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CELL CYCLE REGULATION AND DNA DAMAGE RESPONSE: A RECORD OF POLO-LIKE KINASE 1 ACTIVITY

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Cell cycle regulation and DNA damage response: a record of Polo-like kinase 1 activity
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Дорогій бабці Ліді

An meine liebe Oma Lydia

та моїй родині

and to my family

ABSTRACT

Division and cell proliferation is an essence of life. A human cell has in its core a very simple yet a very complex machinery to coordinate cell cycle activities and events. A somatic human cell has as a base four different cell cycle stages: a preparatory cell growth stage (G1) a synthetic stage where genomic material is replicated (S) a second growth and preparation stage (G2) and a mitotic stage, where genetic material is segregated in two new cells (M). The main driver of these phase shifts is the oscillatory behavior of cell cycle proteins called Cyclins, which are being produced and degraded in a periodic manner. Cyclins steer kinase activity, and function together with other cell-cycle kinases as Polo-like kinase 1 (Plk1). On top of the cell cycle regulation a cell has important mechanisms to sense and repair DNA damage, a so-called DNA damage response. DNA damage occurs regularly because of intrinsic factors related to cellular activities e.g. genome replication, metabolism or exogenous factors like solar radiation. Therefore, the response to DNA damage is an inherent part of the cell cycle and its main action is to halt cell cycle progression and establish a checkpoint. A cell has several checkpoints throughout the cell cycle: a G1/S checkpoint, an intra-S checkpoint, a G2/M checkpoint and a M-checkpoint. At these positions a cell can stop or slow down in case of unfavorable conditions, stress or DNA damage. To take care of DNA damage, repair mechanisms are put in place and if possible, a cell eventually continues proliferation (checkpoint recovery) or exits the cell cycle.

In this thesis I focused on the regulation that precedes mitotic entry – the regulation of G2 phase during both normal mitotic entry and after checkpoint activation. I further focused on the activity of the protein Plk1 that is important but not essential to enter mitosis in a normal cell cycle, but becomes indispensable for mitotic entry after DNA damage.

For this study I employed a biosensor for Plk1 activity, Plk1-FRET, and developed a setup that allows to follow single cells expressing the sensor over several cell cycles and later quantify the signals. To study protein behaviors we further developed a technique that allows to elucidate dynamics of the cell cycle proteins from fixed cells growing on micropatterns. Using this approach combined with endogenously tagged Cyclin A and Cyclin B cell lines and a Plk1-FRET biosensor, we find that activities that precede mitotic entry are in place several hours before mitosis, at the completion of S phase, contrary to the previous belief that the mitotic entry network is activated less than an hour before mitosis. We further employed two different model systems and find that Cdk1 and Plk1/Plx1 coordinate degradation of Bora, a protein important for Plk1 activation. We find that in human cells Plk1-induced Bora degradation starts about two hours before mitosis, at the time when Plk1 activity reaches the cytoplasm. Moreover, a small pool of Bora is not degraded and is stabilized in mitosis, providing the possibility to keep Plk1 active in mitosis.

Lastly, using a micropatterning approach and Plk1-FRET biosensor in combination with a probe for APC/C activation I show that upon checkpoint activation in G2 there is a decision point marked by a threshold of Plk1 activity. Activity above this threshold correlates with progression to mitosis, whereas activity below it correlates with cell cycle exit. Furthermore, cells damaged in S phase can exit the cell cycle in two positions in G2, with and without upregulating Plk1 activity, indicating that Plk1 activity is not required for cell cycle exit. Likewise, G1 cells that crossed the G1/S border after receiving DNA damage can exit the cell cycle in G2 phase, in a similar manner as cells receiving DNA damage in S-phase.

Taken together our results shed light on the activities underlying the G2/M transition both in an unperturbed cell cycle and after DNA damage.

LIST OF SCIENTIFIC PAPERS

I. **Hukasova E**, Silva Cascales H, Kumar SR, Lindqvist A. Monitoring Kinase and Phosphatase Activities Through the Cell Cycle by Ratiometric FRET. *J. Vis. Exp.* 1–6 (2012)
II. Akopyan K*, Silva Cascales H*, **Hukasova E***, Saurin AT, Müllers E, Jaiswal H, Hollman DA, Kops GJ, Medema RH, Lindqvist A. Assessing kinetics from fixed cells reveals activation of the mitotic entry network at the S/G2 transition. *Mol. Cell* 53, 843–853 (2014).
III. Feine O, **Hukasova E**, Bruinsma W, Freire R, Fainsod A, Gannon J, Mahbubani HM, Lindqvist A, Brandeis M. Phosphorylation-mediated stabilization of bora in mitosis coordinates Plx1/Plk1 and Cdk1 oscillations. *Cell Cycle* 13**,** 1727–1736 (2014).
IV. **Hukasova E**, Lindqvsit A. Cell cycle exit after DNA damage can occur at two positions in G2 phase. *Manuscript*

* Equal contribution

ADDITIONAL PAPERS

Mäki-Jouppila JH, Laine LJ, Rehnberg J, Narvi E, Tiikkainen P, **Hukasova E**, Halonen P, Lindqvist A, Kallio L, Poso A, Kallio MJ. Centmitor-1, a novel acridinyl-acetohydrazide, possesses similar molecular interaction field and antimitotic cellular phenotype as rigosertib, on 01910.Na. Mol Cancer Ther. 2014 May;13(5):1054-66

CONTENTS

LIST OF ABBREVIATIONS

Plk1	Polo-like kinase 1
Cdk	Cyclin-dependent kinase
DDR	DNA damage response
PBD	Polo-box domain
FRET	Förster resonance energy transfer
Plk1-FRET	FRET-based reporter for kinase activity of Polo-like kinase 1
DSB	Double strand break
ssDNA	Single stranded DNA
Checkpoint recovery	A re-entry into the cell cycle after genotoxic stress
Checkpoint control	A system of checkpoints to sense DNA damage or unfavourable conditions during cell cycle progression

1 INTRODUCTION

1.1 THE CELL CYCLE

Cell cycle as we define it today is an order of events that lead to cell division. These events include cell growth, replication of genetic material and organelles, segregation of genetic material and organelles and separation of two daughter cells[1]. Dependent on the context of cell cycle it can occur in a "fast mode" and alternate only between replication and division cycles like in early embryonic cycles of fertilized egg[1,2]. Here a cell is of a big size at start and has all the nutrients to skip growth phases and focus on cell division, resulting in many small daughter cells from one big mother cell. In somatic cells, however, cells are of a standard size at the start of each cycle and therefore need time to grow and accumulate proteins to enter replication and division, thus event occur in a "slow mode"[1,2].

Every event in the cell cycle constitutes a corresponding phase: G1, S, G2 and M. G1-preparation for division, S-replication of chromosomes, G2-preparation for mitosis, M-mitosis, segregation and separation of chromosomes to two daughter cells (Figure1)[1]. In early days G-phase was standing for Gap phase, periods where visually nothing happened, and a cell rested from active division. Later G-phases were renamed Growth phases, as we learned that cells use this time for growth. However, what exactly happens in G-phases is not very obvious. Why coming from embryonic cycles, a cell evolves such long gap phases? Somatic cells can remain in G1 stage for a half of the cell cycle time in favorable conditions[1]. In stress conditions this time is even longer, and some cells remain outside active division for a long time, entering so-called G0.[1]

G1 phase is finished once a cell initiates replication of its genetic material. In case of G2 phase the definition is more vague, as it is defined as a time between completion of DNA synthesis and initiation of mitosis. For a long time this stage has been overlooked and its importance emerged at the event of genotoxic stress. G1 and G2 stages are places where cells arrest after damage occurs to the genetic material[3]. Besides, for many transformed cells G2 is the only possible stop point before mitosis[4,5]. In epithelial cells G2 phase is estimated to last approximately 4-6 hours. So why is G2 phase necessary, when does it start and why does it take so long? The author hopes that her work presented in this thesis contributed to a better understanding of the regulation and importance of G2 phase in a somatic cell.

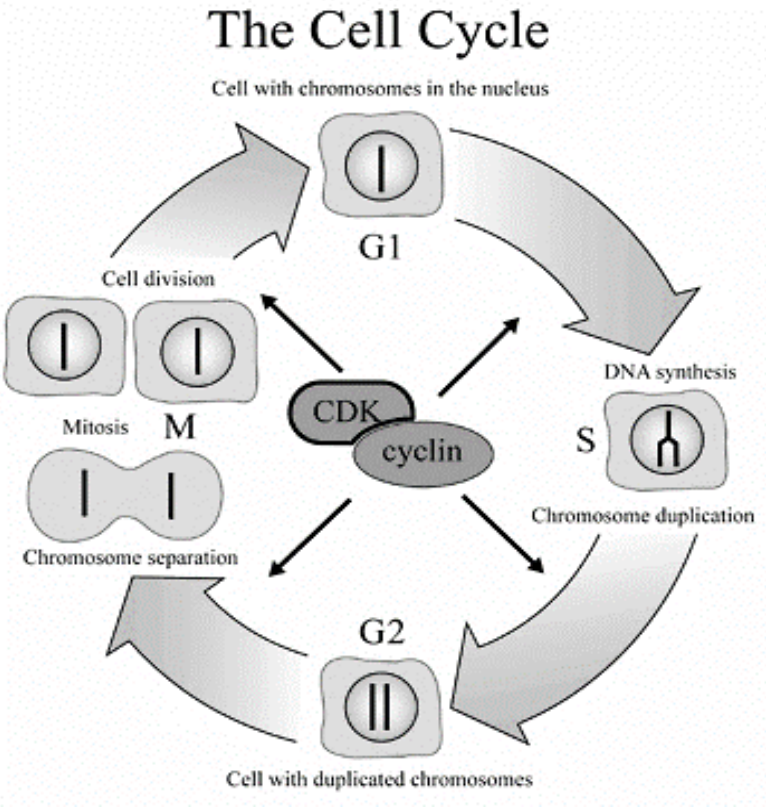

Figure 1: A schematic of the cell cycle progression (from nobelprize.org)

1.1.1 REGULATORS OF THE CELL CYCLE

Since cell proliferation is an essence and a definition of life, it must be regulated by a fundamental and well-conserved mechanism. In fact, it is. In 2001 a Nobel prize was awarded for the discovery of key regulators of the cell cycle in three different species: a bakers yeast *Saccharomyces cerevisiae*, a fission yeast *Schizosaccharomyces pombe* and a sea urchin *Arbacia*[6]. Together these discoveries brought to the identification of a protein Cyclin and a Cyclin dependent kinase (Cdk) as main regulators of the cell cycle. Today we identify several Cyclin-Cdk complexes as regulators of distinct stages of the cell cycle. Moreover, over a dozen of cyclins and Cdks were identified over the years[7].

In mammalian cells at least two Cyclins are present in G2 phase: Cyclin A2, its expression is induced at the start of S phase and Cyclin B1, production of which is induced in G2 preceding mitotic entry. At the same time there are two Cdks that can form complexes with either of the cyclins: Cdk2, essential for S phase progression as Cdk2-Cyclin A complex and Cdk1, essential for triggering mitosis as Cdk1-Cyclin B complex. Although in some organisms Cdk-Cyclin A is essential to promote mitosis[8], its importance in human cells is still debated.

A key feature of the cell cycle regulation is that it needs to occur in a periodic manner. One cycle has to end for another one to start. One phase needs to precede the other and progression has to be unidirectional. This is achieved by cycles of production and degradation of Cyclins, creating an oscillator of Cdk activity – a cell cycle oscillator[9].

This mechanism puts timely protein degradation on important place in regulation of cell cycle exit and re-start of the cell cycle. It is achieved through the function of anaphase-promoting complex/cyclosome (APC/C), an E3 ubiquitin ligase that is activated and targeted to its substrates by the specific subunits Cdc20 and Cdh1 and by SCF (Skp1/cullin/F-box protein)-related complexes[10]. In early *Xenopus* embryos the system works as a simple oscillator: Cyclin-Cdks at the peak of their activity phosphorylate and activate APC, which after short delay degrades cyclins and resets the system[11]. Thus, Cdks regulate their own degradation.

However, in somatic cells introduction of a G1-phase and a presence of several Cyclin-Cdks require additional level of regulation. Here mitotic Cdk activation has to be prevented during G1 phase, therefore APC/C regulated by the Cdh1 subunit is active from the end of mitosis and throughout G1[11]. Additionally, APC/C-Cdc20 degrades distinct Cyclins at defined times during mitosis[12]. Moreover, a large number of mitotic substrates are degraded in mitosis in somatic cells.

However, Cyclin-Cdk complexes do not regulate the cell cycle on their own. Today more cell cycle regulators have emerged that play crucial functions in mitosis and interphase and further regulate, boost or repress the activity of Cyclin-Cdk complexes. All of cell cycle regulators are highly interconnected by feed-forward and feedback loops that make a robust system with a certain level of redundancy[13]. In particular two recently identified kinase families are essential to cell cycle regulation: Polo-like kinases and Aurora kinases. Both of them received names from mutant phenotypes in mitosis and will be discussed in more detail below. Further, a protein Aurora Borealis has important function in the cell cycle.

1.1.2 REGULATION OF THE MITOTIC ENTRY

Whereas timely Cyclin/protein degradation is important for cell cycle exit and for switching between cell cycle phases, regulated Cyclin/protein production is important for entry into distinctive phases. The Cyclin B-Cdk complex was identified in *Xenopus* eggs as a factor that drives G2 cells to mitosis (Mitosis Promoting Factor)[14]. Mechanistically, rapid accumulation of cyclins triggers Cdk activation and mitotic entry[15]. Similarly, in somatic cells mitotic entry from G2 is induced by a rapid increase in Cyclin B/Cdk1 activity[16]. However, to control mitotic entry, a crucial balance between activation and inhibition of Cdk has to be reached.

The Cdk1-Cyclin B complex is inhibitory phosphorylated by Wee1 and Myt1 kinases on T14 and Y15 residues and is re-activated through dephosphorylation by Cdc25 phosphatases[13]. Interestingly, Cdk1 activity itself can regulate these feedback loops; active Cyclin B-Cdk1 complexes can phosphorylate Wee1 and Myt1 to inactivate them, and can activate Cdc25 phosphatases to further boost its own activity (Figure 2)[13]. Once triggered, this mechanism creates a self-amplifying loop of Cdk activity that leads to mitosis. Moreover, there is another level of Cdk activity regulation; Polo-like kinase 1 (Plk1) is involved in feedback loops contributing to Cdk1 activation by targeting Wee1

and Myt1 for degradation and by activating Cdk-activating Cdc25C phosphatase[17,18,19]. Further, Aurora A kinase promotes recruitment of Cdk1 to centrosomes in late G2 and phosphorylates Cdk-activating Cdc25B[20]. Interestingly, Plk1 can further boost Cdk1 and Plk1 activity by directly activating FoxM1, a transcription factor that regulates multiple genes in mitotic entry among others Cyclin B, Cdc25 phosphatases and Plk1[21].

Thus, accumulation of Cdk1 activity is supported by multiple redundant feedback loops to ensure a robust mechanism for mitotic entry.

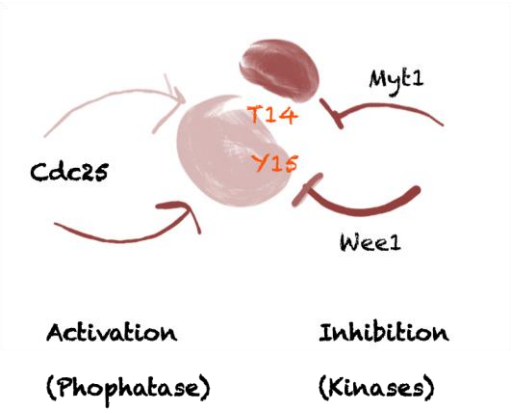

Figure 2. Activation and inhibition of Cyclin-Cdk1 complex

Rapid increase in Cyclin B/Cdk1 activity occurs shortly before mitosis, however low levels of Cyclin B/Cdk1 activity can already be detected in early G2[16,22]. Therefore, mitotic entry activation is most likely a gradual process. Mechanistically, it is possible that S-phase activities, e.g. DNA replication and S phase Cyclins actively repress Cdk1 activity and delay accumulation of Cyclin B. Theoretically, in human cells this mechanism would allow a sufficient time for repair of replication-induced DNA damage and therefore would prevent mitotic errors caused by untimely mitotic entry.

1.1.3 POLO-LIKE KINASE FAMILY: STRUCTURE AND FUNCTIONS

Human Polo-like kinase 1 was first characterized by Erich Niggs group in 1994[23]. Plk1 exhibited homology to the previously described polo from *Drosophila melanogaster*[24,25] and Cdc5 in *Saccharomyces cerevisiae*[26,27]. In Drosophila lack of polo produced mutants with defective spindle orientation[24]. Similarly, in *S. cerevisiae* CDC5 mutants showed mitotic spindle anomalies. Golsteyn and colleagues have noticed that Plk1 levels fluctuate in a cell cycle dependent manner similarly to Cyclin A and are highest in mitosis; moreover Plk1 levels are high in tissues with dividing cells[23]. Ever since Plk1 has been recognized as a kinase important in cell division for timely mitotic entry, mitosis and cytokinesis[28]. However, our understanding of Plk1 functions in interphase is still limited. Since 2004 Plk1 has emerged as a kinase that is indispensable for mitotic entry after DNA damage in G2[29]. And in 2011 Plk1 was suggested to have functions in regulation of S phase under stress[30]. These findings challenge a long-standing view of Plk1 as exclusively mitotic kinase.

Plk1 belongs to the family of serine-threonine kinases that includes four other members Plk2, Plk3, Plk4 and latest discovered Plk5[31]. A special feature of these kinases is the presence of two polo box regions that form a polo box domain (PBD) with exception of Plk4, that has only one polo box[32,33] and Plk5 that lacks kinase domain[34]. Plk1 consists of a N-terminal kinase domain and a C-terminal PBD (Figure3). PBD is an important

regulatory domain that mediates Plk1 localization to different substrates and allows Plk1 to perform its function in the cell cycle[19,32]. Despite structural similarity between polo-like kinase family members, they all have mostly non-redundant functions in cell cycle and differentiation[31,35].

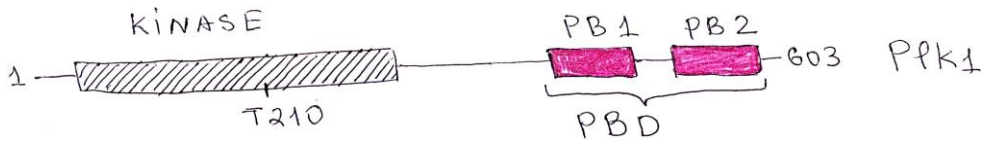

Figure 3. Structure of Polo-like kinase 1.

The polo box domain of Plk1 is a functional domain that binds phosphorylated peptides. The sites recognized by PBD are those phosphorylated by Cdk1 and Plk1 itself[19,32]. Hence through prime-phosphorylation of substrates Cdk1 is involved in spatio-temporal regulation of Plk1 activity. Interestingly, the polo-box domain of Plk1 in a basal state is thought to inhibit the kinase domain leaving the kinase in an inactive conformation (Figure 4). Upon phosphorylation in the T-loop, the PBD is dissociated from the kinase domain bringing the kinase into an open active conformation[19]. Therefore, activation of Plk1 is essential to allow Plk1 recruitment to different sub-cellular localizations to phosphorylate distinct substrates. It is worth a notice that Plk1 does not interact through its PDB with all substrates. In particular, association with the mitotic spindle can occur through direct protein interaction with Map205[36].

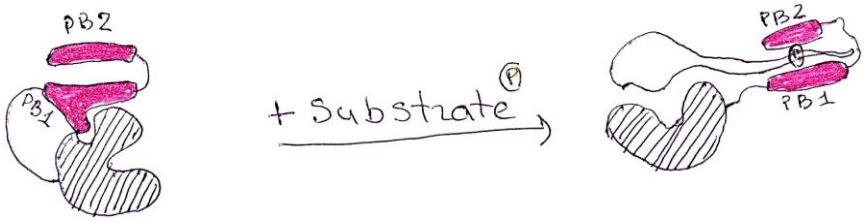

Figure 4. Interaction between a kinase domain and a polo box domain of Plk1.

Plk1 is recruited in a timely manner to different substrates within the cell (Figure 5). In S phase Plk1 is localized to centrosomes[37] and is involved in centriole duplication[37,38]. In G2 Plk1 is detected on kinetochores, centrosomes and in cytoplasm in late G2[22]. Plk1 is recruited to kinetochores by pre-phosphorylating centromeric protein PBIP1 and regulates kinetochore function until anaphase[39]. Plk1 is involved in centrosome maturation and is recruited to pericentrin together with Aurora A, g-tubulin and CEP192[40]; as well as centrosome separation mediated by Eg5 in accordance with Cdk1[41–43]; and microtubule nucleation by phosphorylating Nlp[44]. Further, Plk1 is involved in feedback loops boosting Cdk1 activity to promote mitotic entry[13].

Plk1 has prominent functions in mitosis that led to its discovery in the first place. In mitosis Plk1 is localized to kinetochores, mitotic spindle, and mitotic poles. As the cell approaches cytokinesis Plk1 is localized to the mid-body.

After early recruitment Plk1 targets PBIP1 for degradation[39] and further remains on kinetochores through interactions with Bub1, Bubr1 and INCENP to regulate kinetochore-microtubule attachments[45–47]. Further Plk1 is involved in activation of the anaphase-promoting complex/cyclosome by phosphorylating its inhibitor Emi1 and

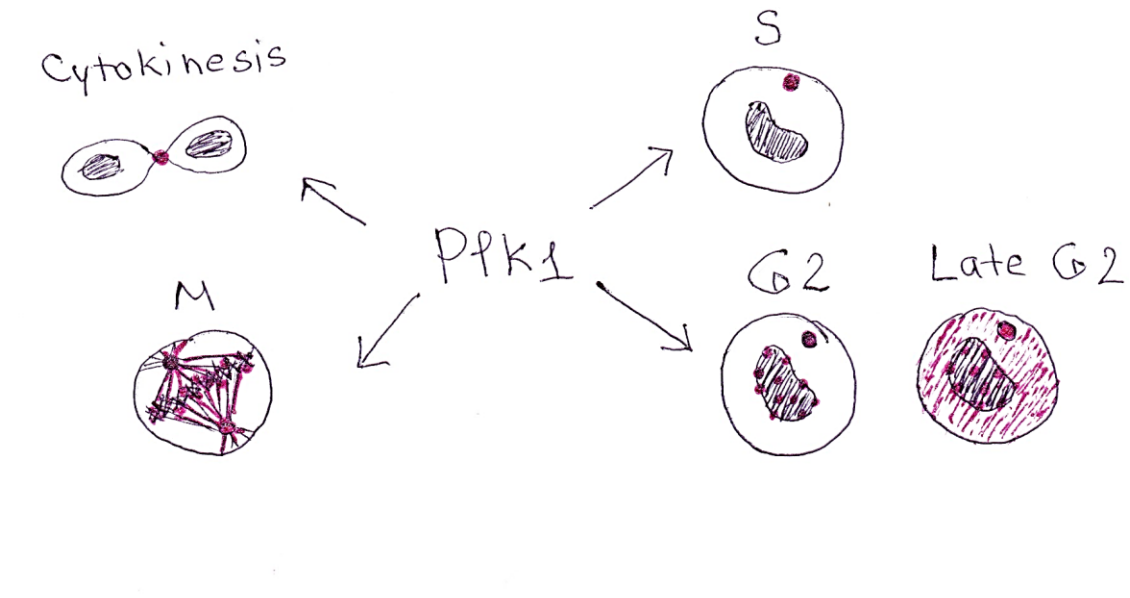

Figure 5. A schematic of Polo-like kinase 1 localization during the cell cycle.

targeting it for SCF-bTrCP-dependent degradation[48]. Plk1 has functions in positioning of the mitotic spindle by controlling switching of dynein/dynactin and phosphorylation of LGN/NuMa proteins[49]. For regulation of cytokinesis and abscission Plk1 translocate to the spindle midzone through interaction with PRC1[50].

First substrate phosphorylation by Plk1 can be recorded as early as 5 hours before mitosis, at the completion of S-phase[22,51]. Plk1 is activated in G2 through phosphorylation of its T-loop on threonine 210 (T210)[51,52]. This action is performed by the mitotic kinase Aurora A in complex with a co-factor scaffolding protein Bora[51,53]. Mechanistically it is thought that Bora binds to the PBD of Plk1 and provides a platform for the interaction between Plk1 and Aurora A[53]. In the absence of Bora, Plk1 T210 is phosphorylated by Aurora A very inefficiently, but addition of Bora greatly enhanced the activity of Aurora A towards Plk1[51,53]. Cdk1 is found to phosphorylate Bora to promote its binding to Plk1 through PBD and facilitate Aurora A–dependent activation of Plk1[54,55]. Therefore, Cdk1 activity is underlying Plk1 activation. It is unclear whether Cdk1 in complex with Cyclin B is responsible for pre-phosphorylation leading to Plk1 activation, as new evidence emerges showing that Cyclin A-Cdk1 complex could be involved in it [56,57].

1.1.4 AURORA BOREALIS (BORA)

Aurora Borealis or Bora was first identified in *D. melanogaster* as a binding partner and activator of Aurora A[58]. Aurora A mutants similarly to *polo* mutants exhibited centrosome maturation effects and asymmetric spindles. Overexpression of Bora rescued these phenotypes to some extend[58]. Bora levels fluctuate throughout the cell

cycle: low at G1/S border, increase in S phase, peak in G2 and then drop in mitosis. Structurally Bora is a protein that does not possess any known regulatory domains[58]. Bora is regulated through post-translational modifications by among others Cdk1, Aurora A and Plk1[53,58–60] and recently three new functional Cdk1 sites on Bora were discovered[54].

Bora is essential for Plk1 activation in G2, however once Plk1 is activated it phosphorylates Bora and targets it for b-TrCP dependent proteasomal degradation[55,60,61]. Interestingly, experiments with overexpressed Bora in human cells show that Bora needs to be downregulated in mitosis, as overexpression leads to significant mitotic delay[55,60]. Since Plk1 degrades its activator at the entry in mitosis it has long remained unclear what kept Plk1 active. One possibility was suggested that TP2AX**,** a different co-factor of Aurora A could be involved. Recently we and others have shown that a small pool of Bora remains present in mitosis and continues to activate Plk1[60,61]. Bora presence in mitosis is in agreement with a requirement for its function, since Bora depletion also leads to metaphase arrest due to presence of unaligned chromosomes[55]. Thus, Bora functions in mitosis to regulate Plk1 and Aurora A kinases.

1.2 DNA DAMAGE RESPONSE

We are surrounded by endogenous and exogenous factors that can and do inflict damage to our DNA on everyday basis. Reactive oxygen species, replicative errors along with UV-radiation, tobacco, IR-radiation just to name a few[62,63]. However most of the damage occurring on a cellular level goes by unnoticed for an organism as a whole for many years. The reason for this is that organisms, both eukaryotic and prokaryotic, possess mechanisms that detect and process DNA damage and constantly repair it. However, DNA damage although a threat is also a part of a healthy functioning organism, as it is a crucial part of the immune response (e.g. lymphocyte maturation and antibody production) and meiotic recombination[63]. The importance of DNA damage and repair pathways is highlighted when some of the components don't work. Patients carrying mutations in the essential proteins in these pathways have higher sensitivity to DNA- damaging agents and higher incidence of cancer[63–65].

DNA damage response (DDR) is a collective term that describe sensing, processing and eventual repair of the DNA damage lesion. Dependent on the type of the lesion there are differences in processing and a choice of repair mechanisms. However, the overall DNA damage response pathway is thought to look the same: a signal is sensed by a sensor, transduced by a transducer and appropriate measures are put in place by effectors[65,66].

To sense the DNA damage different sensors are recruited to different lesions. By homology to *S. pombe* and *S. cerevisiae* Rad1, Rad9, Hus1, Rad17 are involved in sensor complexes in human cells[65]. Double strand breaks (DSB) are sensed by Mre11-Rad50-Nbs1 (MRN) complex that further activates and rapidly recruits a transducer to the sites of damage. Whereas the border of single and double stranded DNA (ssDNA/dsDNA) at breaks can be sensed by Rad17-RFC2-5 clamp loader that further recruits a Rad9-Rad1-Hus1 complex. Further, the RPA protein that coats ssDNA can also act as a sensor and recruit transducer to the site of DNA damage or to a stalled replication fork[66].

Key signaling transducers in mammalian cells are protein kinases ATM (ataxia-telangiectasia mutated), ATR (ataxia telangiectasia and Rad3-related protein) and DNA-PK (DNA-dependent protein kinase catalytic subunit)[62]. ATM and DNA-PK are activated and recruited to double-strand breaks (DSBs) after e.g. exposure to genotoxic agents. ATR is activated and recruited to RPA-coated single-stranded DNA, which can occur at stalled replication forks or as a result of processing of DSBs[62]. ATM and ATR kinases upon activation phosphorylate hundreds of substrates and facilitate rapid signal transduction either direct to the effectors or through the downstream signaling pathways[62,63]. One of the best-studied downstream targets of ATM and ATR are Chk2 and Chk1 kinases. ATM activates Chk2 through direct phosphorylation. Similarly, ATR phosphorylates Chk1, stimulated by the adaptor protein Claspin[67]. Activation of these kinases aims to decrease the activity of the cyclin-dependent kinases (Cdks) and other mitotic kinases of the cell cycle and impose a **checkpoint**[63,68]**.**

Checkpoint activation is thought to give a cell time to repair DNA damage[69]**.** In parallel ATM-, DNA-PK- and ATR-mediated chromatin remodeling of histone H2AX at Ser139 (gH2AX) at and around the site of damage promotes DNA repair and further signal

amplification[62,70]. Once activated, a cell cycle checkpoint can be sustained by ATM, ATR, and Chk2-mediated phosphorylation of the transcription factor p53. P53 transcriptionally regulates the CDK inhibitor p21 and proapoptotic proteins e.g. BAX and Puma and can therefore induce cell cycle arrest, senescence or cell death[62,71]. DDR is a very rigid and robust response, at the same time as it is a dynamic process; since not only DNA damage signaling can modify cell cycle proteins, but cell cycle proteins can modify the DNA damage signaling.

1.2.1 REPAIR OF DSBs

DSBs are considered one of the most toxic DNA lesions as they can cause large chromosome re-arrangements and require immediate attention[72]. DSBs can be repaired by four different mechanisms: homologous recombination (HR), non-homologous end joining (NHEJ), alternative-NHEJ (alt-NHEJ), and single-strand annealing (SSA)[72]. Dependent on the cell cycle stage and DNA end processing requirement different mechanisms can be employed. The most common repair mechanisms are HR and NHEJ. NHEJ is an error-prone but effective mechanism that can be employed in any stage of the cell cycle, with preference towards G1 and M phases. At the same time HR is a more complex repair mechanism, but less error prone. It is limited to S and G2 phases of the cell cycle, as it requires a homologous chromosome.

1.2.2 CHECKPOINT CONTROL: CELL CYCLE CHECKPOINTS

Cells respond to DNA damage by activating cell cycle checkpoints[69,73]. This mechanism allows cells to halt the cell cycle progression, evaluate damage and eventually repair it or initiate cell death. Checkpoints are important to avoid propagation of lethal mutations and avoid genome instability, which is a hallmark of cancer[74]. Moreover checkpoint control is often compromised in a series of hereditary diseases, which further underlines the importance of cell cycle control[75–77]. Dependent on when DNA damage was inflicted during the cell cycle, cells pull different types of breaks. Therefore, we distinguish G1/S checkpoint, intra-S checkpoint, G2/M checkpoint and M checkpoint (spindle assembly checkpoint) (Figure 6).

Although the DDR response to the DNA damage is in essence the same, the checkpoint is established differently in different cell cycle phases. As different protein complexes and activities drive distinct stages of the cell cycle[78].

When DNA damage occurs in G2, cells need to inhibit quickly rising levels of Cyclin-Cdk activities, in particular Cyclin B-Cdk1, to prevent mitotic entry[79,80]. The immediate checkpoint break targets feedback loops that keep Cdk1 activity: Cdc25 phosphatases are inactivated while Wee1 kinase is activated[81,82]. At the same time Plk1 and its activator Aurora A are inhibited[83,84]. Further, the immediate checkpoint is sustained by slower transcriptional regulation through activation and stabilization of p53[71]. P53 promotes transcription and accumulation of p21, which binds Cyclin/Cdk complexes and continues cell cycle arrest[85,86]**.** Furthermore, although ATM/Chk2 signaling is essential in establishing a G2 response, ATR/Chk1 signaling is an essential contributor to maintain a G2 arrest. Since it is involved in degradation of Plk1, Bora, Cyclin B, Cdc25A for the sake of repair or cell cycle exit[81,87–89].

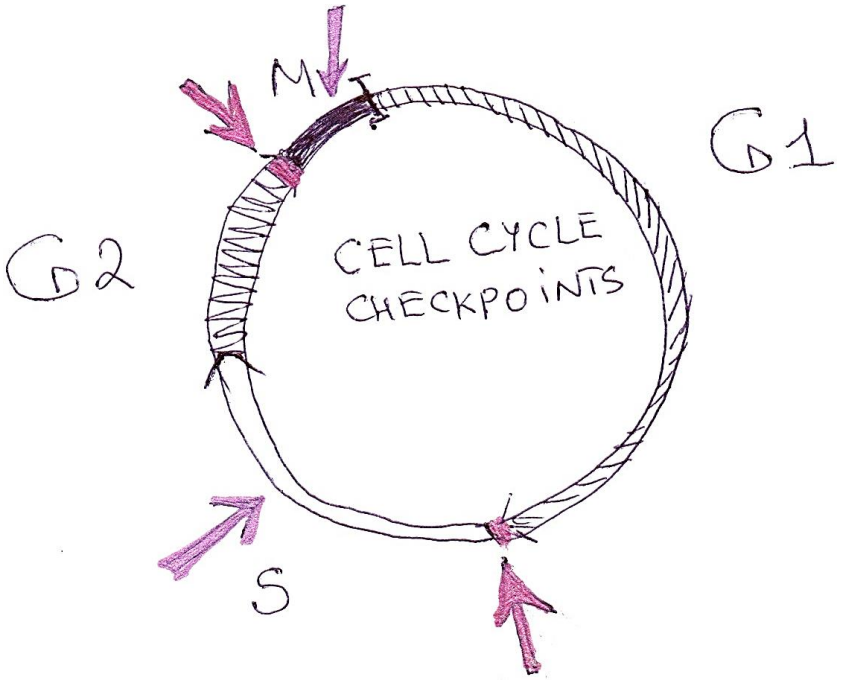

Figure 6. Cell cycle checkpoints: G1/S, S, G2/M and M (spindle assembly checkpoint).

1.2.3 CHECKPOINT RECOVERY COMPETENCE

Once DDR has been established and a cell has repaired the damage it can continue the cell cycle. However, in order to resume the cell cycle a cell needs to maintain a certain level of Cdk activity (Figure 7)[90]. Failure to keep the Cdk activity leads to inability to re-enter the cell cycle after the damage was repaired and checkpoint silenced[90]. At the same time, failure to establish a checkpoint and repress Cdk activity after damage leads to mitotic entry with unrepaired DNA, a so-called checkpoint adaptation or checkpoint override[91]. Therefore, to keep the checkpoint recovery competence, an ability to continue the cell cycle after arrest, a cell needs to maintain a low level expression of G2-specific genes[90]. This is achieved by juggling the transcriptional repression and continuous expression of the mitotic entry proteins.

A way to keep this tricky balance is provided by the intrinsic nature of the DNA-damage response. It is not linear or switch-like, but instead is non-linear and provides pulses of DNA damage activity[92–94]. The tumor suppressor p53 that is a main transcriptional repressor in G2 is in the heart of these pulses. P53 pulses are sustained through a feedback mechanism, by cycles of Mdm2 activation and de-activation regulated by p53 itself[92]. Further, wild-type p53 induced phosphatase 1 (Wip1) regulates the pulses by timely dephosphorylation of p53 targets and de-phosphorylation of p53 at S15. Wip1 depletion results in stable transcriptional repression of the cell cycle regulators Cyclin B

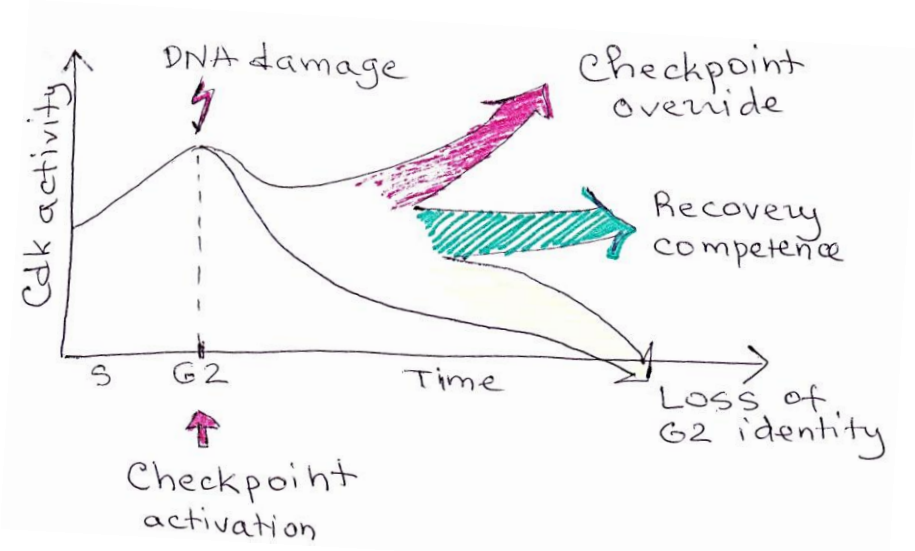

Figure 7. Cell cycle outcomes after checkpoint activation.

and Plk1, which leads to G2 arrest and inability to recover after the checkpoint silencing[95]. Moreover, ATM, an upstream regulator of p53 has been shown to display oscillations in the activity[93,94].

The pulses of p53 activity after DSBs are suggested to be a mechanism that determines cells fate after damage. In particular, cells that exhibit pulses are more likely to enter mitosis after arrest than those that don't[96]. However, how these pulses are mechanistically coupled to the cell cycle regulators is not explicitly shown. One prospect is that they provide a possibility for a checkpoint-arrested cell to continue transcription of G2-specific proteins to maintain G2-identitity[97].

To continue transcription of pro-mitotic genes as Cyclin B and Plk1 a cell relies on the transcription factors FoxM1 and B-Myb[90,98]. In a feedback manner, Cdk and Plk1 activity promote expression of FoxM1 and B-Myb to further boost their own activity[21,99,100]. Therefore, functions of FoxM1 and B-Myb are essential to maintain checkpoint recovery competence.

However, as mentioned earlier regulation of protein expression is only one part of the DDR. Equally important is regulation of protein degradation after DNA damage. Upon DNA damage APC/C is prematurely activated and targets several proteins including Plk1, Cyclin B and Cyclin A for degradation[87,101,102]. To perform its function after DNA damage APC/C depends on its co-activator Cdh1.

Degradation of Plk1 by APC/C-Cdh1 is essential to maintain an efficient G2/M checkpoint, suggesting that Plk1 degradation in G2 can be associated with loss of checkpoint recovery competence[87]. However, induction of APC/C-Cdh1 does not happen immediately after damage, but rather in the later stage of a DDR and marks cell cycle exit. In G2, extended p21 induction leads to either irreversible cell cycle exit several hours past damage (through nuclear Cyclin B degradation by APC/C-Cdh1) or reversible G2 arrest (without Cyclin B degradation and APC/C-Cdh1 activation)[86,103]. Therefore, APC/C activation in G2 marks loss of recovery competence.

1.2.4 APC/C-Cdh1 ACTIVATION UPON DNA DAMAGE

In a normal cell cycle expression and degradation of proteins in a timely manner guarantees unidirectionality of the cycle. Activity of Cyclin-dependent kinases inhibits APC/C and vice versa APC/C inhibits Cdk activity by degrading Cyclins[104]. Additionally, Emi1, an inhibitor of APC/C that is expressed from late G1 to mitosis, is essential for regulation of APC/C activity. Emi1 allows for accumulation of S phase-specific proteins and initiation of S phase. In normal conditions APC/C Cdh1 is inactivated upon entry into S phase and becomes active during mitotic exit. In the event of DNA damage there are several pre-requisites for ACP/C Cdh1 activation. The DNA damage response, in particular p53 activity preconditions APC/C activation by a) enforcing decrease in Cdk activity[102] b) down-regulating APC/C inhibitor Emi1[102]. Next, phosphatases involved in mitotic exit become active at DNA damage. In particular, Cdc14 phosphatase is released from the nucleolus to nucleoplasm and removes inhibitory phosphorylation on Cdh1 leading to APC/C activation[87].

1.2.5 PLK1 ACTIVATION AFTER DNA DAMAGE

Similarly as in an unperturbed condition, Plk1 is activated by phosphorylation on T210 by Aurora A kinase in a complex with Bora to promote G2/M checkpoint recovery[51]. Cdk1 activity is the pre-requisite for this activation by enabling Aurora A/Bora complex formation through pre-phosphorylation of Bora[54,59]. In unperturbed mitotic entry Plk1 activity is redundant and cells are able to enter mitosis in the absence of Plk1 activity. However, upon DNA damage in G2 Plk1-depleted or Plk1-inhibited cells are unable to enter mitosis[51,105].

1.2.6 PLK1 AS A TARGET OF DNA DAMAGE SIGNALING

Upon DNA damage Plk1 is inhibited by several mechanisms. As discussed earlier APC/C Cdh1 is activated upon DNA damage in G2 and degrades Plk1[87]. However, early upon checkpoint activation Plk1 is not degraded, but its further activation by phosphorylation at T210 is prevented. This is achieved by inhibition of Aurora A phosphorylation on Plk1/Bora[106]. Further mechanism of Plk1 inhibition upon DNA damage is SCF-bTrCP dependent-degradation of Bora by ATR upon UV-damage[88].

DNA damage signaling inhibits Plk1 to prevent untimely mitotic entry. In response, Plk1 inhibits DNA damage signaling to keep the checkpoint recovery competence and

promote the recovery. In relation to cell cycle DNA damage response is established throughout several independent axes. First, is immediate activation of Wee1 kinase to inhibit Cdk activity, then ATM-Chk2 axis, ATR-Chk1 and direct p53 axis (Figure 8). Wee1 kinase axis: Cdk1 together with Plk1 target Wee1 for SCF-bTrCP dependent proteasomal degradation[17,107]. Interference with this degradation leads to inability to silence the checkpoint[105].

ATR-Chk1 axis: Active ATR requires adaptor protein Claspin to efficiently phosphorylate Chk1 together with *Rad9-Rad1-Hus1* complex[108,109]. Claspin is a cell-cycle regulated protein which levels peak in S and G2 phases. Upon DNA damage Claspin is transiently stabilized[110]. Active Plk1 phosphorylates Claspin and targets it for degradation by SCF-bTrCP, preventing full Chk1 activation in response to DNA damage[110–112]. Plk1-dependent Claspin degradation is an important step towards checkpoint recovery since expression of non-degradable Claspin inhibits mitotic entry.

ATM-Chk2 axis: Plk1 further counteracts DDR signaling by inactivating Chk2 and preventing further activation of 53BP1[113]. Plk1 phosphorylation of 53BP1 appears to cause its dissociation from chromatin and failure to localize to DNA breaks. Plk1 phosphorylation of Chk2 occurs in its phospho-binding domain and deactivates the kinase[113].

P53 axis: Plk1 phosphorylates GTSE1, an Mdm2-dependent negative regulator of p53, and causes its translocation to the nucleus[114]. In the nucleus GTSE1 binds p53 and shuttles it out of the nucleus, thereby inhibiting its function[114]. Furthermore, Plk1 destabilizes p53 through phosphorylation of Topors, causing increased ubiquitination of p53[115]. Thus, Plk1 modifies and is modified by DNA damage signaling during DNA damage arrest in G2.

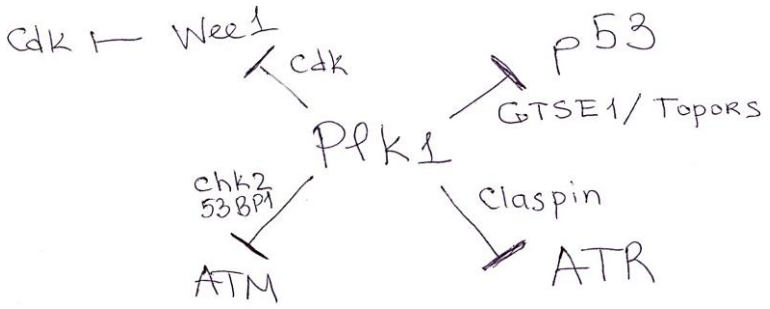

Figure 8. A schematic of Plk1 counteracting the DNA damage response signaling.

1.3 PLK1 AND CANCER

Plk1 function in checkpoint recovery after DNA damage would suggest that it has an oncogenic potential. However, its position on the chromosome has no recorded amplifications and the only gene mutation reported leads to Plk1 instability[116]. And several studies, except for one, have shown that its overexpression does not lead to cell transformation[117–119]. Nevertheless, Plk1 is well exploited by proliferating cancers. Plk1 overexpression has been reported in tumors of colon[120–123], lung[124], breast[125,126], ovary[127,128] as well as in leukemia[129] and this correlates with poor prognosis. In normal conditions Plk1 is expressed only in highly proliferative tissues like testis and bone marrow (https://www.proteinatlas.org/ENSG00000166851-PLK1/tissue) suggesting that its high levels in various cancer tissues is a sign of proliferation.

Since Plk1 function can contribute to tumorigenesis, it is likely that high Plk1 levels in tumors are not just attributed to high proliferation index, but rather reflects deregulation of its activity. Something that supports this idea is that Plk1 in cancer cells is already expressed at the G1/S border[130]. Furthermore AURKA, the gene for Aurora A, an activator of Plk1, is found to be amplified in human cancers and is associated with poor prognosis[131]. High levels of Aurora A could be contributing to increased Plk1 activity in malignancies. On the other hand, Plk1 needs to be actively inhibited by DNA damage signaling to halt cell cycle progression. A majority of tumors are compromised in the DNA damage response through mutations in key proteins e.g. p53, p21, BRCA1 or BRCA2 and through failures in signal transduction[132,133]. This can in turn lead to inability to establish or sustain a checkpoint.

Plk1 is an attractive anticancer target, especially supported by the fact that it has unique functions in DNA damaged cells. Several Plk1 kinase inhibitors, among others BI-2536, Volasertib, Rigosertib, and GSK461364 were developed and their anti-cancer potential was confirmed in pre-clinical studies[130,134]. However, as a common theme for small molecule kinase inhibitors, most of them performed poorly in clinical studies, with exception for Rigosertib[130,134]. First problem encountered is the lack of specificity, that leads to off target effects and toxicity. Another issue is that one inhibitor as a monotherapy is rarely enough. Cancer cells quickly adapt and find another way to proliferate, relapsing in new, usually more resistant tumors.

However, Plk1 inhibitors are still attractive for cancer treatment, especially in combination with other small molecule inhibitors e.g. Aurora A to achieve synergistic effects or with classical cancer therapies like irradiation and chemotherapy[130,131,134]. To battle the kinase specificity issues, new PBD inhibitors are in pre-clinical trials (e.g. poloxin and purpurogallin)[130]. Moreover, Plk1 inhibitors can be used as mitotic poisons where resistance to traditional cell cycle toxins like vinca alcaloids has been acquired.

1.4 METHODS

Since establishment of the cell cycle field multiple experiments were performed in a variety of organisms to elucidate components and mechanisms. Classical examples of the cell cycle model systems are simple eukaryotes like yeast *S. cerevisiae*, *S. pombe*, a sea urchin *A. punctulata*, a fly *D. melanogaster,* eggs from a frog *Xenopus laevis* and immortalized human cell lines[1].

Lower eukaryotes and Drosophila revealed through functional mutagenesis new proteins and their functions. Xenopus eggs provided a biochemical test tube, a simple yet elegant system for understanding protein interactions[1]. Main components were elucidated, purified, enzymatic interactions were tested and phenotypes verified by microscopy. Further, diversity of cell cycle phases in population of human cells brought the development of synchronization techniques to elucidate averages of protein behaviors. Thus, we have learned a lot about cell cycle components from simple organisms and translated these findings to humans where system got more complex and single discovered proteins were replaced by families of proteins.

The study of biochemical reactions in a test tube constitutes a complex but very controlled process, however chemical reactions inside of living cells are even more complex. Availability of compartments and subcellular structures in combination with a multitude of substrates allows for difference in speed, dynamics and location of the interaction. Recent work in mammalian cell populations revealed the heterogeneity in cell cycle decisions and a need for more nuanced understanding of the cell cycle choices[135]. Thus, starting from averages of the cell populations we emerged to single cell approaches. A single cell approach would not be possible without the underlying technological advances in microscopy, in flow cytometry, in the field of fluorescence proteins and biosensors and in single-cell genomics and proteomics.

1.4.1 FRET-BASED BIOSENSORS

In this thesis I have broadly employed biosensor technology that with the help of a physical phenomenon allows measuring kinase activity directly inside a living cell. Biosensors for different kinases have been generated among others for ATM, Aurora B, Cdk1 and Plk1[16,51,136–138].

The biosensor for Plk1 activity like most other kinase biosensors consists of two fluorophores, CFP and YFP, joined by a linker with a consensus sequence for the kinase and a phospho-binding domain[139]. In unphosphorylated state CFP and YFP are in close proximity and Förster resonance energy transfer (FRET) occurs between the two fluorophores. Upon phosphorylation, the probe changes its conformation, since the phospho-binding domain binds to the phosphorylated motif, and CFP and YFP lose close proximity making FRET very inefficient. With the help of fluorescence microscopy, the difference in FRET efficiency can be measured and quantified.

When it comes to the design of the probe there are several important aspects to consider. Fluorophores used in the design of the probe need to have overlapping spectra for energy transfer to occur, however they should not dimerize as it will influence the dynamics of the probe. The binding of the phospho-binding domain to the phosphosite should be optimized, so that the probe can be phosphorylated and dephosphorylated. Next important part is the consensus sequence itself, a difference in timing and strength of the signal can occur dependent on the target that is being phosphorylated by the kinase. Moreover, if specificity is important a sequence also needs to be unique for the kinase of interest, which sometimes can be difficult to achieve. Furthermore, a FRET biosensor is a sensor for both kinase and phosphatase activities, therefore phosphatase activity can influence the kinetics of the signal. Finally, the three-dimensional structure of the probe should allow a conformational change in the probe that should lead to as large as possible difference in FRET-efficiency upon phosphorylation. All these aspects need to be taken into consideration when using and interpreting results obtained by a FRET probe.

In the Plk1-FRET probe, originally cloned by M. Lampson, the phospho-binding domain FHA2 derives from the *S. cerevisiae* Rad51 protein. Plk1 is a serine-threonine kinase, however the FHA2 domain binds phosphorylated threonine more strongly than serine[140]. The consensus sequence for Plk1 in the probe is present in Myt1, but modified so that a threonine replaces a serine as phospho-acceptor and an isoleucine is inserted at +3 position to enhance binding to FHA2[138]. This sequence is unique for Plk1 in G2, however in mitosis another kinase, Mps1, can marginally contribute to the phosphorylation of the probe, since it has a similar consensus site[141]. Therefore, a FRET signal needs to be carefully interpreted. In spite of their limitations, biosensors are powerful tools to study kinase activities in live cells with high spatial and temporal resolution.

1.4.2 FLUORESCENCE LIVE CELL IMAGING

Microscopy is an indispensable tool for cell biology as it allows to study events inside living cells. However, in live cell imaging a caution needs to be taken with microscopy conditions and exposures[139,142]. Since cell cycle wiring seems to be different in unperturbed and perturbed conditions, it is important to put an effort to minimize stress in live cell microscopy. The main sources of stress in live cell imaging are environmental factors e.g. temperature, media, CO_2 supply and phototoxicity from live imaging itself.

Long light exposures during acquisition can be damaging to cells. Even more so are free radicals and reactive compounds generated by fluorophore photobleaching. Moreover, prolonged exposure of the fluorophore to the light source causes fluorophore bleaching that diminishes the strength of the signal[139,142].

Thus, for an optimal signal-to-noise ratio, light and exposure conditions need to be adjusted, so that they would allow to study the process without creating a noticeable

stress response. The length of the cell cycle is among others a good indicator of stress conditions[139].

When it comes to image analysis, the property of light creates uneven background distribution further away from the source of light. Unless a cell is present in the precise angle straight under the objective, these differences in light distribution needs to be compensated by background subtraction. Furthermore, to get a precise quantification of the signal, a background subtraction is necessary to further remove autofluorescence signals and improve the signal-to-noise ratio[22].

1.5 GENERAL DISCUSSION

1.5.1 PLK1 IMPORTANCE FOR MITOTIC ENTRY

Plk1 activation is necessary to perform its functions in the cell cycle. One main of these functions in G2 is boosting Cdk1 activity to facilitate entry into mitosis. Interestingly, however, depletion experiments performed for Plk1 in normal conditions resulted in cells entering mitosis, although with a delay[29], indicating that Plk1 activity may speed up activation of Cdk1.

However, recent data using a small molecule ATP-competitive inhibitor of Plk1 kinase show that acute inhibition of Plk1 with high dose of BI2536 in G2 abrogates mitotic entry in a majority of cells[57]. Further cells rely on regaining of Plk1 activity several hours after treatment to enter mitosis, since repeated inhibitor treatments decreased the resumption of mitotic entry. These data could indicate that once Plk1 gets engaged in feedback loops in G2, its activity cannot be easily replaced, since it takes time to rewire the network.

Furthermore, since RNAi usually does not deplete all the protein, the small amount of Plk1 activity could be sufficient for the mitotic entry in unperturbed cell cycle. However, siRNA treatment blocks checkpoint recovery in the cells subjected to genotoxic stress showing a higher requirement for Plk1 activity after DNA damage than during unperturbed growth.

In accordance with Plk1 functions in mitosis, Plk1-inhbited cells do not complete mitosis. Instead they arrest in prometaphase with immature centrosomes and, if going through mitosis, display chromosome segregation defects

1.5.2 WHERE IS PLK1 ACTIVATED?

Although mechanistically and biochemically a model is suggested for Plk1 activation, the question remains unsolved where exactly Plk1 is activated. The predominantly cytoplasmic localization of Aurora A and Bora makes it apparent to suggest that Plk1 is activated in cytoplasm. Using a biochemical approach hBora has been detected only in the cytoplasmic fraction. Aurora A is known to localize to centrosomes in interphase as does Plk1, providing a place and time for Plk1 activation. However, something that doesn't quite fit with the model is that Plk1 activity is first detected in the nucleus 5-6 hours before mitosis using Plk1-FRET probe. At this point there is no activity detected in the cytoplasm and only after 2-3 hours the probe is also phosphorylated there.

Since the dynamics of Plk1-FRET probe reflects the balance of phosphorylation and dephosphorylation, one possibility arises that the phosphatase activity for this particular Plk1 substrate is higher in the cytoplasm and therefore Plk1 activity cannot be detected there.

Another possibility is that Plk1 is indeed activated in the nucleus and a kinase to perform this function in the nucleus is Aurora B[143]. However, recent evidence supports the notion that Aurora B is not involved in Plk1 activation in human cells[141]. Alternatively, a small pool of Aurora A can localize to the nucleus in interphase, and overexpressed hBora can be also detected in the nucleus making it plausible that at least some level of Plk1 activation could occur there.

2 AIMS

The overall goal of my thesis was to study Polo-like kinase 1 activity in G2 during unperturbed mitotic entry and after DNA damage. Plk1 is essential for checkpoint recovery and it means its activity lies on the crossroads of the DNA damage response and cell cycle regulation. Specifically, the respective studies aimed to:

- develop conditions for using ratiometric imaging to follow a FRET-based biosensor for Plk1 activity over several cell cycles with minimal stress induction.
- develop a setup to follow human cells on micropatterns to detect temporal information from both fixed and live cells. Use this setup to detect when mitotic kinases, in particular Plk1, is activated.
- study how degradation of Bora is coordinated with Plk1 activation in nucleus and cytoplasm.
- follow Plk1 activity during a checkpoint response to study possible involvement in cell fate decsisions.

3 RESULTS AND DISCUSSION

3.1 MONITORING KINASE AND PHOSPHATASE ACTIVITIES THROUGH THE CELL CYCLE BY RATIOMETRIC FRET (PAPER I)

In this paper we describe and visually demonstrate a setup that allows to perform ratiometric imaging of a Förster Resonance Energy Transfer (FRET)-based biosensor throughout the cell cycle. We further show how to validate and quantify acquired FRET signals using microscopy software. A FRET sensor for kinase activity consists usually of two fluorophores, as CFP and YFP, joined by a linker with a consensus sequence and a phospho-binding domain. Upon phosphorylation, CFP and YFP lose close proximity, which results in loss of FRET. Due to the design, this kind of probe can be both phosphorylated by a kinase and dephosphorylated by a phosphatase, reflecting a balance of antagonizing activities. Since the cell cycle is likely to be regulated differently in unperturbed conditions and during recovery from stress, a caution needs to be taken when following the activity of cell cycle proteins by a FRET-probe over time. When it comes to stress in ratiometric imaging it can be caused by several factors. Firstly, the expression levels of the FRET-probe can interfere with the cell cycle progression by competing with a substrate and titrating away binding sites. Secondly, the exposure conditions and the nature of ratiometric imaging, that requires acquisition of two images at a time, can cause a stress response. Therefore, filming of a FRET-probe during at least one cell cycle and comparing cell cycle timings to the normal growing cells gives a good preliminary assessment of stress conditions. In case of deviating cell cycle timings, the expression level or exposure conditions need to be adjusted.

With the help of microscopy, a difference in the efficiency of FRET can be measured by exciting a donor fluorophore and measuring emission from the acceptor fluorophore. This value is then compared to the excitation and emission ratio of the donor fluorophore itself. A FRET ratio is calculated between these two excitations-emissions with the help of microscopy software. During analysis, a special caution needs to be taken in estimating the background value and clipping value. To achieve desired visualization of the ratio, an inverted FRET ratio can be calculated.

Here we discussed how to monitor a FRET-based probe without noticeably perturbing the cell cycle and still retain an acceptable signal-to-noise ratio. However, this may not be the issue for the probes that study fast responses and short-lived events, but mostly has implication for long-term imaging. In this article we have used a diffusible Plk1-FRET probe, however in case of targeted biosensors, issues with photo-toxicity can have stronger impact. In case of H2B-coupled probes, close proximity to DNA can cause DNA damage upon excitation of fluorophores.

3.2 ASSESSING KINETICS FROM FIXED CELLS REVEALS ACTIVATION OF THE MITOTIC ENTRY NETWORK AT THE S/G2 TRANSITION (PAPER II)

In this research paper we report a micropatterning method, that allows to extract kinetics of G2-proteins from fixed cells. Immunofluorescence is a powerful tool that allows to visualize the spatial distribution of cell cycle proteins, however it is limited by the lack of temporal resolution. Here we developed a method, that allows to extract temporal information from a large number of fixed cells, based on the knowledge of protein dynamics in the cell cycle. Assuming a gradual increase of the cell cycle components[144] and having an estimate of the cell cycle duration, allows to assign timings to the cells based on strength of the cell cycle signal. Since precise signal quantification is essential for the accuracy of this approach, we have employed a system that allows to grow cells individually on fibronectin-coated micropatterns. This approach reduces cell-to-cell variation in shape and cell-to-cell contact and allows for optimal objective angle in image acquisition, which simplifies background subtraction. Using our quantitative immunofluorescence approach we find that early activation of the mitotic entry network occurs already at the S/G2 border. We detect Cdk1 target phosphorylation and a marker for Cdk1-Cyclin B1 activity as early as 5 hours before mitosis. We further verify our approach in live cells, by endogenous tagging and following of accumulation and degradation of Cyclin A and Cyclin B along with monitoring of Plk1 activity using a biosensor. Tracking of Plk1 activity together with the dynamics of PCNA foci reveals activation of Plk1 at the S/G2 border, 5 hours before mitosis. Similar to Cdk1 activity, Plk1 activity remained coupled to S-phase even after prolonged thymidine treatment. Thus, our results show that contrary to previous findings[16], the mitotic entry network is activated at the S/G2 border, several hours before mitosis. Furthermore, our results indicate that S phase can play an important role in the regulation of G2 phase.

In embryonic cycles G2 phase is mostly absent and mitotic entry can occur shortly after DNA replication. This would require a rapid activation of Cyclin-Cdk complexes that leads to mitosis. In somatic cells G2 phase is rather long, 5-6 hours, however a rapid increase in activation of Cyclin-Cdks is reserved for last 40 min before mitotic entry. Artificial extension of S phase leads to a delay in G2-specific target phosphorylation. Therefore, a mechanism can exist that allows for S phase to regulate the speed of accumulation of mitotic proteins and cyclins, by actively inhibiting them. Since S-phase progression in the absence of DNA damage requires ATR activity through Chk1 and its regulatory protein Claspin[109], a possibility exists that ATR-Claspin-Chk1 could actively counteract Plk1 accumulation and activation in S phase.

3.3 PHOSPHORYLATION-MEDIATED STABILIZATION OF BORA IN MITOSIS COORDINATES PLX1/PLK1 AND CDK1 OSCILLATIONS (PAPER III)

In this paper we and our collaborators have employed two different approaches in two different model organisms, Xenopus egg oocytes and human somatic cells, to study Bora degradation. In Xenopus oocytes periodical inactivation of Cdk1 through APC/C-mediated degradation of Cyclins creates an oscillator. Plk1/Plx1 is an important cell cycle regulator that facilitates Cdk1 activation and entry into mitosis. In somatic cells Bora/Aurora A activate Plk1, which upon activation targets Bora for proteasomal degradation. This activation is coordinated by Cdk1, that initially pre-phosphorylates Bora and creates a docking site for Plk1/Aurora A interaction. Plk1 in somatic cells is degraded by APC/C-Cdh1 upon completion of mitosis. However, in Xenopus oocytes Plx1 levels are stable, but Plx1 activity is regulated by that phosphorylation oscillates. How these oscillations of Plx1 activity in early cycles were generated and how Plk1 activity was sustained during mitosis remained unclear.

Here we find that in oocytes, degradation of Bora regulates oscillations of Plx1 activity between mitosis and interphase. Embryonic Bora degradation, similar to in somatic cells, requires phosphorylation of Bora by Cdk1 and Plk1. In arrested oocyte extracts Bora is phosphorylated on the T52 consensus site of Cdk1. This phosphorylation stabilizes Bora by blocking its degradation. Calcineurin in a calcium-dependent manner dephosphorylates the T52 site and Bora can be degraded, thus the calcium surge that occurs at fertilization can trigger Plx1 oscillations.

In somatic cells we have followed the dynamics of degradation of GFP-Bora. We record that GFP-Bora degradation slowly starts approximately 2 hours before mitosis when Plk1 activity monitored by Plk1-FRET probe begins to build up in the cytoplasm. Interestingly, this degradation stops as the cells enter mitosis, and GFP-Bora becomes stabilized in mitosis when Cdk1 is fully active. Taken together our results suggest that Cdk1 controls Bora degradation and stabilization in an incoherent feed-forward manner that coordinates Plk1/Plx1 and Cdk1 activity.

With regard to Bora degradation and Plk1 activity it is tempting to speculate that spatio-temporal coordination of these events is not a coincidence. GFP-Bora localizes predominantly to the cytoplasm in human cells, and our results indicate that there is no active shuttling to the nucleus. Plk1 activity measured by a diffusible Plk1-FRET probe is first detected in the nucleus as early as 5 hours before mitosis, suggesting that this is where Plk1 phosphorylates its first substrates. However, the cytoplasmic activity of Plk1 becomes apparent later, approximately 2 hours before mitosis. Although it is not clear where Plk1 is activated, this spatial resolution could allow to preserve Bora in the cytoplasm after Plk1 activation and contribute to a delay for the Plk1-Bora negative feedback loop.

3.4 CELL CYCLE EXIT AFTER DNA DAMAGE CAN OCCUR AT TWO POSITIONS IN G2 PHASE (PAPER IV)

In this manuscript we followed single RPE cells after infliction of DNA damage to checkpoint recovery or cell cycle exit. After DNA damage, activation of the checkpoint leads to two different outcomes: cell cycle resumption or cell cycle exit. However, the decision depends on the cell cycle stage when damage was inflicted and the amount of phase-specific activity. Moreover, a checkpoint is established differently in different cell cycle stages. Here we monitored Plk1 activity in live cells by the Plk1-FRET sensor to follow cell cycle activities and simultaneously followed a Geminin-RFP probe to monitor APC/C-Cdh1 activity. We find that cells damaged in all cell cycle stages can recover or exit cell cycle, however they do it with different dynamics. In case of G2, late G2 cells do not arrest after damage, but continue into mitosis, whereas early G2 cells exit the cell cycle by premature activation of APC/C-Cdh1. Interestingly, there is a point marked by a threshold level of Plk1 activity in G2, where a direct decision to enter or exit the cell cycle seem to be taken.

Furthermore, cells damaged in S phase can exit the cell cycle with or without upregulation of Plk1 activity. Interestingly, both G2 damaged cells and S damaged cells that upregulated Plk1 activity could reach approximately the same threshold of Plk1 activity. We conclude that cell cycle exit is possible at two positions in G2 and that Plk1 activity is not required for cell cycle exit after damage in S phase. Moreover, cells damaged in G1 phase and overcoming the G1/S checkpoint do not necessary commit to mitosis, as we find that they can also exit the cell cycle after S phase in a similar manner as cells damaged in S phase.

Interestingly, our results fit well with the observation that after genotoxic stress cells accumulate in G1 and G2 phases of the cell cycle. Moreover, a checkpoint can be efficiently established in G1 and G2 phases, but in S phase accumulation of p21 is inhibited by replication-coupled degradation[78]. The same might be true for late G2, where high Cdk and Plk1 activity inhibit the checkpoint. The fact that premature activation of APC/C-Cdh1 in G2 leads to cell cycle exit has been already reported[86,103]. Cyclin B translocation to the nucleus marks a point of no return for the cell cycle progression. P53 induction and p21 accumulation in the nucleus are mechanistical clues to cell cycle exit in G2. However, what triggers APC/C-Cdh1 activation after damage is unknown. Lowered Cdk1 activity and degradation of the APC/C inhibitor Emi1 could be contributing factors[104]. Plk1 is an established target for APC/C-Cdh1 activity, and several hours after DNA damage Plk1 can be degraded.

4 FUTURE OUTLOOK

Life is simple and complex at the same time. Although, the life basics can be simple, the individual processes can evolve in complexity the more we learn about them.

The more we learn about cell cycle networks the more complex they become. Cell cycle regulations seems to be intertwined with the majority, if not all, essential processes within the cell. Cell cycle is connected to metabolic processes, various stress responses, DNA damage response, DNA repair and others. On one hand, some of these connections are expected e.g. those of metabolic processes that regulate cell growth[145]. On the other hand, they highlight how much is still there to explore.

Cell cycle pathways are interlinked thought a plethora of feedback and feedforward loops that regulate signal transduction. However, the more we learn about contribution of the individual loops to the whole pathway, the less intuitive an understanding of the process becomes. Therefore, a mathematical modeling could be a future way to approach the rising complexity of the networks. A modelling approach could allow to incorporate single processes, characterized by single interaction loops into a model of the bigger process and predict behaviors for the whole network.

Furthermore, advances in microcopy and other screening approaches allow for fast accumulation of a big number of data. However, the speed of data processing is limited. Thereof there is a need for efficient processing of the large data sets and a better way to present them. Computer learning and artificial intelligence could contribute to the solution in the future.

Single cell approaches are essential in understanding of the somatic cell cycle networks. Since we are all individuals. However, we are also a system. Rules within a system could be different than those for single individuals, moreover, a signal transduction between cells may change the way individual cells respond to the stimuli. For example, cells can communicate DNA damage through bystander communication and the effects are increasingly obvious in cancers[146]. Therefore, it is important to extend the knowledge obtained in singe cells to the tissue or organ. These studies, could be really important in the understanding of complex processes like tumorigenesis.

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