The molecular basis of chromosome segregation regulation by the p97$^{Ufd1-Npl4}$ complex.

A dissertation submitted to

ETH ZURICH

for the degree of
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presented by

GRZEGORZ DOBRYNIN

M.Sc. Eng., Technical University of Lodz

born on the 3rd of April 1984

citizen of Poland

accepted at the recommendation of:

Prof. Dr. Ulrike Kutay, examiner
Prof. Dr. Hemmo Meyer, co-examiner

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Contents
Contents ................................................................................................................................................. 0
Summary ................................................................................................................................................ 3
Zusammenfassung .................................................................................................................................... 5
1 Introduction .......................................................................................................................................... 7
  1.1 Mammalian cell-cycle regulation .............................................................................................. 7
    1.1.1 Mitosis .................................................................................................................................... 9
    1.1.2 DNA damage response (DDR) .............................................................................................. 17
  1.2 The AAA ATPase p97 ............................................................................................................... 21
    1.2.1 The p97 structure and ATPase activity ............................................................................... 21
    1.2.2 The general mode of p97 function as an ubiquitin-dependent chaperon ....................... 23
    1.2.3 The p97 cofactors and substrate adaptors .......................................................................... 24
    1.2.4 Cellular functions of p97 ..................................................................................................... 29
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1.2.5 p97 functions in mitosis................................................................. 35
1.3 The aim of the thesis ............................................................................. 43
2 Results........................................................................................................... 44
2.1 Part I: p97$^{\text{Ufd1-Npl4}}$ antagonizes Aurora B during chromosome segregation in HeLa cells........................................................................................................ 44

2.1.1 Ufd1-Npl4 depletion leads to elevated levels of Aurora B and survivin association with chromosomes during mitosis................................................................. 44

2.1.2 Proper chromosome congression and Aurora B levels on chromatin in Ufd1 S2, but not V-S2 siRNA are restored by overexpression of the siRNA resistant Ufd1-GFP construct ........................................................................................................ 50

2.1.3 Ufd1-Npl4 reduces the chromosome-associated Aurora B activity early in mitosis. 52

2.1.4 Ufd1-Npl4 functionally antagonizes Aurora B during chromosome congression......................................................................................................................... 55
The molecular basis of chromosome segregation regulation by the p97\textsuperscript{Ufd1-Npl4} complex.

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2.1.5 Depletion of Ufd1 does not affect chromosome condensation in prometaphase cells................................. 58

2.1.6 Inhibition of p97 with DBeQ affects chromosome segregation during mitosis. 60

2.2 Part II: The role of p97 in the G2/M checkpoint ..................................... 62

2.2.1 Depletion of p97Ufd1-Npl4 complex leads to mitotic defects after ionizing radiation (IR) .................................................. 62

2.2.2 Depletion of Ufd1-Npl4 induces micronuclei formation in HeLa cells upon ionizing radiation treatment. ........................................... 69

2.2.3 Ufd1 or Npl4 depletion leads to compromised Cdc25A degradation upon IR-induced DNA damage. ......................................................... 71

2.2.4 The p97\textsuperscript{Ufd1-Npl4} complex binds the ubiquitinated form of Cdc25A............ 74

2.2.5 p97 siRNA-mediated depletion leads to reduced phosphorylation of Cdk1 at tyrosine 15 and decreased levels of Wee1, but not Cdc25A stabilization. .......... 77
The molecular basis of chromosome segregation regulation by the p97\textsuperscript{Ufd1-Npl4} complex.

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2.2.6 Depletion of Ufd1, Npl4 or DVC1, but not p97 leads to moderate stabilization of Cdc25A upon doxorubicin or cisplatin-induced DNA damage. ........ 79

3 Discussion.......................................................................................................................... 81

3.1 Part I – The p97\textsuperscript{Ufd1-Npl4} complex antagonizes Aurora B activity during chromosome segregation in HeLa cells ................................................................. 82

3.2 Part II – The p97\textsuperscript{Ufd1-Npl4} complex is involved in regulation of the G2/M checkpoint in human somatic cells. ................................................................. 91

4 Materials and Methods.................................................................................................. 97

4.1 Cloning ......................................................................................................................... 97

4.2 Generation of plasmid constructs ................................................................................ 97

4.3 Maintenance of the cell lines ...................................................................................... 98

4.4 Transfections .............................................................................................................. 98

4.5 Immunofluorescence staining .................................................................................... 99

4.6 Antibodies and other reagents .................................................................................. 100

4.7 Fluorescence imaging ................................................................................................. 101
The molecular basis of chromosome segregation regulation by the p97$^{Ufd1-Npl4}$ complex.

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born on the 3"d of April 1984

4.8 Preparation of cell extracts ................................................................. 101
4.9 Immunoprecipitation (IP) experiments ................................................. 102
4.10 SDS PAGE and Western blotting ........................................................ 102
4.11 Karyotyping ......................................................................................... 103
References ..................................................................................................... 104
Abbreviations .............................................................................................. 119
Acknowledgments .......................................................................................... 126
Curriculum Vitae .......................................................................................... Error! Bookmark not defined.
Summary

Cell division is a fundamental process that leads to a faithful propagation of the duplicated DNA into two daughter cells. Before the division can occur, cells have to ensure that the genetic material was properly replicated during S phase and that the DNA is not damaged. Only then, the duplicated DNA material can be equally separated between the daughter cells. The fidelity of chromosome segregation during mitosis depends on proper bioriented attachments of the sister kinetochores to the microtubules emanating from the opposing spindle poles. Many of the processes during cell division are governed by the ubiquitin-proteasome system (UPS). A central component of the UPS is the AAA+ ATPase p97 chaperone which recognizes the ubiquitylated substrate proteins and targets them for proteasomal degradation. During cell division, p97 has been implicated in regulation of many processes, including DNA replication, DNA damage signaling as well as chromosome segregation. This work aimed at elucidating the molecular basis of the role of p97^{Ufd1-Npl4} complex in chromosome segregation in HeLa cells. During our study we discovered two cellular mechanisms by which the p97 ATPase ensures faithful segregation of the DNA material.

In the first and main part of this thesis, we demonstrate that Ufd1-Npl4 complex antagonizes Aurora B on chromosomes already during early stages of mitosis and that this is crucial for proper chromosome segregation. Depletion of the Ufd1-Npl4 heterodimer by siRNA caused chromosome alignment defects, resulting in missegregated chromosomes and multi-lobed nuclei. Ufd1-Npl4 depletion also led to increased levels of Aurora B on prometaphase and metaphase chromosomes. Importantly, this increase in protein levels was also associated with higher Aurora B activity, as evidenced by hyperphosphorylation of the Aurora B substrate CENP-A, as well as by partial restoration of chromosome alignment in Ufd1-depleted cells by low concentrations of Aurora B inhibitor hesperadin. These results establish p97^{Ufd1-Npl4} as a crucial negative regulator of the Aurora B kinase early in mitosis of human somatic cells and indicated that the activity of Aurora B on chromosomes needs to be restrained to ensure faithful chromosome segregation.

In the second part of this study, we present evidence that the p97^{Ufd1-Npl4} complex is involved in G2/M checkpoint regulation. Depletion of p97, Ufd1 or Npl4 led to G2/M
checkpoint abrogation after ionizing-radiation (IR). Moreover, depletion of Ufd1-Npl4 resulted in compromised Cdc25A phosphatase degradation upon IR-induced DNA damage. Importantly, these findings were further supported by the fact that Cdc25A physically interacted with p97 ATPase, as well as its adapter protein Npl4. Taken together, these findings implicate a novel role of p97$^{Ufd1-Npl4}$ in regulating the cell cycle progression by controlling the degradation of one of the main G2/M checkpoint effector protein, Cdc25A.
Zusammenfassung

Zusammenfassung

den Chromosomen reguliert werden muss, um eine exakte Chromosomensegregation zu gewährleisten.

1 Introduction

1.1 Mammalian cell-cycle regulation

The cell cycle is a tightly regulated process that leads to faithful segregation of DNA material into two daughter cells and is controlled by the oscillating activities of the cyclin-dependent kinases (CDKs) that are activated by cyclins and inhibited by the CDK inhibitors (CKIs) (Murray 2004). This oscillating activity of the CDKs is regulated by diverse mechanisms, including transcriptional and translational levels, posttranslational modifications (especially phosphorylation) as well as the periodical degradation of cyclins and the CKIs by the ubiquitin-proteasome system (Glotzer et al. 1991; Skaar and Pagano 2009). In specific phases, different CDKs form distinct complexes with different cyclins and drive the cell through the cell cycle (Bicknell et al. 2003; Nurse 2000). Cyclins are a family of proteins that are periodically synthesized and degraded during each cell cycle. So far, eight cyclins have been shown to be directly involved in regulation of cell cycle progression: cyclins A1 and A2, B1, -2 and -3, C, D1, -2 and -3, E1 and -2, F, G1 and -2, H. They all share an 150-amino acid region of homology called the cyclin box that binds to the N-terminal end of the different CDKs (Bicknell et al. 2003). The prototypical cyclins driving the cell cycle progression are cyclins A, B and E, which are expressed in a cell cycle dependent manner and associate with Cdk1 or Cdk2 to mediate downstream events, including DNA replication and mitosis. The cyclin-dependent kinases are a family of the serine/threonine protein kinases each consisting of the catalytic CDK subunit and an activating cyclin subunit. The CDKs activity drives the cell cycle phase transitions in a timely and accurate manner, and these transitions can be transiently arrested by multiple checkpoint mechanisms that monitor fidelity and completion level of each of the cell cycle phases (Satyanarayana and Kaldis 2009). To date, at least eleven CDKs have been characterized (Malumbres and Barbacid 2005) and named with subsequent numbers from Cdk1 to Cdk11. The CDK complexes are key regulators of cell cycle progression, and are held inactive by phosphorylation of two residues located within the ATP binding loop, by the Wee1 and Myt1 kinases (Malumbres and Barbacid 2005). For instance, Cdk1, the main kinase involved in mitosis is kept inactive by inhibitory phosphorylation at threonine 14 and tyrosine 15. When the CDK activity becomes required for progression into the next cell
Introduction

cycle phase, the Cdc25 phosphatases dephosphorylate these two residues, thereby activating the CDK-cyclin complex. The Cdc25 phosphatases and their role in the cell cycle regulation will be discussed in more detail below.

In contrast to the reversible modifications, such as association with the CKIs or phosphorylation, ubiquitin-mediated proteasomal degradation is an irreversible mechanism. As such, the UPS plays a critical role in cell cycle regulation by mediating the precise spatial and temporal proteolysis of the key components that drive the cell cycle machinery, and thus assures unidirectionality of the cell cycle. The high specificity of the ubiquitylation process is ensured by more than 600 different E3 ligases that are encoded in the human genome (W. Li et al. 2008). These ligases are subdivided into two major classes, the HECT (homologous to E6-AP C-terminus) family E3 ligases and the RING (really interesting new gene) finger family E3 ligases (Metzger et al. 2012). One of the best described E3 families within the multi-subunit RING ligases is the cullin RING ligase (CRL) superfamily (Petroski and Deshaies 2005), which includes the SCF (Skp1-Cul1-F-box protein complex, also known as CRL1) and the APC/C (anaphase promoting complex/cyclosome) E3 ligases. Both complexes have well established cell cycle-regulatory roles and are essential for the cell division (Peters 2006; Reed 2003; Thornton and Toczyski 2006; Willems et al. 2004). The SCF complex consist of scaffold protein Cul1, Ring protein Rbx1 and S-phase kinase associated protein 1 (Skp1), which serves as an adaptor to bind the substrate-binding subunit, F-box protein (FBP) (Cardozo and Pagano 2004; Petroski and Deshaies 2005; Zheng et al. 2002). So far, 69 different FBPs were found in the human genome, and each is thought to recruit a specific set of substrates to the core SCF complex, determining its broad functional range (Silverman et al. 2012; Skaar and Pagano 2009). The APC/C is another prominent E3 ubiquitin ligase that is involved in cell cycle regulation. It has two substrate-specific adaptor proteins, Cdc20 and Cdh1 that associate in a cell cycle dependent manner. While the SCF ligases can be active throughout the whole cell cycle, the APC/C activity is restricted to the period between metaphase and the end of G1. During this time APC/C$^{Cdc20}$ initiates anaphase onset and coordinates mitotic exit, while APC/C$^{Cdh1}$ contributes to the final stages of mitosis and establishment of a stable G1 state (Acquaviva and Pines 2006; M. Li and Zhang 2009).
1.1.1 Mitosis

Mitosis is an elaborate process resulting in division of a cell into two daughter cells with identical genetic material. To ensure genetic identity as well as integrity, the genome must be replicated and segregated prior to the actual division process. Chromosome segregation is carried out by the mitotic spindle, a highly dynamic structure consisting of microtubule polymers and hundreds of other regulatory proteins (Walczak and Heald 2008). The fidelity of chromosome segregation is governed by the specialized protein complexes such as the chromosomal passenger complex (CPC) or the anaphase promoting complex/cyclosome (APC/C) as well as two cell-cycle checkpoints - the G2/M and the spindle assembly checkpoint (SAC). The following sections will discuss in more detail the key players of mitosis, including the crucial cellular function of the Aurora B kinase, the enzymatic core of the CPC, in chromosome segregation.

Mitotic entry

The decision to enter mitosis is mediated by a network of proteins that regulate the activation of the cyclin B-Cdk1 complex. In Xenopus egg extracts, cyclin B-Cdk1 is activated when the level of cyclin B reaches a threshold concentration (Solomon et al. 1990). Thus, a critical regulatory step in the activation of cyclin B-Cdk1 is the amount of cyclin B available to form a complex with Cdk1. In human somatic cells, cyclin B levels are temporally confined to G2 phase and early mitosis by regulated transcription and protein degradation (Fung and Poon 2005). The transcription of cyclin B starts in S phase and peaks in late G2 (Fung and Poon 2005). In mid G2 phase, concomitantly with centrosome maturation, cyclin B starts to accumulate at the centrosomes, where it reaches its highest concentration by late G2 what leads to its activation and binding to Cdk1 (Jackman et al. 2003). The ubiquitin-dependent degradation of cyclin B is regulated by the anaphase promoting complex/cyclosome (APC/C), a multisubunit E3 ligase. Poly-ubiquitylation of cyclin B starts in metaphase, when the spindle assembly checkpoint is silenced (Acquaviva and Pines 2006; van Leuken et al. 2008) and continues till early S phase (Lukas et al. 1999).

However, mere association of cyclin B to Cdk1 is not sufficient to form an active complex, as the Cdk subunit is subjected to posttranslational modifications that affect the kinase activity. As mentioned above, in addition to the activation by cyclin B binding,
Cdk1 activity is negatively regulated by the phosphorylation on the two inhibitor residues - threonine 14 (Thr14) and tyrosine 15 (Tyr15), which is controlled by the balance between the Wee1/Myt1 kinases and the Cdc25 phosphatases. Wee1 is a tyrosine kinase that phosphorylates Tyr15 (Parker and Piwnica-Worms 1992), and Myt1 is a dual-specificity kinase that can phosphorylate both sites (Kornbluth et al. 1994; Mueller et al. 1995), with a higher affinity towards Thr14 (F. Liu et al. 1997). These inhibitory phosphorylations are removed by the Cdc25 phosphatases. The functions of Wee1, Myt1 and Cdc25, in turn, are regulated by the Cdk1 activity itself. During the transition from G2 phase to mitosis, Myt1 and Wee1 kinases become phosphorylated and inactive when Cdk1 activity rises (Mueller et al. 1995; Watanabe et al. 2005), and the Cdc25 phosphatases become phosphorylated and active (Hoffmann et al. 1993). Thus, through the inner feedback loop, cyclin B-Cdk1 can stimulate its further activation by directly activating its activators and deactivating its inactivators. These two mechanisms serve as positive feedback that induces efficient autoamplification of Cdk1 activity (O’Farrell 2001), and they are responsible for the switch-like activation of the cyclin B-Cdk1 complex which leads to initiation of mitosis (Pomerening et al. 2005).

**Chromosomal Passenger Complex (CPC)**

One of the key regulators of mitosis is the chromosomal passenger complex (Carmena et al. 2012). It is composed of four proteins – the Aurora B kinase and three non-enzymatic subunits – inner centromeric protein (INCENP), survivin and borealin (also known as DASRA) (Adams et al. 2000; Cooke et al. 1987; Kelly and Funabiki 2009; Ruchaud et al. 2007; Terada et al. 1998; van der Waal et al. 2012). The non-enzymatic members of the complex control the targeting, enzymatic activity and stability of the Aurora B kinase (Lens et al. 2006). It was shown that siRNA-mediated knockdown of any member of the complex delocalizes the others and disrupts mitotic progression (Adams et al. 2001; Carvalho et al. 2003; Gassmann et al. 2004; Honda et al. 2003; Lens et al. 2003; Vader et al. 2006).

INCENP was the first member of the complex to be identified in a screen for novel components of mitotic chromosomes and acts as a scaffold on which the CPC assembles (Cooke et al. 1987). The INCENP C terminus is involved in binding and regulation of Aurora B (Adams et al. 2000; Kaitna et al. 2000). The INCENP N terminus
residues 1-58 form a triple-helix bundle with survivin and borealin that is required for the localization of the CPC to the centromere, anaphase spindle midzone and telophase midbody (Ainsztein et al. 1998; Jeyaprakash et al. 2007; Vader et al. 2006). Additionally, INCENP also interacts with heterochromatin protein 1 (HP1), and this binding is important for the CPC localization to the chromatin during interphase (Ainsztein et al. 1998; Kang et al. 2011; Nozawa et al. 2010).

Survivin is a conserved member of the inhibitor of apoptosis protein (IAP) family and contains a baculovirus IAP repeat (BIR) domain which is crucial for its dimerization (Chantalat et al. 2000; Muchmore et al. 2000). Survivin binds the other three components of the CPC and is phosphorylated by Aurora B (Wheatley et al. 2004) as well as Cdk1 (O'Connor et al. 2000; O'Connor et al. 2002) and Plk1 (Chu et al. 2011; Colnaghi and Wheatley 2010). It has been proposed that the binding of the CPC to the centromere is dependent on ubiquitylation of survivin (Vong et al. 2005). Vong and colleagues suggested that the lysine 63-linked ubiquitylation mediated by Ufd1 promotes the centromeric localization of survivin, whereas deubiquitylation, mediated by USP9X (also known as hFAM), is required for its dissociation (Vong et al. 2005).

The N terminus of mammalian borealin participates in the three-helix bundle formation that is crucial for proper localization of the CPC (Jeyaprakash et al. 2007). In humans, the central region of borealin (amino acids 110-207) interacts with the ESCRT-III (endosomal complexes required for transport III) subunit CHMP4C (charged multivesicular body protein 4C), and this interaction is important for regulation of daughter cells abscission at the final stages of mitosis (Carlton et al. 2012).

Finally, Aurora B belongs to the family of Ser/Thr kinases and is highly conserved from yeast (known as Ipl1) to mammals (C. S. Chan and Botstein 1993; Glover et al. 1995; van der Waal et al. 2012). This family consists of three members: Aurora A, which localizes to the mitotic spindle poles; Aurora B, which functions at the centromere, anaphase spindle and cell cortex; and Aurora C, which shares high resemblance with Aurora B but regulates mitosis and meiosis at early developmental stages (Carmena et al. 2009). As mentioned above, the Aurora B activity is tightly regulated at multiple levels, including INCENP binding, localization, post-translational modification and degradation. Below, cellular functions of Aurora B in mitosis are discussed in more detail.
**Aurora B functions in mitosis**

The Aurora B kinase is the catalytic core of the chromosomal passenger complex and plays a pivotal role in the cell cycle, ensuring correct chromosome segregation and execution of cytokinesis. To fulfill these critical functions, the kinase needs to be in its active conformation at the right place at the right time. And indeed, the localization of Aurora B is very dynamic which directly correlates with its multiple distinct functions during mitosis (Figure 1.1) (Carmena and Earnshaw 2003).

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**Figure 1.1 Localization of Aurora B during mitosis.** Indirect immunofluorescence (upper panels) and schematic representation (lower panels) of Aurora B localization (green) in HeLa cells during mitosis with kinetochores (pink), α-tubulin (red) and DNA (blue). In prophase, Aurora B localizes to the chromosome arms and starts to accumulate at the centromeres between kinetochores (panels Aa and Ab). In metaphase, Aurora B is found solely at the centromeres (panels Ba and Bb), whereas in anaphase it relocates to the spindle midzone (panels Ca and Cb). In telophase, Aurora B concentrates at the midbody (panels Da, Db) (adopted from (Ruchaud et al. 2007)).
The activation of Aurora B is a complex, multistep process that begins with Aurora B interaction with the IN box of INCENP. This initial low level activation of Aurora B allows for its subsequent auto-phosphorylation at Thr232, which results in its further activation (Yasui et al. 2004). Furthermore, it was recently described that for full activity, Aurora B also requires an additional phosphorylation at Ser311 which is mediated by checkpoint kinase 1 (Chk1) (Petsalaki et al. 2011; Zachos et al. 2007). At the beginning of mitosis Aurora B together with other components of the CPC localizes to chromatin and catalyzes the phosphorylation of histone H3 at serine 10, as well as centromeric histone H3 homologue CENP-A at serine 7 (Adams et al. 2001; Giet and Glover 2001; Hsu et al. 2000; Murnion et al. 2001; Zeitlin et al. 2001). It was reported that in some organisms phosphorylation of histone H3 at the very first stages of mitosis is important for proper chromosome compaction (Neurohr et al. 2011; Wei et al. 1999), however role of this phosphorylation in higher eukaryotes remains to be established. One proposed function of Aurora B in mitotic chromosome compaction is regulating the binding of the multimeric protein complex condensin (Collette et al. 2011; Giet and Glover 2001; Lipp et al. 2007; Morishita et al. 2001; Ono et al. 2004). In fission yeast and humans, Aurora B-dependent phosphorylation of the kleisin Cnd2 (known as CAPH in mammals) leads to recruitment of condensin to chromosomes (Nakazawa et al. 2011; Tada et al. 2011). Indeed, chromosome condensation in yeast Ipl1 mutants was strongly impaired (Morishita et al. 2001; Nakazawa et al. 2008; Petersen and Hagan 2003; Tada et al. 2011), however this effect was much less pronounced in vertebrates.

As mitosis progresses to metaphase, Aurora B dissociates from the chromosomal arms and concentrates at the centromeric region of the chromosomes where it regulates the binding of microtubules to the kinetochores (Adams et al. 2001; Cimini et al. 2006; Giet and Glover 2001; Hauf et al. 2003; Kaitna et al. 2000; Kelly and Funabiki 2009; Tanaka et al. 2002). Faithful segregation of chromosomes depends on the establishment of correct, biorientated (amphitelic) attachments of kinetochores to the mitotic spindle. During mitosis in vertebrates, proper, amphitelic binding is often established through a trial and error method that can result in chromosome malorientation (Figure 1.2) (Nicklas 1997; Rieder and Salmon 1998). Therefore, the kinetochore-microtubule attachments must be carefully regulated; incorrect attachments are destabilized and repaired, while correct attachments are stabilized.
Figure 1.2 Chromosome biorientation. (a) Correctly aligned chromosomes with sister kinetochores attached to microtubules emanating from opposing spindle poles (bioriented). Incorrect attachments can occur if both kinetochores establish microtubule connections to the same pole (syntelic) (b), or if one kinetochore is attached to both poles (merotelic)(c) (adopted from (Ruchaud et al. 2007)).

The Aurora B kinase activity is required for the selective destabilization of these improper microtubule attachments so the bi-orientation may be achieved (Tanaka et al. 2002). In vertebrates, the chemical inhibition or depletion of Aurora B leads to stabilization of the incorrect kinetochore-microtubule attachments which in turn causes a dramatic increase in merotelic and syntelic attachments (Cimini et al. 2006; Hauf et al. 2003; Lampson et al. 2004). Aurora B regulates chromosome-spindle attachments by phosphorylating several of the kinetochore substrates that bind microtubules, which results in destabilization of the kinetochore-microtubule bindings. The two kinetochore substrates that are negatively regulated by Aurora B phosphorylation include Ndc80/Hec1, a key microtubule-binding component of the kinetochore, and the Dam1 complex (in higher eukaryotes known as the spindle and kinetochore-associated complex, SKA) (Y. W. Chan et al. 2012; J. G. DeLuca et al. 2006; K. F. DeLuca et al. 2011). Additionally, Aurora B regulates the localization and activity of the kinesin 13 family member MCAK (mitotic centromere-associated kinesin; also known as Kif2), which functions as an important microtubule depolymerase. Aurora B phosphorylation recruits MCAK to the centromere by promoting its interaction with shugoshin 2 (Sgo2)(Andrews et al. 2004), which simultaneously suppresses both the MCAK microtubule-depolymerizing activity (Lan et al. 2004) as well as its accumulation at the microtubule plus-ends (Tanenbaum et al. 2011).
At anaphase onset a population of Aurora B relocalizes from the centromeres to the central spindle microtubules where it orchestrates contraction of chromosome arms, stabilizes the mitotic spindle and recruits factors that are important for late telophase and cytokinesis (Kaitna et al. 2000). The exact mechanism of the Aurora B spatial translocation is not clear, however it is initiated by a decrease in Cdk1 activity and requires both phosphatase and Aurora B activity (Hummer and Mayer 2009; Pereira and Schiebel 2003; Xu et al. 2009). It is postulated that active removal of Aurora B from the chromosomes may enhance its relocalization to the spindle midzone. Aurora B is ubiquitylated by two midzone-associated E3 ubiquitin ligase complexes, the CUL3-Kelch-like protein 9 (KLHL9)-KLHL13 (Sumara et al. 2007) and the CUL3-KLHL21 (Maerki et al. 2009). At the final stages of mitosis in cytokinesis, Aurora B accumulates at the midbody where its kinase activity is required for assembly and constriction of an equatorial contractile ring, that leads to the formation of the two daughter cells (Carmena et al. 2009; Ruchaud et al. 2007; van der Waal et al. 2012).

**Spindle assembly checkpoint (SAC)**
The SAC is a feedback control mechanism whose activity ensures accurate chromosome segregation during mitosis. The spindle assembly checkpoint is triggered by unattached kinetochores, and its cellular function is to delay the sister chromatid separation as well as the cell cycle progression until all kinetochores attain bioriented microtubule attachments (van der Waal et al. 2012). The key components of this checkpoint are the protein kinases Aurora B, Bub1, BubR1 and Mps1 and the non-kinase proteins Bub3, Mad1 (mitotic arrest deficient 1) and Mad2. These checkpoint proteins collectively ensure that the anaphase-promoting complex/cyclosome (APC/C) is inactive until all kinetochores are properly attached to the opposing spindle poles. As mentioned above, the APC/C is an ubiquitin ligase that targets securin and cyclin B for proteolytic degradation. Cyclin B degradation inactivates the protein kinase Cdk1 and promotes mitotic exit. The ubiquitin-dependent removal of securin releases separase, which triggers anaphase by cleaving kleisin, a subunit of cohesin, that keeps the sister chromatids together at the centromeres. Checkpoint signaling, induced by improperly attached sister kinetochores, promotes the accumulation of the mitotic checkpoint complex (MCC) that consist of BubR1, Bub3, Mad2 as well as Cdc20, and thus inhibits
the activation of $\text{APC/C}^{\text{Cdc20}}$. The cross talk between spindle microtubule binding and checkpoint signaling occurs at the kinetochores, large complex structures compromising at least 80 different proteins assembled at the centromere of each sister chromatid (Cheeseman and Desai 2008; Santaguida and Musacchio 2009). The inner kinetochore consists of the CENP proteins, including the histone H3 centromeric variant CENP-A, that organize the underlying chromatin and are associated with the centromeres throughout the cell cycle. In contrast, the outer kinetochore consists of proteins that are only assembled on the inner kinetochore at the beginning of mitosis. The core of the outer kinetochore is formed by the KNL1/Mis12/Ndc80 (KMN) protein complex. Ndc80 forms a direct attachment site for the spindle microtubules, and also binds the checkpoint kinase Mps1 (Saurin et al. 2011). KNL1 (known as Blinkin in vertebrates) binds spindle microtubules as well, and additionally interacts with the checkpoint proteins BubR1 and Bub1.

Aurora B is a master regulator of the spindle checkpoint signaling and its activity promotes kinetochore recruitment of the key SAC components Mad1, Mad2, Mps1, Bub1, BubR1 and CENP-E (Ditchfield et al. 2003; Santaguida et al. 2011; Saurin et al. 2011; Vazquez-Novelle and Petronczki 2010; Vigneron et al. 2004). Moreover, Aurora B phosphorylates multiple subunits of the KMN network, including Ndc80/Hec1, KNL1 or Mad3p (in yeast), which destabilizes erroneous kinetochore-microtubule attachment (Cheeseman et al. 2006; J. G. DeLuca et al. 2006; Welburn et al. 2010). It is assumed that once all the sister kinetochores achieve bipolar orientation and the chromosomes align at the metaphase plate, the kinetochores stretch by the pulling forces exerted by attached microtubules emanating from the opposing centrosomes (D. Liu et al. 2009; Maresca and Salmon 2009, 2010; Uchida et al. 2009). This force spatially separates the subset of the Aurora B kinase, which is accumulated at the centromeric regions of the chromosomes, from its outer-kinetochore substrates. In this hypothetical model, physical distance between Aurora B and its substrates (for e.g. Ndc80) prevents them from being phosphorylated and the microtubule-kinetochore interaction is stabilized. When biorientation of the chromosomes is achieved, Aurora B substrates are dephosphorylated and the $\text{APC/C}^{\text{Cdc20}}$ complex initiates the anaphase. In mammalian cells, Aurora B activity is counterbalanced by both PP1 and PP2A phosphatases, as well as the regulatory subunits of these proteins. The regulatory subunits of PP1 and PP2A
define their substrate specificity and cellular targeting (Barr et al. 2011; Foley et al. 2011; Sun et al. 2008). It is postulated that depending on the regulatory subunit that PP1 is bound to, it can antagonize the Aurora B function in at least three distinctive ways (Qian et al. 2011): (a) by direct modulation of the Aurora B activity through dephosphorylation of Thr232 in the T-loop of the kinase (Sds22-PP1), (b) by Repo-man-PP1, Knl1-PP1 or CENP-E-PP1-dependent dephosphorylation of Aurora B substrates, or (c) indirectly, by dephosphorylating of Haspin-mediated phosphorylation of histone H3 at Thr3, thus inhibiting proper localization of Aurora B to the centromere (Repo-man-PP1). Therefore, it is now clear that proper balance between Aurora B kinase and PP1 phosphatase activity is crucial for faithful chromosome segregation. Furthermore, it has been reported that the excess of Aurora B activity leads to a failure in stabilization of correctly attached spindle microtubule, and thus a checkpoint-dependent mitotic delay (D. Liu et al. 2009; Ricke et al. 2011); whereas too little kinase activity increases the chance of attachment errors and weakens both, the mitotic and abscission checkpoint (van der Waal et al. 2012).

1.1.2 DNA damage response (DDR)

Maintenance of the genomic integrity, and thus viability of the living organism, is an essential part of the cellular physiology. The DNA damages, induced by genotoxic insults, must be repaired in order to prevent the propagation of mutations that may lead to cancer formation. The genotoxic insults that cause DNA damage comprise a variety of stimuli, including ultraviolet (UV) and ionizing (IR) radiation, chemicals from the environment, or reactive oxygen species that are the byproduct of a cellular metabolism. The processes by which cells sense and repair damaged DNA are collectively known as DNA damage response (DDR) (Zhou and Elledge 2000). In mammals, the critical regulators of the cellular DDR are the ATM (ataxia-telangiectasia-mutated) and ATR (ATM and Rad3-related) Ser/Thr protein kinases. ATM/ATR accumulate at the damage sites where they are subsequently activated (Bartek and Lukas 2007; Delacroix et al. 2007; J. H. Lee and Paull 2005; J. Lee et al. 2007). The activated ATM/ATR kinases transduce the DNA damage signal to its downstream targets, including Chk1 and H2AX (Sancar et al. 2004). The Chk1 kinase was originally identified in yeast as a protein that is essential for the DNA damage-induced cell cycle arrest (Walworth et al. 1993). Further
studies identified Chk1 homologues in Drosophila, Xenopus, mouse and human (Fogarty et al. 1994; Kumagai et al. 1998; Peng et al. 1997) and established Chk1 as a crucial effector kinase that sustains and amplifies the DDR signal in mammals. In response to DNA damage, ATM/ATR rapidly phosphorylates Chk1 at Ser317 and Ser345 which leads to the activation of DNA damage checkpoints and cell cycle arrest (Q. Liu et al. 2000; Tapia-Alveal et al. 2009). Chk1 induces the cell cycle arrest in DDR, mainly by phosphorylation of the Cdc25 family phosphatases, Wee1 kinase and controlling Plk1 (Fig. 1.9).

**Figure 1.3 Regulation of the cell cycle progression by Chk1 kinase.** Chk1 regulates the progression of the cell cycle by inhibiting polo-like kinase 1 (Plk1) and Cdc25 family phosphatases, and activating Wee1 kinase. The Cdc25 phosphatases are activators of Cdk1, whereas Wee1 regulates Cdk1 by inhibitory phosphorylation at Thr15. Plk1 can activate Cdk1 by inhibiting Wee1 or also directly promote cell cycle progression (Patil et al. 2013).

The cell division cycle 25 (Cdc25) family is dual-specificity phosphatases that can dephosphorylate CDKs in their ATP-binding loop, resulting in the activation of CDKs complexes and cell cycle progression (Donzelli and Draetta 2003; Galaktionov and Beach 1991; Nagata et al. 1991; Sadhu et al. 1990). Mammalian cells have three Cdc25 isoforms: Cdc25A, Cdc25B and Cdc25C, all of which are phosphorylated by Chk1 in DDR (Figure 1.4). Cdc25A controls the progression through S phase and G2/M

**Figure 1.4 Chk1-mediated Cdc25 regulation in response to DNA damage.** Phosphorylation of Cdc25 family phosphatases by Chk1 leads to their inhibition. Chk1 can also activate Nek11, which further phosphorylates Cdc25A at multiple sites to target it for ubiquitin-dependent degradation. Cdc25A is crucial for the G1/S transition, S phase progression, and mitotic entry; as a result, the inhibition of Cdc25A leads to G1 arrest, slowed down replication, and G2 phase arrest. Chk1 phosphorylates Cdc25B to induce its binding to the regulatory protein 14-3-3 and sequestration from the centrosome. Chk1 also phosphorylates Cdc25C to promote its association with the 14-3-3 protein and the subsequent nuclear exclusion. Chk1-mediated inhibition of Cdc25B and Cdc25C leads mainly to the G2 arrest (Patil et al. 2013).

The negative regulation of Cdc25 phosphatases by Chk1 leads to the activation of G1/S, intra-S, and G2/M checkpoints. Interestingly, Chk1-mediated phosphorylation of Cdc25A, B and C activates various mechanisms of their inhibition (Donzelli and Draetta 2003; Uto et al. 2004). For Cdc25A, phosphorylation by Chk1 targets it for
ubiquitin-dependent proteasomal degradation (Jin et al. 2003), which leads to the inhibition of Cdk1 resulting in cell cycle arrest at G1/S transition, S phase and G2/M transition (Mailand et al. 2000; Sanchez et al. 1997). In S and G2 phases, Cdc25A is targeted for degradation by the Skp1-Cul1-Fbox(SCF)β-TrCP, a component of the SCF ubiquitin ligase (Busino et al. 2003). Interestingly, besides direct phosphorylation of Cdc25A, Chk1 can additionally activate the Nek11 kinase, which in turn also phosphorylates Cdc25A and thus, further amplifies the DDR-mediated cell cycle arrest (Melixetian et al. 2009). In contrast to Cdc25A, phosphorylation of Cdc25B and Cdc25C does not lead to their degradation, but rather spatial translocation. Chk1-dependent phosphorylation of these two phosphatases leads to their binding to the regulatory molecule 14-3-3 and subsequent centrosomal sequestration of Cdc25B or nuclear exclusion of Cdc25C (Kramer et al. 2004; Peng et al. 1997). In addition to the Cdc25 phosphatases, Chk1 phosphorylates Wee1, the protein kinase that executes the inhibitory phosphorylation of Cdk1 at Thr15 (Fig. 1.10). As a result, in response to DNA damage, the phosphorylation and activation of Wee1 by Chk1 leads to the inhibition of Cdk1 activity and cell cycle arrest in G2 phase (J. Lee et al. 2001; O'Connell et al. 1997). Chk1 is also a negative regulator of polo-like kinase 1 (Plk1), a mitotic kinase that contributes to centrosome maturation, spindle formation and cytokinesis (Tang et al. 2006). Plk1 phosphorylates Wee1 and targets it for degradation, leading to the cyclin B-Cdk1 complex activation and initiation of the G2/M transition (Watanabe et al. 2005). Upon DNA damage, Chk1-mediated phosphorylation of Plk1 leads to its inhibition, thus Wee1 is stabilized and cell cycle arrest in the G2 phase.
1.2 The AAA ATPase p97

The p97 chaperone – also called valosin-containing protein (VCP) in mammals, Cdc48p in yeast, or CDC-48 in C. elegans – is an essential and highly abundant ATPase that accounts for about 1% of the total cellular protein contents. p97 is well conserved among all eukaryotes and plays a critical role in broad range of diverse cellular processes, including membrane fusion, ubiquitin-dependent protein degradation, cell cycle regulation as well as recently discovered functions in DNA repair and the control of the condensation state of the chromatin. To fulfill its different cellular functions, p97 converts chemical energy generated from ATP hydrolysis into mechanical force used to structurally remodel or unfold target proteins. It cooperates with diverse partner proteins to recognize and bind ubiquitin-labeled clients in order to target them for proteasomal degradation or recycling. This remarkable feature of p97, to assemble into alternate multiprotein complexes with different substrate-specific cofactors, determines its functional specificity as well as its precise temporal and spatial localization. Considering the broad range of substrates and processes in which p97 is involved, it is not surprising that p97 mutations have been implicated in the pathogenesis of several disorders, ranging from cancer to a wide variety of degenerative diseases.

1.2.1 The p97 structure and ATPase activity

The p97 ATPase is a homohexameric complex composed of six protomers arranged in a ring around a central channel. Each protomer contains three domains: the N-terminal domain and two ATP binding domains termed D1 and D2. The six D1 and D2 domains of oligomeric p97 form two stacked rings (Figure 1.5). The D1 domain is believed to be primarily structural, facilitating the hexameric complex assembly (Q. Wang et al. 2003b) while D2 domain is responsible for the majority of p97 ATPase activity (DeLaBarre et al. 2006; Song et al. 2003). The globular N-terminal domain that resides at the periphery of D1 domain has been shown to interact with many of the p97 cofactors required for cellular positioning and activity of p97 (Beskow et al. 2009; DeLaBarre and Brunger 2005; Pye et al. 2006). Some cofactors were also found to bind the disordered C-terminal region, including substrate modifying cofactors that alter the post-translational modification state of the client proteins (Jentsch and Rumpf 2007). Additionally, the C-terminal tail of p97 can be modified by phosphorylation and acetylation. These
modifications influence the p97 ATPase activity, localization as well as its binding to partner proteins (Ewens et al. 2010; Mullally et al. 2006; G. Zhao et al. 2007).

Figure 1.5 Structure of the AAA+ p97 ATPase. Crystal structure and schematic illustration of the p97 hexamer with color coded domains, N domain in green, two ATPase domains D1 (cyan) and D2 (blue) and the highly unstructured C region in grey. The N-terminal globular domain is the major binding site of p97 cofactors followed by the ATPase domains, D1 responsible for the formation of the hexamer and D2 that catalyzes the hydrolysis of ATP (adopted from (Meyer et al. 2012)).

The AAA ATP binding cassettes consist of six elements: the Walker A and B motif, the second region of homology (SRH), sensors 1 and 2, and the pore loops (Hanson and Whiteheart 2005). The Walker A and Walker B motifs are responsible for ATP binding and hydrolysis, respectively. It was shown, that the mutation of the conserved glutamate (E) residue to glutamine (Q) in the Walker B motif of the D2 domain, abolishes the ATP hydrolysis and leads to a dominant negative variant of p97, termed p97EQ, that bind but cannot release substrates (DeLaBarre and Brunger 2005; Pye et al. 2006). Downstream of the Walker B motif is the SRH motif that is also required for ATP hydrolysis. Sensor 1 interacts with the Walker B motif and the γ-phosphate of ATP. Sensor 2 (also called arginine fingers (R-fingers)) interacts directly with the γ-phosphate of ATP on a neighboring subunit. It is suggested, that these R-fingers are the primary line of communication between the protomers.
1.2.2 The general mode of p97 function as an ubiquitin-dependent chaperon

p97 has many roles within the cell, most of them linked to ubiquitin signaling or to the ubiquitin-proteasome system (UPS). Ubiquitin (Ub) is a small 76 amino acid protein that is highly conserved in all eukaryotes (Glickman and Ciechanover 2002). It is covalently attached to the target protein by an isopeptide linkage between the C-terminal glycine of Ub and a lysine in the target protein. Conjugation of ubiquitin to the substrates proceeds via a three-step mechanism. In the first step, ubiquitin is activated in its C-terminal glycine by the ubiquitin-activating enzyme, E1. Following activation, one of the several E2 enzymes (ubiquitin-conjugating enzymes, UBCs) transfers the ubiquitin from E1 to a member of the ubiquitin-protein ligase family, E3, to which the substrate protein is specifically bound. The E3 ligase (described in more detail above) catalyzes the last step in the conjugation process, the covalent attachment of the ubiquitin to the substrate. In addition to monoubiquitylation, substrates can be ubiquitylated on numerous lysines, resulting in multiubiquitylation (Petroski and Deshaies 2003, 2005). Furthermore, some E2/E3 combinations can utilize lysines on the substrate-conjugated ubiquitin, to catalyze further cycles of ubiquitylation, resulting in the target protein polyubiquitylation (Petroski and Deshaies 2005; Pickart and Eddins 2004). The ability to generate various substrate-ubiquitin structures is important for targeting proteins to different fates. For instance, monoubiquitylation is usually implicated in the regulation of gene expression and DNA repair, polyubiquitylation through lysine 48 generally targets a protein for proteasomal degradation whereas K63-linked Ub chains can regulate DNA damage tolerance, kinase activation, signal transduction as well as endocytosis (Passmore and Barford 2004).

p97, through its ability to structurally remodel ubiquitylated client proteins and alter the ubiquitin modification with the help of associated cofactors, has introduced an important additional level of regulation and plasticity to the ubiquitin-mediated processes (Jentsch and Rumpf 2007; Stolz et al. 2011; Ye 2006). p97 recognizes and binds ubiquitylated substrates, both in the context of proteasomal protein degradation and in other pathways of the ubiquitin system. Next, utilizing its segregase activity (Rape et al. 2001), p97 extracts the substrate proteins from interaction partners, cellular structures or the chromatin (Hanson and Whiteheart 2005; Pye et al. 2006; White and Lauring 2007). Then, the substrate is either targeted for proteasomal degradation, or, with the help of
deubiquitylating enzymes (DUBs), the ubiquitin is removed and the protein is recycled (Figure 1.6). In order to provide specificity to its various cellular functions, p97 is stringently controlled by numerous adaptors and substrate-processing cofactors. In the next sections, these will be discussed in more detail.

**Figure 1.6 General model of the p97 function in substrate segregation and ubiquitin chain editing.** A p97 target substrate (S) is post-transnationally modified with ubiquitin (orange) by the enzymatic cascade comprising the Ub-activating (E1), Ub-conjugating (E2) and the substrate-specific E3 ubiquitin ligase. p97 binds to ubiquitinated substrates with the help of ubiquitin-binding cofactors (C). p97 then converts the energy of ATP hydrolysis to structurally remodel the target in order to segregate it from the binding partners or cellular structures. The extracted substrate is then targeted for the proteasomal degradation (Pr) or recycled with the help of p97 Ub-editing cofactors, including the deubiquitylating enzymes (DUBs) or the chain modifying enzymes (E4) (adopted from (Meyer et al. 2012)).

### 1.2.3 The p97 cofactors and substrate adaptors
The involvement of p97 in multiple cellular processes (as described below) requires an extremely tight spatial and temporal regulation of its specificity as well as activity. This crucial regulation of the p97 functions is achieved by an elaborate system of p97 cofactor proteins, which assemble into distinct multiprotein p97 complexes which are involved in different cellular pathways. The vast majority of the cofactors bind to the N domain of p97 by interacting with either the UBX domain, UBX-like domain, SHP box (also called Binding Site 1), VCP binding motif (VBM) or the VCP interacting motif (VIM) (Buchberger 2010; Kloppsteck et al. 2012; Meyer et al. 2002; H. Meyer 2012). A small
subset of cofactors has been shown to bind with the C-terminal tail of p97 by interacting with the PUB or PUL domains (Madsen et al. 2009). p97 may directly associate with ubiquitin and unfolded proteins in vitro (Rape et al. 2001; Song et al. 2007; Thoms 2002), however this binding is weak (Dai and Li 2001; Ye et al. 2003), therefore it is believed that for optimal-activity in vivo, p97 requires certain substrate-recruiting cofactors. Most of the substrate-recruiting adaptor proteins have the ability to simultaneously bind ubiquitin, and thus ubiquitylated substrates, and p97 (Meyer et al. 2002). They recognize and bind client proteins carrying ubiquitin chains (Rape et al. 2001; Rumpf and Jentsch 2006), allowing for p97 association. The largest group of cofactors binds to the p97 N domain by a ubiquitin regulatory X (UBX) domain or structurally related UBX-like domains (Alexandru et al. 2008; Decottignies et al. 2004; Hartmann-Petersen et al. 2004; Schuberth et al. 2004; Schuberth and Buchberger 2008). The UBX domain has an ubiquitin-like fold as well as high overall structural homology to ubiquitin and it belongs to the first p97 domains that were identified (Buchberger et al. 2001; Yuan et al. 2001). So far, 13 UBX domain-containing domains have been identified in mammals, all of which have been demonstrated to bind p97 (Alexandru et al. 2008).

A hierarchical system of the p97 interaction with the adaptor proteins was suggested, where p97 forms core complexes with mutually exclusive major cofactors, including the Ufd1 (ubiquitin fusion degradation 1)-Npl4 (nuclear protein localization homolog 4) heterodimer, p47 or UBD1 (Alexandru et al. 2008; Meyer et al. 2000; Neuber et al. 2005; Ritz et al. 2011; Schuberth and Buchberger 2005, 2008; Totsukawa et al. 2011; Wang et al. 2004). Major cofactors have been shown to modulate p97 activity or govern assembly of additional cofactors (Bruderer et al. 2004; Hanzelmann et al. 2011). Each core complex can then act in several pathways by associating with alternative sets of accessory proteins that determine localization or provide additional enzymatic activities (Schuberth and Buchberger 2008; Yeung et al. 2008). Below, p47 and Ufd1-Npl4 adaptor proteins are described in more detail as the prototypical examples of substrate-recruiting cofactors.
p47 (known as Shp1) is the most abundant and the first identified cofactor of p97 (Kondo et al. 1997). Binding of p47 to p97 is mediated by a SHP box (also called BS1) as well as conserved loop region of the p47 UBX domain that is inserted into the hydrophobic pocket of the p97 N domain (Dreveny et al. 2004). On the N-terminal site of p47, a ubiquitin-associated (UBA) domain is located, which binds to the ubiquitylated substrate. So far, p47 was mostly implicated in the p97-regulated reassembly of the Golgi apparatus, endoplasmic reticulum (ER) membranes, nuclear envelope vesicles and the yeast vacuolar membranes (Acharya et al. 1995; Hetzer et al. 2001; Kano et al. 2005; Kondo et al. 1997; Latterich et al. 1995; Patil and Latterich 1998; Rabouille et al. 1995; Roy et al. 2000; Seeley et al. 2002). Interestingly, recently, p$^{7}_{97}$Shp1 complex was shown to play a role in mitosis in yeast. Two independent studies, reported that p97 together with the cofactor p47/Shp1 regulates the localization and activity of the PP1 phosphatase during mitosis what is crucial for the proper chromosome segregation in yeast (Bohm and Buchberger 2013; Y. W. Chan et al. 2012).

_Ufd1-Npl4 complex_

Ufd1 and Npl4 are present in the cytosol either as a heterodimer with a 1:1 stoichiometry or bound to p97 (Bruderer et al. 2004; Meyer et al. 2000; Pye et al. 2007). It was shown that Ufd1 is unstable in the absence of Npl4 (Bruderer et al. 2004), and that the heterodimer Ufd1-Npl4 is the only essential cofactor in yeast (DeHoratius and Silver 1996; Johnson et al. 1995). The Ufd1 N-terminal domain shares a high similarity to the N domain of p97 and contains binding sites for both mono- and poly-ubiquitin, with the higher affinity for the latter (Park et al. 2005). The C-terminal domain of Ufd1 is flexible and comprises a SHP box through which it can bind to the N domain of p97 (Bruderer et al. 2004). The structure of Npl4 is less well understood although two domains, the N-terminal UBX-like domain and the C-terminal zinc finger (NZF), have been solved by NMR studies (Isaacson et al. 2007; B. Wang et al. 2003a). The interaction between the Ufd1-Npl4 heterodimer and p97 has been described as bipartite with two different p97-interacting sites located at the C-terminal domain of Ufd1 and the UBX-L domain of Npl4 (Bruderer et al. 2004), and it has been shown that Ufd1-Npl4 binds p97 either via a single or double binding site. It was speculated that the UBX-L domain in Npl4 is a
general p97 binding domain and that the binding site in Ufd1 provides higher affinity towards the ubiquitylated substrates (Ramadan et al. 2007). Recently, however, it was reported that both binding modes can occur within the same population of p97\textsuperscript{Ufd1-Npl4} complexes, suggesting a high degree of conformational flexibility (Figure 1.7) (Bebeacua et al. 2012).

**Figure 1.7** EM reconstructions of p97\textsuperscript{Ufd1-Npl4} complex. (A) Mapping of the different domains in the 3D reconstruction of p97\textsuperscript{Ufd1-Npl4} in the confirmation where Ufd1-Npl4 interacts with p97 via two binding sites, and (B) via a single binding site. In both cases, the first and second images (top and side view, respectively) shows a fit of the crystal structure of p97 and of the negative-stain reconstruction obtained from cross-linked Ufd1-Npl4 (pink) into the 3D reconstruction of p97\textsuperscript{Ufd1-Npl4}. The third and fourth images (side and top view, respectively) are colored by domain according to the sequence representation below. (scale bar: 20 Å) (Bebeacua et al. 2012)

The p97\textsuperscript{Ufd1-Npl4} complex has a well-established function in ERAD, where it is involved in the retrotranslocation of emerging ERAD substrates through the interaction both before
and after their ubiquitylation and subsequent their targeting to the cytosolic 26S proteasomes (Bays et al. 2001; Braun et al. 2002; Jarosch et al. 2002; Ye et al. 2001, 2003). In addition, the Ufd1-Npl4 heterodimer was also implicated to play a role in the mitotic progression (Hetzer et al. 2001; Ramadan et al. 2007), transcription factor activation (Hoppe et al. 2000; Rape et al. 2001) or chromosome segregation (Ufd1 alone) (Vong et al. 2005). Recently, it was shown in our laboratory in Xenopus egg extracts, that the p97^{Ufd1-Npl4}-dependent extraction of Aurora B from chromatin during exit from mitosis is crucial for the nuclear envelope reassembly and chromatin decondensation (Ramadan et al. 2007). Moreover, in course of this study, it was reported that Ufd1-Npl4 plays a role in DNA replication (Franz et al. 2011; Mouysset et al. 2008; Raman et al. 2011) as well as in the DNA damage repair (Davis et al. 2012; Meerang et al. 2011; Mosbech et al. 2012). These data implicate the Ufd1-Npl4 heterodimer together with the p97 ATPase in the cell cycle regulation. As describe above, Ufd1-Npl4 has been shown to be involved in chromosome segregation during mitosis of human somatic cells, however, so far no systematic study was undertaken to elucidate the molecular basis of its role in this process.

Accessory p97 cofactors

After the recruitment of specific ubiquitylated substrates to p97 by adaptor proteins, the second group of p97 cofactors takes action. This group of p97 cofactors consists of proteins that alter the post-translational modifications of the p97 substrates. Many proteins emerging from the ubiquitin field such as E3 ubiquitin ligases (HRD1, gp78, Dorfin, Cullins and others) (Fang et al. 2001; Gardner et al. 2001; Y. Huang et al. 2006; X. Zhong et al. 2004), E4 enzymes (Ufd2) (Koegl et al. 1999) and deubiquitylating enzymes (VCIP135, Ataxin-3) (Burnett et al. 2003; Rumpf and Jentsch 2006; Wang et al. 2004) are found among these cofactors, but also deglycosylation enzymes such as PNGase belong to this group (Y. Yoshida et al. 2005). By influencing the degree of ubiquitylation of the bound client protein, substrate-processing cofactors can directly decide upon fate of the substrate protein. For instance, Ufd2 (U-box-containing polyubiquitylation enzyme) adds further Ub molecules to a p97-bound substrate, targeting it for degradation (Richly et al. 2005). Interestingly, Ufd3 (also known as Doa1), a WD-40 repeat-containing protein that associates with ubiquitin through a PFU (PLAA
family ubiquitin-binding) domain, binds to the same region at the C-terminus of p97 as Ufd2 and thereby blocks Ufd2-catalysed polyubiquitylation (Mullally et al. 2006; Rumpf and Jentsch 2006). Ubiquitylation of bound substrates can be additionally further antagonized by the deubiquitylating enzymes (DUBs), like VCIP135, Otu1 or Ataxin-3 (Burnett et al. 2003; Doss-Pepe et al. 2003; Mao et al. 2005; Uchiyama and Kondo 2005; Wang et al. 2004) that also interact with p97 and catalyze the release of ubiquitin from the substrate. Other p97 substrate-processing cofactors include the peptide N-glycanase (PNGase) enzyme that removes oligosaccharides from the ERAD substrates before their proteasomal degradation (Allen et al. 2006) and the HDAC6 deacetylase, which promotes accumulation of poly-ubiquitylated protein aggregates and inclusion body formation (Boyault et al. 2006).

1.2.4 Cellular functions of p97

*Activation of the transcription factor Spt23*

One of the best understood cellular functions of the p97<sup>Ufd1-Npl4</sup> complex is its involvement in the yeast OLE pathway, that regulates the synthesis of unsaturated fatty acids, which are important for membrane fluidity and thus cell viability (Hoppe et al. 2000). The key enzyme of this lipid and membrane synthesis pathway is Ole1, a fatty acid desaturase which is localized at the ER membrane. Transcription of this gene is activated by the transcription factor Spt23 (Zhang et al. 1999), a distant homologue of mammalian NF-κB (Aravind et al. 1999). Spt23 is an integral membrane protein that localizes to the ER membrane in form of the inactive precursor p120. When the levels of unsaturated fatty acids in the cell decrease, fluidity and thickness of the membrane change (Stukey et al. 1989). This change induces homodimerization of two p120 molecules and in turn one of the two gets mono- or poly-ubiquitylated by the RSP5 ubiquitin ligase. Subsequently, the ubiquitylated p120 molecule is endoproteolytically cleaved, resulting in a transcription factor called Spt23 p90. After cleavage, p90 remains bound to its unmodified p120 partner until the p90-p120 dimer is disassembled by the p97<sup>Ufd1-Npl4</sup> complex. Then, the free Spt23 enters the nucleus and activates the transcription of the OLE1 gene (Rape et al. 2001). Rape and colleagues suggest that p97 catalyzes the mobilization of mono-ubiquitylated p90 from the p90-p120 complex and as ubiquitin-chaperone remains bound to it, thereby preventing the re-association of
**Introduction**

p90 with another p120 on the membrane. However, a recent study showed that p97 interacts rather with the poly-ubiquitylated membrane-bound p120 and segregase p90 away from it (Shcherbik and Haines 2007), which is in line with the notion that the p97 has a higher affinity towards the poly-ubiquitylated substrates (Meyer et al. 2002). The activation of Spt23p90 in the OLE pathways is an example of p97-mediated regulatory extraction in which the p97 substrate protein becomes activated instead of being targeted for the proteasomal degradation.

**ER-associated degradation (ERAD)**

As a central element of the UPS system, p97 protects the cells from the cytotoxic effects of damaged or misfolded proteins, and thus it is essential for cellular homeostasis, especially under stress conditions. The role of p97 in bulk degradation of misfolded proteins can be illustrated by its well described function in ER-associated degradation. About 20% of the proteins encoded by the human genome are predicted to be secretory proteins (Lander et al. 2001). The proteins of the secretory pathway are imported into the ER lumen where the proper folding of these proteins is achieved and controlled. The translocation through the membrane occurs via a conserved protein Sec61, a component of a large multiprotein complex providing the channel for the entering protein (Rapoport 2007). As a protein folding is a complex and an error prone process, folding mistakes resulting in unfolded or misfolded proteins are inevitable. Approximately one third of all newly synthesized proteins are degraded co-transnationally or destroyed within minutes of their synthesis (Schubert et al. 2000). Proteins committed for degradation are transported across the ER membrane, ubiquitylated and degraded by the 26S proteasome, by a process referred to as ER-associated degradation (ERAD) (Stolz and Wolf 2010). This ubiquitin proteasome system (UPS), as described above, constitutes a protein quality control mechanism, which failure leads to the formation of proteins aggregates that in turn can cause severe disorders, such as neurodegenerative diseases (e.g. Alzheimer or Parkinson disease), diabetes as well as liver, heart and kidney malfunction (Hoseki et al. 2010; H. Yoshida 2007).

In the first step of the ERAD pathway, misfolded proteins are recognized by a set of ER lumen proteins, including EDEM1-3, OS9 and XTP3B, which discriminate properly folded from unfolded proteins (Oda et al. 2003; Szathmary et al. 2005). The misfolded
proteins are then translocated across the ER membrane and poly-ubiquitylated at lysine 48 mostly by one of the two RING-finger type ER membrane embedded ubiquitin ligases, Hrd1/SYVN1 (Kikkert et al. 2004; Nadav et al. 2003) or gp87/AMFR (Fang et al. 2001). Then, the ternary complex of p97\textsuperscript{Ufd1-Npl4} docks to the ER membrane with the help of the membrane-embedded ERAD components, which have p97-binding motifs and extracts the target proteins into the cytosol, where they are subsequently degraded by the proteasome (Wolf and Stolz 2012; H. Yoshida 2007). The p97-binding motifs that recruit the ATPase to the ER membrane include UBX domains (UBXD2 and UBXD8), VIM motifs (gp78 and VIMP), SHP boxes (Derlin-1 and Derlin-2) and yet undefined cytosolic regions of Hrd1 and VIMP (Ballar et al. 2006; Greenblatt et al. 2011; Liang et al. 2006; Suzuki et al. 2012; Ye et al. 2004; Ye et al. 2005).

In addition to its role in substrate extraction, p97 functions as a hub that links dislocated substrates to the cytoplasmic cofactors involved in substrate modification and processing. These include the deubiquitylating enzymes (Yod1, VCIP135, Usp19 and Ataxin-3), E4 ubiquitin extension enzymes (Ube4a) or PNGase that removes sugar residues from glycosylated ERAD substrates before degradation (Elsasser et al. 2002; G. Li et al. 2006; Romisch 2006; Wolf and Stolz 2012).

**DNA damage response**

Recently, p97 has been also implicated to play a role in the signaling pathways in response to DNA damage. Upon DNA damage, ATM (ataxia telangiectasia mutated) phosphorylates histone H2AX (γ-H2AX) at the sites of lesions what initiates an extensive signaling cascade of the DNA damage response (DDR). The ATM-mediated phosphorylation of γ-H2AX is followed by the association of the large adaptor protein MDC1 (Stucki et al. 2005), which, in turn facilitates the binding of two E3 ubiquitin ligases, RNF8 and RNF168 (Doil et al. 2009; Kinner et al. 2008; Mailand et al. 2007; Stewart et al. 2009). Crucially, the RNF8- and RNF168-mediated ubiquitylation of substrates at the sites of DNA damage orchestrates the accumulation of checkpoint and repair proteins such as RAD18, BRCA1 and 53BP1 (J. Huang et al. 2009; Huen et al. 2007; Marteijn et al. 2009; B. Wang and Elledge 2007). Several lines of evidence showed that p97 is involved in the signaling events of the DNA damage response. It was reported that RNF8-mediated Lys48 ubiquitylation triggers the association of p97
together with the ubiquitin-binding Ufd1-Npl4 cofactor to the DSBs (Meerang et al. 2011). Subsequently, p97 applies its segregase activity to mediate the removal of Lys48-Ub conjugates from the chromatin, and thus allowing the proper assembly of downstream factors at the site of the DNA damage. However, the molecular mechanism underlying the p97-dependent recruitment of these DDR downstream factors was not fully addressed. Recently, Dantuma and colleagues observed that the RNF8/RNF168 pathway mediates the DNA-damage induced chromatin extraction of the tumor suppressor L3MBTL1 (Acs et al. 2011), which interacts with the same binding site as 53BP1, namely H4K20me (histone H4 methylated on Lys20) (Min et al. 2007). It was shown that DNA damage-induced ubiquitylation of L3MBTL1 led to its increased interaction with p97. Additionally, both the extraction of L3MBTL1 from the chromatin and the recruitment of 53BP1 to the damage sites were impaired in cells expressing a dominant-negative mutant of p97 (Acs et al. 2011). Together, this data suggest the p97\textsuperscript{Ufd1-Npl4} complex helps to release L3MBTL1 from H4K20me at DSBs allowing the association of 53BP1 to newly exposed histone methyl marks.

**DNA replication**

Furthermore, in course of this study, several lines of evidence implicated p97 ATPase in the regulation of DNA replication. The efficient duplication of the genome and its equal distribution into two daughter cells is an essential process for all dividing organisms. To ensure that DNA replication occurs only once per cell cycle, the initiation of the replication must be tightly controlled. The errors, resulting from either incomplete or over-replicated genome, have severe consequences on the cell cycle progression and increase cancer susceptibility. The first evidence, that p97 plays a general role in the DNA replication came from the nematode *Caenorhabditis elegans*, where the inactivation of the p97\textsuperscript{Ufd1-Npl4} complex led to a hypersensitivity toward the HU treatment, a decreased DNA synthesis, and replication-dependent delay in the S phase progression (Mouysset et al. 2008). Recently, two independent studies shed more light on the molecular mechanisms of the p97 involvement in the DNA replication and linked these phenotypes to a new role of CDC-48/p97 in the degradation of the replication licensing factor Cdt1 (Franz et al. 2011; Raman et al. 2011). A critical step in the initiation of the DNA replication is the recruitment of the licensing factor Cdt1 to the
origins of the replication during G1 phase by the multisubunit origin recognition complex (ORC). Cdt1 primes origins by recruiting the MCM2-7 helicase that unwinds the double-stranded DNA at the start of S phase (Arias and Walter 2007). Upon initiation of the replication in S phase, Cdt1 is destroyed in order to prevent re-firing of the licensed origins. The lack of Cdt1 degradation in S phase leads to the DNA re-replication, aberrant chromosomal ploidy, genomic instability and tumorgenesis (Jin et al. 2006; Lovejoy et al. 2006; Melixetian et al. 2004; W. Zhong et al. 2003). To ensure a single round of DNA amplification, the protein levels of Cdt1 are tightly regulated by the Cul4 ubiquitin ligase at the onset of S phase (W. Zhong et al. 2003). Additionally, the Cdt1 degradation is induced upon DNA damage to immediately stop the DNA replication (Hu et al. 2004). Interestingly, recent genome-wide siRNA screen identified p97 and its cofactor Ufd1 to be involved in the DNA damage-induced degradation of Cdt1 in human cells (Raman et al. 2011). Raman and colleagues presented a model in which the p97Ufd1-Npl4 complex binds to the ubiquitylated Cdt1 and facilitates an ATP-dependent unfolding of Cdt1, followed by its release from the PCNA and subsequent transfer to the proteasome (Figure 1.8).

Figure 1.8 A model for p97-mediated degradation of Cdt1 on damaged DNA. A major ubiquitylation site on Cdt1 is located near the PIP-box (Havens and Walter 2009), which is tightly associated with the PCNA on the chromatin. Upon DNA damage, Cdt1 is ubiquitylated by Crl4Cdt2 what leads to recruitment of the p97Ufd1-Npl4 complex. After binding, p97 facilitates the ATP-dependent unfolding of Cdt1, followed by its release from PCNA and subsequent capture by, or facilitated transfer to, the proteasome (Raman et al. 2011).
The role of \( \text{p97}^{\text{Ufd1-Npl4}} \) in licensing of the DNA replication was not exclusively confined to the cellular response to DNA damage, because depletion of p97 also caused a general increase in Cdt1 levels in cells arrested in the G1/S phase. The role of p97 in the Cdt1 degradation was confirmed in another independent study, where Hoppe and colleagues showed that C. elegans embryos lacking CDC-48 or its cofactors UFD-1/NPL-4 accumulated CDT-1 on the mitotic chromatin (Franz et al. 2011).

All cells are continuously exposed to a multitude of genotoxic insults, which, if not properly repaired, represent a serious threat to the maintenance of genomic stability. Genetic material is particularly susceptible to DNA damage encountered during DNA replication, because the replicative DNA polymerases cannot accommodate damaged or modified nucleotides in their active sites. The presence of unrepaired DNA lesions during DNA replication may lead to stalling or even collapsing of the advancing replication machinery, yielding in highly cytotoxic DNA double-strand breaks (DSBs) (Branzei and Foiani 2010; Ciccia and Elledge 2010). Fork stalling at DNA lesions triggers the mono-ubiquitylation of PCNA (proliferating cell nuclear antigen) and the binding of the translesion synthesis (TLS) DNA polymerase \( \eta \) (Pol \( \eta \)) that can replicate past the damaged DNA (Arossi et al. 2010). The TLS polymerases have extended active sites that can accommodate the damaged bases (Yang and Woodgate 2007) and continue DNA replication in the presence of the DNA lesions, however they are error prone and introduce mutations to the genome. Therefore, it is important that the TLS polymerases are extracted from the chromatin as soon as the replication of damaged DNA is accomplished in order to limit the incidence of mutations. Two independent studies have recently identified a novel p97 adaptor protein DVC1, which recruits the p97 protein segregase to the DNA-damage sites and helps to facilitate the dissociation of the translesion polymerase Pol \( \eta \) during DNA replication (Davis et al. 2012; Mosbech et al. 2012). The recruitment of DVC1 to the stalled replisomes requires both its PCNA-binding PIP box and the DVC1 C-terminal ubiquitin-binding UBZ motif that binds to a yet unidentified poly-ubiquitylated factor. The chromatin-bound DVC1 specifically targets p97 to the damage site to facilitate the ubiquitin- and p97-dependent displacement of Pol \( \eta \) from replication forks after lesion bypass (Figure 1.9).
Figure 1.9 Model of p97<sup>DVC1</sup> function in cellular response to the DNA damage. DVC1 recruits p97 to the DNA damage site via interaction with ubiquitin and PCNA. Subsequently, p97, by means of its ATPase activity, catalyzes the segregation of Pol η from mono-ubiquitylated PCNA, restraining the access of Pol η to the replication machinery and promoting a polymerase switching during TLS-mediated bypass of DNA damage (Mosbech et al. 2012).

Whether p97<sup>DVC1</sup> only extracts Pol η from the chromatin or promotes its ubiquitin-dependent degradation remains to be solved. Interestingly, the exact molecular mechanism of p97 binding to DVC1 is also yet to be identified. Mailand and colleagues suggest that p97 interacts directly with DVC1 via the SHP motif (Mosbech et al. 2012), whereas Rouse and colleagues speculate that DVC1 specifically recruits the p97<sup>Ufd1-Npl4</sup> complex and binds to it via the SHP motif-mediated interaction between Ufd1 and DVC1 (Davis et al. 2012).

1.2.5 p97 functions in mitosis

The role of p97 in mitosis has been implicated for a long time. The very first evidence of p97 involvement in the cell division was discovered in yeast, where cdc48 mutants of <i>Saccharomyces cerevisiae</i> arrested in cell cycle, with large buds and the nucleus located in the neck between the mother- and daughter cells (Moir et al. 1982). Interestingly, similar phenotypes were observed in mutants of the two genes coding for the Ufd1-Npl4 heterodimer (DeHoratius and Silver 1996; Johnson et al. 1995), which, as mentioned above, are the only essential Cdc48/p97 cofactors in yeast identified so far. In recent years, p97 has emerged as an important factor involved in mitotic progression, including regulation of the sister chromatid disjunction and separation, spindle disassembly, chromatin decondensation and organelle membrane fusion in late mitosis (Cao et al. 2003; Frohlich et al. 1991; Hetzer et al. 2001; Ikai and Yanagida 2006; Kondo...
et al. 1997; Rabouille et al. 1998; Ramadan et al. 2007; Sasagawa et al. 2007; Uchiyama et al. 2002; Vong et al. 2005; Yuasa et al. 2004). However, in contrast to the p97 function in chromatin decondensation (Ramadan et al. 2007), which is well described, detailed functions of p97 in other mitotic processes, still remain poorly understood.

Role of p97 in chromatin decondensation

The so far best understood mitotic function of p97 is the regulation of Aurora B kinase during exit from mitosis, what is crucial for the nuclear envelope reformation and chromatin decondensation (Ramadan et al. 2007). The chromatin-bound mitotic kinase Aurora B, next to other processes, controls the chromosome condensation state during exit from mitosis. During the final stages of mitosis, Aurora B is poly-ubiquitylated possibly by the E3 ligase Cullin-3 (Sumara et al. 2007). This ubiquitylated form of Aurora B is then targeted by the p97\textsuperscript{Ufd1-Npl4} complex. Utilizing the mechanistic force derived from ATP hydrolysis, p97 removes Aurora B from the chromatin, and targets it for proteasomal degradation or recycling by deubiquitylating enzymes (Figure 1.10). The p97\textsuperscript{Ufd1-Npl4}-mediated extraction of Aurora B kinase decreases its chromatin-associated activity, thus allowing for chromatin decondensation and subsequent nuclear envelope reformation (Ramadan et al. 2007).
Figure 1.10 Model for the role of p97 in regulating Aurora B and chromatin condensation during exit from mitosis. During mitosis, Aurora B kinase activity inhibits the formation of the nucleus by preventing chromosome decondensation and nuclear envelope formation. In telophase, Aurora B is modified by the ubiquitin system, possibly involving a Cullin 3-based ubiquitin ligase. The p97-Ufd1-Npl4 complex binds to poly-ubiquitylated Aurora B and utilizing energy derived from ATP hydrolysis extracts it from the chromatin. In analogy to other p97-mediated processes, Aurora B could then be targeted for proteasomal degradation or recycling by deubiquitylating enzymes. The drop of chromatin-bound Aurora B activity allows for chromatin decondensation and nuclear envelope formation.

Role of p97 in sister chromatid disjunction and separation

A genetic interaction between Cdc48/p97 and Cut1/separase has been found in Schizosaccharomyces pombe fission yeast. Separase is a large protease that is essential for sister chromatid disjunction by cleaving the cohesion subunit Scc1/Rad51 what leads to the dissociation of the link between the sister chromatids (Ikai and Yanagida 2006). Overexpression of Cut1/separase suppresses the mitotic defects of cdc48 mutant whereas overexpression of securin, an inhibitor of separase, aggravates the phenotype (Yuasa et al. 2004). This result implicated p97 in the sister chromatid disjunction and segregation, as well as suggested that p97 is a positive regulator of separase and thus cohesion cleavage. The destabilization of Cut1 in cdc48 mutants is proteasome independent, and it was therefore suggested that p97 directly stabilizes Cut1 (Ikai and Yanagida 2006). However, no direct interaction between these proteins was found, and therefore it was speculated that p97 might control Cut1 indirectly via the regulation of an yet unidentified factor upstream in the signaling pathway (Meyer and Popp 2008).
Role of p97 in spindle function and disassembly

The first evidence implicating p97 in the spindle function came from the observation that mutations in the yeast cell cycle gene, cdc48, led to an accumulation of cells with bundles of microtubules spreading throughout the cytoplasm that were originating from an unseparated spindle pole body (Frohlich et al. 1991). Further analysis revealed that the mutant cells arrested in metaphase, possibly due to a spindle checkpoint activation caused by the spindle malfunction. Additionally, in Xenopus laevis egg extracts, the inactivation of p97 or the depletion of its major adaptor Ufd1-Npl4 disturbed spindle disassembly during exit from mitosis, indicating that the p97^{Ufd1-Npl4} complex may regulated the spindle disassembly (Cao et al. 2003). In the absence of the p97^{Ufd1-Npl4} activity, spindle regulators including XMAP215 (Xenopus microtubule-associated protein 215), TPX2 (targeting protein for Xklp2) and Plx (Polo-like kinase of Xenopus laevis) remain on the spindle. Therefore, it was suggested that p97 may extract one or more of these factors from the spindle, and that this extraction triggers the spindle disassembly. However, this role of p97 was questioned by Heubes and colleagues, which observed that p97^{Ufd1-Npl4} is involved in ERAD, but could not reproduce the requirement for either p97 or the Ufd1-Npl4 heterodimer in the spindle disassembly in Xenopus laevis egg extracts (Heubes and Stemmann 2007).

Role of p97 in the nuclear envelope, ER and Golgi membrane reformation at the end of mitosis

Among the first activities that p97 was implicated in, was its involvement in the homotypic membrane fusion (Woodman 2003). As such, p97 regulates the reassembly of the Golgi apparatus, reformation of the endoplasmic reticulum, as well as growth of the nuclear envelope at the end of cellular division (Hetzer et al. 2001; Kondo et al. 1997; Latterich et al. 1995; Rabouille et al. 1995). At the onset of mitosis, these organelles are fragmented and equally distributed between the daughter cells, where they reassemble into functional organelles at the end of mitosis (Rothman and Warren 1994). All of these reactions are mediated by p97 in co-operation with the major adaptor p47, and the best studied of these processes is the Golgi apparatus reassembly at the end of mitosis in mammalian system (Uchiyama and Kondo 2005). In this processes, the p97^{p47} complex binds and regulates syntaxin 5, a SNARE protein that mediates
Introduction

membrane fusion (Rabouille et al. 1995). However, it still remains unclear how p97\textsuperscript{p47} activates syntaxin 5 in this process, because so far no direct evidence was presented that p97p47 induces structural changes in syntaxin 5. Essentially, the reassembly of the Golgi requires ubiquitin, the ubiquitin-binding by p47 and the de-ubiquitylating activity of an additional p97 cofactor VCIP135 (VCP/p47 complex-interacting protein of 135 kDa) (Wang et al. 2004). Summarizing, these findings suggest that the p97\textsuperscript{p47} complex activates syntaxin 5 indirectly through a yet unknown ubiquitylated regulator of the syntaxin 5-mediated fusion (Meyer 2005), but the exact mechanism is still to be found.

Role of p97 in chromosome segregation

A critical step in mitosis is the assembly of the kinetochores at the centromere and the binding of sister kinetochores to the microtubules emanating from the opposite spindle poles. The attachment of kinetochores to microtubules is governed by the Aurora B protein kinase that promotes the turnover of the microtubules from improperly attached kinetochores (Pinsky et al. 2006; Tanaka et al. 2002). Until now, no systematic study of the role of the p97 system during mitosis in mammalian somatic cells has been carried out. However, the available evidence from HeLa cells and budding yeast so far suggest a role of p97 in the chromosome segregation. Zheng and colleagues observed that Ufd1-depleted cells displayed a delay in mitosis indicating that the spindle assembly checkpoint was activated. The activation of the spindle checkpoint was due to the chromosome segregation defects that additionally led to chromosome lagging in anaphase (Vong et al. 2005). The depletion of Ufd1 resulted in a reduced recruitment of survivin to the kinetochores. Further analysis revealed that Ufd1 is required for the K63 ubiquitylation of survivin in mitosis which appears to be crucial for the proper targeting of survivin to the kinetochores. However, there is no evidence that Ufd1 itself is an ubiquitin ligase so even though that the physical interaction between Ufd1 and survivin was detected, it is still unclear in what way Ufd1 is needed for the regulatory ubiquitylation of survivin, and whether Ufd1 co-operates with p97 and Npl4 in this process.

Recently, however, the initial data obtained in our laboratory also demonstrated that the p97\textsuperscript{Ufd1-Npl4} complex may in fact play a role in the mammalian chromosome segregation. HeLa Kyoto cells were depleted of Ufd1, Npl4 or p47 for 48 hours, their progression was monitored by time-lapse microscopy and the time between nuclear envelope breakdown
Figure 1.11 The p97 adaptor Ufd1-Npl4 is required for normal progression through mitosis of HeLa cells. (A) Images from representative confocal time-lapse fluorescence microscopy movies of unsynchronized HeLa Kyoto cells progressing through mitosis, after treatment with the indicated siRNA oligonucleotides for 48 hours. The cells are stably expressing histone H2B-mRFP to visualize the chromatin. The time (minutes) after nuclear envelope breakdown is indicated, closed arrowheads point to anaphase onset. Note that some Npl4-depleted cells did not enter anaphase (as shown in the bottom series of images). The open arrowhead in Npl4-depleted cells indicates a chromosome bridge in anaphase. Scale bars: 5 µM. (B) Quantification of the movies, as in B, from 5 independent experiments. The time between nuclear envelope breakdown (NEBD) and anaphase onset was determined and is represented in a box-and-whisker plot [Ctrl, median: 30.0 min, mean: 31.3 min (n=48); Ufd1, median: 43.3 min, mean: 44.9 min (n=28); Npl4, median: 43.3 min, mean: 46.3 minutes (n=12); p47, median: 29.5 min, mean: 31.0 min (n=12)]. Only Npl4 siRNA-treated cells that entered anaphase within 170 minutes were quantified. Statistical significance was tested with the Mann-Whitney U-test (n.s., not significant) (adopted from Dobrynin et al. 2011).

(assessed according to (Beaudouin et al. 2002)) and anaphase onset was measured (Figure 1.11A). Ufd1- or Npl4-siRNA-treated cells displayed a delay of anaphase onset (median of ~43 minutes), compared with that of control or p47-depleted cells (median of ~30 min), suggesting that the spindle assembly checkpoint was activated, probably due to compromised spindle-kinetochore attachments. Indeed, inspection of the movies revealed misaligned chromosomes at the spindle pole, as well as a delay in congression
and formation of the metaphase plate in Ufd1- or Npl4-depleted cells (Figure 1.11B). Additionally, when cells eventually proceeded into anaphase, segregation defects were observed, with the separating chromosomes remaining entangled and delayed retraction of chromosome arms from the spindle midzone. These findings were then confirmed in fixed HeLa cells, where colleagues from my laboratory observed that depletion of Ufd1 or Npl4, but not the alternative p97 cofactor p47, led to congression defects in metaphase (Figure 1.12 A and C) as well as segregation errors in anaphase (Figure 1.12B and D). The segregation defects in anaphase included chromosome bridges between the separating chromatin masses, as well as lagging and entangled chromosomes. In addition, they observed that the nuclei of Ufd1- or especially Npl4-depleted cells were multilobed (Figure 1.12E), a phenotype known to result from segregation errors and defective axial compaction of anaphase chromosome, which is associated with the inhibition of Aurora B (Hauf et al. 2003; Mora-Bermudez et al. 2007). Taken together, these findings suggest that the p97\textsuperscript{Ufd1-Npl4} chaperone complex plays an important role in chromosome segregation of human somatic cells, however the exact molecular mechanism of p97 involvement in this process remain unsolved.
Figure 1.12 Chromosome congression and segregation defects, but not multinucleation, upon Ufd1-Npl4 depletion. (A, B) Representative confocal fluorescence images of unsynchronized metaphase and anaphase HeLa Kyoto cells that had been treated for 48 h with the indicated siRNAs. Cells were formaldehyde fixed and stained with DAPI. (C) Quantification of the experiment described in A. More than 200 metaphase cells were scored and congression defects were analyzed. Quantification based on three independent experiments. Columns and error bars represent means and standard deviation, respectively. Statistical significance was analyzed with Student’s t-test. (D) Quantification of the experiment described in B. More than 200 anaphase cells were scored and segregation defects were analyzed. Quantification based on three independent experiments. Columns and error bars represent means and standard deviation, respectively. Statistical significance was analyzed with Student’s t-test. Both quantifications, C and D, were performed by Oliver Popp as described in Dobrynin et al., 2011. (E) Representative confocal fluorescence images of interphase cells treated with indicated siRNAs. Cells were fixed with formaldehyde and stained with DAPI.
1.3 The aim of the thesis

The chromosome segregation errors result from defects in mitotic as well as pre-mitotic mechanisms. The mitotic mechanisms, which induce chromosome alignment defects, include defective mitotic checkpoint function or erroneous chromosome attachment to the mitotic spindle. The pre-mitotic causes of chromosome missegregation result from a cumulative effect of an abrogated G2/M checkpoint and unrepaired DNA damage. Many of the processes during cell division are governed by the ubiquitin-proteasome system (UPS) and its central component, the AAA+ ATPase p97 chaperone. p97 has been implicated in regulation of many processes, including chromosome segregation as well as a recently discovered involvement in DNA replication and DNA damage signaling. Previous experiments in Xenopus egg extracts conducted in our laboratory identified the p97\textsuperscript{Ufd1-Npl4} complex as a negative regulator of Aurora B during exit of mitosis (Ramadan et al. 2007). The p97-mediated removal of Aurora B from chromatin during the final stages of mitosis was crucial for timely chromatin decondensation and nuclear envelope reformation. In addition, our initial experiments performed in HeLa cells indicated that the Ufd1-Npl4 heterodimer is required for proper chromosome segregation in HeLa cells. Therefore, the main goal of this work was to elucidate the molecular basis of the chromosome segregation defects in human somatic cells. Specifically, we aimed to identify whether the p97\textsuperscript{Ufd1-Npl4} complex is involved in regulation of Aurora B during early stages of mitosis in HeLa cells. We addressed the question whether the potentially antagonistic role of p97 towards Aurora B kinase activity might have any functional relevance in chromosome congression and segregation during mitosis of mammalian cells. Furthermore, due to a fact that chromosome segregation defects may also be caused by pre-mitotic mechanisms, we additionally aimed at elucidating whether the p97\textsuperscript{Ufd1-Npl4} complex plays a role in regulation of G2/M checkpoint in human somatic cells.
2 Results

2.1 Part I: p97^Ufd1-Npl4 antagonistizes Aurora B during chromosome segregation in HeLa cells

2.1.1 Ufd1-Npl4 depletion leads to elevated levels of Aurora B and survivin association with chromosomes during mitosis.

The chromosome congression defects and segregation errors upon Ufd1-Npl4 depletion resembled the phenotype of Aurora B kinase depletion (Figure 1.12A) or Aurora B chemical inhibition (Ditchfield et al. 2003; Hauf et al. 2003). Therefore, as a first step of our investigation we set out to inspect whether Ufd1-Npl4 depletion influences the Aurora B kinase cellular localization or its protein levels during mitosis. Unsynchronized HeLa Kyoto cells were treated with control or Ufd1 siRNA for 48 hours, formaldehyde fixed and stained by indirect immunofluorescence for Aurora B and the centromeric antigen CREST. The DNA was stained with DAPI. The cells in different mitotic phases were visually inspected and representative wide field fluorescence microscopy images were acquired with identical exposure settings (Figure 2.1). Interestingly, the siRNA-mediated depletion of Ufd1 led to increased levels of Aurora B on the chromatin throughout mitosis. While in prometaphase and metaphase in control cells Aurora B localized exclusively to the centromeric region of the chromosomes (co-localizing with CREST staining), in Ufd1-depleted cells we observed increased levels on the centromeres as well as on the chromosome arms. Similarly, while in control cells in late anaphase and telophase Aurora B localized solely to the equatorial cortex and midbody, respectively, in Ufd1-depleted cells it was still present on the chromatin. These results showed that the depletion of Ufd1 in HeLa cells led to an increased association of Aurora B to the chromatin during mitosis in human somatic cells.
### Figure 2.1 Increased Aurora B association with the chromatin throughout mitosis upon Ufd1 depletion.

Unsynchronized HeLa cells were treated with control or Ufd1 S2 siRNA for 48 h. Then, the cells were formaldehyde fixed, stained by indirect immunofluorescence with the indicated antibodies and imaged with identical exposure settings by wide field fluorescence microscopy. The DNA was stained with DAPI. Representative cells from different mitotic stages are presented. Note the increased levels of Aurora B on the chromatin in Ufd1 depleted cells.
To confirm this observation, we set out to compare and quantify the Aurora B and survivin protein levels on the chromatin during mitosis in control and Ufd1-Npl4-depleted cells. To do so, we treated unsynchronized HeLa cells with the indicated siRNA for 48 hours. The cells were then either fixed and stained by indirect immunofluorescence with Aurora B and survivin antibodies, or lysed and extracts were prepared for Western Blot analysis. The depletion for 48 hours was efficient and did not affect the p97 levels (Figure 2.2A). Importantly, depletion of Npl4 also specifically destabilized Ufd1, but not vice versa, consistent with previous biochemical data showing that Ufd1 is unstable in the absence of Npl4 (Bruderer et al. 2004). Interestingly, depletion of Npl4 with the S2, but not S1 oligonucleotide additionally led to decreased levels of BubR1 protein (data not shown). In cells prepared for microscopy, the localization of Aurora B and survivin was examined. Images of prometaphase cells with a visible metaphase plate were taken and the regions of interest (ROIs), depicted with dashed circles, were marked on metaphase chromatin and the image background for further analysis of the Aurora B and survivin intensity. The intensity of the Aurora B and survivin signal within the ROI on the chromatin was measured with ImageJ. After the background signal intensity subtraction, the resulting values for the indicated siRNAs were plotted on graphs (Figure 2.2C and D). The signal intensity measurement revealed that the levels of chromosome-associated Aurora B increased by more than 2.5-5-fold both in Ufd1- and Npl4 depleted cells, except of the cells depleted with siUfd1 V-S1 and V-S2, were the levels remained the same or even decreased. In addition, the levels of survivin on the chromosomes were also increased upon Ufd1-Npl4 depletion (Figure 2.2C and D). Therefore, the morphological data gathered in this experiment indicate that the impairment of Ufd1-Npl4 leads to elevated levels of Aurora B associated with chromosomes.
Figure 2.2 Elevated levels of Aurora B and survivin associated with the chromosomes in Ufd1-Npl4 depleted cells. (A) Western blot analysis of HeLa Kyoto cells treated with non-silencing control (Ctrl) siRNA, two independent oligonucleotides targeting Npl4, five siRNAs targeting Ufd1 or two additional oligonucleotides targeting Ufd1, V-S1 and V-S2, from (Vong et al., 2005) for 48 h. The membranes were probed with the indicated antibodies. Note that Npl4 depletion also specifically destabilizes its binding partner Ufd1. (B) Cells were treated for 48 h with control (siCtrl), Ufd1 S2 or Npl4 S2 siRNA, were pre-extracted to remove soluble protein and fixed. Aurora B and survivin was detected by indirect immunofluorescence using wide-field fluorescence microscopy with identical settings. Regions of interest (ROI) (depicted as dashed circles) were chosen on metaphase chromatin and image background for further analysis of Aurora B and survivin intensity. (C and D) The intensity of Aurora B and survivin on metaphase chromatin of cells treated with the indicated siRNA oligonucleotides was determined and background subtracted as shown in B ROIs. Panels C and D from two sets of experiments, with n>21 and n>62 for each condition, respectively. Columns represent means and error bars represent standard deviation.
Next, we asked whether Aurora B accumulates at a specific region on the chromosome in the absence of p97\(^{Ufd1-Npl4} \) function. Unsynchronized HeLa Kyoto cells were treated for 48 hours with control or Ufd1 S2 siRNA. Then, they were formaldehyde fixed and stained with Aurora B as well as centromeric antigen CREST antibodies in order to identify centromeres of the mitotic chromosomes. The DNA was stained with DAPI. More than 385 images of cells in prometaphase were acquired in three independent experiments and representative wide field fluorescence microscopy images are shown. For the Aurora B intensity measurements, we chose ROIs depicted by the dashed circles on the micrographs (Figure 2.3A) corresponding to the centromeric region (as defined by CREST counterstain), the areas on distal chromosome arms and finally the image background. The Aurora B signal intensity was measured with ImageJ. White squares indicate magnified regions of the chromatin. During prometaphase of Ufd1-depleted cells, Aurora B localized along the entire chromosome arms, whereas in control depletion it was confined to the centromeric regions of chromosomes (Figure 2.1 and Figure 2.3A). The fluorescence intensity measurement revealed that the Aurora B levels were significantly increased in the centromeric regions as well as on the chromosomal arms in Ufd1-depleted cells comparing to the control siRNA-treated cells. This result confirmed that the Ufd1-Npl4 depletion leads to an increased association of Aurora B to the centromeres and the chromosome arms during mitosis in HeLa cells.
Figure 2.3 Ufd1-Npl4 depletion leads to increased levels of Aurora B on chromosome arms and centromeres in mitotic cells. (A) HeLa Kyoto cells were treated for 48 h with control or Ufd1 S2 siRNA. Then, they were formaldehyde fixed and stained with Aurora B and centromeric antigen CREST antibodies. The DNA was stained with DAPI. Representative wide-field fluorescence microscopy images of prometaphase cells were acquired. ROIs corresponding to the centromere (assessed by CREST counterstain), the chromosome arm and the image background were chosen as depicted by the dashed circles. White squares indicated magnified sections of the images presented in the lower row for each depletion. Scale bar: 10 µM (B) Quantification of Aurora B intensities on the chromosome arms and centromeres. The intensities of Aurora B in the ROIs were determined and the background subtracted as visualized in A. Columns and error bars represent means and standard deviation, respectively. Data obtained from 3 independent experiments. n=385 or n=416 for centromere and chromosome arm, respectively.
2.1.2 Proper chromosome congression and Aurora B levels on chromatin in Ufd1 S2, but not V-S2 siRNA are restored by overexpression of the siRNA resistant Ufd1-GFP construct.

As shown above, all our siRNAs targeting Ufd1 upregulated the localization of Aurora B to the chromosomes, although to a different degree (Figure 2.2C and D). This finding is in sharp contrast to the observation of Zheng and colleagues, who reported a loss of chromatin-associated Aurora B upon Ufd1 depletion (Vong et al. 2005). We confirmed the result obtained by Zheng and colleagues by depleting the cells with the two siRNA oligonucleotide sequences, Ufd1 V-S1 and Ufd1 V-S2, which were used in their experiments. Intriguingly, only Ufd1 V-S2 siRNA caused the reported decrease of Aurora B levels on chromatin (Figure 2.2C). The conflicting results were observed despite the fact that all siRNA oligonucleotides efficiently depleted Ufd1 (Figure 2.2A). In order to solve this discrepancy, we performed a rescue experiment with Ufd1-GFP cDNA to exclude that the siRNA oligonucleotides that were used in our study did not have an off-target effect. Firstly, four silent point mutations in the mouse Ufd1 cDNA were introduced to render it resistant to the published V-S2 as well as our Ufd1 S2 siRNA. Then, we used this modified Ufd1 cDNA to generate a resistant Ufd1-GFP cDNA (reUfd1-GFP). The overexpression of reUfd1-GFP cDNA was efficient in all cell populations without affecting the depletion of Ufd1, either by siUfd1 V-S2 or our S2, as confirmed by western blotting (Figure 2.4A). Importantly, the increased levels of chromatin-associated Aurora B in Ufd1 S2 siRNA treated cells were reduced to almost control levels in cells overexpressing reUfd1-GFP. In contrast, the loss of Aurora B from chromosomes in cells treated with the V-S2 oligonucleotide was not restored (Figure 2.4B and C). Consistently, reUfd1-GFP restored proper chromosome alignment in siUfd1 S2-treated cells, whereas it failed to do so in siUfd1 V-S2-treated cells (Figure 2.4B and D). This result conclusively demonstrates that the phenotype of S2 treatment was specifically caused by Ufd1 depletion, while V-S2 affected mitosis also indirectly through an unknown mechanism.
Figure 2.4 Overexpression of the siRNA resistant Ufd1-GFP construct restores proper chromosome congression and Aurora B levels on the chromatin during mitosis in Ufd1 S2, but not V-S2 siRNA depleted HeLa cells. (A) HeLa cells were treated with non-silencing (siCtrl) siRNA, or the Ufd1 oligonucleotides S2 or the previously reported V-S2 (Vong et al., 2005) for a total of 48 h. After 24 h, they were left untransfected, or transfected with empty GFP vector or a Ufd1-GFP construct rendered resistant to both of the two oligonucleotides (reUfd1-GFP). Lysates were probed with polyclonal antibodies against Ufd1 and p97 in a Western blot analysis. Non-specific bands are marked with asterisks. (B) Representative wide field fluorescence images of cells treated as in A. Cells were stained by indirect immunofluorescence with GFP or Aurora B antibodies and formaldehyde fixed. The DNA was stained with DAPI. Note that reUfd1-GFP restored the Aurora B levels and proper chromosome alignment in Ufd1 S2, but not V-S2 siRNA treated cells. Bar, 5 μm. (C) Quantification of the Aurora B immunosignal on metaphase chromatin (as described in Figure 2.2B) in cells treated as in B. Columns and error bars represent means and standard deviation, respectively. Data obtained from three independent experiments, n>58. (D) Quantification of chromosome misalignment in cells treated as in B. Columns and error bars represent means and standard deviation, respectively. Data obtained from three independent experiments, n=60.
2.1.3 Ufd1-Npl4 reduces the chromosome-associated Aurora B activity early in mitosis.

Next, we asked whether the Aurora B that accumulates on chromosomes in the absence of p97\textsuperscript{Ufd1-Npl4} function is active. In the initial experiments performed in our laboratory, my colleagues checked whether the chromatin-bound Aurora B in Ufd1- or Npl4-depleted cells was active. To do so, HeLa cells were stained with a phospho-specific antibody recognizing the phosphorylation of Aurora B at threonine 232. This phosphorylation site is auto-phosphorylated by Aurora B and is indicative of its activity (Yasui et al. 2004). This experiment revealed that the chromatin-bound Aurora B in Ufd1-Npl4-depleted cells was active in prometa- and metaphase cells, but not in anaphase.

To further address the question whether the increased levels of Aurora B on the chromosomal arms and centromeres also resulted in increased Aurora B activity, we monitored phosphorylation of the Aurora B substrates, histone H3 and centromeric histone H3 homologue CENP-A, by immunofluorescence microscopy. First, unsynchronized HeLa Kyoto cells were treated for the indicated times with 100 nM of the Aurora B inhibitor hesperadin. After formaldehyde fixation, cells were stained by indirect immunofluorescence with antibodies recognizing phosphorylation of histone H3 at serine 10, phosphorylation of CENP-A at serine 7 and antibody against cyclin B1. The DNA was stained with DAPI. Representative wide field microscopy images of prometa- and metaphase cells were taken. The inhibition of Aurora B kinase with hesperadin leads to morphological changes of the chromatin, making it difficult to distinguish between the mitotic phases (Figure 2.5A and (Hauf et al. 2003)). In order to identify the prometa- and metaphase cells, we stained the cells with antibody against cyclin B1. Cyclin B1 forms a complex with Cdk1 that promotes the transition from the G2 phase into mitosis, and is rapidly degraded upon anaphase onset (Murray et al. 1989). Therefore, in this analysis we only scored the cells that had broken down the nuclear envelope (Beaudouin et al. 2002) but not yet degraded cyclin B1 (data not shown). Histone H3 as well as CENP-A were first phosphorylated by Aurora B in late G2 phase. No differences in the levels of the steady-state phosphorylation in control or Ufd1-depleted cells were detected (Figure 2.6A, DMSO control; data not shown), possibly because both substrates were already nearly maximally phosphorylated in control cells. Therefore, we next asked, whether a possible increase in the Aurora B activity would be reflected in a partial resistance to the
Results

chemical inhibitor of Aurora B, hesperadin. To address this question, we assessed whether the phosphorylation of histone H3 or CENP-A is dynamic in prometaphase, as a result of repeated dephosphorylation and subsequent re-phosphorylation by Aurora B, which could be then probed in analysis. To test this, cells were treated with 100 nM hesperadin for between 2 and 60 minutes, to inhibit possible re-phosphorylation. Histone H3 phosphorylation was not affected when cells were treated for up to 15 minutes (Figure 2.5A and B). However, after 30 minutes, mixed population of cells with either fully, partially or non-phosphorylated chromatin was observed. The latter cases most probably reflected inhibition of the initial phosphorylation in those cells that were in prophase during application of the inhibitor. This suggested that histone H3 phosphorylation is not dynamic and that the phosphorylation that occurs in late G2 phase is stable at least until anaphase onset.

Figure 2.5 Phosphorylation state of the Aurora B substrate CENP-A, but not of histone H3, is dynamic during prometaphase in HeLa cells. (A) Unsynchronized HeLa Kyoto cells were treated for the indicated times with 100 nM Aurora B inhibitor hesperadin (Hesp). After formaldehyde fixation cells were stained by indirect immunofluorescence with antibodies against the phosphorylation of histone H3 at serine 10 (pH3) or the phosphorylation of CENP-A at serine 7 (pCENP-A). Wide-field fluorescence microscopy images were taken. Cells were only assessed if they had broken down the nuclear envelope but not yet degraded Cyclin B1 (determined with an Cyclin B1 antibody). (B) Quantification of A. Columns represents percentage of prometa-/metaphase cells with positive staining of the indicated protein. n=40
In contrast, the CENP-A phosphorylation at the centromere was sensitive to 15 min incubation with hesperadin in almost all of the cells, indicating that it is constantly dephosphorylated and needs to be re-phosphorylated by Aurora B to maintain its phosphorylation status during prometaphase. Therefore, we investigated the sensitivity of the CENP-A phosphorylation to 15-minute incubations with increasing concentrations of hesperadin (from 1 to 100 nM) in control or Ufd1-depleted cells (Figure 2.6A and B). As shown in Figure 2.6B, phosphorylation of CENP-A was undetectable at 20 nM hesperadin in the majority of control cells, whereas in Ufd1-depleted cells phosphorylation was resistant to 50 nM and even was detectable in 100 nM hesperadin. This result demonstrates that the impairment of the Ufd1-Npl4 function leads not only to accumulation of chromatin-bound Aurora B, but also in an increase of the Aurora B activity towards its centromeric substrate, CENP-A.

Figure 2.6 Ufd1 depletion leads to increases chromosome-associated Aurora B activity towards a centromeric substrate. (A) Wide-field fluorescence microscopy micrographs of cells that were treated with increasing concentrations of hesperadin for 15 min. Phosphorylation of CENP-A was detected as in Figure 2.5A. (B) Quantification of A. Note that CENP-A phosphorylation is resistant to 20-50 nM hesperadin in Ufd1- but not control-siRNA treated cells. Columns and error bars represent means and standard deviation, respectively. Data from 3 independent experiments. n>137
2.1.4 *Ufd1-Npl4* functionally antagonizes Aurora B during chromosome congression.

Next, we asked whether the regulation of Aurora B activity by p97*Ufd1-Npl4* had any functional relevance for proper progression of mitosis in HeLa cells. To address a possible effect of Ufd1-Npl4 on the chromosome congression, we analyzed the efficiency and timing of chromosome alignment both in fixed and live cells. In the first approach, HeLa Kyoto cells were transfected with control or Ufd1 siRNA for 48 hours and treated with increasing concentrations of hesperadin (from 0.7 to 100 nM) for 1 h. The cells were then formaldehyde-fixed and the DNA was stained with DAPI. Images were acquired by wide field fluorescence microscopy and percentage of prometa- and metaphase cells with fully aligned chromosomes was determined (Figure 2.6A and B). As expected, both depletion of Ufd1 and addition of hesperadin at concentrations equal or above 5 nM caused a marked increase in chromosome misalignment compared with that observed in control cells. Importantly however, chromosome alignment in Ufd1-depleted cells was partially rescued by moderate inhibition of Aurora B, with 1 nM of hesperadin. This result suggested that the alignment defect observed in Ufd1-depleted cells was, at least in part, caused by overactivation of Aurora B. Conversely, Ufd1 depletion restored proper alignment in the presence of 5-15 nM hesperadin, showing that the overactivation of Aurora B due to impaired Ufd1-Npl4 function can overcome partial inhibition of Aurora B.
Figure 2.7 Ufd1-Npl4 functionally antagonizes Aurora B during chromosome congression - evidence in fixed cells. (A and B) Unsynchronized control or Ufd1 S2-depleted cells were treated with the indicated concentrations of hesperadin (Hesp) for 1 hour, fixed and stained with cyclin B1 antibodies. DNA was stained with DAPI. Cells after nuclear envelope breakdown but before cyclin B1 degradation were identified by wide-field fluorescence microscopy as in Figure 2.5A. White arrowheads in (A) indicated the misaligned chromosomes in Ufd1 depleted cells. The percentage of cells with fully aligned chromosomes was determined. Data from three independent experiments (n=100 for each condition). Error bars represent standard deviation.

To confirm this result, we monitored metaphase plate formation in live cells. Control or Ufd1-depleted cells stably overexpressing histone H2B-mRFP were imaged in the absence or presence of 50 nM hesperadin using time-lapse video microscopy as described in Figure 1.11. We measured the time from nuclear envelope breakdown
Results

(scored on the basis of the loss of a defined boundary for the chromatin regions) (Beaudouin et al. 2002) to the formation of a distinct metaphase plate, when no misaligned chromosomes could be detected. Then the percentage of cells that have completed metaphase plates formation was plotted cumulatively as a function of time (Figure 2.8B).

![Image of Figure 2.8 A and B](image-url)

**Figure 2.8 Ufd1-Npl4 functionally antagonizes Aurora B during chromosome congression - evidence from live cell imaging.** HeLa cells stably expressing H2B-mRFP were transfected with control (Ctrl) or Ufd1 S2 siRNA. After 48h, cells were treated with 50 nM hesperadin (Hesp) or equivalent amounts of its solvent DMSO, and imaged by confocal time-lapse microscopy (A) Still images from representative movies. White arrowheads indicate misaligned chromosomes in Ufd1 depleted cells or control depleted cells but treated with 50 nM hesperadin. (B) Quantification of the timing between nuclear envelope breakdown and metaphase plate formation. Individual cells were followed in time-lapse movies and scored when they formed a distinct metaphase plate. Scored cells were plotted cumulatively for each time point. The data is from three independent experiments (siCtrl n=146; siCtrl + Hesp n=138; siUfd1 n=114; siUfd1 + Hesp n=88).

Control cells formed a metaphase plate within 30 minutes. In accordance with the results from Figure 1.11, Ufd1-depleted cells required longer, but eventually nearly all cells
completed congression. On the other hand and as expected, less than 10% of the hesperadin-treated cells managed to form a metaphase plate. Importantly, Ufd1 depletion partially restored the metaphase plate formation in hesperadin-treated cells, as 50% of the cells achieved proper chromosome alignment, though at a lower rate and with metaphase plates occasionally collapsing after formation. In conclusion, these results demonstrate that Ufd1 antagonizes Aurora B during chromosome congression and suggest that misregulation of Aurora B, at least in part, accounts for the congression phenotype in Ufd1-Npl4-depleted cells.

2.1.5 Depletion of Ufd1 does not affect chromosome condensation in prometaphase cells.

Formation of compact mitotic chromosomes is critical for faithful segregation of genetic material (Schmiesing et al. 1998). Therefore, as a next step, we examined whether the congression and segregation defects that were observed in Ufd1-Npl4-depleted cells may be in fact caused by the erroneous condensation of chromosomes. HeLa Kyoto cells were depleted for 48 hours with control or Ufd1 siRNA. 5 min before formaldehyde fixation, the cells were incubated with PBS or the hypotonic buffer RSB (10 mM Tris pH 7.5, 10 mM NaCl, 5 mM MgCl₂), which leads to decomposition of unstable chromosomes (Gassmann et al. 2004). Wide field fluorescence microscopy images were acquired and prometaphase cells were analyzed. In agreement with previously obtained results, Ufd1 depletion led to increased levels of Aurora B on the chromosomes compared to control-depleted cells. More importantly however, treatment of Ufd1-depleted cells with hypotonic buffer did not affect the chromosome morphology (Fig. 12). This result suggests that Ufd1 depletion does not affect proper chromosome condensation.
Figure 2.9 Ufd1 depletion does not affect chromosome condensation in prometaphase cells. Unsynchronized HeLa cells were treated with control siRNA (siCtrl) or Ufd1 S2 siRNA (siUfd1). After 2 days, cells were either incubated with PBS or the hypotonic buffer RSB (10 mM Tris-HCl pH 7.4, 10 mM NaCl, 5 mM MgCl2) for 5 min. After that time, cells were fixed, stained with DAPI or the indicated antibodies, and imaged by wide field fluorescence microscopy. Note that the prometaphase chromatin condensation state remains unchanged in Ufd1 depleted cells upon RSB buffer treatment. Representative cells are presented. Bar, 10 µm.
2.1.6 Inhibition of p97 with DBeQ affects chromosome segregation during mitosis.

Lastly, to study the role of p97 in regulating mitosis of human somatic cells, and at the same time to reduce the pleiotropic effects of siRNA-mediated depletion, we used transient chemical inhibition of the p97 AAA+ ATPase. For the inhibition of p97 we used a first-generation, selective ATP-competitive p97 inhibitor – DBeQ (Chou et al. 2011). HeLa Kyoto cells stably overexpressing H2B-mCherry were treated with increasing amounts of DBeQ (1-20 µM) and followed by video time-lapse microscopy. First, we inspected whether inhibition of p97 affects the mitotic progression of HeLa cells. To do so, we determined the mitotic timing from the nuclear envelope breakdown at the beginning of mitosis to the anaphase onset. The p97 inhibition led to gradual delay of anaphase onset from a median of 33 min in DMSO-treated cells, up to 55 min in 20 µM DBeQ-treated HeLa cells (Figure 2.10A). Further inspection of DBeQ-treated cells revealed that this delay in the anaphase onset was contributed to mitotic spindle defects that were induced by p97 inhibition. During cell division, chromosomes form the metaphase plate at the middle of mitotic spindle and their spatial orientation remains mostly unchanged till anaphase onset (data not shown and (Stevens et al. 2011)). Interestingly, the addition of DBeQ led to an increased number of cells with a metaphase plate that was laterally oscillating towards one or the other centrosome in the cell. On average 16% of 10 µM DBeQ-treated cells displayed a rocking metaphase plate, comparing to ~4.5% in DMSO-treated cells (Figure 2.10B). Additionally, the chemical inhibition of p97 led to a moderate increase, from less than 2% in DMSO-treated to ~7% in DBeQ-treated cells in which the metaphase plate was not parallel to equatorial cortex of the dividing cell (Figure 2.10C). These data suggest that the selective inhibition of p97 leads to a prolongation of anaphase onset as well as multiple defects in the mitotic spindle function of human somatic cells.
Figure 2.10 Pharmacological inhibition of p97 affects chromosomes segregation during mitosis. HeLa cells expressing H2B-mRFP were seeded in 8-well IBIDI chambers for live-cell imaging. On the next day, cells were treated with increasing concentrations of the p97 inhibitor DBeQ or equivalent amount of DMSO solvent and imaged by confocal time-lapse microscopy. Data from 2 independent experiments. (A) Time between nuclear envelope breakdown and metaphase plate formation was measured as in 11B and presented in a box and whisker plot. Boxplots show medians, lower and upper quartiles (line and box), 10th and 90th percentiles (whiskers), and outliers (•). The table below the graph contains the mitotic timing and the number of analyzed cells for each condition. (B) Cells with rocking metaphase plate from A where scored and quantified. Bars represent standard deviation. (C) Quantification of cells from A with spindle orientation defects. Cells were visually examined and scored if opposing centrosomes of a given cell were not at the same focal plane. Bars represent standard deviation.
2.2 Part II: The role of p97 in the G2/M checkpoint

The primary goal of the first part of this thesis was to understand the molecular basis by which the p97\textsuperscript{Ufd1-Npl4} complex regulates mitotic progression in mammalian cells. We have shown that the antagonizing role of p97\textsuperscript{Ufd1-Npl4} towards Aurora B kinase activity is crucial for proper congression and segregation of mitotic chromosomes in HeLa cells. However, p97 plays multiple roles in other chromatin associated processes during the cell cycle. As mentioned above, p97 has been recently implicated to play a general role in S phase during DNA replication (Franz et al. 2011; Raman et al. 2011), as well as in repair of IR-induced DNA damage (Meerang et al. 2011) or in TLS (Davis et al. 2012; Mosbech et al. 2012). Therefore, in the second part of my thesis, I asked whether the observed mitotic phenotypes in Ufd1-Npl4-depleted cells are solely an account of Aurora B misregulation or maybe, additionally due to pre-mitotic defects that occurred in previous cell cycle stages and were passed on into mitosis as a result of an abrogated G2/M checkpoint.

2.2.1 Depletion of the p97\textsuperscript{Ufd1-Npl4} complex leads to mitotic defects after ionizing radiation (IR)

In order to analyze the role of p97\textsuperscript{Ufd1-Npl4} in the G2/M checkpoint we first compared unchallenged mitotic cells with DNA damage-induced cells. DNA damage was induced by exposing the cells to ionizing radiation (IR). IR has been shown to cause a broad spectrum of genetic damage, including gene mutations, micronucleus formation, chromosomal aberrations, single and double DNA strand breaks as well as chromosomal instability (CIN) that leads to ploidy changes. For initial experiments we used an siRNA-mediated approach to deplete control, Npl4 as well as Chk1 and assessed the chromosome morphology for any visible aberrations. Unsynchronized HeLa Kyoto cells were depleted for 48 hours with the indicated siRNAs. After 24 hours, cells were subjected to 3 Gy IR dose or left untreated. 1.5 hours prior to fixation, cells were incubated in medium containing colchicine in order to accumulate mitotic cells. Chromosomal spreads were prepared, representative images were taken by bright field microscopy and the average number of chromosomal aberration (CA) per cell was quantified.
Results

Figure 2.11 Npl4 depletion leads to IR-induced chromosomal aberrations in HeLa cells. HeLa Kyoto cells were treated for 48 h with control, Npl4 S1, Npl4 S2 or Chk1 siRNA. After 24 h cells were treated with a 3 Gy dose of ionizing radiation or left untreated. 1.5 hours prior to fixation, cells were incubated in medium containing colchicine. After fixation, chromosomal spreads were prepared and DNA was stained with Giemsa. Images were taken with bright-field microscopy. (A) Overview images of representative chromosome spreads of prometaphase cells. The black arrowheads indicate chromosomal aberrations (CA). Note the increased number of chromosomal aberrations in Npl4-depleted cells. (B) Magnification of representative Ctrl and Npl4 S2-depleted cells treated with a 3 Gy dose of ionizing radiation. Note the increased number of various types of chromosomal aberrations (double strand brakes, dicentric chromosomes) in Npl4 S2 treated cells. (C) Quantification of B. Columns represent mean values. Data from one representative experiment. n>100 cells per condition.
As presented in Figure 2.11A (0 Gy) and C, a low frequency of chromosomal aberrations were present in unchallenged control or Chk1-depleted cells (average of 0.1 % or 0.2 % for Ctrl or Chk1, respectively). However, in cells depleted with Npl4 S1 or S2-siRNA oligonucleotides, a slight increase of chromosomal aberrations was detected (0.9 % or 0.4 %, respectively). This indicated that the destabilization of the Ufd1-Npl4 heterodimer in unchallenged cells might already, to small degree, affect the G2/M checkpoint function. Therefore, we asked whether a moderate challenge of the system with IR would amplify the observed phenotype. As expected, the frequency of chromosomal aberrations in Npl4-depleted cells increased after a 3 Gy IR dose (6.7 % and 5.1 % in Npl4 S1 and S2, respectively), comparing to control-depleted cells (2.6 %) (Figure 2.11A, B and C). This initial data indicated an additional role of Npl4 in the G2/M checkpoint function as its depletion led to an increased frequency of chromosomal aberrations after IR-induced DNA damage.

IR-induced DNA damage triggers the rapid activation of the DNA-damage response (DDR) which is followed by the activation of the cell-cycle checkpoints and DNA repair pathways (Kastan and Bartek 2004; Shiloh 2003). To further analyze the role of the p97\textsuperscript{Ufd1-Npl4} complex in the G2/M checkpoint, we asked whether the observed mitotic chromosome aberrations in Npl4-depleted cells result from a failure of cell cycle arrest. Unsynchronized HeLa Kyoto cells were depleted with the indicated siRNAs for 48 h, irradiated or left untreated and checked for the presence of mitotic cells. The depletion of Ufd1-Npl4 in non-irradiated cells led to congression and segregation defects in metaphase and anaphase, respectively (Fig. 16B,C left panels). As expected, the 3 Gy IR dose, led to efficient cell cycle arrest in control depletion, where only few mitotic cells were found. Those cells that entered mitosis were γ-H2AX negative and did not display any chromosome segregation defects (Figure 2.12A,B,C). Surprisingly, in Ufd1, Npl4, DVC1 or p97-depleted cells, just like in Chk1-depleted cells, many cells in various stages of mitosis were observed. Importantly, the cells were progressing through mitosis despite of the persistent DNA damage, as multiple γ-H2AX foci on mitotic chromatin were detected (Figure 2.12A,B,C right panels). Finally, the vast majority of these cells exhibited massive chromosome congression and segregation defects, starting already in prometaphase. This data show that the depletion of the p97\textsuperscript{Ufd1-Npl4} complex or the DVC1
Results

adaptor protein leads to abnormal progression through mitosis after IR-induced DNA damage.
Figure 2.12 Depletion of p97\textsuperscript{Ufd1-Npl4} leads to mitotic defects in HeLa cells after ionizing radiation treatment. HeLa Kyoto cells were prepared as described in Fig. 15. Representative confocal fluorescence microscopy pictures were taken of prometa- (16A), meta- (16B) and ana-phase (16C) cells. 3 Gy irradiation induces DNA damage that leads to the accumulation of γ-H2AX foci on the chromatin. Note that 3 Gy IR-treated HeLa cells with depleted Ufd1, Npl4, DVC1 or p97 undergo mitosis even with DNA damage present. In control cells barely any mitotic cells were observed, and those present, were γ-H2AX negative. Cells progressing through mitosis with sustained DNA damage encounter multiple congression (16A and 16B) as well as segregation (16C) defects.
Figure 2.12 Depletion of $p97^{Ufd1-Npl4}$ leads to mitotic defects in HeLa cells after ionizing radiation treatment.
Figure 2.12 Depletion of p97\textsuperscript{Ufd1-Npl4} leads to mitotic defects in HeLa cells after ionizing radiation treatment.
2.2.2 Depletion of Ufd1-Npl4 induces micronuclei formation in HeLa cells upon ionizing radiation treatment.

Due to the fact that unrepaired double strand brakes lead to micronuclei formation, we inspected the nuclear morphology of cells depleted of Ufd1, Npl4 and DVC1 upon IR-treatment. Unsynchronized HeLa Kyoto cells were depleted for 48 hours with the indicated siRNA oligonucleotides and 16 hours before fixation, cells were subjected to a 3 Gy dose of IR, or left untreated. In order to confirm the gamma-radiation-induced DNA damage, cells were stained with γ-H2AX antibody which recognizes phosphorylated histone H2AX at the sites of DNA double strand breaks (DSBs). Confocal images of non-irradiated cells confirmed the previous observation that depletion of Ufd1-Npl4 leads to irregular-shaped and, especially in Npl4 depletion, multilobed nuclei (Figure 1.12E). Interestingly, the depletion of DVC1 with siRNA oligonucleotide S1 but not S2, led to similar phenotypes as observed in Npl4-depleted cells. Moreover, siRNA mediated depletion of Ufd1, Npl4 or DVC1 in non-irradiated cells induced the formation of sporadic γ-H2AX foci (Figure 2.13 and data not shown). For Ufd1-Npl4 depletion this finding is in agreement with the observed increased frequency of chromosomal aberrations in non-irradiated cells (Figure 2.11C) and for DVC1 it confirms its previously described role in the DDR signaling pathway response (Davis et al. 2012; Mosbech et al. 2012). On the other hand, the 3 Gy IR dose efficiently induced the formation of γ-H2AX foci in all cells. Surprisingly, IR-treatment of Ufd1, Npl4 or DVC1-depleted cells led to the formation of micronuclei similar to those observed in Chk1 depletion, where the G2/M checkpoint is compromised.
Depletion of the p97\textsuperscript{Ufd1-Npl4} complex induces micronuclei formation in HeLa cells upon IR-treatment. HeLa Kyoto cells were transfected for 48 hours with the indicated siRNAs. 16 h before fixation, cells were subjected to 3 Gy ionizing radiation (IR). After formaldehyde fixation, cells were immunostained with γ-H2AX and phospho-histone H3 (Ser10) antibody. DNA was stained with DAPI. Representative confocal fluorescence microscopy images were taken. 3 Gy dose of ionizing radiation induces DNA damage that leads to the accumulation of γ-H2AX foci in the nuclei of the cells. Note the micronuclei formation in Ufd1, Npl4 and DVC1 depleted cells after IR-treatment.
2.2.3 Ufd1 or Npl4 depletion leads to compromised Cdc25A degradation upon IR-induced DNA damage.

Presence of mitotic cells after IR-induced DNA damage indicates that the G2/M checkpoint is compromised. Therefore, we followed the downstream substrates of the ATM/ATR signaling pathway in Ufd1 or Npl4-depleted cells in order to examine whether the G2/M checkpoint is properly activated. Unsynchronized HeLa Kyoto cells were depleted for 48 hours with the indicated siRNA and 1 hour prior to lysis cells were subjected to 15 Gy dose of gamma-radiation or left untreated. Extracts were analyzed by Western blot. In all depletions after IR-induced DNA damage we observed phosphorylation of Chk1 at serine 317 and 345 as well as Cdc25C at serine 216 (Figure 2.14). As expected, Cdc25A was efficiently degraded in control-depleted but not in the ubiquitin ligase β-TrCP-depleted cells. In contrast, depletion of Ufd1 or Npl4 led to an increased accumulation and stabilization of Cdc25A in unchallenged and IR-treated cells, respectively (Figure 2.14).
Figure 2.14 Ufd1 or Npl4 depletion compromises Cdc25A degradation upon IR-induced DNA damage in HeLa cells. HeLa Kyoto cells were transfected for 48 hours with indicated siRNAs. Cells were subjected to 15 Gy dose of ionizing radiation 1 h prior to lysis. Protein extracts were prepared for Western Blot analysis. Membranes were probed with indicated antibodies. Phosphorylation of Chk1 at Ser317 and Ser 345 as well as Cdc25C at Ser 216 indicates that upon IR-treatment of Ufd1 or Npl4 depleted cells, the DNA damage response signaling is activated. In spite of that, the Cdc25A degradation is impaired.

In order to examine the functional relevance of the observed Cdc25A stabilization in Ufd1 or Npl4 depleted cells, we checked the phosphorylation state of its substrate, Cdk1. Unsynchronized HeLa Kyoto cells were depleted with two additional siRNA oligonucleotides Ufd1 S3 and Npl4 S1 to confirm the specificity of the depletion. Cells were treated with 10 Gy IR dose 30 min prior to protein extraction. As shown above, the depletion of Ufd1 and Npl4 with all of the siRNA oligonucleotides used led to a stabilization of Cdc25A after IR (Figure 2.15). Interestingly, phosphorylation of Cdk1 at tyrosine 15 was reduced in Ufd1 or Npl4-depleted cells comparing to that in control cells. At the same time, the levels of Wee1 kinase, which is responsible for Cdk1
Results

phosphorylation, remained unchanged (except for Npl4 S1-depleted cells). Polo-like kinase 1 (Plk1) and Cdc25B were unaffected by Ufd1 or Npl4 depletion (Figure 2.15). The data from both experiments show that depletion of Ufd1 or Npl4 does not influence the proper activation of Chk1 and its downstream substrates upon IR-induced DNA damage. However, interestingly, despite of the proper activation of Chk1, Cdc25A is stabilized in Ufd1 or Npl4-depleted cells and in those cells we additionally observed a slight decrease of Cdk1 phosphorylation at tyrosine 15.

Figure 2.15 Ufd1 or Npl4 depletion leads to stabilization of Cdc25A. HeLa Kyoto cells were transfected for 48 hours with indicated siRNAs. Cells were subjected to 10 Gy dose of ionizing radiation 30 min prior to lysis. Protein extracts were prepared for Western Blot analysis. Membranes were probed with indicated antibodies. Note the compromised degradation of Cdc25A in Ufd1 or Npl4 depleted HeLa cells, as well as reduced phosphorylation of Cdk1 at Tyr15 comparing to control (siLuc)-treated cells.
2.2.4 The p97\textsuperscript{Ufd1-Npl4} complex binds the ubiquitinated form of Cdc25A

The stabilization of Cdc25A in Ufd1 or Npl4-depleted cells upon ionizing-radiation suggested that degradation of this protein might be compromised. In order to test whether p97 is involved in the proteasomal degradation of Cdc25A we performed pulldown experiments to examine whether p97\textsuperscript{Ufd1-Npl4} binds to the ubiquitinated form of Cdc25A. To do so, we used HEK293 cells that stably overexpress the ATPase-inactive p97 E578Q mutant. The E578Q (EQ) mutation in p97 abolishes ATP hydrolysis and leads to a dominant-negative variant that traps ubiquitin conjugates along with the substrate proteins (Ramadan et al. 2007; Ye et al. 2003). HEK293 cells were transiently transfected for 24 hours with HA-Cdc25A or empty vector and four hours later expression of p97-mycSTREP WT or EQ was induced with doxycycline. 30 min before extraction, the cells were subjected to a 10 Gy IR dose or left untreated. Then, the cells were lysed with IP buffer which was supplemented with N-ethylmaleimide (NEM) in order to preserve the ubiquitylation of the proteins. NEM is an organic compound that irreversibly inhibits all cysteine peptidases, including deubiquitylating enzymes (DUBs), by alkylating the thiol groups in their active site. HA-Cdc25A was immunoprecipitated using anti-HA antibodies and extracts were analyzed by Western Blot. Staining of the membranes with anti-HA antibodies revealed that equal amounts of HA-Cdc25A were isolated from cells expressing p97 WT and EQ. Probing of the membranes with anti-ubiquitin antibodies showed that ubiquitin-conjugates were specifically co-isolated with HA-Cdc25A in the p97 EQ background (Figure 2.16 – 0 Gy panel). Importantly, treatment of the cells with 10 Gy IR dose led to increased amounts of the ubiquitinated form of Cdc25A that were co-immunoprecipitated with p97 EQ (Figure 2.16 – 10 Gy panel). Furthermore, both p97 and Npl4 were readily co-isolated with Cdc25A in WT and EQ background, independent of the ionizing radiation. The co-immunoprecipitation of Ufd1 with HA-Cdc25A could not be detected in the tested conditions.
Figure 2.16 The p97^Ufd1-Npl4 complex targets ubiquitinated Cdc25A. Stable HEK293 cell line inducibly expressing either mycSTREP p97 (WT) or ATPase inactive p97 E587Q (EQ) were transiently transfected with a vector coding for HA-Cdc25A or empty pcDNA 3.1 HA. Four hours after the transfection cells were induced with doxycycline (DOX+) or left uninduced (DOX-). 30 min before lysis, cells were subjected to 10 Gy dose of ionizing radiation or left untreated. Cells were lysed in IP buffer supplemented with N-ethylmaleimide (an inhibitor of all cysteine peptidases) in order to prevent deubiquitylation of proteins in the extracts. Lysates were subjected to immunoprecipitation with anti-HA antibodies (HA-IP). IP samples together with 10 % inputs and flowthroughs (IN or FT, respectively) were analyzed by SDS PAGE and Western blotting. Membranes were probed with the indicated antibodies. Note the interaction of p97 and Npl4 with Cdc25A in p97 WT as well as EQ cells. In addition, note the increased accumulation of the ubiquitinated form of Cdc25A in the ATPase inactive p97EQ in comparison to p97WT.
In order to further analyze this finding, we directly compared the unchallenged and the 10 Gy IR dose-treated p97 EQ cells. The cells were processed and the extracts were prepared as described above. Immunoprecipitation with the anti-HA antibody led to efficient HA-Cdc25A isolation from the protein extracts (Figure 2.17). Probing of the membranes with anti-ubiquitin antibodies confirmed that upon irradiation increased amounts of the ubiquitinated form of Cdc25A accumulate in p97 EQ background (Figure 2.17). In addition, increased levels of the p97 ATPase itself, that co-immunoprecipitated with the ubiquitinated form of Cdc25A, were observed in the 10 Gy IR-treated p97 EQ cells. Together, this data showed that mutation of p97 ATPase leads to an accumulation of the ubiquitinated, p97-bound form of Cdc25A and that this accumulation is further increased by IR-induced DNA damage. Moreover, our Western Blot analysis revealed that Cdc25A interacts with p97 as well as Npl4.

Figure 2.17 Fig. 20. Inhibition of p97 ATPase activity leads to an accumulation of the ubiquitylated, p97-bound form of Cdc25A. Direct comparison of non-irradiated or 10 Gy---irradiated p97 EQ cells, processed as described in Fig. 19. Note the increased accumulation of the ubiquitinated form of Cdc25A in p97EQ HEK293 after treatment with 10 Gy dose of ionizing radiation. Additionally, note the increased interaction of p97 with Cdc25A upon IR-induced DNA damage.
2.2.5 p97 siRNA-mediated depletion leads to reduced phosphorylation of Cdk1 at tyrosine 15 and decreased levels of Wee1, but not Cdc25A stabilization.

As mentioned above, p97 is involved in plethora of cellular processes that are crucial for the proper cell cycle progression, and thus viability of the cell. To investigate the role of p97 in regulating the G2/M checkpoint systematically, we used a siRNA approach to first deplete one of its main adapters, the Ufd1-Npl4 complex, rather than p97 itself, in order to reduce pleiotropic effects. We showed that the depletion of p97 adaptor proteins Ufd1 or Npl4 leads to stabilization of Cdc25A levels upon IR-induced DNA damage (Figure 2.14 and Figure 2.15). Bearing in mind that the p97 depletion leads to complex alterations of diverse cellular processes, we nevertheless asked how depletion of p97 would affect the G2/M checkpoint. Unsynchronized HeLa Kyoto cells were depleted for 48 hours with the indicated p97 siRNA oligonucleotides and 30 min prior to lysis cells were subjected to 10 Gy IR dose. The extracts were analyzed by Western blot. As previously observed, IR-induced DNA damage led to phosphorylation of the Chk1 kinase at serine 317, and thus its activation. The proper activation of the Chk1 kinase was confirmed by its ability to phosphorylate Cdc25C at serine 216 (data not shown). Intriguingly, in contrast to Ufd1-Npl4 depletion, the depletion of the p97 ATPase itself did not stabilize Cdc25A protein levels in IR-treated cells. On the contrary, three out of four siRNA oligonucleotides that were used for the p97 depletion, led to moderate decrease of the Cdc25A protein levels in irradiated cells, and interestingly also in unchallenged HeLa cells. Nevertheless, the inhibitory-phosphorylation of Cdk1 at tyrosine 15 in all p97-depleted cells was still significantly reduced comparing to control-depleted cells. The phosphorylation of Cdk1 at tyrosine 15 is mediated by the Wee1 kinase. Therefore, we then probed the membrane with anti-Wee1 antibodies and discovered that the levels of that kinase decreased in p97 S4, S5 or S6-depleted cells, which might explain the decreased phosphorylation of Cdk1. Together with the data from the Figure 2.12, these finding implicate a role of the p97 AAA+ ATPase in regulation of the G2/M checkpoint. However, due to the fact that p97 AAA+ ATPase is a critical regulator of many other crucial cellular processes, its function in the G2/M checkpoint seems to be much more complex than that of the Ufd1-Npl4 complex only.
Results

Figure 2.18 p97 depletion leads to reduced phosphorylation of Cdk1 at Tyr15 and Wee1 levels but not Cdc25A stabilization. HeLa Kyoto cells were transfected for 48 hours with the indicated siRNAs. Cells were subjected to 10 Gy dose of ionizing radiation 30 min prior to lysis. Protein extracts were prepared for Western Blot analysis. Membranes were probed with the indicated antibodies. Note that depletion of p97, in contrast to Ufd1 or Npl4 depletion, does not lead to Cdc25A stabilization. However, in p97-depleted cells, reduced levels of Wee1 protein as well as Cdk1 phosphorylation at Tyr15 were observed.
2.2.6 Depletion of Ufd1, Npl4 or DVC1, but not p97 leads to moderate stabilization of Cdc25A upon doxorubicin or cisplatin-induced DNA damage.

p53 is a tumor suppressor protein which is crucial for the regulation of the cell cycle, and thus preventing cancer development (Matlashewski et al. 1984). p53 coordinates cellular responses to different forms of stress e.g., DNA damage. Activation of p53 may trigger apoptosis, cell cycle arrest, or attempts to repair the damaged DNA, depending on how extensive the damage is (Vousden and Lu 2002). The tumor suppressor p53 is mutated in about half of all human malignancies (Bug and Dobbelstein 2011) and due to the loss of p53 function these tumors may be sensitive to treatments that abrogate the G2/M checkpoint (Meng et al. 2013). To further investigate the role of p97 in the G2/M checkpoint, we examined the impact of combining p97\textsuperscript{Ufd1-Npl4} depletion and treatment with therapeutic molecules, on protein levels of the G2/M checkpoint regulator, Cdc25A. In this experiment, we used two potent anti-cancer drugs, doxorubicin (DRB) or cis-Platinum (cisplatin), which induce DNA damage and activate the DDR signaling pathway. Unsynchronized HeLa Kyoto cells were transfected for 48 hours with the indicated siRNAs. After 32 hours, cells were incubated for 2 hours in medium containing either DRB or cisplatin. After a total of 48 hours protein extracts were prepared and the samples were analyzed by Western Blot. Both, DRB as well as cisplatin-treatment led to activation of Chk1 (phosphorylation of Chk1 at serine 317) and Cdk1 at tyrosine 15. Importantly, the siRNA-mediated depletion of Ufd1, Npl4 or DVC1 stabilized Cdc25A in DNA-damaged cells. Interestingly, the exposure of p97-depleted cells to DRB or cisplatin, in contrast to IR-treated cells, also led to a moderate stabilization of Cdc25A when compared to control-depleted cells.
Figure 2.19 The depletion of p97 adaptor proteins leads to a moderate stabilization of Cdc25A upon doxorubicin or cisplatin-induced DNA damage. HeLa Kyoto cells were transfected for 48 hours with the indicated siRNAs. After 32 h cells were incubated for 2 h in medium containing the DNA damaging factors - doxorubicin or cisplatin. After total of 48 hours cells were lysed and extracts were prepared for Western Blot analysis. Membranes were probed with the indicated antibodies. Note that in Ufd1, Npl4 or DVC1-depleted cells in spite of the DNA damage response signaling activation (phosphorylation of Chk1 at Ser317), Cdc25A is not fully degraded.
3 Discussion

The fidelity of chromosome segregation during cell division depends on proper DNA replication in S phase and correct bioriented attachment of the sister kinetochores to the microtubules during mitosis. Many of the processes during cell division are governed by the ubiquitin-proteasome system (UPS). The central component of the UPS is the AAA+ ATPase p97 chaperone which recognizes the ubiquitylated substrate proteins and targets them for proteasomal degradation. During cell division, p97 has been implicated in regulation of many processes, including DNA replication, DNA damage signaling as well as chromosome segregation. Therefore, in this study we addressed the question, what is the molecular basis of chromosome segregations defects in human somatic cells. Our results demonstrate that there are two answers to this question, namely, the p97*Ufd1-Npl4* complex ensures proper chromosome segregation during mitosis by: (a) regulation of the Aurora B kinase activity on the mitotic chromatin and (b) control of the G2/M checkpoint function.

It has been previously shown that during exit from mitosis in *Xenopus laevis* egg extracts, p97 and its adapter Ufd1-Npl4 remove kinase Aurora B from chromatin to allow nucleus formation (Ramadan et al. 2007). Here we present evidence that the p97*Ufd1-Npl4* complex antagonizes Aurora B on chromosomes already in early stages of mitosis and that this is crucial for proper chromosome segregation in HeLa cells. This study revealed that inhibition of the p97*Ufd1-Npl4* complex, leads to increased protein levels of Aurora B on prometaphase as well as metaphase chromosomes of human somatic cells. Moreover, this increase is associated with higher Aurora B activity at the kinetochores which leads to chromosome alignment and anaphase defects, resulting in missegregated chromosomes and multi-lobed nuclei. Furthermore, we also discovered a novel role of p97*Ufd1-Npl4* complex in regulation of G2/M checkpoint. The G2/M checkpoint prevents DNA-damaged cells from entering mitosis and allows for the repair of DNA that was damaged in late S or G2 phases prior to mitosis. Depletion of Ufd1 or Npl4 led to increased incidence of chromosomal aberrations in the cells after ionizing radiation (IR). The cells, in which p97*Ufd1-Npl4* function was inactivated, failed to engage G2/M checkpoint in response to DNA damage and proceed into mitosis with damaged DNA. Importantly, we showed that the attenuation of the mitotic checkpoint upon Ufd1-Npl4
depletion is caused by stabilization of one of the main G2/M checkpoint controllers, Cdc25A phosphatase.

In the following sections I will discuss the function of p97 in early stages of mitosis in human somatic cells, and describe the functional relevance of the antagonistic role of p97 towards Aurora B kinase activity in chromosome segregation. Furthermore, I will also present evidence for a novel function of p97\textsuperscript{Ufd1-Npl4} complex in regulation of the G2/M checkpoint in mammalian cells, and thus implicate the p97 AAA+ ATPase as a potential target for therapy of human malignancies.

3.1 Part I – The p97\textsuperscript{Ufd1-Npl4} complex antagonizes Aurora B activity during chromosome segregation in HeLa cells

\textit{Ufd1-Npl4 is required for proper segregation of the chromosomes during mitosis.}

Two independent studies have previously associated the Ufd1-Npl4 heterodimer with chromosome segregation in HeLa cells. However, the exact molecular mechanism by which Ufd1-Npl4 may regulate chromosome segregation remained unsolved. The RNAi-mediated depletion of Npl4 resulted in anaphase defects with entangled chromosomes (Porter et al. 2007), whereas Ufd1 depletion has been shown to cause chromosome segregation defects (Vong et al. 2005). Along with these findings, initial studies performed in our laboratory also implicated a role of the p97 adaptor Ufd1-Npl4 in chromosome segregation of human somatic cells. Utilizing time-lapse video microscopy, we observed that the siRNA-mediated depletion in HeLa cells of Ufd1 or Npl4, but not an alternative adapter p47, led to a delay of anaphase onset, suggesting that the spindle assembly checkpoint was activated, probably owing to compromised spindle-kinetochore attachments. The congression defects were then confirmed in fixed HeLa cells, where upon Ufd1-Npl4 depletion, we observed an increase of metaphase cells that displayed misaligned chromosomes, often several at time. In addition, in these cells we observed a significant increase in chromosome segregation defects in anaphase, including lagging chromosomes that probably resulted from an incorrect spindle attachment. Moreover, we also observed chromosome bridges between the separating chromatin masses, and often several entangled chromosomes, indicating
defects in sister chromatid resolution. Additionally, the Npl4-treated cells, which are specifically depleted of the entire Ufd1-Npl4 heterodimer, displayed aberrations in nuclear morphology in interphase. The nuclei of Npl4-depleted cells were multilobed, a phenotype known to result from segregation errors and defective axial compaction of anaphase chromosome, which is associated with inhibition of Aurora B (Hauf et al. 2003; Mora-Bermudez et al. 2007). However, in contrast to Aurora B depletion, siRNA-mediated depletion of Ufd1 or Npl4 did not cause polyploidy of the cells, indicating that the cytokinesis after the segregation of the chromosomes in anaphase was not affected. These results confirmed that the Ufd1-Npl4 adapter of p97 is required for proper chromosome segregation in human somatic cells.

The role of the p97$^{\text{Ufd1-Npl4}}$ complex in mitosis was recently questioned, as a mitotic delay could not be observed in C. elegans embryos treated with RNAi against p97 or Ufd1-Npl4 (Mouysset et al. 2008). However, in HeLa cells, due to the monocentric chromosomes, the two kinetochores assemble only on one centromere. In marked contrast, chromosomes in C. elegans are holocentric, which means that the kinetochores assemble along the entire length of the chromosome arms, rather than being localized to a single site. Therefore, it remains unclear whether in holocentric chromosomes of C. elegans the precise spatial regulation of Aurora B is of utmost importance in regulation of its activity towards the centromeric substrates as well as in regulation of chromosome segregation. Moreover, a major delay due to chromosome misalignment is not expected in early embryos, because under normal growth conditions they have a very weak spindle assembly checkpoint, which can only delay anaphase onset moderately and only when the spindle is disrupted severely (Nystul et al. 2003). We suggest, therefore, that the evidence presented by Mouysset and colleagues (Mouysset et al. 2008) does not preclude a role for p97$^{\text{Ufd1-Npl4}}$ in mitosis of human somatic cells.

**What is the molecular basis of Aurora B regulation by the p97$^{\text{Ufd1-Npl4}}$ complex?**

The observed chromosome congression defects in metaphase, and segregation errors in anaphase upon Ufd1 or Npl4 depletion strongly resembled the phenotype that is typically associated with the Aurora B kinase depletion or its chemical inhibition
In apparent accordance, Vong and colleagues reported a decrease of Aurora B and survivin on the chromosomes upon Ufd1 depletion (Vong et al. 2005). This suggested that Ufd1 is required for the proper localization of Aurora B to the mitotic chromosomes and potentially acts as a positive regulator of the Aurora B kinase. By contrast, it was previously shown in our laboratory that the p97$^{Ufd1-Npl4}$ complex negatively regulates Aurora B by removing its ubiquitylated form from the chromatin during exit from mitosis in embryonic systems (Ramadan et al. 2007). The later finding is in agreement with the prototypical function of p97. The AAA+ ATPase p97 binds to the ubiquitylated substrates via the Ufd1-Npl4 adaptor complex, and upon ATP hydrolysis extracts them from the interaction partners or cellular structures in order to target them for recycling by deubiquitylating enzymes or proteasomal degradation. In line with that notion, we showed that depletion of Ufd1 caused an enhanced association of Aurora B to the chromosomes throughout all mitotic stages (Figure 2.1). The increased levels of Aurora B on the chromatin were observed as early as in prophase and were even observed on segregating chromosomes in anaphase, as well as decondensing chromatin in telophase. This finding was confirmed by immunohistochemistry-based quantification of Aurora B and survivin levels on the chromosomes in metaphase cells. The result from a total of seven Ufd1 and Npl4 siRNA oligonucleotides indicated that the Ufd1-Npl4 depletion leads to elevated levels of Aurora B and survivin on mitotic chromosomes (Figure 2.2). We noted, however, that the depletion efficiencies and degree of segregation errors do not completely correlate with the various degrees of Aurora B accumulation for the different siRNA oligonucleotides. Nevertheless, restoration experiments with a siRNA-resistant Ufd1 cDNA demonstrated that both Aurora B accumulation and the segregation defects are a specific consequence of Ufd1 depletion (Figure 2.4). Moreover, the same experiments showed that the effect of an oligonucleotide previously reported to cause loss of Aurora B from chromosomes (Vong et al. 2005) was indirect, as it was not rescued by overexpression of the resistant Ufd1 cDNA.

In *Xenopus* egg extracts, the p97$^{Ufd1-Npl4}$ complex binds to Aurora B directly and removes it from the chromatin at the end of mitosis (Ramadan et al. 2007). This suggests that the p97$^{Ufd1-Npl4}$ complex might act in a similar manner at earlier stages of mitosis in HeLa
Discussion

cells and remove some Aurora B from the chromosomes as cells progress from prophase, where Aurora B localizes along the entire chromosome arms, to metaphase, thereby attenuating its activity. Given that the p97\textsuperscript{Ufd1-Npl4} function is triggered by ubiquitylation (Ramadan et al. 2007; Ye 2006) this implied the requirement for a specific ubiquitin ligase activity early in mitosis. Interestingly, recent studies implicated a role of human Cul3 ligase, in complex with its BTB adaptors Kelch-like (KLHL) 9 and KLHL13, in chromosome attachment and mitotic progression by regulating the Aurora B localization during cell division (Sumara et al. 2007). It was shown that the Cul3\textsuperscript{KLHL9-KLHL13} complex ubiquitylates Aurora B both \textit{in vivo} and \textit{in vitro}, and KLHL9 as well as KLHL13 directly bind to Aurora B (Sumara et al. 2007). Interestingly, the Cul3\textsuperscript{KLHL9-KLHL13} activity is required to remove part of Aurora B from the mitotic chromosomes during prometaphase, what is crucial for proper chromosome congression (Maerki et al. 2009; Sumara et al. 2007). In accordance with this finding, our results showed that depletion of Ufd1 leads to persistence of Aurora B on the chromosome arms, as well as its increased levels on the centromeres of the prometaphase chromosomes (Figure 2.3). In addition, Cul3 physically interacts with p97 (Alexandru et al. 2008) raising the possibility that the two factors cooperate to regulate chromatin-associated Aurora B early in mitosis. These findings imply a model where Cul3-dependent ubiquitylation of Aurora B may prime for the p97\textsuperscript{Ufd1-Npl4}-dependent removal of the kinase to prevent the excessive centromeric kinase activity. The exact fate of Aurora B upon its p97-mediated extraction from the chromatin is still unclear. However, other than in \textit{Xenopus} egg extracts, where Aurora B levels remains stable, the available data suggests that the mobilized fraction of Aurora B in HeLa cells might, at least in part, be degraded. This hypothesis is supported by the increased total levels of Aurora B in both Ufd1-depleted cells (Figure 2.2B, C and D) and Cul3-depleted cells (Sumara et al. 2007). Furthermore, a pharmacological inhibition of the 26S proteasome leads to increased and persisting levels of Aurora B on the mitotic chromosomes (Sumara et al. 2007), as well as increased total levels of cellular Aurora B (Teng et al. 2012).
The p97\textsuperscript{Ufd1-Npl4} complex antagonizes the Aurora B activity during mitosis.

We next asked whether the increased levels of Aurora B on the chromosomal arms and the centromeres also resulted in an increased Aurora B activity associated with the mitotic chromatin. To explore the role of p97\textsuperscript{Ufd1-Npl4} in regulation of Aurora B activity, we monitored the phosphorylation status of its substrate, CENP-A. We found that phosphorylation of CENP-A in Ufd1-depleted cells is more resistant to a partial inhibition of Aurora B, comparing to control cells. This result demonstrated that the impairment of the Ufd1-Npl4 function leads to a shift towards stronger phosphorylation of CENP-A, which is consistent with an elevated Aurora B activity associated with the chromosomes. Therefore, together with the observed increase of Aurora B protein levels, this shows that the Ufd1-Npl4 complex helps to restrain the activity of the Aurora B kinase early in mitosis, by removing a fraction of it from the chromosomes as cells progress to metaphase. This result, in contrast to the findings of Vong and colleagues (Vong et al. 2005), further confirms the role of p97\textsuperscript{Ufd1-Npl4} as a negative regulator of Aurora B. Therefore, the main question arising from this findings is, whether the activity of p97\textsuperscript{Ufd1-Npl4} in antagonizing Aurora B in prometaphase has any functional impact on the chromosome segregation and hence whether the excessive Aurora B activity can explain the defects caused by the Ufd1-Npl4 depletion. The crucial role for Ufd1-Npl4-mediated downregulation of Aurora B in chromosome segregation is supported by the similarity of the phenotypes observed upon Ufd1-Npl4 depletion and inactivation of Aurora B. The siRNA-mediated depletion, or chemical inhibition of Aurora B, leads to severe defects in chromosome alignment in metaphase, as well as lagging and entangled chromosomes in anaphase that lead to the occurrence of micronuclei and multi-lobed nuclei (Ditchfield et al. 2003; Hauf et al. 2003; Mora-Bermudez et al. 2007; Vagnarelli and Earnshaw 2004). The segregation defects in anaphase might also be caused indirectly through a defective mitotic checkpoint function or through pre-mitotic defects affecting chromosome structure, such as faulty DNA repair and replication (Ichijima et al. 2010; Janssen et al. 2011; Mouysset et al. 2008; Thompson and Compton 2011). However, although we do not exclude the possibility that problems in S-phase might contribute to the observed anaphase defects, we also do not detect major inhibition of DNA replication in Ufd1-Npl4-depleted HeLa cells in normal
growth conditions (Alina Dressler and (Dobrynin et al. 2011)). Furthermore, and more importantly, our results draw a clear link between Ufd1-Npl4 and Aurora B during mitosis, as we show that they functionally interact in regulating chromosome alignment. Our experiments revealed that attenuation of Aurora B activity, by chemical inhibition with hesperadin, improved chromosome alignment to the metaphase plate in Ufd1-depleted cells. Moreover, Ufd1-depletion partially rescued chromosome congression and metaphase plate formation in hesperadin-treated cells. Together with our observation that Ufd1-Npl4 depletion leads to chromosome segregation defects and elevated Aurora B activity, this functional interaction again shows that p97\textsuperscript{Ufd1-Npl4} antagonizes Aurora B during chromosome congression and that overactivation of Aurora B accounts, at least in part, for the phenotype of Ufd1-Npl4 depletion in HeLa cells.

The implication that excessive Aurora B activity at the centromere, upon p97\textsuperscript{Ufd1-Npl4} depletion, induces congression defects is in line with the notion that both negative and positive regulation of Aurora B is crucial for its role in controlling bipolar spindle attachments (Kelly and Funabiki 2009). One of the negative regulators of Aurora B activity is Bub1. It was recently observed that Bub1 overexpression resulted in an aberrant Bub1 kinase activity and hyperactivation of the Aurora B kinase, which led to massive chromosome segregation defects. However, when Aurora B activity was pharmacologically suppressed, the chromosome segregation errors caused by Bub1 overexpression were largely corrected (Ricke et al. 2011). Furthermore, repositioning of Aurora B closer to the kinetochore prevented stabilization of bi-oriented attachments and activated the spindle assembly checkpoint (D. Liu et al. 2009). Aurora B localizes to the inner centromere through interactions with INCENP (Adams et al. 2000). Liu and colleagues manipulated the position of Aurora B by creating INCENP fusion proteins in which the inner centromere-targeting domain of INCENP was replaced either with the centromere-targeting domain of CENP-B or Mis12 (D. Liu et al. 2009). This caused Aurora B to localize closer to, or at the kinetochore, and increased phosphorylation of the Aurora B substrates. The excess of Aurora B activity might either overly destabilize microtubule attachments by phosphorylation of Aurora B-controlled anchor points, such as Ndc80/Hec1 (J. G. DeLuca et al. 2006; K. F. DeLuca et al. 2011). Alternatively, Aurora B might prevent adjustment of incorrect spindle attachment to kinetochores by
phosphorylation, and thus inhibition of the microtubule-depolymerizing activity of MCAK (Andrews et al. 2004; Lan et al. 2004; Ohi et al. 2004). Taken together, this data clearly demonstrates that excessive activity of Aurora B leads to chromosome alignment defects, further supporting our results.

The requirement for a negative regulation to limit Aurora B activity has been shown before in the case of protein phosphatase-1 (PP1), which antagonizes Aurora B throughout mitosis at the chromosome arms (Murnion et al. 2001; Sugiyama et al. 2002; W. Wang et al. 2008) and kinetochores, to ensure proper chromosome segregation (Emanuele et al. 2008; Francisco et al. 1994; Pinsky et al. 2006). The Aurora B kinase phosphorylates kinetochore substrates to destabilize the kinetochore-microtubule interactions and eliminate incorrect attachments. These substrates must be dephosphorylated to stabilize correct attachments, which is especially critical in anaphase, when removal of sister chromatid cohesion leads to a drop of tension between the kinetochores. During mitosis, PP1 is targeted to the mitotic chromosomes by several proteins, including KNL1, Sds22 and Repo-man. PP1 localizes to the kinetochores through a direct interaction with a conserved motif RVSF in the kinetochore protein KNL1. Recruitment of PP1 is required to oppose the Aurora B activity at the kinetochores, by dephosphorylating several of its substrate, including Ndc80, and thus stabilizing the microtubule attachments. Interestingly, the interaction between PP1 and KNL1 is regulated through phosphorylation of KNL1 by Aurora B, which provides a mechanism to coordinate and fine-tune the kinase and phosphatase activities at the kinetochores (D. Liu et al. 2010). During anaphase, PP1 is targeted to the mitotic chromosomes by two subunits, Sds22 and Repo-man which contribute to faithful chromosome segregation. Repo-man and Sds22 counteract a sustained activity of Aurora B on anaphase chromatin, and on the kinetochores, after translocation of Aurora B to the central spindle, hence a balance between opposing kinase and phosphatase activities is ensured (Fuller et al. 2008; Wurzenberger et al. 2012).

In parallel to our study on the role of p97\textsuperscript{Ufd1-Npl4} in progression of mitosis, two independent studies revealed that in \textit{Saccharomyces cerevisiae}, Cdc48/p97 also counteracts Aurora B (Ipl1) during chromosome bi-orientation and cell cycle progression (Bohm and Buchberger 2013; Cheng and Chen 2010). Cheng and colleagues implicated that the kinetochore function of Cdc48/p97 is mediated by the cofactor Shp1 (p47)
Both cdc48-3-mutant and Shp1-depleted cells displayed a delay in anaphase onset due to a defect in kinetochore-microtubule attachment that activated the spindle assembly checkpoint. The observed defects resulted from increased Ipl1/Aurora B kinase activity due to an impaired nuclear accumulation of Glc7/PP1 which counteracts the Ipl1/Aurora B kinase. Moreover, it was demonstrated that Shp1 physically interacts with Glc7, and that the Cdc48\textsuperscript{Shp1} complex is required for the activation of Glc7/PP1 by Glc8/I-2, and thus proper regulation of Glc7 function at the kinetochores (Bohm and Buchberger 2013). Together with our data, this suggests that the antagonizing function of Cdc48/p97 towards Aurora B is conserved during evolution. Interestingly, both groups suggested a role of the p47 adapter homologue Shp1/p47 in chromosome segregation. In our study, we did not find a significant increase in chromosome segregation defects in p47-depleted cells, which might point to distinct requirements for adapters in the two systems, possibly owning to differences like the open compared with the closed mitosis. Interestingly, however, a recent study showed that depletion of p47 in HeLa cells results in higher accumulation of Aurora A at the centrosomes in prophase, which led to a delay in the centrosome separation timing as well as the spindle orientation defects (Kress et al. \textit{in press}). This function is also conserved in worms, where depletion of UBXN-2 (a homologue of mammalian p47 and p37) led to an increased recruitment of AIR-1/Aurora A to the centrosomes, their premature maturation as well as impairment of the alignment of the mitotic spindle with the axis of polarity (Kress et al. \textit{in press}). Importantly, we also observed a mitotic spindle defects in the cells that were treated with the first generation inhibitor of p97, DBeQ. The transient inhibition of p97 in HeLa cells led to spindle orientation defects, including rocking metaphase plates and metaphase plates that were not parallel to the equatorial cortex of the cell (Figure 2.10). These data further confirm the role of p97 in regulation of mitosis and show that the antagonizing role of various p97 complexes towards different Aurora kinases is crucial for proper progression of the cell division. Finally, another recent study in \textit{C. elegans}, demonstrated that p97 is required for proper chromosome segregation in meiosis via the regulation of AIR-2/Aurora B localization. In line with our findings, Sasagawa and colleagues found that the depletion of CDC-48 resulted in a significant increase in the amount of chromosome-bound Aurora B and defective meiotic chromosome segregation (Sasagawa et al. 2012). In addition,
depletion of CDC48/p97 in *C. elegans* caused an excessive phosphorylation of the AIR-2 substrate, histone H3, over the entire length of the chromosomes.

Taken together, these data demonstrate that p97 antagonizes Aurora B already during early stages of mitosis which is crucial for proper chromosome segregation. As described above, we observed that the depletion of the Ufd1-Npl4 heterodimer leads to accumulation of Aurora B at the chromosomes, which led to an increase of chromatin-bound Aurora B kinase activity. Therefore, we suggest that the partial removal of the Aurora B kinase from the chromatin by the p97\textsuperscript{Ufd1-Npl4} complex, as cells progresses from prophase to anaphase, ensures proper chromosome segregation in human somatic cells (Figure 3.1). However, at the same time, we do not exclude the possibility that the p97 ATPase also positively regulates the PP1 phosphatase activity. In line with this notion, p97 might promote proper localization of PP1 to the centromeres, which in turn dephosphorylates Aurora B substrates, counterbalancing the Aurora B kinase activity.

![Hypothetical model of the p97\textsuperscript{Ufd1-Npl4} complex function in early stages of mitosis in human somatic cells.](image)

*Figure 3.1* Hypothetical model of the p97\textsuperscript{Ufd1-Npl4} complex function in early stages of mitosis in human somatic cells. The Aurora B kinase is post-translationally modified by the enzymatic cascade of the ubiquitylating enzymes. p97 binds to the ubiquitylated Aurora B with the help of Ufd1-Npl4 adapter complex and upon ATP hydrolysis extracts the Aurora B kinase from the chromatin, restraining its activity in early stages of mitosis. Then, Aurora B is targeted for proteasomal degradation or recycling by de-ubiquitylating enzymes.
3.2 Part II – The p97\textsuperscript{Ufd1-Npl4} complex is involved in regulation of the G2/M checkpoint in human somatic cells.

Depletion of the p97\textsuperscript{Ufd1-Npl4} complex leads to abrogation of the G2/M checkpoint. We showed above that the depletion of the Ufd1-Npl4 heterodimer, leads to a missegregation of mitotic chromosomes due to an increased activity of Aurora B kinase. However, the chromosome segregation errors that occur during mitosis might not only be caused by mitotic, but also pre-mitotic mechanisms (Burrell et al. 2013). The mitotic mechanisms, which induce chromosome alignment defects, include defective mitotic checkpoint function or erroneous chromosome attachment to the mitotic spindle. The pre-mitotic causes of chromosome missegregation result from a cumulative effect of an abrogated G2/M checkpoint and defects affecting chromosome structure, such as faulty DNA repair and replication, which might lead to various types of DNA damage (Ichijima et al. 2010; Janssen et al. 2011; Pampalona et al. 2010; Thompson and Compton 2011). Interestingly, recently, the p97\textsuperscript{Ufd1-Npl4} complex has been implicated to play a role in both, DNA replication as well as DNA repair. Ramadan and colleagues identified the p97\textsuperscript{Ufd1-Npl4} complex as an essential factor of the DNA repair mechanism of double strand brakes after ionizing radiation-induced DNA damage (Meerang et al. 2011). Moreover, two independent studies showed that p97 together with its adaptor protein DVC1 plays an important role in the ubiquitin-dependent regulation of translation synthesis (TLS), highlighting its importance in guarding of genome stability (Davis et al. 2012; Mosbech et al. 2012). Finally, Mouysset and colleagues observed that challenging of the p97, Ufd1 or Npl4-depleted cells with a sublethal doses of the replication blocking drug hydroxyurea (HU), results in embryonic lethality and sterile worms (Mouysset et al. 2008), implicating the role of p97 in DNA replication, which is crucial for cell cycle progression and genome stability. The unrepaired DNA damage imposes one of the greatest challenges that the cell encounters during proliferation. To cope with damaged DNA, for instance induced by ionizing radiation (IR), cells are equipped with a network of proteins that recognize DNA damage and subsequently orchestrate a proper cellular response. Collectively, this network of proteins is called the DNA damage response (DDR) (Kastan and Bartek 2004). In p53-defficient cells, the activation of the DDR results, among other processes, in activation of the G2/M checkpoint that arrests the cell
cycle progression and allows for repair of the damaged DNA. Above, we showed that the chromosome segregation defects observed in the Ufd1-Npl4-depleted cells were in part caused by overactivation of the Aurora B kinase activity due to an impaired p97\(^{\text{Ufd1-Npl4}}\) complex function. However, in order to address the possibility of the involvement of pre-mitotic mechanisms in the observed mitotic phenotypes, we additionally examined the mitotic chromosomes for any damage that may lead to chromosome segregation defects in mitosis. Our results showed, that depletion of Npl4 in cells growing under normal conditions, led only to a moderate increase of chromosomal aberrations (CAs) in mitotic cells (Figure 2.11A 0 Gy panel and C). This finding, further confirmed our observation that the chromosome segregation defects observed in mitosis upon p97\(^{\text{Ufd1-Npl4}}\) depletion were caused by Aurora B misregulation, rather than pre-mitotic defects, that occurred in previous phases of the cell cycle. The slight increase of mitotic chromosome aberrations in Npl4 depleted cells might be explained by an important role of p97 in regulation of DNA replication as well as DNA damage repair (Franz et al. 2011; Meerang et al. 2011; Raman et al. 2011). Interestingly, when cells were challenged with a DNA damage-inducing dose of ionizing radiation, the frequency of chromosomal aberrations in Chk1-, and more importantly, in Npl4-depleted cells significantly increased, comparing to control cells (Figure 2.11A, B and C). As chromosomal aberrations result from a G2/M checkpoint failure due to a defect of the components in the DDR network (Kastan and Bartek 2004; Shiloh 2003), this result indicated that in Npl4-depleted cells, the G2/M checkpoint function might be impaired. Indeed, our further experiments demonstrated that cells depleted of the p97\(^{\text{Ufd1-Npl4}}\) complex failed to arrest the cell cycle upon IR-induced DNA damage, implicating the abrogation of the G2/M checkpoint. Upon siRNA-mediated depletion of p97, Ufd1 or Npl4, cells in all mitotic phases were found, with massive chromosome segregation defects and multiple γ-H2AX foci, whereas control cells arrested cell cycle progression or entered mitosis only when the DNA damage was repaired (Figure 2.12A, B and C). Interestingly, DVC1-depleted cells also progressed into mitosis with persistent DNA damage on the chromatin. The p97\(^{\text{DVC1}}\) complex has been recently implicated in translesion synthesis (Davis et al. 2012; Mosbech et al. 2012), therefore the role of DVC1 in regulation of the G2/M checkpoint has to be further addressed. Taken together, those results indicated that the p97\(^{\text{Ufd1-Npl4}}\) complex is involved in regulation of the G2/M
checkpoint upon DNA damage, and lack of p97 function leads to the entry of cells into mitosis with damaged DNA and formation of micronuclei (Figure 2.13) as a result of misaligned chromosomes.

What is the cellular mechanism of the G2/M checkpoint abrogation upon DNA damage in p97<sup>Ufd1-Npl4</sup>-depleted cells?

In response to DNA damage, Chk1 is rapidly phosphorylated by the ATM/ATR kinases and becomes highly activated (Q. Liu et al. 2000; Tapia-Alveal et al. 2009; H. Zhao and Piwnica-Worms 2001). Subsequently, Chk1 transduces the DNA damage signal to the downstream effectors, including the Cdc25 family phosphatases and the Wee1 kinase. The phosphorylation of the Cdc25 phosphatases by Chk1, targets them either for ubiquitin-dependent degradation (Cdc25A) or nuclear exclusion (Cdc25B and Cdc25C) (Mailand et al. 2002). The degradation of Cdc25A in response to DNA damage is crucial for preserving of the genome integrity and is governed by the SCF<sup>β-TrCP</sup> complex as well as the proteasome system (Busino et al. 2003). Overexpression of Cdc25A leads to enhanced DNA damage and decreases the cell survival (Mailand et al. 2000). Our experiments showed that upon IR-induced DNA damage, the G2/M checkpoint signaling cascade is properly activated. The checkpoint kinase Chk1 was phosphorylated at Ser317 as well as Ser345, and Cdc25C was phosphorylated at Ser216. Strikingly, despite of the proper DDR activation, the degradation of Cdc25A in Ufd1-Npl4 depleted cells upon DNA damage was compromised (Figure 2.14). The Cdc25A phosphatase promotes the entry into mitosis by removal of the inhibitory phosphorylation from Cdk1 at Tyr15 (Strausfeld et al. 1991). Our results showed that impaired degradation of Cdc25A in Ufd1-Npl4-depleted cells, leads to a moderate decrease of Cdk1 phosphorylation, comparing to the control cells. This finding implicate that Ufd1-Npl4 is involved in the degradation of Cdc25A upon DNA damage, and the stabilization of the Cdc25A phosphatase leads to an impaired G2/M checkpoint function, possibly due to an enhanced dephosphorylation of Cdk1.
Discussion

Does the p97\textsuperscript{Ufd1-Npl4} complex target Cdc25A?

As mentioned above, the Cdc25A phosphatase is essential for cell-cycle progression due to its function in dephosphorylating cyclin dependent kinases, including the cyclin B-Cdk1 complex. In response to DNA damage, the ATM/ATR protein kinases activate Chk1 and Chk2, which leads to hyperphosphorylation of Cdc25A (Falck et al. 2001; Sorensen et al. 2003; H. Zhao et al. 2002). Phosphorylation of the Cdc25A phosphatase stimulates ubiquitin-dependent proteolysis of Cdc25A (Mailand et al. 2000; Molinari et al. 2000) and contributes to delaying cell-cycle progression by the activation of the G2/M checkpoint, allowing for the DNA damage repair. The DNA-damage induced ubiquitin-dependent degradation of Cdc25A is governed by the Cul1-Skp1-β-TrCP E3 ligase (Donzelli et al. 2002; Jin et al. 2003). Our results demonstrate that the overexpression of the ATPase inactive p97EQ dominant negative mutant, leads to accumulation of poly-ubiquitylated Cdc25A, and that this accumulation is further enhanced by IR-induced DNA damage. Moreover, Cdc25A interacts with both p97 as well as Npl4 (Figure 2.16 and Figure 2.17) suggesting that the Cdc25A is a direct target of the p97\textsuperscript{Ufd1-Npl4} complex. Furthermore, the cyclohexamide-chase experiments performed in our laboratory demonstrated that the depletion of Ufd1 or Npl4 specifically stabilizes Cdc25A degradation in response to IR-induced DNA damage (Anne Riemer unpublished data). Based on the function of AAA+ ATPase p97 as an ubiquitin-selective chaperone, p97 is thought to provide segregase activity that separates ubiquitylated proteins from the interaction partners or cellular structures (Ye 2006). The best studied segregase-like function of the p97\textsuperscript{Ufd1-Npl4} complex involves translocations of the damaged or unfolded proteins from the ER lumen to the cytosol for proteasomal degradation (Jarosch et al. 2002; Ye et al. 2001). In line with this notion, our results suggest that p97 might facilitate the extraction of the ubiquitylated Cdc25A from the mitotic chromatin or other chromatin-associated interaction partner, resulting in degradation of Cdc25A. Compromised degradation of Cdc25A upon IR-induced DNA damage may interfere with the G2/M checkpoint function and result in failure to arrest cell cycle progression, as observed in p97, Ufd1 or Npl4-depleted cells (Figure 2.12A, B and C).

Our results show that depletion of the p97\textsuperscript{Ufd1-Npl4} complex results in adaptation of the G2/M checkpoint, causing the cells to enter mitosis with unrepaired DNA. The
immunoprecipitation experiments that we performed showed that Cdc25A physically interacts with p97 and Npl4, but so far we could not detect the same interaction for Ufd1. Moreover, the depletion of Ufd1 or Npl4 in IR-challenged cells led to a stabilization of the Cdc25A protein levels. Interestingly, in p97-depleted cells, the degradation of Cdc25A in response to DNA damage was not compromised (Figure 2.18). The lack of the Cdc25A stabilization in p97-depleted cells might be explained by the fact that p97 plays a crucial role in many other cellular processes. In line with this notion, a blockade of multiple cellular pathways at the same time, by p97 depletion, is likely to result in pleiotropic effects that might initiate alternative mechanisms that lead to G2/M checkpoint adaptation. Indeed, we observed that the depletion of p97 leads to decreased levels of the Wee1 kinase which is responsible for the inhibitory phosphorylation of Cdk1 at tyrosine 15 (Figure 2.18). As Cdk1 plays a major role in controlling passage through the G2/M transition point (Takizawa and Morgan 2000), its insufficient phosphorylation due to the decreased levels of the Wee1 kinase in the p97-depleted cells may lead to the observed G2/M checkpoint abrogation. In addition, we observed that the depletion of p97 in unchallenged cells also leads to increased occurrence of DNA damage in HeLa cells, further supporting the role of the p97 ATPase in the G2/M checkpoint regulation.

*What is the functional relevance of the G2/M checkpoint abrogation for the treatment of cancer cells?*

Many of the most malignant human tumors have often lost the G1/S cell cycle checkpoint due to a lack of p53 protein function (Di Leonardo et al. 1994; Levine 1997; C. Y. Li et al. 1995). Consequently, G1/S checkpoint-defective tumor cells are largely dependent on their remaining G2/M checkpoint to repair their damaged DNA and preserve their genomic integrity when treated with radiation or DNA-damaging drugs (Lobrich and Jeggo 2007). Therefore, by depleting the G2/M checkpoint, normal cells can still arrest in the G1 phase and repair the DNA damage; whereas, tumor cells that lack the p53 function, and thus the G1/S checkpoint, will proceed into mitosis and eventually undergo apoptosis due to mitotic catastrophe (Xiao et al. 2003). We showed that the depletion of the p97\textsuperscript{Ufd1\textendash Npl4} complex leads to the stabilization of Cdc25A in response to the IR-induced DNA damage. Interestingly, we obtained similar results after challenging the cells with two chemotherapeutic agents that induce DNA damage,
cisplatin and doxorubicin. It was previously reported that treatment of the cells with doxorubicin or cisplatin, leads to the ubiquitin-dependent proteolysis of Cdc25A (Agner et al. 2005; Xiao et al. 2003). In line with these findings, our preliminary experiments indicated that treatment of the cells with cisplatin or doxorubicin, led to the degradation of Cdc25A. Importantly, despite of the proper activation of Chk1 kinase, degradation of Cdc25A in Ufd1 or Npl4-depleted cells was impaired. Taken together, our results demonstrate that the inactivation of the p97\textsuperscript{Ufd1-Npl4} complex, results not only in the stabilization of Cdc25A in response to IR-induced DNA damage, but also in response to damage induced by chemotherapeutic drugs. This finding, implicates the p97\textsuperscript{Ufd1-Npl4} complex as an attractive target for cancer-specific sensitization to DNA-damaging chemotherapy as well as radiotherapy (Chen et al. 2012). Therefore, it would be interesting to further address the influence of a combined p97\textsuperscript{Ufd1-Npl4} complex inhibition with simultaneous IR or DNA-damaging drugs treatment on the survival of human malignancies.
4 Materials and Methods

4.1 Cloning

PCR reactions were performed using the PfuTurbo® DNA-polymerase (Stratagene) or PfuUltra Fusion HS DNA polymerase (Agilent Technologies) according to manufacturer’s recommendations. For restriction digestion, DNA ligation and oligonucleotide dephosphorylation reactions, enzymes from New England Biolabs were used according to manufacturer’s protocol. To insert point mutations in plasmid DNA, the QuikChange® site-directed mutagenesis kit (Stratagene) was used. DNA was extracted from agarose gels using the QIAquick Gel Extraction Kit (QIAGEN). Plasmids were amplified using the QIAGEN Plasmid Maxi Kit, the Macherey-Nagel NucleoBond® Xtra Maxi Kit and the QIApre Spin Miniprep Kit (QIAGEN). Transformation of E. coli DH5-α cells with plasmid DNA was performed according to standard procedures.

4.2 Generation of plasmid constructs

reUfd1-GFP

To generate reUfd1-GFP, mouse Ufd1 cDNA was cloned into the BamHI site of pEFGP-N3 (Clontech) and the sequence stretch 5’-CTGCGGGTGATGGAGACCAAA-3’ was mutated to 5’-CTGCGGGTAATGGAAACTAAG-3’ by the Quickchange protocol to render it resistant to V-S2 Ufd1 siRNA. Oligos used: 648/649

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Table 1 Oligonucleotides used for cloning.
4.3 Maintenance of the cell lines

All cells were grown and maintained in a 5 % CO2/air incubator at 37 °C. Parental HeLa Kyoto and HEK293 cells were grown in standard D-MEM (Invitrogen) supplemented with 10 % FCS (PAA) and 1 % of penicillin/streptomycin (Gibco). Inducible HEK293 p97WT and EQ were generated by Danilo Ritz and grown in D-MEM (Invitrogen) supplemented with 10 % tetracyclin negative FCS (PAA), 1 % penicillin/streptomycin (Gibco), 15 µg/mL Blasticidin (Invitrogen) and 100 µg/ml Hygromycin B (Sigma). HeLa Kyoto H2B-mCherry were generated by Michael Schmitz and grown in D-MEM (Invitrogen) supplemented with 10 % FCS (PAA), 1 % penicillin/streptomycin and 0.5 µg/mL Puromycin (Invitrogen).

4.4 Transfections

For DNA plasmid transfections, cells were seeded and transfected using JetPrime reagent (Polyplus) according to standard protocol.

For siRNAi-mediated depletion, cells were grown to about 30-40 % density and transfected with siRNA oligonucleotides at final concentration of 10 nM for 48 h using Lipofectamine RNAiMAX (Invitrogen). All siRNA were purchased from Microsynth and diluted to 20 µM stock solutions.
Materials and Methods

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Table 2 Oligonucleotide sequences used in RNAi experiments.

4.5 Immunofluorescence staining

HeLa Kyoto cells were seeded on sterile coverslips and transfected on the next day. 48 h after transfection, coverslips were removed from the cell culture dish, washed once with PBS (Gibco) and fixed for 15 min at RT in 4 % PFA (paraformaldehyde, SIGMA). After fixation, samples were washed once with PBS and permeabilized with 0.1 % Triton X-100 in PBS for 5 min at RT. Then, samples were washed trice with PBS for 10 min each and blocked with 3 % BSA (Applichem) in PBS for 30 min. After blocking, cells were incubated with primary antibodies diluted in blocking solution for 1 h at RT in a wet (humid) chamber. Unbound primary antibodies were removed by washing the samples trice for 10 min with PBS. After washing, samples were incubated with secondary diluted in blocking solution for 30-60 min at RT in a wet (humid) chamber. Unbound secondary antibodies were removed by washing the cells 3 x 10 min with PBS.
Then the microscopic slides with cells were shortly immersed in dH2O to remove PBS prior to mounting on glass slides in Mowiol containing 1 µg/mL DAPI.

### 4.6 Antibodies and other reagents

*Antibodies used for immunofluorescence and Western blotting:*

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Table 3 Antibodies used for immunofluorescence and Western blotting experiments.
Materials and Methods

Other reagents
DBeQ was purchased from Interbioscreen and used at 1-20 µM concentrations. Cisplatin was purchased from Teva and used at the final concentration of 10 µg/mL. Doxorubicin was purchased from AppliChem and used at the final concentration of 1 µM.

4.7 Fluorescence imaging

Confocal live cell imaging (mitotic timing)
Stable H2B-mRFP HeLa Kyoto cells growing on μ-Slide 8 well chambers (Ibidi) were transfected with siRNAs. Cells were cultured, for data acquisition, in minimal essential medium with Hank’s F12 (MEM/F12; Gibco) supplemented with 15 mM HEPES pH 7.4. Confocal fluorescence time-lapse movies were taken with Leica SP2 AOPS confocal microscope equipped with a 20x 0.7 NA ApoFluor objective lens. Cells were kept at 37 °C during imaging. For acquisition, pictures from one focal plane of living cells were taken every 2-3 minutes. Microscope settings were as follows: picture average, 6; 400 Hz scan speed, 8-bit resolution; logical size, 1024x1024, PMT 1: 0.7 Offset, 736.2 volts; PMT 2: 3.5 Offset, 677,60 volts. Images were processed using Imaris (Bitplane) and ImageJ software.

Indirect immunofluorescence
Samples were analyzed with Leica SP2 AOPS, SP1 AOPS confocal microscope, Zeiss Axiovert TV, Leica DM600B epifluorescence microscope or Zeiss Axiovert 200M with a Yokogava CSU10 Spinning Disk Confocal microscope.

4.8 Preparation of cell extracts
All cell lysates were prepared on ice/4 °C with ice-cold buffers. Culture dishes were taken out from incubator and placed on ice. Medium was aspirated and cells were washed once with 1xPBS (Gibco). Then cells were collected by scraping in Extraction buffer (150 mM KCl, 25 mM Tris pH 7.4, 5 mM MgCl₂, 5 % glycerol, 1 % Triton X-100 and 2 mM b-Mercaptoethanol) which was additionally supplemented with Complete EDTA-free protease inhibitor and PhosphoSTOP phosphatase inhibitor (both from Roche and used according to manufacturer’s recommendations). Cells were lysed for 20 min on ice and insoluble material was sedimented for 15 min at 16000 g.
Supernatants were transferred to fresh tubes and snap frozen in liquid nitrogen for later use.

Preparation of cell extracts for immunoprecipitation (IP) experiments.
Cells were collected by scrapping in IP buffer (150 mM KCl, 50 mM Tris pH 7.4, 5 mM MgCl₂, 5 % glycerol, 1 % Triton X-100 and 2 mM b-Mercaptoethanol) supplemented with Complete EDTA-free protease inhibitor, PhosphoSTOP phosphatase inhibitor (both from Roche and used according to manufacturer's recommendations) and additionally 25 mM N-Ethylmaleimide (NEM) in order to preserve protein ubiquitylation. Cells were incubated for 15 min on ice and insoluble material sedimented for 20 min at 16000 g at 4 °C. Then supernatants were transferred to fresh tubes and used for immunoprecipitation experiments.

4.9 Immunoprecipitation (IP) experiments
For the IP experiments, cell lysates were prepared as described above and inputs (IN) were transferred to separate tubes. After the addition of the antibodies (1 µg of antibody per 1 mg of protein), samples were incubated for 1 h at 4 °C on a tube rotator. Then, 20 µL of Dynabeads® Protein G magneticbeads (Life Technologies) (trice pre-washed with IP buffer) were added and the samples were further incubated for 1 h at 4 °C, rotating. After the incubation, flowthrough aliquots were transferred to separate tubes. Then, IP samples were washed trice with 500 µL IP buffer on DynaMag™-Spin magnet. After final wash, IP buffer supplemented with 6 x Laemmli buffer was added, the beads were incubated at 95 °C for 5 min and subjected to SDS PAGE.

4.10 SDS PAGE and Western blotting
Proteins were separated by standard SDS PAGE protocols using SDS Running Buffer containing 200 mM Glycine, 25 mM Tris-HCl and 0.1 % SDS. Gels were run at a constant current of 20 mA per gel. A semi-dry Trans-Blot® SD transfer cell (Bio-Rad) was used for Western blotting. Proteins were blotted for 45 min on nitrocellulose membranes (Hybond-C super, Amersham Biosciences®) with a constant current of 120 mA per gel using a transfer buffer containing 1xSDS Running Buffer supplemented with 20 % methanol (200 mM Glycine, 25 mM Tris-HCl, 0.1 % SDS and 20% Methanol). For blocking, membranes were incubated overnight at 4°C in 4 % fat free milk powder in
PBS, 0.05 % Tween® 20 (Fluka) or in 1xTBS, 0.1 % Tween® 20 (for membranes that were later probed with phospho-specific antibodies).

4.11 Karyotyping

HeLa Kyoto cells were treated for 48 h with siRNA. 24 h after siRNA transfection, cells were treated with 3 Gy IR dose or left untreated. 1.5 hours prior to fixation, cells were incubated in medium containing colchicine (final concentration in medium 0.8 µg/mL). Cells were removed from the culture dishes by jetting off with glass pipette and centrifuged at 100 g for 10 min. Then, culture medium was aspirated and cells were incubated for 5 min at 75 mM KCl (hypotonic buffer) at 37 °C. After the incubation in hypotonic buffer cells were spanned down at 100 g for 10 min and the supernatant was removed. Cells were resuspended in 5 mL 3:1 methanol:acetic acid (fixative solution), spanned down and fixed again in 5 mL fixative solution. Then cells were sedimented by centrifugation at 100 g for 7 min and ~4 mL of fixative solution was aspirated, leaving ~1 mL in which cells were resuspended. Then, cells were dropped with pipette on a moist cold microscopic glass and let dry overnight. The next day, cells were incubated for 10 min in freshly prepared and filtered 5 % Giemsa stain and wash trice in dH₂O. Images were acquired with Olympus CKX41 brightfield microscope.
References


Adams, R. R., et al. (2000), 'INCENP binds the Aurora-related kinase AIRK2 and is required to target it to chromosomes, the central spindle and cleavage furrow', Curr Biol, 10 (17), 1075-8.


Bohm, S. and Buchberger, A. (2013), 'The budding yeast Cdc48(Shp1) complex promotes cell cycle progression by positive regulation of protein phosphatase 1 (Glc7)', PLoS One, 8 (2), e56486.
References


Carrassa, L., et al. (2009), 'U2OS cells lacking Chk1 undergo aberrant mitosis and fail to activate the spindle checkpoint', *J Cell Mol Med*, 13 (8A), 1565-76.


References


Doss-Pepe, E. W., et al. (2003), 'Ataxin-3 interactions with rad23 and valosin-containing protein and its associations with ubiquitin chains and the proteasome are consistent with a role in ubiquitin-mediated proteolysis', Mol Cell Biol, 23 (18), 6469-83.
Emanuele, M. J., et al. (2008), 'Aurora B kinase and protein phosphatase 1 have opposing roles in modulating kinetochore assembly', J Cell Biol, 181 (2), 241-54.
Fang, S., et al. (2001), 'The tumor autocrine motility factor receptor, gp78, is a ubiquitin protein ligase implicated in degradation from the endoplasmic reticulum', Proc Natl Acad Sci U S A, 98 (25), 14422-7.
References


Hanson, P. I. and Whiteheart, S. W. (2005), 'AAA+ proteins: have engine, will work', Nat Rev Mol Cell Biol, 6 (7), 519-29.


Hauf, S., et al. (2003), 'The small molecule Hesperadin reveals a role for Aurora B in correcting kinetochore-microtubule attachment and in maintaining the spindle assembly checkpoint', J Cell Biol, 161 (2), 281-94.

Havens, C. G. and Walter, J. C. (2009), 'Docking of a specialized PIP Box onto chromatin-bound PCNA creates a degron for the ubiquitin ligase CRL4Cdt2', Mol Cell, 35 (1), 93-104.


Hsu, J. Y., et al. (2000), 'Mitotic phosphorylation of histone H3 is governed by Ipl1/aurora kinase and Gli7/PP1 phosphatase in budding yeast and nematodes', Cell, 102 (3), 279-91.


Kang, J., et al. (2011), 'Mitotic centromeric targeting of HP1 and its binding to Sgo1 are dispensable for sister-chromatid cohesion in human cells', Mol Biol Cell, 22 (8), 1181-90.

Kano, F., et al. (2005), 'The maintenance of the endoplasmic reticulum network is regulated by p47, a cofactor of p97, through phosphorylation by cdc2 kinase', Genes Cells, 10 (4), 333-44.


Kress, E., et al. (in press), 'The UBXN-2/p37/p47 adaptors of CDC-48/p97 regulate mitosis by limiting the centrosomal recruitment of Aurora A', JCB.


References


Li, G., et al. (2006), 'The AAA ATPase p97 links peptide N-glycanase to the endoplasmic reticulum-associated E3 ligase autocrine motility factor receptor', *Proc Natl Acad Sci U S A*, 103 (22), 8348-53.


Li, W., et al. (2008), 'Genome-wide and functional annotation of human E3 ubiquitin ligases identifies MULAN, a mitochondrial E3 that regulates the organelle's dynamics and signaling', *PLoS One*, 3 (1), e1487.


Morishita, J., et al. (2001), 'Bir1/Cut17 moving from chromosome to spindle upon the loss of cohesion is required for condensation, spindle elongation and repair', *Genes Cells*, 6 (9), 743-63.

Mouysset, J., et al. (2008), 'Cell cycle progression requires the CDC-48UFD-1/NPL-4 complex for efficient DNA replication', *Proc Natl Acad Sci U S A*, 105 (35), 12879-84.


Nagata, A., et al. (1991), 'An additional homolog of the fission yeast cdc25+ gene occurs in humans and is highly expressed in some cancer cells', *New Biol*, 3 (10), 959-68.

Nakazawa, N., et al. (2011), 'Condensin phosphorylated by the Aurora-B-like kinase Ark1 is continuously required until telophase in a mode distinct from Top2', *J Cell Sci*, 124 (Pt 11), 1795-807.


O'Connell, M. J., et al. (1997), 'Chk1 is a wee1 kinase in the G2 DNA damage checkpoint inhibiting cdc2 by Y15 phosphorylation', *EMBO J*, 16 (3), 545-54.


Pampalona, J., et al. (2010), 'Whole chromosome loss is promoted by telomere dysfunction in primary cells', *Genes Chromosomes Cancer*, 49 (4), 368-78.

Park, S., et al. (2005), 'Ufd1 exhibits the AAA-ATPase fold with two distinct ubiquitin interaction sites', *Structure*, 13 (7), 995-1005.


Petroski, M. D. and Deshaies, R. J. (2003), 'Context of multiubiquitin chain attachment influences the rate of Sic1 degradation', *Mol Cell*, 11 (6), 1435-44.


Richly, H., et al. (2005), 'A series of ubiquitin binding factors connects CDC48/p97 to substrate ubiquitylation and proteasomal targeting', Cell, 120 (1), 73-84.
Santaguida, S., et al. (2011), 'Evidence that Aurora B is implicated in spindle checkpoint signalling independently of error correction', EMBO J, 30 (8), 1508-19.
Schuberth, C. and Buchberger, A. (2005), 'Membrane-bound Ubx2 recruits Cdc48 to ubiquitin ligases and their substrates to ensure efficient ER-associated protein degradation', Nat Cell Biol, 7 (10), 999-1006.
References


Szathmary, R., et al. (2005), 'Yos9 protein is essential for degradation of misfolded glycoproteins and may function as lectin in ERAD', *Mol Cell*, 19 (6), 765-75.


References


Watanabe, N., et al. (2005), 'Cyclin-dependent kinase (CDK) phosphorylation destabilizes somatic Wee1 via multiple pathways', *Proc Natl Acad Sci U S A*, 102 (33), 11663-8.


Ye, Y., et al. (2005), 'Recruitment of the p97 ATPase and ubiquitin ligases to the site of retrotranslocation at the endoplasmic reticulum membrane', *Proc Natl Acad Sci U S A*, 102 (40), 14132-8.


Yuasa, T., et al. (2004), 'An interactive gene network for securin-separase, condensin, cohesin, Dis1/Mtc1 and histones constructed by mass transformation', *Genes Cells*, 9 (11), 1069-82.


Zhang, S., Skalsky, Y., and Garfinkel, D. J. (1999), 'MGA2 or SPT23 is required for transcription of the delta9 fatty acid desaturase gene, OLE1, and nuclear membrane integrity in Saccharomyces cerevisiae', *Genetics*, 151 (2), 473-83.


Abbreviations

53BP1  tumor protein p53 binding protein 1
aa    amino acid
AAA   ATPase associated with various cellular activities
ADP   adenosine 5’-diphosphate
APC/C anaphase promoting complex/cyclosome
ATM   ataxia telangiectasia mutated
ATP   adenosine 5’-triphosphate
ATPase adenosine 5’-triphosphatase
ATR   ataxia telangiectasia and Rad3 related
ATX3  Ataxin-3
AurB  aurora kinase B
BIR   baculovirus IAP repeat
BRCA1 breast cancer 1
Bub1  budding uninhibited by benzimidazoles 1
Bub3  budding uninhibited by benzimidazoles 3
CA    chromosomal aberration
CAV1  caveolin 1
Cdc20 cell division cycle 20
Cdc25 cell division cycle 25
Cdc48 cell division cycle protein 48
Cdh1  cadherin 1
CDK   cyclin dependent kinase
Cdk1  cyclin dependent kinase 1
Cdk2  cyclin dependent kinase 2
<table>
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<td>chromatin licensing and DNA replication factor 1</td>
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<td>CENP-E</td>
<td>centromere protein E</td>
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<td>checkpoint kinase 1</td>
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<td>E2</td>
<td>ubiquitin conjugation enzyme</td>
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<td>inhibitor of apoptosis</td>
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<td>NEM sensitive factor</td>
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<td>DNA polymerase η</td>
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<td>β-TrCP</td>
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Abbreviations

UBXD  ubiquitin regulatory X domain
UBZ  ubiquitin-binding motif
UFD  ubiquitin fusion degradation
Ufd1  ubiquitin fusion degradation 1
UPS  ubiquitin proteasome system
Usp19  ubiquitin specific peptidase 19
USP9X  ubiquitin specific peptidase 9, X-linked
UV  ultraviolet
VCIP  valosin-containing protein VCP/p97-p47 complex-interacting protein
VBM  VCP binding motif
VCP  valosin-containing protein
VIM  p97/VCP-interacting motif
VIMP  VCP-interacting membrane protein
Vms1  VCP/Cdc48-associated mitochondrial stress-responsive protein 1
WT  wild type
Acknowledgments

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