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CYCLINS ON THE MOVE: A TIME AND A PLACE FOR CYCLIN A2 AND CYCLIN B1 IN THE HUMAN CELL CYCLE

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Cyclins on the move: A time and a place for Cyclin A2 and Cyclin B1 in the human cell cycle

THESIS FOR DOCTORAL DEGREE (Ph.D.)

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To Daniel

Man kann alles essen
aber nicht alles wissen

ABSTRACT

The ultimate aim of the cell cycle is to create an identical daughter cell. Therefore, correct progression through the different phases of the cell cycle is crucial to ensure faithful cell division. Successful execution of the different processes in the cell cycle is achieved by the coordinated action of a complex network of protein kinases and phosphatases at the centre of which stand Cyclin-Cdk complexes. Human cells possess a variety of cyclins and Cdks, which form complexes that regulate cell cycle transitions.

In an unperturbed cell cycle, preparing a cell for mitosis requires faithful DNA replication and reorganisation of the cell's structures and organelles. In this scenario, cells initiate successive waves of Cdk activity that orchestrate the timely and spatially controlled phosphorylation of a multitude of targets. In contrast, upon DNA damage cells must halt cell cycle progression in order to prevent mitotic entry of damaged cells and subsequently avoid potential propagation of mutations.

Strict control of Cyclin-Cdk complexes is, therefore, essential both for correct cell division and to maintain genome integrity. However, the exact mechanisms underlying the activation of Cyclin-Cdk complexes in these different scenarios remain largely unknown. In this thesis, I have investigated several aspects of the regulation of Cdk activity both in the unperturbed cell cycle and during a DNA damage response.

To address Cdk activity in the unperturbed cell cycle we established a novel quantitative immunofluorescence method and assessed the dynamics of cyclin accumulation and Cdk target phosphorylation in the unperturbed cell cycle. We found that the mitotic entry network first becomes activated at the S/G2 transition. This finding shifts the classical view of an abrupt Cdk activation at mitotic entry to an earlier and more gradual activation. Furthermore, it provides a potential link between S phase and mitosis, suggesting the existence of a mechanism that maintains pro-mitotic activities under a certain threshold until DNA replication is completed (**Paper I**).

Interestingly, in parallel to an increase of pro-mitotic activities at the S/G2 transition, we observed a change in the localisation of Cyclin A2. Using genome-edited cell lines that express endogenous Cyclin A2-eYFP we were able to determine the cell cycle-dependent localisation of Cyclin A2 to the cytoplasm. Interestingly, despite coinciding with an increase of Cdk activity in the cell cycle

we found that the cytoplasmic accumulation Cyclin A2 is modulated by p21 and the presence rather than activity of Cdk1. These findings suggest that complex formation and interaction with Cdk inhibitor proteins (CKI) might regulate Cyclin A2 localisation throughout the cell cycle (**Paper IV**). Despite not having uncovered a role for cytoplasmic Cyclin A2, we hypothesise that the cell cycle-dependent localisation of cyclins may be an important step to regulate Cdk activity.

In order to understand how cells modulate Cdk activity upon DNA damage we made use of endogenously tagged cell lines expressing Cyclin B1-eYFP. We found that upon DNA damage cells continue to accumulate Cyclin B1 until reaching levels that are normally present in G2 phase. At this point, cells translocate Cyclin B1 to the nucleus in a p21 and p53- dependent manner where it is degraded by APC/C^{Cdh1}. We identified nuclear translocation and degradation of Cyclin B1 as a restriction point in the cell cycle when cells irreversibly exit the cell cycle and become senescent (**Paper II**).

Senescence is regarded as an early barrier for tumorigenesis as it prevents the propagation of cells with damaged DNA. Our findings in Paper II suggested a link between mitotic inducers and the induction of senescence; therefore we decided to investigate the role of Cdk activity in terminal cell cycle exit. We found that upon DNA damage cells preserve low levels of Cdk activity to ensure that damaged cells continue to progress through the cell cycle until they reach a point where they can be forced into senescence. In this context, we found that Cdk activity induces p21 expression in a p53-independent manner to promote nuclear translocation and degradation of Cyclin B1 and other mitotic inducers (**Paper III**).

Altogether, the data presented in this thesis points towards the existence of a link between the mitotic entry network and the DNA damage response to modulate the activity of Cyclin-Cdk complexes in time and space to trigger ensure correct progression to mitosis or, when needed, to trigger senescence.

LIST OF ABBREVIATIONS AND DEFINITIONS

Eukaryote: cell with a nucleus, all multicellular organisms are made of eukaryotic cells.

Cdk: Cyclin-dependent kinase: One of the main kinases that regulate cell cycle progression.

Cks: Cyclin-dependent kinase regulatory subunit. Cks is a small protein that binds Cyclin-Cdk complexes.

MPF: Maturation Promoting Factor or Mitosis Promoting Factor. MPF was the first Cyclin-Cdk complex discovered in Xenopus egg extracts. It was later discovered to be Cyclin B1-Cdk1.

Mitosis entry network: term to define the complex network of proteins that regulate mitotic entry. Cdks and Plk1 are two of the major proteins involved in the mitotic entry network.

DDR: DNA damage response. Term to define the network of proteins and cellular processes that sense DNA damage and activate effectors to generate an appropriate response to the inflicted damage.

CKI: Cdk inhibitor proteins. CKIs are small proteins that inhibit Cdk activity. The family of CKIs includes p21, p27 and p57 among others.

Wee1 and Myt1: Kinases that phosphorylate Cdk1 inhibiting it.

Cdc25: Family of phosphatases that activate Cdk1 and Cdk2 by removing the phosphorylations introduced by Wee1 and Myt1.

Plk1: Polo-like kinase 1. Together with Cdk, Plk1 is one of the main kinases that regulates cell cycle progression. Plk1 becomes essential for cell cycle progression after DNA damage.

PP1 and PP2A: Protein phosphatase 1 or Protein phosphatase 2A. Different Serine/Threonine phosphatases involved in the regulation of the phosphorylation status of Cdk targets.

Rb: Retinoblastoma protein. Family of tumour suppressors that modulates the restriction point in the human cell cycle by restricting the expression of E2F targets.

E2F: family of transcription factors that promote cell cycle progression.

B-Myb, FoxM1, NF-Y: different transcription factors that modulate the expression of cell cycle genes.

APC/C: Anaphase Promoting Complex or Cyclosome. E3 ligase that targets cell cycle regulators for degradation in different phases of the cell cycle. APC/C requires an activator to recognise targets: Cdh1 or Cdc20 are the two activators of human APC/C.

SCF: Skp-Cullin-F-Box-containing protein. SCF is the general name of the other family of E3 ligases that regulate the degradation of cell cycle regulators. Similarly to APC/C, SCF E3 ligases require F box containing proteins to determine substrate specificity; β-TrCP is an example of F-box protein involved in the regulation of cell cycle targets.

RPA: Replication Protein A: it's a protein that binds single stranded DNA preventing the formation of secondary structures.

Senescence: cellular state characterised by the absence of proliferation. Senescence differs from G0 phase in that senescent cells irreversibly exit the cell cycle whereas cells in G0 can resume proliferation.

p53: one of the most famous tumour suppressor genes and it's popularly known as "the guardian of the genome" for its many roles in the DNA damage response.

p21: member of the family of CKIs. p21 is especially important to modulate senescence.

LIST OF SCIENTIFIC PAPERS

I. Akopyan K*, **Silva Cascales H***, Hukasova E*, Saurin AT, Müllers E, Jaiswal H, Hollman D, Kops G, Medema RH, Lindqvist A. (2014) Assessing Kinetics from Fixed Cells Reveals Activation of the Mitotic Entry Network ad the S/G2 Transition

II. Müllers E, **Silva Cascales H**, Jaiswal H, Saurin AT, Lindqvist A. Nuclear Translocation of Cyclin B1 Marks the Restriction Point for Terminal Cell Cycle Exit in G2 phase.

III. Müllers E, **Silva Cascales H**, Macurek L, Lindqvist A. Cdk Activity Drives Senescence from G2 phase. *Manuscript*

IV. **Silva Cascales H**, Müllers E, Stoy H, Macurek L, Lindqvist A. Cyclin A2 Localises in the Cytoplasm at the S/G2 Transition. *Manuscript*

* Equal contribution

CONTENTS

1 INTRODUCTION

"Omnis cellula e cellula"

In 1855, Rudolf Virchow postulated his famous quote "Omnis cellula e cellula", i.e. a cell comes from another cell, pointing already to the idea that cell proliferation is key for multicellular organisms [1]. Cell division is indeed the basis of life development. All multicellular organisms come from one cell and develop to over 30 trillion cells in the case of humans [2]. Cell proliferation lies at the basis of such achievement, and it constitutes the basis of multicellular life.

Eukaryotic cell division is a very complex and tightly regulated process that leads to the generation of a daughter cell that contains the same content and genetic information as the mother cell. Eukaryotic cells manage to create more cells by progressing through the cell division cycle (hereafter referred to as cell cycle), completing a series of tasks in a given order. Originally, the chromosome theory postulated by Boveri and Sutton in 1904 gave importance to the events in the cell that modified chromosomes [3, 4]. The cell cycle was therefore divided in phases according to chromosome behaviour. In this context, two phases are important: synthesis phase, or S phase, during which cells duplicate their entire genome creating a copy of each chromosome; and mitosis, the cell cycle phase where cells divide and segregate a copy of each chromosome into each of the two daughter cells generated. The phases between the completion of mitosis and initiation of S phase and from the end of S phase to the next mitosis were designated as Gap1 and Gap2 phases respectively (Figure 1 and [5]). Ever since, extensive study has focused on each of the phases of the cell cycle, however the exact mechanisms driving progression through the cell cycle remain an open question.

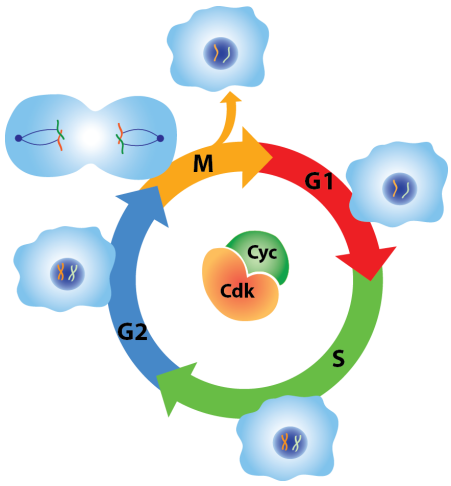

Figure 1. Schematic representation of the human cell cycle.

Conservation in the eukaryotic tree

Progression through the cell cycle is a highly controlled process regulated by multitude of proteins, at the centre of which stands a family of Serine/Threonine heterotrimeric kinases called Cyclin-Cdk complexes (Figure 1). Cyclin-Cdk complexes are composed by a catalytic subunit, a cyclin dependent kinase (Cdk), and a regulatory subunit, a cyclin and an adaptor protein, Cks [6].

During the 1980s, different research groups identified the Maturation Promoting Factor, MPF, as the regulator of mitotic progression [7]. Later, further studies on MPF and cell cycle progression led to the identification of cyclins and Cdks as central elements of the cell cycle engine [8]. Cyclins were first identified in sea urchin eggs as proteins translated from maternal mRNAs that are destroyed at each cleavage division [9]. Later their discovery in other species like yeast, amphibians, *Drosophila* and human cells demonstrated the high conservation through evolution and the importance of cyclins and Cdks in the regulation of the cell cycle [10, 11].

Interestingly, despite presenting slight differences in the amount of proteins involved in the process, the molecular mechanisms underlying the progression through the eukaryotic cell cycle are highly conserved across species from yeast to higher eukaryotes. Indeed, the core machinery of the cell cycle can be traced back to early eukaryotes [12, 13]. Surprisingly, while plants seem to contain homologues of most of the cell cycle regulators found in animals, yeast do not, indicating that the last common ancestor before the bifurcation between plants and animals probably presented these regulators but they were lost in fungi [12]. However, the conservation of cell cycle control mechanisms across the eukaryotic tree points towards Cyclin-Cdk complexes as an efficient way to control cell cycle progression and proliferation [13].

All the work presented in this thesis has been performed in human cell lines, therefore, I will use the human nomenclature for the different players in the cell cycle hereinafter.

The human cell cycle

Different waves of Cdk activity are responsible for the timely phosphorylation of Cdk substrates to complete the different phases of the cell cycle. G1 phase is mostly driven by Cyclin D-Cdk4/6 complexes. Upon passage through the restriction point (see below), Cyclin E-Cdk2 complexes initiate entry into S phase that is maintained by the action of Cyclin A2-Cdk2 complexes. After S-phase completion Cyclin A2 and Cyclin B1 form complexes with Cdk1 to drive progression through G2 phase and induce mitotic entry. Finally, Cyclin B1 regulates correct progression

through mitosis [6]. Waves of Cdk activity are, therefore, key to keep orderly progression through the cell cycle and to ensure that cells execute each process at a specific given time.

Cdk activity is first initiated after commitment to the cell cycle upon mitogenic stimulation. This point in the cell cycle is known as the restriction point and marks the cell's commitment to progress through the cell cycle [14]. In this context, cells respond to extracellular mitogens and start expressing increasing levels of Cyclin D. Once Cyclin D has reached a certain threshold it forms complexes with Cdk4/6 that can phosphorylate the Retinoblastoma proteins (Rb). Rb is a family of tumour suppressor proteins that put a break on the cell cycle by preventing progression from G1 to S phase [15]. During G1 phase, Rb proteins associate with different members of the E2F family of transcription factors to block the expression of cell cycle inducers. Upon phosphorylation by Cyclin D-Cdk4/6, Rb releases E2F leading to the synthesis of several mitotic inducers. One of the targets of active E2F are S phase cyclins, Cyclin E and Cyclin A. Cyclin E forms complexes with Cdk2, which further phosphorylates Rb leading to its full inhibition and releasing the break on E2F-dependent transcription (Figure 2) [16, 17]. This marks the initiation of DNA replication and is accompanied by an increase in the transcription of other E2F targets with important roles in S phase progression like Cyclin A2 and Cdc25A [18-21].

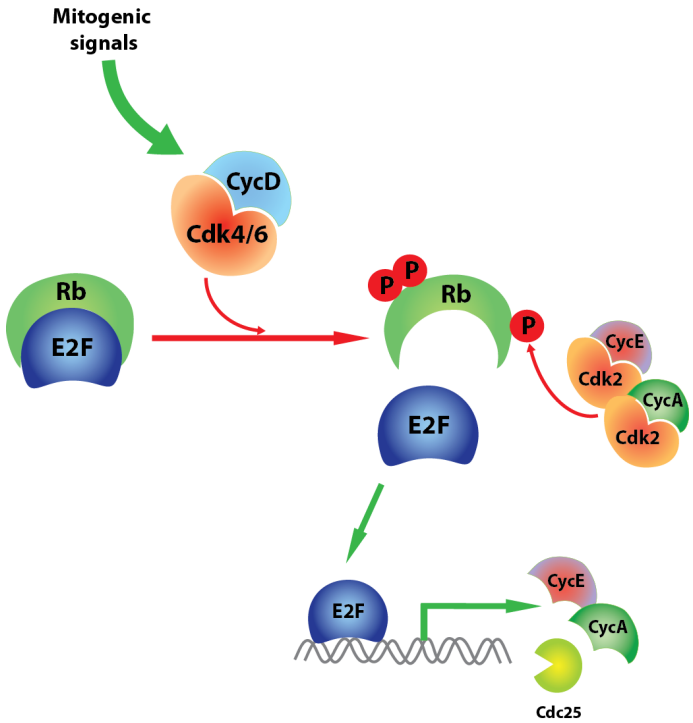

Figure 2. Schematic representation of commitment to the cell cycle in response to mitogens by the action of Cyclin D-Cdk4/6 leading to E2F-dependent transcription of Cyclin E and Cyclin A.

The succession of cyclins leads to a controlled increase of Cdk activity over time providing directionality and robustness to cell cycle progression. In this context, every cyclin leads to the expression of the next one and subsequently the associated Cdk activity (Figure 3).

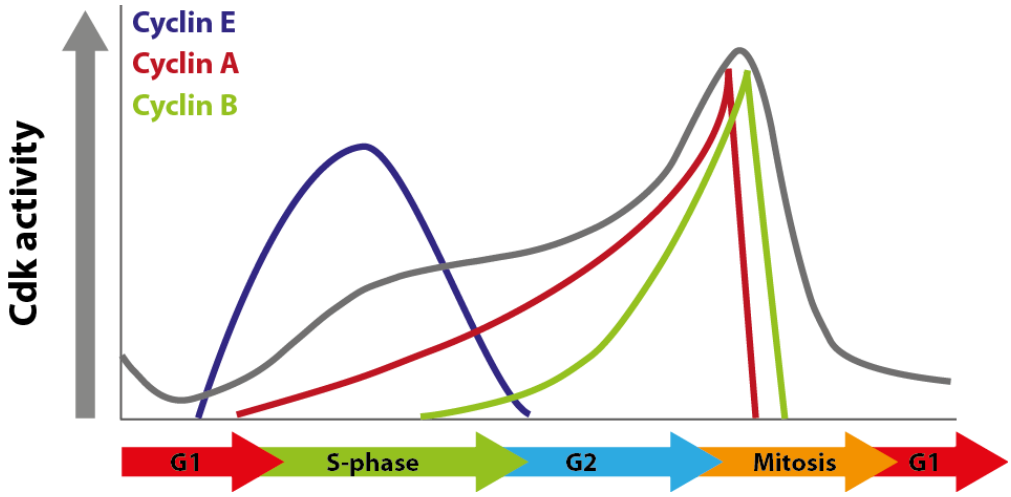

Figure 3. Schematic representation of cyclin dynamics in the human cell cycle and in relation to general Cdk activity.

The discovery that Cdc2 (Cdk homologue in fission yeast) promotes both S phase and mitotic entry led to the idea of a quantitative model for Cdk activity control [22]. In 1996 Stern and Nurse proposed a model, which suggests that in yeast different levels of Cdk activity regulate the order of events in the cell cycle [23, 24]. Therefore, it is not a qualitative difference in Cyclin-Cdk complexes that regulates the different phases of the cell cycle but rather the levels of Cdk activity. In a nutshell, low levels of Cdk activity are needed to trigger and maintain S phase whereas high levels promote mitotic entry.

Despite being a model proposed for yeast, studies conducted in other systems exhibit a similar behaviour. In particular, it has been shown that the G1/S transition in mammalian cells depends on a balance between the degradation of p27 (a small Cdk inhibitory protein) and the accumulation of Cyclins E and A to form complexes with Cdk2. Therefore, once sufficient p27 is degraded, enough Cdk2 activity can build up to trigger S phase initiation [25, 26]. In line, Pomerening and colleagues demonstrated that expression of a constitutively active form of Cdk1 in mammalian cells leads to the shortening of G1 and S phases which translates into failed progression through mitosis, indicating that low levels of Cdk activity are needed for normal progression through S phase [27]. Cyclins are synthesised during S phase, in particular the increase of Cyclin A2 and consequently of Cdk2 activity during S phase has been shown to lead to the

disassembly of pre-replicative complexes thus limiting the use of origins to just once per cell cycle [28]. However, Cyclin A2-Cdk2 and Cyclin E-Cdk2 activity are kept under a certain threshold to prevent premature phosphorylation of mitotic targets as well as DNA damage, indicating that moderate levels are required to maintain S phase (see section "The DNA damage response in the unperturbed cell cycle: a role in S phase"). In late S phase, Cyclin A2 induces FoxM-dependent Cyclin B1 transcription initiating the transition towards G2 phase [29]. At this point, Cyclin A2-dependent appearance of Cyclin B1-Cdk1 complexes leads to an increase in Cdk activity bringing the system to the high Cdk activity that triggers the phosphorylation of a multitude of targets orchestrating the way to mitosis [30-32].

The quantitative model for Cdk activity proposes that cell cycle progression is mediated by increasing Cdk levels, suggesting that cyclin specificity is not essential. Indeed, different cyclins have been shown to be able to replace each other in *Xenopus* or mammalian systems indicating a certain degree of redundancy in the mechanisms of control [33-36]. In addition, two large-scale mass spectrometry studies directed to investigate cell-cycle dependent phosphorylations identified a vast number of phosphorylated targets in mitosis in contrast to interphase. Moreover, they observed that many targets are phosphorylated on multiple sites suggesting that phosphorylation is more efficient during mitosis than in interphase [37-39]. Together, these studies support the basis of the quantitative model of Cdk activity with low Cdk activity during S phase and high during mitosis.

In parallel, a qualitative model has also been suggested which in combination with the quantitative model, gives cyclins a role in modulating the specificity of Cdks towards different targets [40]. In line with this, Loog and Morgan showed that, in yeast, S phase cyclins have higher affinity towards certain S phase-specific targets than M-Cyclins although some substrates are equally recognised by both cyclins [41]. A more recent study reported that cyclins can influence substrate specificity, therefore highlighting the importance of individual cyclins in the normal progression of the cell cycle [42]. Furthermore, Cdk2 has been shown to be a non-redundant regulator of the G1/S transition suggesting that the variety of cyclins and Cdks in mammalian systems might provide the required elements to separate processes in space and time to fine-tune cell cycle progression [43, 44].

Overall, cyclins accumulate during the cell cycle reaching a maximum at mitosis when they are efficiently degraded. This step is crucial to bring the levels of Cdk activity back to a basal level that restarts the cycle. However, Spencer and colleagues recently showed that the levels of p21, a small Cdk inhibitor (CKI) determine cell fate after mitosis. By these means cells either commit to

the next cell cycle and start building up Cdk2 activity directly after mitosis, or remain uncommitted and Cdk levels remain low [45].

The cell cycle control mechanism

The beauty of the orchestrated progression through the cell cycle is the result of a very tight coordination of a complex network of kinases and phosphatases. Due to the scope of this thesis, I will focus on the mechanism of control of Cdk1 and Cdk2 activities mostly during G2 phase. The different layers of control that ensure timely activation of Cdk activity are summarised below.

Cyclin availability

Cdks alone are not active; they require the binding of a cyclin to become activated [46]. Therefore, the presence or rather absence of cyclins poses a first level of regulation on Cdk activity. A transcriptional program driven by E2F, FoxM, NF-Y and B-Myb ensure the timely transcription of specific mRNAs leading to the orderly expression of cyclins in a sequence starting from Cyclin E followed by Cyclin A and last Cyclin B [20, 29, 47, 48]. Together with transcription, E3 ligases are essential regulatory elements of the cell cycle to timely degrade cyclins. Different E3 ligases are responsible for the degradation of cyclins throughout the cell cycle. During early G1 phase, APC/C^{Cdh1} is responsible to prevent premature cyclin accumulation and to maintain a basal low level of pro-mitotic inducing activities [49]. During S and G2 phases, the combined action of SCF E3 ligases and APC/C^{Cdc20} maintain a controlled level of cyclins [50, 51]. Last, APC/C targets Cyclins A and B during mitosis. In detail, APC/C^{Cdc20} times the degradation of Cyclin A at prometaphase while APC/C^{Cdh1} is responsible for the degradation of Cyclin B and securin at anaphase to ensure correct chromosome segregation [52-54].

Interestingly, both the transcription factors and E3 ligases that control Cdk activity are direct targets of Cyclin-Cdk complexes, thus introducing a first feedback loop in the regulatory mechanisms (see below).

Post-translational modifications

A crucial regulatory step of Cyclin-Cdk complexes activity comes from the balanced action of phosphatases and kinases. Upon binding of a cyclin, the conformation of Cdk changes exposing its T loop. At this stage, Cdk-activating Kinase (CAK) phosphorylates Cdk1 and Cdk2 at a conserved threonine residue (T161 and T160, respectively) (Figure 4 and [55, 56]).

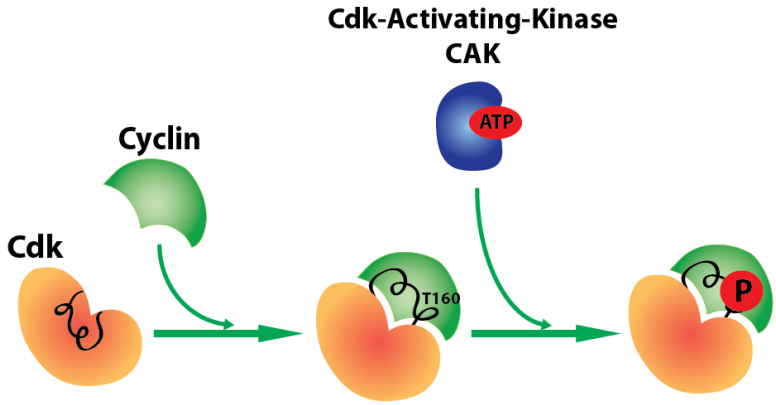

Figure 4. Schematic representation of structural activation of Cyc-Cdk complexes by phosphorylation of the T loop by CAK.

However, this represents only a structural activation. Upon phosphorylation by CAK, Cyclin-Cdk complexes are phosphorylated and inhibited by two kinases: Wee1 and Myt1. These two kinases phosphorylate Cyclin-Cdk complexes at Tyr15 and Thr14 leading to the inhibition of Cdk activity [57, 58]. These inhibitory phosphorylations can be removed by the action of Cdc25, a family of phosphatases with three members, Cdc25A, B and C, each of which play different roles to regulate Cdk activity at different points in the cell cycle (Figure 5 and [59, 60]).

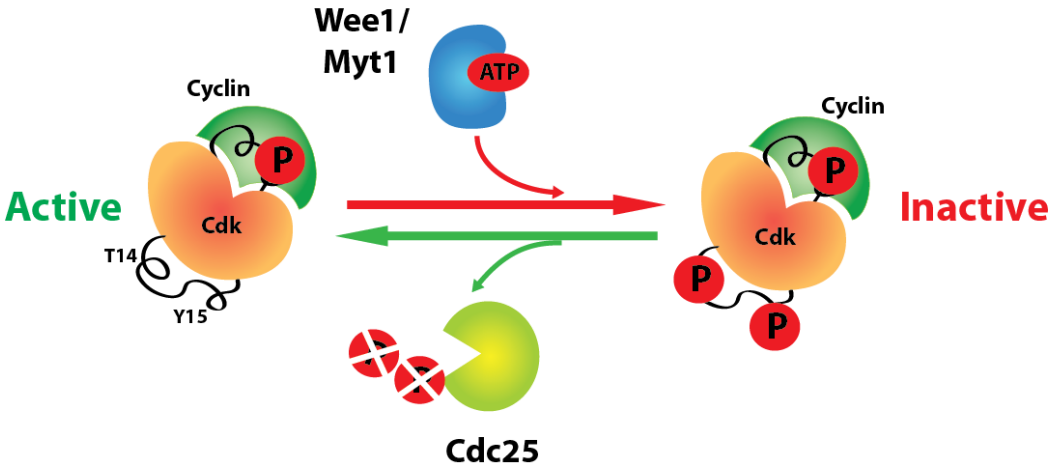

Figure 5. Representation of Cyclin-Cdk activation by the balanced action of Wee1/Myt1 and Cdc25.

Feedback loops

The full activation of Cyclin-Cdk complexes therefore requires (1) a cyclin and (2) the balanced action of Wee1/Myt1 and Cdc25s. However, additional proteins introduce several feedback loops in the system and make the activation of Cyclin-Cdk complexes robust and switch-like in behaviour. In this context, active Cdk1 phosphorylates Wee1 and Cdc25s (Figure 6). Phosphorylation on Wee1 leads to its degradation in a $SCF^{\beta TrCP}$-dependent manner consequently resulting in a decrease in the inhibitory force on Cdk1 activity [61-63]. On the other hand, Cdk1-dependent phosphorylation of Cdc25s leads to their stabilisation, thus translating into a further increase in Cdk1 activity [64]. These events introduce a negative and a positive feedback loop respectively that leads to a rapid amplification of Cdk activity once the first activation is triggered. Interestingly, Cdk1-dependent phosphorylation on Wee1 and Cdc25s involves ultrasensitive multisite phosphorylation, meaning that phosphorylation is rather inefficient at low levels of Cdk activity but increases dramatically in a non-linear manner once Cdk activity starts increasing [65, 66]. This switch-like behaviour of Cdk activity provides directionality and irreversibility to cell cycle transitions [30, 67].

As mentioned above, in addition to a cyclin and a Cdk, Cyclin-Cdk complexes include a small adaptor protein called Cks. Although Cks has been found to have essential roles in cell cycle progression its specific function is not fully clear [68]. However, a recent study revealed that Cks is able to bind phosphoryalated targets in a sequence-dependent manner and direct Cyclin-Cdk complexes to facilitate multisite phosphorylation [69]. In this context, Cks proves to be an essential element to regulate the switch-like behaviour of Cdk activity.

Additional proteins are involved in the feedback loops that activate Cdk complexes. Plk1, for example, phosphorylates Cdc25 activating it, and Wee1, modifying it in a way that leads to its degradation to further promote Cdk activation [70]. On the other hand, several phosphatases exist in cells to oppose Cdk activity by dephosphorylating Cdk targets. Phosphatase activity is essential to ensure correct progression through the cell cycle, and growing evidence suggests that phosphatases are highly controlled to modulate the phosphorylation status of different substrates in the cell cycle [71-73]. Perhaps the clearest example of phosphatase importance for cell cycle control comes in at the end of mitosis, when timely dephosphorylation of Cdk targets by PP1 and PP2A ensures correct completion of mitosis [74, 75].

In the control of interphase, the PP2A family of phosphatases is able to dephosphorylate Cdc25 and Wee1 thus reverting the phosphorylations carried out by Cdk1. Recently, another kinase has come into the feedback loops game. MASTL (or Greatwall, Gwl) in collaboration with two small

regulators Arpp19/Endosulfin-α, phosphorylates and inhibits PP2A at the G2/M transition to boost Cdk activity and ensure timely activation of Cyclin-Cdk complexes prior to mitotic entry. This combination of Gwl, and Cdks has been recently proposed as a more complete description of MPF [74, 76-80]. Interestingly, a common factor in the feedback loops is that elements in the system are direct or indirect targets of Cdk. This provides Cdk the capacity to regulate its own activity and subsequently set phase transitions in a robust and irreversible manner.

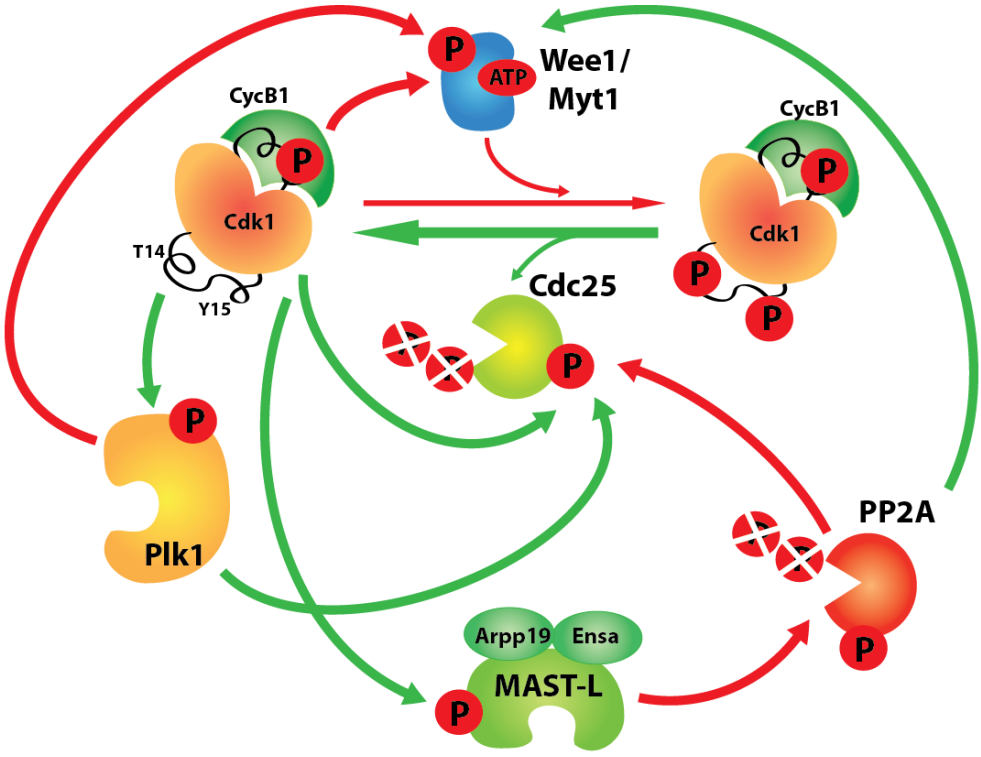

Figure 6. Representation of the feedback loops that modulate Cdk activity. Cdc25s lead to the activation of Cyc-Cdk complexes which promote their own activity by Plk1-dependent Wee1 degradation, Cdc25 further activation and inhibition of PP2A by the MASTL-Arpp19/Ensa pathway.

Spatial regulation

Cyclins, Cdks and the different elements of cell cycle control are restricted to distinct cellular compartments, therefore limiting their activities to a certain extent by the localisation of its components. The clearest example of regulation of activity from a spatial perspective comes from cyclins.

Cyclins E and A are mostly nuclear whereas Cyclin B is mostly cytoplasmic. However, all three cyclins have been shown to shuttle between the nucleus and cytoplasm [81, 82]. Interestingly, the

mechanisms by which they do so are distinct between cyclins. Cyclin E contains a nuclear localisation signal (NLS) in its N-terminus that is required for its transport into the nucleus via interaction with Importin-α/β [83, 84]. Similarly, Cyclin B1 has been found to contain a non-typical NES at its N-terminus named cytoplasmic retention signal (CRS) that favours its localisation in the cytoplasm by the interaction with Exportin1/Crm1 [82, 85, 86]. In this sense, both Cyclins E and B use the Importin/Exportin system to shuttle between nucleus and cytoplasm. In contrast, Cyclin A, which does not contain an NLS, depends on the binding to other proteins to mediate its transport. Maridor and colleagues demonstrated that the capacity of Cyclin A to bind its partner Cdk2 determined the ability to localise to the nucleus [87]. Additional studies reported that proteins like p21 or p107 could interact with Cyclin A to potentially modulate its localisation (discussed in [88]). Further, a screen to identify Cyclin A2 interacting partners revealed SCAPER, an ER-associated protein that directs Cyclin A2 localisation to the cytoplasm [89]. However the exact mechanisms that regulate the localisation of Cyclin A2 remain unclear.

In addition to cyclins, Cdc25s also shuttle between the nucleus and cytoplasm through an NES and an NLS present in their sequences thus providing tools for local activation of Cyclin-Cdk complexes [90, 91]. In addition, Cdc25B localisation is also regulated in response to DNA damage, when it becomes cytoplasmic to prevent nuclear Cyclin-Cdk activity, highlighting the importance of spatial regulation of protein activity [92]. Similarly Plk1 contributes to the spatial control of Cdk1 regulators. Plk1 can phosphorylate the inhibitory kinase Myt1 that resides in the cytoplasm thus promoting the local activation of Cyclin-Cdk complexes. Simultaneously it phosphorylates Cyclin B1 and Cdc25B promoting their transport into the nucleus prior to mitotic entry [93-97].

The dynamic localisation of different cell cycle regulators suggests that spatial control of cell cycle regulators is required to limit certain activities at specific locations in the cell. By these means, cells are able to modulate pro-mitotic inducing activities in a more refined manner [98, 99]. Cyclin B1, for example, can be activated at the centrosome where the restricted localisation might provide a concentration-dependent mechanism to activate Cyclin B1-Cdk1 complexes [97, 100]. On the other hand, Cyclin B1-Cdk1 promotes its own translocation into the nucleus prior to mitotic entry thus introducing a spatial feedback loop to the network that activates MPF [101]. Similarly, Plk1 has been shown to promote its localisation to kinetochores during mitosis to ensure correct chromosome segregation and successful completion of mitosis [102].

Altogether, the discovery of spatial feedback loops during G2 highlights the importance of control of Cdk activity at different locations in the cell to ensure successful progression through the cell cycle.

Cyclin A2 and B1 in particular

Both Cyclin A and Cyclin B are responsible for the progression through G2 phase and to promote mitosis. Despite being similar in structure and presenting significant overlap during the cell cycle their functions are largely different and represent the core components of cell cycle machinery [6, 103]. Both cyclins were identified at a similar time in yeast, sea urchin and different cell lines and several studies determined their mRNA and protein levels, showing that Cyclin A starts to accumulate at the beginning of S phase whereas Cyclin B first appears at the end of S phase. These early studies on cyclins also characterised the differences in their localisation and degradation showing that Cyclin A is predominantly nuclear and degraded earlier than Cyclin B which is mostly cytoplasmic [104-106].

There are two type-A cyclins in higher eukaryotes: Cyclin A1 and Cyclin A2. Cyclin A1 has a role during embryonic development and in the germ line, however its absence only leads to male infertility indicating that Cyclin A1 is not an essential cyclin. On the other hand, Cyclin A2, which is expressed in somatic cells, is essential for the cell cycle and its deficiency leads to early embryonic lethality [107]. A recent study revisited this concept and demonstrated that cells derived from conditional Cyclin $A2^{-/-}$ mice maintain Cyclin E expression throughout S phase to preserve relatively normal progression through the cell cycle. These observations suggest that both Cyclin E and A may have redundant functions in certain cell types [35].

Cyclin A2 is a rather unusual cyclin because it is expressed from S phase to mitosis and can form complexes with both Cdk1 and Cdk2. These unique features give Cyclin A2 the possibility to regulate processes in S and G2 phases and mitosis [108, 109].

During S phase, Cyclin A2 associates with actively replicating DNA and promotes replication by associating and phosphorylating DNA polymerases α and δ and crucial elements for DNA replication like Proliferating Cell Nuclear Antigen (PCNA) [110-113]. It has also been reported that Cyclin A2-Cdk1 complexes may have an important role in regulating the firing of late origins of replication [114]. Later in S phase, Cyclin A2 is responsible for providing directionality to S phase and preventing re-replication by promoting the cytoplasmic localisation of Cdc6 which is needed for initiation of DNA replication [115]. At the same time, Cyclin A2-Cdk activity has been shown to inhibit the APC/C regulatory subunit Cdh1 preventing cyclin degradation and resulting in E2F-dependent Cyclin B accumulation during the last stages of S phase [51]. Last, aside from having key nuclear functions during S phase, Cyclin A2-Cdk2 activity has also been reported to be required for centrosome duplication [116].

In G2 phase, Cyclin A2 has been shown to phosphorylate and activate the transcription factor FoxM1, which leads to the transcription of targets important for G2/M progression, such as Cyclin B1, Cdc25 or Plk1 [29, 47, 117]. Later at the G2/M transition, Cyclin A2-dependent activities coordinate centrosomal and nuclear events acting upstream of Cyclin B1-Cdk1 complexes in a rate-limiting manner [109, 118]. In this context, Cyclin A2-Cdk activity is needed to promote the activation of Cyclin B1-Cdk1 complexes possibly by phosphorylating and inactivating Wee1 and regulating Cdc25B and Cdc25C. Therefore, Cyclin A2 could constitute a trigger to tip the balance of Cdk versus Wee1 towards active Cdk1 [119, 120]. At mitotic entry, Cyclin A2-Cdk is needed to promote CycB1-Cdk1 nuclear translocation, chromosome condensation and nuclear envelope breakdown (NEBD) [121, 122]. Additionally, a recent report showed that Cyclin A2 is also important to promote kinetochore microtubule detachment to ensure correct chromosome segregation [123].

Unlike Cyclin A, Cyclin B expression is restricted to G2 phase; it has been extensively studied due to its implications in the correct execution of mitosis. There are three types of B cyclins in higher eukaryotes: Cyclin B1, B2 and B3. Cyclin B1 and B2 are expressed in somatic cells whereas Cyclin B3 is restricted to embryonic development and the germ line. Cyclin B1 is the main B-type cyclin and it is the one that performs most of the mitotic functions and similar to Cyclin A2, its deficiency leads to early embryonic lethality [124]. Despite shuttling between the nucleus and cytoplasm, Cyclin B1 is predominantly cytoplasmic while Cyclin B2 localisation is more limited to certain subcellular structures like centrioles, centrosomes or the Golgi apparatus [125-127]. Cyclin B1 first appears at the beginning of G2 phase and its levels rapidly increase reaching a maximum at mitosis when it is degraded at the metaphase to anaphase transition by APC/C^{Cdh1} (Paper I and [6, 29]). Cyclins B1 and B2 form complexes with Cdk1 at the beginning of G2 phase, which become rapidly activated by the action of several feedback loops as described above. Cyclin B-Cdk1 complexes accumulate in the cytoplasm during G2 and a small fraction localises in the centrosomes from the moment of centrosome duplication [128]. Accordingly, Cyclin B2 has been recently shown to be required for the initiation of centrosome separation [127]

The ultimate function of CycB1-Cdk1 complexes is to prepare the cell for mitotic entry and to ensure correct progression through mitosis. Cyclin B1-Cdk1 complexes are responsible for the separation of centrosomes to the opposite poles of the cell, to reorganise the microtubule network to form the mitotic spindle, and to activate a myriad of targets that will regulate specific processes in mitosis [100, 129, 130]. One of the main functions is to promote the full activation of APC/C and subsequently its own degradation as well as that of other targets important for chromosome segregation [131]. This step is crucial to reset the system to a basal level of mitotic activities that allow entry and progression through a new cycle.

The DNA damage response

Our genomes are constantly exposed to agents that can damage the DNA. In fact, each cell in the organism experiences an average of 10^5 DNA breaks per day [132, 133].

There are a large variety of agents that can damage DNA, both exogenous and endogenous. One of the most common exogenous physical genotoxic agents is ultraviolet radiation (UV), which usually comes from sunlight. UV radiation causes covalent cross-linking of pyrimidine bases usually leading to the appearance of thymidine dimers that interfere with DNA replication resulting in breaks in the DNA. Similarly, ionising radiation (IR) originating from cosmic rays or medical treatments like X-rays can induce single and double strand breaks [134]. Other examples of exogenous DNA damaging agents fall into the category of chemicals, like the many components of cigarette smoke [135]. In the class of chemical genotoxic agents, chemotherapy drugs cause DNA strand breaks by different means like oxidative damage, cross-linking bases within the same or different DNA strands, or by inhibiting different enzymes like topoisomerases. In this class we find agents such as camptothecin or etoposide, commonly used drugs both in therapy and research [136-138].

In addition to exogenous DNA damaging agents, DNA molecules are constantly exposed to endogenous sources of damage. Here, distinct mechanisms can be highlighted. On the one hand, DNA molecules are exposed to reactions in the cell that produce reactive oxygen species (ROS) or byproducts resulting from cell metabolism which can lead to hydrolysis of bases or DNA oxidation, and eventually lead to DNA damage [139, 140].

On the other hand, the processes of DNA replication and transcription can give rise to Double Strand Breaks (DBSs). Arrested replication forks, active transcription and collision between polymerases transcribing and replicating DNA are clear examples of situations that can cause DNA damage [141-143].

Regardless of the source of DNA damage, any insults to the DNA can cause the appearance and accumulation of mutations, which can eventually lead to the loss of information contained in the genome. Therefore, maintenance of genome integrity is crucial to ensure successful generation of daughter cells. Repair mechanisms and checkpoints have evolved in eukaryotic organisms to ensure that cells repair the lesions in the DNA before they progress into mitosis.

Cells activate different DNA repair mechanisms depending on the type of damage they encounter. The first consideration is the extent of the damage. Single strand breaks or damage that affects only one of the two DNA strands can be repaired by one of the following mechanisms:

- **Base Excision Repair (BER):** this is the mechanism of choice to repair nucleotides with small alterations in their structure or to introduce a missing nucleotide. Cells detect the wrong nucleotide, excise it and use the undamaged DNA strand to correctly introduce the missing nucleotide. BER repairs minor lesions, e.g. alkylations or deaminations of bases [144].
- **Nucleotide Excision Repair (NER):** this pathway detects bulky nucleotides like thymidine dimers caused by UV radiation. Upon detection, cells excise 12-30 nucleotides surrounding the region where the modified nucleotide is detected, and use the undamaged strand as template to repair the DNA [145].
- **Mismatch repair:** this mechanism identifies mismatched pairs of nucleotides, usually during DNA replication [146].

DSBs, on the other hand, represent the most deleterious type of damage and require different mechanisms of repair due to the lack of the undamaged strand to be used as a template. This type of lesions usually arises from physical and chemical genotoxic agents like IR or different drug treatments. Several pathways exist to repair DSBs:

- **Non-homologous end joining (c-NHEJ):** this repair mechanism simply joins the two ends of the broken DNA strand. This mechanism can lead to the loss of nucleotides, which makes NHEJ a mutation-prone repair pathway. However it provides a rapid response to DSBs thus protecting genome integrity [147].
- **Homologous recombination (HR):** perhaps the most complex, yet accurate repair pathway that cells possess. HR is based on the use of the sister chromatid or homologous chromosome to repair the DSB. In this case, cells resect the ends of the DNA strand surrounding the break and introduce the missing nucleotides using the sister chromatid or homologous chromosome as a template. Despite being a more accurate repair mechanism, the use of the homologous chromosome can lead to the loss of heterozygosity in a gene. This pathway is carried out during S and G2 phases due to its requirement for a DNA template to drive DNA repair [148].
- **Alternative non-homologous end joining (alt-EJ):** This pathway was first identified as a back up mechanism for c-NHEJ however growing evidence suggests that this pathway is an independent mechanism [149]. Alt-EJ depends on DSB resection like HR and relies on the detection of microhomology domains to repair the DNA lesion. Contrary to HR, alt-EJ is a mutagenic repair pathway and can lead to chromosome translocations and mutagenic rearrangements due to its preference to ligate DSBs in different chromosomes [150, 151].

The phase during the cell cycle when a cell experiences DNA damage largely affects the repair pathway used. In general, the rapid kinetics of c-NHEJ make it a pathway of choice throughout the whole cell cycle, although it dominates in G1 phase due to the lack of a DNA template to use for the repair. On the other hand, DSB resection-dependent repair pathways are more predominant during S and G2 phases as Cdk activity is needed for efficient DSB resection. Interestingly, it has been recently shown that despite being present during G2 phase, HR occurs mainly in S phase while NHEJ mediates repair throughout the cell cycle [152].

Minor lesions on the DNA can usually be repaired shortly after they occur. This form of repair, usually mediated by BER or NER, does not trigger the activation of the DNA damage checkpoint and is repaired quickly. However, when cells experience extensive damage that requires longer time to be repaired cells initiate and establish a DNA damage checkpoint. The aim of the checkpoint is to slow down cell cycle progression to prevent mitotic entry of cells with damaged DNA and to provide time to repair DNA damage.

Upon DNA damage cells possess a selection of PI3-kinase-like kinases that trigger the activation of a downstream cascade eventually leading to a cell cycle arrest. The MRN complex (composed by Mre11, Rad50 and NBS1) and the Ku complex (composed by Ku70 and Ku80) recruit and activate three different kinases: the kinase Ataxia Telangectasia mutated (ATM), the Ataxia Telangectasia and Rad3-related kinase (ATR) and DNA-PK [153, 154]. While ATM and DNA-PK respond rapidly to DSBs, ATR is recruited mostly to RPA-coated ssDNA, either coming from a minor lesion in itself or as a consequence of a DSB resection, placing ATR between the DDR and the control of DNA replication (Figure 7 and [155]).

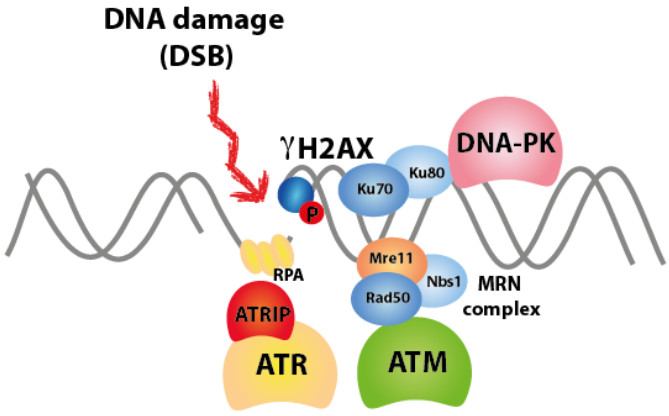

Figure 7. Schematic representation of the recruitment of ATM, ATR and DNA-PK, the three PI3-like kinases in the DDR

Once activated, ATM, ATR and DNA-PK phosphorylate histone H2AX at Ser139, which serves as a platform for the recruitment of repair factors to the site of damage. Therefore, appearance of γ-H2AX marks the establishment of the DNA damage response (DDR) [156, 157]. Other than histone H2AX, ATM and ATR phosphorylate the downstream targets Chk1 and Chk2, which in turn phosphorylate and target Cdc25A for degradation, and activate Wee1 leading to an increase on inhibitory phosphorylations on Cdk1 [158-160]. In parallel, ATM/ATR-dependent p38 activation leads to the phosphorylation and activation of MapKap2 (MK2), which targets Cdc25s for degradation contributing to the cell cycle arrest [161, 162].

These steps are crucial to induce a temporal and reversible arrest of cell cycle progression by negatively regulating the activators of Cdk activity. However, in addition to the rapid response elicited by ATM/ATR and Chk1/2, there are two long-term mechanisms to ensure the maintenance of the DNA damage checkpoint. First, the activation of the DDR kinases leads to the phosphorylation and stabilisation of p53 [163]. This process initiates the p53-dependent transcription program that eventually leads to the repression of several mitotic inducers like Plk1, Cyclin B1 and Cdc25B [164-166]. Second, p53 induces the expression of p21, which inhibits Cdk activity therefore strengthening the cell cycle arrest [167-170]. Last, in parallel to the nuclear checkpoint pathway, p38/MK2 localises to the cytoplasm where it mediates a late response with long-lasting effects. MK2 stabilises certain mRNAs and establishes a feedback loop to maintain p38 expression and subsequently the cytoplasmic component of the DNA damage checkpoint (Figure 8 and [171]).

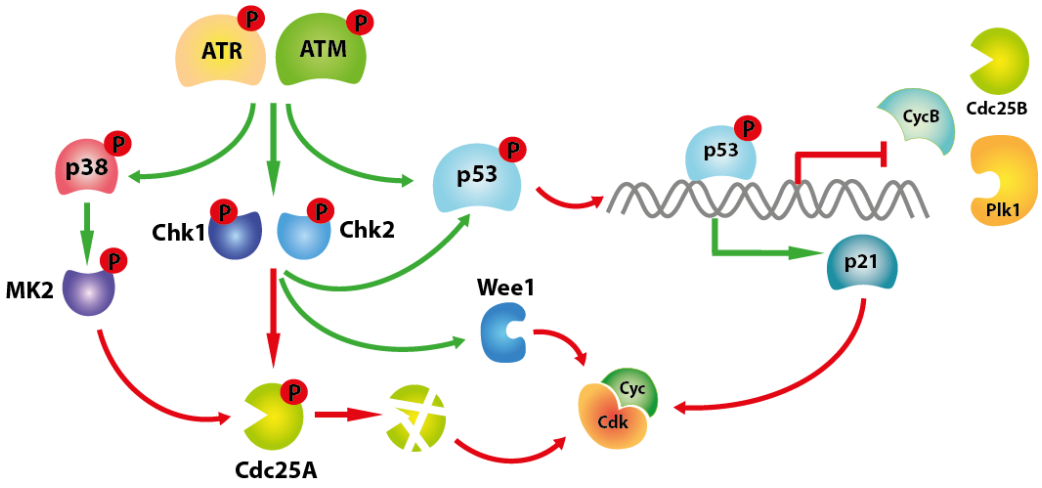

Figure 8. Schematic representation of the downstream targets of ATM and ATR to establish the DNA damage checkpoint. ATM and ATR activate Chk1/2 to downregulate Cdc25A and activate Wee1 to inhibit Cdk activity. In parallel the p53-dependent transcriptional program blocks transcription of cell cycle regulators and promotes expression of p21 that further inhibits Cdk activity.

Other than ultimately promoting a cell cycle arrest, ATM has also been shown to play a role in chromatin status upon DNA damage. In this context, ATM not only acts at a local level but it also induces a general relaxation of chromatin in a Kap1-dependent manner [172]. In line with these findings, our lab recently showed that the spread of ATM signal through the nucleus serves as a timer that restricts pro-mitotic activities during a DDR until DNA repair is completed [173].

Altogether, the combined action of ATM and ATR is essential to establish and maintain the DNA damage checkpoint that restricts mitosis-inducing activities and promotes repair factors.

The DNA damage response in the unperturbed cell cycle: a role in S phase

Despite having a major function to preserve genome integrity upon DNA damage, ATR and Chk1 also become activated during normal S phase progression. In this context, ATR associates with chromatin in a replication-dependent manner suggesting that ATR might be an element of the replication apparatus [174, 175]. Indeed, the role of the ATR-Chk1 pathway is well established as a way to control excessive origin firing during replication [176, 177]. Additionally, the checkpoint adaptor protein Claspin, needed for ATR-dependent Chk1 activation, has been shown to regulate the rate of fork progression during normal S phase [178, 179]. Furthermore, lack or inhibition of ATR or Chk1 leads to chromosome breaks and DNA damage caused by a premature activation of Cyclin A2-Cdk2 [114, 180-182]. Chk1 phosphorylates and inhibits Cdc25A during S phase to maintain Cdk2 activity below a certain threshold during DNA replication (Figure 9 and [183]).

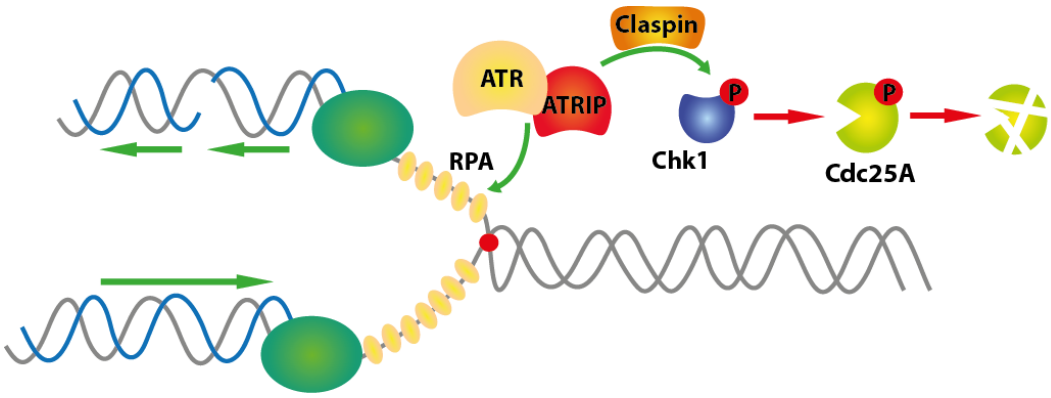

Figure 9. Activation of ATR/Chk1 during normal progression through S phase to control the levels of Cdc25A and subsequently Cyclin A2-Cdk2 activity.

At the late stages of S phase, Cyclin A2-Cdk has been shown to inhibit Chk1 and regulate claspin therefore relieving the inhibition on Cdk activity during DNA replication [184, 185]. However, a tight control of Cdk activity is essential, since unscheduled overexpression of Cdc25s in S phase leads to DNA damage due to an impairment in replication fork progression [186, 187]. This link between ATR/Chk1 and Cdc25A has been recently confirmed highlighting its importance for personalised cancer treatments [188].

Altogether these findings highlight the importance of the delicate balance between the ATR-Chk1 pathway and Cdk activity during S phase to ensure correct DNA replication and prevent accumulation of mutations.

Cell fate after DNA damage

DNA damage can potentially lead to genomic instability and propagation of mutations. Therefore, after detecting a lesion in the DNA a cell must assess the extent of the damage and respond to it, either to repair the DNA or initiate apoptosis [189]. Indeed, the DDR targets and promotes the activation of both repair mechanisms and apoptotic inducers depending on the extent of DNA damage [190, 191]. However, other than cell death, cells can face several different fates upon DNA damage.

Recovery from a DNA damage induced cell cycle arrest

The ultimate aim of the DNA damage checkpoint is to prevent progression of cells with damaged DNA into mitosis and to provide a time window for cells to repair the inflicted damage. The process of checkpoint recovery requires the silencing of the checkpoint proteins as well as the new synthesis and activation of pro-mitotic activities to resume cell cycle progression. The first step to recovery is to reverse the phosphorylations carried out by ATM and ATR. The main phosphatases that revert the action of the DDR kinases are Wip1 and PP2, which remove phosphorylations on H2AX, ATM, Chk2, Chk1, p53 and Mdm2 [170, 192, 193]. The next step towards cell cycle resumption requires the activation of Cdk and Plk1. As described above, the main target of the DNA damage checkpoint is Cdk activity, therefore the levels of Cdk activity during a DDR are strongly reduced but not completely abolished. In line, Alvarez-Fernandez and colleagues showed that the remaining Cdk activity during the DDR is enough to activate FoxM1 and therefore initiate the transcription of cyclins and pro-mitotic inducers [117, 194]. Together with Cdk activity, Plk1 is targeted during a DDR, and its activity needs to accumulate for cells to resume

cell cycle progression. Indeed, Plk1 has been found to be essential to enter into mitosis after checkpoint recovery. In this context, Plk1 phosphorylates and targets Wee1 and Claspin for degradation by $SCF^{\beta TrCP}$, allowing for Cdk activity to accumulate and eventually lead to mitotic entry [195-197].

Checkpoint adaptation

Under certain circumstances cells are able to override the DNA damage checkpoint and progress through mitosis with damaged DNA. This observation was first made in yeast where cells were able to continue to proliferate despite presenting unrepaired DNA. A mutation screen analysis revealed that Cdc5, the yeast homologue of Plk1, is responsible for checkpoint override. This scenario, named checkpoint adaptation, requires a cell cycle arrest upon DNA damage followed by cell cycle progression with unrepaired damage [198]. Importantly, this mechanism is conserved in evolution and Syljuåsen and colleagues demonstrated that human cells are able to override a DNA damage checkpoint in a Chk1 and Plk1-dependent manner [199]. Checkpoint override in human cells implies that cells can progress through mitosis with damaged DNA potentially giving rise to chromosomal breaks, which can lead to genome instability. These observations place the mechanisms regulating checkpoint adaptation in the spotlight as potential targets for cancer treatment.

Terminal cell cycle exit and senescence

As described above, upon DNA damage cells activate a checkpoint that temporally arrests the cell cycle, however when exposed to extensive damage the checkpoint-mediated cell cycle arrest can become irreversible. Cells in these conditions remain metabolically active but they do not proliferate; they become senescent. There are different routes to senescence depending on what induces the terminal cell cycle exit. First, telomere-dependent senescence is a clear example of how the normal progression through several rounds of division can lead to senescence. Cells rely on DNA polymerases to duplicate their DNA, however polymerases are not able to synthesise DNA ends, which translates into the loss of a few nucleotides at the ends of the DNA strands. Human chromosomes have telomeres at the their ends; consequently, cells lose telomeric DNA in every round of replication [200]. Dysfunctional telomeres can trigger a strong DDR leading to the activation of ATM and the DNA damage checkpoint that can eventually lead to cell cycle exit and senescence [201, 202]. Similarly, cells with extensive DNA damage or activated oncogenes can

undergo senescence in a p53-dependent manner [203]. In particular, oncogene-induced senescence has been suggested to be the consequence of an accumulation of DNA damage coming from replication stress or damage in mitosis. Three different reports provided evidence for these models; di Micco and Bartkova showed that replication stress causes DNA damage that triggers the activation of the DDR during S phase eventually leading to induction of senescence; furthermore, Dikovskaya *et al* showed that failed mitosis is key to promote senescence [204-206].

Senescence can therefore be regarded as a barrier to tumorigenesis as it prevents the proliferation of cells that carry potential deleterious mutations [207].

The mechanism by which cells exit the cell cycle has been widely studied and shown to occur both in G1 and G2 phases. Focusing on G2 phase, cells with unrepaired DNA damage in G2 have been shown to become senescent in a p53, p21 and APC/C^{Cdh1}-dependent manner. The activation of this mechanism leads to the degradation of mitotic inducers leading to the loss of G2 identity and checkpoint recovery competence [192, 208, 209]. Interestingly, growing evidence points towards a link between the DDR and the mitotic-inducing activities in order to regulate cell fate upon DNA damage (Papers II and III and [210, 211]). Despite seeming a paradox, this link is likely to be a key regulatory step to decide whether a cell resumes cell cycle progression after DNA damage or permanently exits the cell cycle.

2 AIMS OF THE THESIS

The main aim of my thesis is to understand the molecular mechanisms underlying the regulation of cell cycle progression. In particular, I am interested in understanding how cyclins regulate Cdk activity and allow timely progression through the cell cycle or induce cell cycle arrest and exit when it is needed. In detail, I aim:

- To assess the dynamics of cyclins at the endogenous level and in the different phases of the cell cycle.
- To understand the regulation of Cyclin-Cdk activity after DNA damage and its role in inducing terminal cell cycle exit.
- To shed light on the regulation of Cyclin A2 localisation in the human cell cycle.

3 CHAPTER 1: HOW DO CELLS BUILD UP CDK ACTIVITY TO ENTER INTO MITOSIS?

Cells undergo an extreme reorganisation of the intracellular structures to enter into mitosis. Part of this process depends on the phosphorylation of many proteins in the cell. Correct and timely phosphorylation of many different targets ensures that all the players are in place and ready to act on their substrates at a specific time and location in the cell. However, despite the extensive efforts to resolve the question of Cdk activation during G2, the exact details of this process remain elusive. Therefore, we decided to study the dynamics of Cdk activity in detail to shed light on the time of activation of the mitotic entry network throughout the cell cycle.

When is the mitotic entry network activated?

Cyclin B1-Cdk1 is the core of MPF, in other words, the main driver of mitotic entry. A lot of effort has therefore been dedicated to understand the exact mechanisms that lead to the activation of Cyclin B1-Cdk1 complexes. Gavet and Pines showed that Cyclin B1-Cdk1 becomes activated a few minutes before nuclear envelope breakdown despite being present in the cytoplasm throughout G2 phase [98, 99]. However other reports showed evidence for the presence of active Cyclin B1-Cdk1 complexes earlier than a few minutes before mitosis [100]. Similarly, Plk1 activity has been detected in the nucleus a few hours before mitotic entry suggesting that the activation of the mitotic entry network might be earlier than previously thought [197].

In **paper I** we aimed to clarify this question using a new method based on automated quantification of fluorescence in single fixed cells. Our method uses fibronectin-coated micropatterns in order to homogenise cell shape and size and to be able to have a large number of individual cells with similar cell-to-matrix contacts and reduced extracellular cues that might affect cell cycle progression. We could show that despite being individualised, cells proliferate normally on micropatterns suggesting that the mechanisms of control of cell cycle progression are unaffected and cell cycle dynamics are maintained.

In order to set up an automated microscopy protocol for identification of single cells we used Cy5-labelled fibronectin micropatterns. The recognition of the patterns by the microscope allows for automated identification of the position of each pattern and ensures similar illumination for every cell therefore reducing variability during imaging. By these means we can study several thousands of individual cells with similar shape and size. Furthermore, using DAPI as a nuclear stain and the signal of Cy5-labelled fibronectin we can easily segment images to identify

subcellular locations like the nucleus and cytoplasm. Additionally, the use of specific antibodies allows for more refined image segmentation and identification of smaller cellular structures like centrosomes or kinetochores. Last, the accurate quantification of background fluorescence in the images provides the means to correct the real signal coming from every specific antibody staining.

Using our method we were able to characterise the dynamics of cyclins and Plk1 in fixed cells. Our results showed the expected pattern for the accumulation of cyclins (For details see paper I) thus confirming that our method is a powerful tool to study the dynamics of other proteins. Therefore, we focused our study on the quantification of post-translational modifications on targets of Cdk1 complexes. We found that contrary to what was previously observed, phosphorylation on known Cdk targets starts appearing at the S/G2 transition although at very low levels. These phosphorylations continue to increase throughout G2 phase and until mitosis when we observed a very large increase, corresponding to the time in the cell cycle when a boost in Cdk activity has been reported to occur [67, 98-100]. Even though the targets we monitored most likely reflect Cyclin A2-Cdk1 activity, we provide evidence that demonstrates that a fraction of the phosphorylations are performed by Cyclin B1-Cdk1. In parallel to an activation of Cdk1, we could show that active Plk1 began to accumulate in the nucleus also at the S/G2 transition.

Our study shows that the activation of the mitotic entry network occurs at the completion of S phase rather than only shortly before mitotic entry. These observations point towards a connection between S phase and mitosis implying that cells have a mechanism to sense the end of DNA replication that triggers the initiation of Cdk and Plk1 activities to start phosphorylating mitotic targets. Consequently, this translates into a slower yet perhaps more gradual activation of mitotic kinases rather than an abrupt activation prior to mitotic entry.

Cytoplasmic localisation of Cyclin A2: a layer of Cdk activity regulation?

The use of genome-targeting approaches represents a major advantage in contrast to the expression of fusion proteins. Despite being significantly weaker in fluorescence intensity, endogenously tagged proteins are controlled by the endogenous promoter and signalling thus allowing for the visualisation of endogenous processes in the cell as they occur. Moreover, expression from stably-expressed plasmids is complex since they rely on exogenous promoters that can lead to the overexpression of the tagged protein as well as other artefacts like mislocalisation or differences in regulation [212]. In the case of cyclins this can be problematic since cyclins are known oncogenes and their overexpression may lead to dysregulation of cell cycle

progression and tumorigenesis [213-215]. Endogenous tagging is therefore a good strategy to circumvent potential problems rising from variability in protein expression and regulation. However, careful analysis of the functionality of the tagged endogenous protein is required as both C- and N- terminal tagging can lead to dysfunctional proteins.

Therefore, in order to study cyclin dynamics in live cells we established different cell lines that contain an EYFP tag in the endogenous locus of *CCNA2* and *CCNB1*. Using recombinant adeno-associated virus-mediated homologous recombination we obtained U2OS and RPE cells that express endogenous Cyclin A2-eYFP or Cyclin B1-eYFP (Papers I and IV and [216]).

Characterising the dynamics of Cyclin A2 during the development of **paper I** we observed the accumulation of Cyclin A2-eYFP in the cytoplasm prior to mitotic entry. Despite shuttling between the nucleus and cytoplasm, Cyclin A2 is predominantly nuclear [81, 106]; therefore our observation raised the question of a potential role of Cyclin A2 in the cytoplasm. In **paper IV** we addressed the dynamics of cytoplasmic Cyclin A2 accumulation using cells expressing endogenous Cyclin A2-eYFP as well as untagged cells. We could show that Cyclin A2 first appears in the cytoplasm at the S/G2 transition and continues to accumulate throughout G2 phase in unperturbed cells, indicating that cytoplasmic localisation of Cyclin A2 is a cell cycle-dependent event.

Given that the S/G2 transition corresponds with a time in the cell cycle when changes in kinase activities occur, we reasoned that activities present in either S or G2 phase could regulate the localisation of Cyclin A2 to the cytoplasm. We identified Cdk1 as a regulator of Cyclin A2 localisation. Interestingly, modulation of Cdk1 activity did not lead to major changes in the localisation of Cyclin A2. Rather, its absence after RNAi showed how a subset of cells did not accumulate cytoplasmic Cyclin A2 despite being in G2 phase.

Given that Cdk1 presence does not completely explain how cells modulate Cyclin A2 localisation we decided to investigate if the DDR normally activated during S phase regulates Cyclin A2 cytoplasmic accumulation. We could not identify a specific kinase activity responsible of Cyclin A2 localisation; however inflicting DNA damage induced a rapid loss of cytoplasmic Cyclin A2. Further investigation revealed that p21 or p53 knockdown prevented cytoplasmic loss of Cyclin A2 upon DNA damage suggesting that they could mediate the change in localisation. Interestingly, we found p21, but not p53, to have a role in modulating Cyclin A2 localisation in the unperturbed cell cycle shown by the presence of cytoplasmic Cyclin A2 in S phase in $p21^{-/-}$ cells.

This observation points towards a role for p21 during normal cell cycle progression, and importantly, it implies a decoupling between p53 and p21 suggesting that in the absence of DNA damage cells can induce p21 expression independently of p53 to regulate different events in the cell cycle. Interestingly, p53-independent p21 expression has been shown to be involved in cell growth and differentiation [217].

Despite not having unveiled the role of cytoplasmic Cyclin A2, cell cycle-dependent regulation of cyclin localisation opens a door to a potential additional layer of regulation of Cdk activity, posing a spatial limitation to the activity of Cyclin A2-containing complexes.

4 CHAPTER 2: HOW DO CELLS REGULATE CDK ACTIVITY UPON DNA DAMAGE?

Maintenance of genome integrity is essential to ensure the generation of identical daughter cells with intact chromosomes and to prevent accumulation of mutations. Therefore, upon DNA damage cells activate the DNA damage response and a checkpoint that temporally halts cell cycle progression and provides cells with time to repair DNA lesions. Consequently, one of the roles of the DNA damage response is to counteract Cdk activity. As described above, this is achieved by different means: first, with a fast response inducing the degradation of Cdc25s and second, by stabilisation and activation of p53, that leads to the transcriptional induction of p21 expression. However, low levels of Cdk activity are preserved in cells during an ongoing DNA damage response. It has been shown that in transformed cells, Cdk activity is able to maintain the FoxM-dependent transcription of pro-mitotic proteins like cyclins and Plk1, and effectively to preserve the competence to resume cell cycle progression once DNA damage is repaired [194]. However, not all cells remain competent to resume cell cycle progression. If the damage is too high and cells are not able to repair it, they may trigger apoptosis and die; alternatively they might exit the cell cycle and become senescent. However, how a cell is forced to exit the cell cycle is not fully understood.

Cyclin B1 dynamics and cell fate decisions

Degradation of mitotic inducers is one of the hallmarks of terminal cell cycle exit from G2 phase. In particular, Cyclins A and B form complexes with Cdk1 during G2 to regulate cell cycle progression, therefore studying cyclin dynamics upon DNA damage can shed light on the mechanisms that drive terminal cell cycle exit.

In **paper II** we used endogenously tagged cell lines that express Cyclin B1-eYFP to be able to follow cyclin dynamics during an ongoing DNA damage response in G2 phase. In this context, the levels of Cyclin B1 serve both as a marker for cell cycle exit as well as a cell cycle indicator, with increasing levels of Cyclin B1 indicating progression through G2 phase.

Analysis of transformed cells showed that upon damage Cyclin B1 continues to accumulate exceeding the levels reached during a normal progression through G2 phase and into mitosis. In contrast, we found that untransformed cells translocate Cyclin B1 into the nucleus and initiate its degradation in a p21, p53 and APC/C^{Cdh1}-dependent manner only a few hours after inflicting DNA damage. A long-term study of cells that had lost Cyclin B1 upon DNA damage revealed

that despite being alive these cells had lost the competence to recover from a DNA damage checkpoint-induced cell cycle arrest.

Interestingly, a close look at single untransformed cells revealed different Cyclin B1 dynamics depending on the initial Cyclin B1 level before DNA damage. Cells with low Cyclin B1 continued to accumulate Cyclin B1 up to a certain threshold before initiating translocation and subsequent degradation. In contrast, cells with high Cyclin B1, indicative of later stages of G2 phase, translocated and triggered Cyclin B1 degradation almost immediately after DNA damage. This observation indicates that the moment of the cell cycle when a cell receives DNA damage determines when it initiates terminal cell cycle exit.

Our investigation shows that nuclear translocation of Cyclin B1 and its subsequent degradation marks a decision point in the cell cycle. Interestingly, Cyclin B1 degradation seems to be a cell cycle-dependent event pointing towards Cdk activity as a regulator of terminal cell cycle exit. In this context, cells affected by DNA damage that are unable to repair it initiate a program dependent on p21, p53 and APC/C activities to degrade mitotic inducers like Cyclins A and B and Plk1 that leads to a loss of G2 identity and a terminal cell cycle exit from G2 phase.

Keeping Cdk activity to maintain recovery competence or trigger cell cycle exit

In **paper II** we identified a decision point in the cell cycle when untransformed cells degrade mitotic inducers and permanently exit the cell cycle from G2 phase. Interestingly the fact that induction of senescence upon DNA damage happened when cells reached G2 levels of certain mitotic inducers raised the question whether cell cycle exit correlates with cell cycle regulation. It has been shown previously that the DNA damage checkpoint targets Cdk activity to prevent entry into mitosis with unrepaired damage. However in **paper III** we showed that upon DNA damage cells maintain a certain level of Cdk activity that is needed to induce terminal cell cycle exit. Our study suggests that the DNA damage checkpoint and Cdk activity are interlinked. In line with our findings, it has been previously shown that Cdk activity is needed to maintain checkpoint recovery competence and to mediate repair [117, 218, 219]. Interestingly, we could show that Cdk is involved in a feedback loop with p21 inducing its expression independently of p53. This step contributes to Cyclin B1 nuclear translocation and subsequent degradation therefore initiating the steps to induce senescence.

In summary, Cdk activity has a decision-making role both in unperturbed progression through the cell cycle to induce mitotic entry and upon DNA damage to induce entry into senescence when damage cannot be repaired.

5 CONCLUSIONS

The general aim of this thesis was to explore cyclin dynamics and the regulation of Cyclin-Cdk activity both in the unperturbed cell cycle and upon DNA damage.

In order to study cyclin dynamics we established a quantitative immunofluorescence method to analyse protein levels and post-translational modifications in single cells (**Paper I**). We showed the levels of cyclins E, A and B in an unperturbed cell cycle. In addition we found that the levels of Cdk target phosphorylation started to increase at the transition between S and G2 phases. Using our newly established method we were able to demonstrate that mitosis-inducing activities start earlier than what was previously shown. Therefore, our study can serve as a starting point for a revision of the current model of Cdk activation to explain cell cycle progression.

In parallel, using genome-targeting approaches we established different cell lines that express the ORF for EYFP in the *CCNA2* and *CCNB1* loci. Using these cell lines we were able to confirm and validate the kinetics of cyclin accumulation during the unperturbed cell cycle (**Paper I**). Furthermore, the comprehensive analysis of single cells expressing Cyclin A2-eYFP revealed the cell cycle-dependent localisation of Cyclin A2 to the cytoplasm (**Paper IV**). In particular we found that Cyclin A2 changes its localisation from being only nuclear to both nuclear and cytoplasm at the S/G2 transition and throughout G2 phase. Despite having identified an increase in Cdk and Plk1 activities at the S/G2 transition (**Paper I**), these do not seem to mediate the change of Cyclin A2 localisation. Rather, our findings suggest that a change in binding partner might mediate Cyclin A2 localisation to the cytoplasm. Surprisingly, modulating certain elements of the DNA damage response had the most evident effect on Cyclin A2 localisation. In particular, the absence of p21 led to a marked increase in the number of cells expressing cytoplasmic Cyclin A2 in S phase in contrast to WT cells which express cytoplasmic Cyclin A2 exclusively during G2 phase. Together, our results suggest that both p21 and the presence of Cdk1 might modulate the localisation of Cyclin A2 during G2 phase. We propose that the change in localisation of Cyclin A2 may serve as a regulation layer for Cdk activity in the cytoplasm during G2 phase (**Paper IV**).

Using endogenously tagged cell lines we were also able to study the behaviour of cyclins upon DNA damage (**Papers II and III**). In particular we focused our study in the dynamics of Cyclin B1 during the DNA damage response to understand how cells take cell-fate decisions. Using live-cell imaging on single cells expressing Cyclin B1-eYFP we found that a few hours after DNA damage cells translocate Cyclin B1 to the nucleus in a p53 and p21-dependent manner. Further,

the nuclear translocation of Cyclin B1 led to its degradation by APC/C^{Cdh1} leading to the loss of G2 identity and irreversible cell cycle exit leading cells to senescence (**Paper II**).

Intrigued by the dynamics of Cyclin B1 upon DNA damage as an indicator of cell fate, we decided to investigate the mechanism by which cells withdraw from the cell cycle. In **paper III** we explored the function of Cdk activity during an ongoing DNA damage response and found that Cdk activity is needed to trigger senescence. In line, we revealed a link between p21 and Cdk that seems not to depend on p53. Therefore we propose a model where Cdk activity is maintained to either induce repair and checkpoint recovery or to trigger terminal cell cycle exit.

In conclusion, this thesis addresses the mechanisms of control of cell cycle progression from different angles, showing that Cdk activity works as a regulator both for unperturbed cell growth and cell fate upon DNA damage. In both situations, cells face an irreversible event, that is mitosis or senescence, and Cdk, by modulating the levels and activities of mitotic inducers either in a positive or negative way, can determine the outcome of the cell cycle.

6 FUTURE PERSPECTIVES

Despite being a relatively young field, the knowledge of the cell cycle has expanded extensively since the discovery of cyclins over thirty years ago. However every question answered raises many new ones. How cells regulate Cyclin-Cdk complexes is still one of these many unanswered questions or rather, incompletely answered ones.

In particular I have become very interested in the role of Cyclin A2 in the unperturbed cell cycle. Firstly, Cyclin A2 is the only cyclin known to form active complexes with two different Cdks in unperturbed conditions. Secondly, it localises both to the nucleus and cytoplasm, thus having the potential to regulate processes in both subcellular localisations. And last, deregulated Cyclin A2 is commonly found in several cancers thus making it a potentially interesting target from a clinical perspective.

One of my findings in this thesis is the cell cycle-dependent localisation of Cyclin A2 to the cytoplasm. Therefore my main question is: what is the role of cytoplasmic Cyclin A2? One potential explanation comes from the spatial regulation of the cell cycle. In this context, the cytoplasmic localisation of Cyclin A2 during G2 phase could ensure that phosphorylation of Cyclin A2 targets in the cytoplasm only occurs during G2 phase. A potential cytoplasmic target of Cyclin A2-containing complexes could be the Cyclin B1-Cdk1 complexes residing in the cytoplasm, therefore placing Cyclin A2 in the position to trigger the activation of Cyclin B1-Cdk1 in the cytoplasm.

On the other hand, could cytoplasmic Cyclin A2 have a cell cycle-independent function? A report recently related Cyclin A2 with RhoA and elements of the cytoskeleton thus suggesting a role for Cyclin A2 in modulating cell motility and migration [220].

It will, therefore, be very interesting to continue the study on Cyclin A2 to shed light both on Cdk-dependent and independent functions to understand the full spectrum of processes controlled by Cyclin A2.

7 POPULAR SCIENCE SUMMARY

All organisms have something in common: they are made of cells. To be precise, they all come from one initial cell that gives rise to a large number of cells. The obvious question that rises from this statement is therefore: how does one single cell manage to produce the millions of cells that make up a whole organism? The simple answer would be “by making copies of themselves”. Indeed, cells are able to divide and generate a copy of themselves by going through a process called cell division cycle, or simply cell cycle.

The cell cycle can be divided in four different phases depending on what happens in the cell. The first phase called G1 is the time when cells grow and gather signals from their environment to know if there are enough nutrients for the cell and if the conditions are positive for the cell to make a copy of itself. If a cell receives the appropriate signals, it will start many different processes in order to divide. One of the main processes takes place in the next phase of the cell cycle. This phase, called Synthesis phase or simply S phase is the time when cells make a copy of all the chromosomes present in the nucleus. This will, in practice, create a copy of the instruction book that a cell needs to make copies of itself and work properly in its environment and in accordance to its cell type. Once cells finish copying their chromosomes they are ready to proceed to the next phase. This phase is called G2 and it is a phase for the preparation of the big finale: cell division. During G2 phase, cells make copies of other elements and structures in the cell in order to have enough for the copied cell to contain the same elements as the initial cell. Also, given that cell division is a very complex process, cells need to change their shape and prepare a series of players to make sure that every step needed to make a new cell is in place at a certain given time.

Last, after the 5-6 hours that G2 phase lasts, cells are finally ready to start the making of a new cell. This is the beginning of the last phase of the cell cycle: cell division or mitosis. Once cells enter into mitosis, they experience a change in shape and become spherical. At two opposite sites of the sphere, cells place an organising centre from which a vast number of threads extend towards the centre of the sphere. The main aim of these threads, called microtubuli, is to catch the copied chromosomes from both sides of the cell. Once this is achieved, microtubuli can pull each copy of each chromosome to one side of the cell, which in the end gathers two sets containing all the chromosomes of a cell, one at each side of the cell. A few minutes later the centre of the sphere starts to get squeezed to make two spheres out of this one. This is the final cut that leads to the birth of a new cell that contains the exact same contents as the initial cell.

The cell cycle could be metaphorically compared to a concert. Like at the performance of a symphony, cells count with an orchestra, that is, a series of players that carry out a specific function at

a given time. However, regardless of how skilled each and every player is, they need the leadership of a conductor to know when they need to perform and most important, how they need to do so. In the same way, cells have a group of conductors and assistants, they are called Cyclin-Cdk complexes, and each phase of the cell cycle is led by a specific Cyclin-Cdk complex. Cyclin-Cdk complexes are composed by two players, a Cdk, which is the responsible of executing protein modifications, and a cyclin, which is the partner of Cdk and controls that it does its job at a given time and at a specific place. In addition, cells count with different Cyclin-Cdk complexes to control each phase of the cell cycle. In other words, each movement of the symphony requires a conductor and a specific assistant to make sure the music flows in a beautiful way.

In this thesis we aimed to understand how cells control the activity of Cyclin-Cdk complexes when they are progressing through a normal and healthy cell cycle. Additionally we decided to investigate the role of Cyclin-Cdk complexes when a cell receives DNA damage.

As mentioned above, different Cyclin-Cdk complexes control the series of events occurring during the cell cycle. In particular we were interested in complexes containing Cdk1 that can be in complex with Cyclin A or Cyclin B. These are the Cyclin-Cdk complexes that ensure that cells create a correct copy of the cell, that is, they modulate mitosis. We discovered that cells activate Cdk when they finish making a copy of their chromosomes, meaning that the conductor that directs entry into mitosis becomes active a few hours before mitosis rather than just a few minutes before mitosis. Our discovery revealed that the activation of Cdk1 complexes happens progressively and during a few hours. This is in opposition to previous observations that suggested that Cdk1 complexes are activated just a few minutes before a cell enters into mitosis.

In addition we discovered that the end of DNA replication marks a point in the cell cycle when cells change the localisation of a specific Cyclin. We found that Cyclin A is in the cytoplasm instead of being in the nucleus of all cells before they divide. Bringing back our orchestra example, our findings can be summarised as follows: in the first place, the conductor that makes sure that a cell makes its own copy correctly arrives at the music hall a few hours before the music needs to be played at its maximum volume. Second, the conductor (Cdk) and assistant (Cyclins A or B) start playing their part in the symphony communicating with the musicians so that they start playing their part, but only softly. Last, we discovered that one of the assistants (Cyclin A) that usually sits in the main offices of the music hall (the nucleus) starts to move to other places. Although we still do not understand why Cyclin A changes localisation, we think that it could help control the activity of Cdks in different locations in the cell.

The mechanisms described above portray a healthy cell; however, each and every cell in our organism is exposed to many different types of damage that can harm the DNA and lead to the appearance of mutations. To deal with these situations, cells have different mechanisms to respond and react to DNA

damage. In general when cells are damaged they slow down the processes that drive cells to divide. That is, Cdk activity decreases to avoid that a cell that has problems in its DNA divides. At the same time, cells start to activate repair mechanisms to fix the breaks in the DNA. Once cells have repaired all the damage they are ready to continue preparing for cell division. However sometimes the damage is too extensive and cells are not able to repair it. In these cases, cells exit the cell cycle, that is, they stop proliferating. This mechanism called senescence is a process by which organisms avoid propagation of cells with problems, and, in other words, put a barrier to tumour development.

To understand how cells decide to exit the cell cycle we studied how Cdk activity changes and influences the fate of a cell that experiences damage in its DNA. We found that when cells are damaged they transport Cyclin B (one of Cdk's assistants) into the nucleus where it is destroyed. Interestingly we discovered that Cdk1 is needed to initiate this process. In other words, after DNA damage, even though Cdk activity is decreased, it is enough to activate p21 (a protein that could be regarded as a security guard of the cell) that assists Cyclin B to enter into the nucleus. Once Cyclin B is destroyed cells start to lose their identity, that is, they forget what phase of the cell cycle they were in and they stop proliferating.

Altogether, in this thesis we have explored the mechanisms present in human cells that make sure that cell division happens correctly. One of the hallmarks of cancer is uncontrolled cell division; therefore, understanding how cells regulate Cdk activity will give us the knowledge to understand better what goes wrong in cells that become cancerous.

8 RESUM DE LA TESI

Tots els organismes tenen una característica en comú: estan fets de cèl·lules. En concret, tots vénen d'una cèl·lula que dóna lloc als milions de cèl·lules de les que està fet qualsevol organisme. La pregunta és, per tant, com pot una sola cèl·lula crear la gran quantitat de cèl·lules que necessita cada organisme? La resposta més senzilla és efectivament la realitat: cada cèl·lula és capaç de fer una còpia de si mateixa a través d'un procés anomenat cicle cel·lular.

El cicle cel·lular es pot dividir en quatre fases segons el què succeeix a la cèl·lula a cada fase. En primer lloc la fase G1 és la fase durant la qual les cèl·lules creixen responent a estímuls de l'entorn, per tant podem considerar G1 com una fase de creixement. A més, si una cèl·lula rep les senyals i nutrients necessaris, inicia una sèrie de processos essencials que conduiran a la divisió cel·lular. Un d'aquests processos té lloc a la següent fase del cicle: la fase S. La fase S o fase de síntesi és el moment del cicle cel·lular en què les cèl·lules fan una còpia de l'ADN present al nucli. Aquest procés donarà lloc a la creació d'una còpia de cadascun dels cromosomes al nucli, o en altres paraules, a una còpia del manual d'instruccions amb la informació necessària per tal que la cèl·lula filla fruit de la divisió cel·lular pugui mantenir tots els processos i funcions de la cèl·lula fins que arribi el moment de la següent divisió. Un cop acabada la fase S, comença la fase G2 que és una fase de preparació per mitosi. En primer lloc, les cèl·lules segueixen creixent, però a més fan còpies d'altres elements presents a la cèl·lula per tal de tenir el contingut suficient per la cèl·lula filla. A part de fer còpies del contingut cel·lular, les cèl·lules han de modificar la seva estructura per tal de poder dividir-se. Així, passades unes 5 hores de preparació arriba el moment de fer una còpia de la cèl·lula, o el que és el mateix, dur a terme la mitosi. En primer lloc les cèl·lules canvien de forma i passen de ser relativament planes a ser una esfera. A cada extrem d'aquesta esfera hi ha dues estructures des d'on s'estenen els microtúbuls, la funció dels quals és atrapar cadascun dels cromosomes de la cèl·lula per tal que cada cèl·lula tingui una còpia de cada cromosoma al final de la mitosi. Un cop tots els cromosomes estan enganxats als microtúbuls, aquests comencen a estirar des de cadascun dels pols de la cèl·lula donant lloc a una acumulació de cromosomes a cada extrem de la cèl·lula. En aquest moment un anell comença a constrènyer el centre de la cèl·lula donant lloc a dues esferes al centre de les quals hi ha el nou nucli amb una còpia de cada cromosoma repartit durant mitosi. Finalment, les cèl·lules creen noves membranes per definir els nous nuclis i citoplasmes i la mitosi acaba donant lloc a dues cèl·lules idèntiques que poden tornar a començar el pas pel cicle cel·lular.

Per tal d'entendre els mecanismes de control del cicle cel·lular podem imaginar tot el procés com un concert, on cada fase del cicle cel·lular es pot comparar a un moviment d'una simfonia. De la mateixa manera que a un concert hi ha una orquestra de músics, a la cèl·lula existeix un conjunt de proteïnes

que controlen que cada procés passi en un lloc determinat i a un moment determinat. Però encara que els músics siguin excepcionals, per tal que la simfonia soni bé l'orquestra necessita un director que indiqui quan i com ha de tocar cada músic. Donada la complexitat del cicle cel·lular, les cèl·lules humanes tenen molts "directors d'orquestra" diferents. Un dels més importants és un complex de dues proteïnes anomenat Ciclina-Cdk. Els complexos Ciclina-Cdk estan formats per una quinasa (una proteïna capaç de modificar altres proteïnes i d'aquesta manera, la seva funció) i una unitat reguladora, la ciclina. En l'exemple de l'orquestra es podria veure la quinasa Cdk com el director d'orquestra i les ciclines com assistents del director. Així doncs, la ciclina, dirigeix l'activitat de la quinasa, Cdk. És a dir, l'assistent és l'encarregat de portar al director de l'orquestra al lloc indicat al moment necessari per tal de dirigir al grup de músics que s'encarrega de cada moviment de la simfonia.

Aquesta tesi pretén entendre amb més detall l'activitat dels complexos Ciclina-Cdk. En concret, el nostre objectiu és entendre els mecanismes a través dels quals les cèl·lules regulen l'activitat de Cdk1, la Cdk encarregada de preparar la cèl·lula per la divisió cel·lular conjuntament amb les unitats reguladores Ciclina A i ciclina B.

Estudiant els nivells i l'activitat de diferents proteïnes importants pel cicle cel·lular hem descobert que els complexos Ciclina-Cdk s'activen més aviat del que s'havia establert anteriorment. Hem descobert que els complexos Ciclina-Cdk comencen a activar-se progressivament un cop acabada la fase S enlloc d'activar-se ràpidament minuts abans de la mitosi. Tornant a l'exemple de l'orquestra, hem descobert que el director encarregat de l'últim moviment de la simfonia arriba a l'auditori hores abans que hagi de dirigir el seu moviment. A més hem descobert que el director comença a dirigir als músics per tal que comencin a tocar a un volum molt baix que va incrementant a mesura que avança la simfonia.

A part d'estar interessats en l'activitat de Cdk1 també hem investigat com la Ciclina A es regula durant el cicle cel·lular, i hem descobert que un cop acaba la fase de síntesi, la Ciclina A passa d'estar present només al nucli a trobar-se al nucli i al citoplasma. Malgrat no haver descobert la funció específica de la Ciclina A al citoplasma pensem que aquest canvi de posició podria suposar un canvi en l'activitat de Cdk1 al citoplasma, ja que les ciclines regulen l'activitat de les Cdks.

Tots els mecanismes descrits als paràgrafs anteriors corresponen als mecanismes de control actius en cèl·lules sanes sense cap problema, però cal tenir present que totes les cèl·lules de l'organisme poden patir lesions a l'ADN que en el pitjor dels casos donen lloc a mutacions. Per tal d'evitar aquestes situacions les cèl·lules tenen una sèrie de mecanismes de control que detecten i reparen les lesions a l'ADN. La funció principal dels mecanismes de control és bloquejar la progressió a través del cicle cel·lular mitjançant una reducció de l'activitat de les Cdks, per tal d'evitar que cèl·lules amb mutacions facin còpies d'elles mateixes. Alhora, un cop detectada la lesió, les cèl·lules inicien

diversos processos de reparació de l'ADN. Si les cèl·lules aconsegueixen reparar les lesions poden seguir el seu camí cap a la divisió cel·lular. A vegades, però, l'ADN té massa lesions i les cèl·lules no el poden reparar. Per tal d'evitar que aquestes cèl·lules es divideixin, els organismes han desenvolupat un mecanisme anomenat senescència que impedeix la proliferació de cèl·lules amb lesions a l'ADN. La senescència cel·lular és per tant un dels mecanismes dels organismes per tal d'evitar que una cèl·lula sana es converteixi potencialment en una cèl·lula tumoral.

Per tal d'entendre millor el mecanisme pel qual les cèl·lules decideixen deixar de proliferar hem investigat com l'activitat de les quinases Cdk afecta el destí de les cèl·lules després d'una lesió a l'ADN. Hem descobert que en resposta a una lesió, les cèl·lules transporten la ciclina B des del citoplasma al nucli on es recicla. També hem descobert que l'activitat de la Cdk és necessària per tal que el transport de la ciclina B tingui lloc. És a dir que tot i que l'activitat de Cdk es redueix en resposta a una lesió a l'ADN, encara és suficient per controlar el procés de destrucció de la ciclina B. Un cop s'ha reciclat tota la ciclina B, la cèl·lula perd la seva identitat, és a dir que no recorda en quin moment del cicle cel·lular es trobava abans de patir el dany genètic. Això fa que les cèl·lules amb dany que no s'ha pogut reparar deixin de proliferar i que no puguin contribuir a desenvolupar tumors.

En resum, durant el desenvolupament d'aquesta tesi hem investigat els mecanismes presents a les cèl·lules humanes que permeten que la proliferació de les cèl·lules passi de manera ordenada i controlada. Si tenim present que una de les característiques del càncer és la proliferació descontrolada de cèl·lules amb mutacions, entendre els processos pels quals les cèl·lules controlen de manera natural la divisió cel·lular ens pot ajudar a entendre quin són els mecanismes que estan alterats en els tumors.

9 POPULÄRWISSENCHAFTLICHE ZUSAMMENFASSUNG

Alle Lebewesen haben eines gemeinsam: Sie bestehen aus Zellen. Um genauer zu sein stammen diese alle von einer Ursprungszelle ab, die eine Vielzahl von Tochterzellen bildet. Die naheliegende Frage ist daher: Wie kann eine einzelne Zelle, millionenfache Abkommen produzieren die dann zusammen einen gesamten Organismus bilden? Die einfache Antwort hier wäre: „Sie macht Kopien von sich selber“. Tatsächlich können Zellen sich Teilen und Kopien von sich selber produzieren indem Sie einen Prozess durchlaufen, der sich Zellzyklus nennt.

Der Zellzyklus kann in vier verschiedene Phasen unterteilt werden, je nachdem welche Prozesse gerade in der Zelle ablaufen. Während der ersten, der sogenannte G1-Phase, wächst die Zelle und sammelt Information in Ihrer Umgebung um herauszufinden ob genug Nährstoffe für einen Teilungsprozess vorhanden sind und ob die Bedingungen günstig sind gerade jetzt eine Kopie von sich selber zu produzieren. Wenn die Zelle grünes Licht zum Teilen bekommt, werden viele verschiedene Prozesse eingeleitet. Einer der Hauptprozesse ist die Kopie des Erbmaterials der Zelle, der sogenannten Chromosomen in der zweiten Phase des Zellzyklus, der Synthese oder kurz S-Phase. Die Chromosomen enthalten all die Information die eine Zelle benötigt um Ihre Aufgaben erfüllen zu können um Kopien von sich selber herstellen zu können. Sobald alle Chromosomen kopiert sind geht die Zelle in die G2-Phase über, und bereitet sich auf das große Finale, die Zellteilung vor. Während der G2-Phase kopiert die Zelle alle andern Bestandteile und Strukturen die eine Zelle zum Überleben braucht, damit am Ende jede der Tochterzellen die gleiche Zusammensetzung wie die Ursprungszelle hat. Während dieser komplizierten Prozesse verändert die Zelle Ihre Form und produziert einige Hilfsstrukturen um sicherzustellen, dass jeder einzelne Schritt auf dem Weg zu einer neuen Zelle zum richtigen Zeitpunkt an der richtigen Stelle stattfindet. Nach ungefähr 5 bis 6 Stunden ist die G2 Phase beendet und der letzte Teil des Zellyklus kann beginnen, die Mitose-Phase, also die Zweiteilung des Erbmaterials. Hierzu verformt sich die Zelle zu einer Kugel und auf den sich gegenüberliegenden Seiten der Kugel bilden sich Ankerstrukturen von denen aus Fasern zur Mitte der Kugel gebildet werden. Die Aufgabe dieses Spindelapparats und der Spindelfasern ist es das kopierte Erbmaterial aufzureihen und je eine Kopie eines jeden Chromosoms zu den beiden Seiten der Zelle zu transportieren, sodass sich auf beiden Seiten der Zelle die gleiche Erbinformation wiederfindet. Wenige Minuten danach entfernen sich die gegenüberliegenden Pole der Zelle voneinander und die Mitte der Zellkugel zieht sich zusammen. Die Tochterzellen trennen sich voneinander und zwei neue Zellen mit gleichem Inhalt sind entstanden.

Der Zellzyklus kann im übertragenden Sinne mit einer Symphonie verglichen werden. Wie bei der Aufführung einer Symphonie, ist die Zelle von einem Orchester abhängig in dem eine Reihe von

Musikern ganz spezifische Aufgaben zu ganz spezifischen Zeitpunkten erfüllen müssen. Doch unabhängig davon wie begabt jeder einzelne Spieler ist, brauchen Sie die Führung eines Dirigenten der weiß wann ein jeder zu spielen hat und besonders wichtig mit welcher Lautstärke, welchem Ausdruck und Tempo. In gleicher Weise haben Zellen eine Gruppe von Dirigenten und Assistenten, die sich Cyclin-Cdk Komplexe nennen und jede Phase des Zellzyklus wird von einem spezifischen Cyclin-Cdk Komplex dirigiert. Ein Cyclin-Cdk Komplex besteht aus zwei Bestandteilen, der Cdk, die dafür verantwortlich ist Aufgaben auszuführen und Eiweiße so zu verändern, dass sie Ihre zugedachten Aufgaben erfüllen können und dem Cyclin, dem Partner der Cdk, welches dafür sorgt, dass die Cdk zum richtigen Zeitpunkt am Richtigen Ort aktiv wird. Anders ausgedrückt braucht jeder Satz einer Symphonie einen Dirigenten und einen spezifischen Assistenten der dafür sorgt, dass alles harmonisch und schön erklingt.

In dieser Arbeit haben wir versucht zu verstehen wie gesunde Zellen während des Zellzyklus die Aktivität der Cyclin-Cdk Komplexe kontrollieren. Außerdem möchten wir herausfinden welche Rolle Cyclin-Cdk Komplexe in Zellen mit DNS-Schäden (den Bausteinen der Chromosomen) spielen.

Wie bereits erwähnt kontrollieren verschiedene Cyclin-Cdk Komplexe die verschiedenen Phasen des Zellzyklus. Wir interessieren uns besonders für Cdk1 welche sowohl einen Komplex mit Cyclin A oder Cyclin B bilden kann. Diese sind dafür verantwortlich, dass Zellen eine fehlerfreie Kopie von sich selber bilden können. Wir haben herausgefunden, dass Zellen dann Cdk aktivieren wenn diese damit fertig sind Ihre Chromosomen zu kopieren, also dass der Dirigent der den Beginn der Kernteilung bestimmt schon ein paar Stunden vorher die Bühne betritt. Wir konnten zeigen, dass die Cdk Komplexe teilweise schon Stunden vor der eigentlichen Teilung aktiv werden, im Gegensatz zu bisherigen Beobachtungen nach denen die Cdk Komplexe lediglich Minuten vorher aktiv werden.

Außerdem haben wir herausgefunden, dass am Ende des Kopiervorganges des Erbmaterials Cyclin A den Zellkern verlässt und nicht wie bisher angenommen dort bis zum Ende der Zellteilung verbleibt.

Zurück zu unserem Orchester Beispiel. Zum einen haben wir herausgefunden, dass der Dirigent (Cdk) der für das große Finale am Ende zuständig ist schon einige Stunden vorher in den Konzertsaal kommt und mit Hilfe seines Assistenten (Cyclin A oder B) einigen Musikern die Anweisung gibt ganz leise im Hintergrund den letzten Satz einzuleiten. Außerdem haben wir herausgefunden, dass einer der Assistenten (Cyclin A) die Bühne verlässt und andere Aufgaben im Konzertsaal übernimmt.

Obwohl wir noch nicht verstehen, warum Cyclin A den Zellkern verlässt, vermuten wir, dass es eine Rolle in der Regulation des Zellzyklus spielt.

Die oben beschriebenen Mechanismen beschreiben die Vorgänge in einer gesunden Zelle, aber jede Zelle unseres Köpers ist ständigen Veränderungen und Schäden die zu Mutationen führen können ausgesetzt. Um mit diesen Veränderungen klarzukommen gibt es eine Vielzahl von Mechanismen auf

Schäden des Erbmaterials zu reagieren. Ganz allgemein verlangsamt die Zelle die Prozesse die zur Zellteilung führen solange bis alle Schäden behoben sind. Das heißt die Aktivität der Cdks wird vermindert, sodass Fehler im Erbmaterial nicht weitergegeben werden. Gleichzeitig werden Reperaturmechanismen gestartet welche die Schäden reparieren sollen und sobald alle Schäden behoben sind kann die Zelle Ihren Teilungsprozess fortsetzen. Sind die Schäden allerdings zu viele und nicht reparierbar verlässt die Zelle den Zellzyklus und hört auf sich zu teilen. Diesen Prozess nennt man Seneszenz („alt werden"). Damit wird verhindert, dass sich fehlerhafte Zellen weiterhin vermehren können was andernfalls zur Bildung von Tumoren führen könnte.

Um zu verstehen wie Zellen sich entscheiden den Zellzyklus zu verlassen haben wir die Veränderung der Cdk Aktivität in Zellen mit Schäden im Erbmaterial untersucht. Wir haben dabei herausgefunden, dass geschädigte Zellen Cyclin B (einen der Cdk Assistenten) in den Zellkern transportieren wo es dann unbrauchbar gemacht wird. Interessanterweise ist Cdk1 notwendig um diesen Prozess zu starten. Anders ausgedrückt: also Folge von Schäden im Erbmaterial kann Cdk den Sicherheitschalter der Zelle „p21" aktivieren, welcher dann Cyclin B hilft in den Kern zu gelangen. Sobald Cyclin B dann unbrauchbar gemacht wurde „vergisst" die Zelle in welchem Teil des Zellzyklus sie gerade war und hört auf sich zu teilen.

Zusammengenommen haben wir in dieser Arbeit dazu beigetragen die Mechanismen im Menschen, die für eine funktionierende Zellteilung gebraucht werden genauer zu verstehen. Unkontrollierte Zellteilung ist eines der Hauptkennzeichen von Krebs. Daher ist das Verständnis der Regulation und Funktion der Cdk wichtig um zu verstehen was in Zellen falsch läuft, die unkontrolliert wachsen und Tumore bilden können.

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