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Regulation of G2 Phase and Long-Term Consequences of DNA Damage

THESIS FOR DOCTORAL DEGREE (Ph.D.)

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To my beloved family

"The important thing is to never stop questioning"

- *A. Einstein*

ABSTRACT

Cell proliferation requires the accurate replication of DNA and equal segregation of replicated genes, important for maintaining the integrity of newly formed cells. At the centre of this process is a series of coordinated events termed 'the cell cycle', which ensures cell proliferation proceeds with high fidelity. Cell cycle regulation is driven by the activity of cyclin-dependent kinases (Cdks), which require binding to their regulatory subunit cyclin to become activated. However, the activity of Cdk is regulated by several different mechanisms. Transcription and degradation control mechanisms indirectly affect Cdk activity by modulating the expression of several regulatory proteins, including cyclins, while regulatory phosphorylation and dephosphorylation of cyclin-Cdk complexes provide direct control of Cdk activity. Such post-translational modifications are frequently part of feedback loops, which fine-tune Cdk activity. These mechanisms collectively modulate successive activation of Cdks, and is responsible for timely phosphorylation of Cdk substrates to complete different phases of the cell cycle. This thesis concerns the regulation of G2 phase in the cell cycle, in relation to: 1) the effect of cyclin A2 localisation in G2 phase, 2) the changes in G2 phase regulation in a genetic disorder, and 3) the long-term consequences if G2 phase regulators are completely suppressed.

Although Cdk activity is required for well-delineated cell cycle phase transitions, the spatio-temporal regulation of cyclin is important, as it provides unique substrate specificity and accessibility to the Cdk. Nevertheless, the exact mechanisms underlying the activation of cyclin-Cdk complexes remain largely elusive. The first part of this thesis investigates unknown mechanisms of mitotic kinase activation in G2 phase, by assessing the spatio-temporal regulation of cyclin A2 and its function in G2 phase.

In **paper I**, we observe that nuclear cyclin A2 partially translocates to the cytoplasm at S/G2 phase transition. Interestingly, we reveal that cyclin A2-Cdk2 can initiate the activation of Plk1 through phosphorylation of Bora, but only cyclin A2 localised to the cytoplasm can interact with Bora and Plk1. We find no evidence that the change in localisation of cyclin A2 is involved in feedback loops in G2 phase. Thus, our study strongly supports the notion that cytoplasmic A2 functions as a trigger for the activation of mitotic kinases. Although the precise mechanism that changes the localisation of cyclin A2 to the cytoplasm requires further study, we show that cyclin A2 nuclear localisation until S/G2 phase transition is contributed, in part, by the association of cyclin A2 to chromatin during DNA replication. In addition, our work also

reveals p21 can restrict cyclin A2 to the nucleus, especially after DNA damage. Together, **paper I** expands our understanding of the mechanisms of mitotic kinase activation in G2 phase, and identifies future areas of study to fill in our knowledge gaps of how cyclin A2 changes its cellular localisation.

Cell cycle dysregulation has been implicated in many genetic diseases and disorders. This highlights the importance of understanding cell cycle regulation in certain disease settings. The second part of this thesis is dedicated to studying the role of a non-coding nuclear RNA gene, *RMRP*, that is mutated in the rare genetic disorder, cartilage-hair hypoplasia (CHH). CHH cells show proliferation defects, and studies on yeast suggest that *RMRP* could regulate the accumulation of cyclins. In **paper II**, we reveal *RMRP* has pleiotropic effects on several cell cycle regulatory genes, and the mutation of *RMRP* delays G2 phase progression to mitosis. Furthermore, our work finds evidence of possible impairment in the PI3K-Akt signalling pathway in CHH. These findings contribute to understanding the role of *RMRP* in cell cycle regulation, particularly in relation to CHH, and indicate a possible pathway for therapeutic interventions.

The uncontrolled proliferation of cells with genomic instability can lead to the development of cancer. The cell cycle checkpoint is a mechanism that can restrict cell cycle progression in response to DNA damage and replication blocks. When checkpoint kinases are activated, signals are transmitted to a network of regulatory proteins that increase the inhibitory force and delay cell cycle progression. In the case of persistent DNA damage in G2 phase, p53 and p21-dependent premature activation of APC/C^{Cdh1} mediates cell cycle termination by degrading all cell cycle regulatory proteins. While all these processes ensure genomic integrity, the mechanisms that allow escape from a checkpoint have been the focus of many studies, but whether cell cycle termination in G2 phase can be reversed remains unclear. Therefore, the last part of this thesis investigates the long-term consequences of DNA damage-induced cell cycle termination in G2 phase.

Paper III shows that cells can re-initiate S phase after terminating the cell cycle in G2 phase. Interestingly, expression of p21 persists until cells re-initiate DNA replication and increases further once DNA re-replication is complete. This finding supports our observation of repeated cell cycle termination of re-replicated cells. Furthermore, re-replicated cells can progress to mitosis, which creates a heterogenous cell population, and is linked to genomic instability. Thus, resumption of the cell cycle a long period after termination in G2 phase can give rise to

multiple cell fates. This shifts our current perception of the long-term consequences of cell cycle termination in G2 phase, from a singular outcome of senescence to that of multiple cell fates, possibly alluding to a mechanism by which cells can undergo oncogenic transformation.

In summary, this thesis highlights the importance of the spatio-temporal regulation of cyclin A2 in modulating Cdk to initiate the mitotic entry network in G2 phase, ensuring well-delineated progression to mitosis. Identifying the function of *RPRM* in G2 phase adds to our limited understanding of cell cycle regulation in relation to CHH. Moreover, this thesis reveals that DNA damage-induced cell cycle termination in G2 phase can lead to cell fates other than senescence, an implication that could have relevance in tumourigenesis.

LIST OF SCIENTIFIC PAPERS

I. Silva Cascales H, Burdova K, **Middleton A**, Kuzin V, Müllers E, Stoy H, Baranello L, Macurek L, Lindqvist A. Cyclin A2 localises in the cytoplasm at the S/G2 transition to activate PLK1. *Life Sci Alliance*. 2021 Jan 5;4(3):e202000980.

II. Vakkilainen S, Skoog T, Einarsdottir E, **Middleton A**, Pekkinen M, Öhman T, Katayama S, Krjutškov K, Kovanen PE, Varjosalo M, Lindqvist A, Kere J, Mäkitie O. The human long non-coding RNA gene RMRP has pleiotropic effects and regulates cell-cycle progression at G2. *Sci Rep*. 2019 Sep 24;9(1):13758.

III. **Middleton A**, Suman R, O'Toole P, Akopyan K, Lindqvist A. p53-dependent polyploidisation after DNA damage in G2 phase. *bioRxiv*. 2020. Jun 9:141770; doi:https://doi.org/10.1101/2020.06.09.141770

CONTENTS

LIST OF ABBREVIATIONS

γ-IR	Gamma Ionising Radiation
γH2AX	Phosphorylated (140S) H2A Histone Family Member X
53BP1	p53-Binding Protein 1
APC/C	Anaphase-Promoting Complex/Cyclosome
ASF1	Anti-Silencing Function 1
ATF2	Activating Transcription Factor 2
ATM	Ataxia Telangiectasia Mutated Kinase
ATR	ATM- and Rad3- Related
B55	Regulatory Subunit B55
BCL2L1	BCL2-Like 1 gene
BRCA1	Breast Cancer 1
CAK	Cdk-Activating Kinase
CCNA2	Cyclin Dependent Kinase 2 gene
Cdc	Cell Division Cycle Phosphatase
Cdh1	Cadherin 1
Cdk	Cyclin-Dependent Kinase
CDK2	Cyclin-Dependent Kinase 2 gene
CDKN1A	Cyclin-Dependent Kinase Inhibitor 1A gene
CHH	Cartilage-Hair Hypoplasia
Chk	Checkpoint Kinase
CKI	Cdk Inhibitor Proteins
Cks	Cyclin-Dependent Kinase Regulatory Subunit
CRS	Cytoplasmic Retention Sequence
CtBP	C-Terminal Binding Protein
CtIP	C-Terminal Binding Protein Interacting Protein
DDR	DNA Damage Response
DNA-PK	DNA-Dependent Protein Kinase

DNA-PKcs	DNA-Dependent Protein Kinase Catalytic Subunit
DNA2	DNA Replication Helicase/Nuclease 2
DSB	Double-Strand Break
E2F1	E2 Transcription Factor 1
EdU	5-Ethynyl-2′-Deoxyuridin
Emi1	Early Mitotic Inhibitor 1
EXO1	Human Exonuclease 1
eYFP	Enhanced Yellow Fluorescent Protein
FOXM	Forkhead Box Protein
Gwl	Greatwall Kinase
H2AX	H2A Histone Family Member X
HIRA	Histone Cell Cycle Regulation Defective Homologue A
HR	Homologous Recombination
IFITM1	Interferon Induced Transmembrane Protein 1 gene
IR	Ionising Radiation
KD	Kinase Domain
MDC1	Mediator of DNA Damage Checkpoint 1
Mdm2	Mouse Double Minute Homologue 2
MPF	Maturation Promoting Factor or Mitosis Promoting Factor
MRN	Mre11/Rad50/Nbs1 Complex
NEB	Nuclear Envelope Breakdown
NES	Nuclear Export Sequence
NF-Y	Nuclear Factor Y
NHEJ	Non-Homologous End Joining
NLS	Nuclear Localisation Sequence
p38 MAPK	p38 Mitogen-Activated Protein Kinase
PBD	Polo-Box Domain
PI3K	Phosphatidylinositol-3 Kinase
PIKKs	Phosphatidylinositol-3 Kinase-Like Kinases
Plk1	Polo-Like Kinase 1

PP1	Protein Phosphatase 1
PP2A	Protein Phosphatase 2A
pRB	Phosphorylated Retinoblastoma Protein
QPI	Quantitative Phase Imaging
Rb	Retinoblastoma Protein
RMRP	RNAse for Mitochondrial RNA Processing gene
RNF	RING Finger Protein
RPA	Replication Protein A
RPE	Retinal Pigment Epithelium
SAC	Spindle Assembly Checkpoint
SAHF	Senescence-Associated Heterochromatin Foci
SASP	Senescence-Associated Secretory Phenotype
SCAPER	S Phase Cyclin A-Associated Protein
SCF	Skp-Cullin-F-Box-Containing Protein
Skp2	S Phase Kinase-Associated Protein 2
SP1	Specificity Protein 1
SSB	Single-Strand Break
SUMO	Small Ubiquitin-Like Modifier
TCF	Ternary Complex Factor
TERT	Telomerase Reverse Transcriptase
Ubc9	Ubiquitin-Conjugating Enzyme 9
Wip1	Wild-Type p53-Induced Phosphatase 1
WRN	Werner Syndrome Protein
XRCC4	X-ray Repair Cross-Complementing Protein 4

1 INTRODUCTION

Cell proliferation is fundamental in all organisms, allowing a renewal of living cells. The accurate duplication and segregation of chromosomes are important events in the proliferation of cells. However, these two events constitute only the basic functions of the cell cycle. In 1953, Alma Howard and Stephen Pelc identified the existence of two periods, G1 and G2 phase, in addition to DNA synthesis (S phase) and cell division (mitosis), and proposed that proliferating cells follow successive temporal progression of these phases[1]: G1 phase starts after mitosis is completed and G2 phase begins once S phase is accomplished. This concept of the cell cycle assisted later identification of cell cycle time parameters and the multiple biochemical and molecular events that occur at each cell cycle phase. These discoveries highlight that the cell cycle is a complex, yet highly regulated, series of events which control cell proliferation.

With advancements in molecular biology techniques, cell cycle regulation has been extensively researched over the past few decades, but the regulation of G2 phase remains largely elusive. Nevertheless, the processes involved in G2 phase reinforce cell cycle progression, but critically are also able to restrict the cell cycle when cell integrity is severely compromised[2,3]. This restriction of the cell cycle can lead to cell cycle termination, which often develops into senescence - a state of permanent cell cycle arrest[4]. However, our understanding of cell fate decisions after cell cycle termination is open to question. Dysregulation of the mechanisms that enforce anti-proliferative cell fates can impose genetic instability, a pre-requisite for the development of cancer[5,6]. Interestingly, cell proliferation and its relevance to disease, has been hinted at as early as the initial observations by Robert Remak in 1852, who stated that cells arise from existing cells both in diseased and healthy tissues[7]. Cell proliferation is critical for the maintenance, development and growth of the human body[8], and an accurate and orderly cell cycle lies at the hub of these processes. This highlights that understanding cell cycle regulation is key to uncovering the secrets of human disease and may provide useful targets for future therapeutic interventions.

Thus, the work presented in this thesis explores how the cell cycle is regulated in normal and disease settings, as well as, the long-term effect on cells after DNA damage, with particular emphasis on G2 phase.

1.1 Cell cycle engine: Cyclin and cyclin-dependent kinase complexes

Progression through the cell cycle is primarily controlled by the activity of cyclin-dependent kinases (Cdks). Cdks are a family of conserved serine/threonine kinases that contain a catalytic core comprised of an ATP-binding pocket, PSTAIRE-like cyclin-binding domain and activating T-loop motif[9]. Activation of Cdk requires heterodimeric complex formation with its specific regulatory subunit cyclin and phosphorylation of threonine (Thr160) residue in the T-loop by Cdk-activating kinase (Cak)[10–12]. This cell cycle machinery operates the same way in widely disparate organisms from yeast to higher eukaryotes.

Although the cell cycle control mechanism is conserved, not all eukaryotes depend on the same types of Cdks. In yeast, a single PSTAIRE kinase (Cdc28 in *S. cerevisiae* and Cdc2 in *S. pombe* - homologues of Cdk1), regulates all cell cycle phases by associating with multiple phase-specific cyclins[13]. In contrast, mammalian cells have evolved to have small groups of Cdks, with specific members sequentially activated during the cell cycle to carry out distinct functions[14,15]. Cdk4/6 controls G1 phase, Cdk2 regulates entry into S phase, and Cdk1 activity drives through G2 phase to initiate mitotic entry[16]. However, in mice, mutation of Cdk2, Cdk4 or Cdk6 does not affect viability without major defects in the cell cycle, indicating functional redundancies between the different Cdks[17–19]. Also, while deletion of Cdk1 in Cdk2 mutant chicken DT40 cells prevents the initiation of DNA replication and centrosome duplication, the presence of a single Cdk2 allele renders S phase progression independent of Cdk1, suggesting that Cdk1 and Cdk2 share a function in S phase control[20]. Therefore, these findings challenge the specific function of Cdks at each assigned phase of the cell cycle. Interestingly, knocking out Cdk1 in mice leads to embryonic lethality during the early stages of development, and the functionality of Cdk1 cannot be rescued by other Cdks[21]. However, mice embryos lacking Cdk2, Cdk3, Cdk4 and Cdk6 can undergo embryonic development, and fibroblasts derived from these mice can proliferate *in vitro*[22]. Thus, Cdk1 seems essential in controlling the mammalian cell cycle, which is reminiscent of that in yeast where a single Cdk regulates the entire cell cycle.

The human genome encodes at least 20 proteins that can be considered members of the Cdk family, based on sequence similarities in the conserved domain and an ability to be activated by cyclins[23]. The families of Cdk8, Cdk9, Cdk11 and Cdk20 have been identified to show activities of transcription-related regulation [24,25], but some of these Cdks are also able to directly regulate cell cycle progression. For example, Cdk10 (a subfamily of Cdk11) is thought to be

implicated in regulating the cell cycle through inhibitory regulation of Cdk1. Indeed, overexpression of antisense and dominant-negative mutants of Cdk10 in U2OS cells leads to proliferation inhibition and the dominant-negative mutants halt cell cycle progression in G2/M phase[26]. Also, Cdk10 can restrain Ets2 transcription factor, which binds to the promoter of Cdk1 through its kinase activity, either by complexing with cyclin M to promote ubiquitin ligase/mediated Ets2 degradation[27,28], or by its direct association with the N-terminal pointed domain of Ets2 to inhibit transactivation of Ets2[29]. Similar to Cdk10, which regulates the cell cycle independent of cyclin, canonical or transcriptional cyclins have also been shown to carry out cellular functions that do not require interaction with a Cdk. Independent of Cdk4, cyclin D1 overexpression transcriptionally activates oestrogen receptors in murine derived SCp2 cells[30]. An oestrogen receptor-positive status correlates with cyclin D1 overexpression in human breast cancer[31–33], therefore cyclin D1 may exert an oncogenic potential in breast cancer. Also, cyclin A2 can directly bind to Mre11 transcripts, independent of Cdk, to facilitate polysome loading and translation, which ensures adequate repair of common replication errors[34]. In *S. cerevisiae*, cyclin C translocates to the cytoplasm in response to oxidative stress and directly interacts with Mdv1p, an adaptor protein that is required for mitochondrial fission[35]. Together, these findings suggest that although canonical cyclin-Cdk complexes are undoubtedly core cell cycle engines in cell cycle regulation, the functions and substrates of many cyclins and Cdks remain unknown. Thus, explaining the cell cycle based on canonical cyclin-Cdk complexes is an over simplification.

In summary, the cell cycle truly works like a clock, with canonical cyclin-Cdk complexes representing the cell cycle's movement, while several hidden internal molecular components tightly regulate this movement. While cell cycle functions and mechanisms have been discovered using models across species, including yeast, amphibians and *Drosophila*, the work presented in this thesis focuses on human cell cycle regulation. Therefore, the human nomenclature will be used, herein, to discuss the different cell cycle regulators.

1.2 Cyclin-Cdk activity and substrate specificity

While the concentrations of Cdks remain relatively constant throughout the cell cycle, different cyclins are expressed according to the cell cycle phases[16]. This reflects the oscillatory expression of specific cyclin-Cdk complexes at different cell cycle stages, which serve as molecular switches that regulate cell cycle transitions[36]. In human cells, cyclin D-Cdk4/6 is active in G1 phase, cyclin E-Cdk2 at the G1/S border, cyclin A2-Cdk2 during S phase, cyclin A2-Cdk1 at S/G2 border, and cyclin B1-Cdk1 during G2/M phase transition[37–40]. The timely phosphorylation of target proteins by cyclin-Cdk complexes are essential in cell cycle transition[41–49], and how the different cyclins drive distinct phases of the cell cycle has been previously debated. Since cyclins modulate Cdk substrate specificity or change Cdk subcellular localisations[50,51], a model has been proposed where the intrinsic functional capacities of cyclins drive cell cycle transition. This 'qualitative model' of cyclin function is supported by several findings from the biochemical characterisation of yeast and metazoan cyclin-Cdk complexes[47,52–56]. In *S. cerevisiae*, cyclins exhibit substrate site-specificity and have the ability to compensate, by a docking interaction, for a gradual decrease in the specificity of early cyclin-Cdk1 complexes[54]. Similarly, it has been shown in mammalian cells that cyclin D-Cdk4/6 targets Rb for phosphorylation through recognition of the C-terminal alpha-helix on Rb, the mutation of which leads to arrest in G1 phase. Also, the C-terminal alpha-helix is not recognised by cyclin E-Cdk2, cyclin A-Cdk2, and cyclin B-Cdk1 complexes, suggesting Cdk specificity is determined by intrinsic selectivity of the active site and by the substrate docking site on the cyclin subunit[57]

However, for the qualitative model to work, specific cyclin-Cdks have to be available during the assigned phase in order to transition through the cell cycle without major impact. The observation of mitotic cyclin in *S. pombe*, promoting both S phase and mitosis in the absence of G1 phase cyclins[58], suggests that the substrate specificity of different cyclin-Cdk complexes may be less important for regulating orderly cell cycle transition. This apparent plasticity is also reported in other eukaryotes. As discussed, genetic elimination of specific cyclins-Cdks do not have a major impact on the cell cycle order in *in vivo* and *in vitro* murine systems[22,59–61]. Since the multiple specific docking interactions on Rb are recognised by cyclin D[57], and in the absence of Cdk4/6, the non-consensus interaction between cyclin D and Cdk2 can form and play compensatory roles[18,62]. Thus, the formation of non-canonical cyclin-Cdk complexes could, at least partly, reconcile a qualitative model with the lack of phenotype after depletion

of individual Cdks. In *Xenopus* oocytes, cyclin B1 nuclear translocation induces a gradual increase in Cdk1 activity that first initiates replication and subsequently induces mitotic entry. This suggests Cdk subcellular localisation controls Cdk activity and therefore is a critical factor in determining Cdk specificity[63]. These findings support a model which proposes quantitative changes in the levels of Cdk activity regulate progression of the cell cycle in an orderly manner[64].

In this 'quantitative model', the functional differences between cyclins are primarily explained by differences in their expression levels and timings. It has been shown that Cdh1 ablation lessens the strength of the double-negative feedback loop generated between Cdk2 and Cdk inhibitor, p27, resulting in a more linear response of Cdk2 to cyclin E, with premature entry into S phase, while delaying S phase progression. This demonstrates that a timely increase of Cdk2 activity to its threshold is required for scheduled S phase transition and progression[65,66]. In line with this, expression of constitutively active Cdk1 in human somatic cells shortens the duration of G1 and S phases, allowing progression into a mitotic-like state without proper completion of S or G2 phases, indicating that low Cdk1 activity is required for S phase progression[67]. During S phase, cyclin A2-Cdk2 activity increases as cyclin A2 synthesis increases, which is shown to promote disassembly of the origin recognition complex, thereby helping to restrict origin firing to only once per S phase[68,69]. However, an increase of Cdk activity above a certain threshold induces premature phosphorylation of mitotic targets, which suggests that keeping Cdk2 activity at an intermediate level is required to maintain S phase[70–72]. At the end of S phase, cyclin A2 and B1 transcription starts to increase rapidly, which increases Cdk1 activity to a threshold where it phosphorylates multiple substrates involved in the onset of mitosis[73,74]. Taken together, these findings support a quantitative model of cyclin function, where gradually increasing cyclin-Cdk activity sequentially induces cell cycle events.

While the biochemical characterisation of cyclin-Cdk complexes highlights the qualitative model of cyclin function[47,52–56], the phosphoproteomic-based study of *S. pombe* implicates both the qualitative and quantitative models of cyclin function in controlling the cell cycle[75]. The monotonic rise in activity of cyclin-Cdk complexes induces timely phosphorylation of its substrates, by proceeding through sequential substrate-specific activity thresholds and in part by the affinity of substrates towards the cyclin-Cdk complexes[75]. Furthermore, although genetic elimination studies demonstrate the redundancy of Cdk2, Cdk4 and Cdk6[22,59–61], a chemical-genetic based study reveals that Cdk2 is required for G1/S phase transition when normal cyclin pairing is maintained[76,77]. Together these studies indicate that cell cycle progression is guided

by Cdk activity thresholds, while cyclin-generated specificity contributes to fine tuning of this activity.

1.3 Operations of the cell cycle engine

1.3.1 Temporal regulation: Cyclin synthesis and degradation

Physical association with cyclins is essential for Cdk activation, and the synthesis and degradation of cyclins are regulated throughout the cell cycle. Unbound cyclin D is relatively unstable and its expression levels are adjusted with some precision depending on the presence of extracellular mitogen signals and signalling cascades. Transcription of cyclin D is promoted by several transcription factors, including c-Jun, c-Fos, ATF2, Ets2, SP1, TCFs and Myc, which convey mitogenic signalling cues[78–82]. In contrast, cyclin E, A and B expressions are mostly independent of extracellular mitogenic signalling and regulated at transcriptional or post-transcriptional levels[83–85]. The Rb/E2F transcriptional pathway is most characterised in the G1/S phase transition. In the prevailing model, gradual phosphorylation of Rb by cyclin D-Cdk4/6 releases E2F for transcriptional upregulation of cyclin E, which in turn promotes its own transcription through a positive feedback loop[86,87]. However, a recent study has shown that Rb is only exclusively monophosphorylated by cyclin D-Cdk4/6 in G1 phase, and E2F transcriptional activation is driven by cyclin E-Cdk2-dependent hyper-phosphorylation of Rb at the G1/S phase restriction point[88]. Thus, how cyclin E is activated remains unclear. However, Myc is reported to increase cyclin E gene expression[89], indicating that Cdk activity-dependent transcriptional control of G1/S phase transition could be regulated by a different transcriptional pathway.

Together with transcription, two ubiquitin E3 ligase complexes, SCF and APC/C, play a role in guarding against inappropriate or untimely accumulation of cyclins throughout the cell cycle[90]. In late G1 phase, cyclin E-Cdk2 dependent phosphorylation of Cdk inhibitor p27 at Thr187 residue[91–93] is recognised by F-box protein Skp2, which functions as the receptor component of the SCF ubiquitin ligase complex[94–96]. This recognition consequently promotes ubiquitin-dependent degradation of p27, which prevents inhibition of cyclin A in S phase. Skp2 also targets free cyclin E, which is unbound from Cdk2, for ubiquitylation[97]. As S phase progresses, Cdk2-mediated phosphorylation promotes SCF^{Fbw7}-mediated ubiquitination and degradation of cyclin E[83,98], while E2F-regulated transcription of cyclin A increases[99,100]. Also, factors affecting cyclin transcription are modified post-translationally, including being targeted

for ubiquitin-mediated degradation. At the beginning of S phase, cyclin A2-Cdk phosphorylates activator E2Fs, thereby preventing its ability to bind DNA and mediate transactivation [42,101,102]. In early G2 phase, cyclin A2-Cdk-dependent phosphorylation of B-Myb and FoxM1 promotes cyclin B1 transcription[103,104]. Cyclin F, the founding member of the F-box protein family of the SCF substrate receptor, targets activator E2Fs for proteosomal degradation on entry to G2 phase[105,106]. Also $SCF^{Cyclin\ F}$ is implicated in regulating G2/M phase transition by promoting degradation of atypical repressor E2Fs in G2 phase to ensure the expression of DNA repair genes[107].

Increasing Cdk activity phosphorylates Cdh1 to inhibit its binding to APC/C, which allows accumulation of cyclin A and cyclin B throughout S to G2 phase[108]. SCF may play a role in promoting cyclin A and cyclin B accumulation by promoting the degradation of Cdh1. Cyclin F is also shown to contribute to APC/C inactivation through promoting Cdh1 degradation, providing additional regulation to the G1/S phase transition[109]. In early mitosis, Cdk1-dependent phosphorylation of APC/C increases its binding affinity for Cdc20, mediating degradation of cyclin A at prometaphase, whereas Cdh1 dephosphorylation in anaphase activates APC/C^{Cdh1} for cyclin B degradation[110]. Although APC/C inactivation by the spindle assembly checkpoint (SAC) delays degradation of cyclin B until completion of microtubule attachment to the kinetochores[111], cyclin A degradation is permitted by an active SAC[112]. The temporal and sensitivity differences in these two mitotic cyclins depend, at least in part, on Cks1-mediated recruitment of cyclin A-Cdk1 to the phosphorylated APC/C subunit[112]. APC/C^{Cdh1} remains active during G1 phase, which promotes cyclin B degradation[113], as well as, preventing unscheduled accumulation of cyclin A, thereby maintaining the expression of pro-mitotic regulators at a low level[114].

As discussed, several positive and negative feedback loops are present within the network that controls cyclin expression[111,115], making these feedback mechanisms more important than cyclin expression in understanding cell cycle regulation.

1.4 Regulation of Cdk activity: Post-translational modifications

The activation of Cdk requires more than just the binding of cyclin to Cdk[73]. Post-translational modification is another important regulatory input that regulates Cdk activity. Upon binding of cyclin, the Cdk conformational change allows phosphorylation of the conserved threonine residue in the T-loop by Cdk-activating kinases (CAKs)[10]. At this point Cdk is activated, however, at low concentrations of cyclin, Wee1/Myt1 kinases restrain Cdk activity by phosphorylating the Thr14 and Tyr15 residues[116–118]. When the concentration of cyclin exceeds its threshold Cdc25 phosphatases antagonise Wee1/Myt1, activating Cdk by dephosphorylating the inhibitory phosphates from its aforementioned residues[119,120]. In humans, three isoforms of Cdc25 (Cdc25A,-B and -C) exist, each of which are responsible for influencing the transition between specific phases of the cell cycle[121].

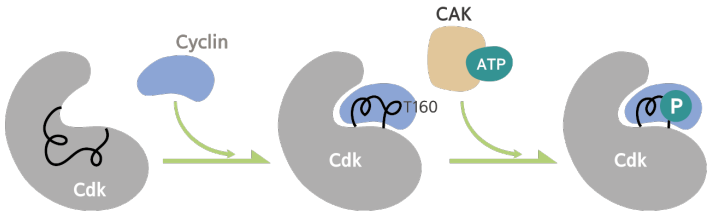

Figure 1. Activation of Cyclin-Cdk complex by phosphorylation of the T-loop by Cdk-activating kinase (CAK).

Cdk1 can also self-promote its own activity by directly regulating Wee1/Myt1 and Cdc25. Active Cdk1 inhibits Wee1 by phosphorylation, which promotes $SCF^{\beta\text{-}TrCP}$-dependent degradation and consequently removes the inhibitory force that was limiting Cdk activity[122–124]. In contrast, Cdc25 is in turn stabilised and activated by Cdk1-dependent phosphorylation, thereby further promoting Cdk activation[125]. These events create double negative and positive feedback loops, respectively, thereby self-promoting phase transition in a robust and irreversible manner[126]. Also, Cdk-dependent phosphorylation of Wee1 and Cdc25C is highly ultrasensitive, generated by multisite phosphorylation over a narrow range of Cdk1 activity in a rapid and switch-like manner[127,128]. In other words, phosphorylation is inefficient at low levels of Cdk activity, thereby maintaining an inactive steady-state, but as Cdk levels start to rise phosphorylation increases abruptly in a non-linear manner, switching Cdk activity to a fully active steady-state. Thus, taken together the feedback mechanisms and ultrasensitivity enable Cdk activity to switch between two steady-states, which explains the highly non-linear and bistable dynamics of Cdk activation at mitotic entry[129].

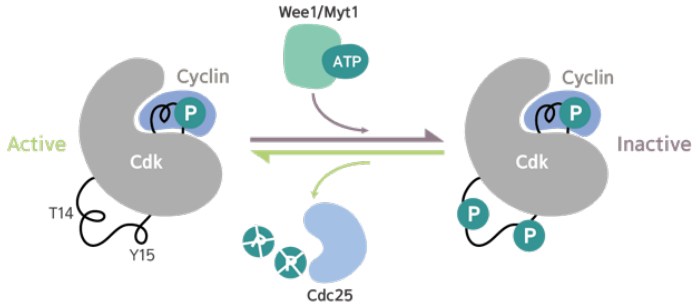

Figure 2. Activation of cyclin-Cdk by the balanced action of Wee1/Myt1 and Cdc25.

In addition to direct regulation of Cdk by phosphorylation at Thr14 and Tyr15, several phosphatases and kinases introduce additional feedback loops that indirectly regulate Cdk activity[130]. In this context, courtesy of Cdk activation, Wee1 and Cdc25 promote Polo-like kinase 1 (Plk1) for phosphorylation, which subsequently activates Cdk, further increasing its activity[131]. In contrast, several phosphatases counteract the activation of Cdk by dephosphorylating Cdk targets. The ability of phosphatases to regulate phosphorylation status has been shown to be crucial for proper progression of the cell cycle[132–134]. The importance of phosphatases in cell cycle regulation is most apparent at mitosis exit, where timely dephosphorylation of Cdk targets by PP1 and PP2A ensures successful completion of mitosis[135,136]. Indeed, depletion of PP2A in *Xenopus* egg extracts has been shown to cause hyperphosphorylation of Cdk targets, leading to premature mitotic entry with low levels of Cdk activity, and a subsequent failure to exit mitosis[132].

In interphase regulation, the PP2A family of phosphatases dephosphorylate Cdc25 and Wee1, thus counteracting Cdk-dependent phosphorylation. At the G2/M phase transition, Cdk-dependent activation of Greatwall kinases (Gwl) promotes phosphorylation of its substrate, Arpp19/Endosulfin-α. This directly inhibits PP2A, thereby preventing dephosphorylation of mitotic substrates during mitosis[132,135,137–139]. PP2A and Gwl are implicated in multi-layer feedback loops, which regulate Cdk activity. Cdk-dependent activation of Gwl inhibits dephosphorylation of Wee1 and Cdc25 by PP2A, resulting in enhanced activation of Cdk, while further promoting inactivation of PP2A[140,141]. This coherent feedforward loop contributes to the non-linear bistability of the mitotic entry network, which reinforces the decision to enter mitosis, thus providing a directionality and irreversibility to cell cycle transitions[134].

1.5 Spatial regulation

Cyclins are essential for activating Cdk, but also provide specificity to the action of Cdks by influencing Cdk subcellular localisation. This change in localisation allows Cdk to access spatially-restricted substrates or regulators in the cell[142–146], and cyclins also provide docking sites for certain Cdk substrates[55,147]. Thus, the spatio-temporal regulation of cyclin localisation determines the successful function of Cdk.

Cyclins localise differently in the cell during interphase; cyclin E and A (S phase onwards) are predominantly localised in the nucleus[99,148], whereas cyclin B mainly resides in the cytoplasm[149]. However, these cyclins are able to move between the nucleus and cytoplasm at specific times and are mediated by distinct shuttling mechanisms. Cyclin E contains a nuclear localisation sequence (NLS) at the N-terminus and its interaction with Importin-α/β drives translocation of cyclin E to the nucleus[144]. This localisation change is essential for the ability of cyclin E to promote the initiation of DNA replication[150].

Similar to cyclin E, translocation of cyclin B relies on the Importin/Exportin system. The interaction between Exportin1/Crm1 and the cytoplasmic retention sequence (CRS), which contains the nuclear export sequence (NES) at the N-terminus of cyclin B, promotes the cytoplasmic localisation of cyclin B[151]. Although cyclin B is constantly shuffled between the cytoplasm and nucleus during interphase, the rate of constitutive nuclear import is slower than the export rate with the net rate reflecting the cytoplasmic localisation of cyclin B[142,143,152]. Auto-phosphorylation of the CRS of cyclin B1 directs cyclin B1 for centrosome accumulation towards late G2 phase, which facilitates the interaction between cyclin B and other cell cycle regulators, including Plk1, Aurora A and Cdc25B/C, creating an active Cdk1 pool at G2/M phase[153,154]. In this sense, the centrosome provides a similar spatial function to that of the nucleus for cyclin B1-Cdk1 activation, and serves as a site where proteins that trigger mitosis can integrate[155]. Immediately preceding nuclear envelope breakdown (NEB), increasing Cdk1 activity modifies the nuclear transport machinery to greatly increase the nuclear import rate, triggering a rapid import of cyclin B1 into the nucleus[156]. Nuclear cyclin B1-Cdk1 is reported to activate the remaining cytoplasmic cyclin B1 by phosphorylation, and as a result further promotes cyclin B1 influx into the nucleus[157]. This spatial positive feedback loop is abolished when phosphorylation sites (Ser116, 126, 128, 133, and 147) in the CRS of cyclin B1 are mutated to alanine, which also delays the timing of nuclear translocation of cyclin B1 concomitant with the NEB[157]. However, these findings are in contradiction with a report where

phosphorylation of cyclin B1 is only required during interphase for initial activation in the cytoplasm, and is not necessary for rapid cyclin B1 nuclear translocation at prophase[156]. Regardless of the mechanism of nuclear translocation, the cytoplasmic retention of cyclin B1 prevents premature mitotic entry in higher eukaryotes, suggesting that the function of cyclin-Cdk localisation control contributes to regulating its activity[63].

Cyclin A also moves dynamically between the cytoplasm and nucleus, but mainly localises in the nucleus due to its steady-state concentration[99]. Since neither cyclin A nor its kinase partner contain an NLS consensus, how cyclin A is spatially controlled remains debatable. However, it has been shown that nuclear localisation of cyclin A correlates with its ability to form a complex with Cdk, suggesting cyclin A nuclear translocation may be subject to the formation of a multiprotein complex, which includes a Cdk catalytic subunit[158]. Cyclin A can bind to a number of NLS-containing proteins including p107, E2F1 and p21 [45,159,160]. Furthermore, ER-associated protein, SCAPER, has been identified to specifically interact with cyclin A in a cell cycle-dependent manner and its association promotes transient cytoplasmic localisation[161]. These findings suggested a mechanism whereby cyclin A binds to NLS-containing proteins to translocate to the nucleus[162]. However, it has been demonstrated that cyclin A is able to correctly translocate to the nucleus in p21-/- mice embryonic fibroblasts[163]. Thus, the idea that cyclin A re-localisation relies on NLS-containing proteins needs to be fully elucidated.

Besides cyclins, other proteins also have an ability to move between the nucleus and cytoplasm. A family of Cdc25 phosphatases containing an NES and NLS in their sequences, serve as a tool for facilitating the local activation of Cdks[164,165]. Cdc25B has been reported to specifically dephosphorylate Cdk inhibitory residues to activate cyclin B1-Cdk1 on the centrosomes, and is implicated in the initial site-specific activation of Cdk[166]. In line with this, Cdc25B is also re-localised to the cytoplasm in response to DNA damage, which creates spatial separation from cyclin-Cdk, and as such prevents nuclear Cdk activity[167]. During prophase, Plk1 phosphorylates the Ser198 residue in the NES of Cdc25C, promoting Cdc25C nuclear translocation where it inhibits Wee1[168]. The cytoplasmic translocation of Plk1 phosphorylates and inactivates Myt1, which promotes the local activation of cyclin B1-Cdk1. This creates a spatial feedback loop, further activating cyclin B1 and Cdc25, thereby contributing to the rapid nuclear import of cyclin B1-Cdk1 prior to mitotic entry[155,169–172].

1.6 G2 phase: Preparing for mitotic entry

1.6.1 Cyclin A2 and cyclin B1: An overview

Both cyclin A and B are accountable for regulating progression through G2 phase to mitosis, but have different characteristics and distinct roles during the cell cycle[39]. In humans, cyclin A (A1 and A2) and cyclin B (B1, B2, and B3) types are expressed in somatic cells, but cyclin A1 and B3 are mainly implicated in meiosis, while cyclin A2 and B1 mostly perform functions in G2/M phase[173–176].

In G2 phase, cyclin A2 facilitates transcriptional activation of kinases that are essential in G2/M phase progression, including cyclin B1, Cdc25 and Plk1[177–179]. At the G2/M phase transition, cyclin A2 coordinates centrosomal and nuclear events as an upstream regulator of cyclin B1-Cdk1[180,181]. In this context, during G2 phase, cyclin A2 regulates activities that are involved in creating positive feedback loops to increase cyclin B1-Cdk1 activity[182,183]. At mitotic entry, cyclin A2-Cdk promotes cyclin B1-Cdk1 nuclear translocation, chromosome condensation and NEB[184,185]. The role of cyclin A2 extends to prometaphase, where it facilitates chromosome segregation[186].

The main functions of cyclin B1-Cdk1 are to initiate mitotic entry and to ensure correct progression through mitosis. Cyclin B1 is first detected at the beginning of G2 phase and abruptly increases to its maximum level at mitosis[39,187,188] with support from the amplifying actions of several feedback loops[111,115]. APC/C-mediated degradation of cyclin B1 takes place at the metaphase/anaphase transition[113]. The catalytic activity of cyclin B1-Cdk1 is inhibited during G2 phase and this complex accumulates in the cytoplasm[189,190]. A small fraction of cytoplasmic cyclin B-Cdk1 translocates to the centrosome at the time of centrosome duplication[191]. This localisation change reflects its role in the initiation and separation of the centrosome, microtubule network reorganisation, and activation of several targets that regulate specific processes in mitosis[192–194]. The final, yet important role of cyclin B1-Cdk1 is to fully activate APC/C to promote its own subsequent degradation, as well as, degrading other regulators responsible for chromosome separation[195]. This step is required to reduce mitotic activities to a basal level, resetting the system so it can initiate a new cell cycle.

Since cyclin A2-Cdk drives G2 phase progression and facilitates the build-up of cyclin B2-Cdk1 activity to initiate mitotic entry, the regulation of G2 phase will focus on cyclin A2-Cdk function.

1.6.2 Cyclin A2-Cdk: The role of G2 phase progression and mitotic entry

Cyclin A2 is unique in that it has biphasic activity owing to its ability to form a complex with both Cdk2 and Cdk1[196,197]. Cyclin A2-Cdk2 is initially activated at the beginning of S phase, while a much stronger activation occurs in early G2 phase. This precedes cyclin B1-Cdk1 activation at G2/M phase, which makes cyclin A a regulator of G2 phase progression[85]. Despite this, the role of cyclin A-Cdk2 in G2 phase progression is relatively less well known than its implications in S phase. However, studies in different systems indicate a function for cyclin A2-Cdk2 in G2 phase progression. Induction of dominant-negative Cdk2 in human cells prevents G2/M phase transition[198], while in *Drosophila* cells cyclin A induces cell cycle arrest in G2 phase after removal of maternal cyclin A[199]. In human cells, siRNA depletion of cyclin A2 and inhibition of Cdk2, delay G2 phase progression and decrease cyclin B1-Cdk1 activity[180]. It has been shown that depletion of cyclin A and inhibition of Cdk2, in early G2 phase, decrease Cdh1 levels by stabilising the APC/C^{Cdh1} target, Claspin, and maintain the levels of activated Chk1, thereby arresting cells in G2 phase[200]. Furthermore, introducing cyclin A2-Cdk2 into early G2 phase Hela cells stimulates premature entry into mitosis[181], while a loss of cyclin A2 in G2 phase prevents mitotic entry[74]. Together, these findings suggest that cyclin A2-Cdk2 is an important rate-limiting component required for progression of G2 phase to mitotic entry.

The involvement of cyclin A in G2/M phase has been the subject of many studies, but its exact role and downstream targets for promoting mitotic entry have only recently started to be identified. Cyclin A2-Cdk2 is recognised as a major regulator of Cdc25C for antagonising Cdk1 Tyr15 phosphorylation[180,182], and stabilising cyclin B1, by phosphorylating Cdh1 for inhibiting APC/C^{Cdh1}-mediated proteolysis [200,201]. Meanwhile, cyclin A2-Cdk1 is initially activated in the middle of S phase and continues to increase until G2/M phase[202]. Recent discoveries have demonstrated the role of cyclin A2-Cdk1 in G2/M phase transition by promoting Plk1 activation[203–206]. Since Plk1 activation involves kinases other than Cdk1, the regulation of Plk1 by cyclin A2-Cdk2 is discussed in the following section. Cyclin A2-Cdk1 is degraded by the APC/C during prometaphase[207], in other words, it is required until that time. Mitotic entry initiates at prophase with centrosomal activation of cyclin B1-Cdk1 promoting microtubule nucleation at the centrosome[155,208]. A study on *Xenopus* egg extracts has shown that cyclin A-Cdk stimulates microtubule nucleating activity in the centrosomes[209]. This indicates that cyclin A-Cdk could have a role beyond promoting mitotic entry and establishes mitosis through affecting the mitotic machinery. In line with this, cyclin A2-Cdk2 depletion in

human cells results in a premature and increased microtubule nucleation at the centrosome with increased cyclin B1-Cdk1 activity[180]. In addition, cyclin A2-Cdk2 translocates to the centrosome shortly after centrosome separation in late G2 phase. Thus, cyclin A2-Cdk2 centrosomal localisation in late G2 phase coordinates nuclear and centrosomal mitotic events[165]. Furthermore, a recent study has shown that cyclin A facilitates kinetochore microtubule attachment and functions as a timer in prometaphase to ensure error correction and faithful chromosome segregation[186].

1.6.3 Polo-like-kinase 1 (Plk1)

Plk1 is a serine/threonine kinase, which contains an N-terminal kinase domain (KD) and a C-terminal domain, termed the Polo-box domain (PBD), which engages in protein interaction[210]. Plk1 is involved in regulating many different functions, including centrosome maturation, chromosome condensation, spindle assembly and cytokinesis[211,212]. In order to regulate these different functions, Plk1 needs to be activated in a timely manner and dynamically recruited to specific locations[213]. Plk1 localisation is controlled by substrate interactions with the PBD, which enables the KD to phosphorylate various effectors[214]. The PBD is a phospho-peptide domain and preferentially binds to phosphorylated targets prior to Plk1 docking, i.e. it requires priming phosphorylation[215]. This priming phosphorylation is usually mediated by Cdk1 during mitosis, however, during G2 phase when Cdk1 activity is low, Plk1 phosphorylates its targets before subsequent binding[216]. Moreover, reciprocal inhibitory action by the interaction between the PBD and KD keeps Plk1 in an inactive state[217,218]. Thus, Plk1 activation relies on post-translational modification and structural changes[210].

1.6.4 Mechanism of Plk1 regulation at the G2/M phase transition

Plk1 is activated by phosphorylation of the Thr210 residue in its T-loop. Phosphorylation at this residue is primarily mediated by Aurora A[219,220]. Aurora A is diffusely distributed throughout the cytoplasm until late G2 phase, and a fraction is enriched in the centrosomes and spindle microtubules, where it rapidly exchanges with cytoplasmic pools[221,222]. It has been reported that the initial phosphorylation of Plk1 occurs in the centrosome during G2 phase, however, concomitant with its activation Plk1 quickly translocates to the nucleus in prophase[223]. Furthermore, restricting Plk1 localisation in either the nucleus or in the cytoplasm

prevents entry into mitosis[223], which highlights the importance of nuclear import as a mechanism of activating Plk1.

Plk1 phosphorylation at the Thr210 residue in the KD has been shown to disrupt binding of the PBD to the KD[217]. This dissociation of the PBD and KD exposes the NLS in the KD, enabling nuclear translocation of Plk1[224]. Thus, Plk1 can rapidly translocate to the nucleus upon its activation. However, SUMOylation of the PBD at Lys492 in human cells is also shown to be responsible for Plk1 nuclear import. This process is facilitated by the SUMO conjugating enzyme, Ubc9. Cdk1-dependent phosphorylation of Ubc9 enhances its binding to Plk1, as well as, increases the SUMOylation activity of Ubc9[225]. Nevertheless, the functional relationship between KD phosphorylation in activating Plk1, and PBD SUMOylation in Plk1 nuclear translocation, requires further exploration.

1.6.5 The contribution of Plk1 in mitotic entry by creating a feedback loop

Plk1 is an upstream regulator of Cdc25B and Cdc25C isoforms in humans[226], and nuclear translocation of Cdc25B and Cdc25C has been observed upon Plk1 activation[168,170]. Plk1 phosphorylation of Cdc25C at multiple residues in its N-terminus during mitotic entry has also been reported. Overexpression of phosphomimetic Cdc25C mutants progress into mitosis, even in the absence of Plk1 activity[203], suggesting that Plk1-dependent phosphorylation of Cdc25C in late G2 phase is essential in promoting a timely G2/M phase transition. Nevertheless, studies around how much Plk1 contributes to promoting mitotic entry by regulating Cdc25A and B are currently limited.

Besides controlling Cdc25, Plk1 also supports transition into mitosis by regulating other kinases. Plk1-dependent phosphorylation of Wee1 paves the way for its proteasome-dependent degradation upon ubiquitination, thereby enhancing the Cdc25 positive feedback loop[227]. Plk1 also regulates Gwl, a kinase that antagonises the dephosphorylating action of PP2A-B55 complex to promote mitotic entry. As mitosis begins, Gwl phosphorylates Arpp19/Endosulfin-α, which in turn selectively inhibits PP2A-B55[132,135,137–139]. Gwl translocates to the cytoplasm in prophase, where PP2A-B55 is also concentrated, and this localisation change is important for Gwl function [228,229]. It has been shown that the cytoplasmic translocation of Gwl just before NEB is facilitated by Plk1 and Cdk1-dependent phosphorylation in *Drosophila* [229]. However, Cdk1 is only responsible for the spatial regulation of Gwl in human cells[228].

1.6.6 Plk1 upstream regulators: Cyclin A2-Cdk2/1 and cyclin B1-Cdk1

Aurora A cofactor Bora is essential in Plk1 activation, and is conserved from *C. elegans* to humans[230]. During G2 phase, Bora exclusively localises in the cytoplasm and facilitates Plk1 phosphorylation at Thr210 on its T-loop by Aurora A at late G2 phase[223]. Importantly, Cdk activity promotes the function of Bora in Plk1 activation . It has been shown that Cdk phosphorylates Bora at multiple serine/threonine residues, but phosphorylation of the three most conserved residues in the its N-terminus are essential for the function of Bora in Plk1 activation[204]. Thus, Cdk activity regulates Plk1 activity by phosphorylating its downstream targets to prime their interaction with the PBD, as well as, activating Plk1 via Aurora A[232].

It has been a long-standing question as to what activates Bora, and thereby Plk1, in G2 phase. Bora can be phosphorylated by cyclin B1-Cdk1 and cyclin A2-Cdk2/1, as it contains a cyclin binding motif[204–206]. Since Plk1 is activated in late G2 phase, shortly before cyclin B1-Cdk1 activation[203], cyclin A2-Cdk2/1 is considered a better candidate for phosphorylating Bora. Indeed, recently we have reported that in human cells *in vitro*, cyclin A2-Cdk2 can activate Plk1 by phosphorylating Bora in G2 phase, and that Bora and Plk1 interacts exclusively with cytoplasmic cyclin A2[205]. On the other hand, others have reported that cyclin A2-Cdk1 can activate Plk1 to promote mitotic entry by phosphorylating Bora in G2 phase[203,204]. A study conducted on *Xenopus* egg extracts has shown that Bora depleted interphase extracts failed to reactivate Plk1, dephosphorylated Cdk1 at Tyr15, and blocked entry into mitosis after adding recombinant hyperactive Gwl[206]. Interestingly, in this study, the addition of the N-terminal fragment (human/*Xenopus*) corresponding to the most conserved phosphorylation sites rescued mitotic entry, but only when the phosphorylation residues and cyclin binding site were preserved. However, endogenous Bora cannot be re-activated in the absence of cyclin A, and introducing Bora that is resistant to endogenous phosphatases solely into a cyclin A depleted extract, rescues Plk1 phosphorylation and mitotic entry, suggesting that cyclin A-Cdk1 is dispensable for mitotic entry once Bora is fully phosphorylated. Taken together, these findings demonstrate that phosphorylation of Bora by cyclin A-Cdk complex in G2 phase is essential and sufficient for Plk1 phosphorylation and mitotic entry[206]. Taken further, another study has reported that cyclin B1-Cdk1 associates with Bora in mitosis[204], which suggests that cyclin B1-Cdk1 likely facilitates the maintenance of Plk1 activity during mitosis by phosphorylating Bora.

1.7 Cell cycle dysregulation and its relevance in disease

Cartilage-hair hypoplasia (CHH) is a skeletal dysplasia inherited as an autosomal recessive trait, arising from mutations in the non-coding RNA component of mitochondrial RNA-processing endoribonuclease, encoded by the *RNase MRP* gene (*RMRP*)[233,234]. CHH is a pleiotropic disorder and affected individuals are characterized by impaired T-lymphocyte function, defects in cell proliferation and an increased susceptibility to developing cancer[235]. It was recently reported that small RNAs derived by the *RMRP* gene display regulatory properties in over 900 genes, including genes that regulate cell proliferation[236]. In line with this finding, others have reported that knockdown of *RMRP* significantly inhibits cell proliferation in *in vitro* and *in vivo*, while over expression of this gene promotes cell growth[237]. In addition, RMRP forms a complex with telomerase-associated reverse transcriptase (TERT) and regulates gene expression through RNA-dependent RNA polymerase activity[238]. This activity has been identified in highly malignant tumour cells. Although, telomerase activity in CHH is not addressed in this study, the interaction of RMRP with TERT raises the possibility that telomere dysfunction forms part of the cellular phenotype of this disorder.

1.8 Cell cycle regulation in response to DNA damage

1.8.1 DNA damage: Double strand breaks

The bases of DNA are highly vulnerable to chemical modifications, which can create numerous lesions[239]. DNA lesions affect essential genomic processes, such as transcription and replication. A normal human cell encounters up to 10^5 spontaneous DNA lesions per day, of which 0.1% are thought to be DNA double-strand breaks (DSBs) [240–242]. While DSBs do not occur as frequently as single-strand breaks (SSBs), DSBs are the most cytotoxic form of DNA lesions and difficult to repair[243]. Moreover, SSBs can lead to DSBs when two SSBs arise in close proximity, or the DNA-replication apparatus encounters an SSB[244]. However, DNA lesions can occur as a side effect of DNA metabolising processes (e.g. replication errors)[245,246], but the highest genomic burden is induced by a variety of agents and processes that either alter the DNA sequence directly or cause mutations when DNA is sub-optimally repaired [242,247]. The most pervasive exogenous DNA damaging agent is ionising radiation (IR), which directly induces DSBs[248]. Imprecise re-joining of the broken DNA ends at DSBs causes mutations and

genomic aberrations, which ultimately lead to cellular dysfunction or cell death[249]. In this respect, DSBs are the most detrimental to genomic stability. Therefore, it is important to understand the intricate regulation of the DNA damage response (DDR) upon DSB formation. To protect genomic stability, cells have evolved mechanisms that recognise and repair different types of DNA lesions. In this thesis, DNA damage was induced by γ-IR and Etoposide (topoisomerase inhibitor II), which create DSBs for inducing apoptosis, and are clinically useful as therapies to treat cancer. Therefore, the following section on DNA damage repair focuses on DSBs.

1.8.2 DNA damage response

The DDR is a large signalling transduction network that senses DNA damage and activates multiple cellular responses, including transcriptional changes, cell cycle transition regulation, recruitment of DNA repair machinery and induction of apoptosis or senescence if the DNA damage is irreparable[250,251]. Activation of a cell cycle checkpoint to regulate cell cycle transition is one of the multifaceted purposes of DDR[252]. However, the main goals of DDR are to repair DNA damage and facilitate DNA replication, while blocking proliferation if the damaged DNA is beyond repair, thereby serving a role in preventing cancer development.

DSBs are detected by Mre11-Rad50-Nbs1 (MRN) and Ku heterodimer (Ku70/ku80) complexes, which subsequently recruit proteins in the apical phosphatidylinositol 3-kinase-like kinases (PIKKs) family. The family of PIKKs, ataxia telangectasia mutated (ATM) and DNA-dependent protein kinase catalytic subunit (DNA-PKcs) are primarily activated at a DSB site, while ATM-and-rad-3-related (ATR) is activated by single-stranded DNA that occurs during the processing of DSBs or DNA replication in S phase[253,254]. Active PIKKs rapidly phosphorylate histone variant H2AX at serine 139 at sites proximal to the DSB[255–257]. Phosphorylated H2AX, which is referred to as γH2AX, serves as a platform for the recruitment of additional DDR factors, such as mediator of DNA damage checkpoint 1 (MDC1)[258–261]. Additional phospho-dependent recruitment of MRN and ATM by MDC1 results in amplification of the ATM signalling[258]. This leads to spread of γH2AX and MDC1 foci over a 2Mb domain of chromatin near the DSB[255]. In addition, phosphorylated MDC1 recruits RING-finger ubiquitin E3-ligases, RNF8 and RNF168, to initiate K63-linked polyubiquitination of H2A and H2AX. These ubiquitin chains are required for binding of BRCA1 and p53-binding protein 1 (53BP1), which activate the DDR[262,263].

1.8.3 The mechanisms of DNA DSB repair

Cells have evolved efficient mechanisms to repair DSBs through multiple pathways of homologous recombination (HR) and non-homologous end-joining (NHEJ) processes. DSBs are predominantly repaired by the canonical NHEJ pathway, which is initiated by binding of Ku heterodimer (Ku70/Ku80) at the DSB DNA ends[264,265]. Ku70/Ku80 protects the DNA ends against nuclease digestion, but does not impede ATM activation or signalling[266,267]. DNA-bound Ku70/Ku80 recruits DNA-PKcs to form a heterotrimeric complex, DNA-PK[268]. This complex formation stabilises the DNA ends at the DSB sites, creating a synaptic complex that holds the two DNA termini together[269]. The catalytic activity of DNA-PK is activated once bound to DNA, phosphorylating the ligation complex, which includes DNA ligase IV, X-ray repair cross-complementation group 4 (XRCC4) and XRCC4 like factor/Cernunnos, for completion of the pathway[270–273]. In addition, DNA-PK activity regulates end processing by recruiting Artemis nuclease when resection is required for re-joining the DNA ends[274]. While NHEJ is a relatively fast and putatively error-prone pathway, which involves minor modification to the DNA ends, a subset of DSBs engage with the HR pathway for extensive processing, which uses an intact homologous template to restore the lost sequence information[240,275,276]. As a result, HR is restricted to S and G2 phase when a sister chromatid is available, while NHEJ is used throughout the entire cell cycle, but preferentially takes place during G0, G1 and early S phase[277–279]. HR is initiated by Mre11 endonuclease, stimulated by CtBP-interacting protein (CtIP) at the broken DNA ends, creating an initial single strand nick[280,281]. CtIP continues end resection, assisted by helicases and exonucleases (i.e. DNA2, BLM, WRN, CtIP and EXO1), promoting the formation of single strand DNA for HR[282,283]. Replication protein A (RPA) rapidly binds to the single strand DNA tail, eliminating secondary structures, and is subsequently replaced by RAD51 through a BRCA2-dependent process[284]. RAD51 replacement promotes invasion onto the intact homologous sequence on the sister chromatid, generating a D-loop necessary for Holliday junction formation[285]. Precise DSB repair ensues using the intact sister chromatid as a template, followed by resolution of the Holliday junctions and ligation of the DNA ends. This resolution process results in either crossover or non-crossover products depending on the direction of resolution[286].

1.8.4 The choice of DSB repair pathway

The DSB repair pathway that cells select is influenced by several factors, such as cell cycle phase, DNA damage complexity or genomic location[274]. HR requires CtIP for resection of the DSB end to generate single strand DNA, which is activated in a Cdk-dependent manner[287]. The timing of Cdk activity corresponds to HR usage in S and G2 phase, which demonstrates that the cell cycle phase is a factor in the choice of repair pathway[280,288–290]. The DNA structure of the DSBs also affects the pathway cells use. During DNA replication in S phase, one-ended DSBs are generated when replication forks encounter SSBs. In this case, HR is promoted for DSB repair, as ligation by NHEJ requires another DNA end[291]. The complexity of DSBs can lead to resection in an ATM-dependent manner. ATM is activated throughout the cell cycle resulting in recruitment of 53BP1 at DSB sites[292]. Tight binding of 53BP1 leads to chromatin compaction and directly or indirectly inhibits DNA nuclease to restrict resection[293]. The tightly bound 53BP1 possibly contributes to the maintenance of the Ku70/Ku80-DNA-PKcs complex at the DNA ends, which promotes NHEJ. Although approximately 70% of DSBs are repaired by NHEJ in G2 phase, cell cycle-dependent activation of BRCA1 antagonises 53BP1 function, conferring 53BP1 repositioning for HR[294–296]. However, the mechanism by which 53BP1 repositioning affects a change in the chromatin environment creating a preference for HR progression is unclear.

1.8.5 The DNA damage checkpoint at G2/M phase

The activation of cell cycle checkpoint regulation and cell fate are strictly reliant on the phase of the cell cycle. Depending on the time that cells encounter DNA damage, these cells will either arrest in G1 or G2 phase, or delay S phase progression. These DNA damage checkpoints provide the time to evaluate the severity of DNA damage and initiate repair or terminal cell cycle exit when the damage is too extensive. After the lesion is repaired, a reversal of these checkpoints is required to restore the cell cycle machinery, allowing resumption of the cell cycle. Therefore, these checkpoints are important for maintenance of the genomic integrity of proliferating cells.

The G2/M checkpoint prevents cells from initiating mitosis when encountering DNA damage in G2 phase, unrepaired lesions sustained from previous cell cycle phases, or when the replicated DNA is suboptimal[297,298]. The critical target of the G2/M checkpoint is the activities

of Cdk1 and Plk1, which are responsible for the onset of mitosis. ATM and ATR both contribute to the checkpoint, together with their downstream target kinases, Chk2 and Chk1, respectively. After the introduction of DNA DSBs, ATM and Chk2 initiate checkpoint activation, which is subsequently followed by ATR and Chk1 mediated cell cycle arrest. As part of rapid-signalling axes, ATM and ATR in turn activate both Chk2 and Chk1, which inhibit Cdc25[299–301]. Chk1-and Chk2-mediated phosphorylation of Cdc25 creates a binding site for 14-3-3 proteins that block substrate access to the catalytic site, thereby preventing the removal of inhibitory phosphates on the Cdks[302–305]. In addition, 14-3-3 proteins stimulate the NES that sequesters Cdc25 in the cytoplasm, causing separation from the nuclear pools of Cdks[306–310], and this compartmentalisation of Cdc25 impedes the activation of Cdks. Although the p38 MAPK family has been primarily described for non-IR types of stress induced cell cycle arrest, it can activate the G2/M phase checkpoint after genotoxic stress caused by IR-induced DSBs[311–313]. p38 also phosphorylates Cdc25 at the same site as Chk1 and Chk2, which results in binding of 14-3-3 proteins. Cdc25A is mainly implicated in G1/S phase transition, but is also involved in mitotic entry. In addition to Chk1 and Chk2-induced 14-3-3 binding, Cdc25A is regulated through ubiquitin-dependent proteolysis[126,314–316]. At the opposite end of the bistable switch system, Chk1 activation has an effect on up-regulating Wee1-dependent inhibitory phosphorylation on Cdks, required for maintaining cell cycle arrest[317]. Activation of a feed forward loop driving Cdk1/2 and Plk1 induces mitotic entry[318,319]. Chk1 inhibits Plk1 activity by targeting the recruitment of Aurora A to Plk1/Bora complex, which prevents phosphorylation of Plk1 at Thr210[320]. However, during checkpoint recovery, Plk1 counteracts Chk1 activity by targeting its co-activator, Claspin, for degradation[321,322]. Plk1 also inactivates Chk2 and 53BP1, which might contribute to inactivation of p53[323,324]. Checkpoint recovery in the absence of p53 is possible in the presence of ATR activity, but is blocked by the ATM spread on chromatin[325].

In unstressed conditions, expression of p53 remains low due to rapid degradation mediated by Mdm2 E3 ubiquitin-protein ligase[326,327]. In response to DNA damage, several checkpoint activators, including ATM, Chk1, Chk2 and p38 phosphorylate p53, thereby preventing recognition by Mdm2[328,329]. ATM phosphorylates Mdm2 directly, promoting Mdm2 for self-ubiquitination[330,331]. These processes stabilise p53, which then allows it to function as a transcription factor. p53 up-regulates expression of p21 and directly suppresses promoters of several key cell cycle regulatory genes, such as cyclin B1 and Plk1, leading to cell cycle arrest[332,333]. Depending on the level of DNA damage generated during S phase, p53-dependent

p21 starts to accumulate in G2 phase and continues into the subsequent G1 phase, where sufficiently high levels of p21 mediate arrest in G1 phase by inhibiting Cdk activity[334,335].

1.9 Cell fate after DNA damage

Unresolved DNA damage is a potential threat to genomic integrity. To ensure the maintenance of this integrity, cells have evolved signalling pathways and mechanisms in addition to the DDR. Depending on the severity of DNA damage, cells can selectively engage different programmes, which decide the fate of the cell. This decision is not simply a choice between 'death' or 'recovery', but a diversity of cell outcomes are possible. Thus, the focus of this chapter is on introducing different cell fate outcomes after DNA damage-induced cell cycle arrest.

1.9.1 Checkpoint recovery

Perhaps the main biological aim of a DNA damage checkpoint is to provide sufficient time to repair the inflicted damage before cells can eventually resume and continue the committed cell cycle. While the process of checkpoint recovery from G2 phase mirrors the initiation of unperturbed mitotic entry[130], it requires a rearrangement of the cell cycle machinery, as the DDR inhibits the activities of pro-mitotic kinases and degrades proteins that promote mitotic entry[336–338]. In this context, Plk1 together with its activator Aurora A and Bora become essential for resuming the cell cycle after DNA damage-induced G2 phase arrest by activating cyclin B1-Cdk1[130,339,340], whereas in unperturbed cells Plk1 is less important for mitotic entry. At the same time, recovery from this checkpoint relies on the stabilised Cdc25 isoform, Cdc25B, in the absence of Cdc25A, reducing the redundancy of Cdc25 phosphatases in normal mitotic entry[340,341].

In addition to its role in re-activating cyclin B1-Cdk1, Plk1 regulates the silencing of DDR signals[342,343]. The checkpoint recovery in G2 phase is driven by the same mechanism that activates Plk1 to prevent checkpoint signalling in mitosis. During checkpoint recovery, Plk1 phosphorylates Claspin and Wee1 for $SCF^{\beta\text{-TrcP}}$-mediated degradation[124,342–344]. While the degradation of Claspin inactivates its co-activator Chk1, allowing the activation of Cdc25B and re-accumulation of Cdc25A, the destruction of Wee1 disposes of direct Cdk

inhibition[340,341,344]. Thus, these processes converge to create feedback loops that activate cyclin B1-Cdk1 (See section 1.4.5). Plk1 also inactivates checkpoint signalling through inhibition of Chk2 by phosphorylation[345,346]. It has been indicated that Plk1 could facilitate inhibition of p53 by either direct or indirect mechanisms[347–350]. Despite a number of findings pointing towards Plk1 as a key regulator that drives the transition from checkpoint arrest to recovery, there is little known about how Plk1 is reactivated at checkpoint recovery. Recently, a mechanism of Plk1 re-activation has been described, where DNA damage-dependent ATM activity across chromatin is counteracted by chromatin-bound Wip1 and re-activated Plk1, even in the presence of active ATM/ATR and DNA break sites[351]. Nevertheless, it should be kept in mind that other factors could be implicated in regulating G2 phase checkpoint recovery. Despite this, the role of Plk1 in activating cyclin B1-Cdk1 is insufficient to account for the entire process. There is emerging evidence that suggests that some phosphatases could counteract ATM/ATR and DNA-PK-dependent phosphorylation, bringing the balance back to the steady-state before DNA damage occurred. Although further supporting evidence is required, Wip1 and PP2A-dependent dephosphorylation of γH2AX, Chk1, Chk2 and p53, are likely to represent additional rate-limiting factors involved in checkpoint recovery[352–355].

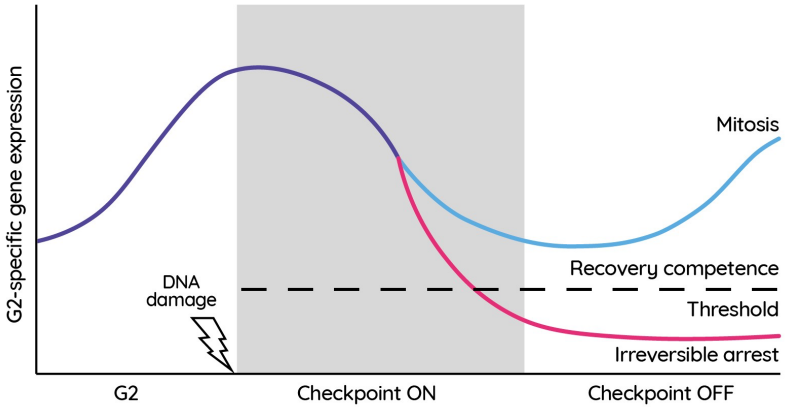

Figure 3. Regulation of recovery competence in G2 phase.

1.9.2 Checkpoint adaptation

Another mechanism that regulates the cell cycle after checkpoint arrest is related to, but conceptually distinct from, checkpoint recovery and termed checkpoint adaptation. Checkpoint adaptation was first described in *S. cerevisiae* where cells overrode checkpoint arrest and entered mitosis in the presence of unrepaired DNA damage[356]. This mechanism is conserved

in evolution, and both human and yeast cells escape checkpoint arrest in a Chk1- and Plk1-dependent manner[356–358]. Degradation of Plk1 upon DNA damage insult is essential for checkpoint arrest, and cells expressing a constitutively active Plk1 mutant increases the fraction of cells proceeding to mitosis in response to DNA damage[338]. It has also been reported that irradiated G2 phase cells progress to mitosis with unresolved DNA damage, indicating sustained G2 phase checkpoint arrest may require a threshold of persistent DNA damage[359–361]. During checkpoint arrest, Plk1 activity continually accumulates and once it reaches a threshold level cells enter mitosis regardless of the presence of DNA damage or a checkpoint signal[361]. In this sense, checkpoint adaptation and recovery is possibly a related process and the duration of checkpoint arrest is not necessarily decided by the DNA repair status. Indeed, after substantial hours of delay in G2 phase, in response to DNA damage insult, cells progress into mitosis in a relatively synchronous manner[362,363]. A study based on mathematical modelling has demonstrated that the G2 phase checkpoint duration does not function by sensing completion of the DNA damage repair, but is controlled by the balance of ATM/Wip1 on chromatin[351]. Thus, checkpoint recovery or adaptation is likely to be driven by Plk1 together with other upstream regulators.

Checkpoint adaptation seems unfavourable for maintaining the genomic integrity of cells. However, as a last resort the presence of a prolonged high level of checkpoint signalling leads to permanent termination of the cell cycle programme[364–366]. In this context, checkpoint adaptation provides an opportunity to repair some DNA damage without too much of a delay in cell cycle progression, while cell fate is decided at the next control mechanism[367–372]. This is most clearly demonstrated by cells that enter mitosis with severe DNA damage, which leads to apoptosis via mitotic catastrophe[370–372]. In cases where cells completed mitosis, the DNA-damage signalling is attenuated, with full DDR activation only taking place when a DSB-containing mitotic cell enters G1 phase[369]. Activation of checkpoint signalling in G2 phase can sensitise checkpoint-abrogated cells for cell cycle arrest or delay at the next G1 phase. It has been reported that p21 regulates the bifurcation in Cdk2 activity at mitotic exit, which dictates the next cell fate. This study has demonstrated that a low level of residual Cdk2 activity establishes a quiescent state in cells that have completed mitosis[373]. Consistent with such a concept, recent work has shown that activation of p53 in G2 phase is a critical step for DNA damage-induced quiescent daughter cells[374]. This study describes that DNA damage increases the half-life of p53 and transmits p53 signalling into the daughter cells, inducing the *de novo* expression of p21 in these cells. Furthermore, p21 outcompetes the mitogen signal, cyclin D1,

leading to the daughter cells becoming quiescent. On another note, PP2A-B56γ-dependent dephosphorylation of pRb in G2 phase reduces Cdk2 activity in T98G cancer cells, which is required for the subsequent development of quiescence after exiting mitosis[375]. As ATM can activate PP2A by direct phosphorylation of B56γ[376], ATM possibly contributes to inhibition of Cdk2 activity, together with p21, in checkpoint-abrogated cells to induce quiescence. Taken together, checkpoint adaptation in G2 phase could be essential for safeguarding genomic stability. Moreover, checkpoint signalling in G2 phase plays a key role in the cell fate decision making of checkpoint-abrogated cells.

1.9.3 Terminal cell cycle exit, all the way to 'late' senescence

In addition to inducing temporal cell cycle arrest, checkpoint activation can induce the terminal cell cycle exit programme when cells are exposed to severe DNA damage. As discussed during DDR, p21 suppresses Cdk activity, but is maintained at a low level, allowing time for repair of the damaged DNA. This remaining Cdk activity is coupled to a negative feedback loop by inducing p21 expression, which is required for sequestration of cyclin B1 in the nucleus and the transition to terminal cell cycle exit[377]. Also, it has been suggested that a change in the oscillating expression of p53 to a continual expression may be critical for p21 expression, driving temporal arrest to terminal cell cycle exit[378]. Nevertheless, cyclin B1 nuclear translocation marks the point-of-no-return during checkpoint arrest, as cells with nuclear cyclin B1 fail to enter mitosis upon checkpoint inhibition[365]. However, subsequent degradation of nuclear cyclin B1 by APC/C^{Cdh1} is required to induce terminal cell cycle exit[365]. p21 is responsible for premature activation of APC/C^{Cdh1} by inhibiting Emi1[379,380], which reinforces the termination of the cell cycle by degradation of all the cell cycle proteins[381].

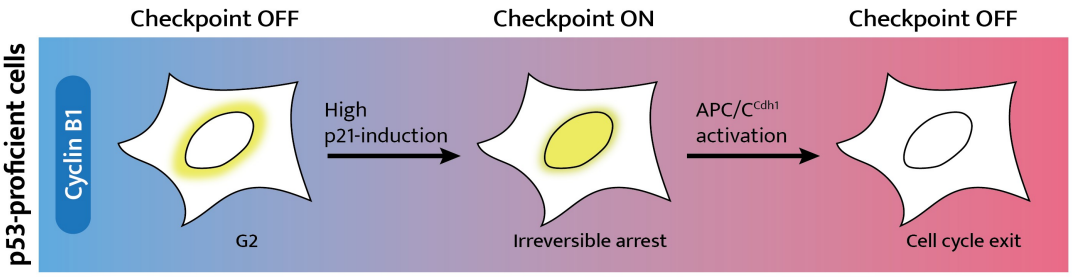

Figure 4. p21-dependent and APC/C^{Cdh1}-mediated cell cycle termination in G2 phase

Cells that terminate the cell cycle eventually become senescent. Senescence is considered a stable and permanent state of cell cycle arrest from which cells are unable to resume the cell cycle, despite optimal growth conditions and mitogenic stimuli[382]. Although senescent cells are insensitive to growth stimuli, these cells are metabolically active and resistant to apoptotic cell death owing to an upregulation of cell survival pathways[383,384]. However, it remains unclear whether this prolonged viability is the result of the selection of cells that are resistant to apoptosis or is an intrinsic property of the senescence programme.

Until now, senescence has been perceived as a static endpoint. However, several recent findings support the notion that senescence can be a highly dynamic and multi-step process, during which the properties of senescent cells evolve and diversify, as in tumourigenesis, but without proliferation as a driving force[385–387]. Progression to full senescence involves global and local modifications in chromatin methylation, which are suggested to be mediated by transcriptional downregulation of lamin B1[388–390]. The global induction of heterochromatin results in senescence-associated heterochromatin foci (SAHF) formation [391–393] , which depends on HIRA, ASF1 [394,395], and p16 [393,396]. SAHF are proposed to be enforcers of senescence by repressing the transcription of pro-proliferative genes [397,398]. Also, senescence-related chromatin modification leads to enormous transcriptional changes[388,399,400], including upregulation of genes that encode for proinflammatory secretory proteins, such as IL-6 and IL-8[401,402]. These proteins are collectively termed as the senescence-associated secretory phenotype (SASP)[401,402], and are suggested to induce local inflammation by attracting immune cells.

The establishment of full senescence in cells grown *in vitro* usually takes several weeks after exposure to the onset of senescence, but variability exists between cells and this process can even take several months[385]. Furthermore, even after cells are fully senescent, these cells continue to evolve and progress to a stage referred to as 'deep' or 'late' senescence[403]. In late-senescence, there is an increase in transcription of transposons and these newly synthesised transcripts accumulate in the cell genome[385,386]. This increasing transposon activity opens up the gene-poor heterochromatin regions, where these elements are located[385]. Another process involved in the development of late-senescent cells is the extrusion of chromatin into the cytoplasm, which is subsequently degraded by lysosomes and results in an overall loss of histones[387]. Thus, genomic and epigenomic re-modelling during transposon activation and the chromatin extrusion processes, contribute to the transcriptome diversity seen in senescent cells.

The dynamic nature of senescence indicates that senescence-associated cell cycle arrest may not necessarily be terminal. Several studies have shown that senescent cells are able to resume proliferation, suggesting that senescence is a biological continuum, at both the cellular intrinsic and extrinsic levels[404–408]. In line with this, recent growing evidence points towards the idea that cells can escape terminal cell cycle arrest after DNA-damage induced cell cycle exit in G2 phase[404,409,410]. However, the mechanism of this escape and consequences on cell fidelity, remain elusive. A recent study suggests that oscillating p53 triggers a sharp switch between p21 and Cdk2, permitting cells to escape after long-term cell cycle arrest[411]. Furthermore, eventual inactivation of APC/C^{Cdh1} has been reported in cells that escape DNA damage-induced terminal cell cycle exit[410]. The low level of p21 and inactivation of APC/C^{Cdh1} may cause cells to be responsive to mitogenic stimuli. Also, these escaped cells re-initiate S phase and progress to mitosis resulting in genomic heterogeneity[409,410], which is often implicated in the progression to malignant transformation in various cancers[412,413]. Thus, escaping terminal cell cycle arrest after DNA damage-induced G2 phase cell cycle exit could lead to oncogenic transformation[414].

2 AIMS OF THE THESIS

The main aim of the work presented in this thesis is to understand the molecular mechanisms of cell cycle regulation in human cells. In particular, my study has focused on G2 phase regulation, including the long-term consequences after DNA damage, and how G2 phase is affected in a human genetic disorder.

The specific aims addressed in this thesis are as follows:

The aim of **Paper I** was to understand the role and mechanism of Cyclin A localisation changes in human cell cycle regulation.

Paper II focused on determining the role of human long non-coding RNA gene, *RMRP*, in regulating cell cycle progression in CHH.

Paper III aimed to elucidate the long-term consequences of cells after DNA damage-induced cell cycle exit in G2 phase.

3 RESULTS AND DISCUSSION

3.1 PAPER I

Cyclin A2 localises in the cytoplasm at the S/G2 transition to activate PLK1

Preparing entry into mitosis requires extensive cellular structural reorganisation, tightly coordinated by a complex network of regulatory proteins that control the timely activation of Cdk1[415]. However, the exact mechanisms that ensure spatio-temporal activation of these regulatory proteins during G2 phase remain elusive. We address this question through our work presented in **Paper I**.

The main drivers of mitotic kinases, Cdk1 and Plk1, can be detected from the S/G2 phase transition[187]. Due to feedback loops, the activities of Cdk1 and Plk1 gradually increase through G2 phase, but the starting points of these feedback loops are difficult to define[416,417]. Given that cyclin A2 is activated before cyclin B1, and necessary for G2 phase progression[85], it is reasonable to think that cyclin A could have a role in initiating these feedback loops. We therefore established an RPE cell line where eYFP is inserted in the endogenous locus of *CCNA2* to characterise the dynamics of cyclin A2 in live cells. We revealed cytoplasmic cyclin A2 first appeared at the S/G2 phase transition and gradually increased throughout G2 phase. This indicates that the cytoplasmic appearance of cyclin A2 is coupled to this cell cycle event. In addition, siRNA-mediated depletion of cyclin A2 delayed S/G2 phase transition, but was partially rescued by induction of siRNA-resistant cytoplasmic cyclin A2. This suggests that cytoplasmic cyclin A2 has a role in stimulating cell cycle progression.

We also observed cyclin A2 depletion impairs Plk1 activation, meaning that either cyclin A2 may regulate Plk1, or the completion of S phase was impaired. We therefore monitored Plk1 activity upon Cdk1 or Cdk2 inhibition in G2 phase to address the contribution that cyclin A2-Cdk complexes make after the S/G2 transition. Inhibition of Cdk1 or Cdk2 decreased phosphorylation of Plk1 at T210, but more prominently when both Cdks were inhibited, indicating Cdk activity is required for Plk1 activation during G2 phase. In previous studies, cyclin A2-Cdk1-mediated activation of Plk1 by Bora has been described[204,206]. However, we identified that cyclin A2-Cdk2 can stimulate Aurora A-mediated activation of Plk1 by phosphorylating Bora. Interestingly, Bora and Plk1 both interact exclusively with cytoplasmic

cyclin A2 in G2 phase, indicating cyclin A2 cytoplasmic localisation is required for Plk1 activation through Bora. This suggests Plk1 activation does not depend on specific Cdk activity, but it is likely that spatial regulation of cyclin A2 contributes to Cdk substrate specificity. This was more clearly demonstrated when we assessed the function of cyclin A2 on Plk1 activation in cell cycle progression. Expression of active Plk1 could partially rescue mitotic entry after depletion of cyclin A2, indicating that Plk1 is an important target for cyclin A2 to promote mitosis. Taken together, cyclin A2 participates in the initial activation of Plk1 by localising in the cytoplasm to interact with cofactor Bora during G2 phase, and thereby promoting mitotic entry.

Since cyclin A2 starts to accumulate in the cytoplasm at the initiation of G2 phase, we reasoned that the cytoplasmic appearance of cyclin A2 could be triggered by mitotic kinase activity. However, we found no evidence that Cdk1 and Plk1 activity regulated cytoplasmic translocation of cyclin A2 at the S/G2 phase transition. This suggests cytoplasmic cyclin A2 translocation is not a component of the feedback loops, but is an important factor for triggering activation of mitotic kinases. We found an indication that Cdk1 may facilitate cytoplasmic cyclin A2 localisation, but the presence of cytoplasmic cyclin A2 with both Cdk1 and Cdk2, suggests an association with Cdk1 is not solely accountable for the cytoplasmic appearance of cyclin A2. Given that cyclin A2 localises in the nucleus while DNA replication occurs, and directly interacts with PCNA, we decided to investigate if cyclin A2 is restricted to the nucleus during S phase. We observed an increased cyclin A2 association with chromatin during replication, and more specifically with replicating chromatin, but there was no clear mobility restriction of chromatin associated cyclin A2 in the nucleus between S and G2 phases. Thus, the decreasing association of cyclin A2 with chromatin is not responsible for the cytoplasmic localisation of cyclin A2 at the S/G2 phase transition.

Activities that promote mitotic entry can be limited during DNA replication by certain signalling components, which resemble a low-level of DDR[418,419]. We therefore assessed the regulation of cytoplasmic cyclin A2 in response to DNA damage. Cytoplasmic cyclin A2 rapidly translocated to the nucleus upon DNA damage and was subsequently degraded, the timing of which was comparable with previously described p53- and p21-mediated degradation of cyclin B1-eYFP. Moreover, cells that received DNA damage in G2 phase showed an apparent nuclear cyclin A2 loss when compared with cells that were targeted in S phase. This finding was consistent with the role of p21, which is active in G2 phase, but its expression is dampened during S phase even in the presence of DNA damage. Interestingly, knockdown of

p21 or p53 prevented cytoplasmic loss of cyclin A2 upon DNA damage, suggesting p21 and p53 can both mediate a change in the localisation of cyclin A2. Since p21 contains an NLS and interacts with cytoplasmic and nuclear cyclin A2 in unperturbed conditions[420], we tested the spatial regulation of cyclin A2 by p53 and p21 without inducing external DNA damage. We revealed that p21 has more of an apparent role than p53 in modulating cyclin A2 localisation in an unperturbed cell cycle, demonstrated by the increase in cytoplasmic cyclin A2 in G2 phase in both p21-/- and p53-/- cells, but more prominently in p21 -/- cells. This observation also highlights a role for p21 in regulating progression of the cell cycle in unperturbed conditions independently of p53.

In conclusion, our findings underscore the importance of the spatial regulation of cyclin A2 in promoting mitotic entry and suggest that activation of Plk1 relies on the cytoplasmic localisation of cyclin A2. Although the exact mechanism for cyclin A cytoplasmic localisation remains elusive, our work excludes possible mechanisms that describe how cyclin A2 localisation changes from the S/G2 transition, and adds to our limited mechanistic understanding of cyclin A2 spatial regulation.

3.2 PAPER II

The human long non-coding RNA gene RMRP has pleiotropic effects and regulates cell-cycle progression at G2 phase

The founder mutation c.71A > G in mitochondrial RNA processing endoribonuclease (*RMRP*) is implicated in several diseases, including the rare autosomal recessive disorder cartilage-hair hypoplasia (CHH)[421]. While studies have highlighted a role for *RMRP* in cell cycle regulation and ribosomal assembly[384,422–424], most studies have been based on experimental systems involving cancer and non-human cells. Interestingly, studies in yeast have indicated that RMRP may be regulating the levels of cyclins[425]. Therefore, we aimed to address the function of *RMRP* in CHH in **paper II**.

Since several data indicate a direct role of *RMRP* in ribosomal RNA processing[422–424], it is reasonable to assume *RMRP* can affect transcriptome balance. Thus, we assessed whole-transcriptomes in CHH patients and control individuals using the STRT RNA-seq method. For this study, we derived fibroblast cells from skin biopsies of CHH patients who have the c.71A > G *RMRP* mutations and healthy individuals for the control. The transcriptome data revealed that 35 genes were significantly upregulated and 130 genes were significantly downregulated in the CHH patient-derived fibroblasts. We confirmed the altered expressions of *CDK2, IFITM1,CDKN1A* and *BCL2L1* genes were comparable with findings of the STRT RNA-seq data. Also, the Gene Ontology category and Kyoto Encyclopaedia of Genes and Genomes pathway analyses of downregulated genes from CHH patient-derived fibroblasts indicate perturbations in the cell cycle and cell cycle progression. Importantly, upregulated genes from CHH patient-derived fibroblasts were strongly associated with PI3K-Akt signalling, suggesting cell cycle regulation is impaired in CHH patient-derived fibroblasts. The PI3K-Akt signalling pathway has been shown to modulate growth plate chondrocyte differentiation and endochondral bone growth in mice, with similarities reflected in CHH phenotypes[426]. The pathway is also implicated in several malignancies, including lymphomas[427], indicating the *RMRP* mutation may contribute to development of non-Hodgkin lymphomas by upregulating the PI3K-Akt signalling pathway in CHH patients[428].

Given that the *RMRP* mutation in CHH patient-derived fibroblasts represents a clear indication of cell cycle impairments, we assessed which specific parts of the cell cycle regulation were defective in CHH patient-derived fibroblasts. In line with the gene expression pattern, CHH

patient-derived fibroblasts had slower growth compared to control cells under the same growth conditions and passage numbers. Given that primary cells with growth defects are difficult to synchronise or create stable cell lines expressing reporters, we directly assessed cell cycle progression by incorporated cells with EdU during time-lapse microscopy monitoring. We found no difference in the proportions of EdU positive cells between the CHH patient-derived and control cells, indicating that the reduced proliferation in CHH patient-derived cells was due to a prolonged cell cycle and not the result of cell cycle termination. We unequivocally showed a delay in the progression from G2 phase to the next G1 phase by monitoring the change in DNA contents of S phase pulse-labelled cells over time. In addition, as the duration of mitosis in the majority of CHH patient-derived cells was similar to the control cells, we reasoned that the prolonged G2 phase progression to mitosis was accountable for the reduction in proliferation. We noted that while positive regulators of Cdk1, such as Cdc25C, Gwl and Cdk2 mRNA were downregulated, the Cdk inhibitor *CDKN1A* mRNA was upregulated in CHH patient-derived cells. Thus, our findings of a delay in G2 phase in CHH patient-derived cells is consistent with the reduced activity and expression levels of the main players promoting G2 phase progression.

In summary, our study revealed that mutations of *RMRP* in CHH alter the expression of multiple genes associated with regulating the cell cycle, which can specifically delay progression from G2 phase to mitosis. Thus, our study expands on our limited understanding of the roles of *RMRP* as an long non-coding RNA, specifically in CHH, and provides possible pathways to develop future therapeutic interventions for this disorder.

3.3 PAPER III

p53-dependent polyploidisation after DNA damage in G2 phase

Cell cycle termination is the first step towards senescence. While senescence has been described as a multistep process and provides protection against genomic instability[385–387], the understanding surrounding the consequences after DNA-damage induced terminal cell cycle exit is limited. Previously reported cell fates after cell cycle termination reflect the data gained from short-term, endpoint outcome or pooled population observations[366,429,430]. Thus, we dedicated our work in **paper III** to delve into the long-term consequences after DNA-damage induced cell cycle exit in G2 phase.

Unlike checkpoint arrest, terminal cell cycle exit in G2 phase is marked by APC/C^{Cdh1}-mediated degradation of all cell cycle regulatory proteins, in a p53 and p21-dependent manner[365,366]. Thus, we characterised in our system the dynamics of key G2 phase regulators, cyclin A2 and cyclin B1, in addition to the promoters of cell cycle exit, APC/C^{Cdh1} and p21. The dynamics of these proteins were consistent with a previous report of cell cycle exit in G2 phase[365], suggesting cell cycle exit in G2 phase had efficiently occurred after DDR activation. Using a combination of EdU pulse-labelling and DNA staining, we identified polyploidisation in a subset of cells 6 days after termination of the cell cycle. We revealed that polyploidisation can spontaneously occur in both an immortalised cell line and primary human fibroblasts, as a consequence of p53-dependent cell cycle exit in G2 phase.

In order to determine the polyploidisation process, we used RPE cells expressing a Cdk1/2 activity sensor and monitored these cells by a combination of live-cell fluorescence microscopy and quantitative phase imaging (QPI). Spatial Cdk dynamics and integrated QPI intensity revealed that cells regain Cdk activity, while continuously increasing cell mass, several days after cell cycle exit in G2 phase. This suggests that cells become polyploid by re-initiating DNA replication with G2 phase DNA contents, upon resumption of the cell cycle several days after cell cycle exit in G2 phase. While p53-dependent premature activation of APC/C^{Cdh1} mediates cell cycle exit[365], it can also target geminin, which is required to prevent DNA replication[431]. Thus, polyploidisation paradoxically depends on p53. Interestingly, cell cycle exit from G2 phase, also led to other cell fates, including mitotic entry after re-replication, repeated cell cycle exit after re-replication, and the development of senescence. This gave rise to cells with various ploidies, suggesting a mechanism for creating a heterogenous population.

DNA damage signalling has been shown to be implicated in proliferation-arrest and proliferation-quiescence decision circuits[78,411,432,433]. However, DNA damage signalling has not been assessed in the context of cell cycle re-entry after long-term cell cycle termination. Thus, we characterised p21 and γH2AX as a DDR profile, and cyclin A2 as a Cdk1/2 activity read-out. Fixed-cell immunofluorescence showed that regained cyclin A expression in a subset of polyploid G2 phase cells (EdU labelled) reflected the reactivation of Cdk1/2 several days after termination of the cell cycle in G2 phase. Cyclin A2 loss in polyploid cells represented repeated cell cycle exit after re-replication. The combination of γH2AX and cyclin A2 protein expressions revealed that DNA damage was continually repaired even after cell cycle exit, but polyploidisation processes introduced additional DNA damage to the cells. p21 expression also supported the ongoing DDR signalling in cell cycle terminated cells. Furthermore, our data suggests that re-initiation of DNA replication suppresses p21, but persistent active DNA damage signalling allows p21 to accumulate in polyploid cells, in turn inducing repeated cell cycle exit. However, the possibility that low p21 permits re-initiation of DNA replication could not be excluded.

Extra centrosomes have been implicated in chromosome instability owing to the formation of asymmetric chromosome segregation[434]. Given that DNA replication involves duplication of centrosomes, we assessed participation of centrosome re-replication in polyploid cells. Immunofluorescent staining of the centrosomal component, pericentrin, revealed that a subpopulation of polyploid cells gained extra centrosomes during re-replication. Thus, this data indicates a possible new mechanism of aneuploidy development.

In conclusion, we conferred a new mechanism of polyploidisation that is paradoxically p53-dependent. Moreover, we shift the notion of the long-term consequence of DNA damage induced cell cycle termination in G2 phase from only senescence to multiple cell fates.

4 METHODOLOGICAL CONSIDERATIONS

Determining the dynamics of cell cycle regulatory proteins

The cell cycle is regulated by a complex network of multiple proteins, which interact over time and space. Fluorescence time-lapse microscopy is one of the most suitable techniques to study the dynamic behaviours and localised interactions of proteins. Fluorescent transcriptional reporters allow immediate visualisation of the temporal gene expression of a target protein from a specific promoter[435,436]. However, endogenous promoters exhibit a low protein expression rate, and the transcriptional reporters generally express a low signal to noise ratio[435]. The plasmid reporter system used to express reporter constructs in cells can increase the signal to noise ratio, but can cause overexpression of the protein of interest, as this expression depends on exogenous promoters[437]. In the context of cell cycle regulation, the overexpression of cyclin A and E could alter the cell cycle and be implicated in cancer[438]. In this respect, a genome-targeting approach is the preferential method to establish cell lines to study cell cycle regulation, despite a low signal to noise ratio. Thus, in **paper I** we introduced an ORF for eYFP in the endogenous locus of *CCNA2* to construct the cyclin A2-eYFP fusion protein in RPE cells, to study the dynamics of cyclin A2. Even with this genome-targeted approach, we observed higher expression of cytoplasmic cyclin A2-eYFP (**paper I**). However, the temporal dynamics were similar between cyclin A2 and cyclin A2-eYFP and sufficient to study the function of cyclin A2. This demonstrates that careful consideration is required when analysing the functionality of a tagged endogenous protein, and the choice of method for creating fluorescent proteins is also of importance.

Characterisation of CHH patient-derived primary fibroblasts

Primary cells have a limited life span and generally have a slow proliferation rate when compared to immortalised cell lines[439,440]. In **paper II**, CHH patient-derived fibroblasts exhibited an even slower proliferation rate than the control fibroblasts. While flow cytometry can carry out high-content analysis, it requires a high number of cells. Thus, our choice of method for characterising CHH patient-derived fibroblast was limited, due to these cells having a restricted proliferation rate. In addition to limited growth, other cell characteristics can influence the design of an experiment. Primary cells may behave differently depending on the

genetic background and age of the individuals from which these cells were derived[441]. Furthermore, the characteristics of primary cells can change over time in cultured conditions[442]. Although our transcriptomic data (**paper II**) were reflective of the clinical manifestations of CHH, discrepancies were exhibited with a previously reported list of genes expressed in CHH. However, this study employed a different type of cell (peripheral blood lymphocytes) and methodology to ours. Thus, careful consideration should be given when defining the control group, and when selecting the method for the experiment. Nevertheless, the method that we selected for our analysis holds a higher sensitivity, thereby ensuring the data quality.

Establishing a system to investigate the long-term consequences of DNA damage-induced cell cycle exit in G2 phase

In RPE cells under unperturbed conditions, G1 phase is the longest phase of the cell cycle, hence the majority of cells are in G1 phase at any given time[40]. Therefore, induction of DNA damage in asynchronous cells can result in the majority of the cell population terminating the cell cycle from G1 phase. The hydroxyurea-induced cell synchronisation method is often used to arrest cells at early S phase. This method is based on reducing DNA replication, by limiting the dNTP pool[443], but can also induce DNA damage[444]. In **paper III**, we used hydroxyurea to synchronise cells to increase the number terminating the cell cycle from G2 phase upon inducing DNA damage, and analysed S phase progression after the cells were release from synchronisation to determine the effects of HU on DNA damage. Nevertheless, this synchronisation strategy does not generate an absolute synchronous cell population and the synchronisation efficiency reduces over time after synchronisation release. Moreover, DNA damage induction in early S phase can disrupt DNA replication, which can create additional factors that may influence the cell fate decision after terminal cell cycle exit in G2 phase. Thus, the method for discriminating cells that exit the cell cycle from G2 phase upon DNA damage is the most critical factor for investigating the long-term consequence of DNA damage in G2 phase. Taking into account these factors, in **paper III** we introduced an EdU incorporation strategy to label the synchronised cells that were at late S or early G2 phase at the time of DNA damage induction. Using this method, we confirmed that the majority of cells contained G2 phase DNA-contents labelled with EdU, after cells had terminated the cell cycle.

5 CONCLUSIONS AND FUTURE PERSPECTIVES

The first part of this thesis was to understand cell cycle regulation with a particular emphasis on G2 phase. G2 phase is characterised by feedback-loops that ensure a gradual build-up of Cdk1 and Plk1 activities, but how these feedback loops are initiated at completion of DNA replication remains unclear. Cyclin A is crucial for G2 progression and has been suggested to initiate mitotic kinase activation. However, as cyclin A-Cdk is active in S phase, why mitotic kinase activities are only detected at the S/G2 phase border is not known. It is therefore of utmost importance to determine the spatio-temporal dynamics of cyclin A2 in order to address our question. Our analysis of cyclin A2 in single-cells reveals cyclin A2 specifically localises in the cytoplasm and this localisation is likely responsible for the initiation of Plk1 activation (**paper I**). This is of significance as other reports on the initial activation of Plk1 by cyclin A2 have focused on the levels of cyclin A2-Cdk activity, rather than the cellular localisation of cyclin A2. Cdk-mediated Plk1 activation in the nucleus by WAC has been reported , but it is unclear how this relates to our finding that nuclear cyclin A2 does not interact with Plk1, and the observation that restricting Plk1 to the nucleus does not result in its activation[223]. A speculative model would be that after initial activation in the cytoplasm, Plk1 activation may be further promoted by other mechanisms in late G2 phase. Indeed, cyclin B1-Cdk1-mediated Plk1 activation has been described in *C. elegans* and human cells[204]. Also, it has been reported that Plk1 can be re-distributed in the cell by exposure of an NLS at the KD upon initial activation in *Drosophila*[224], and by SUMOylation of the PBD at Lys492 in human cells[225]. Thus, future investigations comparing the spatio-temporal activation patterns of Plk1 with cytoplasmic cyclin A2 are needed to test our hypothesis.

The main mechanism of cyclin A2 cytoplasmic translocation remains elusive. However, we revealed that cyclin A2 association with replicating chromatin can facilitate cyclin A2 nuclear retention during S phase (**paper I**). The level of cyclin A2 dramatically rises between early S phase to late G2 phase. It is therefore tempting to postulate that only a limited level of cyclin A2 is required for chromatin association during DNA replication and this retains cyclin A2 in the nucleus. This raises the possibility that cyclin A2 exceeds its level at S/G2 phase transition allowing cyclin A2 to localise in the cytoplasm. Therefore, it would be interesting to follow up whether raising nuclear cyclin A2 increases cytoplasmic cyclin A2.

The localisation change of regulatory proteins, such as Cdc25B, Cdc25C and cyclin B1 can in response to DNA damage delay progression of the cell cycle [446]. The p21-dependent rapid nuclear translocation of cyclin B1 and its subsequent APC/C^{Cdh1}-mediated degradation in response to DNA damage in G2 phase has been reported[365,366,429]. We found that spatio-temporal expression of cyclin A2 after DNA damage was also similar to cyclin B1 (**paper I**). This raises the possibility that restriction of cyclin A2 in the nucleus after DNA damage may prevent Plk1 activation in the cytoplasm. Thus, it may be worthwhile to investigate whether, after DNA damage, expression of cyclin A2 containing the NES can activate Plk1.

The second part of this thesis was aimed at understanding cell cycle dysregulation and its relevance to disease. CHH is known to be caused by mutation of *RMRP*, but its implications in cell cycle regulation have not yet been assessed. In **paper II**, we found that mutation of *RMRP* in CHH patient-derived fibroblasts was implicated in causing dysregulation of cell cycle progression, particularly in G2 phase. We found evidence that *RMRP* is likely to affect major parts of the cell cycle network through acting on several target genes. However, the broad changes made to the cell cycle are coordinated by key cell cycle regulators, such as Cdks. Thus, it would be interesting to assess whether modulating Cdk1/2, Cdc25C and MAST-L could rescue G2 phase delay in CHH patient-derived fibroblasts.

CHH is a rare genetic disorder with multiple clinical presentations including cancer. Our finding supports the notion of a possible impairment in the PI3K-Akt signalling pathway in patients with CHH (**paper II**). It has been reported that PI3K-Akt signalling is dysregulated in lymphomas[427] and could promote the development of non-Hodgkin lymphomas[428]. Thus, direct assessment of the PI3K-Akt signalling pathway in CHH-patient derived cells, and correlation with the development of non-Hodgkin lymphomas in CHH patients, deserves further studies to help develop clinical interventions.

CHH patient-derived cells have a slow growth rate with a delay in G2 phase progression. More interestingly p21 gene expression is upregulated in these cells. This raises the question whether G2 phase delay is the consequence of cell cycle arrest due to replication stress. There is also a possibility that CHH patient-derived cells are more prone to develop senescence, as these cells are difficult to cultivate. Future investigation of determining cell cycle exit and senescence in these cells could explain the G2 phase delay in CHH patient-derived fibroblasts.

The last part of this thesis was to understand the long-term consequences of DNA damage in G2 phase (**paper III**). DNA damage-induced terminal cell cycle exit is perceived as the end of

the cell cycle programme with the only consequence being senescence. Our findings that multiple cell fates can arise a long time after termination of the cell cycle, by cell cycle resumption, raises further questions. One of the most important and fundamental questions is how the cell cycle resumes after terminal cell cycle exit. p21 is implicated in several cell fate decision circuits, therefore characterising p21 and cyclin A2 dynamics in single-cells after cell cycle termination would be a good starting point. Also, APC/C^{Cdh1} prevents re-accumulation of cyclin A2[114], and an eventual inactivation of APC/C^{Cdh1} has been reported in cells that escape DNA damage-induced terminal cell cycle exit[410]. Correlating APC/C^{Cdh1} activity with p21 and cyclin A2 activities would be the next step towards identifying an underlying mechanism. Also, a CRISPR-based gRNA screening approach would be useful to identify other potential regulators in cell cycle re-entry in an unbiased manner.

A particular cell fate deserving future investigation is that of polyploid cells that commit to mitosis (**paper III**). The proliferation capability of polyploid cells has been indicated by these cells committing to mitosis, despite having inherent DNA damage. Oncogenic transformation is achieved through several proliferation events in cells with genetic mutations[447]. Therefore, it would be interesting to follow whether polyploid cells that enter mitosis can maintain a proliferation capability. The identification of the long-term proliferative capability of polyploid cells would be interesting to study further, to determine whether tumour formation could occur *in vivo*.

In conclusion, the work presented in this thesis provides new insights into G2 phase regulation in an unperturbed cell cycle, a compromised cell cycle, and in response to DNA damage. Furthermore, it points to the importance of understanding cell cycle regulation in identifying the pathogenesis of major diseases, such as cancer.

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