 and regulate localization of Aurora B. First, we tested molecular interactions of UBASH3B with Aurora B-CUL3 pathway. Similar to CUL3, UBASH3B did not regulate protein levels of Aurora B or any other components of the CPC (Figure S5A) and strongly interacted with immunoprecipitated GFP-tagged Aurora B (Figure 2A). Importantly, interaction of UBASH3B with Aurora B was dependent on the presence of CUL3 protein (Figure 2B), suggesting that UBASH3B may directly regulate CUL3-modified Aurora B. To corroborate these findings, we performed a pull-down assay, using a short recombinant fragment of UBASH3B corresponding to the ubiquitin-binding domain (UBA) (Figures S5B, C and 2C), which was previously shown to bind ubiquitin and monoubiquitylated proteins *in vitro* (Hoeller et al., 2006). Indeed, isolation of UBA-interacting proteins from lysates of the cells arrested in mitosis revealed many ubiquitylated proteins, suggesting that UBASH3B can act as an intracellular ubiquitin receptor during mitotic progression. Interestingly, only modified, presumably mono- and di-ubiquitylated forms of endogenous and the GFP-tagged form of Aurora B but not GFP alone were found interacting with the UBA domain (Figures 2C and S5D).

To confirm that involvement of UBASH3B in Aurora B-CUL3 pathway is dependent on ubiquitin, we isolated UBA-interacting proteins and subsequently performed *in vitro* deubiquitylation reactions using non-specific, potent recombinant deubiquitinating enzyme (DUB) USP2 (Kim et al., 2011). Indeed, deubiquitylation strongly reduced interaction of UBA domain of UBASH3B with ubiquitylated proteins and Aurora B (Figure 2D). Interestingly, UBA interaction with NEDD8-modified, active form of CUL3 was also abolished upon DUB treatment (Figure 2D). To confirm these observations, we analyzed the UBASH3B sequence and identified two putative ubiquitin-binding motifs. The first one corresponds to the MGF sequence (aa 47, 48, 49) within the UBA domain, previously implicated in ubiquitin binding *in vitro* (Hoeller et al., 2006) and containing highly conserved methionine residue (aa 47) (Figure S5E). The canonical UBA domain of UBASH3B protein is followed by a short C-terminal helical extension, which can be identified in selected UBA domain-containing proteins from different species including USP5/13, UBAC2, UBXN1 (Figure S5E), and will be herein referred to as extended UBA (eUBA). Mutagenesis of both M47 to A and separetely all three conserved residues within the eUBA (WHD aa 72, 76, 79) to alanines dramatically reduced interaction with ubiquitylated proteins in mitotic cells (Figure 2E), suggesting that eUBA domain of UBASH3B contacts ubiquitin molecule at two distinct sites. Importantly, both mutants lost their capacity to interact with GFP-Aurora B and CUL3 as compared to wild type eUBA domain (Figure 2E). No interaction between all forms of the eUBA domain and INCENP, Survivin or PLK1 could be detected in the same experiment. We thus conclude that UBASH3B directly interacts with Aurora B-CUL3 complex in ubiquitin-dependent manner.

UBASH3B is required for microtubule localization of Aurora B.

Since localization of Aurora B was shown to be essential for its function, and Aurora B distribution is regulated by CUL3-mediated ubiquitylation (Maerki

et al., 2009; Sumara et al., 2007), we next analyzed mitotic localization of Aurora B in the presence and absence of UBASH3B. Interestingly, downregulation of UBASH3B by siRNA led to the redistribution of the centromeric Aurora B along the chromosome arms in 74.3 ± 2.03 % of cells arrested in prometaphase by the Eg5 inhibitor STLC as compared to only 23.77 ± 3.12 % of cells treated with control siRNA (Figure 3A and C). This effect was specific to UBASH3B downregulation as re-expression of the nearly endogenous levels of FLAG-tagged UBASH3B reversed the observed phenotype (30.27 ± 0.97 % cells) (Figures 3B and C). Downregulation of UBASH3B in prometaphase-arrested cells also led to chromosomal arm spreading of other components of the CPC including Survivin (Figures S6A and C) and INCENP (Figures S6B and C), consistent with their mutual co-regulation with Aurora B (van der Horst and Lens, 2013). The same localization defects of Aurora B were also observed in prometaphase cells, which were not treated with the mitotic drugs (Figure S6D). Compared to control siRNA cells, chromosome arms contained up to 4 times more Aurora B signals (Figure S6E) and the total chromosome area occupancy by Aurora B increased from 31.53 ± 2.71 % in control cells to 84.93 ± 5.74 % in UBASH3B-downregulated cells (Figure S6F). Because UBASH3B downregulated cells arrested in prometaphase by STLC showed the same results (Figure 3A), which were rescued by expression of UBASH3B protein (Figures 3B and C), we conclude that UBASH3B directly regulates Aurora B localization during prometaphase, reminiscent of CUL3 E3-ubiquitin ligase.

How can spindle associated UBASH3B control centromeric distribution of Aurora B? Interestingly, positive feedback loops (van der Horst and Lens, 2013) and spindle microtubules (Banerjee et al., 2014) were shown to regulate centromeric recruitment of Aurora B. Thus, UBASH3B could act as a molecular link between centromeric and microtubule-associated fractions of Aurora B. To test this hypothesis, we analyzed Aurora B localization in anaphase. Strikingly, downregulation of UBASH3B prevented localization of Aurora B to midzone microtubules. The majority of Aurora B remained associated with segregating chromosomes throughout anaphase, even in

small percentage of UBASH3B-depleted cells with undetectable segregation errors (Figure 3D). To test whether UBASH3B also controls the function of Aurora B in correcting erroneous microtubule-kinetochore interactions, we analyzed localization of a marker of stable microtubule-kinetochore interactions, protein RanGAP1 in cells arrested in metaphase cells by MG132. As expected (Joseph et al., 2004), RanGAP1 was found on the nuclear envelope, mitotic spindles and on stably attached kinetochores in cells treated with control siRNAs (Figure S7). In contrast, downregulation of UBASH3B led to significant, almost a two-fold reduction of the relative kinetochore intensity of RanGAP1 (Figure S7), suggesting that UBASH3B also regulates Aurora B function during mitosis. These results indicate that UBASH3B-depleted cells activate SAC response, which could explain the long delay in anaphase onset (Figure 1D) and death in prometaphase (Figures 1B, C and S2) observed upon downregulation of UBASH3B. Aurora B localization defects are similar to those observed upon inactivation of CUL3 E3 ubiquitin ligases (Maerki et al., 2009; Sumara et al., 2007), which supports the hypothesis that UBASH3B acts as a non-proteolytic ubiquitin receptor directly regulating localization of Aurora B to microtubules and its essential functions during mitosis.

UBASH3B is a rate-limiting factor, which is sufficient for Aurora B localization to microtubules.

Monoubiquitylation can serve as a signal mediating reversible recruitment of the tagged proteins to the specific cellular compartments, as shown for Fanconi anemia (Moldovan and D'Andrea, 2009). In agreement with previous findings (Murata-Hori et al., 2002), regulation of Aurora B localization by ubiquitin is likely to be dynamic and contributes to Aurora B function in faithful chromosome segregation. If this is the case, UBASH3B may regulate the balance of centromeric and spindle associated Aurora B by actively recruiting it to microtubules. To test this assumption, we inducibly overexpressed UBASH3B protein in cells arrested in a prometaphase-like

stage by STLC (Figure 4A). In control cells, the majority of Aurora B localized to the centromeric regions of circularly arranged chromosomes. Strikingly, overexpression of UBASH3B was sufficient to trigger association of endogenous Aurora B with microtubules, and both UBASH3B and Aurora B were localized to the monopolar spindles in cells with fully condensed mitotic chromosomes (Figure 4A). Likewise, the GFP-tagged form of Aurora B was actively recruited to the spindles by UBASH3B (Figures S8A and B), and it could be visualized within the microtubule bundles (Figure S8B) in prometaphase-arrested cells. In agreement with this finding, overexpression of UBASH3B in cells arrested in prometaphase, increased association of Aurora B with stable microtubules, which were detected with the antibodies to the Lysine 40 acetylated- and to detyrosinated forms of tubulin, respectively (Figure S8C). Since a drop in the CDK1 activity at the anaphase onset was reported to contribute to Aurora B microtubule association (Hummer and Mayer, 2009), we sought to analyze the levels of the activatory subunit of CDK1 kinase, Cyclin B. Importantly, the average levels of Cyclin B were not dramatically influenced by overexpression of FLAG-UBASH3B (Figures 4B and C), and both low and high Cyclin B levels could be observed in prometaphase cells with microtubule-associated Aurora B as compared to FLAG-expressing cells (Figure 4D). This result suggests that UBASH3B may target Aurora B to microtubules also in the presence of high CDK1 activity. To corroborate these findings, we have subsequently analyzed localization of Aurora B in prometaphase-arrested cells, in which degradation of Cyclin B was inhibited by the proteasome inhibitor MG132. Indeed, overexpression of GFP-UBASH3B caused relocalization of Aurora B to microtubules as compared to cells expressing GFP only (Figures 4E and Figure S9A). We conclude that UBASH3B is sufficient to determine microtubule affinity of Aurora B prior to anaphase onset.

Aurora B targeting to microtubules by UBASH3B is mediated by MKlp2 prior to anaphase.

Next, we analyzed the precise molecular mechanisms underlying UBASH3B-mediated microtubule targeting of Aurora B. Since MKlp2 kinesin was demonstrated to mediate the midzone localization of Aurora B (Gruneberg et al., 2004), we hypothesized that it may act in concert with UBASH3B prior to anaphase to shift the balance of Aurora B towards microtubules upon chromosome alignment. Moreover, MKlp2 was found on spindle already in metaphase cells (Kitagawa et al., 2014) and UBASH3B is localized preferentially to the metaphase spindles (Figure S4A). To test whether UBASH3B interacts with MKlp2, we have immunoprecipitated GFP-form of UBASH3B or GFP alone from extracts of cells arrested in mitosis by Taxol. Interestingly, similar to Aurora B, endogenous MKlp2 visibly interacted with GFP-UBASH3B but not with GFP control beads (Figure 5A), suggesting that a functional complex of Aurora B, MKlp2 and UBASH3B may exist in pre-anaphase cells. To further corroborate this observation, we subsequently overexpressed GFP-tagged UBASH3B in cells arrested in prometaphase by STLC in the presence of proteasome inhibitor MG132 and analyzed localization of MKlp2. Overexpression of UBASH3B but not the GFP-tag alone was sufficient to prematurely target MKlp2 to the monopolar spindles (Figures 5B and S9B), containing a double amount of MKlp2 as compared to the GFP-control expressing cells (Figure 5C). Accordingly, inactivation of UBASH3B by siRNA prevented microtubule and midbody association of MKlp2 in metaphase and telophase cells, respectively (Figures 5D and S10A and B). Our findings thus suggest that UBASH3B targets Aurora B to microtubules by forming a functional complex with the mitotic kinesin MKlp2 prior to anaphase. Importantly, superresolution microscopy analysis showed that a fraction of Aurora B colocalized with microtubules in a stripe-like pattern already in metaphase cells with fully aligned chromosomes in a distinct manner than a typical centromeric signals of Aurora B found in prometaphase cells (Figure 6 and Figure S10). This indicates that Aurora B switches its affinity to microtubules upon establishment of stable microtubule-kinetochore interactions and this mechanism may control timing and fidelity of chromosome segregation (Figures 1C, D, E, S2 and S3).

Targeting of Aurora B to microtubules by UBASH3B silences SAC in cancer cells.

To understand the role of UBASH3B-mediated targeting of Aurora B to microtubules in the regulation of anaphase, we overexpressed UBASH3B in prometaphase-arrested cells and analyzed the localization of the SAC component BubR1 to kinetochores. In contrast to control-transfected cells, the levels of the kinetochore associated BubR1 were dramatically reduced in UBASH3B overexpressing cells (Figures S11A, B and C), suggesting a role of UBASH3B in SAC silencing. To corroborate these findings, we analyzed protein levels of Securin (Figure S11D), the target of the Anaphase Promoting Complex/Cyclosome APC/C, which is controlled by SAC (Musacchio and Salmon, 2007). Indeed, levels of Securin, but not of Aurora B, were reduced in UBASH3B-overexpressing prometaphase cells. These observations are consistent with the reduced levels of Cyclin B, another target of APC/C, found in approximately 50% of UBASH3B overexpressing cells (Figure 4D). Consistently, overexpression of GFP-tagged UBASH3B induced premature and aberrant chromosome partitioning in prometaphase cells leading to a drastic decrease of mitotic cells (from 67.2 ± 8.26 % in control to 12.1 ± 5.92 % in UBASH3B overexpressing cells, respectively) and a significant increase of cells with multilobed nuclei (13.4 ± 3.7% and 53.3 ± 2.55%) (Figure S11E). Overexpression of UBASH3B in the cells, which were not synchronized in mitosis by drugs, also induced multilobed nuclei in 47.7 ± 2.92 % cells, compared to 11.7 ± 1.25 % in control-transfected cells (Figures 7A and B). These results strongly suggest that UBASH3B controls ploidy of cells by regulating microtubule localization of Aurora B and thereby its essential functions in chromosome segregation (Figure 7C). Importantly, UBASH3B is a rate-limiting factor mediating Aurora B localization to microtubules and thereby SAC silencing and timely onset of chromosome segregation (Figures 7C and D).

Intriguingly, UBASH3B was shown to be elevated in highly aggressive breast and prostate cancers, and to play a role in the promoting malignant growth, invasion, and metastasis (Lee et al., 2013). Cancer cells often display errors during cell division resulting in activation of SAC. We therefore wondered whether cancer cells can utilize UBASH3B to override SAC. If this is the case, depletion of UBASH3B is expected to cause a mitotic delay. We have shown above that downregulation of UBASH3B leads to the mitotic delay in prometaphase/metaphase in cervical cancer-derived HeLa cells, characterized by high levels of SAC due to increased expression of BubR1 (Greene et al., 2008). We next expanded our analysis on other cancer cell lines. Adenocarcinoma-derived MDA-MB-231 cells express high MAD2 and BubR1 levels (Greene et al., 2008), suggesting the presence of strong SAC (Figure 7E, Figure S12). Strikingly and similar to HeLa cells, UBASH3B knockdown in MDA-MB-231 cells markedly increased numbers of prometa- and metaphase-like cells indicating a delay in mitosis (Figure 7F). In contrast, UBASH3B-negative colorectal adenocarcinoma-derived Colo 320 DM cells did not change the numbers of pre-anaphase cells upon downregulation of UBASH3B. Importantly, epidermoid carcinoma-derived A431 cells, characterized by low levels of SAC did not increase pre-anaphase cell numbers upon UBASH3B knockdown (Figure 7F), in line with SAC-dependent mechanism of UBASH3B-mediated mitotic progression (Figure S11). Importantly, protein levels of SAC components were not affected by depletion of UBASH3B in all analyzed cell lines (Figure S12). Interestingly, we observed characteristic differences in localization of Aurora B in the different cell lines. Cells with little UBASH3B displayed diffused Aurora B staining on mitotic chromosomes reminiscent of UBASH3B depletion phenotypes, while cells with high UBASH3B levels showed a strong spindle localization of UBASH3B and a stripe pattern of Aurora B in metaphase cells (data not shown), suggesting that the switch-like mechanism of Aurora B localization by UBASH3B, can be adapted by different cancer cells. Taken together, these observations suggest that mitotic progression in UBASH3B-expressing cancers, characterized by strong SAC, is sensitive to alterations of

UBASH3B levels. Thus, downregulation or inhibition of UBASH3B in such cancer cells could serve as a mechanism to interrupt mitosis in tumors and a novel therapeutic avenue in cancer treatment.

Discussion

Collectively, our data suggest a model in which the initial affinity of Aurora B towards microtubules is achieved prior to anaphase (Figure 7C). This is consistent with the previous findings reporting localization and function of Aurora B on pre-anaphase spindles (Tseng et al., 2010) and with our analysis by super-resolution imaging (Figure 6). Therefore, UBASH3B mediates a switch-like mechanism on ubiquitylated Aurora B, and both loss and gain of function of UBASH3B have dramatic effects on Aurora B function and mitotic progression (Figure 7D). The mitotic cell death and defects in chromosome segregation, observed upon downregulation of UBASH3B, can be explained by compromised centromeric and spindle functions of Aurora B and thereby activation of SAC. Interestingly, spindle microtubules were also implicated in centromeric targeting of Aurora B (Banerjee et al., 2014), which could explain Aurora B spreading to the chromosomal arms in UBASH3B-deficient prometaphase cells.

Importantly, UBASH3B may act as a rate-limiting factor targeting Aurora B to the midzone region in anaphase (Figure 7C) as it is sufficient to target Aurora B to the microtubules even in the presence of fully condensed chromosomes and high CDK1 activity in prometaphase cells (Figure 4). Our data additionally argue that redistribution of Aurora B from centromeres to microtubules controls SAC silencing, timing and fidelity of anaphase and thus euploidy of cells (Figures S11 and 7A and B). Consistent with our observations in cancer cell lines (Figures 7E and F), we speculate that this important mechanism can be utilized by cancer cells with high SAC activity to promote their propagation. Future studies are needed to understand how

precisely the oncogenic potential of this non-proteolytic, intracellular ubiquitin receptor is correlated with its role in chromosome segregation.

How do ubiquitin receptors regulate mitosis?

Directionality of mitotic progression is determined by ubiquitin-dependent degradation of numerous substrates (Min and Lindon, 2012; Sumara et al., 2008). Moreover, emerging non-proteolytic ubiquitin pathways were also shown to control fidelity of mitosis (Beck et al., 2013; Maerki et al., 2009; Sumara et al., 2007). Ubiquitin receptors acting in proteolytic pathways have been shown to transfer substrates to 26S proteasome for degradation or target them to the endoplasmic-reticulum-associated protein degradation (ERAD) and autophagy pathways (Husnjak and Dikic, 2012). Ubiquitin receptors can also decode non-proteolytic ubiquitin signals in regulation of DNA repair (Al-Hakim et al., 2010; Hofmann, 2009) or NF-kB signaling (Husnjak and Dikic, 2012). However, it remained unexplored how the fate of ubiquitylated substrates is determined during mitosis, and very little is known about specific ubiquitin-binding proteins regulating different mitotic stages in mammalian cells (Fournane et al., 2012).

Our work presented here sheds some light on how the versatile ubiquitin code (Komander and Rape, 2012) can be read during mitosis and provides an example of an intracellular ubiquitin receptor controling a non-proteolytic ubiquitylation pathway. Indeed, our data strongly suggest that UBASH3B controls mitotic localization of Aurora B kinase in a non-proteolytic manner. Both downregulation and overexpression of UBASH3B do not affect protein levels of Aurora B (Figures 5A and S11D) but dramatically influence its subcellular localization (Figures 3A, D, 4A and E and S6, S9A). The observed defects in UBASH3B-downregulated cells phenocopy inactivation of mitotic CUL3-based E3-ligases (Maerki et al., 2009; Sumara et al., 2007). Furthermore, UBASH3B strongly interacts with CUL3 and with Aurora B (Figures 2A and B). Our data suggest that interaction of Aurora B with UBASH3B is, at least in part, mediated by ubiquitin recognition. Firstly, Aurora B binding to

UBASH3B depends on presence of CUL3 E3 ligase (Figure 2B) and a short recombinant fragment encompassing ubiquitin-binding moiety, the UBA domain of UBASH3B, is sufficient to isolate modified Aurora B from mitotic extracts (Figures 2C, D, E and S5D). Secondly, this interaction is abolished upon treatment of the isolated UBA-complexes with a potent deubiquitylating enzyme (Figure 2D) and upon mutagenesis of the critical residues within the UBA domain, which contact ubiquitin in cells (Figure 2E). Interestingly, the mode of UBASH3B binding to ubiquitin in cells involves two independent sites, one located within UBA domain and the second one within the extended UBA (eUBA) domain (Figures S5E and 2E). This may therefore provide specificity towards mono-ubiquitylated substrates as sugested previously by the the *in vitro* data (Hoeller et al., 2006). We conlude that UBASH3B directly interacts with Aurora B-CUL3 complex in ubiquitin-dependent manner. Importantly, UBASH3B is a rate-limiting factor determining the outcome of CUL3-mediated ubiquitylation on Aurora B, as its overexpression is sufficient to target Aurora B to microtubules. Thus, our findings uncover an important mechanism how ubiquitin signals can be read within the cells and emphasize the critical role of the ubiquitin-binding proteins in determining the fate of ubiquitylation substrates. In future, it will be important to study the precise roles of other mitotic UBD proteins identified in our study and understand their specificities towards different substrates and ubiquitin modifications.

How does UBASH3B regulate chromosome segregation and genome integrity?

Our data suggest that UBASH3B is critically involved in the regulation of chromosome segregation. Indeed, downregulation of UBASH3B results in a strong delay in anaphase onset (Figure 1D) and leads to death during prolonged prometaphase (Figures 1B, C and S2). Cells surviving depletion of UBASH3B segregate their chromosomes unequally (Figures 1E and S3), which consequently leads to the nuclei of very heterogeneous forms in the

daughter cells (Figures 1A and S1A and D). Our results strongly suggest that these phenotypes are due to defects in mitotic localization of Aurora B following inactivation of UBASH3B. We observe redistribution of the centromeric Aurora B towards chromosome arms upon downregulation of UBASH3B (Figures 3A, C and S6). In accordance with the current knowledge, reduction of the centromere-associated pool of Aurora B compromises its function at the kinetochores and kinetochore microtubules (Funabiki and Wynne, 2013; Lampson and Cheeseman, 2011; Sarangapani and Asbury, 2014). Indeed, downregulation of UBASH3B prevents formation of stable kinetochore-microtubule attachments (Figure S7), which in turn activates the SAC, thus inhibiting chromosome segregation (Figure 1C, D and S2). Interestingly, we found that UBASH3B is localized to spindle microtubules during mitosis, with the strongest signals upon chromosomes reaching the metaphase plates and possibly upon stabilization of the kinetochore-microtubule attachments (Figure S4A). Since microtubule tips of the mitotic spindle can regulate localization of Aurora B to the centromeres (Banerjee et al., 2014), we can speculate that UBASH3B regulates centromeric localization of Aurora B by its association to metaphase spindles and may act at the transition from the lateral to the end-on kinetochore-microtubule attachments. Centromeric recruitment of Aurora B is also controled by histone phosphorylation events enhanced by Aurora B-driven positive feedback loops (van der Horst and Lens, 2013). Intriguingly, a fraction of Aurora B was previously shown to localize to the metaphase spindles and to phosphorylate microtubule-bound substrates (Tseng et al., 2010), suggesting a dynamic feedback between centromeric and microtubule-associated fractions of Aurora B. Our own data obtained using super-resolution imaging confirm these observations and clearly demonstrate that Aurora B localizes to microtubules already during metaphase stage (Figure 6). Strikingly, UBASH3B is sufficient to target Aurora B to microtubules even in cells arrested with monopolar spindles in prometaphase (Figure 4A) and it appears to increase Aurora B binding to less dynamic microtubules (Figure S8B and C). Thus, our findings are in a perfect agreement with the established

role of Aurora B in stabilization of microtubule dynamics at the kinetochores (Andrews et al., 2004; Ems-McClung et al., 2013; Lan et al., 2004). Our data can also explain how Aurora B can contribute to the assembly and stabilization of the mitotic spindle (Kelly et al., 2007; Sampath et al., 2004). Thus, UBASH3B acts as a molecular link between centromeric and spindle-associated fractions of Aurora B in a rate-limiting manner and controls a switch-like mechanisms promoting Aurora B association with microtubules prior to anaphase. Indeed, downregulation of UBASH3B prevents association of Aurora B with the midzone microtubules during anaphase (Figure 3D), similar to inhibition of MKlp2 (Gruneberg et al., 2004; Hummer and Mayer, 2009). Importantly, our data argue that UBASH3B acts in concert with the MKlp2 pathway, as it is sufficient to target both Aurora B and MKlp2 to the monopolar spindles of prometaphase cells even in presence of high CDK1 activity (Figure 4B, C, E and 5B, C and S9). Interestingly, consistent with our own observations (Figure S10A), MKlp2 protein was also previously found to be localized to metaphase spindles (Kitagawa et al., 2014).

Moreover, our findings suggest that relocalization of Aurora B from centromeres to microtubules is sufficient to silence SAC signaling at the kinetochores (Figures S11), which is consistent with the reported role of Aurora B in targeting SAC proteins to kinetochores (Ditchfield et al., 2005; Hauf et al., 2003; Vázquez-Novelle and Petronczki, 2010). Therefore, both loss and gain of function of UBASH3B have strong effects on mitotic progression. Low levels of UBASH3B activate the SAC, which leads to the mitotic death, while elevated levels of this protein silence SAC and cause uncontroled division (Figure 7D). This mechanism can be adopted by cancer cells, which due to high rates of proliferation may accumulate chromosomal abnormalities that under normal circumstances activate SAC leading to apoptosis. It is particularly intriguing that downregulation of UBASH3B in cancer cell lines with high SAC levels leads to de-repression of SAC and accumulation of cells in prometa- and metaphase stages (Figure 7F). High levels of UBASH3B were also observed in highly aggressive forms of breast and pancreatic cancers in humans and in mouse models, in which UBASH3B

promoted metastasis (Lee et al., 2013). We strongly believe that the oncogenic potential of UBASH3B can be, at least partially, explained by its role in chromosome segregation. It will be important to understand the precise molecular mechanism of regulation of UBASH3B protein during mitosis as well as its expression during tumorigenesis.

Experimental procedures

UBASH3B function was assessed in cultured mitotic HeLa cells, plated on glass coverslips or cytospined on glass slides. Direct and indirect immunofluorescence microscopy was used to identify localization of UBASH3B and mitotic markers with the help of Zeiss epifluorescence microscope or confocal microscope Leica/Andor/Yokogawa Spinning Disk. For live-cell microscopy HeLa cells, stably expressing indicated proteins tagged with GFP, mRFP or mCherry, were grown on LabTek II Chambered Slides (Thermo Scientific) or μ-Slide VI 0.4 (IBIDI). Live-cell microscopy was carried out using a 40X objective of confocal microscope Leica/Andor/Yokogawa Spinning Disk. Image analysis was performed using ImageJ or Metamorph. The super-resolution imaging was performed using API OMX "Blaze" system, according to previously established protocols (Sonnen et al., 2012). The complete version of the experimental procedures can be found in the Supplemental online data, accompanying this manuscript.

Author contribution

K.K. and I.S. were responsible for experimental design, data interpretation and writing the manuscript. K.K., C.K., T.M., S.F., S.S. conducted the experiments. K.H. performed bioinformatical analysis of human UBDs and ubiquitin binding by UBASH3B. B.F. and L.B. performed the siRNA screens. N.P. W.R. and O.P. analysed siRNA screening data. The authors declare no competing financial interests.

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Figure legends

Figure 1: UBASH3B is required for chromosome segregation.

(A) HeLa cells were treated with the indicated siRNAs and analyzed by immunofluorescence microscopy (representative images are shown in Figure S1D). Number of cells with multilobed nuclei (n= 664) was quantified in control siRNA and UBASH3B siRNA cells. **(B to E)** HeLa cells expressing H2B-Cherry and EGFP-IBB were treated as in (A) and analyzed by live-cell microscopy. The percentage of cells that die during mitosis (B, n=157) was quantified. The representative frames are depicted in (C and S2). Time (in min) starting from prophase stage is indicated. Bar is 5 µm. The average time (min) from prophase to anaphase (D, n=157) and percentage of anaphase cells with segregation errors (E, n=157) was quantified. Blue bars represent control siRNAs, and red bars - UBASH3B siRNAs, respectively. In all experiments the bars represent the mean of three independent experiments and error bars indicate ± standard error of the mean (S.E.M.)

Figure 2: UBASH3B interacts directly with Aurora B-CUL3 complex in ubiquitin-dependent manner.

(A) HeLa cells expressing GFP alone (3XGFP-NLS) or GFP-Aurora B were arrested in mitosis using Taxol, immunoprecipitated using GFP-Trap beads (GFP-IP) and analyzed by Western blotting. **(B)** HeLa cells expressing GFP-Aurora B were treated with control (-) or CUL3 siRNAs (+) and synchronized and analyzed as in (A). **(C)** Recombinant GST or GST-UBA domain of UBASH3B were incubated with extracts of mitotically synchronized HeLa cells, immunoprecipitated using glutathione-sepharose beads (GST-IP) and analyzed by Western blotting. The positions of the Molecular Weight (MW) markers (in kDa) and short (SE) and long (LE) exposures of the blots are shown. Arrows indicate unmodified and ubiquitin-modified Aurora B. **(D)** Recombinant GST-UBA was incubated with extracts of HeLa cells synchronized in mitosis, immunoprecipitated using glutathione-sepharose beads (GST-IP), treated with buffer (-) or with the recombinant deubiquitylase

USP2 (+) and analyzed by Western blotting. **(E)** Recombinant wild-type (GST-UBA-WT) as well as mutated form with M47A (GST-UBA-Mut) and with W72A/H76A/D79A within extended UBA (GST-eUBA-Mut) were incubated with extracts of mitotically synchronized HeLa cells expressing GFP-Aurora B and analysed as in (A) and by Coomassie blue staining and Western blotting. Short (SE) and long (LE) exposures of the blots are shown.

Figure 3: UBASH3B is required for Aurora B localization in mitosis.

(A) HeLa cells were treated with control and UBASH3B siRNAs, synchronized by Monastrol treatment in prometaphase and analyzed by immunofluorescence microscopy. Bar is 5 µm. **(B)** HeLa cells were treated with control or UBASH3B siRNAs and simultaneously transfected with FLAG and FLAG-UBASH3B cDNAs. Cells were synchronized by Monastrol treatment in prometaphase and analysed by Western blotting. Short (SE) and long (LE) exposures of the blots are shown. **(C)** Number of cells (n= 5295) with Aurora B spread along the entire chromosomes (Aurora B spread, red bars) or Aurora B concentrated in the centromeric regions (Aurora B normal, blue bars) was quantified. Bars represent the mean of three independent experiments and error bars indicate ± standard error of the mean (S.E.M.). **(D)** HeLa cells treated as in (A) were synchronized by double thymidine block and release in anaphase and analyzed by immunofluorescence microscopy. Early (upper two rows) and late (lower two rows) anaphase cells are depicted. Bar is 5 µm.

Figure 4: UBASH3B is sufficient to localize Aurora B to microtubules.

(A and B) HeLa cells inducibly expressing GFP-UBASH3B were treated with doxycycline (DOX) (A) or treated with FLAG or FLAG-UBASH3B cDNAs (B) were synchronized in prometaphase using STLC and analyzed by immunofluorescence microscopy. Stainings of Aurora B and -Tubulin in (A) were pseudocolored in green and red, respectively. Bar is 5 µm. **(C)** Quantification of the relative intensity of Cyclin B in cells (n=131) depicted in

(B) treated with FLAG (blue bars) and FLAG-UBASH3B (red bars), respectively. Bars represent the mean of three independent experiments and error bars indicate ± standard error of the mean (S.E.M.). **(D)** Distribution of relative intensities of Cyclin B in individual cells quantified in (C). FLAG (blue lines) and FLAG-UBASH3B expressing cells (red lines), respectively. **(E)** HeLa cells treated with GFP or GFP-UBASH3B cDNAs were synchronized in prometaphase using STLC in the presence of MG132 and analyzed by immunofluorescence microscopy. The framed regions were magnified and depicted in the corresponding right panels. Other channels depicting UBASH3B and DAPI are shown in Figure S9A. Bar is 5 µm.

Figure 5: Aurora B regulation by UBASH3B is mediated by MKlp2

(A) HeLa cells expressing GFP alone (3XGFP-NLS) or GFP-UBASH3B were arrested in prometaphase using STLC, immunoprecipitated using GFP-Trap beads (GFP-IP) and analyzed by Western blotting. Short (SE) and long (LE) exposures of the blots are shown. **(B)** HeLa cells treated with GFP or GFP-UBASH3B cDNAs were synchronized in prometaphase using STLC in the presence of MG132 and analyzed by immunofluorescence microscopy. Other channels depicting UBASH3B and DAPI are shown in Figure S9B. Bar is 5 µm. **(C)** Quantification of the relative intensity ratios of MKlp2 : □-Tubulin on mitotic spindles in cells (n=277) depicted in (B) treated with GFP (blue bars) and GFP-UBASH3B (red bars), respectively. The antibodies directed to both the N-terminal part (MKlp2 Ab-NT) and to the full-length (MKlp2 Ab-FL) of MKlp2 protein were used for immunofluorescence and quantifications. Bars represent the mean of three independent experiments and error bars indicate ± standard error of the mean (S.E.M.). **(D)** HeLa cells treated with control and UBASH3B siRNAs were synchronized by double thymidine block and release in mitosis and analyzed by immunofluorescence microscopy. Other examples of mitotic stages and other channels depicting Aurora B and DAPI are shown in Figure S10A and B. Bar is 5 µm.

Figure 6: Aurora B/UBASH3B/MKlp2 complex can be found on spindles of metaphase cells.

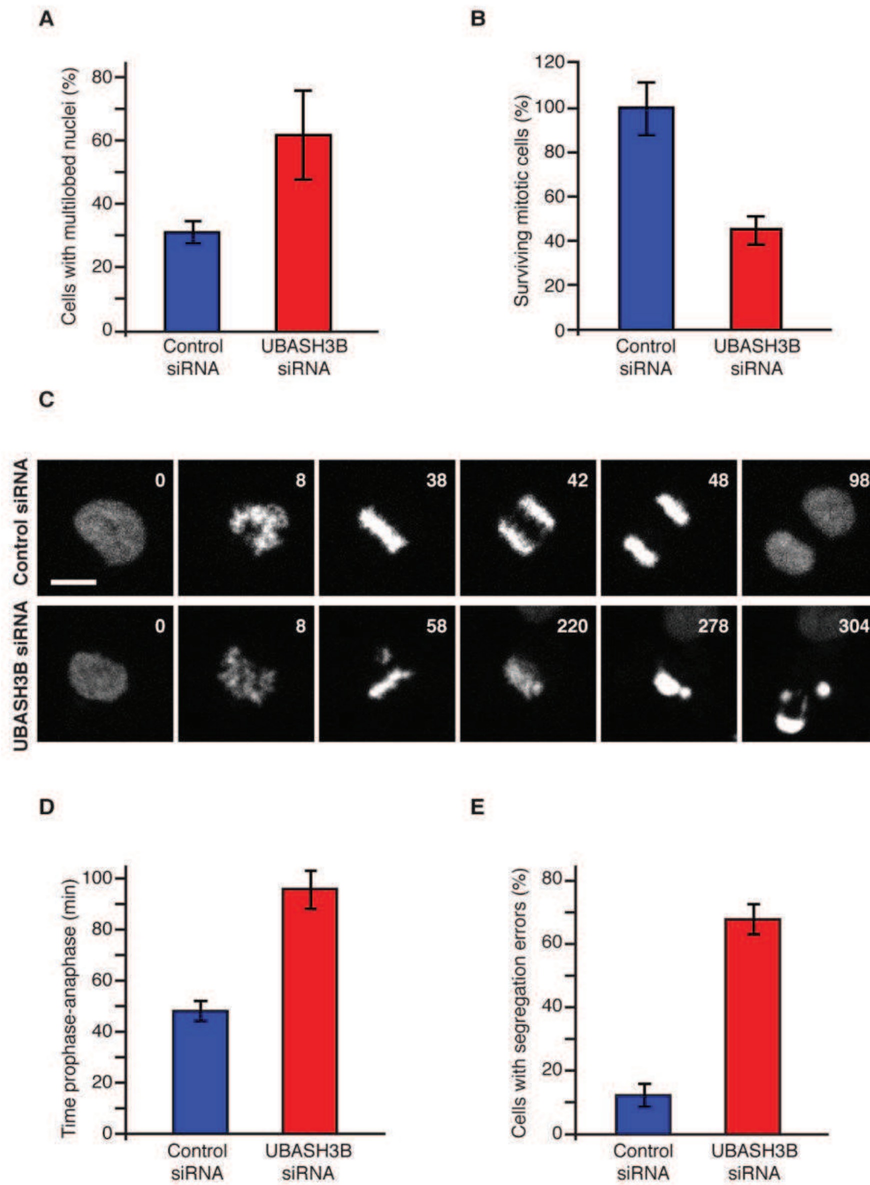
HeLa cells were synchronized by double thymidine block and release in mitosis and analyzed by super-resolution microscopy. The framed regions were magnified and depicted in the corresponding right panels. Bar is 5 µm.

Figure 7: UBASH3B controls euploidy by counteracting SAC in cancer cells.

(A and B) HeLa cells were transfected with FLAG or FLAG-UBASH3B cDNAs and analyzed by immunofluorescence microscopy. Arrowheads and arrows indicate cells with a weak and strong expression of FLAG-UBASH3B, respectively. Bar is 5 µm. **(B)** Number of cells (n=638) with multilobed nuclei was quantified in FLAG- (blue bars) and FLAG-UBASH3B-expressing cells (red bars). Bars represent the mean of three independent experiments and error bars indicate ± standard error of the mean (S.E.M.). **(C)** Model for regulation of localization and function of Aurora B by the ubiquitin receptor UBASH3B. Aurora B (red) is ubiquitylated by CUL3 (light blue) and interacts with microtubule-associated UBASH3B (yellow) prior to anaphase. UBASH3B is required and sufficient to transfer of Aurora B from the centromeric regions of chromosomes (blue) to the spindle microtubules (black lines) thereby controling its centromeric and kinetochore (grey) as well as spindle functions. UBASH3B acts in concert with MKlp2 (green) targeting Aurora B to the future midzone microtubules and controls timing and fidelity of chromosome segregation. **(D)** Schematic model depicting a switch-like mode of regulation Aurora B localization by UBASH3B and thereby SAC signaling. **(E)** Indicated cancer cell lines were analysed by Western blotting. Colo - Colo 320 DM, MDA - MDA-MB-231, HeLa - HeLaWS **(F)** Indicated cancer cell lines were treated by control (blue bars) and UBASH3B siRNAs (red bars), synchronized by double thymidine block and release in mitosis and analyzed by immunofluorescence microscopy. Number of cells (average of n=6200 per cell line) in prometa- and metaphase-like stages were quantified. "***" indicates the P value of less than

0.0001 and N.S. indicates non-significant difference. Corresponding western blots are shown in Figure S12.

Figure 1



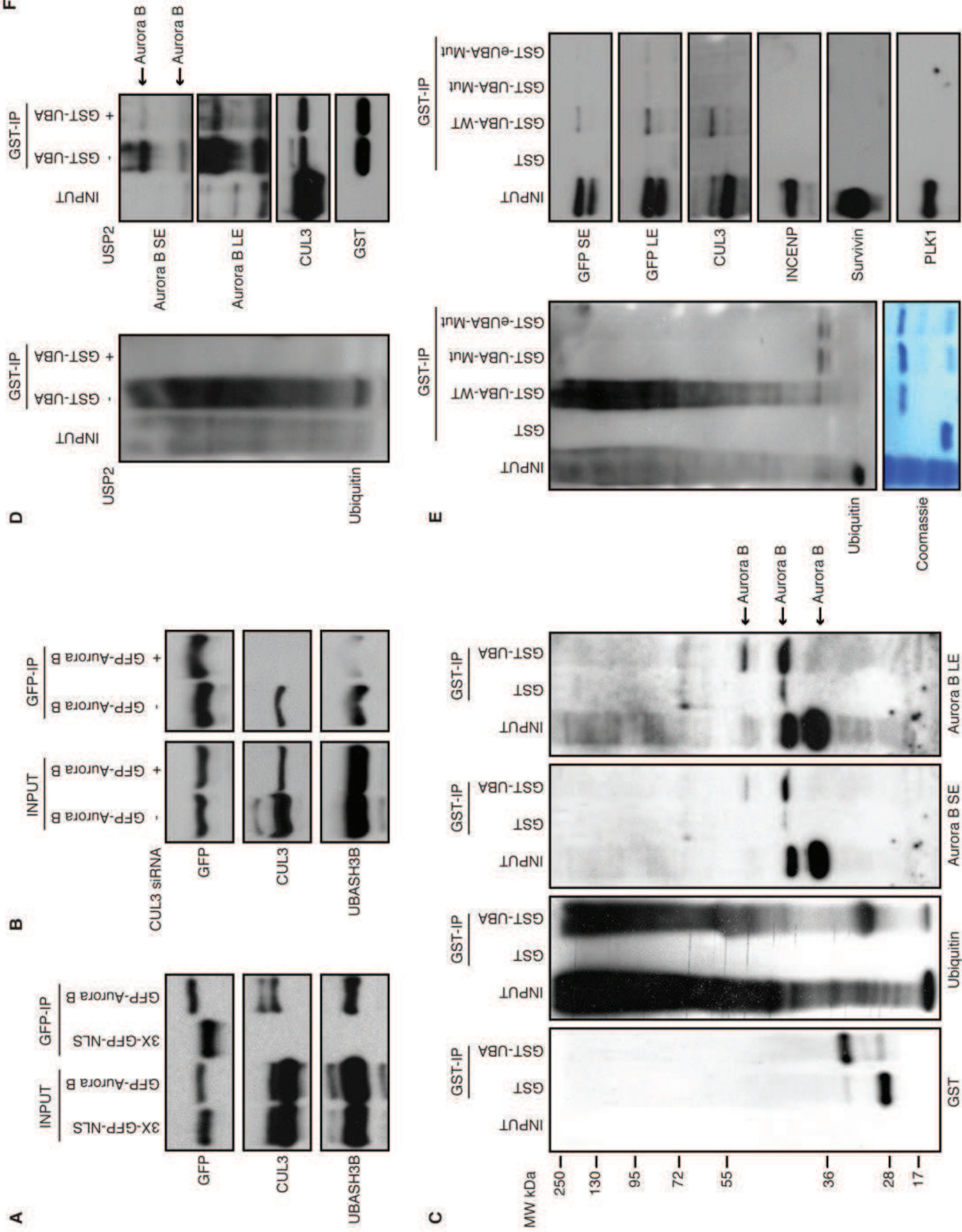


Figure 2
A
INPUT
GFP-IP
3X-GFP-NLS
GFP-Aurora B
GFP
CUL3
UBASH3B
B
CUL3 siRNA
- GFP-Aurora B
+ GFP-Aurora B
C
MW kDa
250
130
95
72
55
36
28
17
GST-IP
GST
GST-UBA
Ubiquitin
Aurora B SE
Aurora B LE
Aurora B
D
USP2
- GST-UBA
+ GST-UBA
E
GST-UBA-WT
GST-UBA-Mut
GST-eUBA-Mut
Coomassie
GFP SE
GFP LE
INCENP
Survivin
PLK1

Figure 3

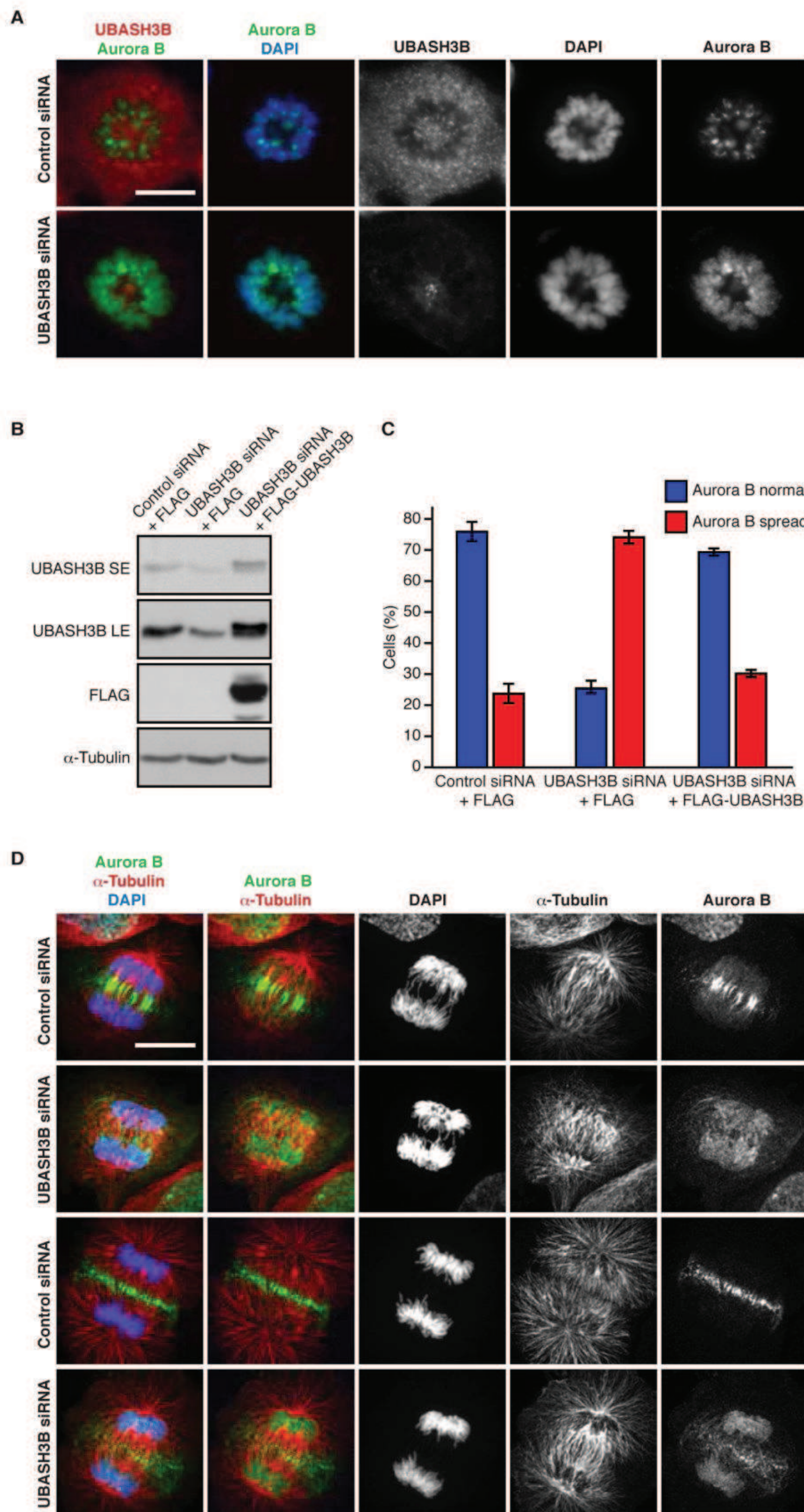


Figure 4

A

DAPI
GFP-UBASH3B
α-Tubulin

Aurora B
α-Tubulin

DAPI

GFP-UBASH3B

α-Tubulin

Aurora B

GFP-UBASH3B - DOX

GFP-UBASH3B + DOX

B

FLAG

FLAG-UBASH3B

Aurora B
Cyclin B

Aurora B

Cyclin B

C

Relative CyclinB intensity

0 1 2 3 4 5 6 7 8

FLAG

FLAG-UBASH3B

D

Relative CyclinB intensity (log)

100 1000

Cells

10 30 50 70 90 110 130

E

Aurora B
α-Tubulin

Aurora B

α-Tubulin

Aurora B

GFP + MG132

GFP-UBASH3B + MG132

Figure 5

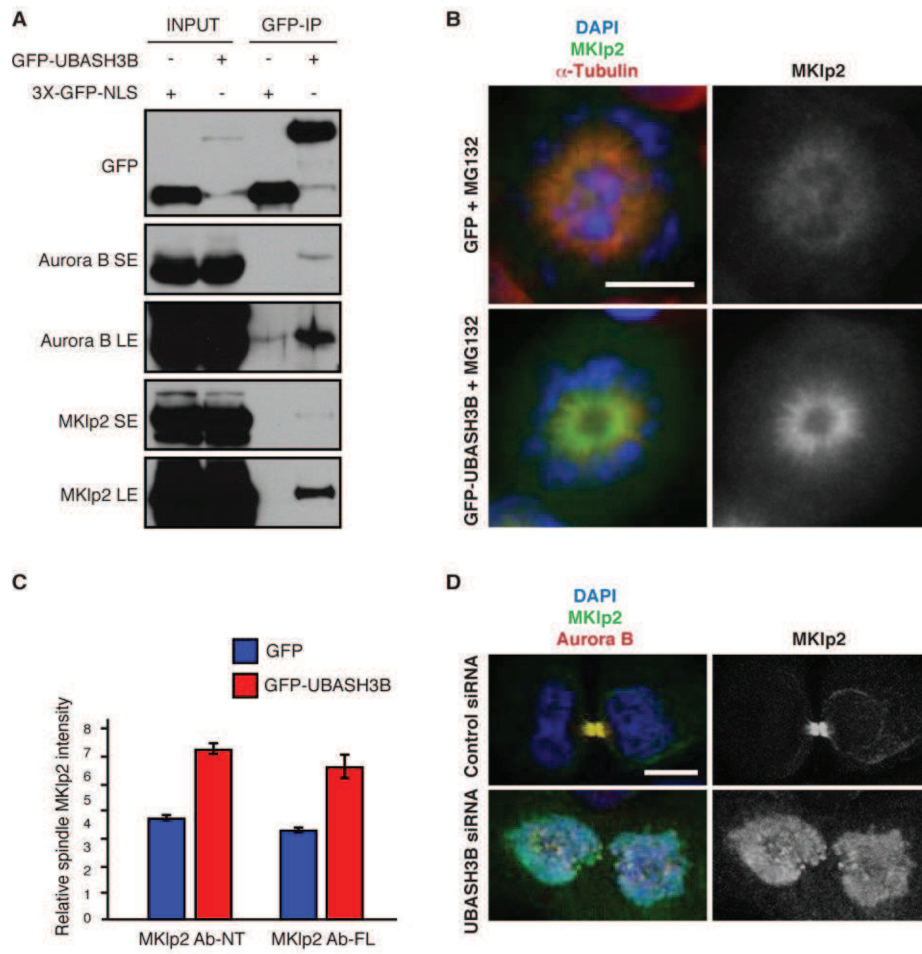


Figure 6

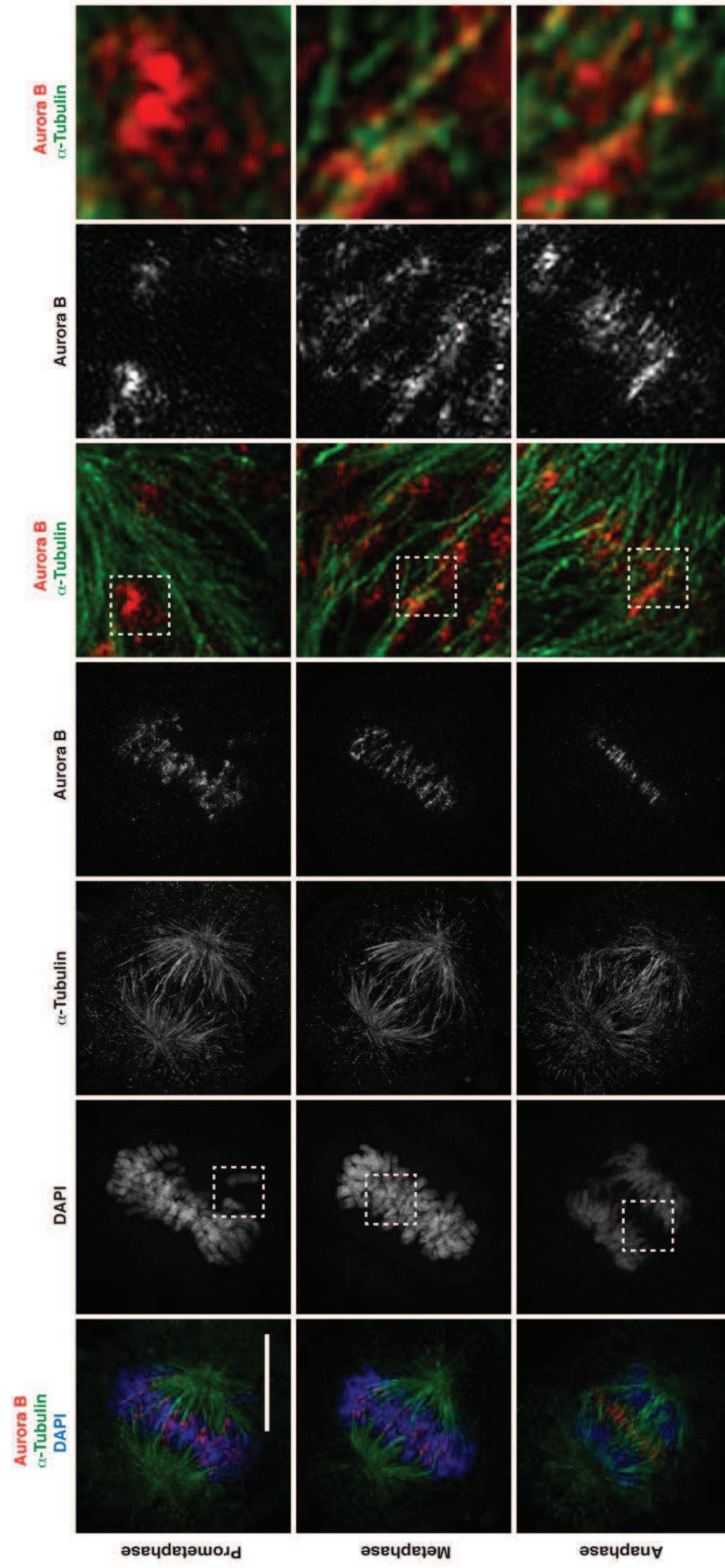
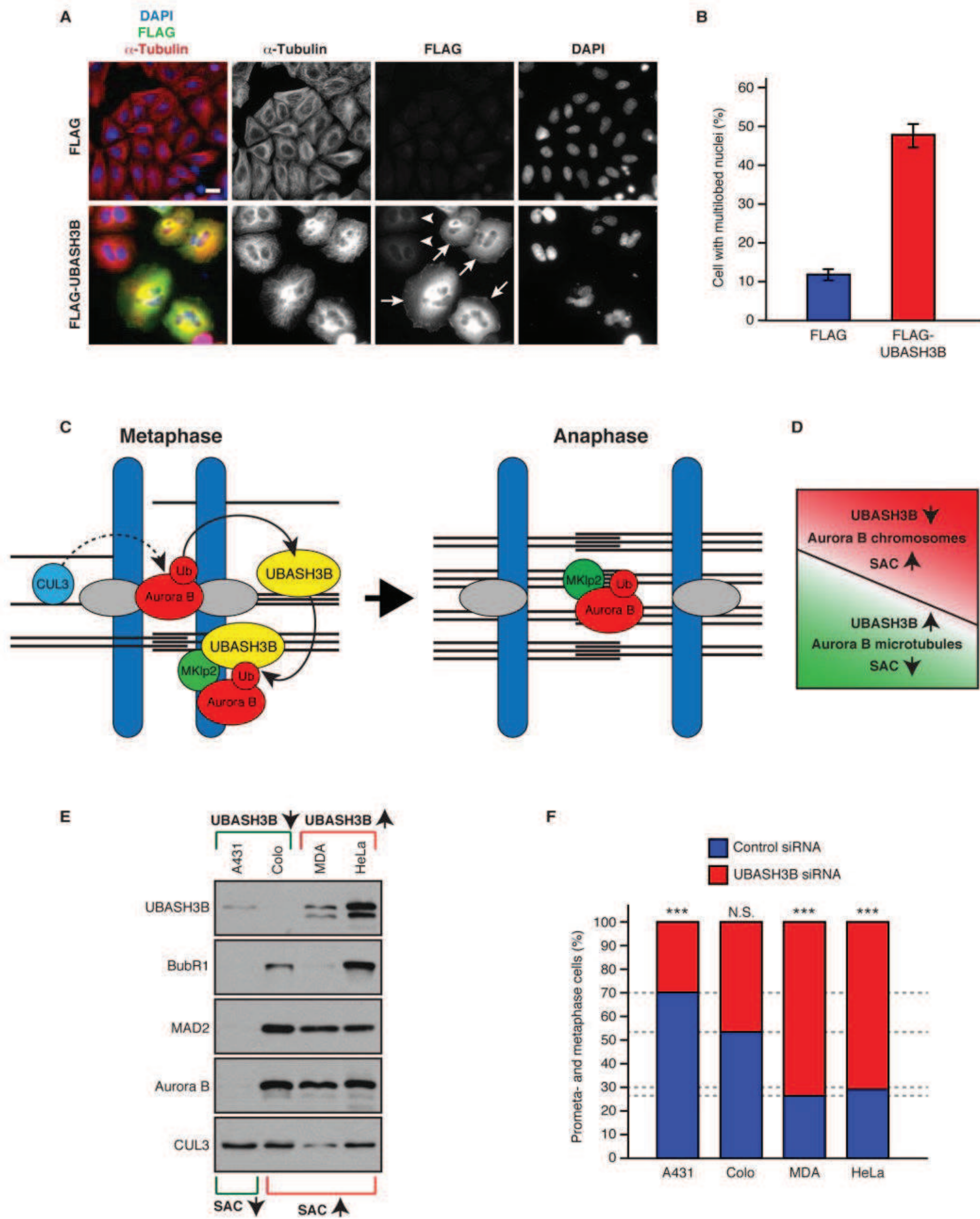


Figure 7



Supplemental Data

Ubiquitin receptor protein UBASH3B determines mitotic localization of Aurora B

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Results

High-content siRNA screen identifies mitotic UBDs

To identify UBD-containing proteins that control euploidy of dividing human cells and coordinate chromosome segregation with cytokinesis, we designed siRNA library, targeting known and predicted human UBDs (Table S1). The list also includes all known Deubiquitinating enzymes (DUBs) as they often contain ubiquitin-binding domains. Then, we performed a high content visual siRNA screen in HeLa cells and analyzed it by a multiparameter software, which was based on the Principle Component Analysis (PCA) (Supplemental experimental procedures). Our screening procedure was designed to identify factors that control fidelity of chromosome segregation, inactivation of which leads to strong terminal phenotypes such as multilobed nuclei and multinucleated cells. The list of primary hits for the putative mitotic ubiquitin receptors (Table S2) was obtained. Importantly, all four positive controls with reported roles in chromosome segregation and cytokinesis scored as hits using this approach (Table S2). Subsequently, all 24 primary ubiquitin receptors hits were confirmed as mitotic factors using a secondary screen with different set of siRNA pools (Table S3). Strikingly, 15 out of 24 (66%) identified genes were reported previously as regulators of

mitosis (table S4), confirming relevance of our approach. For instance, in line with previous studies (Cappell et al., 2012; Garnett et al., 2009; Kittler et al., 2007; Mukherji et al., 2006; Neumann et al., 2010; Whitehurst et al., 2007), inactivation of PRPF8, RAD23A and NXF2 by the siRNA pools from the validation library (table S3) led to multilobed nuclei, reminiscent of a phenotype observed upon downregulation of the key mitotic kinase Aurora B and in strong contrast to control siRNA-treated cells (Figure S1).

Experimental procedures

High-content siRNA-based Gene Knock-Down screening

For the siRNA screens, custom-made libraries were purchased from Dharmacon. 20 nM of siRNA SMARTpools with 4 different siRNAs for each gene (siGENOME for the primary screen, Table S1 and ON-TARGETplus for the secondary screen, Table S3) were transfected into HeLa cell lines (obtained from the German Cancer Research Centre DKFZ, Heidelberg, Germany) grown in Greiner µClear 96-well microplates using a high-throughput (HT) reverse chemical transfection with the INTERFERin delivery reagent (Polyplus-transfection SA, Illkirch France). The HT transfection protocol was optimized for reaching 90-95% transfection efficiency with minimal toxicity on a TECAN Freedom EVO liquid handling workstation. The screens were performed in technical triplicates. To limit biological variability, cell passage (n=3 after thawing), serum batch, transfection agent batch were strictly determined. Internal controls such as multiple positive and negative siRNA controls (Table S1), transfection efficiency control ("PLK1" siRNA that leads to cell death), were added to each microplate to determine parameters for inter-plate and day-to-day variability. Three days post-transfection, the cells were fixed and subjected to immunofluorescence with anti-□-Tubulin antibody (Sigma T5169) allowing labeling of the cytoplasmic ("cell") compartment. Secondary detection was performed with Alexa fluor-488-labeled secondary antibodies (Molecular Probes) and nuclei were stained with DAPI (labeling nucleus compartment "Nuclei"). High-

throughput cell imaging was carried out with the INCELL1000 HCS epifluorescent microscope to collect an average of ~1,000 cells per microwell.

Analysis of the high-content siRNA screening data

Multi Target Analysis parameters measuring nuclei and cell morphology for the non-targeting, control siRNA and for the Aurora kinase B siRNA-treated cells were extracted using the Multi Target Analysis module of the INCELL1000. These parameters describe the DAPI nuclei stain and the cytoplasmic □-Tubulin stain of the two assay conditions. The Principle Component Analysis (PCA) was used to identify parameters that maximize the dynamic range between positive and negative controls. The principal components that best represent these two conditions were identified using variable selection and then used to quantify the dissimilarity between the siRNA targeted genes and the negative control condition. Subsequently, a logistic model was used to identify positive and negative cells. A positive cell is a cell that looks like the cells in the positive control and a negative cell resembles the cells in the negative control. Cells were classified and a proportion of positive cells was calculated for each condition. The PCA analysis of the screened genes was performed using Aurora B kinase siRNA as a positive control and non-targeting siRNA as negative control, respectively. Multi Target Analysis parameters and their descriptions are listed below.

Multi Target Analysis parameters

The term intensity refers to pixel gray levels. The definitions correspond to the individual cell measurements. "Nuclei" measurements correspond to DAPI and "Cell" measurements to the Tubulin staining, respectively. The parameters that were used in PCA to discriminate Aurora B-like from normal cell phenotypes are indicated in bold.

Description of the individual cell measurements made in the "Nuclei" channel:

Nuc/Cell Intensity	Ratio of intensities sampled in the nuclear and cytoplasmic regions
Nuc Area	**Area of identified nucleus**
Nuc cg X	X coordinate of nucleus's center of gravity
Nuc cg Y	Y coordinate of nucleus's center of gravity
Nuc Elongation	**Mean ratio of the short axis of the nucleus to the long axis of the nucleus. If the value is 1 then the object is center-symmetric (not elongated)**
Nuc 1/(Form Factor)	**Mean nucleus roundness index. Value ranges from 1 to infinity, where 1 is a perfect circle**
Nuc Displacement	**Nuclear displacement is the distance between the nucleus's and the cell's centers of gravity, normalized by the gyration radius of the nucleus**
Nuc Intensity	Average nuclear intensity
Nuc Intensity CV	**Coefficient of variation of pixel intensities over the population of pixels comprising the nuclear region.**
Cell Intensity	Average pixel intensity in the cytoplasm region (within the Nuclei channel)
Compactness	**Compactness characterizes shape and is calculated by the formula: 2*PI*(gyration radius*gyration radius^2)/area; Gyration radius is an average radius of a shape**

Light Flux (relative)	The normalized amount of light emitted by the whole nuclei. It is equal to nucleus average intensity multiplied by area and normalized by cytoplasm average intensity. Nucleus area is taken in pixels.
Chord ratio	**Shortest chord to longest chord ratio. Both chords pass through the shape's center of gravity**
Intensity (N+C)	Average intensity in whole cell (nucleus + cytoplasm) (Nuclei channel)
Integrated Intensity (Nuc)	The amount of light emitted by the nucleus. It is equal to nucleus average intensity (Nuclei channel) multiplied by nucleus area.
	Integrated Intensity (Cell) The amount of light emitted by the cytoplasm. It is equal to cytoplasm average intensity (Nuclei channel) multiplied by cytoplasm area.
Integrated Intensity	
(Whole Cell)	The amount of light emitted by the whole cell. It is equal to cell average intensity (Nuclei channel) multiplied by cell area.

<u>Description of the individual cell measurements made in the "Cell" channel:</u>

Nuc/Cell Intensity	Ratio of nuclear to cytoplasmic intensity values (both values obtained from the Cells channel)
Cell/Bckg Intensity	Ratio of cytoplasm to local background intensity values in the Cells channel
Cell Area	Cell area

Nuc/Cell Area	**Ratio of nucleus to cell area**
Nuc Intensity	**Average intensity of pixels within the nuclear region (in the Cells channel)**
Nuc Intensity CV	**Coefficient of variation of pixel intensities over the population of pixels in the nuclear region within the Cells channel.**
Cell Gyration	Average radius of the shape Radius
Cell Intensity	**Average intensity of pixels within the cytoplasm region (in the Cells channel)**
Cell Elongation	**Mean ratio of the short axis of the cell to the long axis of the cell. If the value is 1 then the object is center-symmetric (not elongated). Short and long axes are orthogonal and do not necessarily pass through the shape center of gravity**
Cell 1/(Form Factor)	Mean cell roundness index. Value ranges from 1 to infinity, where 1 is a perfect circle
Intensity Spreading	**Indicator of intensity distribution within the object (cell). If the value is 1, then the intensity is distributed uniformly across the object (cell). If the value is > 1, the intensity concentrates near the periphery of the object . If the value is < 1, the intensity concentrates near the center of the object**
Cell cg X	X coordinate of cell's center of gravity
Cell cg Y	Y coordinate of cell's center of gravity

Cell Intensity CV	**Coefficient of variation of pixel intensities over the population of pixels in the cytoplasm region (within the Cells channel).**
Background Intensity	**Average intensity (Cells channel) of pixels in the background immediately adjacent to the cell. The background mask is obtained by dilation of the cytoplasmic outline.**
Light Flux (relative)	The normalized amount of light emitted by the whole nuclei. It is equal to nucleus average intensity multiplied by area and normalized by cytoplasm average intensity (Cells channel). Nucleus area is taken in pixels.
Intensity (N+C)	Average intensity in whole cell (nucleus + cytoplasm)
Integrated Intensity (Nuc)	The amount of light emitted by the nucleus. It is equal to nucleus average intensity (Cells channel) multiplied by nucleus area.
Integrated Intensity (Cell)	The amount of light emitted by the cytoplasm. It is equal to cytoplasm average intensity (Cells channel) multiplied by cytoplasm area.
Integrated Intensity (Whole Cell)	The amount of light emitted by the whole cell. It is equal to cell average intensity (Cells channel) multiplied by cell area.

Cell lines and cell cycle synchronization

The HeLa cell lines expressing H2B-mRFP and IBB-EGFP, EGFP-tubulin and H2B-mCherry, Aurora B-EGFP and H2B-mCherry markers were kindly

provided by D. Gerlich (Institute of Molecular Biotechnology, Vienna, Austria) (Schmitz et al., 2010; Steigemann et al., 2009). The HeLa cell line expressing three eGFP tags in tandem, fused to a NLS signal was kindly provided by P. V. Lidsky (Russian Academy of Medical Sciences, Russia) (Belov et al., 2004). HeLa Kyoto, HeLa WS and HeLa DKFZ cell cultures were maintained in Dulbecco's modified Eagle Medium (DMEM) supplemented with 10% FCS (PAA) and 1x Penicillin, Streptomycin. HeLa Tet-On 3G cell line was purchased from Clontech (catalog number 631183) and maintained in Dulbecco's modified Eagle Medium (DMEM) supplemented with 10% FBS Tet System Approved, US Sourced (Clontech, catalog number 631105) and 1x Penicillin, Streptomycin. MDA-MB-231 cell line was maintained in RPMI medium without HEPES, supplemented with 10% FCS (Gibco) and 1x Gentamicin (Sigma). Colo 320 DM cell line was maintained in RPMI medium without HEPES, supplemented with 10% FCS inactivated by heating and 1x Gentamicin. A431 cell line was maintained in DMEM (with 1 g/l of glucose), supplemented with FCS and 1x Gentamicin.

Antibiotics for selection were used as follows: hygromycin (Invitrogen) 100 µg ml−1, blasticidin (Invitrogen) 15 µg ml−1 and puromycin (Sigma Aldrich) 0.5 µg ml−1. Doxycycline (Sigma) for induction of protein expression was used at 1 µg ml−1 or 3 µg ml−1.

Chemicals (Sigma) were used as follows: Taxol (paclitaxel) for 16h 1 µM, Thymidine for 16h 2 times with wash-out in between at 2 mM and Monastrol for 16h 100 µM. inhibitor (R&D Systems) was used for 2 hours at 50 µM, STLC (Enzo Life Sciences) for 18h at 5 µM and the deubiquitylase inhibitor PR-619 (TebuBio) for 2 hours at 20 µM.

Plasmid and siRNA transfections

Transient transfections of plasmid DNA in HeLa cells were performed using Lipofectamine 2000 (Invitrogen) according to the manufacturer's instructions. In case of inducible system, plasmid expression was induced with

Doxicycline for 24-72 hours. UBASH3B-GFP-pTRE3G plasmid was transfected into HeLa Tet-On 3G cell line (Clontech, catalog number 631183) together with pCMV-Tet3G plasmid (Clontech, catalog number 631168) using Xfect transfection reagent (Clontech, catalog number 631317) according to the manufacturer's instructions. Expression of UBASH3B-GFP in pTRE3G vector was induced by adding Doxycycline for 24 hours.

Oligofectamine (Invitrogen) was used to deliver siRNAs for gene knockdown according to the manufacturer's instructions at a final concentration of 40-100 nM siRNA. The following siRNA oligonucleotides were used: non-silencing controls 5′ -UUCUCCGAACGUGUCACGU- 3′ (Microsynth), UUCUCCGAACGUGUCACGU (Qiagen); for UBASH3B downregulation pools of siRNA were used: Hs_STS-1_5 FlexiTube siRNA (Qiagen, cat. number SI02647022) and Hs_STS-1_7 FlexiTube siRNA (Qiagen, cat. number SI02647036), siGENOME Human UBASH3B (84959) siRNA SMARTpool (Dharmacon, cat. number M-008533-01-0020), 5′-GAUGCUCCCAUCACUGUGU-3′ and 5′-AUGGGUUGCUGGGAGCACA-3′ (Microsynth); for CUL3 downregulation pools of siRNA were used: 5′-CAAACUAUUGCGGGUGACU-3′ and 5′-CAACACUUGGCAAGGAGAC-3′ (Microsynth).

Primers and molecular cloning

Cloning was performed using New England Biolabs (NEB) restriction enzymes, Taq polymerase (NEB) or Fusion High-Fidelity DNA polymerase (Thermo Scientific) according to the manufacturers' instructions. Flag-UBASH3B was cloned into the vector pcDNA3.1Zeo (+) using BamHI/XhoI restriction enzymes with the primers 5′-GCCGGATCCATGGATTACAAGGATGACGACGATAAGGCTCAGTACGGCCACCCCAGTCCG-3′ (forward) and 5′-CGCCTCGAGTTATTCTTGAAGCAAGGTCTCT-3′ (reverse).

GST-UBA was cloned into the vector pGex6p1 using SalI/NotI restriction enzymes with the primer 5′-AAAGTCGACATGAAAGTCACCCCCCGGA-3′ (forward) and 5′-AAGCGGCCGCTTAGTCATCCAGGAAGGGGTC-3′ (reverse). UBASH3B-GFP was cloned into the vector pEGFP-C1 using XhoI/EcoRI restriction enzymes with the primer 5′-CCCCTCGAGCTATGGCTCAGTACGGC-3′ (forward) and 5′-CCCGAATTCTCATTCTTGAAGCAAGGT-3′ (reverse).

DNA purifications were performed using commercial kits from Macherey-Nagel according to the manufacturer's instructions.

Quantitative Real-Time PCR

RNA of cultured cells was isolated using TRIzol reagent (Sigma) according to manufacturer's instructions. Reverse transcription was performed with random hexamer primers using the SuperScript III First Strand cDNA Synthesis kit (Invitrogen). SYBR Green (Roche Diagnostics) based Real-time PCR was carried out on the LightCycler 480 (Roche Diagnostics) using gene specific primer pairs (http://pga.mgh.harvard.edu/primerbank/):
UBASH3A: 5′-CAATGACCCTTCCCTAGACGA-3′ (forward),
5′-GTACAGGCATTCCACCTTCTG-3′ (reverse);
UBASH3B: 5′-CCATGTCGGTGACCCCTTC-3′ (forward),
5′-GCTGTCCTCGCACATAAAGAA-3′ (reverse);
GAPDH: 5′-GGAGCGAGATCCCTCCAAAAT-3′ (forward),
5′-GGCTGTTGTCATACTTCTCATGG-3′ (reverse).
Analysis of quantitative real-time PCR data was performed using pyQPCR software.

Recombinant protein expression and purification

E. coli BL21 (DE3) bacteria were transformed with the pGex-6P1 constructs. Once cultures reached OD600 = 0.4 – 0.6, they were cooled down at 20 °C for

30 min. Expression was subsequently induced overnight at 20 °C with 1 mM IPTG. Cells were harvested by centrifugation. GST-fusion proteins were resuspended in lysis buffer (PBS, 1% triton X100, 1% NP40, 0.5 mM DTT, Complete Protease Inhibitor Coktail [Roche]), lysed by sonication, and supernatant was cleared by centrifugation at 15 000g for 30 minutes. The supernatant was incubated for 2 h with 500 µl of Glutathione Sepharose 4B (GE Healthcare) per 1 L of culture, previously equilibrated in lysis buffer. Beads were washed with 50 ml of lysis buffer, and elution was done with elution buffer (PBS, 34.8 mM Glutathione, 10% 1.5M Tris HCl ph 8.8).

Pulldowns, immunoprecipitations and western blotting

Preparation of HeLa cell extracts was described previously (Sumara et al., 2007). GFP-fused proteins (UBASH3B-GFP and Aurora B-GFP) were immunoprecipitated using GFP-Trap agarose beads (Chromotek). Beads were incubated with cell extracts for 2 h at 4 °C under constant rotation. Before elution, beads were washed 10 times with lysis buffer (20 mM Tris HCl pH 7.5, 100 mM NaCl, 0.5% NP40, 14 mM β-Glycerophosphate, 10% Glycerol, 1 mM NaF). GST-tagged recombinant proteins were immobilized on Glutathione Sepharose 4B, incubated with cell extracts for 2-3 h at 4°C under constant rotation in lysis buffer supplied with MG-132 (25 µM) and PR-619 (10 µM) and subsequently treated when indicated with 4 µg of Active human USP2 protein fragment (Abcam, ab125735) and USP2 Catalytic Domain (Boston Biochem, E-504) for 30 min each at 30°C in DUB buffer (150 mM NaCl, 25 mM Tris HCl pH 7.5, 10 mM DTT, 1 mM EDTA, Complete Protease Inhibitor Cocktail Tablets (Roche). Beads were washed 10 times with lysis buffer, boiled in Laemmli SDS sample buffer and subjected to SDS-PAGE.

For detection of Aurora B interaction with UBASH3B and CUL3, cells were synchronized by Taxol for 16 h. Expression of Aurora B-GFP was induced for 24 h. To detect the interaction of UBA domain of UBASH3B with Aurora B, cells were synchronized in prometaphase by treatment with monastrol or SLTC for 16-18h and subsequently treated with MG132 and PR-619 for 1 hour

to enrich for ubiquitinated substrates. Ubiquitinated Aurora B was detected using rabbit polyclonal Aurora B (Abcam ab2254).

SDS-PAGE was performed using either 8%, 9% or 10% polyacrylamide gels. Proteins were subsequently transferred from the gel to a PVDF membrane (Millipore) for immunoblotting. Membranes were blocked in 5% non-fat milk powder (BioRad) resuspended in TBS supplemented with 0.1% Tween 20 (TBS-T) for from 30 min to overnight, followed by incubation with antibodies. Membranes were developed with Luminata Forte (Millipore) or SuperSignal West Pico chemiluminescent substrate (Thermo Scientific).

Microtubule Pelleting Assay

Microtubule Pelleting Assay was performed with Microtubule Binding Protein Spin-Down Assay kit (Cytoskeleton, Cat. N. BK029) and according to the manufacturer's protocol. Briefly, a population of stable microtubules (5-10 μm in length and at a concentration of approximately 5x1011 microtubules/ml) was assembled. Microtubule Spin-Down Assay was set up as described in the manufacturer's protocol, either with recombinant proteins or cell lysate, for 30 minutes at the room temperature. Reactions were centrifuged at 100 000 g at room temperature for 40 minutes. The microtubule-containing pellet was resuspended in 1x Laemmli sample buffer and loaded on an SDS-PAGE gel.

Immunofluorescence microscopy and sample preparation

Cells were plated on 9-15 mm glass coverslips (Menzel-Glaser) in 12- or 24-well tissue culture plates. At the end of the experiments cells were washed once with PBS and fixed for 18 minutes with 4% paraformaldehyde (PFA) in PBS at room temperature. The coverslips were rinsed 3 times with PBS and permeabilized with 0.1% Triton X-100 in PBS for 5 minutes, washed with PBS and blocked by Image-iT FX signal enhancer (Life Technologies). Coverslips

were subsequently incubated with primary antibodies in blocking buffer (PBS with 0.1% Tween-20) for 1 hour at room temperature, rinsed three times with PBS and incubated with secondary antibodies in blocking buffer for 1 hour at room temperature in the dark. After incubation, coverslips were rinsed three times with PBS (with DAPI (Life Technologies) added in the 2nd wash step) and mounted on glass slides using ProLong Gold Antifade Reagent (Life Technologies) or Mowiol (Calbiochem) and imaged with a 100X, 63X or 40X objectives using Zeiss epifluorescence microscope or confocal microscope Leica Spinning Disk Andor/Yokogawa.

Live-cell microscopy and image analysis

For live-cell microscopy cells were grown in LabTek II Chambered Slides (Thermo Scientific) or μ-Slide VI 0.4 (IBIDI). Live-cell microscopy was carried out using a 40X objective of confocal microscope Leica/Andor/Yokogawa Spinning Disc. Image analysis was performed using ImageJ or Metamorph.

Super-resolution microscopy

Super-resolution microscopy was performed using API OMX "Blaze" with GE DeltaVision OMX stand and analysed with DeltaVision OMX softWoRx. Cells were grown on #1.5 High Precision Coverslips, fixed, permeabilized and stained according to the protocol for the fluorescent microscopy (see above). Coverslips were mounted on to the microscope slides with VectaShiled H1000 mounting medium (soft setting) and sealed with a nail polish.

Antibodies

The following antibodies were used: rabbit polyclonal UBASH3B (ProteinTech, 1:1000), rabbit polyclonal UBASH3B (IGBMC Polyclonal

Antibody Facility, 1:1000), mouse monoclonal α-tubulin (Sigma T5169, immunofluorescence microscopy 1:4000, western blotting 1:20000), rabbit polyclonal α-tubulin (Abcam ab18251, immunofluorescence microscopy 1:2000, western blotting 1:10000), mouse monoclonal K40-acetylated tubulin (Abcam ab11-323, western blotting 1:1000), rabbit polyclonal detyrosinated tubulin (Abcam ab48389, western blotting 1:500), mouse monoclonal Aurora B (BD Biosciences 611082/3, immunofluorescence microscopy 1:500, western blotting 1:500), rabbit polyclonal Aurora B (Abcam ab2254, immunofluorescence microscopy 1:500, western blotting 1:1000), rabbit polyclonal EGFR 1:1000 (Santa-Cruz SC-03), mouse monoclonal p53 1:1000 (IGBMC Monoclonal Antibody Facility), rabbit polyclonal INCENP 1:500 (Abcam ab36453), mouse monoclonal GFP (Abcam ab3277, 1:2000), rabbit polyclonal CUL3 (Sumara et al., 2007), rabbit polyclonal CUL3 (IGBMC Polyclonal Antibody Facility, 1:1000), mouse monoclonal ubiquitin (Santa Cruz sc-8017, clone P4D1, 1:500), mouse monoclonal GST (IGBMC Monoclonal Antibody Facility, 1:2000), rabbit polyclonal INCENP (Sigma I5283, immunofluorescence microscopy 1:250, western blotting 1:500), mouse monoclonal Borealin (MBL M147-3, clone 1D11, 1:500), rabbit polyclonal Survivin (ab469, 1:1000), mouse monoclonal EB1 (BD Transduction Laboratories 610534, clone 5/EB1, 1:500), human polyclonal CREST (Antibodies Incorporated, 15-234, 1:250), mouse monoclonal BubR1 (BD Biosciences 612502, clone 9/BubR1, 1:1000), rabbit polyclonal phospho histone H3 (Upstate 06-570, 1:500), mouse monoclonal Cyclin B1 (Santa Cruz sc-245, clone GSN1, 1:2000), rabbit monoclonal Securin (Abcam ab79546, clone EPR3240, immunofluorescence microscopy 1:1000, western blotting 1:2000), mouse monoclonal FLAG (Sigma F3165, clone M2, 1:2500), mouse monoclonal CDH1 (Abcam ab3342, clone DCS-266, 1:500), rabbit polyclonal KIF20A/MKlp2 to the N-terminal part (Proteintech 15911-1-AP, immunofluorescence microscopy 1:250, western blotting 1:2000), mouse polyclonal KIF20A/MKlp2 to full length protein (Abnova H00010112-B01, immunofluorescence microscopy 1:250).

Figure legends S1-S12

Figure S1: UBASH3B is required for chromosome segregation and cytokinesis.

(A) HeLa cells were treated with the indicated siRNAs and analyzed by high-content screening and immunofluorescence microscopy. The framed regions were magnified and depicted in panels on the right side of each row. Bar is 5 µm. **(B and C)** HeLa cells were treated with the indicated siRNAs and mRNA levels of UBASH3B (gray bars) and GAPDH (white bars) (B) or protein levels of UBASH3B and □-Tubulin (C) were analyzed by Q-PCR and Western blotting, respectively. **(D)** HeLa cells were treated with the indicated siRNAs and analyzed by immunofluorescence microscopy. Quantification of number of cells with multilobbed nuclei is shown in Figure 1A. Bar is 5 µm. **(E)** HeLa cells treated as in (B), were synchronized by double thymidine block and release and analyzed by immunofluorescence microscopy. Distribution of mitotic stages in control siRNA (white bars) and UBASH3B siRNA cells (gray bars) was quantified (n=664). P - prophase, Pm - prometaphase, M - metaphase, A - anaphase, T - telophase.

Figure S2: UBASH3B controls onset of chromosome segregation.

HeLa cells stably expressing histone marker, H2B-mCherry and the probe for postmitotic nuclear reassembly, IBB–EGFP were treated with indicated siRNAs and analyzed by live video microscopy. Time (in min) starting from prophase stage is indicated. Bar is 5 µm. Quantifications of number of cells dying during mitosis as well as the time from prophase till anaphase and of anaphase cells with segregation errors are shown in Figures 1B, 1D and E, respectively.

Figure S3: UBASH3B controls fidelity of chromosome segregation.

(A and B) HeLa cells stably expressing histone marker, H2B-mCherry and microtubule marker, EGFP-Tubulin were treated with indicated siRNAs and analyzed by live video microscopy. Time (in min) starting from prophase stage is indicated. The arrow head and the arrows indicate the midbody and the spliting daughter cells, respectively. Bar is 5 µm. **(B)** The percentage of anaphase cells with segregation errors in cells (n=148) treated with control (white bars) and UBASH3B siRNAs (grey bars) was quantified.

Figure S4: UBASH3B localizes to the mitotic spindle.

(A) HeLa cells were synchronized by double thymidine block and release in mitosis and analyzed by immunofluorescence microscopy. Bar is 5 µm**. (B and C)** HeLa cell extracts synchronized in mitosis by Taxol (B) and recombinant, full length GST-UBASH3B protein or BSA (C) were analyzed by the microtubule (MT) pelleting assays and both supernatants (S) and pellets (P) were analyzed by Coomassie blue staining and Western blotting. The positions of the Molecular Weight (MW) markers (in kDa) and short (SE) and long (LE) exposures of the blots are shown.

Figure S5: UBASH3B interacts with Aurora B-CUL3 complex in ubiquitin-dependent manner.

(A) HeLa cells were treated with control, UBASH3B and CUL3 siRNAs, synchronized by double thymidine block and release in mitosis and analyzed by Western blotting. **(B)** Schematic representation of domain organization of UBASH3B protein (aa 1-649). GST fusions with UBA domain (red) containing the fragment of the extended UBA (eUBA, orange) (aa 1- 84) were used in this study. The green rectangle depicts SH3 and dark grey rectangle depicts a phosphoglycerate mutase domain. **(C)** 1 mg of recombinant GST and GST fused to the UBA domain of UBASH3B (GST-UBA) were analyzed by SDS-

PAGE and Coomassie blue staining. The positions of the Molecular Weight (MW) markers (in kDa) are indicated. **(D)** Recombinant GST-UBA domain of UBASH3B was incubated with extracts of mitotically synchronized HeLa cells expressing GFP or GFP-Aurora B, immunoprecipitated using glutathione-sepharose beads (GST-IP) and analyzed by Western blotting. The positions of the Molecular Weight (MW) markers (in kDa) and short (SE) and long (LE) exposures of the blots are shown. Arrows indicate unmodified and ubiquitin-modified GFP-Aurora B. **(E)** Schematic representation of the sequence homology of human UBA domains (All UBA domains, above) in comparison to the extended UBA (eUBA) domains (below). Red stars indicate positions of the amino acids, which were mutated in this study and are predicted to contact the ubiquitin molecule.

Figure S6: UBASH3B regulates localization of Aurora B on mitotic chromosomes.

(A to C) HeLa cells were treated with control and UBASH3B siRNAs, synchronized by STLC treatment in prometaphase and analyzed by immunofluorescence microscopy. Bar is 5 µm. **(C)** Quantification of the relative intensity ratios of Survivin : DAPI (n=160) and INCENP : DAPI (n=157) on the entire chromosomal area in prometaphase cells from (A and B). **(D)** HeLa cells were treated with control and UBASH3B siRNAs, synchronized by double thymidine block and release in prometaphase and analyzed by immunofluorescence microscopy. The framed regions were magnified and depicted in the corresponding panels below. Bar is 5 µm. **(E)** Quantification of the relative intensity ratios of Aurora B : DAPI along the individual chromosomes (n=220) from centromeres to chromosome arms in cells form (D). **(F)** Quantification of the relative intensity ratios of Aurora B : DAPI on the entire chromosomal area (n=220) in prometaphase cells from (D).

Figure S7: UBASH3B regulates function of Aurora B on mitotic chromosomes.

(A to C) HeLa cells were treated with control and UBASH3B siRNAs, synchronized by double thymidine block and MG132 in metaphase and analyzed by immunofluorescence microscopy. The framed regions were magnified and depicted in the corresponding panels below in (B). Bar is 5 µm. **(C)** Quantification of the relative intensity ratios of RanGAP1 : CREST on the individual kinetochores (n=157) in cells shown in (A).

Figure S8: UBASH3B is sufficient to localize Aurora B to microtubules.

(A and B) HeLa cells expressing GFP-Aurora B were treated with FLAG or FLAG-UBASH3B cDNAs, synchronized in prometaphase using STLC and analyzed by immunofluorescence microscopy. Bar is 5 µm. The framed regions in (A) were magnified and depicted in the corresponding panels in (B). The arrohead and arrow indicate centromeric and spindle-associated GFP-Aurora B, respectively. **(C)** HeLa cells expressing GFP-Aurora B were treated with FLAG or FLAG-UBASH3B cDNAs, synchronized as in (A), immunoprecipitated using GFP-Trap beads (GFP-IP) and analyzed by Western blotting.

Figure S9: UBASH3B is sufficient to localize Aurora B and MKlp2 to microtubules.

(A and B) HeLa cells treated with GFP or GFP-UBASH3B cDNAs were synchronized in prometaphase using STLC in the presence of MG132 and analyzed by immunofluorescence microscopy. The corresponding selected images for Aurora B are presented in the main Figure 4E (A) and for MKlp2 in the main Figure 5B (B), while corresponding quantifications are presented in the main Figure 5C. Bar is 5 µm.

Figure S10: UBASH3B is required for MKlp2 localization to microtubules.

(A and B) HeLa cells were treated with control and UBASH3B siRNAs, synchronized by double thymidine block in metaphase (A) and in anaphase/telophase (B) stages and analyzed by immunofluorescence microscopy. The framed regions were magnified and depicted in the corresponding right panels. The corresponding selected images are presented in the main Figure 5D. Bar is 5 µm.

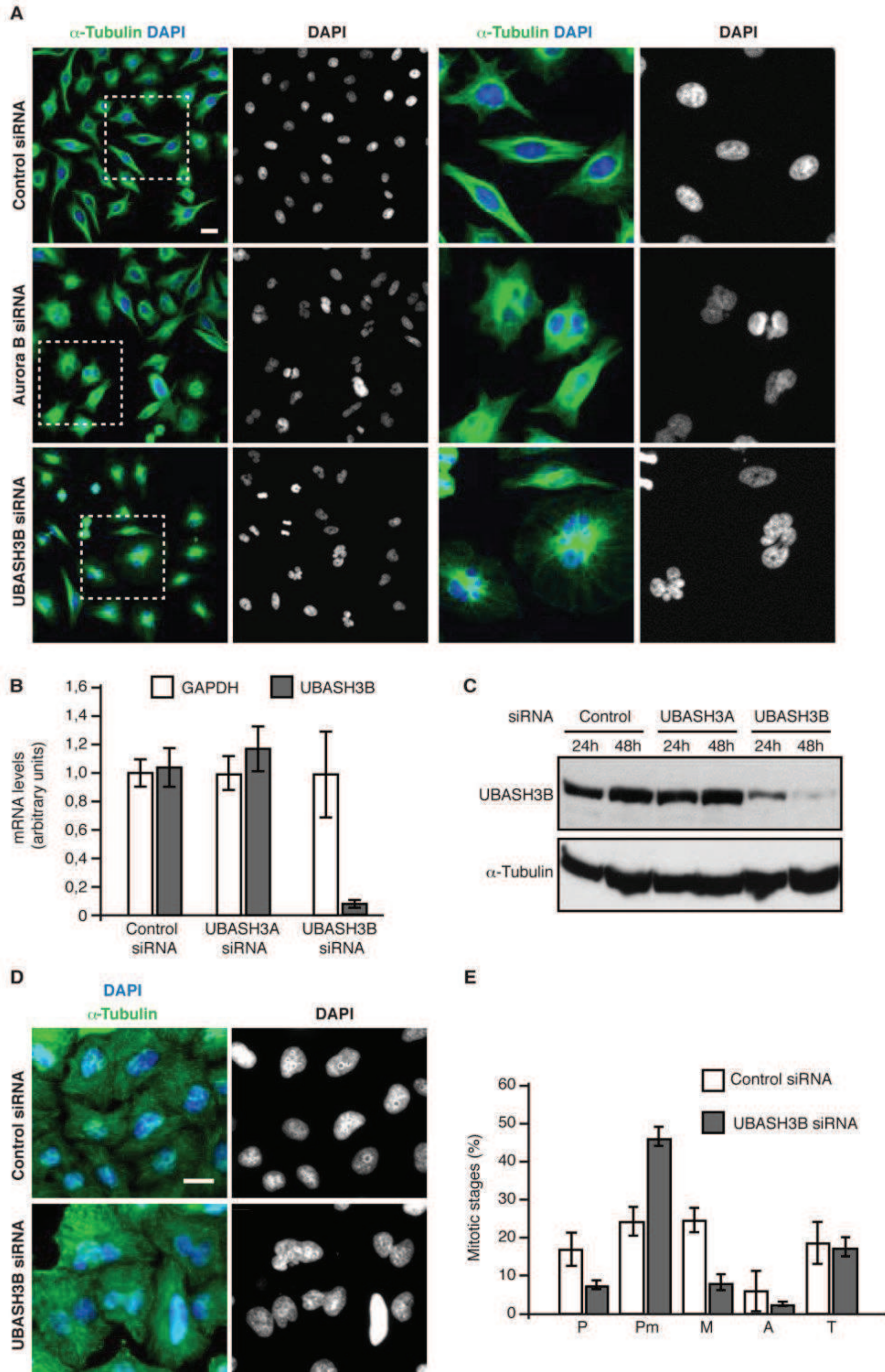
Figure S11: UBASH3B silences the Spindle Assembly Checkpoint (SAC).

(A to C) HeLa cells were synchronized in prometaphase using STLC, treated with FLAG or FLAG-UBASH3B cDNAs and analyzed by immunofluorescence microscopy. The framed regions in (A) were magnified and depicted in the corresponding panels in (B). Stainings of FLAG were pseudocolored in green. Bar is 5 µm. **(C)** Quantification of the relative intensity ratios of BubR1 : CREST on the individual kinetochores (n=380) in cells expressing FLAG (white bar) or FLAG-UBASH3B (grey bar). Bars represent the mean of three independent experiments and error bars indicate ± standard deviation (S.D.) (D) HeLa cells were treated as in (A) and analyzed by Western blotting. The short (SE) and long (LE) exposures of the blots are shown. (E) HeLa cells inducibly expressing GFP-UBASH3B were treated with doxycycline (DOX), synchronized in prometaphase using STLC and analyzed by immunofluorescence microscopy. Number of cells (n= 800) in mitotic state or in interphase with normal nuclei and with multilobed nuclei was quantified in non-expressing cells (-DOX, white bars) and GFP-UBASH3B-expressing cells (+DOX, grey bars). Bars represent the mean of three independent experiments and error bars indicate ± standard error of the mean (S.E.M.).

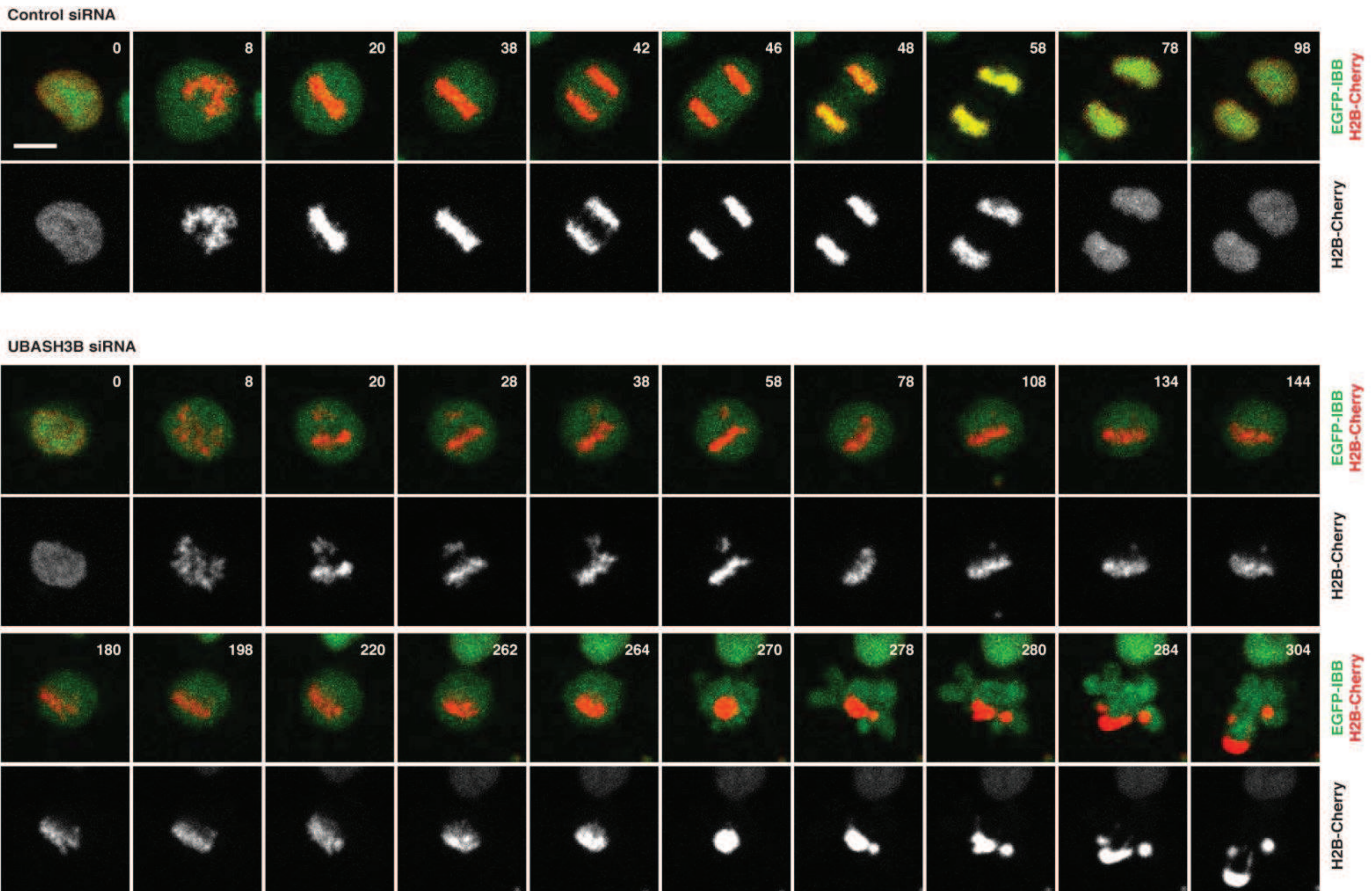
Figure S12: UBASH3B does not regulate levels of Spindle Assembly Checkpoint (SAC) components.

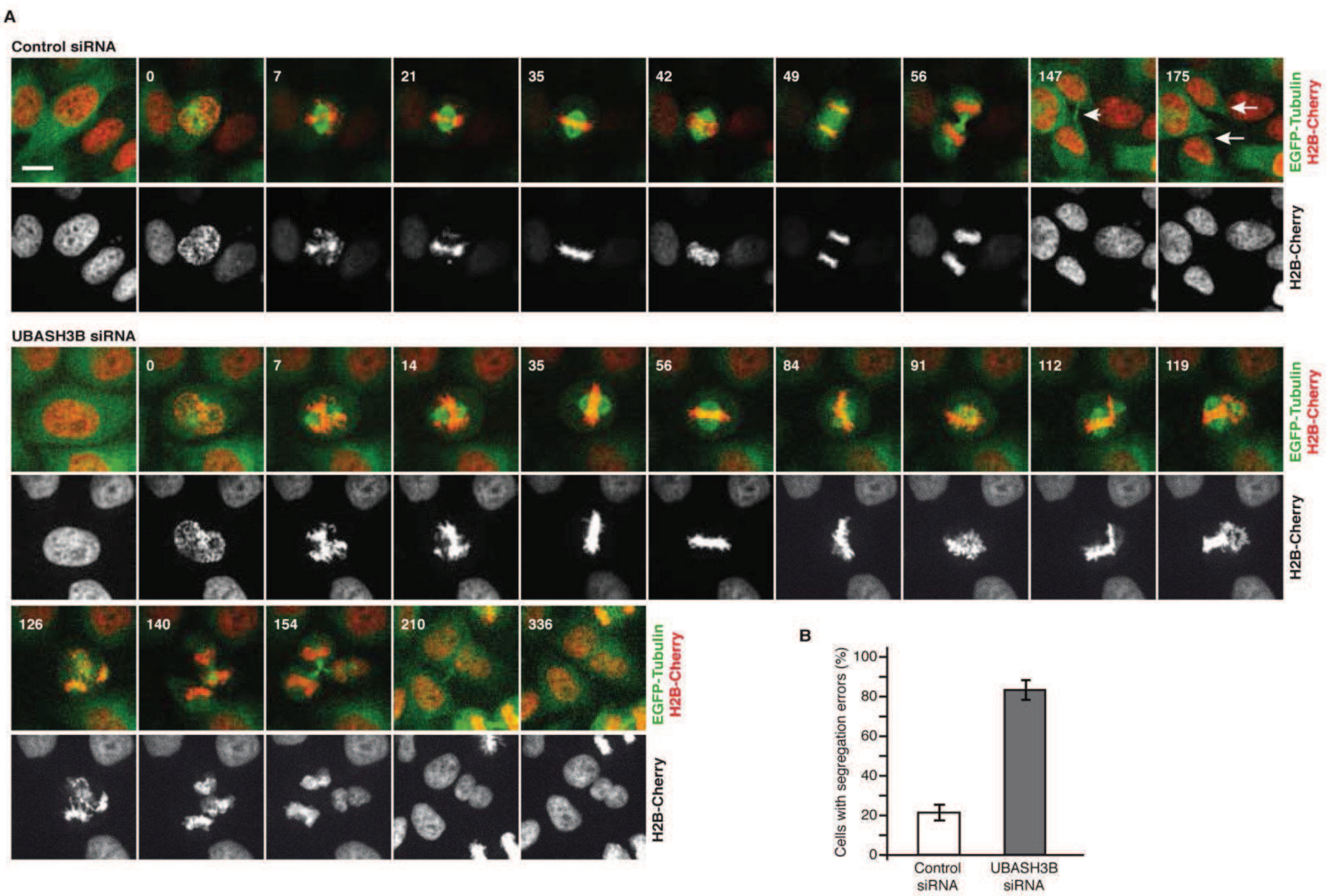
Indicated cancer cell lines were treated by control and UBASH3B siRNAs, synchronized by double thymidine block and release in mitosis and analyzed by Western Blotting and Coomassie blue staining. Colo - Colo 320 DM, MDA - MDA-MB-231, HeLa – HeLaWS. The short (SE) and long (LE) exposures of the blots are shown. The corresponding quantifications of pre-anaphase cells are shown in Figure 7F.

Supplemental Figure 1

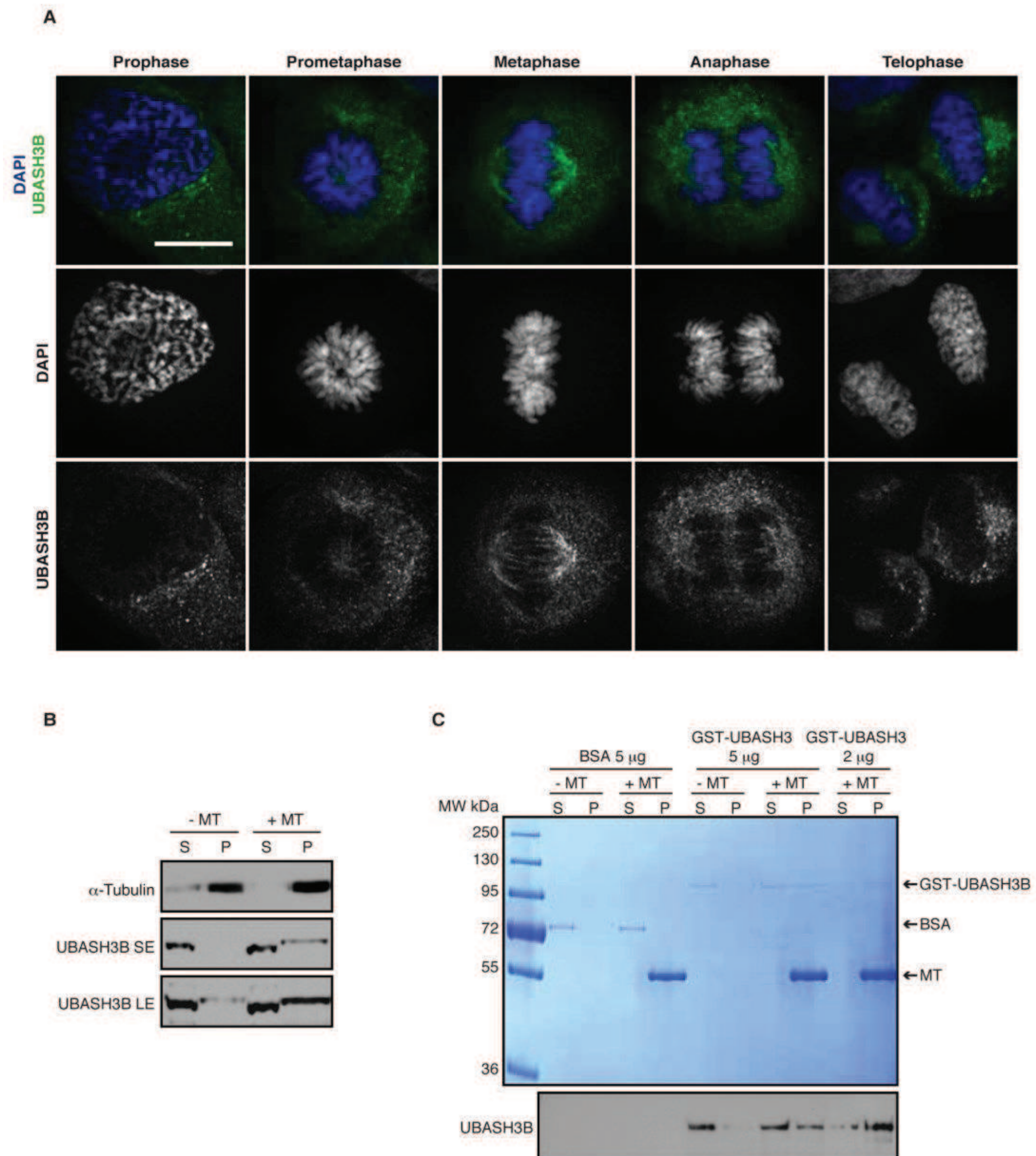


Supplemental Figure 2




Supplemental Figure 3
A
Control siRNA
0
7
21
35
42
49
56
147
175
EGFP-Tubulin
H2B-Cherry
H2B-Cherry
UBASH3B siRNA
0
7
14
35
56
84
91
112
119
EGFP-Tubulin
H2B-Cherry
H2B-Cherry
126
140
154
210
336
EGFP-Tubulin
H2B-Cherry
H2B-Cherry
B
Cells with segregation errors (%)
100
80
60
40
20
0
Control siRNA
UBASH3B siRNA

Supplemental Figure 4



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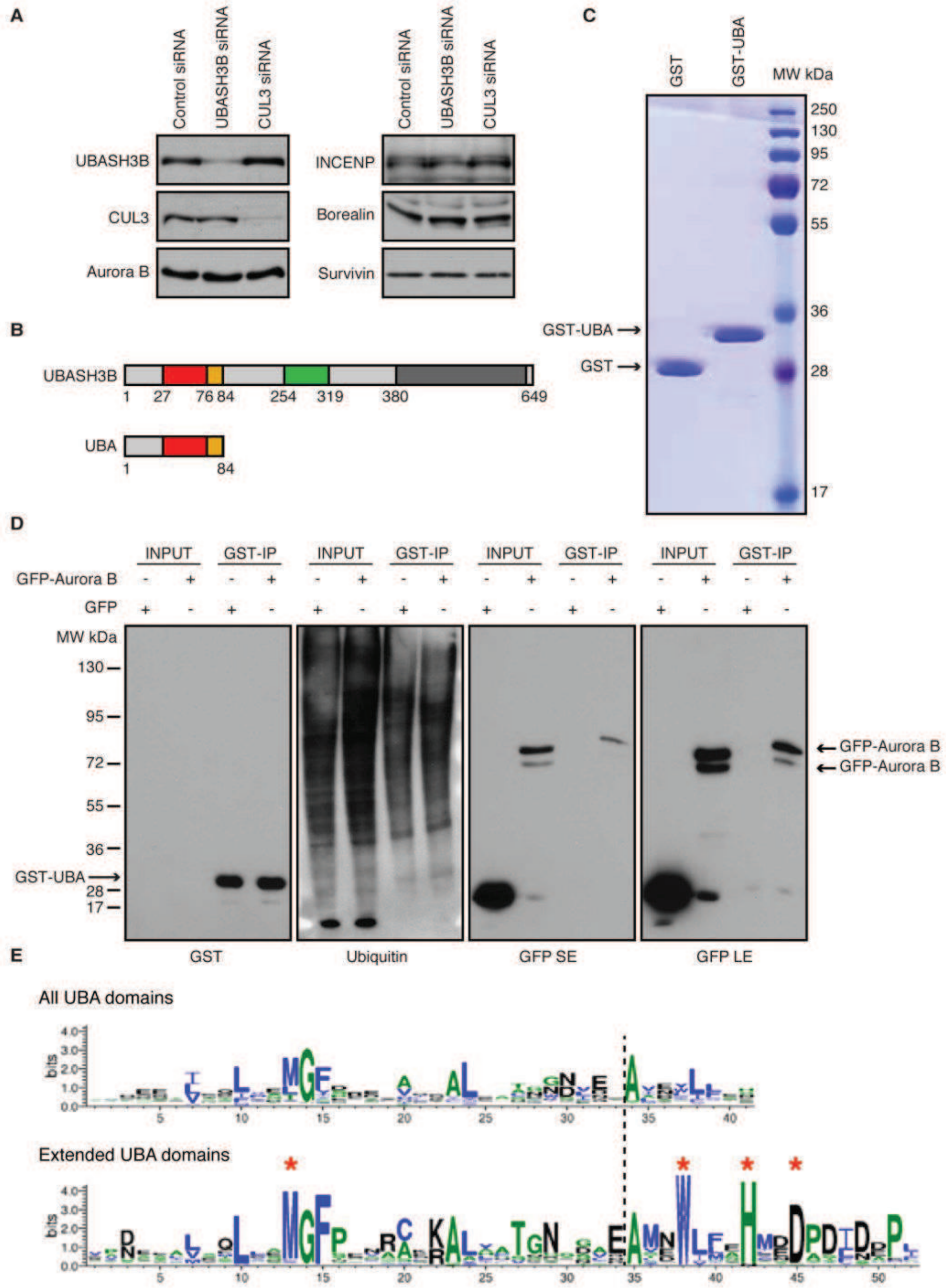
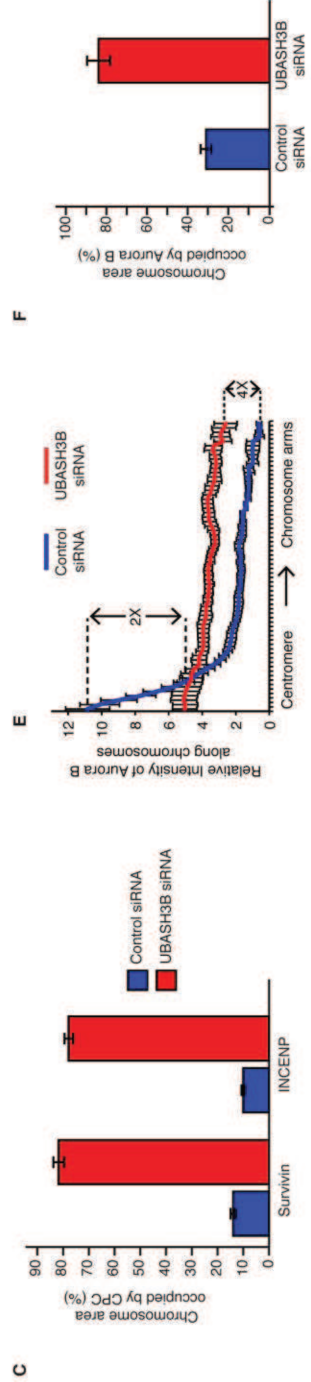
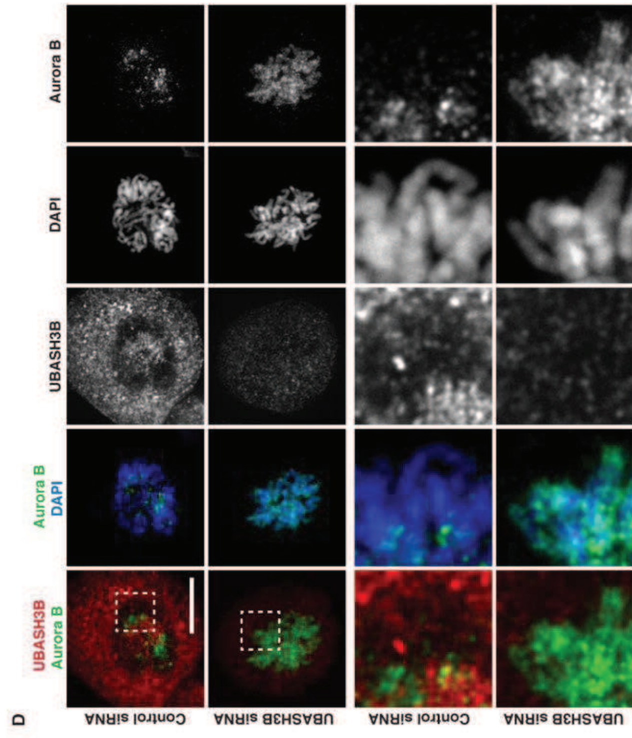
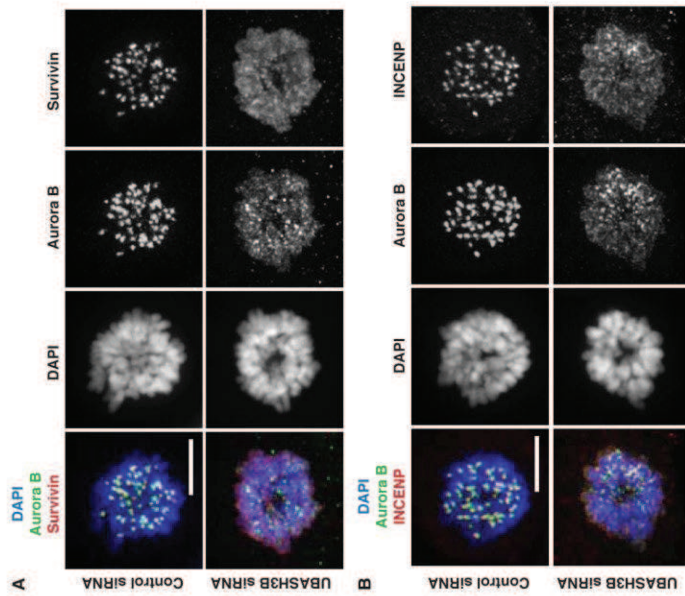


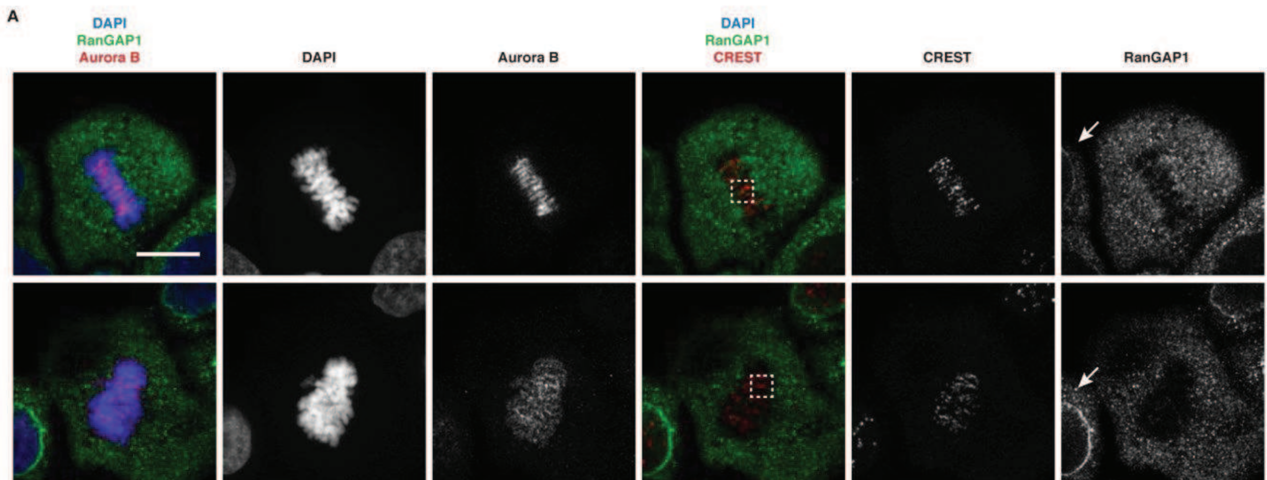
Supplemental Figure 6

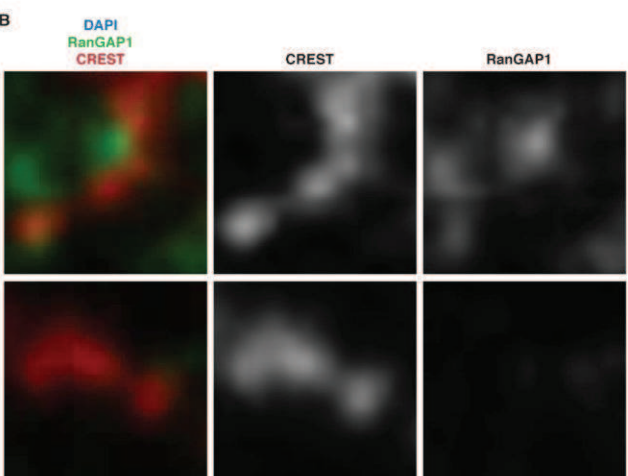


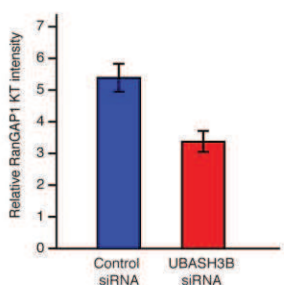




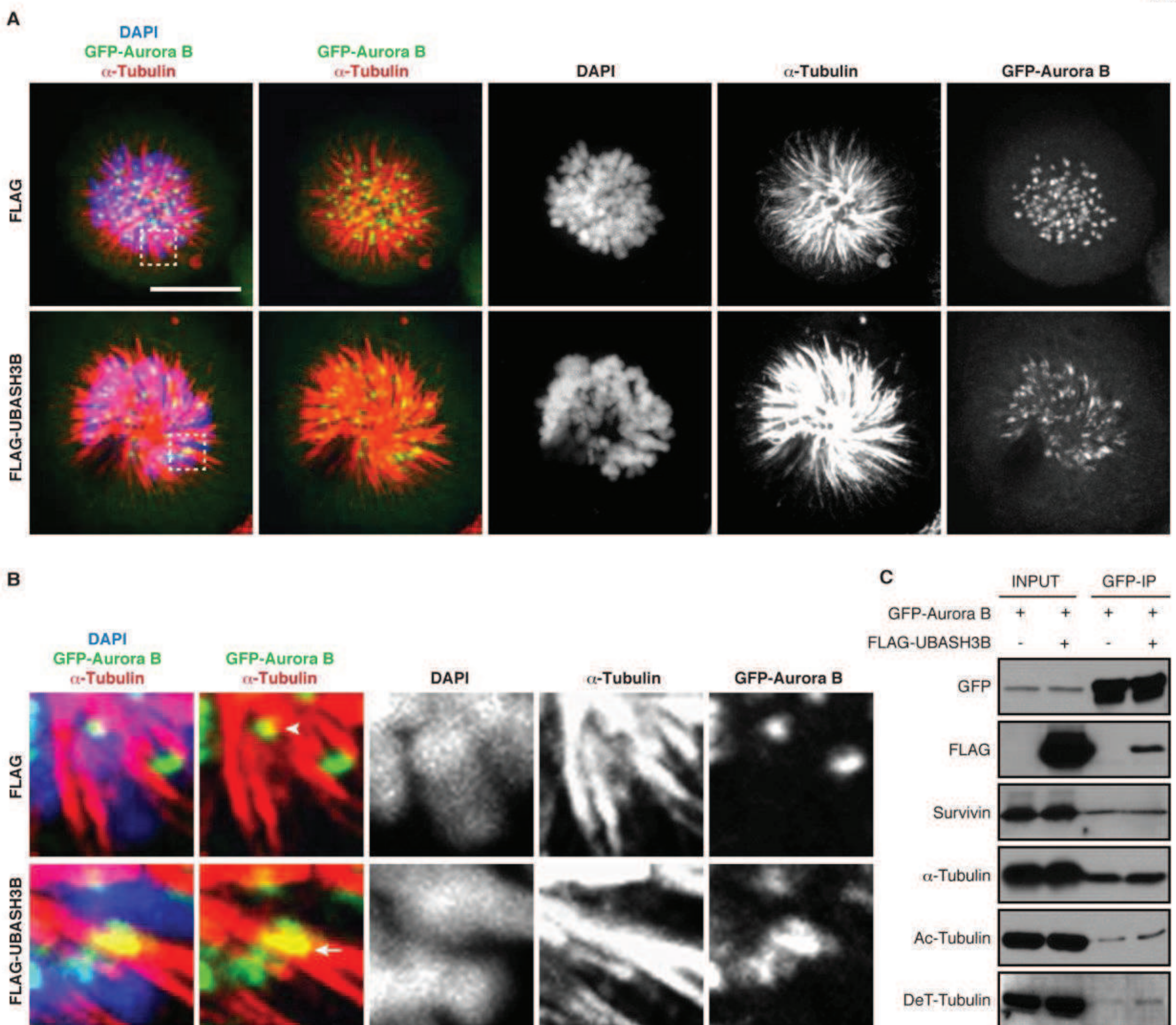
Supplemental Figure 7



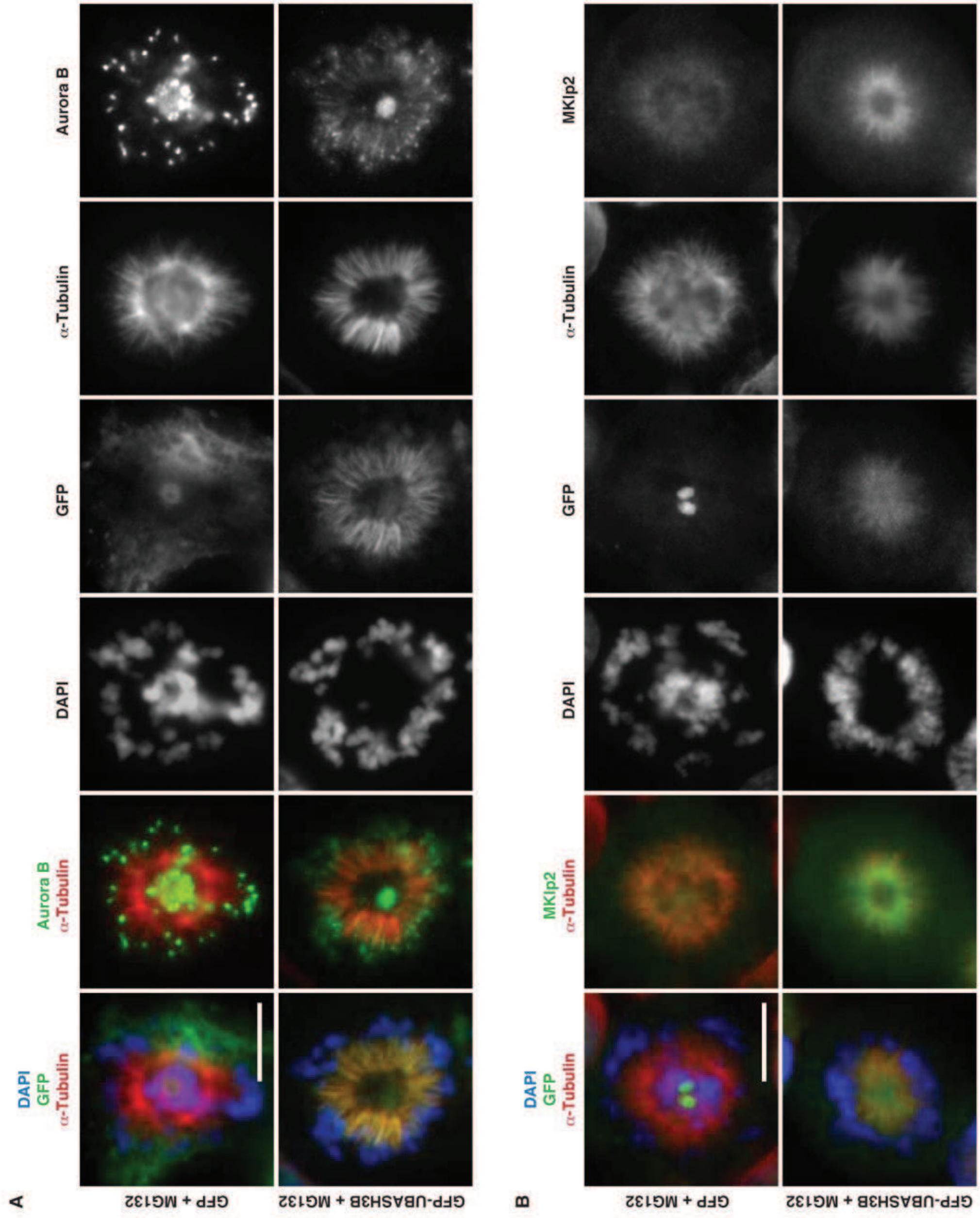




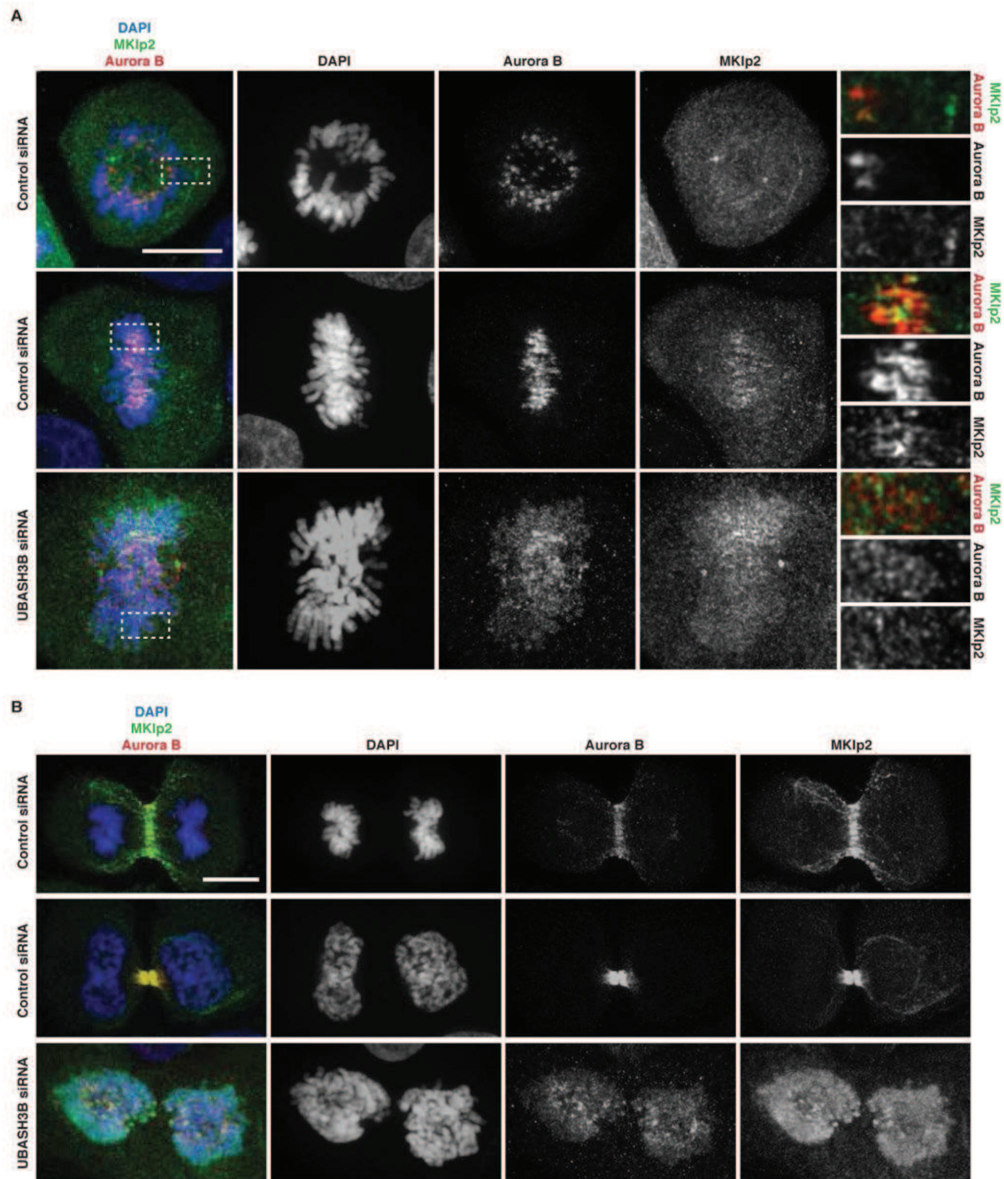
Supplemental Figure 8



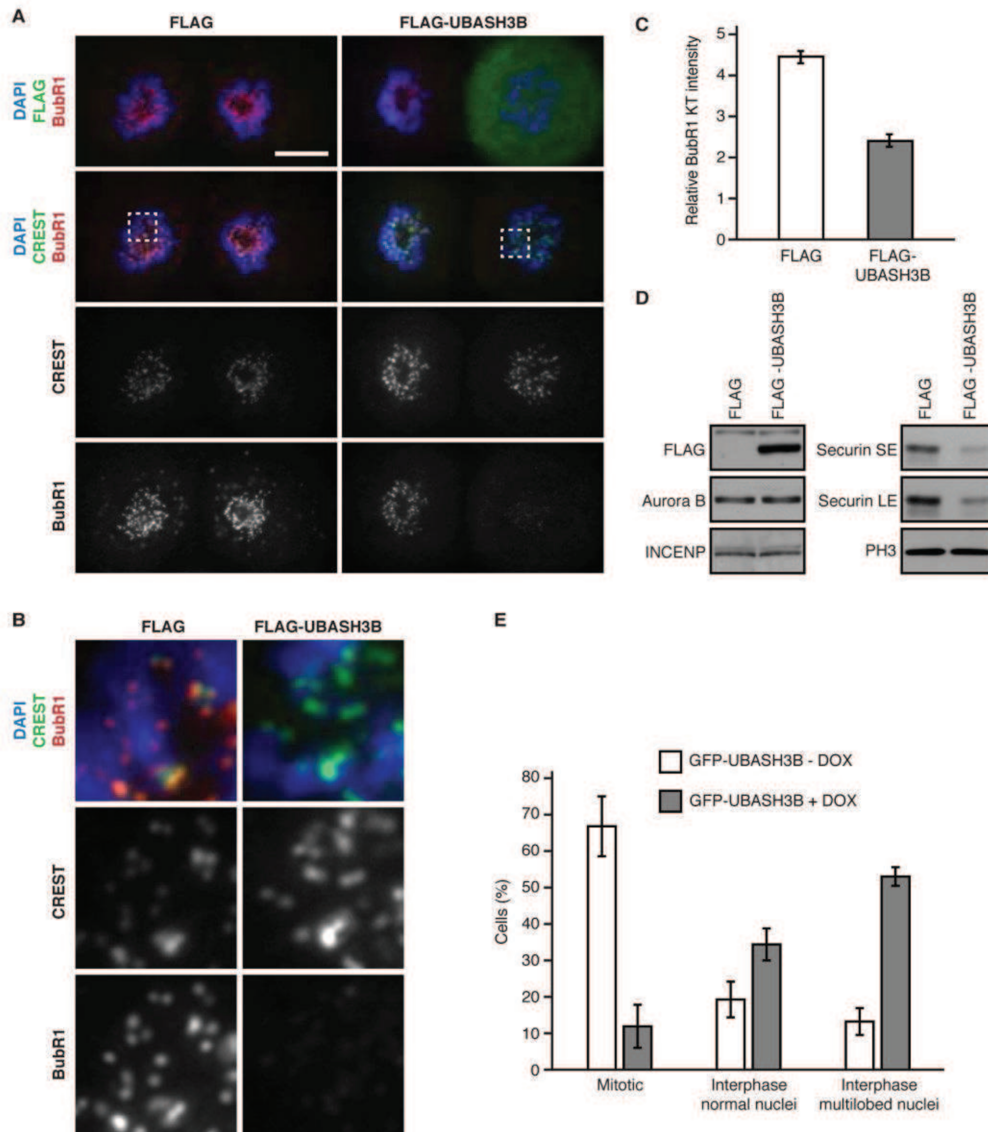
Supplemental Figure 9



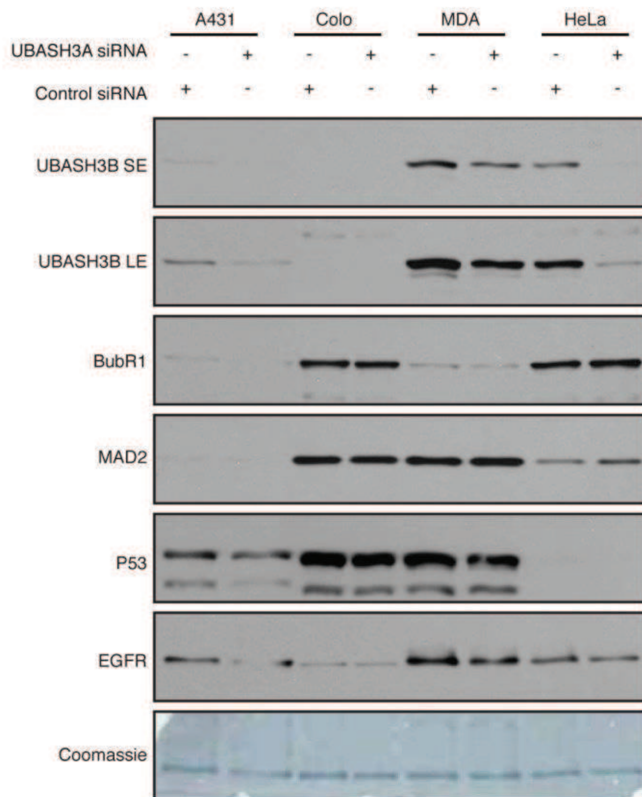
Supplemental Figure 10



Supplemental Figure 11



Supplemental Figure 12



Tables S1-S5 (legends, for tables refer to p. 164)

Supplemental Table 1: Ubiquitin decoders siRNA library.

The list of the individual siRNA oligonucteotides of the siGENOME library used as pools (SMARTpools) for targeting indicated control and targeted genes (Gene Symbol and Gene ID) in the primary siRNA screen. The Dharmacon pool catalog numbers, duplex catalog numbers and the sequences are indicated.

Supplemental Table 2: List of the hits for mitotic ubiquitin receptors identified by the siRNA screens.

The list of genes identified by the primary and secondary siRNA screens. For each hit the gene symbol, gene ID, gene accession number and uniprot number are indicated. Type/(class) depicts the positive controls and ubiquitin (Ub) receptors identified. Domains and proteins family as well as short description of each gene is provided.

Supplemental Table 3: **Ubiquitin decoders validation siRNA library.**

The list of the individual siRNA oligonucteotides of the ON-TARGETplus library used as pools (SMARTpools) for targeting indicated genes (Gene Symbol and Gene ID) in the secondary siRNA screen. The Dharmacon pool catalog numbers, duplex catalog numbers and the sequences are indicated.

Supplemental Table 4: Comparison of the mitotic ubiquitin decoders hits with reported mitotic factors.

Identified ubiquitin receptors (gene symbol, gene ID) were compared to the available published data (reported mitotic roles). Known cellular functions are described.

Supplemental Table 5: List of the CUL3-interacting proteins identified by mass spectrometry analysis.

The list of proteins related to ubiquitin signaling (gene symbol, gene name and uniprot ID) identified by mass spectrometry analysis of CUL3 immunoprecipitations. The BTB domain-containing substrates-specific adaptors for CUL3 are shaded in grey. Red shading indicates the UBA-domain protein UBASH3B.

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3.2. Aurora B interaction profile upon UBASH3B overexpression

As it was shown in the Results Part 3.1. (*Krupina et al.*), UBASH3B regulates the balance of ubiquitinated Aurora B by actively recruiting it to the microtubules. Overexpression of UBASHB leads to premature chromosome segregation without proper alignment, which is correlated with relocalization of ubiquitinated Aurora B to the microtubules of mitotic spindle already in (pro)metaphase. Therefore, I postulate that an interaction profile of Aurora B upon UBASH3B overexpression is changed, reflecting the anaphase or anaphase-like status. It is known that in anaphase Aurora B is localized to the central spindle and associated with midzone microtubule binding proteins, such as centralspindlin (see Introduction, [250,251]).

In order to investigate the mechanism of UBASH3B-mediated relocalization of Aurora B to the microtubules in more details, we performed a mass spectrometry analysis of Aurora B interactors upon UBASH3B overexpression. For this purpose, we transfected Aurora B-GFP expressing cells with flag-tagged UBASH3B or flag as a control, synchronized cells in (pro)metaphase state by Eg5 inhibitor STLC for 16 hours, performed GFP immunoprecipitation and analyzed Aurora B-GFP interacting proteins by mass spectrometry.

The resulting list of Aurora B-interacting proteins contained 70 hits, exclusively found to interact with Aurora B upon UBASH3B overexpression (Appendix I). After analyzing the hit-list and annotating proteins with Reactome online software, I focused my attention on one interesting protein, RACGAP1. This protein is a component of centralspindlin complex, which is recruited to the microtubule bundles during metaphase-anaphase transition, ensuring midzone stability, furrow ingression and abscission (see Introduction). The observation, that Aurora B interacts with RACGAP1 even before anaphase in case of UBASH3B overexpression, opens an interesting prospective about formation of “premature anaphase state” in the cell, with extraction of Aurora B to the microtubules and interaction between Aurora B and centralspidlin.

In order to corroborate this finding, I am confirming the interaction between Aurora B and RACGAP1 upon UBASH3B-flag overexpression by co-immunoprecipitation analysis.

Another interesting hit that was exclusively found in the list of Aurora B interacting proteins upon UBASH3B overexpression, is Tpr, component of nuclear pore complex, which was shown to regulate faithful chromosome segregation. Tpr acts as a spatial regulator of SAC response, which ensures a timely recruitment of spindle checkpoint proteins Mad1 and Mad2 to unattached kinetochore during metaphase-anaphase transition. Tpr also interacts with "-" end-directed motor protein dynein[252–255]. The interaction between Aurora B and Tpr was never reported before, so it would be very interesting to address the functional aspect of this interaction, its specificity to a distinct mitotic stage, possible role of Aurora B phosphorylation in correct Tpr function and potential involvement of UBASH3B in the regulation of Aurora B-Tpr interactions.

Golgi-related protein Golga2 was also found in Aurora B IP upon UBASH3B overexpression. Although there are no published data on Golga2 direct involvement in metaphase to anaphase transition, it could be an interesting candidate to study. Golga2 was shown to regulate centrosomal organization. Its deletion results in cell death in metaphase, aberrant, non functional centrosomes with multiple gamma-tubulin negative foci and centrin2-positive foci, that are unable to organize functional mitotic spindle. It has been shown that Golga2 is associated with Tuba at Golgi apparatus, which activates a subset of Cdc42, which in turn regulates centrosomal organization[256–261].

Other potentially interesting hits, identified by mass spectrometry, include histone binding protein RBBP4, which takes part in the regulation of G2/M transition and nucleosome assembly; and membrane trafficking related protein Rab-5C, which controls clatrin-derived vesicle budding.

It would be important to use slightly different conditions of immunoprecipitation (addition of proteasome inhibitor MG132), which will

allow me to reduce SAC overriding, characteristic for UBASH3B overexpression, and thus enrich for interactors of Aurora B during metaphase-anaphase transition or premature anaphase state. This will shed light on the detailed mechanism of UBASH3B-mediated Aurora B relocalization to the microtubules.

3.3. Potential interactors of UBASH3B in mitosis

The data shown in the Results Part 3.1. (*Krupina et al.*) identified UBASH3B as ubiquitin receptor protein that is critically involved in the regulation of mitosis, as both loss and gain of function of UBASH3B results in strong misregulation of chromosome segregation. Ubiquitin binding domain of UBASH3B interacts with many ubiquitinated proteins in mitosis (*Krupina et al.*, Fig. 2E), suggesting that UBASH3B can be involved in the regulation of several mitotic processes.

In order to characterize other roles of UBASH3B in mitosis, as well as to identify mechanistic aspects of UBASH3B-mediated regulation of mitotic progression, a mass spectrometry analysis of potential binding partners of UBASH3B in mitosis was performed.

Flag-tagged UBASH3B or flag alone as a control were overexpressed in HeLa cells and cells were synchronized in prometaphase by addition of STLC for 12 hours. UBASH3B and its binding partners were isolated from the lysate using anti-flag antibody resin, subsequently digested with trypsin and analyzed by mass spectrometry.

I identified 63 proteins which potentially interact with UBASH3B in mitosis (Appendix II and Table 1). Among them, 18 proteins (29%) were identified as related to mitosis, either involved in the regulation of mitotic progression (for example, Structural Maintenance of Chromosomes protein 3, SMC3; importin beta 1 subunit; moesin), localizing to mitotic structures (telomere-associated protein RIF1) or inducing tumor proliferation (CD44 antigen) (Table 1). Some of potential UBASH3B interacting proteins are important for regulation of cytoskeleton (Ras GTPase-activating-like protein IQGAP1; F-actin-capping protein subunit beta), another group of proteins is related to endocytosis (Annexin A6; Flotillin-2) or to the connection of endosomes to cytoskeleton (Erythrocyte band 7 integral membrane protein STOM; unconventional myosin-Ic and Ie). 7 proteins are directly related to ubiquitin-proteasome system, such as E3 ubiquitin and SUMO ligases (TRIM21, UBR5 and RanBP2).

Importantly, we detected Aurora B among other hits, which additionally confirmed UBASH3B-Aurora B interactions by another method of analysis.

Identification of already established UBASH3B binding partner, Aurora B, serves as a positive control for this experiment, prooving its specificity.

I focused on UBASH3B interacting proteins, related to mitotic progression, as they can provide important information about the mechanism of UBAHS3B-mediated regulation in mitosis. Interestingly, these proteins can be divided into several groups: KIFC1, moesin and importin beta 1 have an established role in the regulation of mitotic spindle organization and dynamics (shown in green, Table 1). I included telomere-associated protein RIF1 in this group as well, based on the very specific and timely organized localization of RIF1 to the midzone micortubules in anaphase. However, RIF1 involvement to the midzone assembly or faithful functioning still needs to be elucidated.

The second group of UBASH3B interacting proteins related to mitosis, includes regulators of sister chromatids cohesion (MYPT1, prohibitin-2, SMC3) or chromosome segregation (DDX21, RanBP2) (Table 1, dark blue). Interestingly, some of identified mitotic hits belong to both spindle organization/dynamics group and chromosome cohesion/segregation group (prohibitin-2, RanBP2) (Table 1, light blue). Knowing that UBASH3B is a spindle-associated mitotic factor, regulating chromosome segregation, these three groups of proteins can be very likely functionally connected to UBASH3B, being either regulated in UBASH3B-dependent manner, like Aurora B, or controlling UBASH3B localization/function in mitosis. Therefore, it will be of the highest priority for me to confirm mass spectrometry results and investigate relations of identified hits to UBASH3B.

To this end, I have already selected one of the interesting candidate proteins, KIFC1, for further analysis. KIFC1 is a “-“ end directed motor protein, which is important for mitotic spindle assembly and stability. The main function of KIFC1 is crosslinking and sliding parallel microtubules, thus regulating the length of the spindle, and focusing “-“ ends of microtubules at the spindle poles in centrosome-independent manner. Recently, it has been shown that regulation of KIFC1 levels is important fo spindle microtubule dynamics. In addition, KIFC1 controls mitotic exit, as it contributes to the formation of the spindle midzone (see Introduction).

Table 1. List of selected proteins related to mitotic progression, identified by Mass Spectrometry analysis of UBASH3B interactors. Confirmed interactor of UBASH3B Aurora B is shown in red. Proteins, involved in the regulation of cytoskeleton during mitotis, are shown in green. Proteins, regulating chromosome cohesion, are shown in dark blue. Hits, involved both in the regulation of cytoskeleton and chromosome cohesion, are shown in light blue. Regulatory subunits of 26S proteasome are shown in orange. Components of TCP chaperonin are shown in grey. Mitotic function of each protein is described.

Protein name	Function in mitosis
Aurora kinase B	Mitotic kinase, component of CPC. One of the key regulators of mitotic progression. Contributes to the loss of cohesion between sister chromatids, regulates kinetochore-microtubule attachments, establishes chromosome biorientation, involved in the midzone formation, cleavage furrow ingression and abscission (see Introduction)
Kinesin-like protein KIFC1	Minus-end directed motor protein, involved in mitotic spindle assembly and stability. Was shown to crosslink and slide parallel microtubules, which regulates spindle length and focuses "-" ends of microtubules at the spindle poles. Regulates formation of the spindle midzone in anaphase (see Introduction)
Moesin, MSN	Moesin is an actin/membrane linker, which integrates these 2 networks to synergize the cortical forces and ensure mitotic cell shape transformation after anaphase. It was shown to regulate spindle organization in metaphase. Activation of moesin upon mitotic entry by Slik stabilizes mitotic cortex, while dephosphorylation by PP1-87B/Sds22 in anaphase induces polar relaxation[262–264]
Telomere-associated protein RIF1	RIF1 is localized in the subset of central spindle microtubules during anaphase and transported to the segregated chromosomes in telophase. Function of RIF1 on these structures is unkown. Note, that no midbody RIF1 localization is detected in telophase[265]
Importin subunit beta-1	After nuclear envelope breakdown, it is involved in the inhibitory binding of mitotic factors, controlling spindle organization and containing NLS, until binding of RANGTP releases these factors. Negatively regulates kinetochore-driven microtubule nucleation by inhibiting TPX2 and HURP. Regulates spindle pole organization, chromosome alignment and mitotic progression. Interacts with RANBP2 SUMO E3 ligase and SUMO-RANGAP1, and negatively regulates RANGAP1 kinetochore localization, interacts with microtubules of mitotic spindle at least in part through dynein. Controls mitotic exit[266–271]
Prohibitin-2, PHB2	PHB2 is required for faithful mitotic progression, chromosome congression and formation of mitotic spindle. It protects centromeric region from premature removal of cohesin by Plk1-mediated phosphorylation. Interacts with cohesin subunit Scc1[272,273]

E3 SUMO ligase RanBP2 (Nup358)	RanBP2/Nup358 SUMO E3 ligases plays a role in nuclear transport and mitosis. It is associated with RanGAP1, which induces activation of a catalytic site of this complex. Knockdown of RanBP2 induced G2/M phase arrest, metaphase catastrophe and mitotic cell death. RanBP2 localizes at the plus ends of spindle microtubules and at kinetochores of chromosomes that have been captured by spindle microtubules. It sumoylates Topo II alpha in mitosis, which is required for its proper localization to inner centromeres and proper sister chromatids separation. In addition, RanBP2 is esseintial for sumoylation of CPC component Borealin. However, CPC complex formation and localization to the centromere and spindle midzone during mitosis occur independently of sumoylation[274–280]
Nucleolar RNA helicase 2, DDX21	Component of toposome, a protein complex, which stimulates the decatenation of condensed mitotic chromatin by TOPOIIα, ensuring chromosome segregation in anaphase. Interacts with PP1 and includes PP1 to the toposome in mitosis to control toposome phosphorylation status[281,282]
Protein phosphatase 1 regulatory subunit 12A, PPP1R12A (MYPT1)	Myosin phosphatase-targeting subunit 1 (MYPT1) is regulator of PP1C. It was shown to bind PP1Cβ and a 20 kDa subunit of unknown function, forming a heterotrimeric holoenzyme. Controls mitosis, antagonizing PLK1 and preventing cohesin subunit SA2 phosphorylation at the centromeres until correct microtubule-kinetochore attachments are formed. MYPT depletion leads to premature chromatid segregation in mitotically arrested cells and increased Plk1 activity[283–286]
Structural maintenance of chromosomes protein 3, SMC3	One of the main components of cohesin, which is important for maintaining of sister chromatids cohesion. Cohesin complex is cleaved and removed from chromatids in anaphase to allow faithfull chromosome segregation. In addition, cohesin complex plays a role in spindle pole assembly during mitosis and in chromosomes movement[287–289]
26S proteasome non-ATPase regulatory subunit 2	Regulatory subunit of 26S proteasome. Involved in the degradation of ubiquitinated substrates, including mitotic cyclins, and ensures separation of chromosomes in anaphase[290–292]
26S proteasome non-ATPase regulatory subunit 3	Regulatory subunit of 26S proteasome. Involved in the degradation of ubiquitinated substrates, including mitotic cyclins, and ensures separation of chromosomes in anaphase[290–292]
26S proteasome non-ATPase regulatory subunit 6	Regulatory subunit of 26S proteasome. Involved in the degradation of ubiquitinated substrates, including mitotic cyclins, and ensures separation of chromosomes in anaphase[290–292]
26S protease regulatory subunit 7 (MSS1)	ATPase, regulatory subunit of 26S proteasome. Inhibition of MSS1 ATPase activity prevents degradation of ubiquitinated substrates, including mitotic cyclins, and negatively influences separation of chromosomes in anaphase[290–292]

T-complex protein 1 subunit alpha, TCP1	TCP1 chaperonin is molecular chaperone specifically required for the folding of tubulin and actin. In addition, TCP1 is associated with centrosome, mediating the growth of microtubules off the centrosome. It is also required for the biogenesis of functional Plk1 and faithful mitotic progression. Promotes correct folding and functioning of the Cdc20 family of APC/C activators[293–297]
T-complex protein 1 subunit beta, CCT2	TCP1 chaperonin is molecular chaperone specifically required for the folding of tubulin and actin. In addition, TCP1 is associated with centrosome, mediating the growth of microtubules off the centrosome. It is also required for the biogenesis of functional Plk1 and faithful mitotic progression. Promotes correct folding and functioning of the Cdc20 family of APC/C activators[293–297]
CD44 antigen	CD44 cleavage in tumors (colon carcinomas, gastrointestinal stromal tumor, breast carcinomas, gliomas, non-small cell lung carcinomas and ovarian carcinomas) is associated with increased mitotic numbers and high rates of tumor proliferation[298,299]
E3 ubiquitin ligase UBR5 (EDD)	E3 ubiquitin ligase that modulates activity of the DNA damage checkpoint kinase, CHK2, and is involved in the maintenance of G2/M arrest after double strand DNA breaks. EDD-depleted cells are characterized by decreased levels of Cdc25A/C and CHK2, UV-resistant synthesis of DNA, premature mitotic entry, accumulation of polyploid cells and cell death via mitotic catastrophe[300]

However, the ability of KIFC1 to serve as a motor protein, ensuring relocalization of Aurora B, in complex with UBASH3B, or for transporting UBASH3B along microtubule bundles, was never investigated. Therefore, it is interesting to disclose possible functional relations between KIFC1 and UBASH3B.

First of all, I confirmed the interactions between KIFC1 and UBASH3B by immunoprecipitation (IP) analysis. For this purpose, I overexpressed UBASH3B-GFP in HeLa cells, which were subsequently blocked at prometaphase/metaphase stage with STLC. UBASH3B-GFP was immunoprecipitated from the lysates, and presence of KIFC1 among UBASH3B-interacting proteins was analyzed by immunoblotting with KIFC1 antibody. Indeed, I observed a significant enrichment of the protein in UBASH3B-GFP IP in comparison to the GFP control, thus confirming mass spectrometry results (Fig 17).

In order to investigate functional relations of KIFC1 and UBASH3B, I plan to perform localization studies of KIFC1 and UBASH3B upon UBASH3B knockdown and overexpression and KIFC1 knockdown and overexpression, respectively.

A third group of UBASH3B-interacting proteins, related to mitosis, includes regulatory subunits of 26S proteasome, which are important for proteasome functioning and timely degradation of its mitotic substrates (fig., shown in orange). Finally, another group included quality-control chaperonin proteins, regulating correct folding and function of PLK1 as well as Cdc20 family of APC/C activators (Fig., shown in grey). To corroborate mass spectrometry results, I am planning to check other selected hits for their involvement in UBASH3B–controlled pathways.

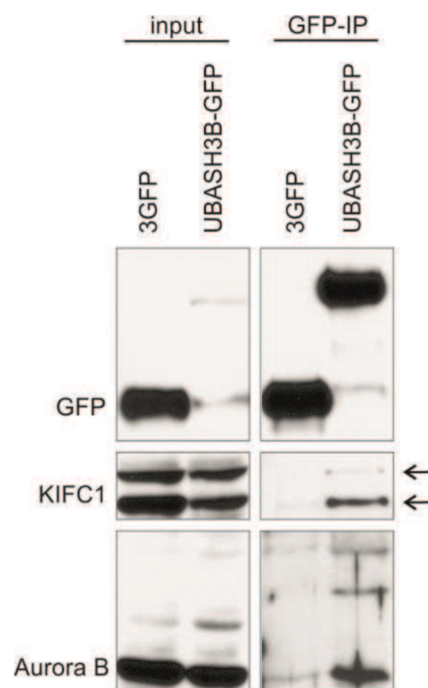

Fig. 17. GFP-IPs of mitotic HeLa cells, expressing 3GFP and UBASH3B-GFP, and visualized by Western Blot with antibodies against GFP, KIFC1 and Aurora B. No interactions of 3GFP control with KIFC1 and Aurora B as positive control was observed. Note that both KIFC1 and Aurora B are detected in UBASH3B-GFP IP.

4. Discussion

4.1. Regulation of Aurora B localization by UBASH3B

The data in the Results part of my Thesis strongly suggest that UBASH3B critically regulates chromosome segregation, serving as a non-proteolytic ubiquitin receptor for Aurora B. UBASH3B acts in the Aurora B-CUL3 pathway, directly interacting with Aurora B in ubiquitin-dependent manner. I showed that UBASH3B is required and sufficient to determine microtubule affinity of Aurora B before the onset of anaphase and chromosome segregation. Taking all these observations into account, I suggest the following model for UBASH3B-mediated regulation of Aurora B localization and correct functioning in mitosis.

During prophase, Aurora B is localized on the chromosomal arms, where it controls the loss of cohesion between arms of sister chromatids[120]. Starting from prometaphase, microtubules of mitotic spindle form initial latheral attachments to kinetochores, which are later transformed into more stable end-on attachments[155]. At this stage Aurora B relocalizes to the centromeric region, this relocalization is ensured by phosphorylation of histones H3 and H2A, presence of spindle microtubules and controlled by Aurora B-dependent positive feedback loops (see Introduction, [121-97,145,146]). At this time, centromeric Aurora B is ubiquitinated by CUL3 E3 ubiquitin ligase, localized on the spindle microtubules[151–154]. This ubiquitination serves as a signal for spindle associated ubiquitin receptor protein UBASH3B to interact with modified form of Aurora B, thus switching Aurora B dynamic balance to the association with spindle microtubules and facilitating its relocalization to the kinetochore-microtubule interface. Therefore, I propose that spindle-associated UBASH3B contributes to the regulation of centromeric localization and function of Aurora B, serving as a molecular link between microtubule-associated and centromeric Aurora.

In other words, the presence of microtubule-kinetochore attachments in prometaphase switches Aurora B function to the correction of these

attachments through its controlled localization to the kinetochore-microtubule interface. Thus, microtubules of mitotic spindle signal the initial establishment of kinetochore-microtubule attachments in ubiquitin-dependent manner: through CUL3 E3 ligase (which generates the signal) and ubiquitin receptor UBAHS3B (which decodes the signal). Histone phosphorylation in the centromeric region defines the exact place for Aurora B localization, and interactions between UBASH3B and ubiquitinated Aurora B direct Aurora B to the kinetochore-microtubule interface, thus facilitating its function in correction of kinetochore-microtubule attachments. This could explain the observed Aurora B spreading to the chromosomal arms and its inability to focus in the centromeric region upon UBASH3B downregulation (*Krupina et al.*, Fig. 3, Fig. S6). Srikingly, I could observe the rescue of Aurora B arm spreading phenotype upon UBASH3B downregulation in the absence of microtubules after nocodazole treatment (data not shown). This observation can be explained by the lack of CUL3-UBASH3B mediated signalling and absence of Aurora B affinity to the kinetochore-microtubule interface.

How does UBASH3B regulate relocalization of Aurora B to the spindle midzone? It is important to note, that full relocalization of Aurora B to the spindle microtubules is coordinated with its complete removal from centromeric region after establishment of correct kinetochore-micortubule attachments. This relocalization switches Aurora B function from error correction to the regulation of midzone structure and mitotic exit. I postulate that relocalization of Aurora B to the microtubules is a continuous process, that starts as soon as stable kinetochore-microtubule attachments are achieved. It is known that biorientation of chromosomes leads to inability of Aurora B to phosphorylate its kinetochore targets, which stabilize correct kinetochore-microtubules attachments (through intra- and inter- kinetochore stretching or/and conformation changes in Aurora B substrates upon biorientation and tension)[175,176]. Lack of Aurora B affinity to the kinetochore substrates upon biorientation leads to its increased affinity to the spindle microtubules through binding to UBASH3B, and finally facilitates Aurora B

relocalization to the spindle midzone to regulate further steps of mitotic progression.

Interestingly, UBASH3B levels on spindle microtubules increase in metaphase, which correlates with stabilization of microtubules, correctly attached to kinetochores. Interestingly, UBASH3B can bind directly to purified microtubules *in vivo* and *in vitro*, and interact with microtubule crosslinker proteins KIFC1 and MKLP2. Although the dynamics of UBASH3B interactions with micortubules still needs to be elucidated, I can speculate that stabilization of microtubules under tension (which correlates with correct kinetochore-microtubule attachments) may lead to more efficient and stable association of UBASH3B with KIFC1 or/and MKLP2-positive microtubule bundles. As a consequence, ubiquitinated Aurora B will be fully removed by UBASH3B from kinetochore-microtubule interface, and relocalized to the forming microtubule bundles of the spindle midzone.

Moreover, I could show that UBASH3B is also sufficient to relocalize Aurora B to the microtubules of mitotic spindle in cells blocked in prometaphase (*Krupina et al*., Fig.4A, Fig. S8). Previous published results confirm our findings as a fraction of Aurora B is able to localize to the microtubules of metaphase spindle and phosphorylate microtubule-binding substrates[301]. UBASH3B downregulation disturbs Aurora B focusing in the centromeric region and compromizes its correct function in this location. At the same time, knockdown of UBASH3B prevents relocalization of Aurora B to the microtubules upon achievement of stable kinetochore-microtubule attachments. Altogether, this is reflected in Aurora B localization on chromosomes and significant misregulation of mitotic progression.

Importanly, both lack and gain of UBASH3B function is critical for proper chromosome segregation. Downregulation of UBASH3B leads to improper localization of Aurora B in mitosis, and either cell death during prometaphase-like stage (in case of full absence of UBASH3B) or significant delay in chromosome segregation together with improper alignment due to compromized Aurora B function (when amounts of UBASH3B are not sufficient for its correct fucntion) (*Krupina et al*., Fig. 1, Fig. S3, Fig. S4). At the

same time, overexpression of UBASH3B induces very rapid extraction of Aurora B to the spindle microtubules, which is sufficient for SAC silencing at kinetochores (see below). Taking these observations into account, I postulate, that UBASH3B levels and localization during mitosis has to be very precisely regulated. It would be interesting to identify molecular mechanisms of this regulation.

Collectively, my data suggest that UBASH3B is critically involved in the regulation of chromosome segregation, acting as a molecular link between centromeric and spindle-associated fractions of Aurora B and controls a switch-like mechanism promoting Aurora B association with microtubules prior to anaphase.

4.2. Spindle assembly checkpoint, Aurora B and UBASH3B

Spindle assembly checkpoint serves as a controller, or « proof-reader », of mitotic progression, preventing anaphase onset until all kinetochores are correctly attached to the microtubules from opposite poles of the cell (see Introduction, [165,188–190]). During prometaphase incorrectly attached kinetochores generate a « wait anaphase » signal, that inhibits degradation of Securin through APC/C E3 ubiquitin ligase and prevents separation of sister chromatids. Inhibited or malfunctioned SAC leads to the premature destruction of Securin and onset of chromosome segregation without their proper alignment. Most of SAC components bind to unattached kinetochores and are released from kinetochores upon establishing of bipolar attachment[188,219–224].

Aurora B is a very important regulator of kinetochore-microtubules attachments, as it phosphorylates components of KMN network and destabilizes kinetochore-microtubule attachments that are not under tension to allow re-establishment of these attachments in the correct manner. Destabilization of kinetochore-microtubule attachments leads to the

activation of SAC through phosphorylation of KNL1 and recruitment of other SAC components to kinetochores[174]. This means that as long as Aurora B is correcting errors in attachments, and until kinetochores are not under tension, SAC prevents the onset of chromosome segregation. Premature removal of Aurora B from centromeres leads to the stabilization of incorrect kinetochore-microtubule attachments and SAC silencing, which allows for chromosome segregation despite of the errors.

How UBASH3B function is related to the activity of SAC? So far, there is no evidence of direct interactions of UBASH3B with SAC components. However, overexpression of UBASH3B in mitosis leads to SAC overriding, and lack of UBASH3B results in the delay of anaphase onset, which can be caused by high SAC activity (*Krupina et al*., Fig. 1D). I postulate that UBASH3B takes part in the regulation of SAC in Aurora B-dependent manner, controlling Aurora B localization and function. In prometaphase UBASH3B ensures correct localization of Aurora B at the kinetochore-microtubule interface and therefore Aurora B functon in destabilizing erroneous kinetochore-microtubule attachments and thereby SAC activation in responce to unattachend kinetochores. Indeed, downregulation of UBASH3B decreases the « focusing » of Aurora B at the centromeres, which leads to diffused prophase-like spreading of Aurora B along chromosomal arms (*Krupina et al*., Fig. 3, Fig. S6). However, Aurora B is not excluded from centromeric region upon lack of UBASH3B, as there are other mechanisms controlling association of Aurora B with centromeres, which are still not sufficient for strong enrichment and full focusing of Aurora B in this region. I predict that activity of Aurora B on centromeres upon UBASH3B downregulation is sufficient (at least partially) for destabilization of erroneours kinetochore-microtubule attachments. Unattached kinetochores in this case induce SAC responce and prevent chromosomes segregation. This is reflected by the prolonged metaphase to anaphase transition and prometaphase cell death upon downregulation of UBASH3B (*Krupina et al*., Fig. 1, Fig. S3, Fig. S4). However, in some cases upon knockdown of UBASH3B, Aurora B activity on centromeres is not able to control all kinetochore-microtubule attachments.

Thus, some of erroneous attachements are stabilized, leading to defects in chromosome segregation. Such unproper chromosome segregation induces formation of multinuceated cells and multilobed nuclei, observed upon UBASH3B knockdown (*Krupina et al*., Fig. 1, Fig. S1, Fig. S3, Fig. S4). Another possibility is that the gradient response of SAC is less sensitive to several unattached kinetochores in case of UBASH3B downregulation.

UBASH3B-mediated removal of ubiquitinated Aurora B from centromere-kinetochore interface and its relocalization to the forming microtubule bundles of spindle midzone collectively ensures timely SAC silencing. This can be explained by the fact that loss of Aurora B affinity to the centromere-kinetochore interface leads to the stabilization of kinetochore-microtubule attachments[22,174]. It is very likely that timely removal of Aurora B from centromeres is as important for SAC silencing as kinetochore stretching and unavailability of Aurora B kinetochore substrates for phosphorylation. Moreover, these processes can happen in parallel to ensure rapid chromosome segregation after establishment of correct and stable kinetochore-microtubule attachments. It is also possible, that the residual Aurora B amounts localized on centromeres upon UBASH3B downregulation, are still able to phosphorylate the kinetochore substrates, independenly of kinetochore stretching, and to activate SAC.

Taken together, UBASH3B controls localization of Aurora B on kinetochore-microtubule interface in prometaphase and its relocalization to the spindle midzone upon establishment of bipolar kinetochore attachments, thus ensuring timely SAC silencing and faithful mitotic progression.

4.3. UBASH3B - a novel regulator of mitosis

Collectively, results presented in my Thesis strongly suggest that UBASH3B is a novel mitotic regulator, which is associated with mitotic spindle and is involved in the control of timing and fidelity of chromosome segregation.

Importanly, mitotic role of UBASH3B was never observed before. In the previous studies, UBASH3B was described as a regulator of the multisubunit T cell antigen receptor (TCR) and T cell tyrosine kinase Zap-70 signalling[302,303,304]. It was also shown to play a role in the inhibition of endocytosis of receptor tyrosine kinases through binding to E3 ubiquitin ligase Cbl[305]. These findings linked UBASH3B to the regulation of some cellular signalling pathways, which involved either the putative phosphatase activity of UBASH3B or its ubiquitin binding domain.

Here, I describe a novel and critical function of UBASH3B in the regulation of mitosis and control of cellular euploidy. Indeed, UBASH3B knockdown results in a strong delay of chromosome segregation and leads to the cell death in (pro)metaphase-like stage without proper chromosome alignment. Moreover, UBASH3B downregulation induces the formation of multilobed nuclei and multinucleated cells, which reflect the unequal chromosome segregation in the surviving cells (*Krupina et al*., Fig. 1, Fig. S3, Fig. S4). How does UBASH3B regulate mitotic progression? In my Thesis, I could show that observed phenotypes can be explained by defective Aurora B localization in the cells lacking UBASH3B. At the same time, heterogenouty of the described phenotypes in the cells lacking UBASH3B provides an evidence, that possibly several pathways controlling faithful chromosome segregation are impaired.

Importantly, UBASH3B overexpression has a critical effect of chromosome segregation as well. This is reflected in a very rapid chromosome decondensation from (pro)metaphase-like state, without correct chromosome alignment, upon UBASH3B overexpression (*Krupina et al*., Fig. S10). The excess of UBASH3B amounts on the mitotic spindle leads to the « anaphase-mimic » state of the cell at least in part through rapid extraction of Aurora B to the spindle micortubules. At the same time, Cyclin B levels are not reduced at the initial moment of UBASH3B-mediated Aurora B tethering to micortubules, making this process independent of CDK1 activity and relying on the presence of spindle microtubules lateraly or bipolarly attached to kinetochores (datat not shown).

An important question to answer is whether relocalization of Aurora B to the spindle microtubules during metaphase to anaphase transition is the only mitotic pathway that is regulated by UBASH3B. To shed some light on this aspect, I performed a mass spectrometry analysis of UBASH3B interacting partners in mitosis. Interestingly, aroung 30% of all identified hits were related to mitosis (Table 1, Appendix II). Based on the functional characteristics of identified hits, as well as the phenotype of UBASH3B overexpression and knockdown, several proteins can serve as candidates involved in the UBASH3B-mediated pathways. For instance, some of the identified hits were shown to play a role in mitotic spindle assembly and dynamics (KIFC1, moesin, importin beta 1), which is critical for proper chromosome segregation. This observation suggests a possible involvement of UBASH3B in the regulation of mitotic spindle structure and/or function. It is important to note that UBASH3B downregulation leads to some changes in the mitotic spindle, such as appearance of microtubule bundles, which can be related to increasing microtubule stability. It is possible that this phenotype reflects an important role of UBASH3B in spindle dynamics.

However, Aurora B was shown to be important for spindle organization as well, so it is difficult to distinguish between « direct » effect of UBASH3B on microtubules (through regulation of microtubule binding proteins) and Aurora B-related effect (mislocalization of Aurora B upon UBASH3B knockdown). To this end, involvement of motor protein KIFC1 in the interaction with UBASH3B can suggest a possible mechanism of Aurora B movement from centromere to the microtubules of mitotic spindle, which in addition involves UBASH3B-medated interaction of Aurora B with motor proteins (MKLP2 and KIFC1). KIFC1 was shown to play a role in the crosslinking of spindle microtubules and formation of more stable microtubule bundles[48]. Association of Aurora B with microtubule bundles upon UBASH3B overexpression can indicate the involvement of KIFC1 in the UBASH3B-mediated relocalization of Aurora B to the microtubules.

At the same time, UBASH3B can regulate KIFC1 in a direct manner, ensuring its spindle localization and function. It can not be excluded, that KIFC1

regulates microtubule-related functions of UBASH3B by transporting it along microtubules. Localization studies of these two proteins upon overexpression and downregulation of the other binding partner will help to clarify functional relations between KIFC1 and UBASH3B.

Additional evidence, suggesting the role of UBASH3B in the regulation of spindle organization/dynamics comes from its interaction with moesin, which is implicated in the control of the metaphase spindle structure and anaphase-induced changes in the shape of the cell[262–264]. Deregulation of moesin during metaphase to anaphase transition due to, for instance, its premature dephosphorylation, may lead to the premature formation of anaphase-like state of the cell by induced polar relaxation. If UBASH3B is involved in the regulation of moesin localization or function, this could be an additional mechanism coordinating metaphase to anaphase transition.

Interaction of UBASH3B with importin beta 1 could also indicate the role of UBASH3B in the spindle organization and dynamics. Importin beta 1 was shown to negatively regulate kinetochore-driven microtubule nucleation by inhibiting TPX2 and HURP[266–271]. Kinetochore-driven microtubules are important for the rapid formation of functional mitotic spindle in mammalian cells, facilitating kinetochore capture by the pole-derived micortubules through formationg of bundles[155]. UBASH3B involvement in this pathway can serve as additional mechanism controlling mitotic spindle formation and ensuring proper and timely chromosome segregation.

Mass spectrometry analysis results suggest the possible involvement of UBASH3B in the regulation of chromosome segregation. The two proteins related to TOPOII alpha function in sister chromatids separation were identified: RanBP2 and DDX21. RanBP2 is E3 SUMO ligase that sumoylates TOPO II alpha in mitotis, which is important for its proper localization at the centromere and faithful chromosome separation. Another protein, DDX21, is a component of toposome, which was shown to stimulate the decatenation of condensed mitotic chromatin by TOPOII alpha[269–277]. It would be very interesting to investigate the potential role of UBASH3B in TOPOII alpha-related regulation of chromosome segregation. For example, UBASH3B can

localize the TOPOII alpha to the centromeric region in close proximity to microtubules, and attract its regulatory components. The phenotype of UBASH3B downregulation of delayed chromosome segregation goes in line with this hypothesis. In addition, one of the main components of cohesin, SMC3, was identified as UBASH3B binding partner, confirming the role of UBASH3B in the regulation of chromosome cohesion/separation, together with TOPOII alpha related proteins. Thus, it would be vey interesting to investigate the role of UBASH3B in this process in details.

Another group of proteins, interacting with UBASH3B in mitosis, includes prohibitin-2 and MYPT1. These proteins are related to cohesin and involved in the regulation of mitotic progression by PLK1-dependent pathway. Both proteins prevent premature cohesin phosphorylation on centromeres, antagonizing PLK1 phosphorylation. Lack of prohibitn-2 and MYPT1 results in the increased PLK1 activity, premature removal of cohesin and chromosome segregation[283–286]. Therefore, potential involvement of UBASH3B in the regulation of PLK1 activity, in a direct or indirect manner,should be addressed in detail. Interestingly, a PLK1 phosphorylation site on UBASH3B was recently identified by mass spectrometry analysis in mitotic celss[306].

To summarize, it would be very important to study the role of UBASH3B in the regulation of different aspects of mitotic progression, such as spindle dynamics, kinetochore-microtubule attachments and sister chromatids cohesion, the function of mitotic kinases (like PLK1 or Aurora B) in the regulation of SAC function.

4.4. Ubiquitin binding domain proteins and their role in mitosis

Ubiquitin-mediated degradation of many mitotic regulators ensures directionality of mitotic progression. At the same time, non-proteolytic ubiquitin-dependent pathways have recently emerged as mitotic regulators, controlling proper localization of mitotic substrates or interactions with other

proteins during mitotic progression[307]. However, the picture of ubiquitin-mediated regulation of mitosis is very far from being complete, as well as the specific roles of ubiquitin modifications during mitosis remain unclear. It would be very important to understand in details how ubiquitin code is read at different mitotic stages, and how exactly ubiquitin modifications influence mitotic progression.

Using a high-throughput siRNA screening approach, we have identified a set of ubiquitin binding proteins that are potentially important for mitotic progression (*Krupina et al*., Suppl. Table 2, Suppl. Table 4). The design of our screen allowed us to identify factors, important for coordination of chromosome segregation with cytokinesis.

In my Thesis I focused on one ubiquitin binding protein, UBASH3B, which serves as an example of ubiquitin receptor controlling mitotic progression in a non-proteolytic manner. I could show that UBASH3B does not change the protein levels of Aurora B in mitosis, but controls Aurora B localization, which is critical for correct function of Aurora B and faithful mitotic progression. Interestingly, UBASH3B downregulation phenotype, which is characterized by increased number of multinucleated cells and multilobed nuclei together with inability of Aurora B to localize in the spindle midzone, resembles the phenotype of CUL3 E3 ligase downregulation[154]. Indeed, I could show that UBASH3B interacts with Aurora B in a CUL3-dependent manner, and directly binds to CUL3 in mitosis (Fig). Moreover, UBA domain of UBASH3B is sufficient to interact with modified Aurora B in mitosis, and this interaction is lost upon treatment of UBA domain interacting proteins with an unspecific deubiquitinating enzyme (*Krupina et al*., Fig. S7C). These observations suggest that interactions between UBASH3B and Aurora B are mediated by ubiquitination, which is specific to mitosis. However, I can not exclude, that SH3 domain of UBASH3B contributes to this interaction, as its binding to modified Aurora B was not fully abolished by DUB treatment. I can speculate that specificity of UBASH3B interactions with CUL3/Aurora B pathway is reinforced by both recognition of ubiquitnated Aurora B by UBA

domain of UBASH3B, and additional signals on CUL3/Aurora B complex by SH3 domain of UBAHS3B.

Strikingly, ubiquitination signal on Aurora B was shown to be required for its correct localization to the spindle midzone in anaphase, but the mechanism of decoding this ubiquitination signal, as well as its spatio-temporal regulation remained unknown. In my Thesis, I could show a critical role of ubiquitin recognition and signal transduction by receptor protein, which shed light on a very important function of components that are downstream of E3 ubiquitin ligases.

Mitosis is a complex process which requires a very strict coordination of numerous events, including chromosome condensation, alignment and segregation, as well as spindle formation and reorganization. The sequence of mitotic events is very consistent, with several checkpoints, which allow to temporaly stop mitotic progression in case of any error, implement appropriate correction mechanisms, and proceed further. Many multifunctional mitotic factors are relocalizing from one mitotic structure to another at different stages, to ensure the correct order of mitotic events and their coordination. Ubiquitination emerges as a very potent modification for the regulation of mitosis, taking into consideration many possible ways to modify substrate proteins (mono-, multi mono-, polyubiquitination, involving different lysins) and many possible ways to decode this signal using specific ubiquitin receptors. Huge variety of existing ubiquitin receptors can be explained by their involvement in a very specific regulation of a large number of events in the cell. It will be very important to investigate the precise mitotic roles of different ubiquitin receptors and their specificity towards different substrates and types of modifications, at different time points of mitosis. It will shed a light on the exact mechanisms of the complex mitotic control machinery function and let us understand the spatio-temporal regulation of mitosis by signaling networks.

4.5. UBASH3B as potential anti-cancer drug target

Most human cancers are characterized by abnormal numbers of chromosomes, which is called aneuploidy. The major cause of aneuploidy is chromosomal instability (CIN), leading to an increase in the rate of gain and lost of whole chromosomes or their large parts, as a result of defective cell division. Several mechanisms are known to induce chromosomal instability and tumor progression. They include multiplication of centrosomes, defects in sister chromatid cohesion, malfunction of SAC, misregulations of kinetochore-microtubule attachments, just to name a few. For instance, in many cancers chromosomal instability is associated with the loss of SAC regulation caused, for instance, by inactivation of Bub1[308]. Another example is cancer-specific overexpression of Separase and, as a consequence, premature sister chromatids separation with many lagging chromosomes and anaphase bridges[309]. Defects in telomere maintainance and persistent telomere dysfunction also lead to aneuploidy[310], as well as replication stress[311]. Finally, incorrect kinetochore-microtubule attachments and stabilization of mitotic spindle, together with inability of the cell to efficiently correct such attachments, will lead to chromosome missegregation and aneuploidy[312].

Strikingly, I could show that UBASH3B is involved in the regulation of chromosome segregation through regulating of Aurora B localization in mitosis. In addition, UBASH3B can be potentially involved in the regulation of mitosis in Aurora B-independent manner. Overexpression of UBASH3B leads to chromosome decondensation without proper alignment and correction of kinetochore-microtubule attachments, and causes formation of multilobed nucei and multinucleated cells (*Krupina et al.*, Fig. S10). Importanly, UBASH3B is overexpressed in a highly aggressive triple-negative breast cancer (TNBC)[313], supporting the tumor growth, invasion and metastasis. Lee et al demonstrated that phosphatase activity of UBASH3B is important for its oncogenic potential, and provided a mechanism of UBASH3B-induced tumor growth, which partially relies on targeting E3

ubiquitin ligase Cbl for inactivation, that causes up-regulation and stabilization of EGFR.

I hypothesise that UBASH3B overexpression induces TNBC invasion and metastasis by combining EGFR-related and Aurora B-related mechanisms, thus increasing tumor proliferation and aggressiveness. Aurora B-related mechanism leads to chromosome instability and aneuploidy, while EGFR-related pathway induces uncontrollable cell growth.

I believe that UBASH3B represents a potentially interesting drug candidate for cancer therapies. First, despite its ubiquitous expression, knockout mice lacking UBASH3B, are viable and have no detectable mitosis- or cancer-related phenotypes[314]. At the same time, it is overexpressed in cancers. Moreover, UBASH3B contains a protein-protein interaction interface (Ubiquitin-associated domain, UBA) required for the recognition of ubiquitinated Aurora B, rending it a druggable target. Finally, my data show that downregulation of UBASH3B in cancer cells leads to their death in prometaphase. It is possible that UBASH3B represents a novel marker, specific for aneuploid and tetraploid cancerous cells, and interfereing with the function of UBASH3B in cancer cells will lead to their reduced growth and mitotic death, but at the same time will not dramaticaly influence normally dividing, primary cells.

In the future, it will be important to compare the phenotype of UBASH3B downregulation in cancer cells and in primary, non-transformed cells, and to analyze expression levels of UBASH3B in cancers of different tissue origin. Finally, it will be important to identify and characterize the chemical compounds that can bind to and interefere with the function of UBASH3B.

Supplemental Tables S1-S5

Supplemental Table 1: Ubiquitin decoders siRNA library.

	Duplex Catalog Number	Gene Symbol	GENE ID	Sequence
Control genes				
M-006823-01	D-006823-01	INCENP	3619	CCACGAUGCUGACUAAGAA
M-006823-01	D-006823-02	INCENP	3619	CAAGAAGACUGCCGAAGAG
M-006823-01	D-006823-03	INCENP	3619	GCAAAGAGCCAGAGCUGAU
M-006823-01	D-006823-04	INCENP	3619	UCUGCAACAUGGAUAAUAA
M-003326-08	D-003326-07	AURKB	9212	AAGGUGAUGGAGAAUAGCA
M-003326-08	D-003326-08	AURKB	9212	CUUCACAAUUGAUGACUUU
M-003326-08	D-003326-09	AURKB	9212	ACAUCCUGCGUCUCUACAA
M-003326-08	D-003326-10	AURKB	9212	UGAUUCACAGAGACAUAAA
M-003290-01	D-003290-05	PLK1	5347	CAACCAAAGUCGAAUAUGA
M-003290-01	D-003290-06	PLK1	5347	CAAGAAGAAUGAAUACAGU
M-003290-01	D-003290-07	PLK1	5347	GAAGAUGUCCAUGGAAAUA
M-003290-01	D-003290-08	PLK1	5347	CAACACGCCUCAUCCUCUA
M-004101-02	D-004101-01	BUB1B	701	GGAAGAAGAUCUAGAUGUA
M-004101-02	D-004101-02	BUB1B	701	CAAGAUGGCUGUAUUGUUU
M-004101-02	D-004101-03	BUB1B	701	CAAUACAGCUUCACUGAUA
M-004101-02	D-004101-04	BUB1B	701	GGAACAACCUCAUUCUAAA
M-010224-02	D-010224-03	CUL3	8452	CCGAACAUCUCAUAAAUAA
M-010224-02	D-010224-04	CUL3	8452	GAGAAGAUGUACUAAAUUC
M-010224-02	D-010224-05	CUL3	8452	GAGAUCAAGUUGUACGUUA
M-010224-02	D-010224-18	CUL3	8452	GCGGAAAGGAGAAGUCGUA
Targeted genes				
M-015375-01	D-015375-01	ATG3	64422	GCAAACAGAUGGAAUAUUC

M-015375-01	D-015375-02	ATG3	64422	CAAGACACUUCACAAUGUA
M-015375-01	D-015375-03	ATG3	64422	GCGGAUGGGUAGAUACAUA
M-015375-01	D-015375-04	ATG3	64422	UGAAGAGAGUGGAUUGUUG
M-020623-01	D-020623-01	UFC1	51506	GGAAGGAACUCGGUGGUUU
M-020623-01	D-020623-02	UFC1	51506	CCAGGAAUGUGCCCAAAUU
M-020623-01	D-020623-03	UFC1	51506	AAAUAUGCCUGACGGAUCA
M-020623-01	D-020623-04	UFC1	51506	AAUAUGAGUUUGACAUCGA
M-003215-02	D-003215-05	CCNF	899	UCACAAAGCAUCCAUAUUG
M-003215-02	D-003215-06	CCNF	899	GAAGUCAUGUUUACAGUGU
M-003215-02	D-003215-07	CCNF	899	UAGCCUACCUCUACAAUGA
M-003215-02	D-003215-08	CCNF	899	GCACCCGGUUUAUCAGUAA
M-014930-01	D-014930-01	FBXL10	84678	GCAAUAAGGUCACUGAUCA
M-014930-01	D-014930-02	FBXL10	84678	GACCUCAGCUGGACCAAUA
M-014930-01	D-014930-03	FBXL10	84678	GGGAGUCGAUGCUUAUUGA
M-014930-01	D-014930-04	FBXL10	84678	CAGCAUAGACGGCUUCUCU
M-012881-00	D-012881-01	GGA3	23163	GAGGAGAAGUCCAAGCUUU
M-012881-00	D-012881-02	GGA3	23163	GUGAGAUGCUGCUUCAUUA
M-012881-00	D-012881-03	GGA3	23163	GAACACGGCUCCCUUACCU
M-012881-00	D-012881-04	GGA3	23163	GAACAAGAGGCGGACUUUA
M-012066-00	D-012066-01	TOM1	10043	GAACAGGCCAGACCACCAA
M-012066-00	D-012066-02	TOM1	10043	UUAGAAACCUGUGUCAAGA
M-012066-00	D-012066-03	TOM1	10043	GGACCGUGUUCAACUCAGA
M-012066-00	D-012066-04	TOM1	10043	UCCCUCAGAUCGCCAAUGA
M-003911-01	D-003911-02	TOM1L1	10040	GAACGAAGAUGUAACUGUU
M-003911-01	D-003911-03	TOM1L1	10040	GAACUCGACUGUUACAUUG
M-003911-01	D-003911-05	TOM1L1	10040	AGAUGUAACUGUUGAGCUA
M-003911-01	D-003911-06	TOM1L1	10040	GGAUGUAAGCGAAGUCAAA
M-018810-01	D-018810-02	TOM1L2	146691	GAGGAUUGGACGUUGAAUA
M-018810-01	D-018810-04	TOM1L2	146691	GGACGUCGUUCGAGGAAAC
M-018810-01	D-018810-17	TOM1L2	146691	ACGAGAGGUUCGAACGAUA
M-018810-01	D-018810-18	TOM1L2	146691	GAGCAGCGCAAGACGGUAA

M-005314-02	D-005314-13	EIF2AK4	440275	CAGAGAAGCUUCCGAUAAU
M-005314-02	D-005314-14	EIF2AK4	440275	GAGCAGGAGCAACGUGAAA
M-005314-02	D-005314-15	EIF2AK4	440275	AUGAGAACAUUGUGCGCUA
M-005314-02	D-005314-16	EIF2AK4	440275	CAGCAGAAAUCAUGUACGA
M-020439-01	D-020439-01	IMPACT	55364	GAGGAGUGGUGUGUCAUUG
M-020439-01	D-020439-02	IMPACT	55364	UAGAAUUAGCGACGAUAUA
M-020439-01	D-020439-03	IMPACT	55364	GGUAGUAUCACGCUGGUAU
M-020439-01	D-020439-04	IMPACT	55364	CAGUGAAACUCGGACAGAA
M-020946-01	D-020946-01	RWDD1	51389	GAAACAACGUGGAGGUAGA
M-020946-01	D-020946-02	RWDD1	51389	CAUCUGAUAUCCAGUUCUU
M-020946-01	D-020946-04	RWDD1	51389	AAGCAAUUAUUCCAUGGUA
M-020946-01	D-020946-17	RWDD1	51389	UCUAGUGACAGCUGUGCAA
M-015117-01	D-015117-01	RWDD2	112611	ACAGAAAGCUUGUAUAUGA
M-015117-01	D-015117-02	RWDD2	112611	CAACAAAGGUCUCACUUCU
M-015117-01	D-015117-03	RWDD2	112611	GAAUGUGGAUCUACAGUCA
M-015117-01	D-015117-04	RWDD2	112611	UGACGAAUAUAAAGAGGUA
M-013856-00	D-013856-01	C21ORF6	10069	UAGCAGAACUGAAAGAUUG
M-013856-00	D-013856-02	C21ORF6	10069	GGAGAUGUUUGUAUACUGA
M-013856-00	D-013856-03	C21ORF6	10069	UGAAAUUACUGUCAGAUCA
M-013856-00	D-013856-04	C21ORF6	10069	CUACAGCCAUCAUAUCUAU
M-016719-01	D-016719-01	RWDD3	25950	GGAUAACUCUUUUGCAUUU
M-016719-01	D-016719-02	RWDD3	25950	GAAUUGGUGUUCCAUUUGC
M-016719-01	D-016719-03	RWDD3	25950	CCUGGUAUCUCGAUUAACU
M-016719-01	D-016719-04	RWDD3	25950	GGAUUUAUGGAUGCGGAUA
M-016803-01	D-016803-01	MGC10198	201965	GCAUUACGCUCUAUUUAUG
M-016803-01	D-016803-02	MGC10198	201965	GAACUGGGUUGAUGUUGUG
M-016803-01	D-016803-03	MGC10198	201965	GCUCUAAGGAUGAUGAGUA
M-016803-01	D-016803-17	MGC10198	201965	GCUUAAUUACUGAACCGUA
M-016816-00	D-016816-01	FLJ32642	137492	CGACAAAGAUGACUUAGUA
M-016816-00	D-016816-02	FLJ32642	137492	CGACAUCACUUAAUGGAUA
M-016816-00	D-016816-03	FLJ32642	137492	GGUACAAGAUGCCAGAUGU

M-016816-00	D-016816-04	FLJ32642	137492	CUCAGUAGCUUCAUGGAAA
M-007193-01	D-007193-01	HACE1	57531	CAACAGAGAUCACUUCUAU
M-007193-01	D-007193-02	HACE1	57531	GUAAAGAAAUACUCAAGGA
M-007193-01	D-007193-03	HACE1	57531	GCACAGAUCCUACUAUUAC
M-007193-01	D-007193-04	HACE1	57531	GGAAUGACUUCACCUCGUU
M-007185-01	D-007185-01	HUWE1	10075	GCAAAGAAAUGGAUAUCAA
M-007185-01	D-007185-04	HUWE1	10075	GGAAGAGGCUAAAUGUCUA
M-007185-01	D-007185-05	HUWE1	10075	UAACAUCAAUUGUCCACUU
M-007185-01	D-007185-06	HUWE1	10075	GAAAUGGAUAUCAAACGUA
M-017674-01	D-017674-01	JOSD1	9929	CCACAAAUCUACCAUGAGA
M-017674-01	D-017674-02	JOSD1	9929	GAGACAAGGCCAAAUCUGA
M-017674-01	D-017674-03	JOSD1	9929	AAACAUCAUUUGCGAGGAA
M-017674-01	D-017674-04	JOSD1	9929	GGAAAUGGCAACUACGAUG
M-015500-01	D-015500-01	SBBI54	126119	GAUGAGAUCUGCAAGAGGU
M-015500-01	D-015500-02	SBBI54	126119	CGGCAACUAUGAUGUCAAU
M-015500-01	D-015500-03	SBBI54	126119	CAGCAGCUCUUUAGCCAGG
M-015500-01	D-015500-04	SBBI54	126119	UGGACGGUGUCUACUACAA
M-012013-01	D-012013-01	MJD	4287	GUACAAAUCUUACUUCAGA
M-012013-01	D-012013-02	MJD	4287	GCUCAGGAAUGUUAGACGA
M-012013-01	D-012013-03	MJD	4287	GCAGGGCUAUUCAGCUAAG
M-012013-01	D-012013-04	MJD	4287	ACGAGAAGCCUACUUUGAA
M-024927-01	D-024927-17	ATXN3L	92552	CCGGAAACACAUCGCAAGA
M-024927-01	D-024927-18	ATXN3L	92552	GCAGAUCAUCAGUGUCGAA
M-024927-01	D-024927-19	ATXN3L	92552	AGUUCAUACCUACACGAAA
M-024927-01	D-024927-20	ATXN3L	92552	GCCAACAACAAGUUCGAGA
M-005798-02	D-005798-08	CXORF53	79184	CAUAAUGGCUCAGUGUUUA
M-005798-02	D-005798-09	CXORF53	79184	CGUCAGAAUUGUUCACAUU
M-005798-02	D-005798-10	CXORF53	79184	GAAGGACCGAGUAGAAAUU
M-005798-02	D-005798-11	CXORF53	79184	GAAUCGAAAUCCCAAUCCA
M-018630-01	D-018630-01	FLJ14981	84954	GGAGUGAGGUCGUGGGUUA
M-018630-01	D-018630-02	FLJ14981	84954	GGUGAACCCUGCCAAGAAG

M-018630-01	D-018630-03	FLJ14981	84954	GACUGCAGCUGCCAUCGAA
M-018630-01	D-018630-04	FLJ14981	84954	AGUCCAAGAUCUCGCCUUU
M-005905-02	D-005905-01	MYSM1	114803	GAAGAGAACUGUACAAAGG
M-005905-02	D-005905-03	MYSM1	114803	CAGAUUACCUGCCUGGUUA
M-005905-02	D-005905-05	MYSM1	114803	GCAGUGAUCUCUUGUUAGA
M-005905-02	D-005905-18	MYSM1	114803	CAGUAAAGUGGACGAUAGA
M-012252-02	D-012252-01	PRPF8	10594	UGAAGCAUCUCAUCUAUUA
M-012252-02	D-012252-02	PRPF8	10594	GCAGAUGGAUUGCAGUAUA
M-012252-02	D-012252-04	PRPF8	10594	GGAAGAAGCUAACUAAUGC
M-012252-02	D-012252-05	PRPF8	10594	GAUAAGGGCUGGCGUGUCA
M-012202-01	D-012202-02	STAMBP	10617	GAGAAGCCCUCCUUAGAUG
M-012202-01	D-012202-03	STAMBP	10617	UCACUGCUCUUACCAGAUG
M-012202-01	D-012202-04	STAMBP	10617	GCAAGGAUCCACCUCUGUU
M-012202-01	D-012202-17	STAMBP	10617	AGACCUUCGAUGAGCGUUU
M-005783-02	D-005783-01	STAMBPL1	57559	GGACUGCGAUGUGUAGUUU
M-005783-02	D-005783-03	STAMBPL1	57559	UAACGUAGAAUACCAAGAA
M-005783-02	D-005783-04	STAMBPL1	57559	GAGAUUGCAUUCCCAAGGA
M-005783-02	D-005783-05	STAMBPL1	57559	CGUAGAAUACCAAGAAUAU
M-005814-01	D-005814-01	COPS5	10987	GGACUAAGGAUCACCAUUA
M-005814-01	D-005814-03	COPS5	10987	CUACAAACCUCCUGAUGAA
M-005814-01	D-005814-05	COPS5	10987	GCAGGAAGCUCAGAGUAUC
M-005814-01	D-005814-06	COPS5	10987	GGAGUUUCAUGUUGGGUUU
M-006024-00	D-006024-01	PSMD14	10213	CAGAAGAUGUUGCUAAAUU
M-006024-00	D-006024-02	PSMD14	10213	GAACAAGUCUAUAUCUCUU
M-006024-00	D-006024-03	PSMD14	10213	GGACUAAACAGACAUUAUU
M-006024-00	D-006024-04	PSMD14	10213	GUACUUAUGACCUCAAAUA
M-019535-02	D-019535-01	EIF3S5	8665	GCACAAUGAGUCAGAAGAU
M-019535-02	D-019535-03	EIF3S5	8665	ACAAUGAGUCAGAAGAUGA
M-019535-02	D-019535-05	EIF3S5	8665	GUACAGUGUUGCAAUAUGC
M-019535-02	D-019535-06	EIF3S5	8665	GGACCAUGGGAGUGAUGUU
M-003883-01	D-003883-01	EIF3S3	8667	GAAGAUCGGCUUGAAAUUA

M-003883-01	D-003883-02	EIF3S3	8667	GAAGUGCCGAUUGUAAUUA
M-003883-01	D-003883-03	EIF3S3	8667	UACUAUGGCUCAUUCGUUA
M-003883-01	D-003883-04	EIF3S3	8667	AAGGAUCUCUCUCACUAAA
M-017017-00	D-017017-01	COPS6	10980	GAAGAGAAGAUUAUCAUUG
M-017017-00	D-017017-02	COPS6	10980	GCAACACCAUGAACCAGUU
M-017017-00	D-017017-03	COPS6	10980	CCAAGGAGGAGCAGUUUAA
M-017017-00	D-017017-04	COPS6	10980	CCACGUAGCCCGAAUGACA
M-009621-01	D-009621-01	PSMD7	5713	GAAAGUACUUGAUGUAUCG
M-009621-01	D-009621-02	PSMD7	5713	CAAAGACGAUUCUGUAUGG
M-009621-01	D-009621-03	PSMD7	5713	GCCCUAAACUACACAAGAA
M-009621-01	D-009621-05	PSMD7	5713	CUACAGAAGCGUACAUUUC
M-004771-01	D-004771-02	MAP3K7IP2	23118	UAAGGGAAGUGCUGAAAUA
M-004771-01	D-004771-03	MAP3K7IP2	23118	GGAACAAGGUUUAACUAUU
M-004771-01	D-004771-04	MAP3K7IP2	23118	CCUCAAGGCUUUAAUGUUU
M-004771-01	D-004771-17	MAP3K7IP2	23118	UUACAUGACCUGCGACAAA
M-015572-01	D-015572-01	TAB3	257397	GUACAUAGCUCAAGUGAUG
M-015572-01	D-015572-02	TAB3	257397	GAAAGAAGUUGACCUCCUU
M-015572-01	D-015572-03	TAB3	257397	CAAAGCAACUGAAACUUGA
M-015572-01	D-015572-04	TAB3	257397	GAAUAGAAAUCGCCUUUUA
M-020939-02	D-020939-01	NEIL3	55247	GAUGCUAGGUGAUGUGCUA
M-020939-02	D-020939-02	NEIL3	55247	GCAAGCUACCGACUAGAAA
M-020939-02	D-020939-03	NEIL3	55247	UUAAUGAAGUACCCGUGUA
M-020939-02	D-020939-04	NEIL3	55247	GGACCUGUGUGGUGUGUAC
M-005283-00	D-005283-01	NUP153	9972	GAGGAGAGCUCUAAUAUUA
M-005283-00	D-005283-02	NUP153	9972	GGAAGAAAGCUGACAAUGA
M-005283-00	D-005283-03	NUP153	9972	CAAUUCGUCUCAAGCAUUA
M-005283-00	D-005283-04	NUP153	9972	GAAGCGAGCCCUUACAUUG
M-004746-02	D-004746-05	RANBP2	5903	CGAAACAGCUGUCAAGAAA
M-004746-02	D-004746-06	RANBP2	5903	GAAAGAAGGUCACUGGGAU
M-004746-02	D-004746-07	RANBP2	5903	GAAAGGACAUGUAUCACUG
M-004746-02	D-004746-08	RANBP2	5903	GAAUAACUAUCACAGAAUG

M-009065-00	D-009065-01	RBM10	8241	GCAUGGAUCUCCCGAAAUU
M-009065-00	D-009065-02	RBM10	8241	GGACAUGGCCUCCAAUGAA
M-009065-00	D-009065-03	RBM10	8241	AGACCGGCCUCUACUAUGA
M-009065-00	D-009065-04	RBM10	8241	AGAAUGACAUGGAGCAAAU
M-020032-01	D-020032-01	RBM6	10180	GAAAUCCACCGGAAGAUAA
M-020032-01	D-020032-02	RBM6	10180	GCAAGAAGGAAAUGUCUAA
M-020032-01	D-020032-04	RBM6	10180	CAGCAGGCUUAUUCGAUUA
M-020032-01	D-020032-17	RBM6	10180	CUAUCAUGCUAAAGCGUAU
M-015936-01	D-015936-01	RYBP	23429	GAAAGAUCCUCCUAGUGAA
M-015936-01	D-015936-02	RYBP	23429	GCACAGCAGUUGGCAGUAA
M-015936-01	D-015936-03	RYBP	23429	CGACAUGUCAGCAGUCAAU
M-015936-01	D-015936-04	RYBP	23429	GAAAACCUCGGAUCAAUUC
M-018575-02	D-018575-01	SHARPIN	81858	UAGGAGCCCUGGAAACUUG
M-018575-02	D-018575-03	SHARPIN	81858	CCGCAGUGCUCUUGGCUGU
M-018575-02	D-018575-04	SHARPIN	81858	CCACCCAGCACGAGCUACA
M-018575-02	D-018575-17	SHARPIN	81858	GGUCACACUUGAAGACGCU
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M-006037-00	D-006037-02	SOLH	6650	CCAAGUGCACGCUCAGAAA
M-006037-00	D-006037-03	SOLH	6650	GUGAGGGCAUGACCUGCUA
M-006037-00	D-006037-04	SOLH	6650	GGAGACACCGUGCGUUACA
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M-009265-01	D-009265-03	YAF2	10138	GAUAACACAGAGAGAGGAA
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M-010158-00	D-010158-02	ZNF265	9406	GGAGAGAAGUGGGAUAUAU
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M-010158-00	D-010158-04	ZNF265	9406	CAAAAUAGAUGUUCUGUCA
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M-010025-01	D-010025-02	ZRANB3	84083	CAAUAUCGCUCACUGAUUU
M-010025-01	D-010025-03	ZRANB3	84083	GAUCAGACAUCACACGAUU

M-010025-01	D-010025-04	ZRANB3	84083	CAGAUGAGCUGUAAUUUCA
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M-020796-01	D-020796-03	NPL4	55666	GGACACCUAUUUCCUAAGU
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M-010983-01	D-010983-03	OTUB2	78990	AGAGAAGGAUGGCUCAGUG
M-010983-01	D-010983-04	OTUB2	78990	AAAGAACGCGUACUGCAGA
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M-026487-01	D-026487-04	OTUD1	220213	UAUCAUCGCUGCUGCCCAA
M-026487-01	D-026487-17	OTUD1	220213	ACGCAAGCUUUCCGAGCAU
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M-009927-00	D-009927-03	OTUD4	54726	AGAGAAAUUUGAAGCGUUU
M-009927-00	D-009927-04	OTUD4	54726	AAGAAUAUUUAAAGCGUUU
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M-013823-00	D-013823-02	OTUD5	55593	CAACAGUGAGGACGAGUAU
M-013823-00	D-013823-03	OTUD5	55593	AAUCCUACCUGCAGUGGUU
M-013823-00	D-013823-04	OTUD5	55593	AAACGAGGACGAACCCAUU
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M-008553-01	D-008553-03	OTUD6B	51633	AAAGAAAGCUGCAUUGGAA
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M-016115-01	D-016115-02	PARP11	57097	GAGAUUACAUAAACGGAGA
M-016115-01	D-016115-03	PARP11	57097	GACGGGAGCUAUGUGAAUU
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M-009270-01	D-009270-03	ZRANB1	54764	CAGCAGAUAUUGAAGAUUU
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M-027369-00	D-027369-02	YOD1	55432	GAAUGAGGGUUGAAGCCUA
M-027369-00	D-027369-03	YOD1	55432	GAUCCAGACUUCUAUAGUG
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M-003966-05	D-003966-11	MAP2K5	5607	GUUAAAGGCCUUACUUAUU
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M-006932-02	D-006932-08	C20ORF18	10616	UCACCUGCCCUGUGUGUUU
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M-019984-00	D-019984-03	ARIH1	25820	ACACUUAUGUCUUCGCUUU
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M-020104-01	D-020104-03	ARIH2	10425	GUAUGCAGUUUGUGCGAAA
M-020104-01	D-020104-04	ARIH2	10425	AUGCAAUGCUCCAAAUGUA
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M-028950-01	D-028950-03	ANKIB1	54467	GAGCAUAGUUAUCAGCUAG
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M-003603-00	D-003603-03	PARK2	5071	UUAAAGAGCUCCAUCACUU
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M-010993-01	D-010993-04	AIRE	326	CAACAGUCCAGGAGGUGCA
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M-018070-00	D-018070-03	FLJ32440	286053	GGUUGAAUUUGCUACAUUG
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M-003279-04	D-003279-26	MDM2	4193	AAAGUCUGUUGGUGCACAA
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M-006536-03	D-006536-05	MDM4	4194	UGAUACCGAUGUAGAGGUU
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M-004591-00	D-004591-02	RAD18	56852	GCAGGUUAAUGGAUAAUUU
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M-004591-00	D-004591-04	RAD18	56852	GCAGUUUGCUUUAGAGUCA
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M-005345-02	D-005345-05	MARK4	57787	GGAUCAACAUCGGCUAUGA
M-005345-02	D-005345-06	MARK4	57787	GCUGUACUCUCGAGCAAAU
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M-019752-00	D-019752-02	RHBDD3	25807	CGGCAGCUGUGGAUACAUG
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M-019752-00	D-019752-04	RHBDD3	25807	ACUGUUGGUUGGAGGACAA
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M-003959-05	D-003959-03	SNF1LK	150094	ACGAUUAGAUUCAAGCAAU
M-003959-05	D-003959-08	SNF1LK	150094	GAAAAUCUAUCGUGAGGUU
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M-004778-03	D-004778-23	SIK2	23235	GGUGUGUGCUAUUGCAUAU
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M-004322-05	D-004322-04	SNRK	54861	GUGGAUCUCUUGCAUAUUC
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M-008533-01	D-008533-04	KIAA1959	84959	GGUGAAGCCUUAUUAGAGA
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M-014655-00	D-014655-04	TDRD3	81550	GAAUCCAGUUCGAAGUAAU
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M-003102-04	D-003102-12	TNK2	10188	CAUCCUACCUGGAGCGCUA
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M-019399-00	D-019399-03	KIAA1582	57690	GGAGUGACCGACCAUAAUG
M-019399-00	D-019399-04	KIAA1582	57690	ACAAUGGCGUUGGUAAUAU

M-017914-00	D-017914-01	PHGDHL1	337867	GAACCCAUCUUCUCUUCUU
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M-017914-00	D-017914-03	PHGDHL1	337867	GCACAAGGGAGGCGACAGA
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M-017474-00	D-017474-03	UBAP1	51271	GUACGAGUGUGUCCUCAGA
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M-013168-00	D-013168-03	UBAP2	55833	CCAGUGGACUACUAUGGAA
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M-021220-01	D-021220-17	NICE-4	9898	CCUGGGAGAUGGUCGGGAA
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M-008616-00	D-008616-02	UBASH3A	53347	UGAGAGCCCUAUUCCAGUA
M-008616-00	D-008616-03	UBASH3A	53347	CACGGAAGCAUCUCUCUUA
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M-021567-01	D-021567-01	VPS13D	55187	GAGCGUCGAUUGAUUUAUA
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M-021567-01	D-021567-03	VPS13D	55187	GCAAUUAGACGUAGCAGAA
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M-019158-01	D-019158-02	NYREN18	51667	CGAUGGUGCUUGAACUAAA
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M-019158-01	D-019158-17	NYREN18	51667	GAACAGAAAUAGCGCUAAU
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M-021178-00	D-021178-04	C1ORF6	56893	GAGCCUCGGUCAAGGAGUU
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M-012410-01	D-012410-04	AUP1	550	GAUGUGGUACAACCUCUUA
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M-027332-03	D-027332-04	DUB3	377630	CCAAGAAUGUGCAAUAUCC
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M-008570-00	D-008570-02	TEX27	60685	CAACAACAAUACCUCGAUA
M-008570-00	D-008570-03	TEX27	60685	GCGGUUAUGUGUUCUGUAU
M-008570-00	D-008570-04	TEX27	60685	GAUGAUUCCGCUCCAAGUA
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M-009701-01	D-009701-02	ZNF216	7763	UAACUCAGCAAAUGACAGA
M-009701-01	D-009701-03	ZNF216	7763	GAGAGCAGACACUAGCUUA
M-009701-01	D-009701-04	ZNF216	7763	AAUGGAAUGUGUUCAGUUU
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M-006061-02	D-006061-03	USP1	7398	GAAAUACACAGCCAAGUAA
M-006061-02	D-006061-05	USP1	7398	CAUAGUGGCAUUACAAUUA
M-006061-02	D-006061-19	USP1	7398	GCACAAAGCCAACUAACGA

M-006062-02	D-006062-05	USP10	9100	CCAUAAAGAUUGCAGAGUU
M-006062-02	D-006062-06	USP10	9100	CAAACAAGAGGUUGAGAUA
M-006062-02	D-006062-08	USP10	9100	CCACAUAUAUUUACAGACU
M-006062-02	D-006062-21	USP10	9100	GAGUUGCACACCACGGAAA
M-006063-01	D-006063-01	USP11	8237	GGGCAAAUCUCACACUGUU
M-006063-01	D-006063-02	USP11	8237	GAACAAGGUUGGCCAUUUU
M-006063-01	D-006063-03	USP11	8237	GAUGAUAUCUUCGUCUAUG
M-006063-01	D-006063-04	USP11	8237	GAGAAGCACUGGUAUAAGC
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M-027148-00	D-027148-03	USP12	219333	GAAACUCUGUGCAGUGAAU
M-027148-00	D-027148-04	USP12	219333	GAAGAAUUCUACGGGUUGA
M-006066-01	D-006066-01	USP15	9958	GAAGAAGGCUCACCAAGUG
M-006066-01	D-006066-02	USP15	9958	GAACGCACCUUGGAAGUUU
M-006066-01	D-006066-04	USP15	9958	GCAGAUAAGAUGAUAGUUA
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M-004236-03	D-004236-04	USP18	11274	GGAAGAAGACAGCAACAUG
M-004236-03	D-004236-19	USP18	11274	CGUUUGGGCUCCUGAGGCA
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M-006068-02	D-006068-05	USP19	10869	UGGCGGAGGUAAUUAAGAA
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M-006071-00	D-006071-02	USP21	27005	GAACCUGAGUUAAGUGAUG
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M-006071-00	D-006071-04	USP21	27005	GAGCAGCACUCGACCUCUU
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M-006073-02	D-006073-05	USP24	23358	AGGGAAACCUUACCUGUUA
M-006073-02	D-006073-06	USP24	23358	CCACAGCUUUGUUGAAUGA
M-006073-02	D-006073-07	USP24	23358	GUAGAAGCCUUGUUGUUCA
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M-006075-01	D-006075-03	USP26	83844	GAAGAUACCUCACUUUGUC
M-006075-01	D-006075-04	USP26	83844	GCACAAGACUUCCGUUGGA
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M-031532-01	D-031532-02	USP27X	389856	UCAUGUGCCCUAUAAGUUA
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M-006077-01	D-006077-01	USP29	57663	GAAAGAAGCUCUCAUUGAA
M-006077-01	D-006077-02	USP29	57663	GAAUAUAACUGUCAGAUGU
M-006077-01	D-006077-03	USP29	57663	GUACAAAGAUCAAGAGAGA
M-006077-01	D-006077-04	USP29	57663	GAAGAACCCAUCAAGUUUA
M-021294-03	D-021294-03	USP30	84749	ACAGGAUGCUCACGAAUUA
M-021294-03	D-021294-04	USP30	84749	GCCAAGAAGUUACUGAUGA
M-021294-03	D-021294-05	USP30	84749	GAGCAGCAGUCAGAAAUAA
M-021294-03	D-021294-18	USP30	84749	CCAGAGUCCUGUUCGAUUU
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M-022513-02	D-022513-02	USP31	57478	GUAGACAGCUCUCCAGUCA
M-022513-02	D-022513-03	USP31	57478	CCUCAAACCUGCACUUUAU
M-022513-02	D-022513-04	USP31	57478	CAGCAUACAUCCUCUUCUA
M-006082-01	D-006082-01	USP34	9736	GAAAUUGACUCUCCUUAUU
M-006082-01	D-006082-02	USP34	9736	UAACAUGGCUGACUUAAUG
M-006082-01	D-006082-03	USP34	9736	GCAAUGAGGUUAAUUCUAG
M-006082-01	D-006082-04	USP34	9736	GGACCAAAUUUACAUAUUG
M-006083-02	D-006083-02	USP35	57558	AGAGCGAGCUGGCGGGUUU
M-006083-02	D-006083-03	USP35	57558	GCUCGGAGUAUCUGAAGUA

M-006083-02	D-006083-04	USP35	57558	CAACAUCCUUUACCUACAG
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M-006084-02	D-006084-01	USP36	57602	GCACACCACUGAAGAGAUU
M-006084-02	D-006084-02	USP36	57602	GAAAGCAGAUGUCCUGAGU
M-006084-02	D-006084-04	USP36	57602	GGACUCGGCUGAUGAUGGA
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M-006086-01	D-006086-02	USP38	84640	GGAGACAAGUAUUAUCUUU
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M-006086-01	D-006086-05	USP38	84640	GCAUAGUACUAAUGGUUUA
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M-004974-01	D-004974-03	USP4	7375	GGAAUAAACUACUAAACUG
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M-006088-01	D-006088-01	USP40	55230	GAAACUAGCUGUUAUACAU
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M-006088-01	D-006088-03	USP40	55230	GCAGAGAGUUGCCGAUUUC
M-006088-01	D-006088-04	USP40	55230	GAACGAGCCUGCGCAAGUU
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M-031434-01	D-031434-04	USP41	373856	CCAGGGAGUUAUCAAGCAA
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M-031434-01	D-031434-18	USP41	373856	GCGAGAGUCUUGUGAUGCU
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M-006089-01	D-006089-02	USP42	84132	UUAGAGACCUUCAGGCUUA
M-006089-01	D-006089-04	USP42	84132	CAACCUAACCUUCAUAGUA
M-006089-01	D-006089-17	USP42	84132	GAAUGGGAUUGGUACGAUU
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M-023019-03	D-023019-02	USP43	124739	GUGAAAGGCAGAAGCAUUA
M-023019-03	D-023019-03	USP43	124739	GAAGAGGACCUGAAUACCA
M-023019-03	D-023019-04	USP43	124739	GUGCUAAUCCUCUUCUGUA
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M-006092-03	D-006092-04	USP46	64854	CCAGAGCAGUUUCCAAUCA
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M-006092-03	D-006092-18	USP46	64854	UGAACGAACCUGCGGAAAA
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M-006093-01	D-006093-02	USP47	55031	CCUGAAAGCUGAAGGAUUU
M-006093-01	D-006093-03	USP47	55031	GAGAGAAGCUUAGUGAAAU
M-006093-01	D-006093-04	USP47	55031	GCAACGAUUUCUCCAAUGA
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M-027186-01	D-027186-02	USP53	54532	GAAAGGACCAGCUAAGUUA
M-027186-01	D-027186-03	USP53	54532	GCAAUGAGGUUGAAAGAAU
M-027186-01	D-027186-04	USP53	54532	GAAAAGAACUCAGGAAUUU
M-016853-01	D-016853-01	USP54	159195	GAUAGAAGUUUGUCAGGUA
M-016853-01	D-016853-02	USP54	159195	GAAUUUACGUCGCAUCUCA
M-016853-01	D-016853-04	USP54	159195	GGGAAGAGUCCACUGAACA
M-016853-01	D-016853-17	USP54	159195	GGUCACUGAUAGAGCGCAA
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M-006096-03	D-006096-07	USP6	9098	GCGGAGAGGUUCACAACAA
M-006096-03	D-006096-08	USP6	9098	GAGCGGAAGGACAUACUUA
M-006096-03	D-006096-09	USP6	9098	GAACCUGAUUGACGGGAUC
M-006097-01	D-006097-01	USP7	7874	CUAAGGACCCUGCAAAUUA
M-006097-01	D-006097-02	USP7	7874	GUGGUUACGUUAUCAAAUA
M-006097-01	D-006097-03	USP7	7874	UGACGUGUCUCUUGAUAAA
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M-005203-01	D-005203-02	USP8	9101	UGAAAUACGUGACUGUUUA
M-005203-01	D-005203-03	USP8	9101	GGACAGGACAGUAUAGAUA
M-005203-01	D-005203-04	USP8	9101	AAAUAAAGCUCAACGAGAA
M-005203-01	D-005203-05	USP8	9101	GGCAAGCCAUUUAAGAUUA
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M-017741-00	D-017741-02	C13ORF22	10208	GGACACAGCUGUAACUCAU
M-017741-00	D-017741-03	C13ORF22	10208	UCAAUUGAGUGGUGUUAAA
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M-006067-01	D-006067-02	USP16	10600	GGAACAAGGUAAUUUGAAA
M-006067-01	D-006067-03	USP16	10600	GAACACAGUGGUACUAUGA
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M-006070-02	D-006070-01	USP20	10868	GCGAGUGGCUCAACAAGUU
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M-006070-02	D-006070-03	USP20	10868	UCUGAAAGCUGUUCCUAUU
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M-006072-01	D-006072-01	USP22	23326	GGAGAAAGAUCACCUCGAA
M-006072-01	D-006072-02	USP22	23326	CAAAGCAGCUCACUAUGAA
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M-006081-00	D-006081-02	USP33	23032	GACAGUGGCUUAAUAAAUU
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M-010054-01	D-010054-16	USP45	85015	UGGCUUGAGUCUUCGUAAA
M-005945-01	D-005945-02	USP49	25862	CCAAACAGGUCUUAAAGGU
M-005945-01	D-005945-04	USP49	25862	GGAUAGAUGCAAACAUGUA
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M-005945-01	D-005945-06	USP49	25862	ACAAGGAGCCGAGUUCAAA
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M-032247-01	D-032247-02	USP51	158880	AAACAAAGCAGCACCAUUU
M-032247-01	D-032247-04	USP51	158880	GCUUUAAAGUAGGUAAGAA
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M-006064-00	D-006064-01	USP13	8975	GAAGAUGGGUGAUUUACAA
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M-006076-01	D-006076-07	USP28	57646	UCAGGUGCCUUAUCGCUUG
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M-021192-00	D-021192-03	USP52	9924	GAACAAGGAAUUUGCUUUG
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M-023237-01	D-023237-02	DKFZP761G 2113	56970	GGUCGAGGAUUCUUGUUUG
M-023237-01	D-023237-03	DKFZP761G 2113	56970	UGAAUAAGUCUGAGAGUGA
M-023237-01	D-023237-04	DKFZP761G 2113	56970	GAGGCGAACCGUACGGAUU
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M-015442-02	D-015442-03	DKFZP547N 043	83932	GUAUUACGGCUAUGUCAAA
M-015442-02	D-015442-04	DKFZP547N 043	83932	GUGGUAAUGAUCCAAAGUA
M-015442-02	D-015442-18	DKFZP547N 043	83932	UGAAGUGGAGCGUGCGAAU
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M-018651-02	D-018651-03	FLJ31031	199990	GCCCAGUGCUUGGCCGAAA
M-018651-02	D-018651-04	FLJ31031	199990	CUGUCGGACCCAAGACCUU
M-018651-02	D-018651-18	FLJ31031	199990	CCGCAUGCGCAUUAAAGCA
M-024738-01	D-024738-01	LOC153918	153918	CGAAUGAAGUCCCAACCAA
M-024738-01	D-024738-02	LOC153918	153918	GCGUAAACCUUUCAGUUCU
M-024738-01	D-024738-03	LOC153918	153918	GCUGAGCGACAUACUAAUU
M-024738-01	D-024738-04	LOC153918	153918	GCAGAUGGCAAUCAGGAAU
M-021000-00	D-021000-01	CGI-62	51101	GAACAGGCAGCACGUAUUA
M-021000-00	D-021000-02	CGI-62	51101	GAAACAAACUUCAGACCUU
M-021000-00	D-021000-03	CGI-62	51101	GGUAAAGUGUCUUCAAGUA
M-021000-00	D-021000-04	CGI-62	51101	CCAAAGAAACCAUCUAAUU
M-007038-00	D-007038-01	KIAA1536	57658	GGAGUCAACUUUCUCAAUG
M-007038-00	D-007038-02	KIAA1536	57658	GGAAGUGGCUGAAGUUAAC
M-007038-00	D-007038-03	KIAA1536	57658	GUACAGUUGUCAGAAAGUA
M-007038-00	D-007038-04	KIAA1536	57658	ACACGGAGCUGAUGGAACA

M-010637-01	D-010637-01	NDP52	10241	GACAAGAUCUUCCCAGCUA
M-010637-01	D-010637-03	NDP52	10241	GAAGACAACCCGUGAGUAU
M-010637-01	D-010637-04	NDP52	10241	CCAAGGAUGAUGAGUAUUA
M-010637-01	D-010637-17	NDP52	10241	AGACUGAGUGAGAACGAAA
M-004012-02	D-004012-01	EEA1	8411	AGACAGAGCUUGAGAAUAA
M-004012-02	D-004012-02	EEA1	8411	GAGAAGAUCUUUAUGCAAA
M-004012-02	D-004012-03	EEA1	8411	GAAGAGAAAUCAGCAGAUA
M-004012-02	D-004012-05	EEA1	8411	GAACAAGACUAUACUAAGU
M-003767-02	D-003767-01	IKBKG	8517	AACAGGAGGUGAUCGAUAA
M-003767-02	D-003767-02	IKBKG	8517	UGGAGAAGCUCGAUCUGAA
M-003767-02	D-003767-03	IKBKG	8517	GGAAGAGCCAACUGUGUGA
M-003767-02	D-003767-04	IKBKG	8517	AAACAGGAGGUGAUCGAUA
M-020327-01	D-020327-01	KIAA1018	22909	GUAAGGCUCUUUCAACGUA
M-020327-01	D-020327-02	KIAA1018	22909	GUAAAAUGGUGCCUAGAUA
M-020327-01	D-020327-03	KIAA1018	22909	UCAAAUGUGUCUAUGGUAG
M-020327-01	D-020327-04	KIAA1018	22909	CAACCGGUCAUCCUUACUA
M-016269-02	D-016269-01	OPTN	10133	GCACGGCAUUGUCUAAAUA
M-016269-02	D-016269-02	OPTN	10133	GAAGUUUACUGUUCUGAUU
M-016269-02	D-016269-03	OPTN	10133	GAAGGAAGAACGCCAGUUU
M-016269-02	D-016269-04	OPTN	10133	CCAUGAAGCUAAAUAAUCA
M-015381-01	D-015381-01	PCF11	51585	GGAAUGAGGUUUACAACAU
M-015381-01	D-015381-02	PCF11	51585	AGUAUGAGGUUGUCUGAUA
M-015381-01	D-015381-03	PCF11	51585	UCAAGGUGGUCCAAGAUUU
M-015381-01	D-015381-04	PCF11	51585	GGAUAAGACCGAUGGCAAA
M-006454-01	D-006454-05	POLH	5429	AAACUGGCCUGUGGACUAA
M-006454-01	D-006454-06	POLH	5429	GAAGUUAUGUCCAGAUCUU
M-006454-01	D-006454-07	POLH	5429	GCACUUACAUUGAAGGGUU
M-006454-01	D-006454-08	POLH	5429	GCAAUUAGCCCAGGAACUA
M-021038-00	D-021038-01	POLK	51426	GUACAGAACUUUACCAACA
M-021038-00	D-021038-02	POLK	51426	GAUGAAGCCUACUUGAAUA
M-021038-00	D-021038-03	POLK	51426	GGAUGGGACUUAAUGAUAA

M-021038-00	D-021038-04	POLK	51426	GUAGAACUGUUACCAUUAA
M-005255-02	D-005255-02	TANK	10010	GGAUAGAGAUUCUGCAGUA
M-005255-02	D-005255-20	TANK	10010	GCAACUCAAUAAAGCGUAU
M-005255-02	D-005255-21	TANK	10010	CAACAGACUAUUAUUGACA
M-005255-02	D-005255-22	TANK	10010	GAGCAGAGAAUACGUGAAC
M-016892-01	D-016892-01	TAX1BP1	8887	GAUAUUACCUUCCAAAUGA
M-016892-01	D-016892-02	TAX1BP1	8887	AAAGAGAACUUAACCAUGA
M-016892-01	D-016892-03	TAX1BP1	8887	GAGAACAACUUGAAUGUCA
M-016892-01	D-016892-04	TAX1BP1	8887	GUACUGCUCGUGAUUAUUA
M-020406-01	D-020406-01	PROSAPIP2	9755	GGGAUGAUCAGGUGAAUUU
M-020406-01	D-020406-02	PROSAPIP2	9755	GAGAUCAAGUACCCACUGA
M-020406-01	D-020406-03	PROSAPIP2	9755	CCAGUUCCAGCAUGAGUUA
M-020406-01	D-020406-04	PROSAPIP2	9755	AGACAUGGCGUGGGUGAAA
M-014328-00	D-014328-01	TNIP2	79155	GAGCAAAACUGCCAAGUAU
M-014328-00	D-014328-02	TNIP2	79155	AACAGCAGAUUCUCGCUUA
M-014328-00	D-014328-03	TNIP2	79155	GAAGAAAAUCGACUGUUAA
M-014328-00	D-014328-04	TNIP2	79155	CAGAAAGGGCCGAUCGGGA
M-010072-02	D-010072-01	WRNIP1	56897	CAACAAAUGCCAAGACAAA
M-010072-02	D-010072-02	WRNIP1	56897	CUAGGAAGAUGUUCUGUAA
M-010072-02	D-010072-04	WRNIP1	56897	GCAUAAGGUUUGUGACAUU
M-010072-02	D-010072-18	WRNIP1	56897	AGGCGCAGUUCUUUCGAAU
M-005067-01	D-005067-01	XPA	7507	GAAAGACUGUGAUUUAGAA
M-005067-01	D-005067-02	XPA	7507	GCCAUGAACUGACAUAUGA
M-005067-01	D-005067-03	XPA	7507	GGGAAAGAAUUUAUGGAUU
M-005067-01	D-005067-04	XPA	7507	GAUGACAUGUACCGUAAGA
M-014036-01	D-014036-01	ZFYVE20	64145	UCGAUAACAUCAAGGCAUA
M-014036-01	D-014036-02	ZFYVE20	64145	GCUGAUAGACGCUUUAAGU
M-014036-01	D-014036-03	ZFYVE20	64145	GACCGGAUCCGCUGCUGUA
M-014036-01	D-014036-04	ZFYVE20	64145	GAACCCACCUGUAUCAACC
M-005791-00	D-005791-01	BAP1	8314	GAGUUCAUCUGCACCUUUA
M-005791-00	D-005791-02	BAP1	8314	CCACAAGUCUCAAGAGUCA

M-005791-00	D-005791-03	BAP1	8314	GAUGAUACGUCCGUGAUUG
M-005791-00	D-005791-04	BAP1	8314	GAGCAAAGGAUAUGCGAUU
M-004309-00	D-004309-01	UCHL1	7345	UAGAUGACAAGGUGAAUUU
M-004309-00	D-004309-02	UCHL1	7345	CCGAGAUGCUGAACAAAGU
M-004309-00	D-004309-03	UCHL1	7345	UCAAGCCGAUGGAGAUCAA
M-004309-00	D-004309-04	UCHL1	7345	GAAGGGACAAGAAGUUAGU
M-006059-02	D-006059-02	UCHL3	7347	GGAGGAAUCUGUGUCAAUG
M-006059-02	D-006059-03	UCHL3	7347	GCGCGACCCUGAUGAACUA
M-006059-02	D-006059-04	UCHL3	7347	GAAGUUUGCAAGAAGUUUA
M-006059-02	D-006059-18	UCHL3	7347	AUGAAUUAGAUGGGCGGAA
M-006060-03	D-006060-01	UCHL5	51377	CCACCAGGAUGUCCAUUUA
M-006060-03	D-006060-03	UCHL5	51377	GUUUAGAGCCUGAGAAUUU
M-006060-03	D-006060-04	UCHL5	51377	GCAAAGAAAGCUCAGGAAA
M-006060-03	D-006060-05	UCHL5	51377	GAUCAAGGUAAUAGUAUGU
M-008768-03	D-008768-15	AKTIP	64400	AGAAAACAGUGGCGACUUA
M-008768-03	D-008768-16	AKTIP	64400	AGGCGGAACCAUAAUCAUA
M-008768-03	D-008768-17	AKTIP	64400	AGUAAUAUUCAUACGGCAU
M-008768-03	D-008768-23	AKTIP	64400	CUAAUGAAGUCCGGUAUUU
M-003549-01	D-003549-01	TSG101	7251	AAACUGAGAUGGCGGAUGA
M-003549-01	D-003549-02	TSG101	7251	GAACCUCACUGGAACAAUC
M-003549-01	D-003549-04	TSG101	7251	CCGUUUAGAUCAAGAAGUA
M-003549-01	D-003549-05	TSG101	7251	UCCCACAGCUCCCUUAUAC
M-010064-03	D-010064-02	UBE2V1	7335	GGACAGUGUUACAGCAAUU
M-010064-03	D-010064-21	UBE2V1	7335	GUGGAUGCAUACCGAAAUA
M-010064-03	D-010064-22	UBE2V1	7335	GCCGAAGCAUAGAUUGUAA
M-010064-03	D-010064-23	UBE2V1	7335	UGAGAUUGGCCUUCGGUGA
M-008823-00	D-008823-01	UBE2V2	7336	GCUAAGACGUCUAAUGAUG
M-008823-00	D-008823-02	UBE2V2	7336	GGACAGGCAUGAUUAUUGG
M-008823-00	D-008823-03	UBE2V2	7336	GAGUUAAAGUUCCUCGUAA
M-008823-00	D-008823-04	UBE2V2	7336	GCAUACCAGUGUUAGCAAA
M-008494-02	D-008494-01	UEVLD	55293	AGAAAGACCUGCUGAAUUU

M-008494-02	D-008494-03	UEVLD	55293	UCACAGAGAUUACAGUAUA
M-008494-02	D-008494-04	UEVLD	55293	GGGCAAAUCAUGAGAAUAA
M-008494-02	D-008494-17	UEVLD	55293	GCAAAUCGAGUGAUCGGAA
M-027120-00	D-027120-01	ANKRD13	88455	GGAAUCUGCUCGAGUCUUA
M-027120-00	D-027120-02	ANKRD13	88455	CCCACGAGGUCGAACAUUA
M-027120-00	D-027120-03	ANKRD13	88455	GCGCGUCGAUAUCACAUUG
M-027120-00	D-027120-04	ANKRD13	88455	GGGCGGAGUUAAUGGAAGU
M-018787-01	D-018787-02	FLJ25555	124930	GAAUGGGUAUGAAGCUAAG
M-018787-01	D-018787-03	FLJ25555	124930	GUAAGACACCUUUGCAGUC
M-018787-01	D-018787-04	FLJ25555	124930	CGAUGGAACUGUCGGCGCA
M-018787-01	D-018787-17	FLJ25555	124930	CCGCAUCACCUUCGGGAAC
M-026603-01	D-026603-02	LOC338692	338692	CGAGAUGUCCAGCAAAGUA
M-026603-01	D-026603-03	LOC338692	338692	GCUACGAGGCCAAGGUGUA
M-026603-01	D-026603-04	LOC338692	338692	GGCCACGGUUUAUGAGGAA
M-026603-01	D-026603-17	LOC338692	338692	CCACCUGGACACUCGUAAU
M-017685-01	D-017685-01	DNAJB2	3300	GAAGUGCGUCCGCUGAUGA
M-017685-01	D-017685-02	DNAJB2	3300	GCACUCAGGUCCAGCAGAC
M-017685-01	D-017685-04	DNAJB2	3300	AGCACAAGCGGGAGAUUUA
M-017685-01	D-017685-17	DNAJB2	3300	CCGCAUCACCACACGCAGA
M-004724-00	D-004724-01	EPN1	29924	GGAAGACGCCGGAGUCAUU
M-004724-00	D-004724-02	EPN1	29924	GAACUGGCGUCACGUUUAC
M-004724-00	D-004724-03	EPN1	29924	GGACCUUGCUGACGUCUUC
M-004724-00	D-004724-04	EPN1	29924	GAACGUGCGUGAGAAAGCU
M-004725-01	D-004725-01	EPN2	22905	AGACUACGCUGUUGGAUUU
M-004725-01	D-004725-03	EPN2	22905	CCACUGGGCCCUGCAAUGA
M-004725-01	D-004725-04	EPN2	22905	CCACCAAGCCCGUGUCUGU
M-004725-01	D-004725-17	EPN2	22905	AUUAAAUCCACUAGAGCGA
M-021006-01	D-021006-01	EPN3	55040	GCGAGAACCUCUACACCAU
M-021006-01	D-021006-02	EPN3	55040	UAACAUUGCUGGACUACCU
M-021006-01	D-021006-03	EPN3	55040	UCGCUGACCUGACCUUCAA
M-021006-01	D-021006-04	EPN3	55040	UGCCAAACCUCCAGAAUCC

M-004005-01	D-004005-01	EPS15	2060	CCACCAAGAUUUCAUGAUA
M-004005-01	D-004005-02	EPS15	2060	GAUCGGAACUCCAACAAGA
M-004005-01	D-004005-03	EPS15	2060	AAACGGAGCUACAGAUUAU
M-004005-01	D-004005-04	EPS15	2060	CCACACAGCAUUCUUGUAA
M-004006-00	D-004006-01	EPS15L1	58513	GAAGUUACCUUGAGCAAUC
M-004006-00	D-004006-02	EPS15L1	58513	GGACUUGGCCGAUCCAGAA
M-004006-00	D-004006-03	EPS15L1	58513	GCACUUGGAUCGAGAUGAG
M-004006-00	D-004006-04	EPS15L1	58513	CAAAGACCAAUUCGCGUUA
M-016835-00	D-016835-01	HGS	9146	GCACGUCUUUCCAGAAUUC
M-016835-00	D-016835-02	HGS	9146	AGAGAGCGAUGCCAUGUUU
M-016835-00	D-016835-03	HGS	9146	GAUAUUCUGUGGAAAGUGU
M-016835-00	D-016835-04	HGS	9146	GUAAACGUCCGUAACAAGA
M-011423-00	D-011423-01	STAM	8027	GAACGAAGAUCCGAUGUAU
M-011423-00	D-011423-02	STAM	8027	CCACAAAGAUCCUCACGUU
M-011423-00	D-011423-03	STAM	8027	GAACCAGCCUUUAUUGAUG
M-011423-00	D-011423-04	STAM	8027	CAACCACCCUUUCCACUUU
M-017361-01	D-017361-02	STAM2	10254	CAAAGAGGAUGAAGACAUA
M-017361-01	D-017361-03	STAM2	10254	GAACUCACCUUUAAACAUG
M-017361-01	D-017361-04	STAM2	10254	GAACACUACUUCCAAUUUG
M-017361-01	D-017361-05	STAM2	10254	GAAGUAUGUUCCCGUGAUU
M-016586-01	D-016586-02	LOC130617	130617	CCAAAAGGAUAUCCAGGUA
M-016586-01	D-016586-03	LOC130617	130617	GCGCAGACCAUGUGGCCUA
M-016586-01	D-016586-04	LOC130617	130617	UAAGUGUGAUGCCUGCUCA
M-016586-01	D-016586-17	LOC130617	130617	GGCAGUUGAUAAAACGUAA
M-011365-01	D-011365-01	PSMD4	5710	CAACAUAGUUUGUCAUUCA
M-011365-01	D-011365-02	PSMD4	5710	GGACAACAGUGAGUAUAUG
M-011365-01	D-011365-03	PSMD4	5710	GGAAAGCACUAUGGUGUGU
M-011365-01	D-011365-04	PSMD4	5710	GAGAACAACGUGGGCCUUA
M-006995-03	D-006995-03	RAP80	51720	AGAGGCAGCUCCUUAAUAA
M-006995-03	D-006995-04	RAP80	51720	GCACAAGACUUCAGAUGCA
M-006995-03	D-006995-20	RAP80	51720	CCACAAAGAUUGAACGACA

M-006995-03	D-006995-21	RAP80	51720	AAGACAAAACAGUCGAAUA
M-006357-00	D-006357-01	SENP1	29843	GGAAAGAGUUUGACACCAA
M-006357-00	D-006357-02	SENP1	29843	GCAAAUAUGCUGACUGUAU
M-006357-00	D-006357-03	SENP1	29843	GAUCAUCAAUUUCUACAUG
M-006357-00	D-006357-04	SENP1	29843	GCAUUUCGCCUGACCAUUA
M-006033-01	D-006033-01	SENP2	59343	GGACAAACCUAUCACAUUU
M-006033-01	D-006033-02	SENP2	59343	GGUAAUAAAUCUCCUAAUG
M-006033-01	D-006033-03	SENP2	59343	GAGGAGAUAUUCAGACAUU
M-006033-01	D-006033-04	SENP2	59343	UCAGAAGGCUGUAAUAGAA
M-006034-01	D-006034-01	SENP3	26168	GUGCAUUUCUUCAAUAGUU
M-006034-01	D-006034-02	SENP3	26168	CUGGAAAGGUUACUUCAAA
M-006034-01	D-006034-03	SENP3	26168	GGAAGGGCCUGGUGUUGCA
M-006034-01	D-006034-04	SENP3	26168	GGAUGCUGCUCUACUCAAA
M-005946-01	D-005946-01	SENP5	205564	GUACAGAGCUGAUUCAUGA
M-005946-01	D-005946-02	SENP5	205564	GAAAGUAUUUGCUGACUGA
M-005946-01	D-005946-03	SENP5	205564	GCAAGGAGCUUAGUUUAGA
M-005946-01	D-005946-04	SENP5	205564	CAAAGGAUAUAAUGGAGUA
M-006044-01	D-006044-02	SENP6	26054	GAACGUCAAUUCAUCAGAA
M-006044-01	D-006044-03	SENP6	26054	CAACUAAUCUGUCAAUACA
M-006044-01	D-006044-04	SENP6	26054	GCACAGAUACCAGUAGUAA
M-006044-01	D-006044-17	SENP6	26054	CCAAGGUGUUGAACGUAUA
M-006035-01	D-006035-01	SENP7	57337	GAAGAUGACUCAACAAUAU
M-006035-01	D-006035-03	SENP7	57337	GAAUUGAAGCUGAAAGAUA
M-006035-01	D-006035-04	SENP7	57337	AGAGAAGGCUUAGAAAUAA
M-006035-01	D-006035-17	SENP7	57337	CAAAGUACCGAGUCGAAUA
M-004071-00	D-004071-01	SENP8	123228	AAUCAGAUGUCUCACUAUU
M-004071-00	D-004071-02	SENP8	123228	GAUCACGUCAGUUUCAUCA
M-004071-00	D-004071-03	SENP8	123228	UGGUCUACCUCCAAGAUAA
M-004071-00	D-004071-04	SENP8	123228	GGUUUGCGUUUGAGUACUU
M-029321-01	D-029321-01	LOC392188	392188	CAAGAAGGCCAGUGGUAUA
M-029321-01	D-029321-02	LOC392188	392188	CCAAGAAUGUGCAAUAUCC

M-029321-01	D-029321-03	LOC392188	392188	GGAAAGACACAGUGAGAGU
M-029321-01	D-029321-04	LOC392188	392188	CCAACGUACUUGUGAUUCA
M-009477-01	D-009477-01	ZFAND6	54469	GAAUGCCGGUGUGGAAAUG
M-009477-01	D-009477-02	ZFAND6	54469	GUACACAAUUGCUCUUACA
M-009477-01	D-009477-03	ZFAND6	54469	GCCCAGCCCUGUAUCAAAU
M-009477-01	D-009477-04	ZFAND6	54469	UAGUCUGUCUGAAUCUUUA
M-036937-00	D-036937-09	LOC645402	645402	GGAGUUGUCACGACGGAUA
M-036937-00	D-036937-10	LOC645402	645402	CGAUGAACUCGACAGAUCA
M-036937-00	D-036937-11	LOC645402	645402	GAGGUGAGUGGCAGUUCAA
M-036937-00	D-036937-12	LOC645402	645402	GAUCUCUGCUUGUGUGCCA
M-183279-00	D-183279-01	LOC645836	645836	AGACAGGCGAGCAAAGCAA
M-183279-00	D-183279-02	LOC645836	645836	GCCACAAGCAGGUAGAUCA
M-183279-00	D-183279-03	LOC645836	645836	GGGCUCUGCUUGUGUGCCA
M-183279-00	D-183279-04	LOC645836	645836	CGGGAGCACUCUCAAACAU
M-028352-02	D-028352-01	DUB1A	402164	GCAGGUAGAUCAUCACUCU
M-028352-02	D-028352-02	DUB1A	402164	CAACAAGAUUGCCAAGAAU
M-028352-02	D-028352-19	DUB1A	402164	GCAAGCAGGAAGAUGCCCA
M-028352-02	D-028352-20	DUB1A	402164	ACUGGAGAUCUCAAAUCAA
M-014021-01	D-014021-01	RBAF600	23352	GAAGUACCGUUCUGUUAUA
M-014021-01	D-014021-02	RBAF600	23352	GAACAAAUUUGCCGAUAAG
M-014021-01	D-014021-03	RBAF600	23352	UAAACAAGCUCUAAUUCGA
M-014021-01	D-014021-04	RBAF600	23352	GUAAAGCUAUGGAGGAGUU
M-017918-01	D-017918-02	UFD1L	7353	AAUCAAGCCUGGAGAUAUU
M-017918-01	D-017918-03	UFD1L	7353	GACCAAACCCGACAAGGCA
M-017918-01	D-017918-04	UFD1L	7353	GAGCGUCAACCUUCAAGUG
M-017918-01	D-017918-17	UFD1L	7353	GAGGCAGAUUCGUCGCUUU
M-008727-01	D-008727-05	VCP	7415	GUAAUCUCUUCGAGGUAUA
M-008727-01	D-008727-06	VCP	7415	AAACAGAUCCUAGCCCUUA
M-008727-01	D-008727-07	VCP	7415	GAGAGCAACCUUCGUAAAG
M-008727-01	D-008727-08	VCP	7415	GCACAGGUGGCAGUGUAUA
M-004701-02	D-004701-01	C13ORF9	51028	CAAAGAACAUGGCCAGAUU

M-004701-02	D-004701-02	C13ORF9	51028	CAAAGAACCUGGCCCAUUC
M-004701-02	D-004701-03	C13ORF9	51028	GGGAAUAGCUAACCCAGUU
M-004701-02	D-004701-19	C13ORF9	51028	CGACUGAUUUGGAGAGAUC

Supplemental Table 2: List of the hits for mitotic ubiquitin receptors identified by the siRNA screens.

#	Gene Symbol	Gene ID	Gene Accession	Type (class)	Family/ Domains	Gene description
1	INCENP	3619	NM_020238	Control	INCENP	Inner Centromere Protein, component of the chromosomal passenger complex (CPC)
2	AURKB	9212	NM_004217	Control	Kinase	Aurora kinase B, component of the chromosomal passenger complex (CPC)
3	PRPF8	10594	NM_006445	Ub Receptor	MPN	Pre-mRNA-processing-splicing factor 8
4	RHBDD3	25807	NM_012265	Ub Receptor	UBA	Rhomboid domain-containing protein 3 (C22ORF3)
7	MARK3	4140	NM_002376	Ub Receptor	UBA/Kinase	MAP/microtubule affinity-regulating kinase 3
8	BUB1B	701	NM_001211	Control	Kinase	Mitotic checkpoint serine/threonine-protein kinase BUB1 beta (BUBR1)
9	RAD23A	5886	NM_005053	Ub Receptor	UBA/UBL	UV excision repair protein RAD23 homolog A
10	NXF2	56001	NM_022053	Ub Receptor	UBA-like	Nuclear RNA export factor 2
11	SNRK	54861	NM_001100594	Ub Receptor	UBA/ Kinase	SNF-related serine/threonine-protein kinase
12	UBASH3B	84959	NM_032873	Ub Receptor	UBA	Ubiquitin-associated and SH3 domain-containing protein B (KIAA1959/STS1/TULA2)
13	CUL3	8452	NM_003590	Control	Cullin	Cullin-3
14	UBAC1	10422	NM_016172	Ub Receptor	UBA/UBL	Ubiquitin-associated domain-containing protein 1 (UBADC1)
15	RWDD1	51389	NM_015952	Ub Receptor	GI-UEV	RWD domain-containing protein 1
16	NXF1	10482	NM_006362	Ub Receptor	UBA-like	Nuclear RNA export factor 1
17	VCP	7415	NM_007126	Ub Receptor	AAA ATPase	Transitional endoplasmic reticulum ATPase (Valosin-containing protein/VCP/p97)
18	MARK1	4139	NM_018650	Ub	UBA/	Serine/threonine-protein

				Receptor	Kinase	kinase MARK1
19	WDR59	79726	NM_030581	Ub Receptor	WD repeats/ GI-UEV	WD repeat-containing protein 59
20	UBE4B	10277	NM_006048	Ub Receptor	U-box	Ubiquitin conjugation factor E4 B (Ubiquitin fusion degradation protein 2/UFD2)
21	RWDD4	201965	NM_152682	Ub Receptor	GI-UEV	RWD domain-containing protein 4 (MGC10198)
22	TNK2	10188	NM_005781	Ub Receptor	UBA/ Kinase	Tyrosine kinase non-receptor protein 2 (Activated CDC42 kinase 1/ACK1)
23	PRPF19	27339	NM_014502	Ub Receptor	U-box/ WD repeats	Pre-mRNA-processing factor 19
24	EEA1	8411	NM_003566	Ub Receptor	UBZ	Early endosome antigen 1
25	XPA	7507	NM_000380	Ub Receptor	UBZ	DNA repair protein complementing XP-A cells (Xeroderma pigmentosum group A-complementing protein)
26	ATXN7L2	127002	NM_153340	Ub Receptor	UBZ	Ataxin-7-like protein 2 (MGC46534)
27	CALCOCO1	57658	NM_020898	Ub Receptor	UBZ	Calcium-binding and coiled-coil domain-containing protein 1 (KIAA1536)
28	NICE-4	9898	NM_014847	Ub Receptor	UBA	Ubiquitin-associated protein 2-like (UBAP2L)
29	AMFR	267	NM_001144	Ub Receptor	RING UBA-like	E3 ubiquitin-protein ligase AMFR (Autocrine motility factor receptor)
30	NACAD	23148	NM_001146334	Ub Receptor	UBA-like	NAC-alpha domain-containing protein 1 (KIAA0363)

Supplemental Table 3: Ubiquitin decoders validation siRNA library.

Pool Catalog Number	Duplex Catalog Number	Gene Symbol	GENE ID	Sequence
L-012252-00	J-012252-06	PRPF8	10594	UGACGGGCAUGUAUCGAUA
L-012252-00	J-012252-07	PRPF8	10594	GCAGAUGGAUUGCAGUAUA
L-012252-00	J-012252-08	PRPF8	10594	GAUAAGGGCUGGCGUGUCA
L-012252-00	J-012252-09	PRPF8	10594	GAACAGACGCCUGACUUUA
L-019752-02	J-019752-13	RHBDD3	25807	CCUAGUACGUGUUUAGAAU
L-019752-02	J-019752-14	RHBDD3	25807	ACUGUUGGUUGGAGGACAA
L-019752-02	J-019752-15	RHBDD3	25807	GCUGGAACCCUCAGAGCGA
L-019752-02	J-019752-16	RHBDD3	25807	GCACCAUGGCUGUCCAAGU
L-003517-00	J-003517-11	MARK3	4140	GCGGUAAACUCGACACGUU
L-003517-00	J-003517-12	MARK3	4140	GCUAGUGAUUCCAGUUCUA
L-003517-00	J-003517-13	MARK3	4140	CCAAUAACUGCGACUAUGA
L-003517-00	J-003517-14	MARK3	4140	GGAGCUCGGUGUAGAAACU
L-005231-00	J-005231-05	RAD23A	5886	GCUCUGAGUAUGAGACGAU
L-005231-00	J-005231-06	RAD23A	5886	GAAGAUAGAAGCUGAGAAG
L-005231-00	J-005231-07	RAD23A	5886	GAUCUUGAGUGACGAUGUC
L-005231-00	J-005231-08	RAD23A	5886	GAAGAACUUUGUGGUCGUC
L-010445-00	J-010445-05	NXF2	56001	AGAAUGAAAUGGCAUAGUG
L-010445-00	J-010445-06	NXF2	56001	GAGGAUAGCAGGAAUAUGA
L-010445-00	J-010445-07	NXF2	56001	GGGAGUUCUUUCCGGGAUA
L-010445-00	J-010445-08	NXF2	56001	GCUCUACUCUAAAGAAGUG
L-004322-00	J-004322-19	SNRK	54861	GCUACAAAGUAUAACAUUC
L-004322-00	J-004322-20	SNRK	54861	GGGAGCACCAAGUACAUUA
L-004322-00	J-004322-21	SNRK	54861	GAAGUGAGAUGCAUGAAAC
L-004322-00	J-004322-22	SNRK	54861	GCUCAGAUAGUUCAUGCUA
L-008533-00	J-008533-05	UBASH3B	84959	CACAUUACCUGCAUGGAUA
L-008533-00	J-008533-06	UBASH3B	84959	GCAAUACCAUUAUCGAUCA

L-008533-00	J-008533-07	UBASH3B	84959	CCACCAAUCCUUCCUCUUA
L-008533-00	J-008533-08	UBASH3B	84959	GUCAGUCGCUGGAAAUGUA
L-020776-01	J-020776-09	UBAC1	10422	GCUAAUUGAACACGCAGAA
L-020776-01	J-020776-10	UBAC1	10422	GCACGUAGGUGGCGUUGUU
L-020776-01	J-020776-11	UBAC1	10422	CAGAAUGCCGCGUGCGAGU
L-020776-01	J-020776-12	UBAC1	10422	AGAGAUGAGCUGACGGAAA
L-020946-02	J-020946-18	RWDD1	51389	GGCUAUGCUCAGAGGGUUA
L-020946-02	J-020946-19	RWDD1	51389	UGAAGAUGAUCCAGACUAU
L-020946-02	J-020946-20	RWDD1	51389	GCAGAACUCUUGGAAAUUA
L-020946-02	J-020946-21	RWDD1	51389	UCUAGUGACAGCUGUGCAA
L-013680-01	J-013680-09	NXF1	10482	GGGAAGUCGUACAGCGAAC
L-013680-01	J-013680-10	NXF1	10482	GCGCCAUUCGCGAACGAUU
L-013680-01	J-013680-11	NXF1	10482	AAUUGAAGUCUGAGCGGGA
L-013680-01	J-013680-12	NXF1	10482	CGAUGAUGAACGCGUUAAU
L-008727-00	J-008727-09	VCP	7415	GCAUGUGGGUGCUGACUUA
L-008727-00	J-008727-10	VCP	7415	CAAAUUGGCUGGUGAGUCU
L-008727-00	J-008727-11	VCP	7415	CCUGAUUGCUCGAGCUGUA
L-008727-00	J-008727-12	VCP	7415	GUAAUCUCUUCGAGGUAUA
L-004259-00	J-004259-08	MARK1	4139	GGACAUCUAUUGCCUUUAA
L-004259-00	J-004259-09	MARK1	4139	GACCACAGAUCGAUACGUA
L-004259-00	J-004259-10	MARK1	4139	ACAAAGAUGUGGCUCGAAA
L-004259-00	J-004259-11	MARK1	4139	UAACUGUGAUUAUGAGCAA
L-022683-00	J-022683-05	WDR59	79726	GAGCUGAAGUGUUGAAGUU
L-022683-00	J-022683-06	WDR59	79726	CAUAUUAGAUGGUGUUGAU
L-022683-00	J-022683-07	WDR59	79726	GAGCGGAAAUCAAGACGAU
L-022683-00	J-022683-08	WDR59	79726	AAACGUGGUUGUAGAGUUC
L-007202-00	J-007202-06	UBE4B	10277	GCAACUAGACACCGCGAAA
L-007202-00	J-007202-07	UBE4B	10277	GGACUUGAUUGGCCAGAUU
L-007202-00	J-007202-08	UBE4B	10277	GCUAAACACUGGCUCCAAU

L-007202-00	J-007202-09	UBE4B	10277	CAAGAACGCACGCGCAGAA
L-016803-02	J-016803-18	RWDD4	201965	CUCAUAGAUUACAGAGAAU
L-016803-02	J-016803-19	RWDD4	201965	UCUGGUAGGAAGAUUGUUA
L-016803-02	J-016803-20	RWDD4	201965	GCUUAAUUACUGAACCGUA
L-016803-02	J-016803-21	RWDD4	201965	CCGCUAUGACCUAUACAUU
L-003102-01	J-003102-18	TNK2	10188	GGUGUUCAGUGGAAAGCGA
L-003102-01	J-003102-19	TNK2	10188	CCUAUGACCCUGUGAGCGA
L-003102-01	J-003102-20	TNK2	10188	CGGACAGGAUUGACGAACU
L-003102-01	J-003102-21	TNK2	10188	CGAAGUUGCUGCUCCGGCA
L-004668-00	J-004668-05	PRPF19	27339	GAUAACAACUUUGAGGUAA
L-004668-00	J-004668-06	PRPF19	27339	GCACGGAUGUCCAGAUCUA
L-004668-00	J-004668-07	PRPF19	27339	GUACUAAUGUGGCCAACUU
L-004668-00	J-004668-08	PRPF19	27339	GAUCUGCGCAAGCUUAAGA
L-004012-00	J-004012-06	EEA1	8411	GCAGUCAGCUGGAAAGUCA
L-004012-00	J-004012-07	EEA1	8411	GAAGCAACGGUUCAGAAUA
L-004012-00	J-004012-08	EEA1	8411	GUUCAAACACUAAUGGAUA
L-004012-00	J-004012-09	EEA1	8411	GAACCUUGAAGCUUUAUUA
L-005067-00	J-005067-05	XPA	7507	CGUAAGACUUGUACUAUGU
L-005067-00	J-005067-06	XPA	7507	GGAGACGAUUGUUCAUCAA
L-005067-00	J-005067-07	XPA	7507	GAGCCACCUCUUAAAUUUA
L-005067-00	J-005067-08	XPA	7507	GGAGGCAUGGCUAAUGUAA
L-018376-02	J-018376-17	ATXN7L2	127002	CCAGAAGCACUGCGAAAGA
L-018376-02	J-018376-18	ATXN7L2	127002	UCGAUGACUUCGCGGGACA
L-018376-02	J-018376-19	ATXN7L2	127002	GCAGAGAGAACAUCGAGAU
L-018376-02	J-018376-20	ATXN7L2	127002	GCAAGGAGCAAGUUCUCGA
L-007038-01	J-007038-09	CALCOCO1	57658	GUGCAGAGAUACUUCGAUU
L-007038-01	J-007038-10	CALCOCO1	57658	GGACAUCCUGAGCCGGCAA
L-007038-01	J-007038-11	CALCOCO1	57658	UGACAGACUCAGAGGACGA
L-007038-01	J-007038-12	CALCOCO1	57658	UGUCAGAAAGUAAGCGGGA

L-021220-01	J-021220-09	NICE-4	9898	CAACACAGCAGCACGUUAU
L-021220-01	J-021220-10	NICE-4	9898	GUGUGGAGAGUGAGGCGAA
L-021220-01	J-021220-11	NICE-4	9898	CAACAGAACCAGACGCAGA
L-021220-01	J-021220-12	NICE-4	9898	CCUGGGAGAUGGUCGGGAA
L-006522-00	J-006522-05	AMFR	267	GCAAGGAUCGAUUUGAAUA
L-006522-00	J-006522-06	AMFR	267	GGACGUAUGUCUAUUACAC
L-006522-00	J-006522-07	AMFR	267	GAAUUCGUCGGCACAAGAA
L-006522-00	J-006522-08	AMFR	267	GUAAAUACCGCUUGCUGUG
L-025327-02	J-025327-17	NACAD	23148	CUAGAGACCUGGAGACCGA
L-025327-02	J-025327-18	NACAD	23148	UGAGGAGACCAUCGCCAAA
L-025327-02	J-025327-19	NACAD	23148	CUUAUGUGGUCUUUGGCGA
L-025327-02	J-025327-20	NACAD	23148	GCUCCUACAUUACGGCCGA

Supplemental Table 4: Comparison of the mitotic ubiquitin decoders hits with reported mitotic factors.

#	Gene Symbol	Gene ID	Reported mitotic roles	Function
1	PRPF8	10594	(Garnett et al., 2009; Kittler et al., 2007; Neumann et al., 2010)	Functions as a scaffold that mediates the ordered assembly of spliceosomal proteins and snRNAs
2	RHBDD3	25807	(Neumann et al., 2010)	Predicted multi-pass membrane protein
3	MARK3	4140	(Peng et al., 1998)	Phosphorylates microtubule-associated proteins tau, MAP2 and MAP4. Phosphorylates Cdc25C and may act as a negative regulator of mitosis
4	RAD23A	5886	(Mukherji et al., 2006; Neumann et al., 2010)	Involved in global genome nucleotide excision repair (GG-NER) by association with XPC
5	NXF2	56001	(Cappell et al., 2012; Whitehurst et al., 2007)	Involved in the export of mRNA from the nucleus to the cytoplasm.
6	SNRK	54861	(Draviam et al., 2007)	May play a role in hematopoietic cell proliferation or differentiation
7	UBASH3B	84959		Interferes with CBL-mediated down-regulation and degradation of receptor-type tyrosine kinases
8	UBAC1	10422		Non-catalytic subunit of the KPC complex that acts as E3 ubiquitin-protein ligase. Required for poly-ubiquitination and proteasome-mediated degradation of CDKN1B during G1 phase of the cell cycle
9	RWDD1	51389		Interacts with DRG2 (developmentally regulated GTP binding protein 2), protects DRG2 from proteolytic degradation.
10	NXF1	10482	(Kittler et al., 2007)	Involved in the nuclear export of mRNA species bearing retroviral constitutive transport elements (CTE) and in the export of mRNA from the nucleus to the cytoplasm

11	VCP	7415	(Dobrynin et al., 2011; Ramadan et al., 2007)	Binds ubiquitinated proteins and exports misfolded proteins from the ER to the cytoplasm, where they are degraded by the proteasome. Necessary for the fragmentation of Golgi stacks during mitosis
12	MARK1	4139	(Draviam et al., 2007)	Serine/threonine-protein kinase involved in cell polarity and microtubule dynamics regulation
13	WDR59	79726		May interact with DDB1-CUL4A/B E3 ligase complexes
14	UBE4B	10277	(Garnett et al., 2009; Liu et al., 2011; Spinette et al., 2004)	Binds to the ubiquitin moieties of preformed conjugates and catalyzes ubiquitin chain assembly in conjunction with E1, E2, and E3. Interacts with VCP (UBE4B/UFD2)
15	RWDD4	201965		RWD domain-containing protein 4 (MGC10198)
16	TNK2	10188		Non-receptor tyrosine-protein and serine/threonine-protein kinase that is implicated in cell spreading and migration, cell survival, cell growth and proliferation (Activated CDC42 kinase 1/ACK1)
17	PRPF19	27339	(Neumann et al., 2010; Song et al., 2010)	Component of the PRP19-CDC5L complex that forms an integral part of the spliceosome and is required for activating pre-mRNA splicing.
18	EEA1	8411	(Kittler et al., 2007)	Binds phospholipid vesicles containing phosphatidylinositol 3-phosphate and participates in endosomal trafficking.
19	XPA	7507	(Kittler et al., 2007)	Involved in DNA excision repair. (Xeroderma pigmentosum group A-complementing protein)
20	ATXN7L2	127002		Ataxin-7-like protein 2 (MGC46534)
21	CALCOCO1	57658	(Hasegawa et al., 2013)	Calcium-binding and coiled-coil domain-containing protein 1 (KIAA1536). Functions as a coactivator for aryl hydrocarbon and nuclear receptors (NR).
22	NICE-4	9898	(Hasegawa et al., 2013)	Ubiquitin-associated protein 2-like (UBAP2L)
23	AMFR	267	(Garnett et al., 2009)	E3 ubiquitin-protein ligase.Component of a VCP/p97-AMFR/gp78 complex that participates in the final step of endoplasmic reticulum-associated

				degradation (ERAD).
24	NACAD	23148		May prevent inappropriate targeting of non-secretory polypeptides to the endoplasmic reticulum (ER).

Supplemental Table 5: List of the CUL3-interacting proteins identified by mass spectrometry analysis.

#	Gene Symbol, Name	Uniprot ID
1	CUL3, Cullin-3	Q13618
2	KBTBD6, Kelch repeat and BTB domain-containing protein 6	Q86V97
3	IBTK, Inhibitor of Bruton tyrosine kinase	Q2QKU3
4	KLHL15, Kelch-like protein 15	Q96M94
5	KLHL9, Kelch-like protein 9	Q9P2J3
6	KLHDC5, Kelch domain-containing protein 5	Q9P2K6
7	KLHL22, Kelch-like protein 22	Q53GT1
8	BTBD2, BTB/POZ domain-containing protein 2	Q9BX70
9	KCTD10, BTB/POZ domain-containing protein KCTD10	Q9H3F6
10	KLHL20, Kelch-like protein 20	Q9Y2M5
11	KCTD3, BTB/POZ domain-containing protein KCTD3	Q9Y597
12	KLHL7, Kelch-like protein 7	Q8IXQ5
13	KCTD6, BTB/POZ domain-containing protein KCTD6	Q8NC69
14	KBTBD7, Kelch repeat and BTB domain-containing protein 7	Q8WVZ9
15	KLHL12, Kelch-like protein 12	Q53G59
16	KCTD5, BTB/POZ domain-containing protein KCTD5	Q9NXV2
17	KLHL13, Kelch-like protein 13	Q9P2N7
18	KBTBD8, Kelch repeat and BTB domain-containing protein 8	Q8NFY9
19	BTBD1, BTB/POZ domain-containing protein 1	Q9H0C5
20	KLHL36, Kelch-like protein 36	Q8N4N3
21	KLHL21, Kelch-like protein 21	Q9UJP4
22	KCTD9, BTB/POZ domain-containing protein KCTD9	Q7L273
23	BTBD9, BTB/POZ domain-containing protein 9	Q96Q07
24	SHKBP1, SH3KBP1-binding protein 1	Q8TBC3
25	KCTD18, BTB/POZ domain-containing protein KCTD18	Q6PI47

26	TNFAIP1, BTB/POZ domain-containing protein TNFAIP1	Q13829
27	KCTD3, BTB/POZ domain-containing protein KCTD3	Q9Y597
28	PSMC4, 26S protease regulatory subunit 6B	P43686
29	KCTD17, BTB/POZ domain-containing protein KCTD17	Q8N5Z5
30	KBTBD4, Kelch repeat and BTB domain-containing protein 4	Q9NVX7
31	KCTD17, BTB/POZ domain-containing protein KCTD17	Q8N5Z5
32	KLHL24, Kelch-like protein 24	Q6TFL4
33	CAND1, Cullin-associated NEDD8-dissociated protein 1	Q86VP6
34	TCEB2, Transcription elongation factor B polypeptide 2, Elongin B	Q15370
35	PSMB5, Proteasome subunit beta type-5	P28074
36	PSMB5, Proteasome subunit beta type-5	P28074
37	KLHL8, Kelch-like protein 8	Q9P2G9
38	RBX1, RING-box protein 1	P62877
39	COPS2, COP9 signalosome complex subunit 2	P61201
40	KLHL26, Kelch-like protein 26	Q53HC5
41	LGALS3BP, Galectin-3-binding protein	Q08380
42	TCEB1, Transcription elongation factor B polypeptide 1, Elongin-C	Q15369
43	KLHL25, Kelch-like protein 25	Q9H0H3
44	ANKFY1, Ankyrin repeat and FYVE domain-containing protein 1	Q9P2R3
45	UBXN1, UBX domain-containing protein 1	Q04323
46	RPS27A, Ribosomal protein S27a, UBC, Ubiquitin C	Q5RKT7
47	IPP, KLHL27, Actin-binding protein IPP	Q9Y573
48	KCTD7, BTB/POZ domain-containing protein KCTD7	Q96MP8
49	PSMD2, 26S proteasome non-ATPase regulatory subunit 2	Q13200
50	KBTBD2, Kelch repeat and BTB domain-containing protein 2	Q8IY47
51	LZTR1, Leucine-zipper-like transcriptional regulator 1	Q8N653
52	PSMB2, Proteasome subunit beta type-2	P49721
53	RRCBTB1, RCC1 and BTB domain-containing protein 1	Q8NDN9

54	RHRHOBTB3, Rho-related BTB domain-containing protein 3	O94955
55	UBASH3B, Ubiquitin-associated and SH3 domain-containing protein B	Q8TF42
56	Kelch/BTB protein, Q8IWW9_HUMAN	Q8IWW9

Appendix I. List of Aurora B-GFP interacting proteins identified by mass spectrometry analysis upon UBASH3B-flag overexpression

This list excludes proteins which are present in control Aurora B-GFP IP upon FLAG overexpression, and proteins which are present in control GFP IP upon FLAG-UBASH3B overexpression. Hits are sorted by decreasing number of peptide spectrum mass (PSM). Score, coverage, number of peptides and number of PSM are shown for each identified protein.

Accession	Description, Protein name, Gene name	Score	#Pept	#PSM
Q13885	Tubulin beta-2A chain, TUBB2A, [TBB2A_HUMAN]	39,96	9	13
Q96GD4	Aurora kinase B, AURKB, [AURKB_HUMAN]	20,55	5	7
Q13310	Polyadenylate-binding protein 4, PABPC4, [PABP4_HUMAN]	18,55	5	6
P50990	T-complex protein 1 subunit theta, CCT8, [TCPQ_HUMAN]	16,83	6	6
Q12965	Unconventional myosin-Ie, MYO1E, [MYO1E_HUMAN]	13,47	5	5
Q00325	Phosphate carrier protein, mitochondrial, SLC25A3, [MPCP_HUMAN]	13,56	3	4
P61221	ATP-binding cassette sub-family E member 1, ABCE1, [ABCE1_HUMAN]	7,69	3	3
Q9UPY3	Endoribonuclease Dicer, DICER1, [DICER_HUMAN]	9,74	3	3
Q9Y608	Leucine-rich repeat flightless-interacting protein 2, LRRFIP2, [LRRF2_HUMAN]	10,05	3	3
P11177	Pyruvate dehydrogenase E1 component subunit beta, mitochondrial, PDHB, [ODPB_HUMAN]	9,33	3	3
Q9H0H5	Rac GTPase-activating protein 1, RACGAP1, [RGAP1_HUMAN]	9,77	3	3
P31151	Protein S100-A7, S100A7, [S10A7_HUMAN]	7,80	2	3
Q9Y5M8	Signal recognition particle receptor subunit beta, SRPRB, [SRPRB_HUMAN]	11,05	3	3
Q08188	Protein-glutamine gamma-glutamyltransferase E, TGM3, [TGM3_HUMAN]	9,51	3	3
Q4KMQ1	Taperin, TPRN, [TPRN_HUMAN]	7,73	3	3
Q5BKZ1	DBIRD complex subunit ZNF326, ZNF326, [ZN326_HUMAN]	9,03	3	3
P07951	Tropomyosin beta chain, TPM2, [TPM2_HUMAN]	6,67	2	2
P52294	Importin subunit alpha-5, KPNA1, [IMA5_HUMAN]	5,32	2	2
P61106	Ras-related protein Rab-14, RAB14, [RAB14_HUMAN]	5,69	2	2

Q9Y295	Developmentally-regulated GTP-binding protein 1, DRG1, [DRG1_HUMAN]	5,80	2	2
Q09028	Histone-binding protein RBBP4, RBBP4, [RBBP4_HUMAN]	5,32	2	2
P62888	60S ribosomal protein L30, RPL30, [RL30_HUMAN]	5,81	2	2
P62277	40S ribosomal protein S13, RPS13, [RS13_HUMAN]	5,99	2	2
P63220	40S ribosomal protein S21, RPS21, [RS21_HUMAN]	5,35	2	2
Q01130	Serine/arginine-rich splicing factor 2, SRSF2, [SRSF2_HUMAN]	6,52	2	2
Q9H568	Actin-like protein 8, ACTL8, [ACTL8_HUMAN]	5,62	2	2
O00116	Alkyldihydroxyacetonephosphate synthase, peroxisomal, AGPS, [ADAS_HUMAN]	4,96	2	2
Q92667	A-kinase anchor protein 1, mitochondrial, AKAP1, [AKAP1_HUMAN]	6,19	2	2
P02768	Serum albumin, ALB, [ALBU_HUMAN]	5,00	2	2
O94973	AP-2 complex subunit alpha-2, AP2A2, [AP2A2_HUMAN]	5,34	2	2
P05089	Arginase-1, ARG1, [ARGI1_HUMAN]	6,98	2	2
O95816	BAG family molecular chaperone regulator 2, BAG2, [BAG2_HUMAN]	5,30	2	2
Q96A33	Coiled-coil domain-containing protein 47, CCDC47, [CCD47_HUMAN]	5,34	2	2
P31040	Succinate dehydrogenase [ubiquinone] flavoprotein subunit, mitochondrial, SDHA, [SDHA_HUMAN]	5,80	2	2
Q96HP0	Dedicator of cytokinesis protein 6, DOCK6, [DOCK6_HUMAN]	8,12	2	2
Q16610	Extracellular matrix protein 1, ECM1, [ECM1_HUMAN]	4,95	2	2
O15372	Eukaryotic translation initiation factor 3 subunit H, EIF3H, [EIF3H_HUMAN]	5,77	2	2
Q13347	Eukaryotic translation initiation factor 3 subunit I, [EIF3I_HUMAN]	5,88	2	2
Q9P0I2	ER membrane protein complex subunit 3, EMC3, [EMC3_HUMAN]	5,17	2	2
P29317	Ephrin type-A receptor 2, EPHA2, [EPHA2_HUMAN]	6,12	2	2
O75477	Erlin-1, ERLIN1, [ERLN1_HUMAN]	6,38	2	2
Q9NZB2	Constitutive coactivator of PPAR-gamma-like protein 1, FAM120A, [F120A_HUMAN]	7,42	2	2
Q14344	Guanine nucleotide-binding protein subunit alpha-13, GNA13, [GNA13_HUMAN]	6,53	2	2
Q08379	Golgin subfamily A member 2, GOLGA2, [GOGA2_HUMAN]	7,17	2	2
P20839	Inosine-5'-monophosphate dehydrogenase 1, [IMPDH1, IMDH1_HUMAN]	5,97	2	2
P17301	Integrin alpha-2, ITGA2, [ITA2_HUMAN]	7,13	2	2
P31153	S-adenosylmethionine synthase isoform type-2, MAT2A, [METK2_HUMAN]	6,73	2	2
P43121	Cell surface glycoprotein MUC18, MCAM, [MUC18_HUMAN]	6,03	2	2

Q9H8H0	Nucleolar protein 11, NOL11, [NOL11_HUMAN]	5,08	2	2
Q9H6R4	Nucleolar protein 6, NOL6, [NOL6_HUMAN]	5,94	2	2
O00567	Nucleolar protein 56, NOP56, [NOP56_HUMAN]	5,61	2	2
P39656	Dolichyl-diphosphooligosaccharide-protein glycosyltransferase 48 kDa subunit, DDOST, [OST48_HUMAN]	5,41	2	2
Q9UQ80	Proliferation-associated protein 2G4, PA2G4, [PA2G4_HUMAN]	5,48	2	2
Q9NRX1	RNA-binding protein PNO1, PNO1, [PNO1_HUMAN]	5,77	2	2
Q8WVV4	Protein POF1B, POF1B, [POF1B_HUMAN]	4,85	2	2
P51148	Ras-related protein Rab-5C, RAB5C, [RAB5C_HUMAN]	5,26	2	2
Q9BWF3	RNA-binding protein 4, RBM4, [RBM4_HUMAN]	6,85	2	2
P42285	Superkiller viralicidic activity 2-like 2, SKIV2L2, [SK2L2_HUMAN]	6,64	2	2
Q96P63	Serpin B12, SERPINB12, [SPB12_HUMAN]	4,97	2	2
Q13247	Serine/arginine-rich splicing factor 6, SRSF6, [SRSF6_HUMAN]	5,76	2	2
P14868	Aspartate-tRNA ligase, cytoplasmic, DARS, [SYDC_HUMAN]	4,95	2	2
Q9NSD9	Phenylalanine-tRNA ligase beta subunit, FARSB, [SYFB_HUMAN]	5,76	2	2
Q03518	Antigen peptide transporter 1, TAP1, [TAP1_HUMAN]	6,05	2	2
P53999	Activated RNA polymerase II transcriptional coactivator p15, SUB1, [TCP4_HUMAN]	6,09	2	2
P49368	T-complex protein 1 subunit gamma, CCT3, [TCPG_HUMAN]	5,34	2	2
O14925	Mitochondrial import inner membrane translocase subunit Tim23, TIMM23, [TIM23_HUMAN]	7,92	2	2
P12270	Nucleoprotein TPR, TPR, [TPR_HUMAN]	5,64	2	2
Q9Y2W1	Thyroid hormone receptor-associated protein 3, THRAP3, [TR150_HUMAN]	5,95	2	2
Q8TED0	U3 small nucleolar RNA-associated protein 15 homolog, UTP15, [UTP15_HUMAN]	5,36	2	2
Q96KR1	Zinc finger RNA-binding protein, ZFR, [ZFR_HUMAN]	5,81	2	2
P68871	Hemoglobin subunit beta, HBB, [HBB_HUMAN]	6,09	2	2

Appendix II. List of potential interactors of UBASH3B in mitosis, identified by Mass Spectrometry

This list excludes proteins which are present in control FLAG IP. Hits are sorted by decreasing number of peptide spectrum mass (PSM). Score, coverage, number of peptides and number of PSM are shown for each identified protein, as well as protein function, mitotic roles of the proteins ("YES" for positive, "ND" for not detected), and number of ubiquitination sites identified in GGBase (https://gygi.med.harvard.edu/ggbase/)

Name, Description, Accession	Score	**# Pept**	# PSM	Function	Mit funct	Ubi sites
Ubiquitin-associated and SH3 domain-containing protein B, UBASH3B, [UBS3B_HUMAN] Q8TF42	39,61	**8**	13	Interferes with CBL-mediated down-regulation and degradation of receptor-type tyrosine kinases. Promotes accumulation of activated target receptors, such as T-cell receptors and EGFR, on the cell surface. Exhibits tyrosine phosphatase activity toward several substrates including EGFR, FAK, SYK, and ZAP70. Down-regulates proteins that are dually modified by both protein tyrosine phosphorylation and ubiquitination. The mitotic function of UBASH3B is shown in this Thesis for the first time.	YES	3
Filaggrin-2, FLG2, [FILA2_HUMAN] Q5D862	32,69	**7**	9	Intermediate filament-associated and psoriasis-susceptibility protein, precise biological role is still unknow, Filaggrin-2 protein was detected in the granular and horny layers of normal stratified epithelium. Recently, the expression of filaggrin-2 was shown to be decreased in patients with atopic dermatitis	ND	ND
Annexin A6, ANXA6, [ANXA6_HUMAN] P08133	16,91	**5**	5	Belongs to a family of calcium-dependent membrane and phospholipid binding proteins, has been implicated in mediating the endosome aggregation and vesicle fusion in secreting epithelia during exocytosis	ND	6
E3 ubiquitin-protein ligase TRIM21, TRIM21, [RO52_HUMAN] P19474	18,59	**3**	5	E3 ubiquitin-protein ligase whose activity is dependent on E2 enzymes, component of cullin-RING-based SCF (SKP1-CUL1-F-box protein) E3 ubiquitin-protein ligase complexes. A TRIM21-containing SCF(SKP2)-like complex is shown to mediate ubiquitination of CDKN1B ('Thr-187' phosphorylated-form), thereby promoting its degradation by the proteasome. Monoubiquitinates IKBKB that negatively regulates Tax-induced NF-kappa-B signaling. Negatively regulates IFN-beta production post-pathogen recognition by polyubiquitin-mediated degradation of IRF3. Mediates the ubiquitin-mediated proteasomal degradation of IgG1 heavy chain, which is linked to the VCP-mediated ER-associated degradation (ERAD) pathway. Promotes	ND	ND

				IRF8 ubiquitination, which enhanced the ability of IRF8 to stimulate cytokine genes transcription in macrophages.		
Voltage-dependent anion-selective channel protein 1, VDAC1, [VDAC1_HUMAN] P21796	12,84	**4**	4	Forms a channel through the mitochondrial outer membrane and also the plasma membrane. The channel at the outer mitochondrial membrane allows diffusion of small hydrophilic molecules; in the plasma membrane it is involved in cell volume regulation and apoptosis	ND	5
26S protease regulatory subunit 7, PSMC2, [PRS7_HUMAN] P35998	12,10	**4**	4	Belongs to AAA-ATPase family, involved in the ATP-dependent degradation of ubiquitinated proteins. The regulatory (or ATPase) complex confers ATP dependency and substrate specificity to the 26S complex	YES	24
Methylosome subunit pICln, CLNS1A, [ICLN_HUMAN] P54105	13,16	**3**	4	Chaperone that regulates the assembly of spliceosomal U1, U2, U4 and U5 small nuclear ribonucleoproteins (snRNPs), the building blocks of the spliceosome. Thereby, plays an important role in the splicing of cellular pre-mRNAs	ND	2
Coiled-coil-helix-coiled-coil-helix domain-containing protein 3, mitochondrial, [CHCH3_HUMAN] Q9NX63	9,57	**3**	3	Required for maintenance of mitochondrial crista integrity and mitochondrial function. May act as a scaffolding protein that stabilizes protein complexes involved in crista architecture and protein import. Has also been shown to function as a transcription factor which binds to the BAG1 promoter and represses BAG1 transcription	ND	5
Mitogen-activated protein kinase kinase kinase 7, MAP3K7, [M3K7_HUMAN] O43318	11,16	**3**	3	Serine/threonine kinase which acts as an essential component of the MAP kinase signal transduction pathway. Plays an important role in the cascades of cellular responses evoked by changes in the environment. Mediates signal transduction of TRAF6, various cytokines including interleukin-1 (IL-1), transforming growth factor-beta (TGFB), TGFB-related factors like BMP2 and BMP4, toll-like receptors (TLR), tumor necrosis factor receptor CD40 and B-cell receptor (BCR). Ceramides are also able to activate MAP3K7/TAK1. Once activated, acts as an upstream activator of the MKK/JNK signal transduction cascade and the p38 MAPK signal transduction cascade through the phosphorylation and activation of several MAP kinase kinases like MAP2K1/MEK1, MAP2K3/MKK3, MAP2K6/MKK6 and MAP2K7/MKK7. These MAP2Ks in turn activate p38 MAPKs, c-jun N-terminal kinases (JNKs) and I-kappa-B kinase complex (IKK). Both p38 MAPK and JNK pathways control the transcription factors activator protein-1 (AP-1), while nuclear factor-kappa B is activated by IKK. MAP3K7 activates also IKBKB and MAPK8/JNK1 in response to TRAF6 signaling and mediates BMP2-induced apoptosis. In osmotic stress signaling, plays a major role in the activation of MAPK8/JNK1, but not that of NF-kappa-B. Promotes TRIM5 capsid-specific restriction activity	ND	1
Erythrocyte band 7 integral membrane protein, STOM, [STOM_HUMAN]	9,42	**2**	3	Regulates ion channel activity and transmembrane ion transport. Regulates ASIC2 and ASIC3 channel activity, colocalizes with actin and lipid rafts	ND	ND

P27105						
Tight junction protein, TJP2, [ZO2_HUMAN]	8,84	**3**	3	Plays a role in tight junctions and adherens junctions	ND	1
Protein PRRC2A, PRRC2A, [PRC2A_HUMAN] Q9UDY2	9,57	**3**	3	May play a role in the regulation of pre-mRNA splicing	ND	ND
Ras GTPase-activating-like protein IQGAP1, IQGAP1, [IQGA1_HUMAN] P46940	10,20	**3**	3	Binds to activated CDC42 but does not stimulate its GTPase activity. It associates with calmodulin. Could serve as an assembly scaffold for the organization of a multimolecular complex that would interface incoming signals to the reorganization of the actin cytoskeleton at the plasma membrane. May promote neurite outgrowth	ND	7
Thioredoxin domain-containing protein 12, TXNDC12, [TXD12_HUMAN] O95881	8,67	**2**	3	Possesses significant protein thiol-disulfide oxidase activity	ND	ND
40S ribosomal protein S24, RPS24, [RS24_HUMAN] P62847	8,73	**2**	3	Required for processing of pre-rRNA and maturation of 40S ribosomal subunits	ND	3
Splicing factor 3A subunit 3, SF3A3, [SF3A3_HUMAN] Q12874	9,86	**3**	3	Subunit of the splicing factor SF3A required for 'A' complex assembly formed by the stable binding of U2 snRNP to the branchpoint sequence (BPS) in pre-mRNA. Sequence independent binding of SF3A/SF3B complex upstream of the branch site is essential, it may anchor U2 snRNP to the pre-mRNA.	ND	ND
Tryptophan-tRNA ligase, cytoplasmic, WARS, [SYWC_HUMAN] P23381	10,65	**3**	3	Isoform 1, isoform 2 and T1-TrpRS have aminoacylation activity while T2-TrpRS lacks it. Isoform 2, T1-TrpRS and T2-TrpRS possess angiostatic activity whereas isoform 1 lacks it. T2-TrpRS inhibits fluid shear stress-activated responses of endothelial cells. Regulates ERK, Akt, and eNOS activation pathways that are associated with angiogenesis, cytoskeletal reorganization and shear stress-responsive gene expression	ND	ND
Arf-GAP with coiled-coil, ANK repeat and PH domain-containing protein 2, [ACAP2_HUMAN] Q15057	6,02	**2**	2	GTPase-activating protein (GAP) for ADP ribosylation factor 6 (ARF6)	ND	ND
Aurora kinase B, AURKB, [AURKB_HUMAN] Q96GD4	6,13	**2**	2	Serine/threonine-protein kinase component of the chromosomal passenger complex (CPC), a complex that acts as a key regulator of mitosis. The CPC complex has essential functions at the centromere in ensuring correct chromosome alignment and segregation and is required for chromatin-induced microtubule stabilization and spindle assembly. Involved in the bipolar attachment of spindle microtubules to kinetochores and is a key regulator for the onset of cytokinesis during mitosis. Required for central/midzone spindle assembly and cleavage furrow formation. Key component of the cytokinesis checkpoint, a process required to delay abscission to prevent both premature resolution of intercellular chromosome bridges and	YES	10

				accumulation of DNA damage: phosphorylates CHMP4C, leading to retain abscission-competent VPS4 (VPS4A and/or VPS4B) at the midbody ring until abscission checkpoint signaling is terminated at late cytokinesis. AURKB phosphorylates the CPC complex subunits BIRC5/survivin, CDCA8/borealin and INCENP. Phosphorylation of INCENP leads to increased AURKB activity. Other known AURKB substrates involved in centromeric functions and mitosis are CENPA, DES/desmin, GPAF, KIF2C, NSUN2, RACGAP1, SEPT1, VIM/vimentin, GSG2/Haspin, and histone H3. A positive feedback loop involving GSG2 and AURKB contributes to localization of CPC to centromeres. Phosphorylation of VIM controls vimentin filament segregation in cytokinetic process, whereas histone H3 is phosphorylated at 'Ser-10' and 'Ser-28' during mitosis (H3S10ph and H3S28ph, respectively). A positive feedback between GSG2 and AURKB contributes to CPC localization. AURKB is also required for kinetochore localization of BUB1 and SGOL1. Phosphorylation of p53/TP53 negatively regulates its transcriptional activity. Key regulator of active promoters in resting B- and T-lymphocytes: acts by mediating phosphorylation of H3S28ph at active promoters in resting B-cells, inhibiting RNF2/RING1B-mediated ubiquitination of histone H2A and enhancing binding and activity of the USP16 deubiquitinase at transcribed genes		
CD44 antigen, CD44, [CD44_HUMAN] P16070	7,33	**2**	2	Receptor for hyaluronic acid (HA). Mediates cell-cell and cell-matrix interactions through its affinity for HA, and possibly also through its affinity for other ligands such as osteopontin, collagens, and matrix metalloproteinases (MMPs). Adhesion with HA plays an important role in cell migration, tumor growth and progression. In cancer cells, may play an important role in invadopodia formation. Also involved in lymphocyte activation, recirculation and homing, and in hematopoiesis. Altered expression or dysfunction causes numerous pathogenic phenotypes. Big protein heterogeneity due to numerous alternative splicing and post-translational modification events	promo prolifera	5
Eukaryotic translation initiation factor 3 subunit J, EIF3J, [EIF3J_HUMAN] O75822	6,04	**2**	2	Component of the eukaryotic translation initiation factor 3 (eIF-3) complex, which is required for several steps in the initiation of protein synthesis. The eIF-3 complex associates with the 40S ribosome and facilitates the recruitment of eIF-1, eIF-1A, eIF-2:GTP:methionyl-tRNAi and eIF-5 to form the 43S preinitiation complex (43S PIC). The eIF-3 complex stimulates mRNA recruitment to the 43S PIC and scanning of the mRNA for AUG recognition. The eIF-3 complex is also required for disassembly and recycling of post-termination ribosomal complexes and subsequently prevents premature joining of the 40S and	ND	ND

				60S ribosomal subunits prior to initiation. This subunit binds directly within the mRNA entry channel of the 40S ribosome to the aminoacyl (A) site. It may regulate the interaction between the 43S PIC and mRNA		
Flotillin-2, FLOT2, [FLOT2_HUMAN] Q14254	5,89	**2**	2	May act as a scaffolding protein within caveolar membranes, functionally participating in formation of caveolae or caveolae-like vesicles. May be involved in epidermal cell adhesion and epidermal structure and function	ND	2
Kinesin-like protein KIFC1, KIFC1, [KIFC1_HUMAN] Q9BW19	5,52	**2**	2	Minus end-directed microtubule-dependent motor required for bipolar spindle formation. May contribute to movement of early endocytic vesicles	YES	ND, bu was shown to be a target APC/
Unconventional myosin-Ie, MYO1E, [MYO1E_HUMAN] Q12965	6,35	**2**	2	Myosins are actin-based motor molecules with ATPase activity. Unconventional myosins serve in intracellular movements. Their highly divergent tails bind to membranous compartments, which are then moved relative to actin filaments. Binds to membranes containing anionic phospholipids via its tail domain. Required for normal morphology of the glomerular basement membrane, normal development of foot processes by kidney podocytes and normal kidney function. In dendritic cells, may control the movement of class II-containing cytoplasmic vesicles along the actin cytoskeleton by connecting them with the actin network via ARL14EP and ARL1	ND	2
Polymerase I and transcript release factor, PTRF, [PTRF_HUMAN] Q6NZI2	8,82	**2**	2	Plays an important role in caveolae formation and organization. Required for the sequestration of mobile caveolin into immobile caveolae. Termination of transcription by RNA polymerase I involves pausing of transcription by TTF1, and the dissociation of the transcription complex, releasing pre-rRNA and RNA polymerase I from the template. PTRF is required for dissociation of the ternary transcription complex	ND	11
Cytochrome b-c1 complex subunit 2, mitochondrial, UQCRC2, [QCR2_HUMAN] P22695	5,59	**2**	2	This is a component of the ubiquinol-cytochrome c reductase complex (complex III or cytochrome b-c1 complex), which is part of the mitochondrial respiratory chain. The core protein 2 is required for the assembly of the complex.	ND	ND
Protein-glutamine gamma-glutamyltransferase K, TGM1, [TGM1_HUMAN] P22735	5,51	**2**	2	Catalyzes the cross-linking of proteins and the conjugation of polyamines to proteins. Responsible for cross-linking epidermal proteins during formation of the stratum corneum	ND	ND
E3 ubiquitin-protein ligase UBR5, UBR5, [UBR5_HUMAN] O95071	5,62	**2**	2	E3 ubiquitin-protein ligase which is a component of the N-end rule pathway. Recognizes and binds to proteins bearing specific N-terminal residues that are destabilizing according to the N-end rule, leading to their ubiquitination and subsequent degradation. Involved in maturation and/or transcriptional regulation of mRNA by activating CDK9 by polyubiquitination. May play a role in control of cell cycle progression. May have	YES	14

				tumor suppressor function. Regulates DNA topoisomerase II binding protein (TopBP1) in the DNA damage response. Plays an essential role in extraembryonic development. Ubiquitinates acetylated PCK1. Also acts as a regulator of DNA damage response by acting as a suppressor of RNF168, an E3 ubiquitin-protein ligase that promotes accumulation of 'Lys-63'-linked histone H2A and H2AX at DNA damage sites, thereby acting as a guard against excessive spreading of ubiquitinated chromatin at damaged chromosomes		
4F2 cell-surface antigen heavy chain, SLC3A2, [4F2_HUMAN] P08195	7,08	**2**	2	Required for the function of light chain amino-acid transporters. Involved in sodium-independent, high-affinity transport of large neutral amino acids such as phenylalanine, tyrosine, leucine, arginine and tryptophan.	ND	16
Neutral amino acid transporter B, SLC1A5, [AAAT_HUMAN] Q15758	6,35	**2**	2	Has a broad substrate specificity, a preference for zwitterionic amino acids, and a sodium-dependence. It accepts as substrates all neutral amino acids, including glutamine, asparagine, and branched-chain and aromatic amino acids, and excludes methylated amino acids, anionic amino acids, and cationic amino acids. Acts as a cell surface receptor for feline endogenous virus RD114, baboon M7 endogenous virus and type D simian retroviruses.	ND	5
Inosine-5'-monophosphate dehydrogenase 2, IMPDH2, [IMDH2_HUMAN] P12268	6,57	**2**	2	Catalyzes the conversion of inosine 5'-phosphate (IMP) to xanthosine 5'-phosphate (XMP), the first committed and rate-limiting step in the de novo synthesis of guanine nucleotides, and therefore plays an important role in the regulation of cell growth. Could also have a single-stranded nucleic acid-binding activity and could play a role in RNA and/or DNA metabolism. It may also have a role in the development of malignancy and the growth progression of some tumors	Has a progres	12
Phosphoribosyl pyrophosphate synthase-associated protein 1, [KPRA_HUMAN] Q14558	5,89	**2**	2	Seems to play a negative regulatory role in 5-phosphoribose 1-diphosphate synthesis.	ND	3
Leucine-rich repeat flightless-interacting protein 1, LRRFIP1, [LRRF1_HUMAN] Q32MZ4	5,91	**2**	2	Transcriptional repressor which preferentially binds to the GC-rich consensus sequence (5'-AGCCCCCGGCG-3') and may regulate expression of TNF, EGFR and PDGFA. May control smooth muscle cells proliferation following artery injury through PDGFA repression. May also bind double-stranded RNA. Positively regulates Toll-like receptor (TLR) signaling in response to agonist probably by competing with the negative FLII regulator for MYD88-binding	ND	ND
Moesin, MSN, [MOES_HUMAN] P26038	5,82	**2**	2	Probably involved in connections of major cytoskeletal structures to the plasma membrane. May inhibit herpes simplex virus 1 infection at an early stage	YES	18
Unconventional myosin-Ic OS, MYO1C PE,	6,05	**2**	2	Myosins are actin-based motor molecules with ATPase activity. Unconventional myosins serve in intracellular movements.	ND	3

[MYO1C_HUMAN] O00159				Their highly divergent tails are presumed to bind to membranous compartments, which would be moved relative to actin filaments. Involved in glucose transporter recycling in response to insulin by regulating movement of intracellular GLUT4-containing vesicles to the plasma membrane. Component of the hair cell's (the sensory cells of the inner ear) adaptation-motor complex. Acts as a mediator of adaptation of mechanoelectrical transduction in stereocilia of vestibular hair cells. Binds phosphoinositides and links the actin cytoskeleton to cellular membranes.		
Protein phosphatase 1 regulatory subunit 12A, PPP1R12A, [MYPT1_HUMAN] O14974	5,59	**2**	2	Key regulator of protein phosphatase 1C (PPP1C). Mediates binding to myosin. As part of the PPP1C complex, involved in dephosphorylation of PLK1. Capable of inhibiting HIF1AN-dependent suppression of HIF1A activity	YES	ND
Prohibitin-2, PHB2, [PHB2_HUMAN] Q99623	5,40	**2**	2	Acts as a mediator of transcriptional repression by nuclear hormone receptors via recruitment of histone deacetylase. Functions as an estrogen receptor (ER)-selective coregulator that potentiates the inhibitory activities of antiestrogens and represses the activity of estrogens. Competes with NCOA1 for modulation of ER transcriptional activity. Probably involved in regulating mitochondrial respiration activity and in aging	YES	11
Pre-mRNA-processing factor 6P, RPF6, [PRP6_HUMAN] Q99623	6,00	**2**	2	Involved in pre-mRNA splicing as component of the U4/U6-U5 tri-snRNP complex, one of the building blocks of the spliceosome. Enhances dihydrotestosterone-induced transactivation activity of AR, as well as dexamethasone-induced transactivation activity of NR3C1, but does not affect estrogen-induced transactivation	ND	1
26S proteasome non-ATPase regulatory subunit 6, PSMD6, [PSMD6_HUMAN] Q15008	5,76	**2**	2	Acts as a regulatory subunit of the 26S proteasome which is involved in the ATP-dependent degradation of ubiquitinated proteins	YES	8
E3 SUMO-protein ligase RanBP2, RANBP2, [RBP2_HUMAN] P49792	6,23	**2**	2	E3 SUMO-protein ligase which facilitates SUMO1 and SUMO2 conjugation by UBE2I. Involved in transport factor (Ran-GTP, karyopherin)-mediated protein import via the F-G repeat-containing domain which acts as a docking site for substrates. Binds single-stranded RNA (in vitro). May bind DNA. Component of the nuclear export pathway. Specific docking site for the nuclear export factor exportin-1. Sumoylates PML at 'Lys-490' which is essential for the proper assembly of PML-NB	YES	3
Telomere-associated protein RIF1, RIF1, [RIF1_HUMAN] Q5UIP0	6,43	**2**	2	Required for checkpoint mediated arrest of cell cycle progression in response to DNA damage during S-phase (the intra-S-phase checkpoint). This checkpoint requires activation of at least 2 parallel pathways by the ATM kinase: one involves the MRN (MRE11A-RAD50-NBS1) complex, while the second requires CHEK2. RIF1 seems to act independently of both these pathways. Seems to play no role in either the G1/S or G2/M DNA	localiz microtu	ND

				damage checkpoints		
Structural maintenance of chromosomes protein 3, SMC3, [SMC3_HUMAN] Q9UQE7	5,69	**2**	2	Central component of cohesin, a complex required for chromosome cohesion during the cell cycle. The cohesin complex may form a large proteinaceous ring within which sister chromatids can be trapped. At anaphase, the complex is cleaved and dissociates from chromatin, allowing sister chromatids to segregate. Cohesion is coupled to DNA replication and is involved in DNA repair. The cohesin complex plays also an important role in spindle pole assembly during mitosis and in chromosomes movemen	YES	15
Ribose-phosphate pyrophosphokinase 1, PRPS1, [PRPS1_HUMAN] P60891	5,88	**2**	2	Catalyzes the synthesis of phosphoribosylpyrophosphate (PRPP) that is essential for nucleotide synthesis	ND	4
40S ribosomal protein S21, RPS21, [RS21_HUMAN] P63220	7,22	**2**	2	Belongs to the ribosomal protein S21e family.	ND	3
F-actin-capping protein subunit beta, CAPZB, [CAPZB_HUMAN] P47756	5,89	**2**	2	F-actin-capping proteins bind in a Ca2+-independent manner to the fast growing ends of actin filaments (barbed end) thereby blocking the exchange of subunits at these ends. Unlike other capping proteins (such as gelsolin and severin), these proteins do not sever actin filaments. Plays a role in the regulation of cell morphology and cytoskeletal organization	ND	3
Dermcidin, DCD, [DCD_HUMAN] P81605	6,73	**2**	2	DCD-1 displays antimicrobial activity thereby limiting skin infection by potential pathogens in the first few hours after bacterial colonization. Highly effective against E.coli, E.faecalis, S.aureus and C.albicans. Optimal pH and salt concentration resemble the conditions in sweat. Also exhibits proteolytic activity	ND	ND
Nucleolar RNA helicase 2, DDX21, [DDX21_HUMAN] Q9NR30	6,18	**2**	2	Can unwind double-stranded RNA (helicase) and can fold or introduce a secondary structure to a single-stranded RNA (foldase). Functions as cofactor for JUN-activated transcription. Involved in rRNA processing	interac mitotic	ND
Putative pre-mRNA-splicing factor ATP-dependent RNA helicase, DHX15, [DHX15_HUMAN] O43143	5,70	**2**	2	Pre-mRNA processing factor involved in disassembly of spliceosomes after the release of mature mRNA. In cooperation with TFIP11 seem to be involved in the transition of the U2, U5 and U6 snRNP-containing IL complex to the snRNP-free IS complex leading to efficient debranching and turnover of excised introns	ND	10
Eukaryotic translation initiation factor 3 subunit G, EIF3G, [EIF3G_HUMAN] O75821	6,83	**2**	2	Component of the eukaryotic translation initiation factor 3 (eIF-3) complex, which is required for several steps in the initiation of protein synthesis. The eIF-3 complex associates with the 40S ribosome and facilitates the recruitment of eIF-1, eIF-1A, eIF-2:GTP:methionyl-tRNAi and eIF-5 to form the 43S preinitiation complex (43S PIC). The eIF-3 complex stimulates mRNA recruitment to the 43S PIC and scanning of the mRNA for AUG recognition. The eIF-3 complex is also required for disassembly and recycling of post-termination ribosomal complexes and subsequently	ND	3

				prevents premature joining of the 40S and 60S ribosomal subunits prior to initiation. This subunit can bind 18S rRNA		
Heat shock protein 105 kDa, HSPH1, [HS105_HUMAN] Q92598	6,26	**2**	2	Prevents the aggregation of denatured proteins in cells under severe stress, on which the ATP levels decrease markedly. Inhibits HSPA8/HSC70 ATPase and chaperone activities	ND	10
Importin subunit beta-1, KPNB1, [IMB1_HUMAN] Q14974	6,37	**2**	2	Functions in nuclear protein import, either in association with an adapter protein, like an importin-alpha subunit, which binds to nuclear localization signals (NLS) in cargo substrates, or by acting as autonomous nuclear transport receptor. Acting autonomously, serves itself as NLS receptor. Docking of the importin/substrate complex to the nuclear pore complex (NPC) is mediated by KPNB1 through binding to nucleoporin FxFG repeats and the complex is subsequently translocated through the pore by an energy requiring, Ran-dependent mechanism. At the nucleoplasmic side of the NPC, Ran binds to importin-beta and the three components separate and importin-alpha and -beta are re-exported from the nucleus to the cytoplasm where GTP hydrolysis releases Ran from importin. The directionality of nuclear import is thought to be conferred by an asymmetric distribution of the GTP- and GDP-bound forms of Ran between the cytoplasm and nucleus. Mediates autonomously the nuclear import of ribosomal proteins RPL23A, RPS7 and RPL5. Binds to a beta-like import receptor binding (BIB) domain of RPL23A. In association with IPO7 mediates the nuclear import of H1 histone. In vitro, mediates nuclear import of H2A, H2B, H3 and H4 histones. In case of HIV-1 infection, binds and mediates the nuclear import of HIV-1 Rev. Imports SNAI1 and PRKCI into the nucleus	YES	10
DNA replication licensing factor MCM3, MCM3, [MCM3_HUMAN] P25205	5,70	**2**	2	Acts as component of the MCM2-7 complex (MCM complex) which is the putative replicative helicase essential for 'once per cell cycle' DNA replication initiation and elongation in eukaryotic cells. The active ATPase sites in the MCM2-7 ring are formed through the interaction surfaces of two neighboring subunits such that a critical structure of a conserved arginine finger motif is provided in trans relative to the ATP-binding site of the Walker A box of the adjacent subunit. The six ATPase active sites, however, are likely to contribute differentially to the complex helicase activity. Required for DNA replication and cell proliferation.	ND	3
Methylosome protein 50, WDR77, [MEP50_HUMAN] Q9BQA1	6,29	**2**	2	Non-catalytic component of the 20S PRMT5-containing methyltransferase complex, which modifies specific arginines to dimethylarginines in several spliceosomal Sm proteins and histones. This modification targets Sm proteins to the survival of motor neurons (SMN) complex for assembly into small nuclear ribonucleoprotein core particles. Might	ND	ND

				play a role in transcription regulation. The 20S PRMT5-containing methyltransferase complex also methylates the Piwi proteins (PIWIL1, PIWIL2 and PIWIL4), methylation of Piwi proteins being required for the interaction with Tudor domain-containing proteins and subsequent localization to the meiotic nuage		
Plasminogen activator inhibitor 1 RNA-binding protein, SERBP1, [PAIRB_HUMAN] Q8NC51	6,76	**2**	2	May play a role in the regulation of mRNA stability. Binds to the 3'-most 134 nt of the SERPINE1/PAI1 mRNA, a region which confers cyclic nucleotide regulation of message decay	ND	6
26S proteasome non-ATPase regulatory subunit 2, PSMD2, [PSMD2_HUMAN] Q13200	5,40	**2**	2	Acts as a regulatory subunit of the 26 proteasome which is involved in the ATP-dependent degradation of ubiquitinated proteins. Binds to the intracellular domain of tumor necrosis factor type 1 receptor. The binding domain of TRAP1 and TRAP2 resides outside the death domain of TNFR1	YES	9
26S proteasome non-ATPase regulatory subunit 3, PSMD3, [PSMD3_HUMAN] O43242	5,94	**2**	2	Acts as a regulatory subunit of the 26 proteasome which is involved in the ATP-dependent degradation of ubiquitinated proteins	YES	14
U2 small nuclear ribonucleoprotein A', SNRPA1, [RU2A_HUMAN] P09661	5,79	**2**	2	This protein is associated with sn-RNP U2. It helps the A' protein to bind stem loop IV of U2 snRNA	ND	5
Staphylococcal nuclease domain-containing protein 1, SND1, [SND1_HUMAN] Q7KZF4	6,00	**2**	2	Functions as a bridging factor between STAT6 and the basal transcription factor. Plays a role in PIM1 regulation of MYB activity. Functions as a transcriptional coactivator for the Epstein-Barr virus nuclear antigen 2 (EBNA2)	ND	11
Serine/threonine-protein kinase 38-like, STK38L, [ST38L_HUMAN] Q9Y2H1	6,83	**2**	2	Involved in the regulation of structural processes in differentiating and mature neuronal cells	ND	3
Arginine--tRNA ligase, cytoplasmic, RARS, [SYRC_HUMAN] P54136	6,53	**2**	2	Forms part of a macromolecular complex that catalyzes the attachment of specific amino acids to cognate tRNAs during protein synthesis. Modulates the secretion of AIMP1 and may be involved in generation of the inflammatory cytokine EMAP2 from AIMP1	ND	8
T-complex protein 1 subunit alpha, TCP1, [TCPA_HUMAN] P17987	6,76	**2**	2	Molecular chaperone; assists the folding of proteins upon ATP hydrolysis. As part of the BBS/CCT complex may play a role in the assembly of BBSome, a complex involved in ciliogenesis regulating transports vesicles to the cilia. Known to play a role, in vitro, in the folding of actin and tubulin	YES	11
T-complex protein 1 subunit beta, CCT2, [TCPB_HUMAN] P78371	5,69	**2**	2	Molecular chaperone; assists the folding of proteins upon ATP hydrolysis. As part of the BBS/CCT complex may play a role in the assembly of BBSome, a complex involved in ciliogenesis regulating transports vesicles to the cilia. Known to play a role, in vitro, in the folding of actin and tubulin	YES	17

Tropomyosin alpha-4 chain, TPM4, [TPM4_HUMAN] P67936	6,48	**2**	2	Binds to actin filaments in muscle and non-muscle cells. Plays a central role, in association with the troponin complex, in the calcium dependent regulation of vertebrate striated muscle contraction. Smooth muscle contraction is regulated by interaction with caldesmon. In non-muscle cells is implicated in stabilizing cytoskeleton actin filaments. Binds calcium	ND	5

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// Résumé

La mitose assure la répartition égale du génome. La kinase mitotique Aurora B y joue un rôle majeur en contrôlant la fidélité de la ségrégation des chromosomes de par sa localisation aux centromères et aux microtubules, qui nécessite son ubiquitination par CUL3. Cependant, le mécanisme conduisant la forme ubiquitinée d'Aurora B sur ces structures mitotiques reste à déterminer. Dans ce contexte, j'ai pu identifier la protéine UBASH3B, qui contient un domaine de liaison à l'ubiquitine (UBD) comme un régulateur essentiel de la ségrégation chromosomique, agissant comme un récepteur de l'ubiquitine pour Aurora B. UBASH3B interagit directement avec Aurora B et cette interaction est dépendante de la modification d'Aurora B par l'ubiquitine ainsi que de CUL3. UBASH3B ne régule pas le niveau d'expression d'Aurora B. En revanche, UBASH3B se localise aux fuseaux mitotiques et est à la fois nécessaire et suffisant pour transférer Aurora B aux microtubules. De plus, la redistribution d'Aurora B des centromères vers les microtubules contrôle le déroulement et la fidélité de la ségrégation des chromosomes et donc le contenu correct du matériel génétique des cellules. Ainsi, mes résultats expliquent comment la modification par l'ubiquitine régule la localisation et la fonction d'Aurora B, reliant une voie de signalisation impliquant un récepteur à l'ubiquitine à la mitose.

Mots clés : mitose, ubiquitin, Aurora B, CUL3, ségrégation des chromosomes, fuseau mitotique

Résumé en anglais

Mitosis ensures equal segregation of the genome. The major mitotic kinase Aurora B controls fidelity of chromosome segregation by its localization to centromeres and microtubules, which requires CUL3-mediated ubiquitylation. However, it remains unknown how ubiquitylated Aurora B is targeted to mitotic structures. Here, I identify ubiquitin-binding domain (UBD) protein UBASH3B that critically regulates chromosome segregation, acting as ubiquitin receptor for Aurora B. UBASH3B directly binds Aurora B, and this interaction is dependent on CUL3 and on ubiquitin recognition. UBASH3B does not regulate protein levels of Aurora B. Instead, UBASH3B localizes to the mitotic spindle and is both required and sufficient to transfer Aurora B to microtubules. Moreover, redistribution of Aurora B from centromeres to microtubules controls timing and fidelity of chromosome segregation and thereby euploidy of cells. Thus, my findings explain how ubiquitin attachment regulates localization and function of Aurora B, linking receptor-mediated ubiquitin signaling to mitosis.

Keywords : mitosis, ubiquitin, Aurora B, CUL3, chromosome segregation, mitotic spindle