

Unveiling a new role for Wee1 during S-phase entry

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Declaration

I, Lucas Georges José Morales confirm that the work presented in this thesis is my own. Where information has been derived from other sources, I confirm that this has been indicated in the thesis.

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Abstract

Progression through the cell cycle requires temporal and spatial control of cyclin-dependent kinase (CDK) activity. Wee1 kinase and Cdc25 phosphatases play a key role in CDK regulation through inhibitory phosphorylation and activating desphosphorylation of CDKs. Wee1 regulates a G2 DNA damage checkpoint, through CDK1 inhibition. Therefore, Wee1 inhibitor in combination with DNA damaging agents, is in clinical trials aiming to promote mitotic catastrophe and cell death in TP53 mutant cancer cells that rely on the Wee1 checkpoint. However, the role of Wee1 in regulating earlier cell cycle transitions and the potential impact of Wee1 inhibitors on cell cycle entry is poorly understood.

I investigated the role of Wee1 in cell cycle entry using chemical inhibitors, siRNA and inducible degradation combined with quantitative single-cell imaging of live and fixed cells. I found that inhibition of Wee1 kinase during cell cycle re-entry from quiescence leads to a delay in S-phase entry, with many cells failing to exit quiescence. Moreover, cells that do enter S-phase after Wee1 inhibition have abnormal DNA replication and the CDK inhibitor protein, p21, abnormally accumulates during DNA replication, ultimately leading to a p21-dependent G2 arrest. My investigations revealed that inhibition of Wee1 leads to the hyperactivation of APC/C^{Cdh1}, causing the observed delay in S-phase entry. In addition, using phosphoproteomics analysis following Wee1 inhibition, I unveiled distinctive alterations in the phosphorylation landscape, providing valuable information for new Wee1 substrates. My work highlights a novel role for Wee1 during cell cycle entry and has implications for the use of Wee1 inhibitors in cancer treatment.

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Abbreviations

5-ethynyl-2'-deoxyuridine	EdU
5-iodo-2'-deoxyuridine	IdU
5-chloro-2'-deoxyuridine	CIdU
Alanine	Ala
Anaphase promoting complex	APC
APC complex 1	APC1
APC complex 3	APC3
APC complex 6	APC6
APC complex 7	APC7
APC complex 8	APC8
Ataxia Telangiectasia and Rad3-related	ATR
Ataxia telangiectasia mutated	ATM
Autonomously replicating sequences	ARS
Auxin-inducible degron	AID
Auxin/Doxycycline	ID
Casein kinase 1	CK1
Casein kinase 2	CK2
cyclin dependent Kinase	CDK
CDK1 non phosphorytable	CDK1 AF
CDK2 non phosphorytable	CDK2 AF
CDK1 inhibitors	CDK1i
CDK4/6 inhibitors	Palbociclib
CDK7 analogue-sensitive mutant	CDK7as/as
Cell division cycle	Cdc
Checkpoint kinases 1	CHK1
Checkpoint kinases 2	CHK2
Co-activators Cdc20	APC/CCdc20
Co-activators Cdh1	APC/CCdh1
Contact inhibition	CI
Deoxynucleotides+24:80	dNTPs
DNA damage response	DDR
Double strand breaks	dNTPs
Doxycycline	DOX
Drosophila Wee1	DWee1
Estrogen receptor	ER
Fluorescence activated cell sorting	FACS
Gap phase 1	G1
Gap phase 2	G2
Greatwall kinase	Gwl
Growth factor- β	TGF- β
hTert-RPE1 mRuby-PCNA	RPE1 mRuby-PCNA

hTert-RPE1 mTurquoise2-PCNA mCherry-Geminin PIP-Venus	PIP-fucci
Interfering RNA	RNAi
Interstream crosslinks	ICLs
Mammalian target of rapamycin complex 1	mTOR1
Mass spectrometry	MS
Messenger RNA	mRNA
Microtubule-associated protein	MAPs
Mitosis	M
Mouse Embryonic Fibroblasts	MEFs
Mouse Embryonic Fibroblasts lacking Wee1	Wee1Co/ -; Tm-Cre MEFs
Neocarzinostatin	NCS
Nuclear localisation signal	NLS
Non-phosphorylatable CDK1	CDK1AF
Non-phosphorylatable CDK2	CDK2AF
Non-small cell lung cancer	NSCLC
Nuclear envelope breakdown	NEBD
Origin recognition complex	ORC
<i>Oryza sativa</i> TIR1	OsTIR1
p21Cip1	p21
p27Kip1	p27
p53 binding protein 1	53BP1
p57Kip2	p57
pBluescript	pSKII
Phosphoserine	pSer
Phosphothreonine	pThr
Phosphotyrosine	pTyr
Polo like kinase 1	PLK1
pre-replication complex	preRC
Proliferating cell nuclear antigen	PCNA
Propidium iodide	PI
Recombinant adeno-associated virus mediated	rAAV
Replication complexes	RCs
Replication protein A	RPA
Resistance marker	RM
Restriction point	RP
Retinoblastoma	Rb
Serine	Ser
Single strand DNA	ssDNA
Skp1-Cul1-F-Box	SCF
Standard deviation	STDEV

Tensin-1
Threonine
Tyrosine
Valine
Wee1 inhibitors

TNS1
Thr/T
Tyr/Y
Val/V
Wee1i

Chapter 1. Introduction

1.1 Historical background

All living organisms on earth are made up of cells, which are the functional units of life. Eukaryotic organisms can consist of a single cell (unicellular) or a group of either identical or different cells (multicellular). The first description of a cell was by Robert Hooke in the late 17th century (Hooke 1635-1703). By observing cork samples, he was able to describe structural shapes which looked like boxes. He named them *cellula*, or cell, in reference to the shape of his monk chamber. It is only much later, in the 19th century, that Walther Flemming revealed cell division's intricacies (Flemming, 1879). By using optical microscopy, he was able to describe cells undergoing division for the first time. He successfully monitored how chromosomes are distributed between daughter cells. This period marked the first understanding of how cells reproduce. However, the exact understanding of cell cycle dynamics was still lacking. The 20th century brought significant advancements in the understanding of cell cycle dynamics, notably through the work of Alma Howard and Stephen R. Pelc in the 1950s, which delineated interphase into distinct phases: one pre-replicative phase called G1, one replication phase, or S-phase, and one pre-mitotic period, called G2. Only then, the cell can enter the mitotic phase called M, where the cell undergoes nuclear and cellular division (Howard & Pelc, 1951). This period also witnessed the ground-breaking discovery of the structure of DNA by Francis Crick and James Watson in 1953, revolutionising the understanding of genetic transmission and replication (Watson et Crick, 1953). These cumulative discoveries, each building upon the last, have shaped our current understanding of the cell cycle, highlighting its complexity and the critical role of collaborative scientific exploration across various disciplines.

1.2 Cell cycle overview

Cellular reproduction is a fundamental process of all living organisms. From single cell to complex multicellular organisms, cell division is a key factor for harmonious development. Alterations in components of the cell-division machinery occur in most human tumours (Visconti et al., 2016). The complex network of regulatory elements

that form the cell cycle has one goal: the timely and accurate duplication and segregation of the genomic DNA. The series of events that lead to cellular reproduction are called the cell cycle, which is composed of four proliferative phases, G1, S, G2, M, and one quiescent phase G0 (Howard & Pelc, 1951; Howard. Alma, 1953; Lajtha, 1963). In G1, cells grow in size and produce proteins and some organelles required for cellular division such as mitochondria and Golgi apparatus. The initiation and completion of DNA replication occurs in S-phase, ensuring that the genome is duplicated in its entirety and only once per cell cycle. During G2, the cell undergoes further cell growth and organises the cellular contents necessary for chromosome segregation. Finally in M-phase, nuclear and cellular division occurs, leading to the formation of two identical daughter cells (Schafer, 1998). The M phase is separated into 5 phases: prophase, prometaphase, metaphase, anaphase, and telophase. During prophase, the genomic material condenses into chromosomes and the mitotic spindle starts to form. As cells progress through prometaphase, a rapid and abrupt nuclear envelope breakdown (NEBD) occurs. Once these chromosomes achieved their most condensed and organised state, they are aligned in a single, orderly row along the equator of the cells during the metaphase. The transition from metaphase to anaphase is regulated by the anaphase promoting complex/cyclosome (APC/C) (Exact function and regulation of APC/C be further detailed in section 1.6). APC/C targets for degradation securin that in return releases separase that triggers the cleavage of cohesin, a protein which binds sister chromatid and allow their movement to the opposite poles of the cell. This step ensures the accurate distribution of genetic material to the daughter cells. As the chromosomes reach the poles, the nuclear membrane reforms and the chromosomes begin to decondense in telophase. Telophase is followed by cytokinesis or the division of the cytoplasm into two daughter cells. Cytokinesis is not mandatory for completion of mitosis and can happen outside of the cell cycle in other organisms. Hence it is not defined as a phase of mitosis.

While these stages are described as “proliferative”, cells can also enter a state of reversible exit from the cell cycle known as quiescence or G0. G0 is still a poorly understood cellular state that is characterised by a lack of proliferation (Coller et al., 2006). In quiescence, cells are both poised to re-enter the cell cycle under mitogen activation and produce specific factors fundamental for the survival of the cells (Mitchell, 2001). As cells remain quiescent for longer durations, they move progressively deeper into G0 and display a reduced sensitivity to growth signal (Llorens-

Bobadilla et al., 2015; Narita et al., 2003; Soprano, 1994). The quiescent state is a fully reversible arrest and therefore distinct from senescence, which is a terminal cell cycle arrest (Narita et al., 2003). However, more recently it was observed that senescent cells were also able to re-enter the cell cycle upon signal integration (Beauséjour et al., 2003; Kumari & Jat, 2021; Milanovic et al., 2018). As a result, the boundary between quiescence and senescence is becoming increasingly nuanced, and the characteristics of these cellular states may overlap. The key takeaway is that the reversibility of both quiescence and senescence depends on various factors, including the cell type, context, and the specific triggers involved.

The identification of the key molecules involved in the process of regulating the cell were first discovered in yeast. For this the cell division cycle (Cdc) genes were mutated in *Saccharomyces cerevisiae* and *Saccharomyces pombe* which resulted in cells, which are unable to divide. For this reason, Cdc's were identified as key genes essential for cellular division (Hartwell et al., 1970; Nurse et al., 1976a; Nurse & Bissett, 1980). These proteins were later termed cyclin-dependent kinases (CDKs) after cyclins discoveries. cyclins were first observed in sea urchins (Evans et al., 1983). In this study, authors were able to observe proteins levels varied in a cyclical manner and peaking at different times during the cell cycle. Even with no clear sense of the mechanism, it was suggested to be linked to the cell cycle cell cycle oscillator. Afterwards it was shown that cyclins bind to CDKs in order to regulate cell cycle progression. Furthermore, the different phases of the cell cycle are regulated by specific CDKs and regulated through levels variations of cyclins (Fig. 1.1).

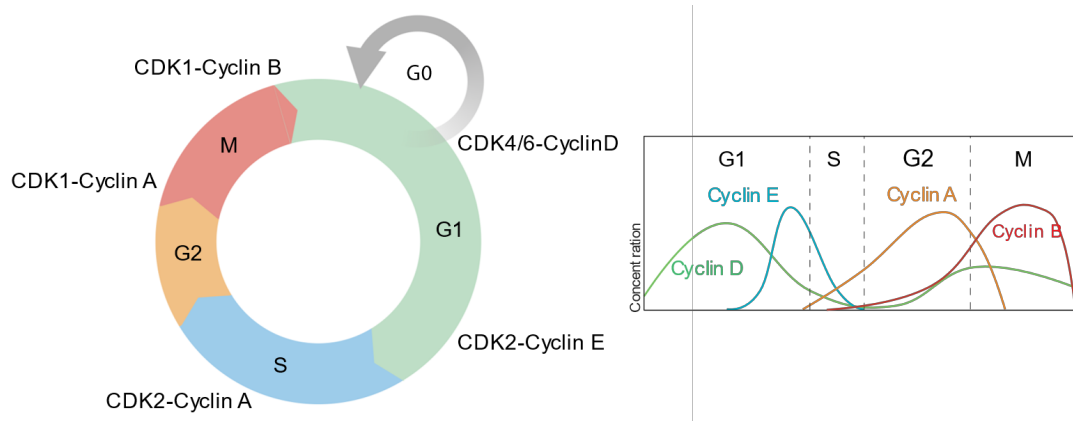


Figure 1-1. Regulation of the cell cycle through CDK-cyclin complexes.

Different cyclins are expressed at different times during the cell cycle. They form a complex with different CDKs, and this drives the different cell cycle phases. CDK4 and CDK6 bind to D-type cyclins in order to regulate events in early G1-phase; cyclin E binds to CDK2 and triggers DNA replication at the beginning of S-phase; CDK2-cyclin A and CDK1-cyclin A regulate the completion of S-phase and G2; and CDK1-cyclin B1 drives mitotic entry.

1.3 CDK regulation

CDKs are crucial enzymes in the regulation of the cell cycle, playing a key role in controlling the progression of cells through various phases of growth and division. Overexpression and/or dysfunction of CDKs or cyclins have been reported in a very large number of human cancers and other diverse pathologies (Hanahan & Weinberg, 2011). These kinases are highly regulated by several mechanisms (Fig. 1.2). The first layer of regulation is through the binding with the sub-unit cyclin. CDK activity is also modulated by phosphorylation, which can activate or inactivate the kinase, respectively. Additionally, other mechanisms such as CDKs dephosphorylation also regulates CDKs activity. These different regulations are performed by several proteins such as Wee1/Myt1, Cdc25 and CDK-activating kinase (CAK). Furthermore, CDK inhibitors, which are proteins that bind to and inhibit CDKs, provide an additional layer of control. This intricate regulation ensures that cell division occurs accurately and at the appropriate time, which is critical for normal growth, development, and maintenance of tissue homeostasis in multicellular organisms.

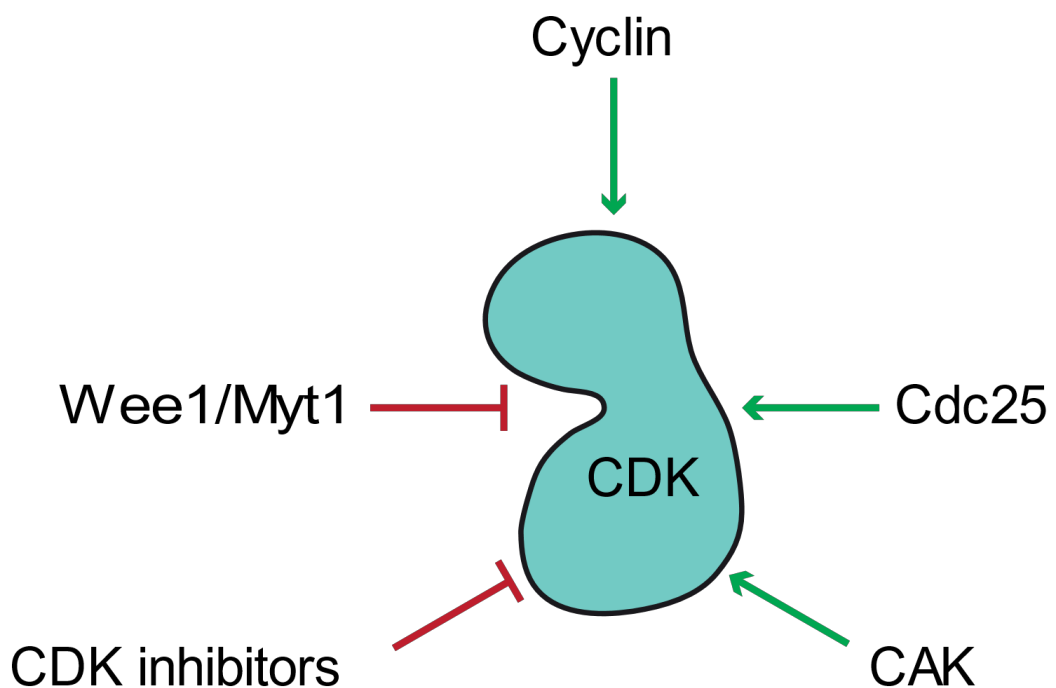


Figure 1-2. Schematic regulation of CDKs integrating multiple layer of regulation.

CDK is regulated by integrating multiple signal over time. Red lines represent negative regulation by CDK inhibitors and Wee1/Myt1 whereas green lines represents positive regulation by cyclin, Cdc25 and CAK.

1.3.1 Cyclin-dependent activation of CDKs

CDKs are small serine/threonine kinases, which are constitutively expressed throughout the cell cycle. In eukaryotic cells, 20 CDKs have been identified, yet only a sub-set was shown to have a function in cell cycle control. The CDKs in charge of the cell cycle control are CDK1, CDK2, CDK4, CDK6, CDK7. CDK1 plays a major role in the regulation of the G2/M transition. CDK2 controls the G1/S transition, whereas CDK4 and CDK6 regulate early G1. On the other hand, CDK7 is required for the activation of other CDKs (Fisher, 2005; Larochelle et al., 1998, 2007). While the expression of CDKs is constant, their activation relies on the binding to cyclins. In the absence of cyclin binding, the CDK active site is blocked (De Bondt et al., 1993). To date, 29 cyclins have been identified (Malumbres & Barbacid, 2005). Each phase of the cell cycle is driven by the expression of cell cycle stage-specific cyclins. Hence, the expression level of cyclins represents the primary mechanism by which CDK activity is regulated in a timely manner (Fig. 1.1)(Bloom & Cross, 2007). It is worth noting that cyclins not only activate CDKs but also direct CDKs kinase activity to specific target. Briefly (further explanation will be provided in this chapter), the first CDKs to be activated during cell cycle re-entry and at the beginning of the cell cycle are CDK4 and CDK6 (CDK4/6). CDK4/6 bind to the D-type family cyclins (D1, D2, D3) (Baldin et al., 1993.; Depoortere et al., 1998; Meyerson & Harlow, 1994). Although these proteins are highly homologous and conserved, they contain distinct structural motif and are differentially regulated across the cell cycle. cyclin D1, D2 and D3 promotes proliferation, however it seems that only cyclin D1 regulates transcriptional pathways involved in the metabolism of carbohydrates (Mullany et al., 2008). Furthermore, although cyclin D2 and D3 can also activate CDK4/6 and promote proliferation they are less frequently implicated in human cancers. CDK4-cyclin D activity not only increase in early G1 but has also been observed to increase during G2 (Gabrielli et al., 1999).

In late G1, CDK2 binds to cyclin E to drive cell cycle progression further (Arooz et al., 2000; Koff et al., 1992).

In mammalian cells, two E-type cyclins, E1 and E2, activate CDK2. cyclin E1 and E2 have differences in tissue expression, transcription and post-transcriptional regulation. Next, as cells progress through S and G2 phase, CDK2 binds to cyclin A (cyclin A1, A2), forming the CDK2-cyclin A complex (Elledge & Spottswood, 1991; Tsai et

al., 1991). Last, as cells prepare for mitosis, cyclin B binds to CDK1 to form the CDK1-Cylin B complex (Nurse, 1990). CDK1 also binds to cyclin A and form the CDK1-cyclin A complex and together with CDK1-cyclin B regulate critical events in mitosis (Hégarat et al., 2018; Nurse, 1990).

As seen with cyclin A which can binds to CDK1 and CDK2, a single mammalian cyclin can interact with multiple CDKs.

1.3.2 T-loop phosphorylation

Even if the binding of cyclins to CDKs is an important step, it is not sufficient to drive CDK activation. Another layer of CDK-cyclin regulation occurs through the phosphorylation of CDKs by CAK (Fesquet et al., 1993; Fisher & Morgan, 1994; Poon et al., 1993; Solomon et al., 1992, 1993). CAK itself is a cyclin-dependent kinase composed of CDK7, cyclin H and MAT1 (ménage à trois) (Fesquet et al., 1993; Fisher & Morgan, 1994; Larochelle et al., 2007). CAK contributes to CDK kinase activation by phosphorylating a threonine residue within the T-loop of CDKs. This induces a conformational change in the C-terminal T-loop domain of the CDKs that makes their active site more accessible. This structural alteration is essential for the kinase activity of CDKs allowing them to bind and phosphorylate substrate proteins. In the absence of phosphorylation, the T-loop occludes CDK substrate interaction (Martinez, 1997).

CDK4 is phosphorylated on threonine 172 (Thr172 or T172) whereas CDK6 is phosphorylated on Thr177 by CAK. Furthermore, both residues are more efficiently phosphorylated in CDK-cyclin dimers than in CDK4/6 monomers (Bockstaele et al., 2006; Jeffrey et al., 1995; Kato et al., 1994). CDK2 is phosphorylated on Thr160 and finally CAK phosphorylates Thr161 in CDK1. *In vivo*, both CDK1 and CDK2 bind cyclin A, but unlike CDK2, CDK1 cannot form stable complexes with cyclin A *in vitro* without T-loop phosphorylation (Desai et al., 1992, 1995; Pagano et al., 1992). Conversely, phosphorylation of CDK1 by CAK requires the presence of a cyclin whereas, like for CDK4/6, CAK can efficiently phosphorylate monomeric CDK2 (Desai et al., 1995; Fisher & Morgan, 1994; Wohlbold et al., 2006). Consistent with the more stringent requirements for assembly into stable complexes, mutant forms of CDK1 with the Thr residue phosphorylated by CAK mutated to Alanine (Ala) or Valine (Val) were defective for cyclin-binding (Ducommun et al., 1991; Larochelle et al., 1998; T. H. Lee et al., 1994).

It is interesting to note that in quiescent cells CAK activity was also detected (Matsuoka et al., 1994). This indicates that quiescence cannot be considered as an inactive state and that CDK regulation can occur in it.

Additionally, research on human cell lines expressing a CDK7 analogue-sensitive mutant (CDK7^{as/as}) suggests that T-loop phosphorylation increases, and thus the CAK's activity increases, during the transition from G0 to G1 in the cell cycle (Schachter et al., 2013). However, this data is subject to controversy since another group was unable to validate this finding (Bisteau et al., 2013).

1.3.3 Cyclin-dependent kinase inhibitors

CDK activity is also under the negative control of inhibitors known as cyclin-dependent kinase inhibitors (CKIs). These CKIs fall into two structural families: the Cip/Kip proteins and the INK4 family. The Cip/Kip family includes three proteins in human cells: p21Cip1 (p21), p27Kip1 (p27), and p57Kip2 (p57) (Gu et al., 1993; Harper et al., 1993; M.-H. Lee et al., 1994.; Polyak et al., 1994; S Matsuoka, 1992.; H. Toyoshima & Hunter, 1994; Xiong et al., 1993a, 1993b). Their primary function is to inhibit CDK-cyclin activity in response to inhibitory signals from the environment or DNA damage (Dulić et al., 1994). Cip/Kip inhibitors have the ability to halt the cell cycle progression. Cip/Kip inhibitors are CDK2 and CDK1 inhibitors (CDKi) *in vitro* and in cells under conditions of growth arrest but also extend their inhibitory action to CDK4 and CDK6 (Reynisdóttir et al., 1995; Sherr & Roberts, 1999). In CDK1/2 and CDK4 complexes, Cip/Kip proteins physically block the substrate-binding site (via their RXL motif) on the cyclin subunit and destabilise ATP binding by remodelling the N lobe of the CDKs (Mitrea et al., 2012). Furthermore, mice lacking p21 or p27 are susceptible to tumourigenesis which is consistent with the important role of CIP and KIP proteins in negatively regulating the cell cycle (Fero et al., 1996; Martín-Caballero et al., 2001). p57 loss is also linked to altered cell differentiation and proliferation leading to muscle defects (P. Zhang et al., 1997).

These proteins are highly flexible and intrinsically disordered, meaning they lack a fixed three-dimensional structure until they interact with CDK-cyclin complexes (Yoon et al., 2012). Besides the inhibitory effect, Cip/Kip proteins can also exert activating effects on CDK-cyclin activity, depending on the context (Cerqueira et al., 2014; Cheng et al., 1999; Guiley et al., 2019; Labaer et al., 1997; Sugimoto et al.,

2002). They can stabilise the formation of CDK-cyclin complexes, particularly those involving cyclin D and CDK4/6 (Guiley et al., 2019). Moreover, they contribute to the nuclear localisation of cyclin D and CDK4 (Alt et al., 2002; Cheng et al., 1999). In the absence of Cip/Kip proteins, cyclin D levels may decrease, suggesting that they have stabilising effect on cyclin D levels (M. Sugimoto et al., 2002). This stabilisation effect was furthermore observed by looking at the influence of DYRK1 on cyclin D level (J.-Y. Chen et al., 2013). DYRK1 is a dual specificity tyrosine-(Y)-phosphorylation family proteins that as a role, among other, to regulate cell proliferation. It has been shown that DYRK1 directly degrades cyclin D and thus play a role in G1 regulation (J.-Y. Chen et al., 2012; Massagué, 2004). Thus, it has been shown when cyclin D form a complex with p21 and confer a degree of resistance against the degradation mediated by DYRK1 and hence showing that p21 can stabilise cyclin D levels (J.-Y. Chen et al., 2013).

While previous work suggests that p21 and p27 promote CDK4/6 activity, recent new study challenged this consensus (Cheng et al., 1999; Pennycook & Barr, 2021). In embryonic fibroblasts that lack both p21 and p27, CDK4-cyclin D failed to assemble in an active complex, whereas in RPE1 cells, it is shown that p21 or p27 has little effect on CDK4/6 activity (Pack et al., 2021; Pennycook & Barr, 2021).

It has also been observed that the CDK4-cyclinD-p27 complex can exist in both active and inactive conformations (Blain, 2008). The switch between the inactive and active CDK4-cyclinD-p27 complex is due to the phosphorylation of Y74 on p27 (Guiley et al., 2019). Intriguingly, the regulation of tyrosine phosphorylation appears to be specific to p27 and not p21 (Guiley et al., 2019). p21 lacks a similar phosphorylation site, preventing CDK4 activation through p21 upon DNA damage (Pennycook & Barr, 2021; Swadling et al., 2022). It also appears that this activation mechanism through CKIs is CDK4 specific (versus CDK2) (Guiley et al., 2019a).

Whereas Cip/Kip proteins have an activating and inhibiting function on CDKs activity, the family of INK4 proteins was shown to uniquely inhibit CDKs (Malumbres, 2014). Members of the INK4 family, p16^{Ink4a}, p15^{Ink4b}, p18^{Ink4c} and p19^{Ink4d}, specifically bind CDK4 and CDK6, inhibiting their catalytic activity by allosteric competition of their binding with cyclins (Ortega et al., 2002). In contrast to other CDK inhibitors, the INK4 family members are differentially expressed during development. The expression pattern of INK4 suggested that this family of cell cycle inhibitors might have cell

lineage-specific or tissue-specific functions. The INK4 proteins are commonly lost or inactivated by mutations in diverse types of cancer, and they serve as therapeutic targets for existing and developing cancer treatments (Ortega et al., 2002; Pei & Xiong, 2005). Apart from their capacity to arrest cells in the G1-phase of the cell cycle they have been shown to participate in an increasing number of cellular processes such as aging or cellular differentiation (W. Y. Kim & Sharpless, 2006; Rowell et al., 2014).

1.3.4 Inhibitory phosphorylation

Another layer of regulation of the CDK-cyclin complexes is through inhibitory phosphorylation (Gu et al., 1992; Morgan, 1995) (Fig. 1.3). CDK1 and CDK2 are restricted by inhibitory phosphorylation in the active site on Y15 and to a lesser extent on T14, respectively performed by Wee1 and Myt1 (Booher et al., 1997; Draetta et al., 1987; M. G. Lee & Nurse, 1987; F. Liu et al., 1997; Norbury et al., 1991; L. L. Parker et al., 1992; Swadling et al., 2022). The adding of a phosphate group to a critical tyrosine residue in the ATP-binding region of the CDK alter its conformation reducing its kinase activity (De Bondt et al., 1993; Jeffrey et al., 1995).

Indeed phospho-mimetic substitutions of CDK1 on T14 and/or Y15 produce dominant negative, kinase-dead mutants that block cells in interphase, indicating that negative charges at either position inhibited CDK1 activity (Krek & Nigg, 1991). In line with this, the expression of non-phosphorylatable CDK1 mutants (CDK1AF) accelerate cell cycle division timing (Jin et al., 1996; Krek & Nigg, 1991; Su et al., 1998). Additionally, it has been shown that Y15 phosphorylation more potently inhibits CDK1 and CDK2 activity than T14 phosphorylation (Fletcher et al., 2002; Potapova et al., 2009). Furthermore, the activities of Wee1 and Myt1 kinases are intricately regulated through a combination of phosphorylation and subcellular localisation, which varies throughout the cell cycle (McGowan & Russell, 1995).

The CDK4/6-cyclin D complex is kept inactivated through phosphorylation of their Y17 and Y24 residues respectively. Phosphorylation on Y17 of CDK4 was shown to occur in mammalian cells that are entering the cell cycle from quiescence (Jinno et al., 1999; Terada et al., 1995). In these studies, the activation of wild-type CDK4 was inhibited by UV irradiation, whereas a Y17F non-phosphorylatable mutant of CDK4 was activated. Phosphorylation of CDK4 on Y17 also occurs in cells entering

quiescence in response to contact inhibition (CI), serum starvation and treatment with transforming growth factor- β (TGF- β) (Iavarone & Massagué, 1997). The obvious candidate kinases for CDK4/6 inhibitory phosphorylation role are Wee1 and Myt1 due to their similar role on CDK1 and CDK2, however, neither is able to phosphorylate CDK4 on Y17 *in vitro* (Booher et al., 1997; Watanabe et al., 1995). Instead, it has been shown that phosphorylation of CDK4 on Y17 is mediated by Src family kinases: C-YES family (N. G. Martin et al., 2008). Indeed, when C-YES is mutated to a kinase dead (C-YES^{k305R}), CDK4 was not being phosphorylated on Y17 and an over-activation of this same kinase led to an increase in Y17 phosphorylation. Due to their similar structure, it is believed that CDK6 is also being phosphorylated on Y24 by a member of the Src-family. It is to be noted that this is not the first report of Src family kinases phosphorylating CDKs. The Src kinase LYN is already known to phosphorylate CDK1 and CDK2 on Y15 in response to DNA damage, and it is plausible that LYN may also phosphorylate CDK4 on Y17 in this context (Kharbanda et al., 1996; Uckun et al., 1996; Z. M. Yuan et al., 1996).

The inhibitory phosphorylation of CDK is removed by the action of the Cdc25 phosphatase family, of which there are three members. Cdc25A removes inhibitory phosphorylation on CDK4/6 and CDK2 during G1 while Cdc25B and Cdc25C are required for M phase entry (Hoffmann et al., 1994a). It was demonstrated that downregulation of Cdc25A causes accumulation of inhibitory phosphorylation, p-Y17, on CDK4 and consequent G1 arrest (Terada et al., 1995). Consistently, *in vitro* studies also showed that GST-bound Cdc25A can remove inhibitory phosphorylation on CDK4 confirming Cdc25A as a positive regulator of CDK4 (Z. Wang et al., 2001). In addition, inhibition of Cdc25A expression also results in accumulating inhibitory phosphorylation Y24, on CDK6, decreasing CDK6 activity (Iavarone & Massagué, 1997). Besides CDK4/6-cyclin D, Cdc25A is also able to dephosphorylate T14/Y15 on CDK2 (Hughes et al., 2013). An additional study showed that overexpression of Cdc25A can accelerate G1/S transition by prematurely upregulating the CDK2 activity (Blomberg & Hoffmann, 1999). However, this showed that the activities of CDK4/6-cyclin D complexes are not affected by overexpression of Cdc25A and thus in direct contradiction with the expected role of Cdc25A in CDK4/6 de-phosphorylation mentioned before.

Cdc25B and Cdc25C are the phosphatases removing inhibitory phosphorylation on CDK1. Indeed, Cdc25B and Cdc25C appears to be necessary for cells to enter

mitosis, because perturbations of both of them block cells in G2 (Gabrielli et al., 1997b; Lammer et al., 1998; Millar et al., 1991). It has also been shown that overexpression of Cdc25B leads to an increase in CDK1 phosphorylation (Timofeev et al., 2010). Wee1 and Cdc25 are in turn also regulated by CDK activity. For example, CDK1 regulates Wee1 and Cdc25B/C through a double-negative feedback loop, in which CDK1 phosphorylates and inactivates Wee1, and a positive feedback loop, in which CDK1 phosphorylates and activates Cdc25 (Hofmann & Beach, 1994; Kumagai & Dunphy, 1992a; McGowan & Russell, 1995; P. R. Mueller, Coleman, & Dunphy, 1995a; Okamoto & Sagata, 2007). Due to feedback regulation, Wee1 and Cdc25 phosphorylation have been shown to be ultrasensitive as a function of CDK1 activity (S. Y. Kim & Ferrell, 2007; Trunnell et al., 2011). CDK2 also form a positive feedback loop with Cdc25A but it is still unknown if a negative feedback loop exists between CDK2 and Wee1.

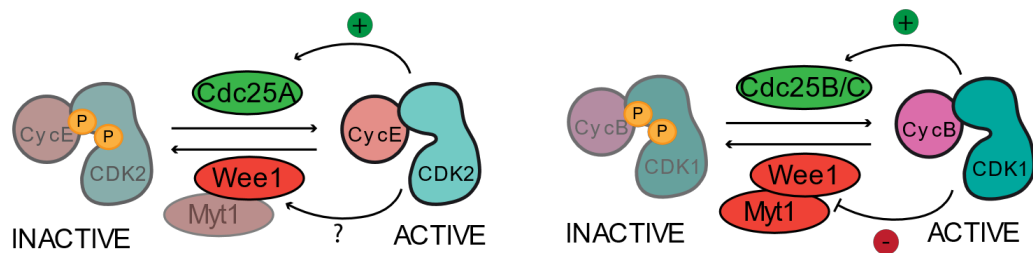


Figure 1-3. CDK-cyclin inhibitory phosphorylation.

Diagram showing a simplified CDK regulatory network, including positive and negative feedback loops. CDK-cyclin are being kept inactivated by phosphorylation on specific residues by Wee1 and Myt1. Once cells need to progress through the next phase of the cell cycle, phosphatases of the Cdc25 family remove the phosphorylation leading to the activation of the CDKs-cyclins complex. CDK1 form a positive feedback loop with Cdc25B/C and a double negative feedback with Wee1/Myt1. CDK2 form a positive feedback loop with Cdc25A.

1.4 Cell cycle progression.

The integration of multiple internal and external signals regulating CDK-cyclin activation allow the proper progression through each phase of the cell cycle. If these criteria are not fulfilled cell cycle can be halted to protect the cells and maintaining genome stability.

1.5 G1/S transition.

1.5.1.1 *Original model.*

The competition between mitogens and DNA damage plays a critical role in regulating the G1/S transition, impacting the balance of cyclins and CDKi (Heldt et al., 2018; Moser et al., 2018; H. W. Yang et al., 2017). Ultimately, this competition dictates whether cells progress into S phase or enter a quiescent state. In G1, the point where S-phase entry becomes independent from growth signal factors is the point of no-return, also called restriction point (RP). At this point cells are committed to enter S-phase and cannot arrest in G1.

The first description of the RP described a time during the G1 phase of the cell cycle when a cell becomes committed to entering S phase and is no longer dependent on growth factors for completing the cell division cycle (Pardee, 1974).

The RP in eukaryotic cells occurs during the late G1. The prevailing model is that under mitogen activation, the MAP-kinase pathway is activated and ultimately leads to *CCND* transcription, encoding cyclin D (Balmain & Cook, 1999; Lavoie et al., 1996). cyclin D then binds to CDK4/6 increasing the phosphorylation of the retinoblastoma (Rb) protein causing its hypophosphorylation (Connell-Crowley et al., 1997; Lees et al., 1991; Lundberg & Weinberg, 1998; Zarkowska et al., 1997). Rb is a tumour suppressor protein that is a master regulator of biological pathways including cell growth, cell-cycle checkpoints, differentiation, senescence, self-renewal, replication, genomic stability and apoptosis (Calo et al., 2010; Cress et al., 2014; Manning & Dyson, 2011; D. M. Thomas et al., 2003). Additional studies revealed that Rb acted together with two related proteins, p107 and p130, to regulate proliferation in most if not all cell types (Cobrinik et al., 2006; Dyson, 1998; E. J. Smith et al., 1998). RB, p107, and p130, together known as the “pocket proteins” are central participants in a gene regulatory network and its deregulation constitutes one of the hallmarks of

cancer (Hanahan & Weinberg, 2000). The function of Rb in cell cycle control is mediated through its interaction with the E2F family of transcription factors. Rb binds to E2F family members at the promoters of genes important for S phase progression and cell proliferation. The binding of Rb to E2F proteins either blocks the recruitment of transcriptional co-activators or recruits transcriptional co-repressors to these promoters, thus repressing the expression of these genes and halting the G1/S cell cycle transition (Chinnam & Goodrich, 2011; Dick & Rubin, 2013; van den Heuvel & Dyson, 2008). The hypophosphorylation of Rb is thought to cause its partial release from E2F (Bertoli et al., 2013; Chellappan et al., 1991; Hiebert et al., 1992; Nevins, 1998; Weinberg, 1995; Weintraub et al., 1992).

E2F proteins are a family of transcription factors playing a crucial role in the control of cell cycle transcription. This family comprises nine transcription factors; E2F1-6 (with E2F3A and 3B) are the best characterised, whilst E2F7 and 8 more recently discovered and structurally different from the other family members (Christensen, 2005; Di Stefano, 2003). E2F family is generally grouped based on their functions: E2F1-3A as transcriptional activators and E2F3B-8 as repressors (Cartwright et al., 1998; Christensen, 2005; Di Stefano, 2003). Whilst this functional distinction is generally true, other studies has suggested this may be too simplistic a view (J.-L. Chong et al., 2009; DeGregori & Johnson, 2006).

E2F transcription genes comprise key genes in cell cycle regulation such as *CCNE1*, *Skp2*, *Cdc6*, *MCM Cdc25A* and other members of the E2F family (Cartwright et al., 1998; Helin et al., 1993; Müller & Helin, 2000; Petersen et al., 1999). Furthermore, E2F also regulate many genes with functions in processes that are unrelated to cell cycle progresses such as DNA damage repair apoptosis cellular differentiation and development (Müller et al., 2001; B. Ren et al., 2002).

In G0/G1 phases, E2F1-3A binding with pRb has been shown to mask the transactivation domain in E2Fs responsible for transcription activation (Helin et al., 1993). At this stage E2F4-8, in complex with p130, is bound to E2F target promoters where it represses transcription (Takahashi et al., 2000). Hypophosphorylation of Rb through CDK4/6 activation allow its partial dissociation from E2F. This partial dissociation allows cyclin E to be transcribed, which increases CDK2 activity and results in further phosphorylation of pocket proteins and p27, targeting it for degradation (Bertoli et al., 2013). Both effects create a positive feedback loop, further increasing CDK activity and ultimately leads to an entry into S-phase. Once CDK2-cyclin E is

fully activated, it inhibits APC/C^{Cdh1} (Pesin & Orr-Weaver, 2008). Once APC/C^{Cdh1} is inactivated, cells can enter S-phase (Fig 1.4). These mechanisms are essential to ensure that cell cycle commitment is switch-like, and progression is unidirectional (Barr et al., 2016).

1.5.1.2 *Alternative model.*

Interestingly, other alternative models have been suggested for RP passage (Narasimha et al., 2014).

In this new model, CDK4/6-cyclin D activity only induces a mono-phosphorylation on Rb at one of 15 possible CDK sites. Hyperphosphorylation then only occurs when CDK2 is activated and not before, as it was thought in the original model. E2F-dependent gene expression remained low while Rb was monophosphorylated by CDK4/6-cyclin D, leading to the conclusion that only CDK2 directly inactivates Rb. Monophosphorylated Rb can still bind to E2F and inhibit its activity. Next, an unidentified process initiates *CCNE* transcription, which in turn activates CDK2 activity and converts monophosphorylated Rb to inactive hyperphosphorylated Rb. Like in the prevailing model, hyperphosphorylation of Rb fully release E2F. cyclin E is then transcribed and binds to CDK2 leading to further increase in Rb phosphorylation which increases *CCNE1/2* transcription and activate furthermore CDK2. This new perspective alters our understanding of Rb phosphorylation, indicating that Rb primarily exists in discrete states, such as unphosphorylated, monophosphorylated (in the presence of cyclin D), and hyperphosphorylated (once CDK2 is activated). The complexity of Rb phosphorylation and the multiple monophosphorylated forms of Rb discovered raise intriguing questions about the functional diversity of these different states. However, several questions emerge from these findings. If CDK4/6 activity does not inactivate Rb, then why is CDK4/6 activity important for cell-cycle progression and proliferation. One possibility is that CDK4/6 mono-phosphorylation is required for priming Rb for CDK2 hyperphosphorylation. Such priming mechanisms for CDK hyperphosphorylation have been observed in other cell-cycle substrates in yeast though they have not yet been implicated in the mechanism of Rb hyperphosphorylation (Kõivomägi et al., 2013). Another intriguing question remains regarding the selectivity of the mono-phosphorylation of just one out of the 15 sites on Rb by CDK4/6, while the others remain unphosphorylated. This phenomenon hints to a

possible alteration in the structure of Rb induced by mono-phosphorylation and could inhibit further CDK4/6 phosphorylation (Burke et al., 2010, 2012; Rubin et al., 2005). Importantly such mechanisms are not mutually exclusive and could concurrently operate in tandem. Another question emerging is how CDK2-cyclin E is activated if E2F is still inhibited by mono-phosphorylation of Rb. Some theories suggest that CDK2-cyclin E activation may be downstream of CDK4/6 phosphorylation of metabolic enzymes or regulated by E2F-independent *CCNE* transcription factors (S. Kim et al., 2006; J. Lukas et al., 1997; Santoni-Rugiu et al., 2000; H. Wang et al., 2017). The last question concerns the relationship between Rb mono-phosphorylation by CDK4-cyclin D and the role of cyclin D1 levels in G1 progression. While *CCND1* is often overexpressed in many cancers, excessive D-type cyclins accelerate G1/S progression (Bahnassy et al., 2004; P. Gao, 2004; Inomata et al., 1998; Quelle et al., 1993). If CDK4/6-cyclinD is not sufficient for inhibiting Rb then it remains unclear how excessive cyclin D can accelerate G1/S transition.

In contrast to mechanisms supporting that, CDK4/6-cyclin D and CDK2-cyclin E regulates the progression through G1/S by Rb phosphorylation, new evidence challenges this notion (Chung et al., 2019; H. W. Yang et al., 2020). Instead, these studies propose that CDK4/6-cyclin D is exclusively responsible for hyperphosphorylation of Rb to activate E2F. Using live cell reporter to monitor CDK2 and CDK4/6 activity, it was concluded that CDK4/6, not CDK2, is both necessary and sufficient for Rb phosphorylation during G1. Subsequently, it was proposed that the function of CDK2 becomes crucial only once cells entered S phase to maintain Rb phosphorylation and trigger a positive feedback regulation on E2F expression. The observation that CDK2 is dispensable for Rb hyperphosphorylation contradicts other experiments demonstrating that acute CDK2 inhibition leads to G1 accumulation (K. A. Merrick et al., 2011; Narasimha et al., 2014).

As for today no consensus on a final model of activation of the RP exist.

1.5.1.3 *Alternative restriction point.*

The definition of what constitute the RP has also been challenged in recent studies (Johnson & Skotheim, 2013; Pennycook & Barr, 2020; Rubin et al., 2020). The classical model placed the commitment point during late G1 phase driven by Rb

hyperphosphorylation, activation of E2F-dependent transcription, and a requirement for sustained growth factor signalling. Traditionally, the inactivation and hence hyperphosphorylation, of Rb was considered a pivotal step during commitment to S phase. Collectively, these feedback loops serve to strengthen the initial rise in CDK2 activity and the hyperphosphorylation and subsequent inactivation of Rb. This ensures that CDK2 activity can be sustained independently of decreases in growth factor signalling. Therefore, CDK2 activity threshold is suggested to be the defining feature of RP passage (Dong et al., 2014). This threshold is reached several hours before full activation of the Rb-E2F pathway, indicating that it reflects the onset of E2F activation rather than its peak (Kolupaeva & Janssens, 2013). It's worth noting that the CDK2 activity threshold for RP passage may vary depending on the cell type and exposed stimuli (Schwarz et al., 2018).

However, recent studies defined a new RP independent function of CDK2 activity (Cappell et al., 2016). It was shown that cells with hyperphosphorylated Rb can still arrest their cell cycle before entering the S phase, when exposed to DNA damage (Cappell et al., 2016; Chung et al., 2019). Therefore, it was proposed that APC/C^{Cdh1} inactivation represents the real RP in cells. APC/C^{Cdh1} inhibits S-phase entry, this is achieved by degrading cyclins, Cdc25, and components of the Skp1-Cul1-F-Box (SCF) complex. The degradation of Skp2 inhibits the degradation of p21 and p27 (Bornstein et al., 2003; Carrano et al., 1999; Kamura et al., 2003). APC/C^{Cdh1} inactivation is a rapid, bistable switch initiated shortly before the start of DNA replication by CDK2-cyclin E and made irreversible by Emi1. Emi1 is a direct target of E2F and a competitive inhibitor of APC (Di Fiore & Pines, 2007; Machida & Dutta, 2007). At the end of G1, Emi1 switch from being an APC/C^{Cdh1} substrate to being its inhibitor (Cappell et al., 2018). Exposure to stress between Rb phosphorylation and APC/C^{Cdh1} inactivation, but not after APC/C^{Cdh1} inactivation, reverted cells to a mitogen-sensitive quiescent state regulated by Rb, from which they can later re-enter the cell cycle. Thus, APC/C^{Cdh1} inactivation is the commitment point when cells lose the ability to return to quiescence and decide to progress through the cell cycle. It is essential to highlight that the inactivation of Cdh1 follows Rb inactivation but precedes the initiation of DNA replication.

Additionally, the window of reversibility between the RP and S-phase entry may not only be determined by APC/C^{Cdh1} inactivation but also by the rapid degradation of p21 at S-phase entry by CRL4^{Cdt2} (Barr et al., 2017; Heldt et al., 2018; K. A. Merrick

et al., 2011). Importantly, Skp2 itself is targeted by Cdh1, suggesting that p21/p27 inactivation likely occurs downstream of Cdh1 inactivation. Notably, p21 degradation is stimulated by the ubiquitin ligases SCF^{Skp2} and CRL4^{Cdt2} and generate a bistable switch and prevents p21 upregulation in S phase, which could otherwise promote premature S-phase exit. First, CDK2 activity phosphorylates p21 and targets it for SCF^{Skp2}-mediated degradation, while p21 inhibits CDK2 activity and next p21 is degraded by CRL4^{Cdt2} following recruitment to the proliferating cell nuclear antigen (PCNA) in active replication complexes (aRCs), whereas p21 binds to PCNA and prevents the activation of replication complexes (RCs) (Barr et al., 2017). Once this bistable switch is engaged, the persistent activity of CRL4^{Cdt2} at a RCs during S phase generates a persistent degradation of p21, preventing it from interfering with DNA replication and protecting S phase progression from the influence of DNA damage (Chao et al., 2017).

However, it is still unclear, if cells arrested during the window of Rb hyperphosphorylation and APC/C^{Cdh1} inactivation is a proper quiescent arrest.

Due to these complexities, the timing of the RP should be described in a general sense rather than pinpointed to an exact moment.

1.5.1.4 Cell size.

Another factor required to ensure a proper progression through G1, is the ability for cells to respond to various growth signals and regulate cell growth (Cadart et al., 2019; Dolznig et al., 2004; Killander & Zetterberg, 1965). Indeed, cell size at birth directly correlates with the time cells spend in G1 (Cadart et al., 2018; Ginzberg et al., 2018; Varsano et al., 2017).

It has been hypothesised that cells are able to measure their size through size-dependent concentration changes of proteins (Carlson et al., 1999; S. G. Martin & Berthelot-Grosjean, 2009; Moseley et al., 2009). This change in concentration regulates the progression across G1. The first molecular mechanism of size sensing was first discovered in budding yeast. It was observed that the concentration of the cell cycle inhibitor Whi5 is low in larger cells compared with smaller cells (Schmoller et al., 2015). Smaller-born daughter cells beginning the cell cycle with higher concentrations of Whi5. Because the synthesis of Whi5 is restricted to the S/G2/M phase of the cell-division cycle, cell growth dilutes Whi5 in G1 to trigger progression into the

cell cycle. In human cells, similar mechanisms have been unveiled, where the concentration of the functional ortholog of Whi5 (Rb) also regulates cell size and hence the cell cycle (Zatulovskiy et al., 2020). Rb inhibition upon phosphorylation was shown to be linked to smaller cell size, whereas its over-expression leads to larger cells (D. A. Foster et al., 2010; Saxton & Sabatini, 2017; Zwang et al., 2011). In addition to Rb concentrations, other factors such as p38 also play a role in cell size regulation. p38 is activated in small cells to prevent entry into S-phase (S. Liu et al., 2018). However, why, and how the p38 pathway is activated in these small cells remains unclear. Other pathways implicated in cell size sensing is the mammalian target of rapamycin complex 1 (mTORC1). mTORC1 is a key regulator of cellular growth. Recent studies suggested that CDK4/6 activity promotes mTORC1 allowing cells to grow to the correct size in order to progress to S-phase (Schmoller et al., 2015).

In summary, while the relationship between cell size, growth, and the G1/S transition has been thoroughly studied, the exact molecular mechanisms behind cell size sensing in eukaryotes remains unclear.

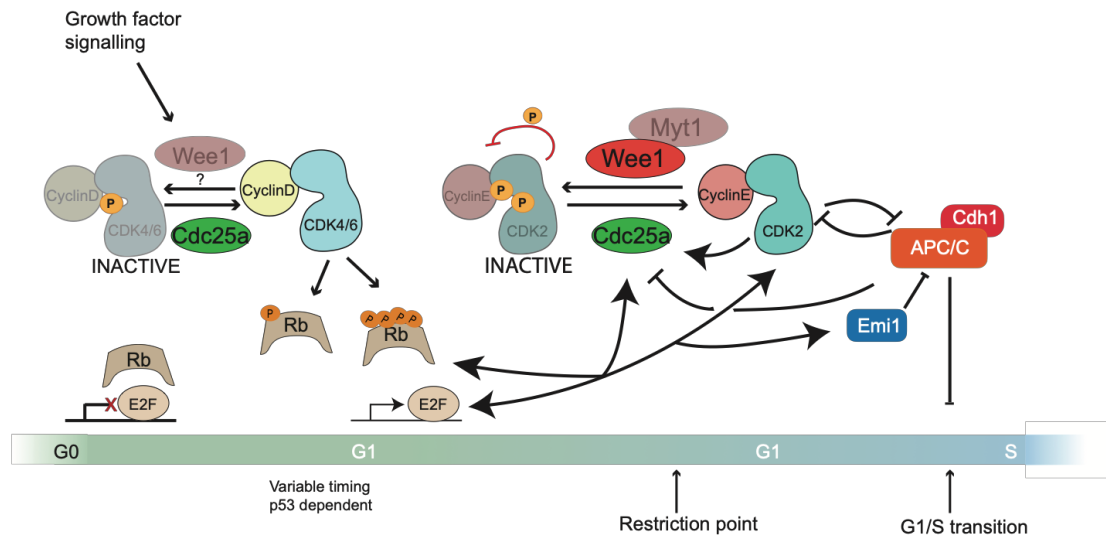


Figure 1-4. Regulation of the G1/S transition.

Adapted from (Pennycook & Barr, 2020). Upon mitogen signals, CDK4/6-cyclin D is activated allowing the partial release of Rb (Mono-phosphorylation or Hypophosphorylation) from E2F. Following Rb activation and E2F release, transcription factor essential for S-phase are being transcribed such as Cdc25A, Emi1 and cyclin E (E2F pointing arrow to CDK2-cyclin E, represents the increase in the complex activity). cyclin E binds to CDK2 and form an activated complex, essential for the G1/S transition. Once CDK2-cyclin E reaches a sufficient activity threshold, it inhibits APC/C^{Cdh1} in combination with Emi1, which switches from an APC/C^{Cdh1} substrate to an APC/C^{Cdh1} inhibitor. It is only when APC/C^{Cdh1} is inactivated that the cell transitions to S-phase.

1.5.1.5 *Restriction point dynamics.*

The definition of the exact timing of the RP has been defined as a precise point in late G1. However recent advancements in single-cell analysis have unveiled a much more intricate and context-dependent scenario (Barr et al., 2017; Martinsson et al., 2005; Naetar et al., 2014; Schwarz et al., 2018; Spencer et al., 2013). While primary fibroblast cell lines tend to adhere to the classical restriction point, many commonly cultured cell lines do not conform to this pattern.

The vast majority of the biochemical analysis to study commitment mechanisms has been done using chemical or synchronisation methods to obtain more homogeneous populations. However, these techniques do not take into account previous cell division. Recent studies on cycling cells highlighted the role of the cellular memory in the commitment decision (Barr et al., 2017; Spencer et al., 2013; T. Zhang, 2013). In this study it is shown that the cells undergo a pre-committed state, which can be caused by cellular memory mechanisms transmitted from the mother cell. Here, high levels of active CDK2 and phosphorylated Rb can occur even before the cell enter the cell cycle. Hence, the cell loses its RP and the cell rushes through the cell cycle phase into S-phase (Spencer et al., 2013; T. Zhang, 2013). On the other hand, the presence of DNA damage in the previous cycle can also impact the timing of RP passage in the subsequent cycle. Inherited DNA damage activates p21 and arrests the cells, preventing them from entering S-phase (Arora et al., 2017; Barr et al., 2017).

In summary, the classical model of the RP in the cell cycle, traditionally associated with Rb hyperphosphorylation and growth factor signalling in late G1, is being re-evaluated in light of recent discoveries. The observed variability in cell behaviour, the existence of pre-committed states in certain cell lines, and the influence of cellular memory from previous cycles challenge the simplicity of the traditional model, highlighting the need for a more nuanced and context-specific understanding of how cells commit to division. The intricate nature of the CDK network, the precise methods of perturbation, and subtle disparities in cellular model systems may all contribute to shaping these varying outcomes. Importantly, it is recognised that the requirements for crossing the G1/S differs in cells re-entering the cell cycle from quiescence compared to continuously cycling cells (Matson and Cook, 2017).

1.6 APC/C regulation in G1 phase.

APC/C is an E3 ubiquitin ligase complex, which is important for protein degradation by the 26S proteasome during G1 phase and mitosis. The activity of APC inhibits the several stages of the cell cycle. For this reason, its activity is tightly regulated during cellular division, which relies on the binding of the co-activators Cdh1 (APC/C^{Cdh1}) and Cdc20 (APC/C^{Cdc20}). Without the co-activator, the core APC/C is inactive as an E3 ubiquitin ligase and is unable to bind most APC/C substrates. The co-activators promote the interaction of the substrate to the APC/C to form a stoichiometric ternary complex of APC/C–co-activator–substrate, dependent on the D box (RxxLxxxxN), ABBA motif and KEN box degrons (Di Fiore et al., 2015; Eytan et al., 2006; Passmore, 2003; Passmore & Barford, 2005).

Indeed, numerous studies demonstrate direct and D box- and KEN box-dependent binding of APC/C substrate proteins to co-activators (Bashir et al., 2004; Burton et al., 2005; Burton & Solomon, 2001; Hilioti et al., 2001; Pflieger & Kirschner, 2000; Zachariae et al., 1998). Chemical cross-linking of substrates to the WD40 domains of Cdc20 and Cdh1 in a D box- and KEN box-dependent manner confirms a role of the co-activators to contribute towards the recruitment of the substrate to the core APC/C (Kraft et al., 2005; Passmore & Barford, 2005).

Where Cdc20 activates APC/C during early mitosis, Cdh1 plays an essential role from later mitosis to the G1/S transition (Fig. 1.5) (Visintin et al., 1997).

In late anaphase the decrease in CDK1 activity results in the partial activation of Cdh1 (Crasta et al., 2008; J. M. Peters, 2006). This process is further augmented when Cdh1 is liberated from Mad2 inhibition. In this case Cdh1 takes over the role of Cdc20 in activating the APC/C complex, which leads to a further decline in CDK1 activity and the entry to the next G1 (Jaspersen et al., 1999; Kramer et al., 2000; Zachariae et al., 1998).

The Cdh1 helix is a structural motif within the Cdh1 protein formed by a sequence of amino acids within the Cdh1 protein, and its three-dimensional structure enables it to bind to a specific region of the APC complex (APC1) domain via its N-terminal Met1, as well as Tyr5, Leu9 and Ile residues on site of APC. On the opposite side of Cdh1 other residues such as Asp2, Asp4, Tyr5, Arg8, Arg11 and Gln12 form an interaction with APC1 and APC8 (Importance on APC/C^{Cdh1} residues conformation will be further discussed in Chapter 7: Discussion) (Anna Höfler et al., 2023).

Following mitosis, the primary function of APC/C^{Cdh1} is to sustain low levels of S-phase proteins during G1. APC/C^{Cdh1} targets several substrates for destruction by the proteasome, including, Cdc25A, Ets2, cyclin A, Geminin and Skp2 (Geley et al., 2001; M. Li et al., 2008; McGarry & Kirschner, 1998; Pesin & Orr-Weaver, 2008; Wei et al., 2004). Skp2 is an F-box protein responsible for the recognition of p21 and p27 by the SCF complex and their subsequent ubiquitylation and Ets2 has been reported to induce cyclin D expression. Thus, APC/C^{Cdh1} maintains cells in G1 by accumulating p21 and p27 through Skp2 proteolysis and by limiting the expression of cyclin D1 via promoting Ets2 degradation (Bornstein et al., 2003; Budhavarapu et al., 2012; Carrano et al., 1999; Eguren et al., 2011; Kamura et al., 2003). Accordingly, depletion of Cdh1 by siRNA stabilises Skp2 and Ets1, resulting in p21 and p27 degradation and cyclin D1 elevation in G1, followed by premature entry into S phase (Eguren et al., 2011).

Besides inhibiting the G1/S transition, APC/C^{Cdh1}, in collaboration with CKIs, plays an important role in maintaining cells in the G0/G1 phase. cycling cells activate APC/C^{Cdh1} at the end of mitosis, while quiescent cells keep APC/C^{Cdh1} active to avoid re-entering the cell cycle.

For cells to commence DNA replication, APC/C^{Cdh1} activity must be switch off. This is facilitated by a combination of APC/C^{Cdh1} inhibitors and phosphorylation by CDK-cyclin complexes (Choudhury et al., 2016a). As CDK1 and CDK2 activity levels increase from the G1/S transition to anaphase, Cdh1 undergoes phosphorylation by CDK2, which impedes its interaction and binding with APC/C (Kramer et al., 2000; C. Lukas et al., 1999; Zachariae et al., 1998). Phosphorylated Cdh1 is also targeted for degradation by the SCF ligase, limiting APC/C^{Cdh1} activity (Choudhury et al., 2016a). Towards the end of G1-phase, E2F activates the transcription of Emi1, which binds to APC/C as a pseudo-substrate, inhibiting Cdh1 binding during interphase (L. Chang et al., 2015; Grosskortenhaus & Sprenger, 2002; Hsu et al., 2002; Reimann et al., 2001). Recent results indicate that Emi1 accelerates APC/C^{Cdh1} degradation (Cappell et al., 2016, 2018). The subcellular localisation of Cdh1 represents another important regulatory layer in activating APC/C^{Cdh1}. In G1-phase, Cdh1 is located in the nucleus, whereas some studies suggest that it migrates to the cytosol between S-phase and anaphase (Jaquenoud, 2002; Zhou et al., 2003). CDK2 phosphorylation promotes the nuclear export and, sequestering Cdh1 to the cytosol hindering its binding to APC/C and other nuclear targets. Furthermore, Cdh1 orchestrates its own

degradation through its own interaction with APC/C (Listovsky et al., 2004; Rape & Kirschner, 2004).

1.7 G1 DNA checkpoint.

The G1/S checkpoint represents a critical control mechanism which the cell uses to ensure genome integrity before entering DNA replication and segregation. It is one of the key checkpoints which is activated by DNA damage during the cell cycle and aimed at halting or delaying cell cycle progression to allow DNA repair. At its core is the Ataxia telangiectasia mutated (ATM) kinase, which triggers a checkpoint signalling pathway upon detecting DNA damage (Abraham, 2001; Hirao et al., 2000; Khanna et al., 2001; J. Smith et al., 2010; Yan et al., 2008). This involves the phosphorylation of targets like checkpoint kinases 1 and 2 (CHK1 and CHK2), leading to the degradation of the Cdc25 phosphatase family and stabilisation of the tumour suppressor p53. As, p21 has been identified as a p53 target, consequently p53 induction increase p21 level (El-Deiry et al., 1993; El-Deiry et al., 1994; Warfel & El-Deiry, 2013). Consequently, p21 levels rise, reducing CDK activity and playing a vital role in genomic integrity. Hence, dysfunction in the G1/S checkpoint is common in oncogenesis due to the bypass of surveillance mechanisms, leading to uncontrolled cell growth.

Despite having functional checkpoint proteins, the effectiveness of the G1 DNA damage checkpoint can be limited and variable between the same populations. This variability in cellular responses to DNA damage has been investigated in recent studies (Englund et al., 2023; Nathans et al., 2021). These results confirm that all cells in G1 phase are able to initiate activation of the ATM-p53-p21 signalling pathway in response to DNA damage, independently of where they lie in G1 phase (Englund et al., 2023; Nathans et al., 2021). However, the data suggests that regarding when the checkpoint is activated during G1, cells can either route back to G0, where the DNA damage can be repaired, or continue to S phase, where the damage results in permanent cell cycle arrest. If the checkpoint is activated within the last 2 hours of the G1 phase, the cells will enter S-phase. This is caused by a delay of 2 hours, which is required between DNA damage sensing and p21 induction, leading to CDK2 inactivation. This shows that the cell cycle checkpoint not only pauses the cell cycle, but

instead can lead to a prompt return to the quiescent state in response to DNA damage (Heldt et al., 2018; Nathans et al., 2021).

1.8 S-phase entry.

Entry into S-phase is a pivotal event in the cell cycle, ensuring the accurate and faithful replication of genetic material. To guarantee equal transmission of genetic material to offspring, the initiation occurs at autonomously replicating sequences (ARS) in yeast, is tightly regulated throughout the cell cycle (Stinchcomb et al., 1979). Interestingly, the exact sequence of the replication origins in mammals remain unknown. However, studies suggest that a AT-rich sequence is preferred to start replication (Vashee et al., 2003). In 1994, Diffley and his research team discovered that replication origins exist in two distinct states during the cell cycle: pre-replicative and post-replicative. The pre-replicative state spans from the end of mitosis to the start of the S phase, while the post-replicative state occurs during the S phase, G2, and mitosis (Diffley et al., 1994). DNA footprinting conducted at ARS sites revealed that regulatory proteins are recruited to replication origins during the pre-replicative state, enabling the subsequent recruitment of protein during the S phase (Diffley et al., 1994b). The loading and activation of these origins is regulated through two distinct steps, ensuring that each origin undergoes replication only once per cell cycle. The initial step, known as origin licensing, takes place in the G1 phase, where replication proteins are loaded onto the origins. The subsequent step, termed the origin activation step, occurs during the S phase, permitting only licensed origins to initiate DNA replication (Fig 1.5).

1.8.1 DNA licensing.

Heavily studied in yeast, several proteins are recruited to ARS to form pre-replication complex (pre-RCs), including origin recognition complex (ORC), cell division cycle 6 (Cdc6) cdc10-dependent cell division cycle transcript 1 (Cdt1) (Hofmann & Beach, 1994) and mini-chromosome maintenance (MCM)(Bell & Stillman, 1992; Cocker et al., 1996; Diffley et al., 1994; Hofmann & Beach, 1994; Speck et al., 2005). Origin licensing is fundamental cellular process, which is highly conserved among eukaryotes. In mammalian cells, pre-RC assembly begins with ORC loading onto presumptive origins of replication. ORC recruits Cdc6 to chromatin where it acts as a clamp

loader to assemble the six-membered of the MCM complex (Borlado & Méndez, 2008). Next, the complex is furthermore stabilised by the binding of Cdt1 to the MCM complex to form a pre-RC (Machida & Dutta, 2005; Masai et al., 2010; Siddiqui et al., 2013a).

The concerted action of ORC, Cdc6 and Cdt1 results in loading of an MCM hetero-hexamer onto DNA with double-stranded DNA passing through the MCM central channel (Remus et al., 2009; Riera et al., 2013; Yardimci & Walter, 2014). Cdc6 and then Cdt1 are released, followed by a second round of Cdc6 and Cdt1-MCM recruitment (Ticau et al., 2015). Origin firing requires phosphorylation events from CDK2 and the replication-specific kinase, Dbf4-dependent kinase. This kinase promotes the recruitment of additional essential helicase components to activate DNA unwinding (Ilves et al., 2010; N. Li et al., 2015). Additionally, it is noteworthy that CDK1 also promotes the activation of the helicase (Suski et al., 2022). Origin licensing can only occur upon low CDK activity (Fisher & Nurse, 1996; Masai et al., 2010; Stern & Nurse, 1996). Thus, it begins in telophase, as soon as the nuclear envelope forms around the segregated mitotic chromosomes, and throughout G1, before ceasing at the G1/S transition (Dimitrova et al., 2002; Symeonidou et al., 2013).

1.8.2 Activation of licensed origins for replication.

After origins have been licensed in G1, only a subset of them are activated in S phase by the G1/S kinases CDK2 and Dbf4-dependent Cdc7. After the phosphorylation and hence activation of the replication origins, DNA polymerases are recruited to the selected origins. Among other crucial player, PCNA is recruited to initiate DNA synthesis (Amasino et al., 2023; Bravo et al., 1987; Feng et al., 2021; G. L. Moldovan et al., 2007; Prelich et al., 1987; C. K. Tan et al., 1986). PCNA is a ring-shaped homotrimer that encircles the DNA and enhance the processivity of the DNA polymerase (Choe & Moldovan, 2017; De March & De Biasio, 2017; González-Magaña & Blanco, 2020; O'Donnell et al., 2013; Siddiqui et al., 2013b; Strzalka & Ziemienowicz, 2011). Ensuring the precision of DNA replication during normal mitotic cell cycles is crucial for maintaining genome stability and preventing potential adverse effects on cell proliferation (Arias & Walter, 2007). The occurrence of DNA re-replication, even from a single origin, can lead to genome instability, DNA damage, cell cycle arrest, and apoptosis (Davidson et al., 2006; Neelsen et al., 2013; Nguyen et al., 2001).

The over-lapping effects of multiple mechanisms prevent re-licensing at the scale necessary in mammalian cells, in which DNA is replicated from tens of thousands of origins (Fig. 1.6). These mechanisms include CDK2-dependent phosphorylation of Cdt1, targeting it for degradation through SCF^{Skp2} E3 ubiquitin ligase (Nguyen et al., 2001; N. Sugimoto et al., 2004). Additionally, the protein geminin binds and sequesters Cdt1 in an inactive complex (Xouri et al., 2004; K. Yoshida & Inoue, 2004). As geminin is an APC/C substrate, this inhibition is relieved upon mitotic exit through to G1, when APC/C activity is high. During S phase, Cdt1 is targeted for degradation through interaction with PCNA and Cul4-Ddb1^{Cdt2} E3 ubiquitin ligase (Arias & Walter, 2007). Together, these mechanisms ensure that Cdt1 protein levels oscillate during the cell cycle and are only high during late M/G1 phase. In addition, re-replication is prevented through inactivation of pre-RC proteins by phosphorylation and their degradation. ORC is inhibited during mitosis by CDK-mediated phosphorylation (Nguyen et al., 2001; Diffley, 2004). Other member of the pre-RC such as Cdc6 can still be targeted for degradation by the activities of APC/C and CDK2 (Arias & Walter, 2007).

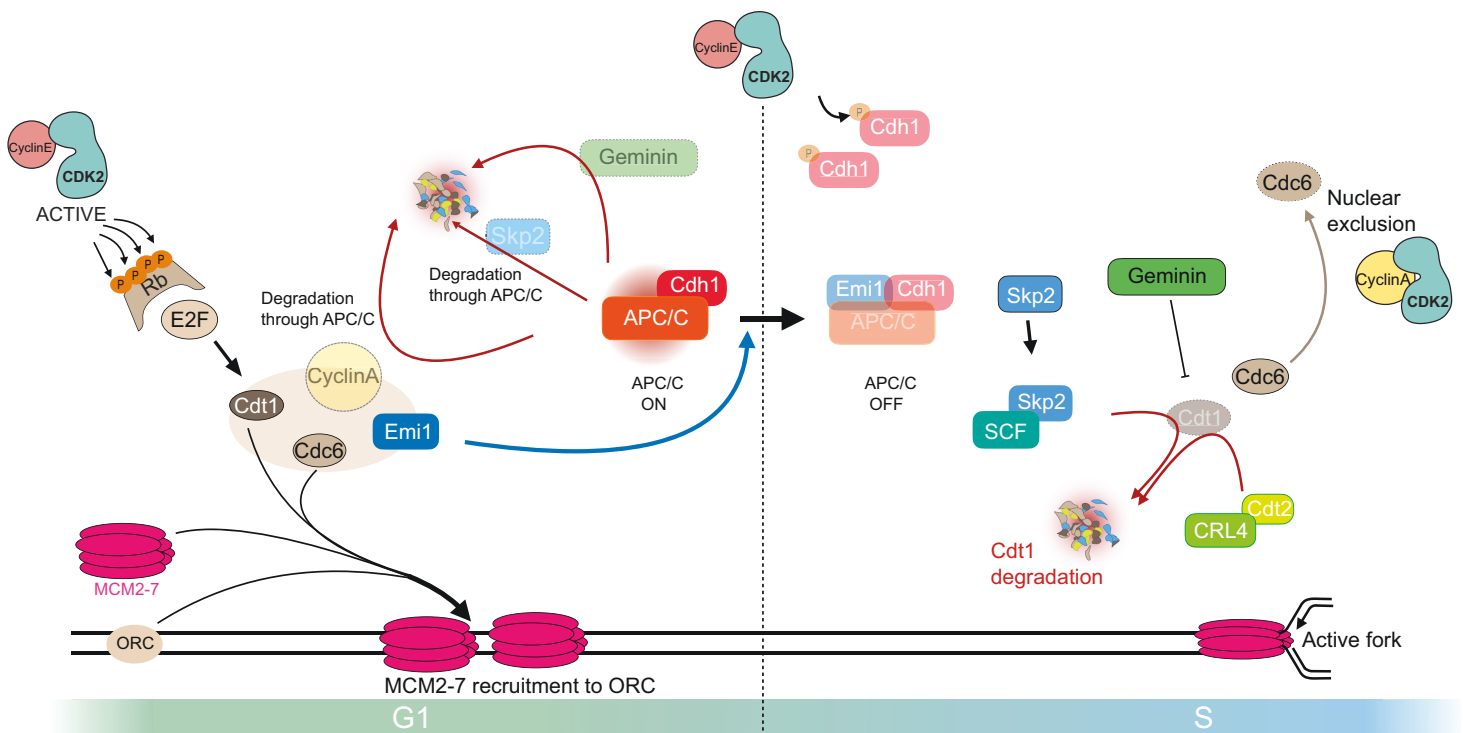


Figure 1-5. Schematic diagram of the main steps in DNA replication.

Rb phosphorylation through CDK2-Cyclin E induces the release of E2F and the expression of target genes, notably Cdt1 and Cdc6. Cdt1 and Cdc6 participate with ORC 1-6 complex to the recruitment of MCM2-7 helicase to chromatin. The release of E2F also enables the expression of Emi1, the accumulation of which will induce inactivation of APC/C^{Cdh1} (in addition to Cdh1 phosphorylation by CDK2-Cylin E). Inactivation of APC/C^{Cdh1} allows the stabilisation of geminin, Skp2 and Cyclin A.

1.8.3 Replication stress and DNA damage during S-phase.

During S-phase of the cell cycle, the process of DNA replication can be hindered by the stall or the collapse of the replication fork. When the replication forks encounter such impediments to their progression, DNA replication stress often ensues. Whilst the precise definition of DNA replication stress is variable, it will be defined here as the stalling or slowing of replication fork progression, exposing single strand DNA (ssDNA), which may activate a specific checkpoint response (Muñoz et al., 2017; Zeman & Cimprich, 2014). The cellular response to DNA replication stress is mediated by the DNA replication stress checkpoint, which depends on the sensor kinase Ataxia Telangiectasia and Rad3-related (ATR) kinase, which phosphorylates and activates downstream effectors such as CHK1. Activated CHK1 promotes cell cycle arrest by deactivating Cdc25 phosphatases and by inhibiting CDKs, allowing time for DNA damage repair. Additionally, the ssDNA is coated by the replication protein A (RPA), initiating the stabilisation of stalled replication forks.

The DNA replication stress checkpoint has a dual role with respect to its effect on DNA replication origin activation. Whilst the activation of new origins is inhibited at a global level by the checkpoint, local activation of dormant origins is promoted when necessary to rescue stalled forks (Ge & Blow, 2010; Yekezare et al., 2013).

Additionally, the over-activation of replication origins can cause replication stress. Over-usage of origins can refer to both increasing the number of origins which are active at one time during S and mis-regulation of DNA replication timing. An increase in the number of active replication forks during S phase could mean that replication factors are exhausted. Excessive origin firing can deplete RPA or deoxynucleotides (dNTPs), for example, leading to replication stress and double strand breaks (DSBs) (Bester et al., 2011; Toledo et al., 2013). In this way, over-replication can result in slower replication fork progression and an increased chance of fork stalling. In addition, an increase in the number of active forks also increases the chance of conflicts with transcription. Indeed, cyclin E overexpression has also been shown to result in origin over-activation and transcription-dependent replication stress in some contexts (Jones et al., 2013).

The DNA replication stress pathway is a specialised aspect of a broader DNA damage response pathway. Upon exogenous or endogenous signals such as radiation,

chemical perturbations or persistence of unsolved replication stress, can lead to DSBs resulting in genome instability (Giglia-Mari et al., 2011). To counteract this, cells activate a complex signalling network called the DNA damage response (DDR). Upon DDR, the levels of the tumour suppressor p53 increase, allowing the induction of genes involved in DNA repair and cell cycle arrest. In fact, p53 increases the production of p21, and inhibits CDK2-cyclin A leading to a halt in the G2 phase of the cell cycle (Ekholm & Reed, 2000; El-Deiry et al., 1993; El-Deiry et al., 1994). p21 has also been implicated in regulation of replication fork progression, although different results were reported as to whether p21 promotes DNA elongation or suppresses fork speed (Mansilla et al., 2020; Maya-Mendoza et al., 2018).

Additionally, p21 was shown to regulate PCNA, a key player in DNA replication (Cayrol et al., 1998; De March et al., 2017; Dovrat et al., 2014; Krishna et al., 1994; G.-L. Moldovan et al., 2007; Waga et al., 1994). PCNA contributes to the processivity of the DNA polymerase, essential for DNA replication and DNA repair. However, p21 can interfere with these functions by obstructing the recruitment of these replication factors by PCNA. Various studies have attributed different functions to p21 ranging from a role in promoting DNA elongation to slowing down the replication fork.

Additionally, a new study observed that DNA damage occurring during S-phase leads to activation of p21 and an arrest in G2 or in the subsequent G1 (in daughter cells) via CDK2 inactivation (Barr et al., 2017). This study also shows the importance of cellular memory to understand cell cycle regulation and progression (explained in section 1.5.1.5).

1.9 G2/M checkpoint.

Mitosis is the most dramatic event in the cell cycle, where cells undergo both biochemical and morphological changes. The regulation of the G2/M transition is dictated by CDK1-cyclin B and CDK1-cyclin A activity. (Fung et al., 2007; Hégarat et al., 2020; Hochegger et al., 2008). It has been shown that cyclin B is sufficient to allow proliferation while cyclin A is essential for viability and mitotic entry in human cells (Hégarat et al., 2020).

Mitosis is driven by two major switches: the first one pushes cells into mitosis by controlling CDK1 activity, and the second one triggers mitotic exit by controlling CDK1-counteracting phosphatase PP2A-B55 by Greatwall kinase (Gwl) (Álvarez-

Fernández et al., 2013; Burgess et al., 2010; Castilho et al., 2009; Cundell et al., 2013; Vigneron et al., 2009). PP2A-B55 is serine/threonine phosphatase and shown to counteract CDK1-cyclin B activity to obtain the temporal pattern of protein phosphorylation and physical changes required for the cell to divide correctly (Mochida et al., 2009). Indeed PP2A-B55 is thought to directly phosphorylates Wee1 and Cdc25 thereby negating regulating CDK1. Gwl kinase pathway regulates PP2A-B55 activity by phosphorylating two proteins, (Arpp19, ENSA) turns these proteins into potent inhibitors of PP2A-B55, thereby promoting a correct timing and progression of mitosis (Castilho et al., 2009; Gharbi-Ayachi et al., 2010; M.-Y. Kim et al., 2012; Lorca et al., 2010; Mochida et al., 2009; Rangone et al., 2011; Vigneron et al., 2009; Y. Zhao et al., 2008).

Cyclin A is critical to trigger mitosis by counteracting Wee1 and PP2A-B55 and contrary to cyclin B, cyclin A can activate both CDK1 and CDK2 (Pines & Hunter, 1991). The levels of cyclin A increase throughout S phase and peak in G2. It is subsequently degraded from prometaphase onwards (den Elzen & Pines, 2001; Jacobs, 2001). Similarly, to cyclin B, cyclin A also shuttles between cytoplasm and nucleus, but in contrast to cyclin B the rate of nuclear import exceeds its export, so it is predominantly nuclear (Jackman et al., 2002). Overexpression of CDK2-cyclin A in G2 phase cells promotes mitotic entry while its depletion delays entry into mitosis arising from a delay in the nuclear translocation and activation of CDK1-cyclin B complex (De Boer et al., 2008; Gong et al., 2007). Additionally, both cyclin B and cyclin A are necessary for mitosis (Brandeis et al., 1998; Murphy et al., 1997). It is likely that cyclin A contributes to the initial activation of CDK1-cyclin B in G2 phase (Fung et al., 2007; Guadagno & Newport, 1996; Mitra & Enders, 2004).

In G2, new cyclin B is synthesised and binds to CDK1 to form the CDK1-cyclin B complex. CDK1-cyclin B accumulates in the cytoplasm during interphase as it is constantly exported from the nucleus (Hagting et al., 1998; J. Li et al., 1997; F. Toyoshima, 1998; J. Yang et al., 1998). Upon mitotic entry, CDK1-cyclin B is rapidly moved to the nucleus. Once in the nucleus, CDK1-cyclin B needs to be dephosphorylated in order to form an active CDK1-cyclin B complex, which promotes mitotic entry (Gould & Nurse, 1989). Dephosphorylation on both residues (Y15 and T14) is carried out by the Cdc25 phosphatases (B and C) (Gautier et al., 1991; Kumagai & Dunphy, 1991, 1992b; Strausfeld et al., 1991). Wee1 and Cdc25 regulate CDK1 activity and are in turn regulated by CDK1, establishing a network of feedback loop

regulations (Fig 1.3). This consists of a double negative feedback loop, in which CDK1 phosphorylates and inactivates Wee1, and a positive feedback loop, in which CDK1 phosphorylates and activates Cdc25 (Fig. 1.3) (Daya-Makin et al., 1992; Hoffmann, 2000; Kumagai & Dunphy, 1991, 1992b; L. X. Lu et al., 2012; McGowan & Russell, 1995; Okamoto & Sagata, 2007; Tang et al., 1993). Cdc25 in turn activates more CDK1-cyclin B1 by dephosphorylating T14 and Y15 in CDK1 (Hoffmann, 2000; Izumi et al., 1992; Izumi & Maller, 1993; Kumagai & Dunphy, 1991, 1992).

Due to feedback regulation, Wee1 and Cdc25 phosphorylation was shown to be highly sensitive in regulating CDK1 activity (Goulev & Charvin, 2011; S. Y. Kim & Ferrell, 2007; Trunnell et al., 2011). It is this regulation involving double positive feedback loops that include Wee1-CDK1-Cdc25 which makes the activation of CDK1 switch-like and mitotic entry an irreversible decision (Novak & Tyson, 1993; Pomerening et al., 2005; Sha et al., 2003). It is worth noting that CDK1-cyclin B is itself subject to positive feedback. Nuclear CDK1-cyclin B promotes the translocation of more CDK1-cyclin B to the nucleus (Gavet & Pines, 2010). The nuclear import of CDK1-cyclin B1 is promoted by the phosphorylation of serine residues in cyclin B1 (Santos et al., 2012). In mammalian cells, cyclin B shuttles between the nucleus and cytoplasm in interphase before rapidly accumulating in the nucleus at prophase. Nuclear CDK1-cyclin B1 promotes the disassembly of the nuclear lamina and the nuclear envelope breakdown (NEBD) (Ookata et al., 1992; Pines & Hunter, 1991). This positive feedback is aborted when cyclin B is degraded during NEB (Santos et al., 2012).

Different levels of CDK1 activity play distinct roles in driving various stages of mitosis. The levels of CDK1-cyclin B1 activity required to initiate mitosis are lower than those needed for advancing through the mitotic process. This implies that a gradual rise in CDK1-cyclin B activity is crucial for coordinating mitotic progression following centrosome separation (Gavet & Pines, 2010). As CDK1-cyclin B activity increases it phosphorylates Gwl that in turns will inhibits PP2A-B55 allowing mitosis completion.

CDK1-cyclin B and Gwl activity will be maintained high until anaphase when the activation of the APC will induce the progressive ubiquitination and degradation of cyclin B (Echard & O'Farrell, 2003). CDK1-cyclin B complex does not only directly phosphorylate Gwl to maintain its activity but also phosphorylates and inhibits PP1, the phosphatase responsible of the dephosphorylation of Gwl. At anaphase, the

progressive decrease of CDK1-cyclin B activity ensues with a gradual auto-dephosphorylation and reactivation of PP1 (J. Q. Wu et al., 2009). Active PP1 induces dephosphorylation of Gwl enabling the activation of a threshold level of PP2A-B55 that will subsequently dephosphorylate Gwl on its activatory sites (Heim et al., 2015; Lacroix et al., 2022). Gwl will be now completely inactivated and will trigger a negative feedback loop resulting in the overall reactivation of PP2A-B55 and mitotic exit (Heim et al., 2015; Ma et al., 2016; D. Ren et al., 2017; Rogers et al., 1986).

1.9.1 APC/C regulation during G2.

In G2, APC/C is activated through the binding to Cdc20, which is responsible for degrading cyclin B. Hence, the G2/M transition is inhibited through APC/C activity. For this reason, the activity of APC/C^{Cdc20} needs to be restrained in order to allow build-up of cyclin B levels, which promotes together with CDK1 G2/M transition (Lara-Gonzalez et al., 2017). Besides cyclin B, APC/C^{Cdc20} also tags securin for proteolytic degradation. Securin is an inhibitor of sister chromatid separation and needs to be degraded during mitosis (Alfieri et al., 2017; J.-M. Peters, 2006; Pines, 2011).

During mitosis, APC/C^{Cdc20} must also be restrained until all chromosomes are connected to the spindle, in order to prevent errors in chromosome segregation. Furthermore, unattached chromosomes can also inhibit APC/C^{Cdc20} during mitosis via the spindle checkpoint pathway that is activated at kinetochores (Musacchio, 2015). In contrast to APC/C^{Cdc20} regulation by kinetochores in mitosis, how APC/C^{Cdc20} activity is restrained during G2 to allow cyclin B accumulation and the G2/M transition is not well understood. The APC/C inhibitor Emi1 was initially proposed to block APC/C^{Cdc20} activity in G2 (Reimann et al., 2001). However, subsequent work showed that Emi1 targets APC/C bound by its other co-activator, Cdh1, and that its primary function is to prevent re-replication of DNA (Cappell et al., 2018; Machida & Dutta, 2007). Other research suggests that restraining the activity of APC/C^{Cdc20} includes the phosphorylation of Cdc20 to reduce its binding affinity with APC (Shevah-Sitry et al., 2022).

1.9.2 G2 DNA Damage Checkpoint.

The G2 DNA damage checkpoint is a critical control mechanism in eukaryotic organisms that ensures that cells with damaged or incomplete DNA replication do not proceed into mitosis. This checkpoint prevents mitotic initiation until DNA is adequately repaired, thus maintaining genomic stability, and avoiding the proliferation of damaged cells. As explained earlier, the transition between G2/M is regulated by CDK1-cyclin B. Upon DNA damage sensing, the G2 checkpoint is activated. The checkpoint activation comprises the activation of the ATM pathway resulting in the degradation of Cdc25B/C, the activation of Wee1 and thereby halting the cell cycle. Concomitantly, the ATM pathway also induces expression of the tumour suppressor p53, which induces activation of the CKI p21. The presence of DNA damage can also activate ATR protein kinase pathways (Brown & Baltimore, 2003; Lavin, 2008). ATR phosphorylates and activates CHK1, which then phosphorylates Wee1 and Cdc25C, thereby both activating Wee1 kinase activity and inactivating Cdc25C phosphatase activity. The activation of ATM and/or ATR depends on the type of genotoxic stress. ATM is primarily activated by DSBs whereas ATR responds to a broad spectrum of DNA damage, including DSBs and a variety of DNA lesions that interfere with replication (Brown & Baltimore, 2003; Lavin, 2008; Marechal & Zou, 2013; Shiloh, 2003; H. Wang et al., 2004).

This also leads to an arrest in the cell cycle as this leads to the inactivation of CDK1 inactivation (O'Connell, 1997). In human cancers, p53 is frequently mutated highlighting the critical role of a functional G2 checkpoint in tumour suppression (Calonge & O'Connell, 2008; Taylor & Stark, 2001).

The induction of p53 also induce several others mechanism such as CDK1 nuclear exclusion through 14-3-3 protein and preventing of the interaction between CDK1 and cyclin B through GADD45-CDK1 competitive binding (E. Tamura et al., 2012; Hermeking et al., 1997).

1.10 Wee1 and cell cycle control.

1.10.1 Wee1 discovery.

Wee1 and Cdc25 are pivotal regulators of cell cycle progression, serving as gatekeepers that coordinate the intricate transition between cell cycle phases. Wee1 kinase, acts as a critical inhibitory regulator, phosphorylating and thereby inactivating CDKs (see section 1.4, 1.7). Cdc25 operates as a dual specificity phosphatase, removing inhibitory phosphate groups from CDKs, thus counterbalancing the activity of Wee1, and promoting CDKs activation (see section 1.4, 1.7, Fig.1.2). The dynamic interplay between CDK inhibitory phosphorylation by Wee1 and the activating dephosphorylation by Cdc25 are important regulatory control mechanisms of the cell cycle, guaranteeing the fidelity of cell division and maintaining genomic stability.

Originally Wee1 was isolated as a gene responsible for the “wee” (Scottish for small) phenotype in fission yeast (P. Russell & Nurse, 1987). Fission yeast lacking Wee1 is characterised by a smaller cell size, caused by the inability of the mutant to negatively regulate the activity of Cdc2 (Gould & Nurse, 1989). These observations led to an important notion that Wee1 kinase is a negative regulator of mitotic initiation and thus of the cell cycle. In line with this, over-expression of the wild type Wee1 protein leads to a pronounced mitotic delay (P. Russell & Nurse, 1987). The budding yeast analogue of Wee1 (Swe1) also delays entry into mitosis and is required for cell-size control (Harvey et al., 2005; Jorgensen et al., 2002).

The Wee1 kinase is a highly conserved protein among a wide range of species including human, frog, mouse, *Saccharomyces cerevisiae*, *Drosophila* (De Schutter et al., 2007; P. R. Mueller, Coleman, & Dunphy, 1995a).

In *Xenopus*, cDNAs encoding a Wee1 homolog have been isolated from an oocyte cDNA library (P. R. Mueller, Coleman, & Dunphy, 1995a; Murakami & Woude, 1998). Wee1 protein encoded by the oocyte cDNA is absent or very scarce in immature or maturing (meiosis I) oocytes but is rapidly synthesised during meiosis II and stably present in mature oocytes arrested at metaphase II (Iwabuchi, 2000; Murakami & Woude, 1998; Nakajo et al., 2000). The presence of Wee1 protein in mature oocytes is required for the recognisable G2 phase or CDK1 Tyr15 phosphorylation in the first

embryonic cell cycle (Murakami & Woude, 1998; Walter et al., 2000). As in yeast, Wee1 loss of function causes premature entry into mitosis.

Drosophila Wee1 (DWee1) is closely related to *Xenopus* Wee1 and also causes premature entry into mitosis (Mueller, Coleman, & Dunphy, 1995b; Price et al., 2000; Stumpff et al., 2004). To isolate DWee1, cDNA clones were screened that rescue *S. pombe* wee1-mutants from lethal mitotic catastrophe (Price et al., 2000). DWee1 has an essential maternal function during the nuclear division cycles of embryogenesis and also implicates zygotic *Dwee1* function in a cell cycle checkpoint that responds to inhibition of DNA replication.

Further investigation in mice shown that, Wee1 ^{-/-} embryos were defective in the G2/M checkpoint induced by γ -irradiation and died of apoptosis before embryonic (E) day 3.5 (Tominaga et al., 2006).

To investigate the human orthologue of Wee1, functional complementation assays were carried out where the cDNA of the human Wee1 was expressed in fission yeast lacking Wee1 (Makoto Igarashi et al., 1991). The results showed that human Wee1 rescued the deficiency in Wee1 in fission yeast. In addition, Wee1 in these cells (p49^{Wee1}) showed to phosphorylate and hence deactivate CDK2-cyclin B on Y15 *in vitro* (McGowan & Russell, 1995b; L. Parker & Piwnica-Worms, 1992). Moreover, the ectopic over-expression of human Wee1 cDNA arrested cell division in Hela Cells. Taken together these results confirmed that human Wee1 plays a similar role in cell cycle regulation as in other organisms (McGowan & Russell, 1995).

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Wee1-S.C      TSSKVSSSTSHLLTRFRNVTLGSGEFSEVFQVEDPVEKTLKYAVKKLVKFSGPKERNR
Wee1-Xenopus   FVLRETNMGSRKYKTEFLEIEKIGAGEFGSVFKCIKRLDG-CFYAIKRSKKPLAGSTDEQL
Wee1-Drosphila.M LALHDTN-ISRFKREFMQVNVIGVGEFGVWFQCVNRLDG-CIYAIKRSKKPVAGSSFEKR
Wee1-Mus Musculus ITITESNMKSRYTTEFHELEKIGSGEFGSVFKCVKRLDG-CIYAIKRSKKPLAGSVDEQN
Wee1-Homo sapiens ITITESNMKSRYTTEFHELEKIGSGEFGSVFKCVKRLDG-CIYAIKRSKKPLAGSVDEQN

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Wee1-S.C      LLQEVSIQRALKGHDHIVELMDSWEHGGFLYMQVELCENGSLDRFLEEQG-QLSRLDEFI
Wee1-Xenopus   ALNEVWAHVLGKHNDVRYSSAWAEDDHMLIQNEFCDDGSLHARIQDH-----CLGEAE
Wee1-Drosphila.M ALREVYAHAVLGHHPHVRYSSAWAEDDHMLIQNEYCNGGSLQDLIVDNNKEGQFVLEQE
Wee1-Mus Musculus ALREVYAHAVLGQHPHVRYSSAWAEDDHMLIQNEYCNGGSLADAISENYRMSYLTEVE
Wee1-Homo sapiens ALREVYAHAVLGQHSHVRYSSAWAEDDHMLIQNEYCNGGSLADAISENYRIMSYFKEAE

      * . * . * . * . * . * . * . * . * . * . * . * . * . * . * .

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*Indicates positions which have a fully conserved residue
:Indicates conservation between groups of strongly similar residue
.Indicates conservation between groups of weakly similar residue

Figure 1-6. Multiple sequence alignment of Wee1 in different species.

Wee1 protein alignment from position 610 to 669 between four different species: *Saccharomyces cerevisiae*, *Xenopus*, *Drosophila melanogaster*, *Mus musculus* and *Homo sapiens* using Clustal Omega.

1.10.2 Wee1 structure.

Work on *S. pombe* allowed describing the structure of this protein, distinguishing a catalytic domain of about 350 amino acids at its C-terminal end and a N-terminal domain of about 550 amino acids, containing the necessary sequences for its negative regulation (NRD, amino-terminal regulatory domain), through phosphorylation by CDK1 at Thr104 and 105 in *Xenopus laevis* and Tyr239 in human somatic cells (Aligue, Wu, & Russell, 1997; Harvey, Charlet, Haas, Gygi, & Kellogg, 2005; S. Y. Kim & Ferrell, 2007; S. Y. Kim, Song, Lee, & Ferrell, 2005). Additionally, in the central region of the protein, between residues 363 and 408, sequences important for its activity were identified, which are believed to be related to substrate recognition (Aligue et al., 1997).

The human Wee1 kinase, composed of 646-amino-acids, can be divided into three distinct domains: an N-terminal regulatory domain, a central kinase domain, and a C-terminal regulatory domain (McGowan & Russell, 1995b; Russell & Nurse, 1987; Squire et al., 2005). The initial observation of Wee1 mitotic regulation set the stage for subsequent studies.

The N-terminal domain, which is the largest, includes two significant phosphorylation sites (Ser53 and Ser123). The phosphorylation of these sites generates a phosphodegron, which allows recognition by an F box protein and targets Wee1 for ubiquitination and degradation by the 26s proteasome (Watanabe et al., 2004). This domain also contains three PEST regions, which is often contained in proteins which have a short intracellular half-life and hence are rapidly degraded. Wee1 also contains a nuclear localisation signal (NLS) at its N-terminus, crucial for nuclear localisation of the protein (Fig. 1.7) (Rogers et al., 1986; Squire et al., 2005).

The central kinase domain functions as a tyrosine kinase, despite its greater resemblance to serine/threonine kinases. This suggests an evolutionary adaptation that enables its action on tyrosine residues (Squire et al., 2005).

The succinct C-terminal domain includes a binding site at Ser642, which upon phosphorylation recruits the 14-3-3 chaperone proteins (Esposito et al., 2021). These are necessary in stabilising Wee1 and thus amplifying its kinase activity (Honda et al., 1997). The binding of the 14-3-3 proteins to Wee1 induces structural change that shields the degradation site, thus leading to protein stabilisation. Moreover, it was

shown that these changes influence the intracellular distribution of Wee1 (J. Lee et al., 2001; Y. Wang et al., 2001). It has been observed that wild type Wee1 is localised evenly throughout the nucleus when an adequate supply of 14-3-3 is available however in non-phosphorytable mutant Wee1 (Ser549), Wee1 loses this uniform distribution and associate with punctate structures in the nucleus.

Further reports also described that Wee1 localises in the nucleus and shuttling back to the cytoplasm during prophase (Baldin & Ducommun, 1995).

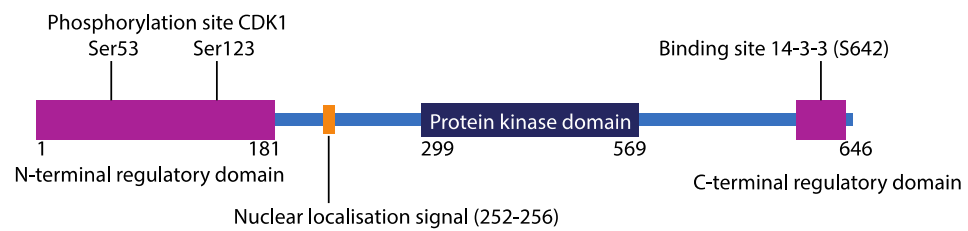


Figure 1-7. Schematic representation of Wee1 domain structures, interaction, and post-translational modifications sites.

1.10.3 Wee1 role and regulation.

Studies conducted in both *S. pombe*, *Xenopus* and human cells conclude that the G2/M checkpoint is primarily induced by the activity of the CDK1 kinase complexed with cyclin B (see section 1.9) (Nurse, 1990). In *S. pombe*, CDK1 is negatively regulated by phosphorylation at tyrosine 15 (p-Y15) Wee1 and Myt1 while in higher eukaryotes, including mammalian cells, phosphorylation of threonine 14 (p-T14) is required (Gould & Nurse, 1989; Lundgren et al., 1991; Norbury et al., 1991; L. Parker & Piwnicka-Worms, 1992; P. Russell & Nurse, 1987).

1.10.3.1 Wee1 substrates.

In humans, Wee1 constitutively phosphorylates CDK1 and CDK2 at Y15 whereas Myt1 is able to also phosphorylate CDK1 on T14, Y15 (Lundgren et al., 1991). Although both Wee1 and Myt1 are necessary for a timely mitotic entry, Myt1 has a more important role in meiosis and in the assembly of the Golgi and ER (Kishimoto, 1998; Murakami & Woude, 1998; Nakajima et al., 2008; Nakajo et al., 2000; Okamoto et al., 2002). During interphase Wee1 and Myt1 have different intracellular localisations. Wee1 is nuclear and Myt1 is associated with the membrane of the Golgi apparatus and of the endoplasmic reticulum (F. Liu et al., 1997; McGowan & Russell, 1993; P. R. Mueller, Coleman, Kumagai, et al., 1995; Russell & Nurse, 1987). CDK1 mutant cells which cannot be phosphorylated on Y15 and T14 undergo rapid cycle and exit M-phase without cytokinesis and karyokinesis, displaying a similar phenotype as seen in Wee1 KO cells (Pomerening et al., 2008). This study shows the interplay between CDK1 and Wee1 in regulating cell cycle timing in human cells. Similar studies using a CDK2 mutant where T14 and Y15 sites were changed to alanine and phenylalanine (CDK2AF) showed that the loss of inhibitory phosphorylation causes premature S-phase entry, abnormal DNA replication and rapid cyclin E degradation which could reflect either decreased transcription or increased degradation (Hughes et al., 2013; Zhao et al., 2012). CDK2AF do not affect cyclin E mRNA abundance supporting the idea that CDK2 inhibitory phosphorylation stabilise cyclin E (Hughes et al., 2013).

Besides Y15 within CDK1 and CDK2, only a few other Wee1 substrates are currently known. Wee1 plays a crucial role in the regulation of histone synthesis by

phosphorylating histone H2B at position Y37 within the nucleosomes located upstream of the histone gene cluster. This phosphorylation event leads to the suppression of histone transcription during the late S phase (Mahajan & Mahajan, 2013).

Furthermore, recent study identified Y15 on the CDK5 subunit of a newly identified CDK5-cyclin B complex as a novel substrate for Wee1 (Andrew V. Grassetti et al., 2021). CDK5 has an essential role in the central nervous system development (Dhavan & Tsai, 2001). A hypothesis arising from this paper is that CDK5-cyclin B do not form an active protein kinase complex but instead act as a reservoir for Wee1 binding and phosphorylation, resulting in an increased pool of unphosphorylated CDK1-cyclin B.

Wee1 has also emerged as a key player in regulating histone synthesis. It phosphorylates histone H2B at tyrosine 37 in the nucleosomes found upstream of the histone gene cluster, which suppresses histone transcription in late S phase (Mahajan & Mahajan, 2013).

After mitotic entry, the regulatory role of Wee1 persists throughout mitosis. As inter-phase microtubules disassemble for chromosomal movements, Wee1 plays a pivotal role in regulating CDK1 localised at centrosomes and their nucleating microtubules. High activity of CDK1-cyclin B is responsible for the remodelling of the cytoskeleton by inhibiting microtubule-associated protein (MAPs) and hence inhibiting tubulin growth (Chang et al., 2001; Mchedlishvili et al., 2018). MAPs are required for the growth of spindle microtubules and spindle assembly. Wee1 has been found to suppress a pool of CDK1 localised at centrosomes. This specific pool of CDK1 manages to evade the action of Cdc25, remaining inhibited by Wee1-mediated phosphorylation (David et al., 2019; Serpico et al., 2022). Consequently, MAPs are locally liberated from their inhibitory phosphorylation, enabling the formation of spindle assembly. In addition to its regulatory role in the G2-M checkpoint, other studies have shown that Wee1 is important for DNA replication during S phase, through CDK-dependent mechanisms.

During S-phase, when fork stalling occurs, the endonuclease MUS81 is required to catalyse the cleavage of stalled fork structures. For efficient substrate targeting, MUS81 needs to form a complex with another protein called SLX4 (Duda et al., 2016). The formation of the MUS81-SXL4 complex is mediated by CDK1-dependent phosphorylation of SXL4 (Duda et al., 2016). It has been shown that premature activation of CDK1 and excessive CDK1/2 activity triggers uncontrolled fork degradation

through MUS81 (Elbæk et al., 2022; Szakal & Branzei, 2013). Wee1 can restrain MUS81 activity through CDK1/2 regulation and potential direct binding with MUS81 (Domínguez-Kelly et al., 2011; Duda et al., 2016). It has been shown that Wee1 controls dNTPs availability by limiting CDK-driven origin firing pool (D'Angiolella et al., 2012; Pfister et al., 2015), CDK1/2 activity regulates the synthesis of dNTPs (D'Angiolella et al., 2012). Indeed, ribonucleotides reductase subunit RRm2, which catalyses the conversion of ribonucleotides to deoxyribonucleotides, is degraded via the CDK-SCF^{cyclin F} axis. Wee1 inhibition leads to higher CDK activity leading to an exhaustion of dNTPs.

Another role of Wee1 can be linked to immunity (Hamilton et al., 2014; Rao et al., 1996). In several cancer types such as lung cancer and pancreas, loss of CDK1 activity has been monitored inducing inadequate lamin phosphorylation (Rao et al., 1996). Lamin phosphorylation and hence degradation is CDK1-dependent, which is an important step during apoptosis (Broers & Ramaekers, 2014; Eldeeb et al., 2018; Jeong et al., 2022). This process induces resistance to lysis by antigen-specific T-cells and natural killer cells. Accordingly, when Wee1 is inactivated, CDK1 activity increases and allow the phosphorylation of lamin and its degradation leading to an increase in apoptosis of cancer cell death.

Furthermore, evidence shows that Wee1 levels are controlled by components of the circadian clock, revealing a direct cross-talk between the cell cycle and the circadian clock (Matsuo et al., 2003). Indeed, a circadian transcription factor, BMAL1/CLOCK, directly binds to the E-box elements of *WEE1* elements and activates the transcription of *WEE1*. Little is known about other Wee1 transcriptional regulation mechanisms.

1.10.3.2 Wee1 regulation.

The activity of Wee1 was shown to be regulated by phosphorylation and proteolytic degradation. At mitosis entry, Wee1 is negatively regulated through phosphorylation. The kinase partially responsible for the phosphorylation of Wee1 at M-phase is CDK1 (McGowan et al., 1995; Mueller, Coleman, & Dunphy, 1995a; Tang, Coleman, & Dunphy, 1993; Watanabe et al., 2005).

Additional phosphorylation events are also known to regulate the activity of Wee1. Wee1 is phosphorylated on S123 by CDK1 thereby generating a binding motif for polo kinase 1 (PLK1) and casein kinase 2 (CK2). Both proteins in turn phosphorylate Wee1 on S53 and S21 (Toyoshima-Morimoto et al., 2001; van Vugt et al., 2004). Together, the phosphorylation of these three serine residues serves as a tag for Wee1 degradation by the ubiquitin ligase SCF β -TrCP. This hyperphosphorylation and subsequent proteolytic degradation collaboratively leads to the inactivation of Wee1 during mitosis (Watanabe et al., 1995). Moreover, catalytic inactivation of Wee1 is observed when phosphorylated by the CDK-cyclin A complex at its inhibitory site T239 (Li et al., 2010). Additionally, spatial regulation occurs through AKT-mediated phosphorylation of Wee1 at S642, creating a 14–3–3 peptide-binding site, facilitating Wee1 translocation from the nucleus to the cytoplasm and abolishing Wee1-induced G2/M arrest (Katayama et al., 2005).

Initially identified through a yeast two-hybrid screen, in mice the protein 14-3-3 emerged as a potential binding partner for the C-terminus of Wee1 (Honda et al., 1997). It has been demonstrated that also the human homologue of Wee1 interacts with 14-3-3 via its C-terminal motif between amino acids 639-646 (Wang et al., 2000). Wee1 protein stability and its ability to maintain interphase was enhanced by co-expression of 14-3-3 in human cells (Wang et al., 2000). This positive regulatory effect was corroborated in *Xenopus* and human cells, where 14-3-3 binding to Wee1 during interphase at the phosphorylated residue S549 (S642 in human Wee1), was found to be essential (Lee et al., 2001; Wang et al., 2000). Chk1-mediated phosphorylation of S549 and subsequent 14-3-3 binding is crucial for optimal Wee1 activity. As the diminished activity of a phospho-mutant Wee1 (S549A) in phosphorylating CDK1-cyclin B *in vitro* did not affect the intrinsic kinase activity of Wee1 (Lee et al., 2001; Wang et al., 2000b). In humans, Wee1 is also positively regulated by auto-phosphorylation during interphase on two sites (Y295 and Y362) which were shown to promote Wee1 activity (Katayama et al., 2005; McGowan & Russell, 1993).

1.10.4 Cdc25 role and regulation.

As mentioned previously, Wee1 acts as a major kinase within the cell cycle, whereas Cdc25 acts as its counteracting phosphatase activity. Hence, it acts as a cell cycle

activator. In mammalian cells, three members of the Cdc25 gene family have been described, which functionally complement *S. pombe* which is lacking *Cdc25* (Nagata et al., 1991; Sadhu et al., 1990). These include the three isoforms of Cdc25: Cdc25A, Cdc25B and Cdc25C.

Experiments performed using tissue culture cells have revealed several distinguishing characteristics of individual Cdc25 family members. Microinjection of Cdc25A antibodies arrests cells prior to S phase, and ectopic expression of Cdc25A shortens the G1 phase, thereby accelerating entry into S-phase (Blomberg & Hoffmann, 1999; Hoffmann et al., 1994b; Jinno et al., 1994; Sexl et al., 1999). These results suggest a role for Cdc25A at the G1/S transition, although Cdc25A is active throughout all stages of the cell cycle (Blomberg & Hoffmann, 1999; Hoffmann et al., 1994a). Microinjection of antibodies specific for either Cdc25B or Cdc25C arrests cells in G2 suggesting roles for these proteins at the G2/M transition (Lammer et al., 1998; Millar et al., 1991). Cdc25B has been shown to activate CDK2-cyclin A and CDK1-cyclin B complexes *in vitro* (Gabrielli et al., 1997a; Honda et al., 1997; Sebastian et al., 1993). Unlike Cdc25B, the phosphatase activity of Cdc25C is low during the S and G2 phases of the cell cycle and is high in mitosis (Ducommun et al., 1990; Hoffmann et al., 1993; Honda et al., 1993; Izumi & Maller, 1993; Kakizuka et al., 1992; Villamoruzzi, 1993).

Interestingly, mice with *Cdc25C* disrupted are healthy, and cells derived from these mice exhibit normal cell cycles and checkpoint responses. *Cdc25B*^{-/-} mice are also generally normal (although females are sterile), and cells derived from *Cdc25B*^{-/-} mice have normal cell cycles. Furthermore, cell cycles, DNA damage responses, and Cdc25A regulation are normal in cells lacking *Cdc25B* and *Cdc25C*. However, mice lacking Cdc25A are embryonic lethal (Bahassi et al., 2011; Solc et al., 2008). These findings indicate that *Cdc25A*, or possibly other phosphatases, is able to functionally compensate for the loss of *Cdc25* and *Cdc25C* in mice.

Cdc25 phosphatases are regulated at transcription, translation, or the post-translational level. Cdc25A is a transcriptional target of the E2F–RB1 pathway (Ray & Kiyokawa, 2008). The Myc transcription factor initiates Cdc25A protein expression and both Myc and Cdc25A are expressed at elevated levels in tumours (Boutros et al., 2007a; Zörnig & Evan, 1996). Transcription factors, E2F1/2/3 and STAT3, activate the transcription of Cdc25. In human cells the activity of Cdc25 phosphatases is

induced through the MAPK signalling (mitogen-activated protein kinases) p38 and ERK1/2 (extracellular signal-regulated kinase) (Brancho et al., 2003; R. Wang et al., 2007; Yan et al., 2008). Indeed, p38 and its downstream target, MAPKAPK2 were initially shown to phosphorylate Cdc25B and Cdc25C *in vitro* and *in vivo* after UV-induced DNA damage or induce Cdc25A degradation after cisplatin treatment (Bulavin et al., 2001; Lemaire et al., 2006; Manke et al., 2005; Reinhardt et al., 2007). Cyclin B accumulates before and/or mitosis entry. Before mitotic entry, cyclin B accumulates in the nucleus and increases the activity of CDK1. Increasing activity of CDK1 triggers the activation of Cdc25 B/C, further enhancing CDK1 activity. This forms a double positive feedback loop where activated CDK1 stimulates more Cdc25B/C activation, amplifying the cycle. Cdc25 B/C level may also be affected by other factors (Hégarat et al., 2016; Kishimoto, 2015; Qian et al., 2013). Upon entry into mitosis PLK1 is activated by Aurora in a CDK1-cyclin A-dependent manner, and in turn phosphorylates Cdc25 to trigger activation of CDK1-cyclin B (O'Farrell, 2001; Rieder, 2011; Y. Thomas et al., 2016). During interphase, Cdc25 is inactivated by phosphorylation at Ser287 leading to the binding of 14-3-3 proteins and Cdc25 degradation (Kumagai et al., 1998).

The importance of Ser287 in maintaining Cdc25 inactivation during interphase was confirmed by experiments in which Ser287 was changed to a non-phosphorylatable Ala, thereby driving premature entry into mitosis, even in the presence of Cdc25B and C. Additionally, Cdc25 was shown to be phosphorylated by Chk1 and 2, which equally results in binding of the 14-3-3 proteins (Furnari et al., 1997, 1999; Graves et al., 2001; Y. Zeng et al., 1998). At the end of mitosis, Cdc25A is degraded via APC/C^{Cdh1}, thus further promoting CDK1 inactivation (M. Donzelli et al., 2002). Other mechanisms independent upon APC/C complex also play a role in the degradation of Cdc25A. It has been monitored that when interfering with the SCF function, it prevent the degradation of Cdc25A and thus confirming its role in this process (M. Donzelli et al., 2002).

Cdc25A/B messenger RNA (mRNA) have been reported to be overexpressed in various human cancer including breast, lung and colorectal cancer (Bonin et al., 2006; Cangi et al., 2000; Galaktionov, Lee, et al., 1995; Ito, Yoshida, Uruno, et al., 2004; Ngan et al., 2003; Ozen & Ittmann, 2005). However, the lack of correlation between mRNA level and protein levels reports is one of the limiting utility of Cdc25

overexpression as a prognostic indicator of cancer. Indeed, *Cdc25B* overexpression could be associated with higher grade tumours while in other study it was also associated with lower grade tumours (Galaktionov et al., 1996; Ito, Yoshida, Matsuzuka, et al., 2004). A similar case has been observed for *Cdc25A* mRNA overexpression. *Cdc25A* mRNA was observed to be overexpressed and associated with poor prognosis in one study whereas no association could be done between *Cdc25A* protein overexpression and clinicopathological (Cangi et al., 2000; Ito, Yoshida, Uruno, et al., 2004). Furthermore, the mechanisms by which *Cdc25* become overexpressed is not fully understood. At the moment there is no significant evidence that it's a results from gene amplification or rearrangements or any other specific genetic mutations. Some studies reported that *Cdc25A/B* could be direct transcriptional targets of the MYC pathway. Activation of MYC resulted in increased *Cdc25A* and *Cdc25B* mRNA levels, which correlated with an increase in protein expression (Galaktionov et al., 1996). Additionally, ATM mutant cells, were also shown to result in increased *Cdc25A* stability in both unperturbed cell division and in response to UV radiation (Alderton et al., 2006).

1.11 Cell cycle deregulation and cancer.

Uncontrolled cell proliferation due to constitutive mitogenic signalling or due to the lack or defective responses to anti-mitogenic stimuli, leads to a deregulation of the cell cycle, which is a hallmark of human cancer (Hanahan & Weinberg, 2011). Cells showing mutations affecting CDKs, cyclins and checkpoint regulators have an increased proliferative potential compared to non-mutated cells. In humans, the vast majority of tumours shows alterations in in the CDK4/6-cyclinD-INK4-Rb axis.

CDKs are often targeted by cancer strategies to increase cellular proliferation. It was shown that the CDK4 gene undergoes amplification or over-expression in various cancer types (An et al., 1999; Tsutsui et al., 1999; Yu et al., 2006). Similar observations concerning CDK6 have been made. CDK6 gene amplification or over-expression was reported in different lymphoma leukaemia and glioma (Corcoran et al., 1999; Mendrzyk et al., 2005). CDK2 is usually not mutated in human cancers. However, amplification of cyclins E1 and E2 genes is common in cancers, notably in uterine and ovarian cancers (Caldon & Musgrove, 2010; Giannone et al., 2019;

Gorski et al., 2020). CDK2 can also be aberrantly activated due to changes in CDK inhibitors and other regulatory proteins. CDK2 activity is regulated by the tumour suppressor p53 upon DNA damage. This occurs through increasing levels of the CKI p21, which in turn inhibits CDK2, contributing to cell cycle control. Loss or mutation of p53, which is common in cancers, thus indirectly affects CDK2 activity (Ozaki & Nakagawara, 2011).

p21 is hardly ever mutated in cancer whereas p27 is often downregulated in several cancers, leading to increased cyclin E activity and initiation of the S-phase independently of CDK4/CDK6 (Abbas & Dutta, 2009; Chu et al., 2008). Unlike CDK2-4/6, genetic dysregulation of CDK1 isn't commonly linked to cancer. However more recent studies on CDK1 showed that its over-expression is associated with poor prognosis in colorectal cancer (J. Li et al., 2020). Additionally, alterations in the p53, p21, p27 pathways, and DNA damage checkpoints can indirectly influence CDK1. Increased CDK activity can also result from amplification in CDK-activating enzymes, particularly Cdc25 phosphatases, and alterations in pathways involving C-myc or RAS oncogenes (Boutros et al., 2007b; M. Donzelli & Draetta, 2003; Galaktionov, Jessus, et al., 1995).

Besides CDKs, cyclins are also often deregulated in cancer. Overexpression of cyclin D was observed in variety of cancers including breast, colorectal and prostate cancer (H. Gao et al., 2004; Hoechtlen-Vollmar et al., 2000; Yu et al., 2001). Also, gene amplification of cyclins E1 and E2 genes is common in cancers, notably in uterine and ovarian cancers (Caldon & Musgrove, 2010; Giannone et al., 2019; Gorski et al., 2020). Similar observations concerning CDK6 has been monitored where CDK6 gene amplification or over-expression have been reported in different lymphoma leukaemia and glioma (Corcoran et al. 1999, Mendrzyk et al., 2005).

Among *INK4*, p16 is the most commonly altered tumour suppressor gene in human cancer (Serra & Chetty, 2018). The loss of p16 determines the escape of cells from senescence driven by the sustained activity of CDK4/6 activity (LaPak & Burd, 2014; Serra & Chetty, 2018). The protein Rb, which is the most relevant target of CDK4/CDK6 and CDK2, is also often altered in cancer, particularly in retinoblastoma and small cell lung carcinomas (Sherr & McCormick, 2002). Moreover, Rb is

specifically targeted by proteins from oncogenic viruses like HPV E7, adenovirus E1A, and SV40 large T antigen, leading to its inactivation (Bourgo et al., 2009; H.-Y. Kim, 2001).

Other prevalent mutations in cancer are mutations in cell cycle checkpoint proteins. The ATM-CHEK2-p53 pathway is a commonly dysregulated checkpoint in cancer, which can be linked to prostate and breast cancers (Cao et al., 2006; Squatrito et al., 2010; Stolarova et al., 2020).

Other mutations such as mutations in BRCA1 and BRCA2 genes, involved in S- and G2/M checkpoints, predispose individuals to breast and ovarian cancers (Roy et al., 2012). Finally, p53 is the most frequently mutated gene in cancer (Hollstein et al., 1991). p53 can also be inactivated by binding to various viral oncoproteins or by mutations affecting its antagonistic regulator Mdm2 (Giono & Manfredi, 2007; Subhasree et al., 2013). This reduction in p53 activity leads to decreased p21 levels, resulting in CDK hyperactivity.

Given the critical role of dysregulated CDKs and cyclins in cancer, their inhibition is used for therapeutic strategies (Canavese et al., 2012; Pennycook & Barr, 2021; M. Zhang et al., 2021). CDKi are currently in clinical trials, with three CDK4/CDK6 inhibitors recently approved for certain breast cancers (O'Leary et al., 2016; Panagiotou et al., 2022; Shah et al., 2018; N. C. Turner et al., 2018; Yap et al., 2023). However, their efficacy in other cancers is limited, possibly due to the functional redundancy of CDKs and the lack of CDK-specific inhibitors. Novel inhibitor classes and alternative strategies, like microRNA, targeting cell cycle regulators, are being explored (Peng & Croce, 2016). Some microRNAs have shown efficacy in lowering cell proliferation and tumour progression *in vitro* and *in vivo* (S. Donzelli et al., 2015; Lehmann et al., 2008; Lujambio et al., 2008; Lujambio & Esteller, 2007).

1.11.1 Wee1 in cancer.

Due to its pivotal regulatory role at the G2/M transition, Wee1 has gathered significant attention in cancer research. Several studies highlighted that Wee1 is implicated in various cancers, including glioblastoma and breast cancer (Iorns et al., 2009; Mag-nussen et al., 2013; Masaki, 2003; Mir et al., 2010; S. Mueller et al., 2014; Music et

al., 2016). A comprehensive CRISPR screen within a multitude of cancer cell lines underlined its essential role in cancer cell survival (Liang et al., 2020).

Interestingly, some cancers, notably colon cancer and non-small cell lung cancer (NSCLC), exhibit reduced or absent Wee1 expression (Egeland et al., 2016; T. Yoshida et al., 2004). The molecular mechanism behind these phenotypes is not fully understood yet.

Interestingly, cancer cells in turn relied on the activity of Wee1. Cancer cells often inactivate the key cell cycle regulator p53, which inactivates the G1/S checkpoint. For this reason, some cancer cells fully rely on the G2 checkpoint to survive, which is regulated by Wee1 (Bridges et al., 2011a; Lamoral-Theys et al., 2010).

It has also been reported that certain cancer cells display an over-expression of Wee1 causing increase in the ATR/CHK1 pathway activity (Harris et al., 2014; Masaki, 2003b; Mir et al., 2010; S. Mueller et al., 2014b). This high Wee1 expression has been hypothesised to protect cells from DNA damage and subsequent death by prolonged cell cycle arrest, providing these cells with an opportunity to repair damaged DNA before proceeding to the next phase of the cell cycle.

Moreover, they observed a notable increase in Wee1 levels after exposure to chemotherapy, suggesting a role for this kinase in mediating the progression of the disease. Thus, increased Wee1 expression may represent an adaptive response to the chemotherapy that allows tumour cells to repair DNA damage and thereby survive.

1.11.2 The use of Wee1 inhibitors.

Given the important role of Wee1 in cancer, numerous strategies have been developed to target this kinase. However, to date only a few Wee1 inhibitors (Wee1i) have been developed. Wee1i are ATP-competitive inhibitors by targeting the ATP-binding pocket, these compounds block the catalytic activity, preventing substrate phosphorylation. One of them is pyridopyrimidine (PD-0166285). This inhibitor was shown to prevent CDK1 Y15 phosphorylation at low concentrations in various cancer cell lines (Panek et al., 1997; Y. Wang et al., 2001). It was also observed that PD-0166285 is a non-selective Wee1i and thus also targets Myt1, CHK1 and EGFR (Hashimoto et al., 2006; Panek et al., 1997; Y. Wang et al., 2001). Hence, its use remains limited.

Another Wee1i screen led to the discovery of Adavosertib or AZD11775 (now known as MK-1775) (Hirai et al., 2009; Krehling et al., 2012). This pyrazol-pyrimidine derivative has been reported to have an IC₅₀ of 5.2 nM *in vitro* (Hirai et al., 2010).

Detailed interaction between Wee1 and AZD-1775 can be found in (Matheson, Venkataraman, et al., 2016).

The molecular mechanisms of Wee1 inhibition rely on its effect on the G2 checkpoint. After treatment with Wee1i, cells do not have the inhibitory Y15 phosphorylation on CDK1-cyclin B, which removes the checkpoint. Hence, the cells are unable to pause the cell cycle in G2 phase to repair DNA damage, despite activated CHK1. When cells start to divide with large amounts of unrepaired DNA lesions, this will lead to aberrant chromosome segregation and loss of genetic information, ultimately causing cell death through mitotic catastrophe (Bridges et al., 2011b; Castedo et al., 2004). Cells with dysfunctional p53 demonstrate increased sensitivity to Wee1 inhibition likely due to a compromised G1 DNA damage checkpoint (Ku et al., 2017; Van Linden et al., 2013). Currently, no Wee1 inhibitors are FDA approved. However, research into Wee1 inhibition has demonstrated promising potential in cancer therapy. Indeed, Wee1i have shown promising results as chemosensitisers. Results showed that Wee1 inhibition can be used in combination with different DNA damaging agents, including radiation, gemcitabine, and ATR inhibitors (Bukhari et al., 2019; C.-P. Chen et al., 2023; Rajeshkumar et al., 2011; Rødland et al., 2021; Yin et al., 2017). This synergy is based on disrupting the DDR pathway upon Wee1i with MK-1775, which abrogates the G2/M checkpoint. Hence, the cells enter mitosis even if the DNA damage, induced by chemotherapy or radiotherapy, remains unrepaired. This results in mitotic catastrophe and cell death. This approach underscores the potential of Wee1i not only as a monotherapy but also as a combinatorial treatment with existing cancer treatments, offering a new avenue for enhancing the efficacy of cancer therapeutics.

Wee1 inhibition was initially thought to be selectively effective in p53-deficient cancer cells. Later studies showed efficacy of the treatment independent of the p53 status, both in monotherapies with MK-1775 and combinatorial treatments (Cuneo et al., 2016; Geenen & Schellens, 2017; M. Zhang et al., 2017). An explanation for this could be that cancers may have a defective G1 checkpoint caused by other factors than mutations in the p53 gene. For example, previous studies have shown that Wee1i not only affects the G2/M checkpoint but also progression through S and G2

phases (Aarts et al., 2012; Lockhead et al., 2020). As mentioned above, checkpoint kinase inhibition by Wee1i causes increased CDK activity and deregulates replication initiation in S phase including origin firing. Furthermore, Wee1 inhibition during S-phase results in the accumulation of DNA DSBs (Beck et al., 2012). This is explained by a new role of Wee1 in regulating the nucleotide pool. Sufficient levels of nucleotides are required to support accurate replication and nucleotide deficiency causes decreased fork speed and ultimately DNA breaks (Beck et al., 2012; Pfister et al., 2015). Notably, Wee1 controls nucleotide pools by limiting CDK-driven origin firing (Domínguez-Kelly et al., 2011; Hughes et al., 2013; Pfister et al., 2015). This relationship is further demonstrated in cancer cells harbouring elevated levels of CDK2 activity because they are sensitive to Wee1i. The current clinical trials investigating Wee1 inhibition and its effect on cancer cells show promising results (Do et al., 2015a; Leijen, van Geel, et al., 2016). In clinical trial phase 1 it has been observed that of 176 patients 94 (53%) had stable disease as best response, and 17 (10%) achieved a partial response. The response rate in *TP53*-mutated patients ($n = 19$) was 21% compared with 12% in *TP53* wild-type patients ($n = 33$). This first trial shows that Wee1i (MK-1775 or AZD-1775) was safe and tolerable as a single agent and in combination with chemotherapy. Cancer cells treated with MK-1775 show an increase in DNA damage markers due to high levels of γ H2AX, reduced levels of pY15-CDK2 and an increase in response rate in p53 mutant cancer cell lines (Leijen, van Geel, et al., 2016). In the clinical trial phase II out of 21 patients five patients (24%) showed progressive disease. Seven patients (33%) experienced stable disease as best response. Eight patients (38%) showed a partial response as best response, and one patient (5%) had a complete response, resulting in an overall response rate of 43% (Leijen, Van Geel, et al., 2016).

1.11.3 Wee1 inhibition sensitivity.

Although Wee1i have been extensively studied the factors that define Wee1i sensitivity or resistance remains elusive. Recent studies have highlighted some factors that explained the response to Wee1i. The most important one, as explained in section 1.11, is the p53 status of the cells. p53KO cells are more sensitive to Wee1i treatment. Moreover, other factors have also been investigated. It has been observed that cells with elevated replication stress are more sensitive to MK-1775.

This sensitivity is exemplified in cells with an overexpression of cyclin E (X. Chen et al., 2018). When overexpressed, cyclin E induces increase replication stress (Kok et al., 2020). Consequently, this increase replication stress renders these cells particularly vulnerable to the inhibitory effects of Wee1i. In addition to sensitivity to cyclin E overexpression, it has been shown that cells displaying KRAS mutation are also more sensitive to Wee1i (Richer et al., 2017).

The Fanconi Anaemia pathway is activated upon interstream crosslinks (ICLs) (Schubert et al., 2022). ICLs are DNA lesions that inhibit essential processes such as replication and transcription (Deans & West, 2011). Mutation of this pathway is also implicated in the sensitivity of Wee1 treatment. It is believed that this sensitivity is due to excessive replication stress (Aarts et al., 2015). Other mutations in the pathway responsible for a correct progression through S-phase leading to higher replication stress (such as RAD18 and Polk) have also been identified as more sensitive to Wee1i treatment (Y. Yang et al., 2017). Finally, as mentioned previously, low nucleotide levels are linked to Wee1 inhibition and some cancer cell lines are more sensitive to this effect (Pfister et al., 2015b). In this study it has been shown that cells with H3K36me3 deficiency produce low level of dNTPs. Indeed, H3K36me3 allow the transcription of RRM2 protein which is important for controlling dNTP pool in cells (Diehl et al., 2022). The combination of Wee1i in H3K36me3 deficient cells leads to a concentration of dNTPs too low for cell survival and thus leads to cell death.

Wee1i clinical trials also led to the discovery of resistance to Wee1i treatment. Decreased CDK activity in the G1 and S phases confers resistance to Wee1i (Heijink et al., 2015). The relation between p21 and Wee1 have been further investigated by Syljuåsen group showing that p21 protects cancer cells against Wee1 inhibition (Hauge et al, 2019).

1.12 Aim of the thesis.

Wee1 kinase is known to play a crucial role in the G2/M checkpoint through inhibitory phosphorylation of CDKs. Its role in the G2/M phase has been thoroughly studied, which resulted in the discovery of Wee1i as a cancer treatment. However, the role of Wee1 in other cell cycle phases requires further investigation. Recently, the use of Wee1i as a mono- or combinatorial therapy was heavily employed to treat several

cancer types. However, resistance mechanisms and insensitivity to Wee1i remain. For this reason, it is important to understand the role of Wee1 across the whole cell cycle and not only during G2/M phase.

In the work presented here, I focussed on the role of Wee1 specifically during G1. The effect of Wee1 on G1 progression and CDK2 regulation in G1 phase is a largely understudied field. Due to the presence of T14 and Y15 residues on CDK2, it is believed that the regulatory role of Wee1 on CDK2 would be similar than the one on CDK1, however such mechanism has not been studied yet. Understanding the exact role of Wee1 during cell cycle re-entry helps to understand how Wee1 affects cell cycle dynamics in healthy and cancerous cells and potentially helps to improve Wee1i cancer treatments.

Therefore, the aim of my thesis was to investigate the cell cycle dynamics upon Wee1 inhibition during G1. My hypothesis is that as Wee1 is a CDK2 negative regulator, then, its perturbation in cells re-entering the cell cycle from quiescence would lead to CDK hyperactivation and accelerated S-phase entry.

In order to assess the role of Wee1 during G1, I made use of single-cell time-lapse imaging. Previous approaches to study cell cycle dynamics upon perturbations included fixed cell and bulk assays, which are little informative about cellular heterogeneity and temporal dynamics of protein expression and localisation (Almendro et al., 2013; Lawson et al., 2018; McClellan & King, 2010). I hypothesised that by using single-cell imaging I would be able to monitor variation in cell cycle dynamics at a single cell level.

First, I followed cell cycle dynamics using live bio-sensors to distinguish the different phases of the cell cycle, upon Wee1 inhibition during G1. By tracking cell cycle length, I was able to monitor the effect(s) of Wee1 inhibition during G1 on cell cycle entry and subsequent cell cycle phase.

Second, I investigated the expression changes and localisation of Wee1 and Cdc25A. To test this, I tagged endogenous Wee1 with GFP and used a Cdc25A-GFP cell line to better understand the dynamics of these regulators at the G1/S transition. I carried out single-cell live imaging to quantify their expression during cell cycle re-entry and understand their regulatory role during this transition.

Third, I determined which other Wee1 substrates exist besides CDKs and how they are important for cell cycle entry. Wee1 is known to phosphorylate CDKs across the cell cycle. However, its role outside of this function has not been studied yet. For this I carried out phospho-proteomics on cells where Wee1 activity was specifically inhibited using MK-1775 and cells where Wee1 was degraded using an auxin-inducible degron system.

Chapter 2. Materials & Methods.

2.1 Cell lines.

Cell	Source	Cat no.	Species	Tissue	Media	Antibiotics
hTert-RPE1	ATCC	CRL-2302	Human	epithelial	DMEM	N/A
hTert-RPE1 mRuby-PCNA	Barr Lab	N/A	Human	epithelial	DMEM	N/A
hTert-RPE1 mRuby-PCNA mTurq2	Barr Lab	N/A	Human	epithelial	DMEM	N/A
hTert-RPE1 mRuby-PCNA Cdc25A-GFP	Barr Lab	N/A	Human	epithelial	DMEM	N/A
hTert-RPE1 mRuby-PCNA PLSD-GFP	Barr Lab	N/A	Human	epithelial	DMEM	N/A
myc-OsTIR1 hTert-RPE1	Hoe-cheger Lab	N/A	Human	epithelial	DMEM	N/A
myc-OsTIR1 hTert-RPE1 mRuby-PCNA p21-GFP	Barr Lab	N/A	Human	epithelial	DMEM	N/A
myc-OsTIR1 hTert-RPE1 Wee1-GFP-AID	Barr Lab	N/A	Human	epithelial	DMEM	N/A
hTert-RPE1 PIP-fucci Cdt1- geminin PCNA-mTurq2	Cook Lab	N/A	Human	epithelial	DMEM	N/A
hTert-RPE1 DHB-mCherry Cdc6-mVenus PCNA-mTurq2	Cook Lab	N/A	Human	epithelial	DMEM	N/A
NCI-H1944	ATCC	CRL-5907	Human	epithelial	EMEM	N/A
NCI-H1944 mRuby-PCNA	Barr Lab	N/A	Human	epithelial	EMEM	N/A
A549	ATCC	CCL-185	Human	epithelial	RPMI-1640	N/A
293T	ATCC	CRL-3216	Human	epithelial	DMEM	N/A
MCF7		HTB-22	Human	epithelial	DMEM	N/A

Table 2-1. List of all cell lines used.

hTert-RPE1 (referred to as RPE1 throughout) is an immortalised human retinal pigment epithelial cell line which evolved from a human cell line that was derived from a primary RPE culture derived by Amy Aotaki-Keen in 1986 (Dunn et al., 1996). RPE1 exhibit the basic phenotype common to all epithelia-forming stable monolayers that exhibits morphological and functional polarity, and also expresses the RPE-specific markers CRALBP and RPE-65. Although h-Tert RPE1 are non-transformed cells, they carry a KRAS mutation (Di Nicolantonio et al., 2008). hTert-RPE1 cells are widely used in cell cycle research and has been used to identify genes and signalling pathways that regulate cell growth and differentiation (Alizadeh et al., 2001.; Zhang et al., 2003). This cell line is diploid with stable 46 chromosomes (source ATCC and (Dunn et al., 1996).

MCF7 was first isolated in 1970 and established in 1973 (Soule et al., 1973). It is a widely used Estrogen receptor (ER+) breast cancer cell line and has been the one of the most frequently used cancer line to produce practical knowledge for clinical applications (E. Leung et al., 2011; Sweeney et al., 2012). These cells present an extensive aneuploidy with an average number of 82 chromosomes (range 66 to 87, source ATCC).

A549 is a non-small lung cancer cell line. It was isolated and established in 1973 (Giard et al., 1973). This cell line is used in a variety of applications such as disease modelling (model the alveolar Type II pulmonary epithelium), drug development and enzymatic studies (K. A. Foster et al., 1998; Juszczak et al., 2016; Lin et al., 1998). NCI-H1944 was first isolated and established in 1988 (Baker et al., 1988). It is a non-small lung cancer cell line and is heavily used in drug screening research (Baker et al., 1988; Tolani et al., 2022).

HEK 293T cell lines are derivated from a human embryonic kidney in 1973 and transformed with sheared adenovirus 5 (Ad5) in 1977 (W. C. Russell et al., 1977).

HEK293T cells are easily transfected, cell line has been extensively used as an expression tool for recombinants proteins (E. Tan et al., 2021).

2.2 Cell culture.

2.2.1 Growth media for cell lines.

All cell lines were routinely tested for mycoplasma using an Enzyme-Linked Immunosorbent Assay (ELISA) test kit (MycoAlert ® Cat Number: LT07-318) and were cultured at 37°C and 5% CO₂ with the corresponding growth conditions:

- RPE1 was cultured in DMEM/F12 (1:1) (Gibco) supplemented with 10% Foetal Bovine Serum (FBS) (Sigma, F7524), 1% Penicillin-Streptomycin (P/S) (15140).
- MCF7 was cultured in DMEM supplemented with 10% Foetal Bovine Serum (FBS) (Sigma, F7524), 1% Penicillin-Streptomycin (P/S) (15140).
- NCI-H1944 was cultured in EMEM supplemented with 10% Foetal Bovine Serum (FBS) (Sigma, F7524), 1% Penicillin-Streptomycin (P/S) (15140).

2.2.2 Cell maintenance.

Cells in culture were routinely split 1:10 every 2-3 days. Confluent cells were washed with PBS and detached by incubating for 3 minutes with Gibco™ Trypsin-EDTA (0.25%), phenol red (ThermoFisher Scientific) before being re-plated in fresh media. To freeze cells, fresh media was added to the cells and then they were centrifuged at 120xg for 5 minutes. The pellet was carefully re-suspended in 1mL of “freezing medium” corresponding to 10% DMSO in growth media.

The cell suspension was then transferred to a cryotube vial and kept at -80°C using a Corning™ CoolCell™ LX Cell Freezing Vial Containers filled with isopropanol. After 24 hours, the cryotube vial was transferred to a liquid nitrogen tank and stored for future use. To thaw cells, a frozen vial kept in liquid nitrogen was incubated in a water bath at 37°C for a maximum of 2 minutes. Immediately after thawing the cell suspension was transferred and centrifuged at 120xg for 5 minutes. “Freezing medium” was removed and substituted with fresh media. Thawed cells in fresh media were transferred into a culture dish containing growth medium.

2.3 Cell synchronisation.

2.3.1 Cell synchronisation via contact inhibition.

RPE1 cells were arrested in G0/G1 by CI so that they reached 100% confluency for 3 days in a 6-well plate. After 6 days cells were re-plated at low cell density in DMEM

serum-free medium for 24h. Cells were released into the cell cycle with addition of appropriate fresh DMEM/F12 (ThermoFisher scientific).

2.3.2 Cells synchronisation using CDK4/6 inhibitors.

RPE1 cells were treated with 1 μ M Palbociclib (Selleckchem) for 24h. Cells were washed three times with PBS before released into the cell cycle with addition of appropriate media plus serum.

MCF7, NCI-H1944 cells were treated with 10 μ M Palbociclib (Selleckchem) for 24h. Cells were washed three times with PBS before release into the cell cycle with addition of appropriate media plus serum.

2.4 RNAi interference.

For depletion by siRNA, Lipofectamine RNAiMAX was used according to the manufacturer's instructions (Invitrogen). Briefly, cells were either reverse or forward transfected.

- **Reverse Transcription (384-well plate):**

Master Mix Preparation:

In a tube, 50 μ l of Opti-MEM per condition was added with 2 μ l of Lipofectamine RNAiMAX to the master mix.

siRNA Dilution:

In separate tubes, 50 μ l of Opti-MEM + 0.4 μ l of siRNA for per conditions was prepared.

Tubes were flicked to mixes and incubated 5 mins at RT.

Transfection:

50 μ l of the master mix were added to the siRNA tube and incubated at 37°C for 15 mins.

10 μ l of the resulting solution was added to each well.

20 μ l cells at a density of 2.5×10^4 cells ml^{-1} were plated on top of this, and cells were incubated at 37°C.

- **Forward transcription (384-well plate):**

One day before transfection, 20 μ l cells at a density of 2.5×10^4 cells ml^{-1} were plated in a 384-well CellCarrierUltra (PerkinElmer).

Master Mix Preparation:

In a tube, 50 μ l of Opti-MEM per condition was added with 2 μ l of Lipofectamine RNAiMAX to the master mix.

siRNA Dilution:

In separate tubes, 50 μ l of Opti-MEM + 0.4 μ l of siRNA for per conditions was prepared.

Tubes were flicked to mixes and incubated 5 mins at RT.

Transfection:

50 μ l of the master mix were added to the siRNA tube and incubated at 37°C for 15 mins.

10 μ l of the resulting solution was added to each well.

- **Western Blot (siRNA for Wb in 24 well plate):**

Master Mix Transfection Reagent:

In a tube, mix 50 μ l of Opti-MEM was added with 1 μ l of Lipofectamine RNAiMAX per condition was prepared.

siRNA Dilution:

In separate tubes, 50 μ l of Opti-MEM + 1 μ l of siRNA per condition was prepared.

Tubes were flicked to mixes and incubated 5 mins at RT.

Transfection:

50 μ l of the master mix were added to the siRNA tube and incubated at 37°C for 15 mins.

100 μ l of final solution was added to each well.

siRNA	Cat. No
NTC	D-001830-01
siFzr1/Cdh1	603619
siWee1	16708
siCdc25A	4390771

Table 2-2. siRNA used.

2.5 Inhibitors and small molecules.

In this study, a variety of inhibitors were used to perturb normal cell cycle dynamics. Also, several small molecules were also used across this study. Any other concentrations used during this work would be stated in the text.

Small molecules	Cat. No	Concentration	Target
MK-1775	Selleckchem: S1525 Dissolved in DMSO	1 μ M	Wee1
PD0166285	Selleckchem: S8148 Dissolved in DMSO	1 μ M	Wee1/Myt1/CHK1
Palbociclib	Selleckchem: S4482 Dissolved in DMSO	0.25 to 10 μ M	CDK4/6
proTAME	MedChem: HY-110287 Dissolved in DMSO	10 μ M	APC/C ^{Cdc20/Cdh1}
APCin	Selleckchem: 25835 Dissolved in DMSO	10 μ M	APC/C ^{Cdh1}
Doxycycline	Sigma Aldrich: D9891	1 μ g ml ⁻¹	N/A
IAA	Sigma Aldrich: 87-51-4	500 μ M	N/A
EdU	ThermoFisher: A10044	10 μ M	N/A
G418	ThermoFisher: 10131027	1mg/ml	N/A

Table 2-3. List of small molecules and inhibitors used.

2.6 Cloning and molecular biology.

2.6.1 Generating Wee1-GFP-Aid cell lines.

The GFP-AID tag (gift from Francis Barr's lab) (Alfonso-Pérez et al., 2019) was inserted at the C-terminus of the endogenous Wee1 coding sequence using CRISPR/Cas9-mediated editing. gRNA was designed using Benchling following two requirements. As the system is based on a U6 promoter the first base has to be a G. The second requirement is that the sequence needs to be flanked in the 3' end by a 3 bases NGG PAM sequence and composed of 20 bp.

gRNA used were gRNA forward 5'-acctcccctgaacactgtg-3', gRNA reverse 5'-cacag-tgttcagggggaggt-3'.

This gRNA sequence was cloned into the pX458 SpCas9 (BB) with resistance marker (RM) using BbsI restriction enzyme as following:

- 1µg DNA
- 5µl 10X NEBuffer 2.1
- 1µl BbsI
- 43µl Nuclease free Water

The genomic region surrounding WEE1 exon11 (C-terminus) was PCR amplified from RPE1 gDNA using forward primer 5'-agtattggcctattaaattgtgtg-3' and reverse primer 5'tgcaaattgaagtgataatgttattc-3' (PCR protocol see section 2.7.2). WEE1 exon 11 was cloned into pJet-vector (ThermoFisher) and the sequence verified by Sanger sequencing. To construct the homology donor plasmid, WEE1 homology arms from pJet-Wee1-Exon 11 was PCR amplified using the high-fidelity Q5 DNA polymerase (NEB), according to the manufacturer's instructions and protocol in 2.6.2. To PCR amplify the Left Homology ARM (LHA), forward primer: 5'-acggatcgcataagcttgatattcttttgattattgcag-3' and reverse primer: 5'gccccaccaccgctccaccgtatatagtaaggctgacag-3' were used.

To PCR amplify the Right Homology ARM (RHA), forward primer 5'-cttctgacgagttctctgagactactccttcAcacctcc-3' (underlined base represents mutated base to change the PAM site in the recombined gene) and the reverse primer: 5'-ccgggctgcaggaattcgatgataaaaatatatttgtg-3' were used.

The pBluescript (pSKII) vector was digested with EcoRV

- 1.5 µg pBluescript
- 5µl 10X NEBuffer:
- 1 µl EcoRV:
- top up to 50µL with H₂O
- 37°C for 2h

The samples were then run on gel and gel band was purified using gel extraction kit (see 2.7.3).

2.6.1.1 Gibson Isothermal Assembly based cloning.

The DNA fragments (LHA, cassette with resistance markers (RM) and RHA plus digested vector pBluescript) were purified and used to perform the Gibson Isothermal assembly cloning (Gibson et al., 2009). For a final volume of 20 µL, 0.5 pmols of DNA fragments were calculated based on the (bp) and concentration (ng/mL) and combined with 10µl Gibson assembly Master Mix (New England BioLabs) and top

up with deionized H₂O for a final volume of 20 µL. After, the combined DNA and assembly master mix and incubated for 1 hour at 50°C. The reaction was then transformed into competent bacteria. To select colonies with integrated DNA, fragment a blue/white selection was used. At the core of this method is the enzymatic action of β -galactosidase, a tetrameric enzyme encoded by the lacZ α gene in *E. coli*. This enzyme metabolizes lactose into glucose and galactose. Alternatively, β -galactosidase can hydrolyse X-Gal, resulting in the formation of a blue pigment. To conduct blue-white screening post-transformation, X-Gal is introduced, an inducer of lacZ gene expression. Blue colonies indicate bacteria with functional β -galactosidase, suggesting that the plasmid taken up during transformation lacks the DNA of interest. In contrast, white colonies cannot metabolise X-Gal to produce the blue colour, signaling the absence of functional β -galactosidase after taking up the plasmid carrying the inserted DNA and disrupting the lacZ gene. These white colonies harbour the recombinant bacteria and can be picked.

2.6.1.2 GFP transfection.

In day 1, RPE1 myc-OsTIR1 (generous gift from Helfrid Hochegger Lab) (Hégarat et al., 2020) cells were seeded in a 6 well plate at 50 000 cells per well. 3 wells per condition (3 well per condition act as replicate in order to increase the chance of positive transfection) (3 well control GFP and 3 well Wee1-GFP-AID). Control well GFP are transfected with a GFP-plasmid (Barr lab), if the transfection is successful, GFP-positive cells would be visible upon microscopy.

Day 2, cells were transfected with Lipofectamine LTX/Plus (ThermoFisher).

GFP control:

- 800 ng GFP positive plasmid
- 150 µl Optimem (Invitrogen)
- 2.5 µl Lipofectamine plus (ThermoFisher)
- Master Mix: 150 µl Optimem (Invitrogen) + 6 µl Lipofectamine LTX (ThermoFisher)

Wee1-GFP AID

- 400 ng gRNA
- 400 ng psk Wee1-GFP AID
- 150 µl Optimem (Invitrogen)

- 2.5 µl Lipofectamine plus (ThermoFisher Scientific)
- Master Mix: 150 µl Optimem (Invitrogen) + 6 µl Lipofectamine LTX (ThermoFisher)

On day 3, cells were trypsinised and seeded in 15 cm dish plate with fresh media. Day 4, 1 mg/ml of G418 (ThermoFisher) was added to the plate, then fresh media plus G418 is added to the plate every 3 to 4 days until single colonies are visible.

Colonies are picked using cloning cylinders (Sigma). Cloning cylinders are placed on the different colonies. 5 µl of trypsin (ThermoFisher Scientific) is added and incubated 5 mins at 37°C. Each colony is grown in a singular well in a 384-well CellCarrierUltra (PerkinElmer). Positive clones are then monitored using microscopy and further tested using western-blot (2.7.6).

2.6.1.3 Purification of gel bands from agarose gel.

1% of agarose was added to 1x TAE (Tris Acetate EDTA) buffer and microwaved until agarose powder was dissolved. After cooling down, 1 µg/mL ethidium bromide was added to agarose and transferred to a gel support. After the gel was solidified, 20 µL of DNA digestion and dephosphorylation reactions was loaded onto the gel using 6x DNA loading buffer and 1Kb ladder (New England Biolabs) for DNA size quantification. DNA gel bands were then excised from the gel and purified using Qiagen Gel Extraction Kit according to manufacturer's instructions.

- 50x TAE buffer was prepared in water accordingly:
- 2M Tris/acetate
- 50mM EDTA
- pH 8.5

2.6.1.4 Ligation reaction of vector and DNA fragments

To insert the DNA fragment of interest into the digested vector backbone, cut and dephosphorylated pJet vector was mixed with DNA insert in 4 µL of 5x T4 DNA Ligation Buffer (Invitrogen), 1 µL of T4 DNA Ligase (Invitrogen) and H₂O for a final volume of 20 µL. The mixture was incubated overnight at 16°C and subsequently transformed into bacteria.

2.6.2 Polymerase chain reaction (PCR).

Polymerase chain reaction (PCR) was used to selectively amplify particular DNA fragments. A reaction mixture usually contained a final volume of 50 μ L consisting of:

- 5 μ L of Q5 DNA polymerase buffer (10x) 5 μ L
- 2.5 μ L Q5 DNA polymerase
- 1 μ L of 10mM dNTPs
- 100ng template DNA
- 25pmol Primer 5' (sense)
- 25pmol Primer 3' (anti-sense)
- top up to 50 μ L with H₂O

For WEE1 exon 11:	For RHA:	For LHA:
<u>Initial denaturation:</u>	<u>Initial denaturation:</u>	<u>Initial denaturation:</u>
95°C-2mins	95°C-2mins	95°C-2mins
<u>30 Cycles:</u>	<u>30 Cycles:</u>	<u>30 Cycles:</u>
95°C-30 seconds	95°C-30 seconds	95°C-30 seconds
58°C-30 seconds	62°C-30 seconds	68°C-30 seconds
68°C-1.30 mins	68°C-1 mins	68°C-1 mins
<u>Final extension</u>	<u>Final extension</u>	<u>Final extension</u>
68°C-5 mins	68°C-5 mins	68°C-5 mins
<u>Hold</u>	<u>Hold</u>	<u>Hold</u>
4°C-∞	4°C-∞	4°C-∞

5µL of PCR reaction was run on an agarose gel to see single bands with the right size and products were purified with Qiagen PCR purification kit (2.6.1.3).

2.6.3 Transformation of E. coli cells.

E. coli DH5a (Sigma) chemically competent cells were used for transformation. Competent cells were thawed on ice for 30 minutes, after which 0.5µg of plasmid DNA of interest was added and incubated on ice for a further 20 minutes. In order to facilitate the uptake of plasmid DNA by the cells, they were subject to a heat-shock for 45 seconds at 42°C in a water bath and subsequent incubation on ice for 2 minutes. After this, 250µL of room temperature S.O.C. Medium was added to the cells and the mixture was incubated for an hour in a horizontal shaker at 37°C. The bacteria were then carefully spread on the appropriate antibiotic-containing LB agar plates (100µg/mL ampicillin) and incubated overnight at 37°C.

2.6.4 Isolation of plasmid DNA.

Bacteria colonies were picked and incubated for 8 to 10 hours in 5mL of LB medium containing the corresponding antibiotic (100µg/mL ampicillin) in a horizontal shaker at 37°C. Bacterial cultures were then transferred to 200mL flasks of LB media topped up with fresh antibiotic and incubated overnight at 37°C in a shaker. Cells were then pelleted by centrifugation at 4000 rpm for 15 minutes at 4°C. To prepare large quantities of highly concentrated plasmid DNA, the HiSpeed-Plasmid Maxi Kit (Qiagen)

was used according to the manufacturer's instructions. DNA concentration was determined using a Nanodrop spectrometer (Fisher Scientific).

2.6.5 PCNA transfection.

To endogenously expressed PCNA reporter, the fluorescent protein mRuby was inserted in the first exon into one allele of the PCNA locus by recombinant adeno-associated virus mediated (rAAV) homologous recombination in RPE1 Wee1-GFP-AID. 293T cells were seeded in a T-75 Flask so that they reach 50-70% confluence at the time of transfection. 3 µg of plasmids pAAV-mRuby-PCNA (Generous gift from Joerg Mansfeld) (Zerjatke et al., 2017), pRC and pHelper (9 µg total) were used and mixed with 3.75 ml of Optimem (Invitrogen) in a tube. 19 µl of PLUS Reagent (Invitrogen) was added and mix was incubated 5-10 min at RT. 47 µl of Lipofectamine LTX (Invitrogen) was added to the tube and was let incubated 30 min. 15 min before the end, T-75 flask were washed twice with PBS, then 7.5ml of Optimem (Invitrogen) was added and was let incubated at 37°C for 15min. 1.5 ml of DNA/lipid mixture was added onto the cells and incubated at 37°C for 4-6 hours and then replaced medium with complete DMEM. Two to three days post-infection, cells in medium (and any floating cells) were transferred to a sterile 50 ml Falcon tube. 5 ml of fresh medium was added to the flask and adherent cells were scrape off and transferred to a 50 ml Falcon tube. Cells were lysed with 3 rounds of freeze-thaw (10min in Dry ice/EtOH + 10 min in 37°C water bath). After each cycle, cells were vortexed at full speed for 1 min. Cells were then spin down at 10.000 x g for 30 min at 4°C. Tube were wiped with EtOH and placed in the tissue culture hood. Cell-free supernatants were collected and transferred to new 50 ml Falcon tube. rAAV preps were dispensed in small aliquots (3-5 ml). Then, cells were seeded in a T-75 Flask so that they reached 30-40% confluence at the time of infection. T-75 flask were washed twice with PBS, then 5/6 ml of complete medium and 5/6ml of rAAV stock were added and let to incubate at 37°C for 4 hrs. Fresh media was added to bring the volume up to 15 ml (or more) and infection was allowed to continue for 48h. After 3 days, cells were trypsinised and expanded into more flasks four to five days post infection.

2.6.6 Western blot.

Samples were directly lysed in Tris-Glycine SDS (Novex) + 2 mM DTT (Sigma). Whole cell lysates were loaded onto 4–20% Tris-Glycine Novex precast gels (ThermoFisher) and run at 115 V for 1h15 and transfer on an activated PVDF membrane (soaked in MeOH for 2 mins) by wet transfer at 110 Amp for 2h. Following by blocking in 5% milk in PBS/0.2% Tween for 1h (Blocking buffer), membranes were incubated in primary antibodies overnight at 4°C with rocking. The next day, membranes were washed three times in TBS 0.05% TritonX-100 (TBS/T) incubated with anti-mouse or anti-rabbit HRP-conjugated secondary antibodies (CST) (diluted 1:1000) in blocking buffer and incubated at RT for 1h. Membranes were washed three times in TBS/T and developed in Clarity Max ECL (Bio Rad) and visualised on an Amersham Imager 680.

2.7 Flow cytometry.

For cell cycle analysis, RPE1 cells were collected by trypsinisation, washed with cold PBS, and centrifuged for 3 minutes at 1000 rpm. Cells were then fixed by adding 1ml of ice cold 70% ethanol drop-wise while vortexing cell suspension. Samples were stored overnight at 4°C. Next, samples were washed once with PBS and centrifuged (3 mins at 1000rpm). Cells were resuspended in 250µl staining solution (1µl of 50mg/ml propidium iodide (ThermoFisher), 2µl of 100mg/ml RNase A (ThermoFisher) in 10ml PBS (Sigma, P4864-10ML). Cells were incubated for 30 minutes at 37°C. The DNA content was analysed with a Fluorescence-activated cell sorting (FACS) machine (Becton Dickinson). Data was then analysed and plotted using FlowJo.

2.8 Fluorescence-activated cell sorting.

Fluorescence-activated cell sorting of cells was performed by Bhavik Patel at the Flow cytometry facility, MRC Laboratory of Medical Sciences (MRC LMS), following trypsinisation and incubation at 37°C for 25 min. Cells were washed with PBS and resuspended in FACS sorting buffer to a concentration of 8-12 million cells/ml. FACS sorting buffer: 1 ml of PBS 2% FBS. Prior to sorting, cells were filtered with a 30-40 µm filter. Cells were single sorted into fractions based on mRuby-PCNA and Wee1-

GFP positive cells. Positive cells were incubated in a 384-well CellCarrierUltra (PerkinElmer) at 37 °C. Cells were incubated in 50 µl of recovery media (50% fresh media with 50% of pre-conditioned media) until individual colonies become apparent in each well.

2.9 Growth curve

Cells were collected by trypsinisation, washed with cold PBS, and centrifuged for 3 minutes at 120 x g. Cells were plated at a density of 20 000 cells per well in duplicate in six-well plates (Costar® 6-well). Brightfield images were taken every 2 h for 5 days and the percentage confluency was calculated using an Incucyte Live-Cell analysis system (Sartorius).

2.10 Live cell imaging

Live cell imaging was performed on the Operetta CLS High-Content Analysis microscope (PerkinElmer), with atmospheric control to maintain cells at 37°C, 5% CO₂ and with a breathable film (PerkinElmer) to stop evaporation. Cells were plated in a 384-well CellCarrierUltra (PerkinElmer) plates at a density of 500 cells/well in 20 µL of media for 24 h (unless otherwise stated) prior to imaging. Cells were imaged using a 20X (N.A. 0.8) objective at 5- and 10-min intervals for 48 h (unless otherwise stated) in 50 µL phenol red-free media. For RPE1 Wee1-GFP mRuby-PCNA and RPE1 mRuby-PCNA Cdc25A-GFP live imaging, cells were imaged on an automated inverted spinning disk confocal microscope system IX83 (Olympus) combined with CellVivo (Olympus). Cells were plated on IbiTreat µ-slide 8well (ibidi) plates at a density of 5000 cells/well in 200 µL of media, 24 h prior to imaging (unless otherwise stated). Images were typically acquired with a 20x plan (UCPLFLN) fluorescence objective (NA 0.7).

2.11 Immunofluorescence

Cells were grown on 384-well CellCarrierUltra (PerkinElmer) plates. Cells were fixed in an equal volume of media to 8% formaldehyde (final concentration 4%) in PBS for 5 min at RT. Cells were permeabilised in PBS/0.2% TritonX-100 for 15 min at RT and the blocked in 2% BSA in PBS (Blocking buffer) for 1h at RT. Cells were

incubated in primary antibody diluted in Blocking buffer for either 2h at RT or overnight at 4 °C. Cells were washed three times in PBS and then incubated with Alexa-conjugated secondary antibodies: Donkey anti-Rabbit IgG 488 or 594 (ThermoFisher) and Donkey anti-mouse IgG 488 or 594 (ThermoFisher) diluted in Blocking Buffer for 1h at RT in the dark. Cells were washed three times in PBS and incubated in 1µg/ml Hoescht 33258 (Sigma) diluted in PBS for 15 min at RT in the dark. All washing steps were done using a Biotek splash washer.

For MCM7 cells have been pre-extracted. Medium was aspirated and cells were permeabilised in PBS/0.2% TritonX-100 for 5min at RT before fixation. Then, proceed with same protocol as before.

For EdU detection, cells were incubated for 30 mins in 100 mM TBS pH 7.5, 4 mM CuSO₄, 5 µM sulfo-cyanine 3 azide and sodium ascorbate 1µg ml⁻¹, and then washed three times with PBS.

2.11.1.1 Antibodies used:

Antibody	Species	Sources	Cat. N°	Dilution
γH2AX	Rabbit	Cell signaling	2577s	IF: 1:500
Y15-pCDK1/2/3/5	Rabbit	Cell signaling	2561s	IF: 1:1000
53BP1	Rabbit	Cell signaling	4937s	IF: 1:1000
CDK2	Rabbit	Abcam	Ab76146	IF: 1:500
p21	Mouse	Santa Cruz	Sc-377515	IF: 1:250

Table 2-4. Antibody used for immunostaining.

Antibody	Species	Sources	Cat. N°	Dilution
GAPDH	Mouse	Cell signaling	2118s	WB: 1:1000
Fzr1/Cdh1	Mouse	Santa Cruz	sc-56312	WB: 1:250
Vinculin	Rabbit	Cell signaling	4650t	WB: 1:1000
Wee1	Rabbit	Cell signaling	4936S	WB: 1:250

Table 2-5. Antibody used for Western Blot.

2.12 Data analysis.

2.12.1 Statistical analysis.

Statistical analyses were performed using GraphPad Prism Version 9.0.0 for Mac, GraphPad Software, www.graphpad.com. One-way or T-tests were carried out, depending on the number of conditions tested. T-test analysis was used when comparing the means of two groups to ascertain if they significantly differ from each other. It evaluates whether the means of the two groups exhibit statistical divergence, considering the variance within each group. One-way ANOVA was utilised when assessing the means of three or more groups to determine if significant discrepancies exist among them. It examines the variability between group means relative to the variability within groups.

2.12.2 Automated image analysis of fixed cells.

All quantitative image analysis on fixed cell assays was performed using Harmony High-content Imaging Analysis Software (PerkinElmer) and individual protocols are described below.

Nuclear segmentation: Nuclei were segmented based on Hoechst intensity. Nuclei at the edge of the image and nuclei $< 80 \mu\text{m}^2$ were excluded.

Measuring cytoplasmic intensities of proteins: Nuclei were segmented as above. To measure cytoplasmic intensities of proteins, a cytoplasmic ring region of two pixels around the nuclear periphery was defined.

Cell classification: To defined EdU+ and EdU- negative cells, EdU ratio between EdU nuclear intensity/EdU ring intensity was measured. Cells with a ratio > 1.2 were considered EdU +.

2.12.3 Automated cell tracking.

Automated live imaging tracking was performed using NucliTrack software(Cooper et al., 2017).

Succinct protocol is described below and can vary upon data:

The data were first loaded and segmented depending on the desire channel.

Clipping limit: This defines a maximal intensity threshold above which the different intensities will not be recognised.

Background blur: 35

Image blur: 1

Threshold: To adjust to obtain only red nuclei.

Smallest object: sets size threshold on objects, around 80.

Distance to intensity: 0.15

Separation distance: Adjust to get 1 dot per nuclei.

Edge blur: 1 or 2

Watershed ratio: 0.02-0.08

Movie is the segmented and features are extracted.

Three events can be labelled and define by the following numbers:

1 Area

2 Eccentricity

3 Major axis Length

4 Perimeter

5 CH1 Mean Intensity

6 CH1 Median Intensity

7 CH1 StdDev Intensity

8 CH1 Floored mean.

9 CH1 Ring Region Mean Intensity (opt)

10 CH1 Ring Region Median Intensity (opt)

11 CH2 Mean Intensity

12 CH2 Median Intensity

13 CH2 StdDev Intensity

14 CH2 Floored Mean

15 CH2 Ring Region Mean Intensity (opt)

16 CH2 Ring Region Median Intensity (opt)

17 CH3 Mean Intensity

18 CH3 Median Intensity

19 CH3 Floored Mean

2.12.4 Manually cell tracking

When automated tracking was not possible due to the lack of proper nuclear marker or to an impossibility from the software to correctly track cells across multiple

timepoint, manually tracking was done. In order to do that, live imaging data were analysed on ImageJ. Only cells staying in the field of view from the first minute to the last one or until completion of a full cell cycle (depending on the experiments) were analysed. For cell cycle dynamics, cells were manually tracked, and the length of the different phases were defined upon PCNA appearance and localisation as explain in 2.13.1.

To manually quantify CDK2-PSLD sensor and for each image, CDK2 levels was manually quantify in a small define square (10pixels/10pixels) in the nucleus and in the cytoplasm. CDK2-PSLD activity was then calculated by the ratio of cytoplasm CDK2-PSLD level on nucleus CDK2-PSLD. S-phase entry and cell cycle dynamics were defined using PCNA appearance and localisation as explain in 2.13.1.

To manually quantified other protein such as Cdc25A-GFP, Wee1-GFP and p21-GFP (see section 2.12), the same strategy was used but as these proteins are strictly nuclear (Except Wee1 just before mitosis), the different levels were manually quantified in a small define square (10pixels/10pixels) in the nucleus. S-phase entry and cell cycle dynamics were defined using PCNA appearance and localisation as explain in 2.13.1.

2.13 Live cell biosensors.

Several biosensors for live-cell timelapse imaging have been used in order to quantify the dynamics of different proteins across the cell cycle.

2.13.1 Measuring cell cycle dynamics using RPE1 mRuby-PCNA.

PCNA can be used to decipher S-phase entry and quantify the length of each phase of the cell cycle (Zerjatke et al., 2017). hTert-RPE1 cell line is expressing one allele of endogenously tagged mRuby-PCNA. In early G1, PCNA is localised in the nucleus before forming speckles/foci upon S-phase entry as it loads to the DNA (Fig. 2.1.A, B). During the S/G2 transition the speckles disappear. M entry is characterised by the nuclear envelope breakdown.

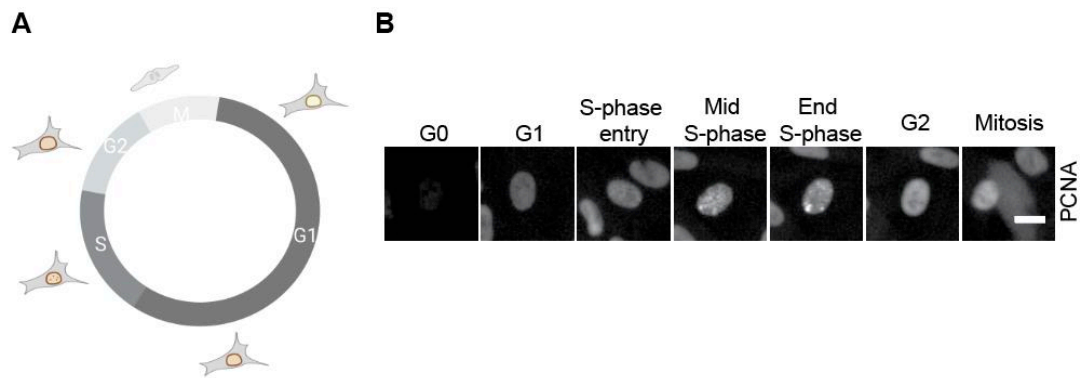


Figure 2-1. Cell cycle dynamics measured in RPE1 mRuby-PCNA.

(A) Schematic of the appearance of mRuby-PCNA proteins during the cell cycle. **(B)** Representative images showing G1-, S-, G2-phase and Mitosis dynamics measured in single cells with cell cycle biosensors. Scale bar 10 μ M.

2.13.2 Measuring cell cycle dynamics using RPE1 mRuby-PCNA Wee1-GFP-AID.

Wee1 has a nuclear localisation across all phases of the cell cycle (Fig. 2.2.A, B) (Baldin & Ducommun, 1995). At the off-set of mitosis, Wee1 migrates to the cytoplasm (Fig. 2.2.B).

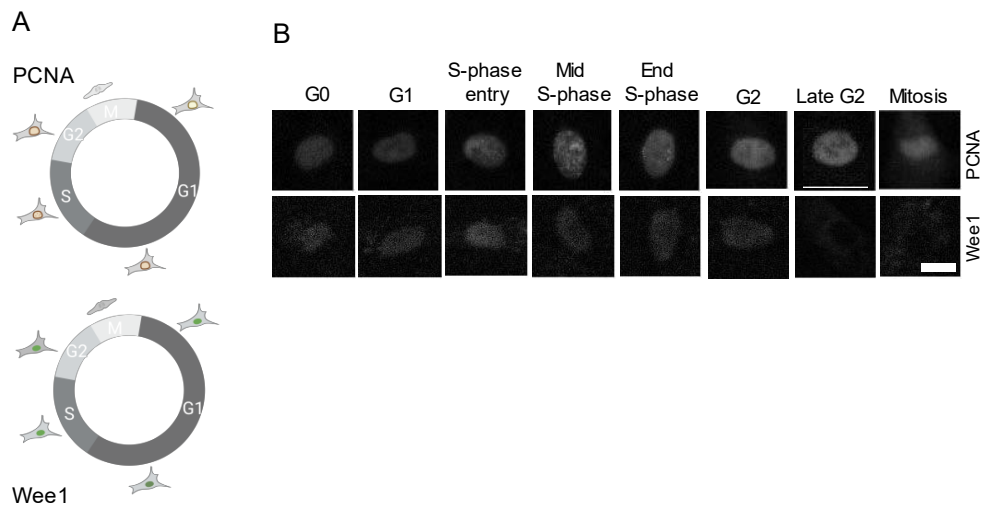


Figure 2-2. Cell cycle dynamics measured in RPE1 with the biosensors mRuby-PCNA Wee1-GFP-AID.

(A) Schematic of the appearance of mRuby-PCNA and Wee1-GFP-AID proteins during the cell cycle. **(B)** Representative images showing G1-, S-, G2-phase and Mitosis dynamics measured in single cells with cell cycle biosensors. Scale bar 10 μ M.

2.13.3 Measuring cell cycle dynamics using RPE1 mRuby-PCNA CDK2-PSLD.

GFP is fused to the CDK2-regulated phosphorylated subcellular localisation domain (PSLD) of human DNA helicase B (Barr et al., 2016). When inactivated, CDK2-PSLD is present in the nucleus and migrates to the cytoplasm upon CDK2 activation. Hence, by measuring the cytoplasmic:nuclear ratio, an estimate CDK2 activity can be calculated from individual cells (Barr et al., 2016) (Fig. 2.4.A, B).

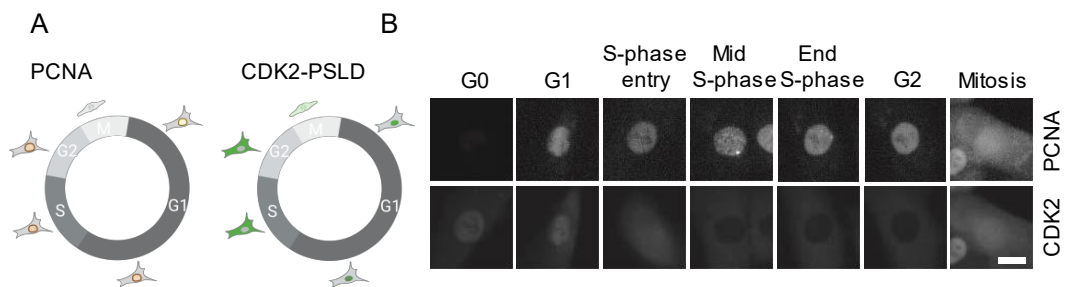


Figure 2-3. Cell cycle dynamics measured in RPE1 with the biosensors mRuby-PCNA and CDK2-PSLD.

(A) Schematic of the appearance of PCNA and CDK2 protein during the cell cycle. **(B)** Representative images showing G1-, S-, G2-phase and Mitosis dynamics measured in single cells with cell cycle biosensors. Scale bar 10 μ M.

2.13.4 Measuring cell cycle dynamics using RPE1 mRuby-PCNA p21-GFP.

To quantify p21 a GFP tag was introduced into the C-terminus of both alleles of the endogenous *CDKN1A* (Barr et al., 2017). In unperturbed cells, p21 intensity remain low in G1 before decreasing during S-phase and G2/M. Upon DNA damage, p21 increase, accumulate to the nucleus, this increase can be monitored and use as a readout for p21 activity (Barr et al., 2017) (Fig. 2.5.A, B).

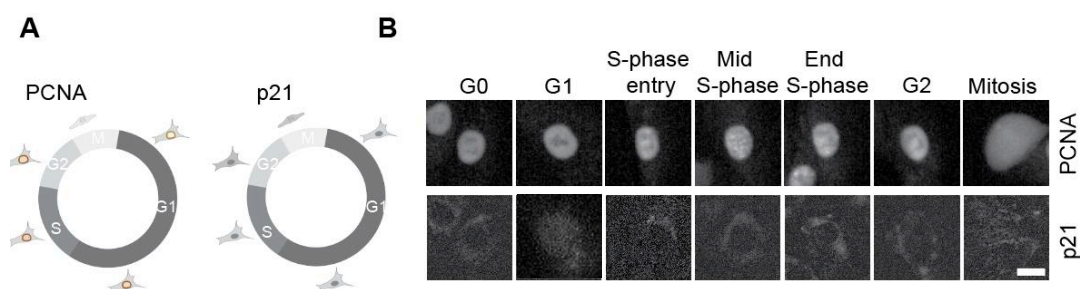


Figure 2-4. Cell cycle dynamics measured in RPE1 with the biosensors mRuby-PCNA and p21-GFP.

(A) Schematic of the appearance of PCNA and p21 protein during the cell cycle. **(B)** Representative images showing G1-, S-, G2-phase and Mitosis dynamics measured in single cells with cell cycle biosensors. Scale bar 10 μ M.

2.13.5 Measuring cell cycle dynamics using RPE1 mRuby-PCNA Cdc25a-GFP.

Cdc25A-GFP is endogenously tagged (Unpublished data). Cdc25A activity increase as a pulse in the nucleus at the G1/S transition (Fig. 2.6.A, B).

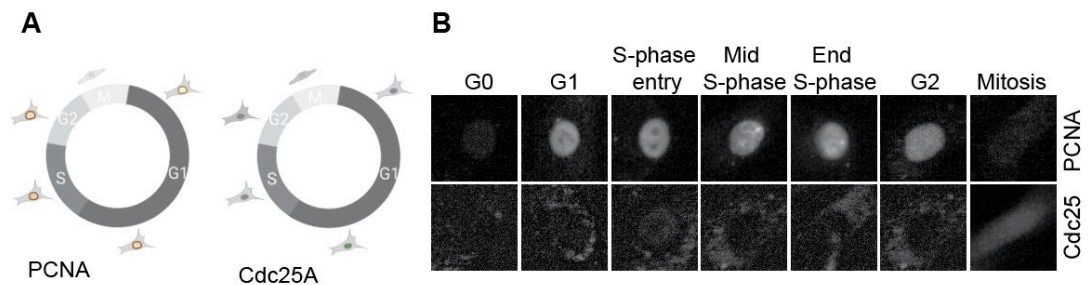


Figure 2-5. Cell cycle dynamics measured in RPE1 with the biosensors mRuby-PCNA Cdc25a-GFP.

(A) Schematic of the appearance of PCNA and Cdc25a protein during the cell cycle.
(B) Representative images showing G1-, S-, G2-phase and Mitosis dynamics measured in single cells with cell cycle biosensors. Scale bar 10 μ M.

2.13.6 Measuring cell cycle dynamics using RPE1 Cdc6-mVenus PCNA-mTurquoise2 DHB-mCherry.

This cell line is used in (Matson et al., 2019). In this cell line, to monitor CDK2, DHB-mCherry is used (Spencer et al., 2013). As in (Figure 2.3), when inactivated, CDK2-PSLD is presents in the nucleus and migrates to the cytoplasm upon CDK2 activation. Hence, by measuring the cytoplasmic:nuclear ratio, an estimate CDK2 activity can be calculated from individual cells (Fig. 2.6.A, B). Cdc6 is presents in the nucleus during G1 and is rapidly degraded and migrates to the cytoplasm at S-phase entry (Matson et al., 2019). In this cell line Cdc6 WT is fused to mVenus for expression under the EF1 promoter. PCNA mTurquoise2 sensor is under the control of the phosphoglycerate kinase and thus is over-expressed in this cell line. However, PCNA behaviour remains the same as in endogenously tagged mRuby-PCNA and is an indicator of phases transition (Bajar et al., 2016a).

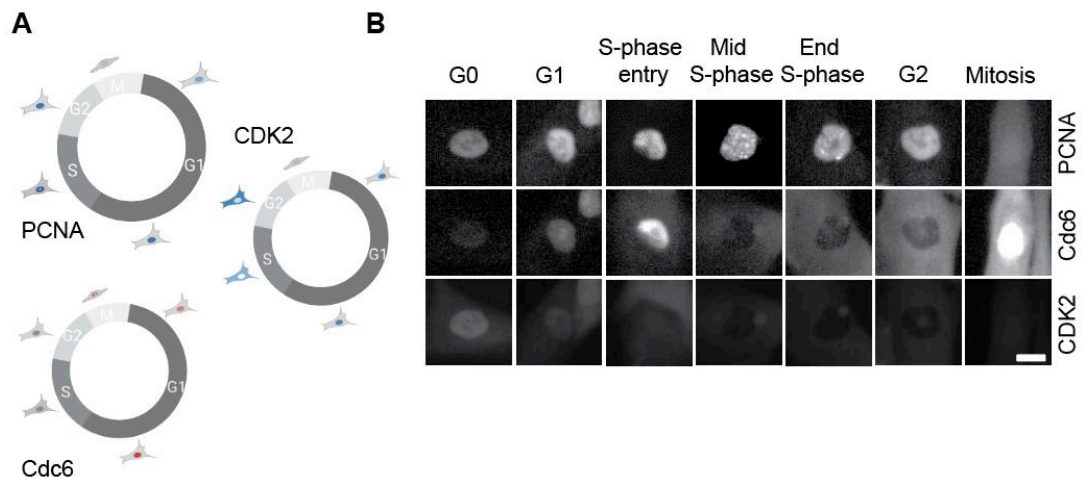


Figure 2-6. Cell cycle dynamics measured in RPE1 with the biosensors PCNA mTurquoise2 Cdc6-mVenus and DHB-mCherry.

(A) Schematic of the appearance of PCNA, Cdc6 and CDK2 protein during the cell cycle. (B) Representative images showing G1-, S-, G2-phase and Mitosis dynamics measured in single cells with cell cycle biosensors. Scale bar 10 μM.

2.13.7 Measuring cell cycle dynamics using RPE1 mRuby-PCNA mCherry-Geminin PIP-mVenus.

RPE1 mRuby-PCNA mCherry-Geminin PIP-Venus cell line is used in (Grant et al., 2018) (Fig. 2.3.A, B). This consist of a minimal version of CRL4^{Cdt2}-dependent FUCCI sensor. PCNA-interacting protein (PIP) degron from human cdt1 fused the mVenus fluorescent protein. PIP-mVenus is rapidly degraded at the onset of DNA replication concurrent with PCNA foci formation following by its steady accumulation during G2. This rapid change is used as a marker of S-phase entry. The changes in PIP-FUCCI protein dynamics at the G1 to S phase transition and the S phase to G2 transition closely corresponded with the changes in PCNA variance associated with DNA replication. Geminin levels responds to APC/C^{Cdh1} ubiquitin ligase activity. When APC/C^{Cdh1} is high during G1, geminin is degraded and only start to increase mid S-phase when APC/C^{Cdh1} is fully inactivated. Geminin expression reaches a peak during G2.

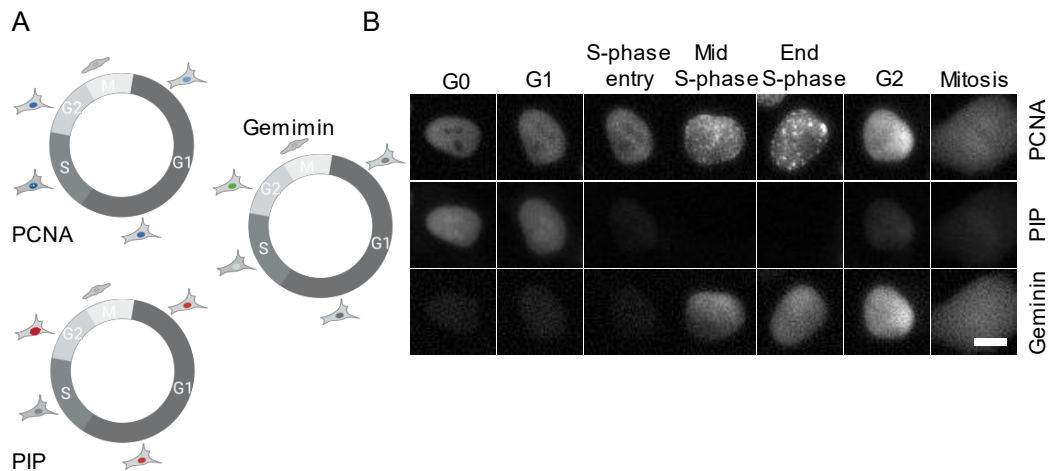


Figure 2-7. Cell cycle dynamics measured in RPE1 with the biosensors mRuby-PCNA PIP-Fucci mCherry-Geminin.

(A) Schematic of the appearance of PCNA, PIP and Geminin during the cell cycle. (B) Representative images showing G1-, S-, G2-phase and Mitosis dynamics measured in single cells with cell cycle biosensors. Scale bar 10 μ M.

2.14 Phospho-tyrosine proteomics.

All sample processing, running of the mass spectrometers and analysis was performed by Alex Montoya, MRC-LMS Mass Spectrometry core facility.

2.14.1 Sample processing.

Samples were processed as recommended in [PMID: 30078316]. Each sample represented a 15 cm plate at confluence, and it was lysed in 1.3ml of 50mM EPPS pH 8.5, 150mM NaCl, 2%SDS, 1% SDC, 2% igepal. 1 tablet of cOmplete Protease Inhibitor Cocktail was added per 50ml of lysis buffer and phosphatase inhibitors 2 and 3 were added at 1 to 100 dilutions. For DNA removal 1ul of 250U/ul benzonase was added to each plate right before the lysis buffer. The samples were incubated at 60C to ensure complete lysis, subjected to BCA assay and diluted to 1.2 g/L in 1.6 mL of lysis buffer. The samples and diluted 1:1 and reduced and alkylated by adding 1600ul of 20mM TCEP and 40mM chloracetomide. The samples were then precipitated with 12.8 mL of ice-cold acetone at -20 for two hours, washed with 10mL of 80% acetone twice. The pellet was digested in 2mL of 50mM EPPS pH 8.5 with trypsin/lysC mix overnight (20ug trypsin and 10ug lysC were added to the sample twice at the beginning of digestion and 4 hours later). 1.3mg of each sample was desalted with 130mg of Oasis HLB in Orochem OF1100 plates: samples were acidified with 0.5% TFA, loaded on beads, washed with 1ml 0.1% TFA three times and eluted first with 1mL of pure MeCN and then with 1mL of 70% MeCN. 20ug of the eluate was speedvaced and resuspended in 60ul 0.1% TFA, to analyse unmodified peptides. For phosphoenrichment 300ug (450 ul) of the eluate was taken and glycolic acid and TFA were added to them to 0.1M and 5% concentration respectively. Phosphopeptides were enriched with 1.2 mg of MagReSyn® Zr-IMAC as recommended by the manufacturer: bound to the resyn for 20min, washed with 100ul of 80% ACN, 1% TFA, 100ul of 10% ACN, 0.2% TFA and eluted with 100ul 1% NH₄OH twice. The samples were then acidified with 5ul of formic acid, speedvaced and resuspended in 40ul of 0.1% TFA. 8ul of sample was taken per injection corresponding to 60 ug of starting material.

2.14.2 Liquid chromatography mass spectrometry (LC-MS) analysis.

Chromatographic separation was performed using an Ultimate 3000 RSLC nano liquid chromatography system (Thermo Scientific) coupled to an Orbitrap exploris 240 mass spectrometer (Thermo Scientific) via an EASY-Spray source. Electro-spray nebulisation achieved by interfacing to Bruker PepSep emitters (PN: PSFSELJ10, 10 μ m).

For unmodified peptide analysis digest was injected directly onto the analytical column (self-packed column, CSH C18 1.7 μ m beads, 150 μ m $\times$ 55cm) at working flow rate of 1.25 μ L/min for 8 minutes. Peptides were then separated using a 120 mins segmented gradient: 0-24% in 100 min, 24-26% in 10 min, 26-29% in 10min, 29-33% in 10 min and 33-41% in 10 min of buffer B (composition of buffer A – 95/5%: H₂O/DMSO + 0.1% FA, buffer B – 75/20/5% MeCN/H₂O/DMSO + 0.1% FA), followed by column conditioning and equilibration. Eluted peptides were analysed by the mass spectrometer in positive polarity using a data-independent acquisition mode as follows: an initial MS1 scan was carried out at 120,000 resolution with an AGC target of 3e6 for a maximum IT of 200ms, m/z range: 410-1650. This was followed by sequential MS2 acquisition and fragmentation of ions at 30,000 resolution over 30 variable windows. AGC target set to 3e6 with maximum IT on auto. Normalised collision energy was set to 27%. Total run acquisition time was 155 minutes.

For phospho peptide analysis digest was injected directly onto the analytical column (self-packed column, CSH C18 1.7 μ m beads, 150 μ m $\times$ 30cm) at working flow rate of 1.25 μ L/min for 8 minutes. Peptides were then separated using a 64 mins stepped gradient: 0-4% in 16 min, 4-12% B in 16 min, 12-24% B in 16 min, 24-45% B in 16 min, 45-99% B in 1 min (composition of buffer A – 95/5%: H₂O/DMSO + 0.1% FA, buffer B – 75/20/5% MeCN/H₂O/DMSO + 0.1% FA), followed by column conditioning and equilibration. Eluted peptides were analysed by the mass spectrometer in positive polarity using a data-independent acquisition mode as follows: an initial MS1 scan was carried out at 120,000 resolution with an AGC target of 3e6 for a maximum IT of 200ms, m/z range: 410-1650. This was followed by sequential MS2 acquisition and fragmentation of ions at 30,000 resolution over 25 variable windows. AGC target set to 3e6 with maximum IT on auto. Normalised collision energy was set to 27%. Total run acquisition time was 79 minutes.

2.14.3 Raw data processing.

Data were processed using the Spectronaut software platform (Biognosys, v 17.7.230531.55965, Bruderer et al., 2015)

Analysis was carried out in direct DIA mode with default settings and the following adjustments as follows:

2.14.3.1 Pulsar Search.

Library generation and database search carried out using default settings for a trypsin/p specific digest as follows - missed cleavage rate set to 3 and fixed modification of cysteine carbamidomethylation. For total protein samples variable modifications allowed for methionine oxidation, protein N-terminal acetylation, asparagine deamidation and glutamine cyclisation to pyro-glutamate. For phospho-enriched samples, variable modifications allowed for methionine oxidation, protein N-terminal acetylation and serine/threonine/tyrosine phosphorylation. PSM, Peptide and Protein group FDR = 0.01. Searches were carried out against the Uniprot homo sapiens protein sequence database (downloaded 20230130, 20,594 entries). Additionally, searches were also carried against a universal protein contaminants database (Frankenfield et al., 2022 – downloaded 20220604, 381 entries).

2. **Direct DIA analysis:** A mutated decoy database approach was employed with protein q-value cut-off for the experiment set to 0.01 at the identification level. Quantification set to MS2 (integrated area) with proteotypicity filter set to 'only proteotypic' for total protein samples. For phospho-enriched samples, proteotypicity filter default setting for PTM workflow was 'none'. Imputation strategy disabled and quantification method set to MaxLFQ for both sample types. PTM workflow option enabled for phospho-enriched samples in order to generate site level tables, with a site localisation probability cut-off set to 0.75 (Cox et al., 2014). PTM analysis settings as follows – multiplicity enabled, flanking region = 14 and with PTM consolidation method set to sum all peptide intensities for a given PTM site group. For total protein samples, intensity normalisation was default setting of local normalisation. For phospho-enriched samples, normalization filter type was set to modification specific, whereby a global median normalisation was applied to site intensities based on phospho-peptides only.

2.14.4 Results processing.

Exploratory and technical analysis of data carried out using in-house developed python pipeline with various visualisations using both the Plotly and Pandas plotting libraries (Sievert, 2020). Spectronaut protein level output tables were filtered to remove hits to the contaminants database.

Further analysis and statistical testing carried out using the Perseus platform (v1.6.15.0, Tyanova et al., 2016). Human ontology annotations GOBP, GOMF, GOCC and KEGG (downloaded through Perseus GO annotation tool) were assigned by Uniprot protein accessions. Data was log2 transformed before additional filtering and statistical testing. For multi-group comparisons, data was filtered for proteins with ≥ 3 replicate intensities per experimental group. Protein intensities of 1-way ANOVA significant hits (multiple testing correction = permutation-based FDR, threshold = 0.05) were then z-scored in order to HCA-heatmap cluster the data. HCA row cluster ID's were assigned manually and subsequently used to carry out GO enrichment analysis (Fishers Exact test, multiple-testing correction = Benjamini-Hochberg, FDR threshold = 0.05). For 2-group comparisons, intensities were filtered as previously described and Student's t-test carried out (multiple testing correction = permutation-based FDR, threshold = 0.05). T-test results visualised as volcano plots. Intensities of significant hits were z-scored and further processed for GO enrichment analysis as previously described.

Phospho-site intensities (pre-normalised to phospho-peptide signal as previously described) were normalised to normalised total protein intensities as follows – protein intensity ratios were calculated by dividing the intensity of a protein per sample by the minimum intensity of the protein across all samples. This generates protein ratios per sample that have equivalent relative levels to the original protein intensity levels. Protein intensity ratios were merged to the phospho-site table by protein accession identifier. Phospho-site intensities were normalised to the total protein ratios by dividing a given phospho-site intensity per sample by its protein ratio. Two phospho-site subsets are therefore generated: **normalised** and **non-normalised** to total protein intensities. Both subsets were further processed for statistical testing as described above. With respect to GO enrichment analysis at the site level, a relative enrichment was carried out, by normalising the data to the protein level (i.e. proteins) with multiple phospho-sites are not counted multiple times so as not to skew GO

enrichment results. Additionally, phospho-site flanking region and protein identifier columns in Spectronaut sites tables, were used to map the following categories to phospho-sites – linear motifs, known kinase sites, kinase-substrate relations, and regulatory sites. Perseus interrogates PhosphoSitePlus databases, from which the different annotations are extracted (Hornbeck et al., 2015).

Chapter 3. Wee1 inhibition during cell cycle re-entry leads to a delay in S-phase entry as well as an arrest in G0/G1 and G2 phases.

3.1 Introduction

Wee1 plays a pivotal role in ensuring genomic stability and proper cell division. It exerts its regulatory role by inhibiting the activity of CDKs, such as CDK1, during the G2 phase or CDK2 during G1 phase (Lundgren et al., 1991; McGowan & Russell, 1993; P. Russell & Nurse, 1987; C. L. Wu et al., 2001). By delaying entry into mitosis, Wee1 helps prevent premature chromosome condensation and DNA replication, allowing for DNA repair and accurate cell cycle progression. Understanding the role of Wee1 in cell cycle control is essential for unravelling the intricacies of cell cycle progression. Additionally, Wee1 inhibitors have emerged as promising therapeutic agents in cancer research, disrupting the G2 checkpoint and increasing the susceptibility of cancer cells to DNA damage, particularly when used in combination with DNA-damaging agents such as chemotherapy or radiation therapy, highlighting the potential of Wee1 inhibition as a targeted cancer therapy. Although the impact of Wee1 inhibition during G2 and in mitotic entry has been extensively characterised, the role of Wee1 during G1 and S-phase entry is less well understood (Bukhari et al., 2019; Kellogg, 2003; Lewis et al., 2017; Lockhead et al., 2020; Matheson, Backos, et al., 2016; Schmidt et al., 2017; Wright et al., 2017; C. L. Wu et al., 2001) (Fig .3.1).

Much of our understanding of the regulatory mechanisms governing the cell cycle in mammals relies on methods that measure cell cycle dynamics within a whole population, which lacks the quantification on a single cell level. Single cell live imaging is a highly valuable approach for quantifying cell cycle length due to its ability to capture the duration of each phase of the cell cycle in individual cells. Furthermore, the possibility of exploiting cell to cell variability allow the identification of potential heterogeneity and rare phenotypes between individual cells. In this chapter, I used single cell live imaging with cells expressing mRuby-PCNA to follow cell cycle dynamics upon Wee1 inhibition.

The study of Wee1 at the G1/S transition raises several challenges. As shown by inhibiting Wee1 in asynchronous populations forces cells to rush through S, G2 and M, before dying from mitotic catastrophe. This leads to a low number of cells undergoing G1/S transitions, upon Wee1 inhibition (Lockhead et al., 2020).

First, to counteract this problem, I used different methods to synchronise RPE1 cells in G0/G1 such as CI and the use of CDK4/6 inhibitors (Palbociclib). This allowed me to monitor cells undergoing the first G1/S transition after Wee1 inhibition.

Previous studies have shown that the use of Wee1i can lead to off-target effects (Wright et al., 2017). To tackle this concern and ensure the specificity of Wee1 inhibition, I also used alternative methods to perturb Wee1 activity at cell cycle re-entry such as siRNA and a degron system. These approaches offer greater precision and control and enhancing the reliability of my results.

Lastly, given the common use of Wee1i in cancer therapeutic contexts, it is justifiable to extend my investigations to assess the consequences of Wee1 inhibition on cancer cell lines. This might give insights into altered regulation mechanisms of Wee1 in cancer cells.

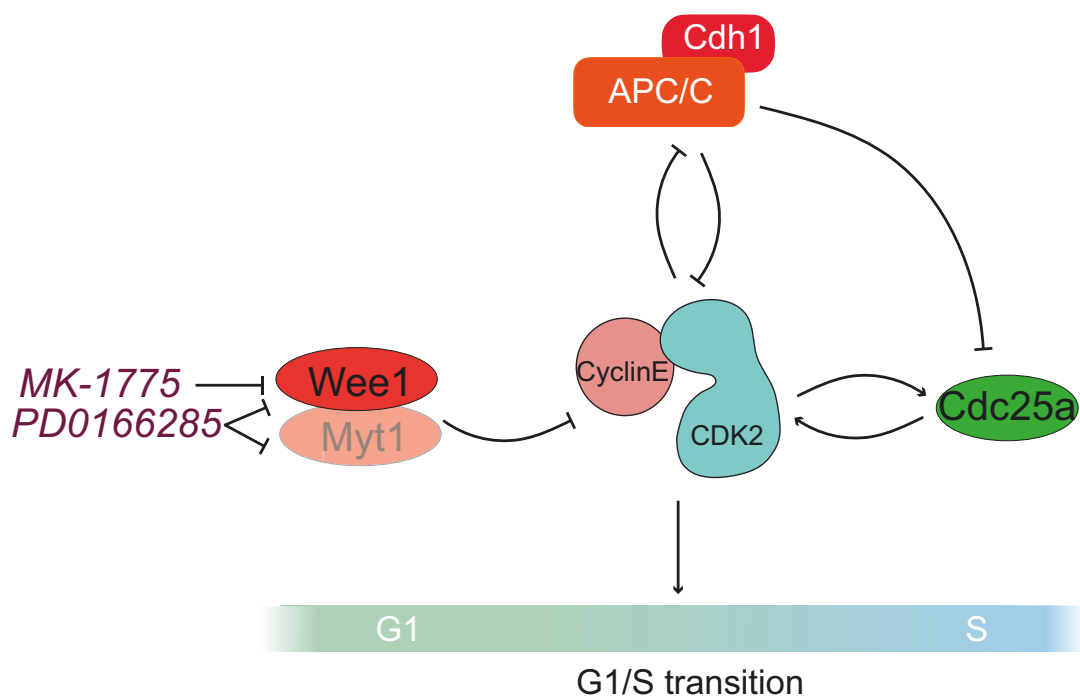


Figure 3-1. Wee1i and cell cycle re-entry.

Simplified schematic illustration of the G1/S transition. CDK2-Cyclin E is inactivated by Wee1 and Myt1 as well as APC/C^{Cdh1}. When cells need to transition to S-phase, CDK2-Cyclin E is activated by the phosphatase Cdc25a and its increase in activity plus the inactivation of APC/C^{Cdh1}, allow the G1 to S transition. I predict, based on this knowledge, that Wee1i (MK-1775 and PD-0166285) should allow a fully activated CDK2-Cyclin E and a quicker transition to S-phase.

3.2 Wee1 perturbation leads to a delay in S-phase entry upon cell cycle re-entry.

3.2.1 Wee1 inhibition leads to a delay in S-phase entry and a G0/G1 arrest.

In order to inhibit Wee1 at cell cycle re-entry, I first made use of pharmacological inhibitors of Wee1. MK-1775 is a selective Wee1i (the term ‘Wee1i’ will be used to describe the Wee inhibitors MK-1775) used in clinical trials for cancer treatment (Hirai et al., 2009) and PD-0166285, is a tool compound that inhibits Wee1 and Myt1 (Wee1i/Myt1i) (Y. Wang et al., 2001). To measure G1 length as a readout for cell cycle entry timing, I used RPE1 cells expressing one allele of endogenously tagged mRuby-PCNA and endogenously tagged mTurquoise2-Histone 3.1 (mTurq2) (RPE1 mRuby-PCNA H3.1-mTurq2) needed for a proper nuclear segmentation (Zerjatke et al., 2017). PCNA expression is tightly coupled to proliferation, peaking at the G1/S transition. For this reason, it can be used as a robust cell cycle phase indicator, based on PCNA expression and its localisation pattern within the nucleus (see: material and methods). RPE1 cells are non-transformed human epithelial cells immortalised with telomerase and they are genetically stable near diploid cells widely used to model cell division, DNA repair, or ciliogenesis in a non-transformed context and thus are commonly used to study cell cycle dynamics (Jiang et al., 1999b). To determine the concentration of Wee1i and Wee1i/Myt1i required to inhibit Wee1 in RPE1 mRuby-PCNA H3.1-mTurq2 cells, I used immunostaining to quantify CDK2 abundance in its phosphorylated (pY15-CDK2) and unphosphorylated state (CDK2). Note that this antibody used for pY15-CDK2 can also recognize pY15-CDK1, pY15-CDK3, pY15-CDK5 due to homology in the region. However, in this experiment this will not affect my assay since I quantified the overall inhibition of Wee1 kinase by Wee1 inhibitors, Wee1i and Wee1i/Myt1i. RPE1 mRuby-PCNA H3.1-mTurq2 cells were treated with Wee1i and Wee1i/Myt1i at various concentrations for 2h before fixation. The results show a significant decrease in the pY15-CDK/CDK2 ratio in Wee1i inhibitors treated cells compared to vehicle-treated cells, in a dose-dependent manner (Fig. 3.2.A). Using 1 μ M Wee1i and Wee1i/Myt1i resulted in a strong decrease of CDK2 phosphorylation with a low toxicity for the cells. I decided for the rest of my experiments to use 1 μ M Wee1i and Wee1i/Myt1i to efficiently inhibit Wee1 (except when indicated).

Synchronous cell populations are commonly used for the analysis of various aspects of cellular metabolism at specific stages of the cell cycle. Cell synchronisation at a chosen cell cycle stage is essential to allow population-wide data to be collected. To study the effect of Wee1 inhibition at cell cycle entry on a large-scale study, I decided to use cells synchronisation in G0/G1. I first arrested cells in G0/G1 using CI. Contact-inhibited cells retained replicative potential, meaning that the cells can restart proliferation after splitting of the culture (Leontieva et al., 2014). Firstly, I wanted to quantify the percentage of arrested cells through CI in my experiments. To distinguish arrested versus non arrested cells, I used 5-ethynyl-2'-deoxyuridine (EdU), to label S-phase (Breinbauer & Köhn, 2003; Cavanagh et al., 2011; Salic & Mitchison, 2008; Q. Wang et al., 2003). I arrested cells through CI by leaving cells at 100% confluency for 3 days. Then, they were replated at lower density in a 384 well plate and serum free media for 24h to prevents cells from proliferating. Cells were pulse labelled with 10 μ M EdU for the last hour. I used immunostaining to analyse EdU incorporation and therefore S-phase entry. As expected, cells arrested using CI show a decrease in EdU ratio compared to the asynchronous population, meaning that a larger percentage of cells were arrested compared to the asynchronous population (Fig. 3.2.B).

Analysis of cell cycle dynamics at single cell resolution was limited to static, fixed tissue methods including flow cytometry BrdU or thymidine incorporation, and staining for cell cycle markers such as Ki67, PCNA, or phospho-H3 (Humbert et al., 1990; Nowakowski et al., 1989; P. J. Smith et al., 2000).

These methods are invasive and require a lot of cell manipulation, such as cell permeabilisation, fixation with aggressive chemicals, and the use of intercalating DNA stains that prevent study of temporal dynamics. This type of approaches shows a snapshot of the cell cycle at a specific time but does not provide temporal information about cell cycle dynamics. Moreover, the majority of high throughput screens provided data for an averaged cell population where information about the variability between cells was discarded. Conversely, single cell analysis by flow cytometry or microscopy provides information about cellular heterogeneity but does not provide insight into cell dynamics. The recent advancement in cell cycle bio-sensor development allow to follow the dynamics of signalling in single cell level time (Abe et al., 2013; Bajar et al., 2016b; Barr et al., 2017; Cooper et al., 2017; Gavet & Pines, 2010;

Heldt et al., 2018; Sakaue-Sawano et al., 2017; Spencer et al., 2013; Sugiyama et al., 2009; Zerjatke et al., 2017; Zielke et al., 2014).

Thus, to monitor the effect of Wee1 inhibition on cell cycle re-entry at a single cell level on a large-scale study, I used single cell live imaging. I arrested RPE1 mRuby-PCNA H3.1-mTurq2 cells in G0/G1 by CI for 3 days and replated in serum-free media for 24h before releasing cells by adding serum with or without Wee1 inhibitors. Cell cycle progression was monitored by time-lapse imaging and tracking to quantify mRuby-PCNA. Our cell tracking software utilises PCNA as a nuclear marker to monitor cells throughout their cell cycle. PCNA distribution across cell cycle is fluctuating across the cell cycle. In G0 and early G1, PCNA remains low and its level increase as cells progress through the cell cycle. This reduced intensity often leads to inaccuracies in the software's ability to consistently track the cells. PCNA intensity across the cell cycle oscillate from low in G0/G1 to a peak intensity in S and G2. In order to monitor cell cycle dynamics, PCNA was used as a nuclear marker however due to its low intensity in G0 and the first 30 minutes of cell cycle re-entry, the tracking software could have difficulties to recognise and track cells. Across my experiments, I noticed that the H3.1-Turq2 marker could be lost across time and thus preventing automated tracking. When automated tracking was not robust or not possible due to the lack of nuclear marker or low signal intensity to segment and track cells, I manually tracked cells and used PCNA intensity and texture of each cell to determine S-phase entry (see materials and methods). I observed that cells treated with Wee1 inhibitors have a delay in S-phase entry compared to DMSO cells. DMSO treated cells enter S-phase at approximately 15.5h (+/- 61min) compared to Wee1 inhibitors treated cells, which enter S-phase only after 18h (+/- 110min) with Wee1i and 20h (+/- 129min) for Wee1i/Myt1i (Fig. 3.2.C). Furthermore, a larger fraction of cells treated with Wee1 inhibitors compared to the untreated cells were not transitioning to S-phase at all, even after 48h serum stimulation (Fig. 3.2.E).

My data from Wee1 perturbation suggests that Wee1 is required for timely S-phase entry and exit from G0. However, kinase inhibitors can produce off-target effects (Wynn et al., 2011). Wee1 inhibitors used in this study have been shown to target other proteins like PLK1 for MK-1775 (Wright et al., 2017) and Myt1/PLK1/CHK1 for Wee1i/Myt1i, PD-0166285 (Delacour & Gavet, 2017). These differences in target could explain the stronger effect observed in Wee1i/Myt1i data.

To confirm that these phenotypes were specific to Wee1 perturbation and not due to an off-target effect of the kinase inhibitors, I used small interfering RNA (siRNA) against Wee1 and quantify S-phase entry. Transient transfection of the siRNA results in a knockdown of the protein of interest, and the resulting phenotypes can be analysed in asynchronous cells or synchronous cells.

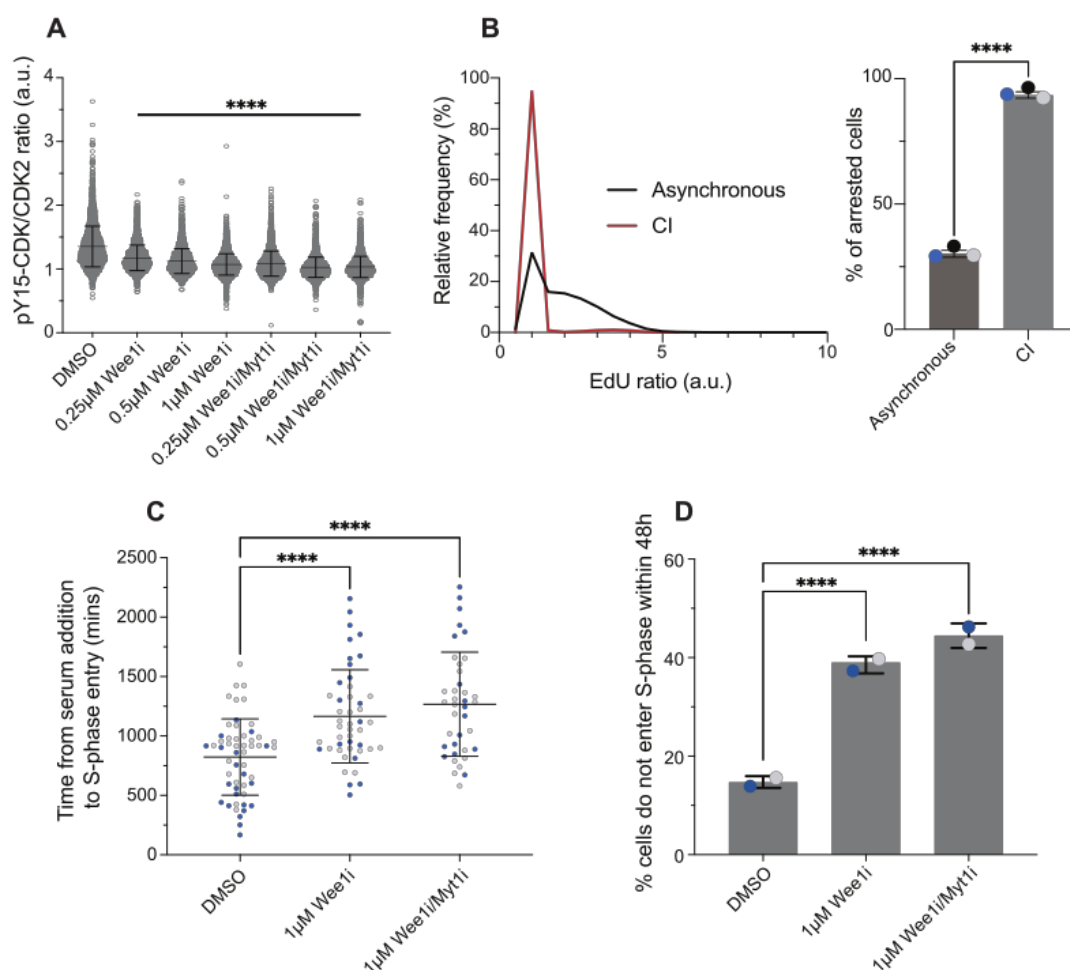


Figure 3-2. Wee1 inhibition leads to S-phase entry and G1 arrest during cell cycle re-entry.

(A) Quantification of the ratio pY15-CDK2/CDK2 in RPE1 mRuby-PCNA mTurq2 cells by immunocytochemistry. Cells were treated with DMSO, Wee1i or Wee1i/Myt1i, at various concentrations for 2h. Single dot represents single cell data. One way ANOVA analysis was performed to calculate p-values (****=p<0.0001). Mean+/- SD are shown. **(B)** Quantification of the EdU ratio of RPE1 mRuby-PCNA by immunocytochemistry. Cells were stopped via CI for 3 days. Cells were pulse labelled for 1h with 10 μ M EdU before fixation. EdU positive cells were quantified as cells with a nuclear:cytoplasmic ratio of EdU signal as greater than or equal to 1.2. Representative frequency distribution of measured intensities from one experimental repeat shown

(left graph). Percentage of arrested cell in asynchronous population and in CI arrested population (right graph). Dots in plot represents replicate wells (3 wells per experiment) colour coded by experimental repeats (n=3). Unpaired T-test analysis was performed to calculate p values (****=p<0.0001) Mean+/- SD are shown. **(C)** Timing of S-phase entry of automated tracked RPE1 mRuby-PCNA mTurq2. Cells were stopped via CI for 3 days and released with serum addition and DMSO, Wee1i or Wee1i/Myt1i, for 48h. Single dots represents single cell data. Each colour represents one replicate (n=2). One way ANOVA analysis was performed to calculate p-values (****=p<0.0001) Mean+/- SD are shown. **(D)** Percentage of RPE1 mRuby-PCNA mTurq2 cells not entering S-phase after 48h, treated with DMSO, Wee1i, or Wee1i/Myt1i. Dots in plot represents replicate wells (3 wells per experiment) colour coded by experimental repeats (n=2). One way ANOVA analysis was performed to calculate p-values (****=p<0.0001) Mean+/- SD are shown.

3.2.2 Wee1 depletion leads to a delay in S-phase entry and G0/G1 arrest.

siRNA can regulate the expression of genes by a phenomenon known as RNA interference (RNAi). RNAi is the biological mechanism by which double-stranded RNA induces gene silencing by targeting complementary mRNA for degradation (Fire et al., 1998). Small molecule inhibitors need to recognise the complicated conformation of certain proteins whereas siRNA executes its function by complete Watson–Crick base pairing with targeted mRNA. This allows a more effective and transient silencing than small kinase inhibitors (Weiss et al., 2007). Validation of siRNA targeting Wee1 (siWee1) were done by Western blot (Fig. 3.3.A). To confirm the phenotypes obtained using Wee1 inhibitors, I repeated the same experiments using this time siWee1. I arrested RPE1 mRuby-PCNA mTurq2 cells in G0/G1 using CI for 3 days before replating them at lower density and reverse transfected them with siRNA for 24h before released. Using siWee1 the phenotype observed was even stronger. G1 length was 27h (+/- 194.4 min) for siWee1 compared to 17.2h (+/-307.1 min) for NTC transfected cells (Fig. 3.3.A). Cells treated with siWee1 also shown a phenotype where a large fraction of cells did not enter S-phase after 48h (Fig. 3.3.C).

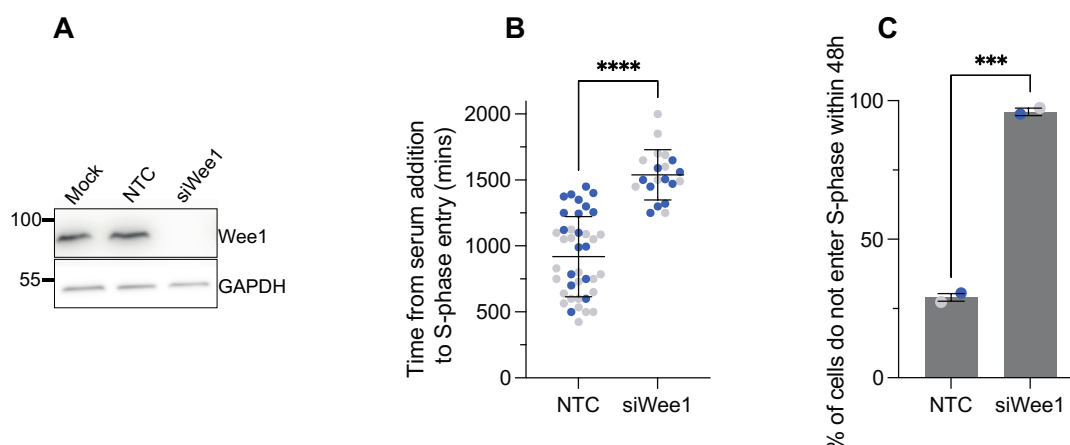


Figure 3-3. Wee1 depletion leads to a delay in S-phase entry and G0 arrest.

(**A**) Western blot showing Wee1 depletion via siRNA. RPE1 mRuby-PCNA mTurq2 cells were transfected with siRNA Wee1 for 24h before collection. Non-targeting Control (NTC) and untreated conditions were used as control. GAPDH is used as loading control. (**B**) Timing of S-phase entry of automated tracked RPE1 mRuby-PCNA mTurq2. Cells were stopped via CI for 3 days and reverse-transfected 24h before released with serum addition and NTC or siWee1 for 48h. Single dots represents single cell data. Each colour represents one replicate (n=2). Unpaired T-test analysis was performed to calculate p-values (****=p<0.0001) Mean+/- SD are shown. (**C**) Percentage of RPE1 mRuby-PCNA mTurq2 cells not entering S-phase after 48h, treated with NTC or siWee1 Dots in plot represents replicate wells (3 wells per experiment) colour coded by experimental repeats (n=2). Unpaired T-test analysis was performed to calculate p-values (****=p<0.0001) Mean+/- SD are shown.

3.2.3 Wee1 inhibition leads to a delay in S-phase entry and a G0/G1 arrest in Palbociclib synchronised cells.

Across my analysis of different experimental repeats, I noticed that arresting cells via CI was not always a robust way to arrest cells and often a non-negligible percentage of cells were still proliferating. As my main goal was to quantify S-phase entry, I had to optimise ways to arrest cells robustly in G0/G1. In the last decade, CDK4/6 inhibitors (CDK4/6i) have been used in cancer treatments to block cell proliferation (O’Leary et al., 2016). Therefore, I decided to use the CDK4/6i Palbociclib, to synchronise cells in G0/G1 as described in (Pennycook & Barr, 2021; Trotter & Hagan, 2020). RPE1 mRuby-PCNA cells were treated with DMSO or 1 μ M Palbociclib for 24h (as described in Pennycook & Barr, 2021). To quantify the percentage of arrested cells through Palbociclib treatment in my experiments, I pulse labelled the cells with 10 μ M EdU for 1h before fixation 23h following drug treatment. After 24h of treatment, 92.5% of Palbociclib treated cells were EdU negative compared to 12% of DMSO treated cells, meaning that cells were able to be arrested upon Palbociclib treatment (Fig. 3.4.A). I then decided to use 1 μ M Palbociclib to robustly arrest my cells in G0/G1 (G0/G1 can also be referred as “quiescence” in the rest of my thesis) for the rest of my experiments.

I synchronised RPE1 mRuby-PCNA in G0/G1 with 1 μ M Palbociclib for 24h and released my cells with or without Wee1i. As before, cells treated with Wee1i show a delay in S-phase entry, 13.74h (+/- 160min) with Wee1i, 15.35h (+/- 219 min) with Wee1/Myt1, compared to DMSO treated cells 11.49h (+/- 150 min) (Fig. 3.4.B). A large fraction of cells also failed to transition into S-phase in the Wee1i treated cells (Fig. 3.4.C). To confirm this phenotype, I also used immunostaining to analyse EdU incorporation and therefore S-phase entry. Cells were arrested using 1 μ M Palbociclib for 24h and released with or without Wee1i or Wee1i/Myt1i plus EdU for 15h. Cells treated with Wee1i or Wee1i/Myt1i showed a decrease in the percentage of EdU positive cells compared to untreated one: 55% EdU+ cells in Wee1i, 48% EdU+ in Wee1i/Myt1i versus 83% in DMSO (Fig. 3.4.D).

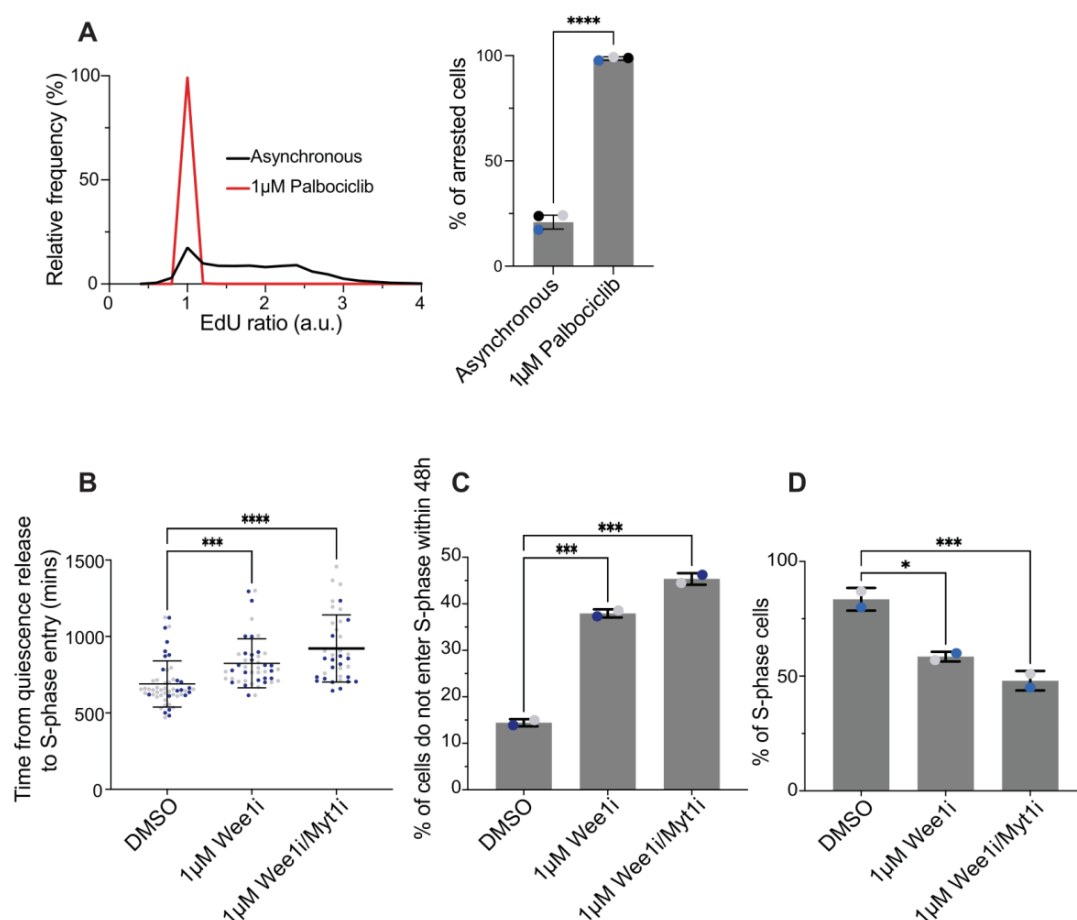


Figure 3-4. Wee1 inhibition leads to a delay in S-phase entry and a G0/G1 arrest in cells synchronised with Palbociclib.

(A) Quantification of the EdU ratio of RPE1 mRuby-PCNA by immunocytochemistry. Cells were arrested in G0/G1 using 1 μ M Palbociclib for 24h with Cells were pulse labelled for 1h with 10 μ M EdU before fixation. EdU positive cells were quantified as cells with a nuclear:cytoplasmic ratio of EdU signal as greater than or equal to 1.2. Representative frequency distribution of measured intensities from one experimental repeat shown (left graph). Percentage of arrested cell in asynchronous population and in palbociclib arrested population (right graph). Dots in plot represents replicate wells (3 wells per experiment) colour coded by experimental repeats (n=3). Unpaired t-test analysis was performed to calculate p-values (****=p<0.0001). Mean $\pm$ SD are shown. **(B)** Timing of S-phase entry of manually tracked RPE1 mRuby-PCNA. Cells were arrested in G0/G1 using 1 μ M Palbociclib for 24h and released with serum addition and DMSO, Wee1i, or Wee1i/Myt1i, for 48h. Single dots represents single cell data. One way ANOVA analysis was performed to calculate p-values (****=p<0.0001, ***=p<0.001). Mean $\pm$ SD are shown. Each colour represents one replicate (n=2). **(C)** Percentage of RPE1 mRuby-PCNA cells not entering S-phase after 48h using data in (B). Dots in plot represents replicate wells (3 wells per experiment) colour coded by experimental repeats (n=2). One way ANOVA analysis was performed to calculate p-values (***=p<0.001). Mean $\pm$ SD are shown. **(D)** Percentage of RPE1 mRuby-PCNA cells not entering S-phase after 15h. Cells were arrested in G0/G1 using 1 μ M Palbociclib for 24h and released with serum addition and DMSO, Wee1i

or Wee1i/Myt1i and 10 μ M EdU for 15h before fixation. EdU positive cells were quantified as cells with a nuclear:cytoplasmic ratio of EdU signal as greater than or equal to 1.2. Dots in plot represent percentage of EdU positive cells per experiment. Each colour represents one experiment (n=2). One way ANOVA analysis was performed to calculate p-values (**=p<0.0001, *=p<0.05). Mean+/- SD are shown.

In both experiments using Wee1i and siWee1 at cell cycle re-entry, leads to a delay in S-phase entry and a G0/G1 arrest. Similarly, to Wee1i, RNAi approaches warrant prolonged treatments, can lead to incomplete protein depletion, and are often associated with off-target effect. Another available option for a most efficient and specific perturbation of Wee1 would be the use of an Wee1 inducible degradation system.

3.2.4 Wee1 degradation leads to a delay in S-phase entry and G0/G1 arrest.

Rapid, precise, and uniform depletion is critical to investigate the functions of regulators within a single cell cycle. The phenotype of the target RNA degradation may not be immediately evident because proteins synthesised prior to degradation may be stable and functional. The time taken for substantial mRNA degradation by RNAi may also affect the interpretation of results. Slow depletion of target proteins provides cells ample time to adapt and either activate compensatory signalling mechanisms or accumulate secondary effects. Since most RNA knockdown studies aim to disrupt the protein function, it is desirable to target the proteins directly. Induced protein degradation via degron tags is probably the most precise and effective tool that is currently available to analyse the consequences of acute loss of a protein of interest in a short time window.

One strategy to specifically degrade a protein is the use of a degron-based system using auxin-inducible degron (AID) technology. This degradation is done using a plant-specific degradation controlled by a phytohormone, auxin, in human cell lines. This technology is based on the expression in human cells of *Oryza sativa* TIR1 (OsTIR1). When expressed OsTIR1 forms a complex with SCF E3 ligase. In plants auxin hormones promotes the interaction between the F-box protein TIR1 and protein containing an AID (Kepinski & Leyser, 2005). In mammalian cells ectopic expression of TIR1 can associate with Skp1 and form a SCF^{TIR1} complex. SCF^{TIR1} has been demonstrated to promote auxin-inducible degradation of proteins tagged with an AID in a variety of animal cells (Nishimura et al., 2009). Therefore, I decided to generate a Wee1-GFP-AID construct This construct will allow me to both understand the spatio-temporal role of Wee1 in regulating cell cycle re-entry and to validate my findings when using Wee1 inhibitors and siRNA in an independent assay.

To tag Wee1, I used an myc-OsTIR1 hTert-RPE1 cell line (Hégarat et al., 2018), where myc-tagged OsTIR1 can be inducibly expressed from the human Rosa26

locus. On the addition of doxycycline (Dox), OsTIR1 forms a complex with SCF. Upon auxin (IAA) induction, the AID interacts with the F-box protein OsTIR1, bringing the AID-tagged protein of interest to the SCF E3 ubiquitin ligase complex for ubiquitination and subsequent degradation by the proteasome (Holland et al., 2012). In this way, the AID system allows a protein of interest to be inducibly and uniformly degraded in cells. The GFP-AID tag was inserted at the C-terminus of the endogenous Wee1 coding sequence using CRISPR/Cas9-mediated gene editing (Fig. 3.5.A, see Materials and Methods). Next, I used western blot to determine homozygous tagging and tested Wee1 degradation by adding IAA and Dox (ID) for 24h (Fig. 3.5.B). In order to analyse Wee1-GFP-AID expression across the cell cycle I wanted to fluorescently label PCNA in this cell line. I infected my cells with a mRuby-PCNA adeno-associated virus (rAAVs, see Materials and Methods). AAV is a single stranded DNA virus that, in recombinant form, can efficiently deliver a targeting construct to the nuclei (Khan et al., 2011; Rago et al., 2007). Once positively infected cells are expressing mRuby-PCNA, I was able to track them across the cell cycle using PCNA intensity. I then, confirmed that myc-OsTIR1 mRuby-PCNA Wee1-GFP-AID cells could be arrested using Palbociclib and are able to normally proliferate after Palbociclib removal. I arrested myc-OsTIR1 mRuby-PCNA Wee1-GFP-AID cells in G0/G1 using 1 μ M Palbociclib for 24h, washed and released cells into the cell cycle by addition of fresh media. Cells were pulse labelled with 10 μ M EdU for 1h before fixation, 23h following drug treatment. I observed that the cells were able to be arrested using 1 μ M Palbociclib (99% of arrested cells) and to correctly re-enter the cell cycle after Palbociclib removal (87.5% of proliferative cells) (Fig. 3.5.D). Furthermore, I tested myc-OsTIR1 mRuby-PCNA Wee1-GFP-AID cells health and viability. Healthy cells follow a predictable growth pattern, while deviations can indicate problems such as genetic instability. To test that, I plated myc-OsTIR1 mRuby-PCNA Wee1-GFP-AID cells low density and followed cell growth for 7 days, until cells reach 100% confluency, using IncuCyte Automated Live-Cell Analysis System. myc-OsTIR1 mRuby-PCNA Wee1-GFP-AID cells achieved 100% confluency after 4.2 days and mirror the growth characteristics of RPE1 mRuby-PCNA cell line (4.8 days) (Fig. 3.5.E). This suggests that the myc-OsTIR1 mRuby-PCNA Wee1-GFP-AID cells lines are healthy, stable and can be used for my experiments. All together these data confirm that myc-OsTIR1 mRuby-PCNA Wee1-GFP-AID cells can be efficiently used to track cell cycle dynamics and follow Wee1 levels across the cell cycle.

To assess the timing of Wee1 degradation upon ID induction, I stopped myc-OsTIR1 mRuby-PCNA Wee1-GFP-AID cells in G0/G1 using 1 μ M Palbociclib for 24h, then washed and released cells into the cell cycle with addition of ID every 30minutes for 2h30 before samples collection. Wee1 partial degradation can be monitored after 30mn of ID treatment whereas complete degradation can be observed after 1h of ID treatment (Fig. 3.5.C).

The dynamics of localisation of Wee1 protein is not clear as no live biosensor has been already described to monitor it. However fixed cell assays described that Wee1 is located in the nucleus to prevent early mitotic entry (Heald et al., 1993). Just before mitosis, Wee1 is redistributed to the cytoplasm (Baldin & Ducommun, 1995).

To assess the localisation of Wee1, I monitored Wee1-GFP-AID levels across the cell cycle using live-cell imaging. Using synchronous population, I also observed that Wee1 is located in the nucleus for all length of the cell cycle with a constant intensity across all phases of the cell cycle and an on-going rapid translocation to the cytoplasm just before mitosis (Fig. 3. 5.F.G).

I then quantified S-phase entry timing upon ID induction in cell cycle re-entry. I arrested myc-OsTIR1 mRuby-PCNA Wee1-GFP-AID cells in G0/G1 using 1 μ M Palbociclib for 24h and released them with or without ID and Wee1i (Fig. 3.5.H). Upon Wee1i treatment, cells were entering S-phase with a delay in S-phase entry (12.75h +/- 135 mins) compared to DMSO (8.5h +/- 85 mins). Upon ID treatment, cells were entering S phase with a stronger delay compared to Wee1i and DMSO (26h +/- 345 mins). Moreover, almost all cells after ID induction were arrested in G0/G1 compared to DMSO, 87% percent of cells with ID compared to 18% of cells for DMSO (Fig. 3.5.I).

These three methods of Wee1 inhibition/degradation (Weei, siWee1, Wee1 degnon) show that perturbation of Wee1 during cell cycle re-entry does not show the expected phenotype of cell cycle acceleration. Instead, perturbation of Wee1 at cell cycle re-entry leads to a delay in S-phase entry and an arrest of cells in G0/G1.

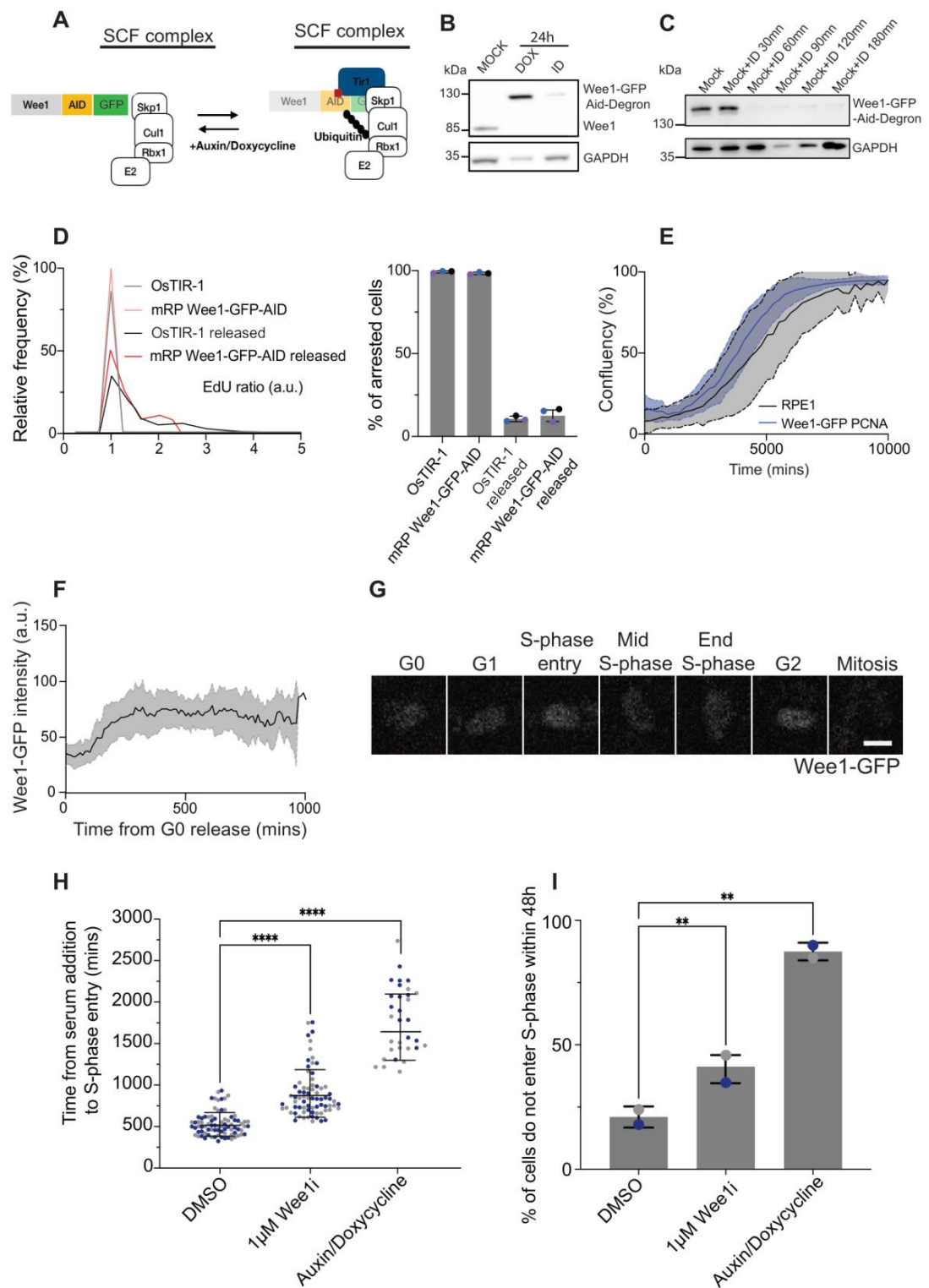


Figure 3-5. Wee1 degradation leads to a delay in S-phase entry and a G0/G1 arrest.

(A) A schematic illustration of the AID system fused with Wee1 protein. Upon doxycycline addition, myc-OsTIR1 is expressed and forms a functional SCF OsTIR1 E3

ligase complex with the endogenous components in human cells. In the presence of auxin, Wee1 is rapidly degraded upon poly-ubiquitylation. **(B)** Western blot showing Wee1-GFP-AID degradation. myc-OsTIR1 mRuby-PCNA Wee1-GFP-AID cells were treated with a combination of Auxin/Doxycycline for 24h before samples collection. GAPDH is used as loading control. **(C)** Western blot showing timing of Wee1-GFP-AID degradation. myc-OsTIR1 mRuby-PCNA Wee1-GFP-AID cells were treated with a combination of Auxin/Doxycycline (ID) every 30min for 2h30 and cell pellets were collected at the end. Images of Wee1-GFP during cell cycle progression. Asynchronous RPE1 Wee1-GFP cells were imaged for a 24h. Single cell is imaged at G1 entry after mitosis and follow to the subsequent mitosis. Scale bar: 10 μ M. **(D)** Quantification of the EdU ratio of myc-OsTIR1 mRuby-PCNA Wee1-GFP-AID cells by immunohistochemistry. myc-OsTIR1 mRuby-PCNA Wee1-GFP-AID cells were arrested in G0/G1 using 1 μ M Palbociclib for 24h and pulse labelled for 1h after 23h of treatment with 10 μ M EdU before fixation. myc-OsTIR1 mRuby-PCNA Wee1-GFP-AID cells were arrested in G0/G1 using 1 μ M Palbociclib for 24h. Cells were washed and released into the cells cycle with fresh media plus EdU for 24h before fixation. EdU positive cells were quantified as cells with a nuclear:cytoplasmic ratio of EdU signal as greater than or equal to 1.2. Representative frequency distribution of measured intensities from one experimental repeat shown (left graph). Percentage of arrested cell in asynchronous population and in Palbociclib arrested population (right graph). Dots in plot represents replicate wells (3 wells per experiment) colour coded by experimental repeats (n=3). **(E)** Wee1-GFP growth curve. Confluency of asynchronous myc-OsTIR1 mRuby-PCNA Wee1-GFP-AID was track over a period of 7days. Mean $\pm$ SD are shown (n=3). **(F)** Quantification of Wee1 expression upon G1 entry using data from (H) **(G)** Images of Wee1-GFP-AID during cell cycle progression. Asynchronous RPE1 Wee1-GFP cells were imaged for a 24h. First image is taken at G1 entry after mitosis and follow to the subsequent mitosis. Scale bar: 10 μ M. **(H)** Timing of S-phase entry of manually tracked myc-OsTIR1 mRuby-PCNA Wee1-GFP-AID cells. myc-OsTIR1 mRuby-PCNA Wee1-GFP-AID cells were arrested in G0/G1 using 1 μ M Palbociclib for 24h and released with serum addition and DMSO, Wee1i, or ID for 48h. Single dots represents single cell data. Each colour represents one replicate (n=2). One way ANOVA analysis was performed to calculate p-values (**=p<0.0001). Mean $\pm$ SD are shown. **(I)** Percentage of myc-OsTIR1 mRuby-PCNA Wee1-GFP-AID cells not entering S-phase after 48h treated with DMSO, Wee1i or ID. Dots in plot represents replicate wells (3 wells per experiment) colour coded by experimental repeats (n=2). One way ANOVA analysis was performed to calculate p-values (**=p<0.0001). Mean $\pm$ SD are shown.

3.2.5 Wee1 is required in early G1.

To further investigate the relationship between Wee1 inhibition and cell cycle re-entry, I monitored when in G1, Wee1 activity is required for timely S-phase entry. I used live imaging to quantify the dynamics of cell cycle re-entry using Wee1i added at different times after release from quiescence. I arrested RPE1 mRuby-PCNA cells in G0/G1 using 1 μ M Palbociclib for 24h and released them by adding Wee1i every 2h for 10h before imaging. I noticed a delay in S-phase entry with cells treated with Wee1i within the first 6h post release. Cells treated with DMSO were entering S-phase after 12.2h (+/- 157 min) whereas cells treated with Wee1i from 0h to 4h post-release entered S-phase with a delay (from 14.36h to 15.01h). G1 wild type length was rescued for cells treated with Wee1i from 6h to 10h post release (Fig. 3.6.A). Interestingly, the percentage of quiescent cells after 48 h also correlates with the time cells spent in G1 before drug addition (Fig. 3.6.B). Cells in G1 between 0h and 6h before drug addition have a higher percentage of cells that did not enter S-phase after 48h compared to cells spending 6h to 10h in G1 before drug addition.

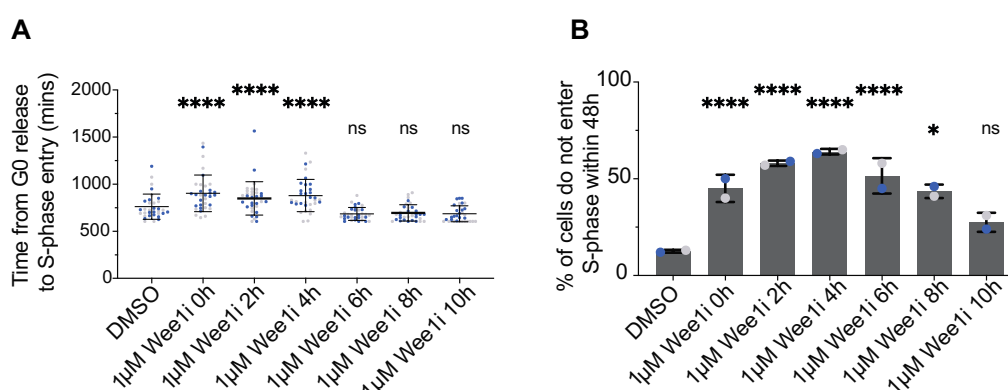


Figure 3-6. Wee1 is required in early G1.

(A) Timing of S-phase entry of manually tracked RPE1 mRuby-PCNA cells treated with Wee1i. Cells were stopped in G0 using 1 μ M Palbociclib for 24h and released for 48h with addition of Wee1i every two hours for 10h. Dots in plot represent single cell. Each colour represents one replicate (n=2). One-way ANOVA analysis was performed to calculate p-values (****=p<0.0001, ns= not significant). Mean+/- SD are shown. **(B)** Percentage RPE1 mRuby-PCNA cells not entering S-phase after 48h treated with DMSO or Wee1i every 2h. Dots in plot represents replicate wells (3 wells per experiment) colour coded by experimental repeats (n=2). One-way ANOVA analysis was performed to calculate p-values (****=p<0.0001, *=p<0.05, ns=not significant). Mean+/- SD are shown.

3.3 Wee1 perturbation in G1 impacts progression through the rest of the cell cycle.

Considering the central role of Wee1 controlling cell cycle progression, I asked whether Wee1 inhibition during cell cycle re-entry has an impact on any other phase of the cell cycle.

3.3.1 Wee1 inhibition in synchronous cells perturbs cell cycle progression.

To monitor, how Wee1 perturbation during G1 impacts the progression through the cell cycle, I arrested RPE1 mRuby-PCNA cells in G0/G1 using 1 μ M Palbociclib for 24h then released cells, with or without Wee1i, for 48h. I manually tracked single cells using PCNA texture and intensity to quantify the length of each phase.

Cells treated with DMSO progressed into each phase with normal duration. Cells were able to progress through several cell cycle across the experiment. (Only the length of the first cell cycle is shown in this graph) (Fig. 3.7.A). Consistent with my previous data, cells treated with Wee1i, have a delayed S-phase entry, 13.1h (+/- 182mins), compared to DMSO treated cells 10.3h (+/- 108.5 mins). No noticeable difference could be noted in the duration of S-phase length between Wee1i treated cells and DMSO. For Wee1i treated cells that completed G2 and progressed into mitosis, I observed a decrease in G2 length in Wee1i treated cells: 2.8h (+/- 36.24 mins) for DMSO and 1.95h (+/- 62.20 mins) for Wee1i. Furthermore, for cells able to progress through mitosis upon Wee1i, I observed an increase in mitosis length compared to DMSO. Indeed, Wee1i treated cells had a mitosis length of 2.5h (+/- 60mins) compared to DMSO with 1.1h (+/- 40mins).

Due to the inhibitory role of Wee1 at the G2/M checkpoint, I was surprised to notice a large fraction of cells treated with Wee1i transitioning through S and G2 (21%) but that did not enter mitosis and arrest in G2, compared to DMSO (0%).

These experiments show that Wee1 perturbation during cell cycle re-entry not only impact the timing in S-phase entry (delay in S-phase entry) but also leads to an arrest in G0/G1 and in G2 preventing a normal progression to the next cell cycle.

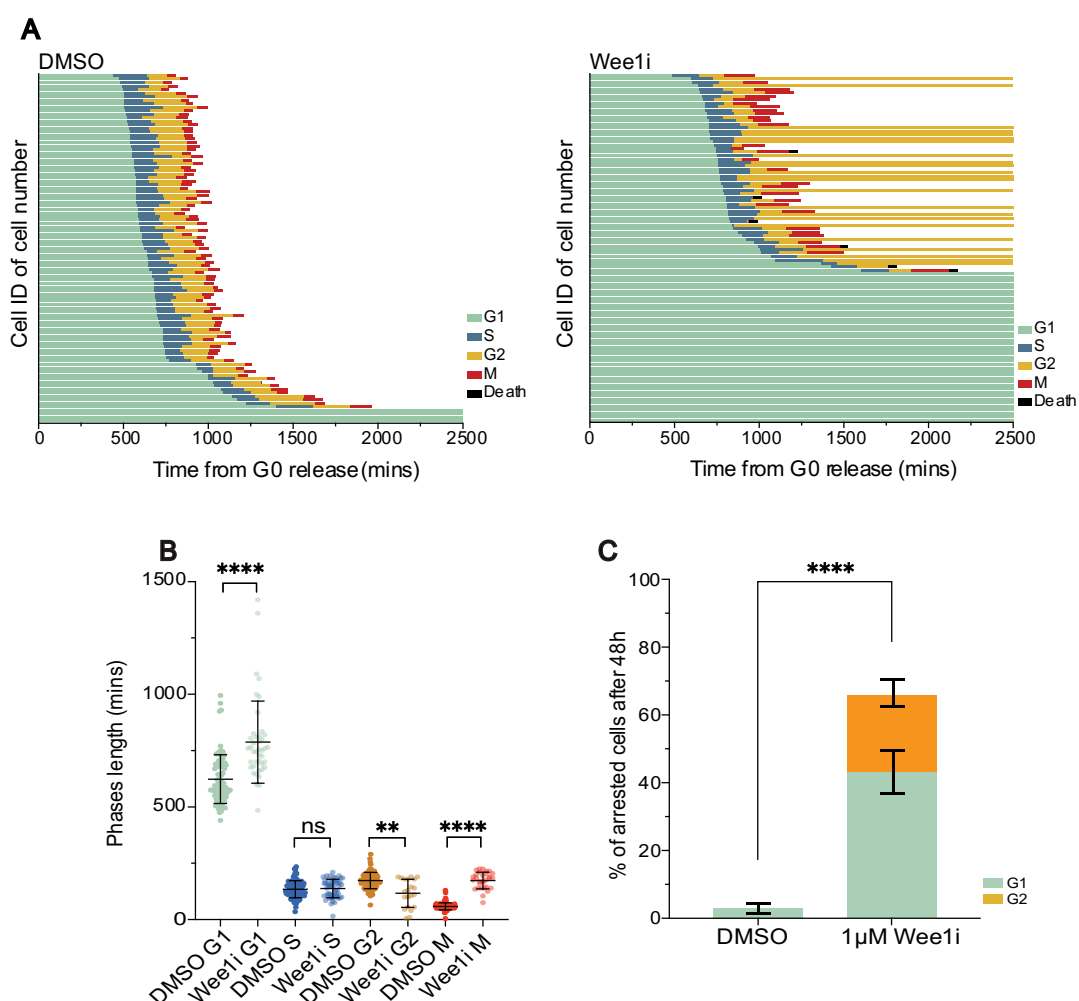


Figure 3-7. Wee1 inhibition during G1 perturbs cell cycle progression.

(A) Manually tracked RPE1 mRuby-PCNA cells cycle progression upon G0 released. RPE1 mRuby-PCNA cells were arrested in G0/G1 using 1μM Palbociclib for 24h and released for 48h with fresh media and DMSO or Wee1i. Each line represents one single cell (DMSO: n=100, Wee1i: n=100). Colours represents different phases of the cell cycle (one experimental repeat is shown). **(B)** Quantification of phase length duration in synchronous RPE1 mRuby-PCNA cells upon DMSO or Wee1i treatment. Data from (A) is used. Single dots represent single cell. Unpaired T-test was performed to calculate p-values (****=p<0.0001, **=p<0.001, ns=not significant). Mean±SD are shown. **(C)** Percentage of RPE1 mRuby-PCNA arrested in G0/G1 and G2 (n=2). Unpaired T-test analysis was performed to calculate p-values (****=p<0.0001). Mean±SD are shown.

3.3.2 Wee1 inhibition during G1 in asynchronous cells perturbs cell cycle dynamics.

Surprisingly, my data suggest that Wee1 perturbation at cell cycle re-entry leads to a S-phase entry delay as well as a G0/G1 and G2 arrest. Current strategies to target cancer cells with Wee1i rely on the fact that Wee1 inhibited cancer cells would rush into mitosis (short S and G2 phases and accumulation of DNA damage) and die from mitotic catastrophe. In patients, Wee1i treatment occurs in an asynchronous population. It was then important to know if cells treated with Wee1i in asynchronously cycling populations that are in G1 could also display a G1 and G2 arrest and potentially create resistance to the treatment.

Hence, I decided to monitor cell cycle dynamics of asynchronous cells that are in G1 at the time of treatment. To understand the effect of Wee1 inhibition during cell cycle entry (G0/G1) in a non-arrested population, I used timelapse imaging to follow asynchronous RPE1 mRuby-PCNA cells 10h prior to drug addition. This allowed me to determine how long cells had spent in G1 phase at the time of drug addition and then determine the length of each phase post-treatment (Fig. 3.8.A). Cells were analysed and grouped into intervals of 2h. I quantified the length of each phase for asynchronous cells treated at different times during G1 (Fig. 3.8.B). I could also observe a delay in S-phase entry after Wee1i treatment in G1. Contrary to data obtained in synchronous cells returning to cycle from G0, S-phase duration and G2 duration were more significantly impacted by Wee1 inhibition.

Cells treated with Wee1i during G1 in asynchronous population showed a reduction of S and G2 length. When I compared G1 length from cells treated at different times with Wee1i, I could see a delay in S-phase entry with cells in G1 for >5h before, Wee1i treatment (Fig. 3.8.C) which is surprising compared to results obtained in synchronous cells and the rescue of a “normal” G1 length in cells treated in late G1 (view Fig. 3.6.A).

Cells treated with DMSO in G1, progressed into S-phase (89%) then G2 and M phases independently from the time spent in G1 before drug addition (Fig. 3.8.D). In contrast to the data obtained during cell cycle re-entry, the percentage of cells arrested in G1 in Wee1i treated cells are overall comparable with DMSO treated cells. However, a large fraction of Wee1i treated cells arrest in G2, also independent of the time spent in G1 before drug addition. A significant portion of cells treated with Wee1i also

arrested in the next G1. In contrast to G2 arrested cells, cells were more likely to be stopped in the next G1 if they were treated with Wee1i in late G1. Out of 25 cells arrested in the second G1, 60% were cells treated with Wee1i in late G1 (5h or plus).

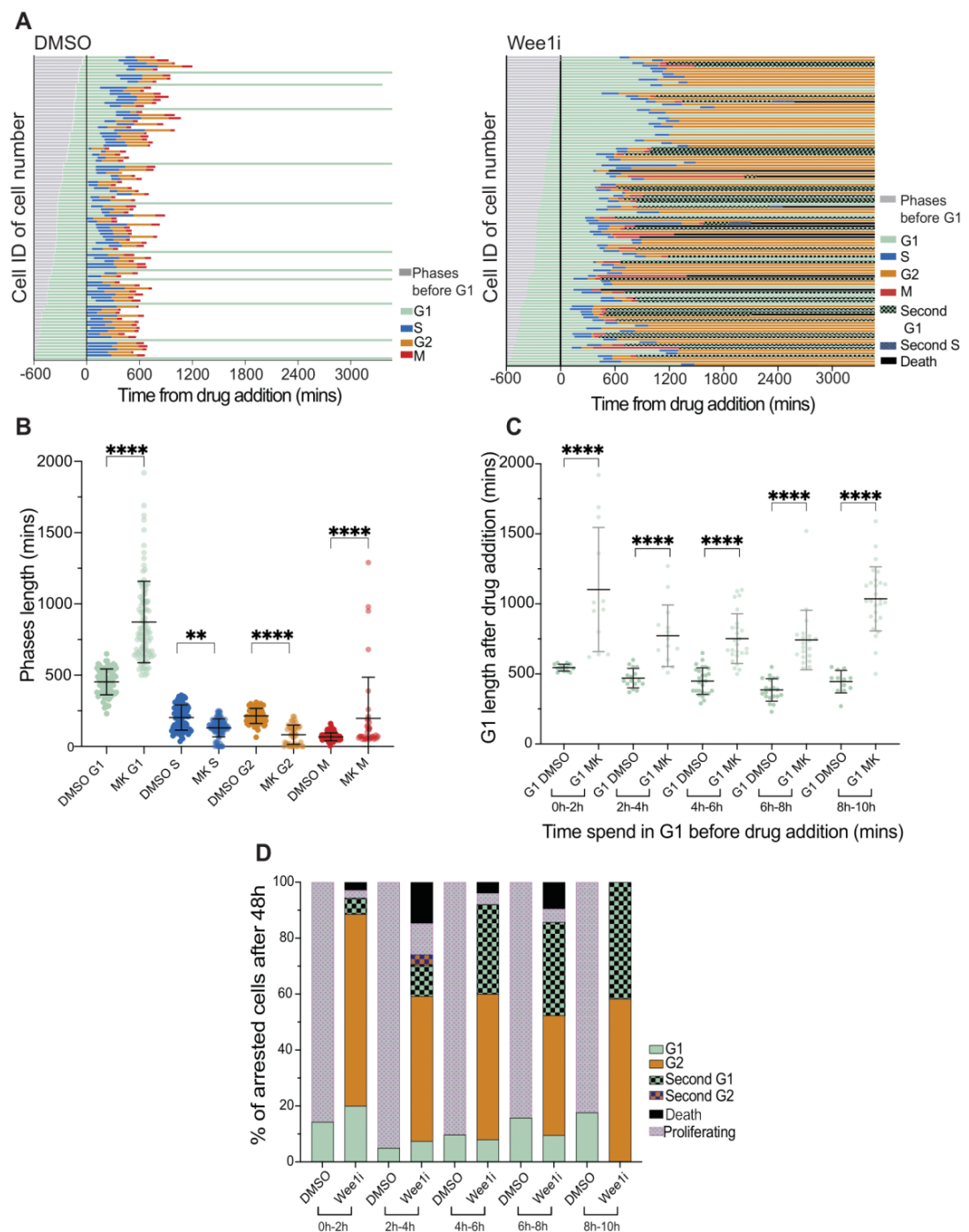


Figure 3-8. Wee1 inhibition during G1 perturbs cell cycle progression in an asynchronously cycling population.

(A) Manually tracked cell cycle progression of asynchronous cells upon DMSO and Wee1i treatment. Asynchronous RPE1 mRuby-PCNA cells were imaged 10h before treatment. Cells were then treated with DMSO or Wee1i for 48h. Only cells in G1 at drug treatment were tracked in this experiment. Each line represents on single cell (DMSO: n=100, Wee1i: n=100). **(B)** Quantification of phase length duration in

asynchronous RPE1 mRuby-PCNA cells upon DMSO or Wee1i treatment. Data from (A) and (B) are used. Single dots represent single cell data with mean and SD shown. Unpaired T-test analysis was performed to calculate p values (****= $p < 0.0001$, **= $p < 0.001$). Mean $\pm$ SD are shown. **(C)** Quantification of G1 length depending on time spend in G1 before drug treatment. Cells were regrouped in 5 groups (0-2h, 2-4h, 4-6h, 6-8h, 8-10h) corresponding to the time spend in G1 before DMSO or Wee1i addition. Data from (A) and (B) are used. Single dots represent single cell dat. Mean $\pm$ SD are shown. **(D)** Percentage of RPE1 mRuby-PCNA arrested in different phases of the cell cycle depending on the time cells spent in G1 before DMSO or Wee1i addition. Data from (A) and (B) are used. Mean $\pm$ SD are shown.

3.4 Wee1 inhibition perturbs PCNA loading.

I noticed that mRuby-PCNA localisation and foci looked different between DMSO and Wee1i treated conditions. PCNA foci emergence, brightness and localisation seems to be disturbed when Wee1 is inhibited during S-phase entry. I quantified this abnormal mRuby-PCNA expression in my live imaging data. PCNA intensity reflects the average signal strength of PCNA across cells, providing an indication of the overall abundance of PCNA. However, in my experiments only the appearance and localisation of the foci look disturbed. It is then more interesting to quantify the standard deviation (STDEV) of PCNA within my experiments. PCNA STDEV intensity provides information about the spread or variability of PCNA signal within a region. A change in its intensity could indicate alterations in the spatial distribution of PCNA such as changes in the formation of PCNA foci.

Upon cell cycle re-entry mRuby-PCNA STDEV intensity is lower with Wee1i compared to DMSO (Fig. 3.9.A).

To confirmed if the perceived S-phase delay after Wee1 inhibition was due to abnormal PCNA dynamics at the G1/S transition, I monitored cell cycle dynamics using additional cell cycle biosensors.

I used hTert-RPE1 mTurquoise2-PCNA mCherry-Geminin PIP-Venus (PIP-FUCCI) cell line to distinguish S-phase entry upon Wee1 inhibition (see: Materials and Methods)(Grant et al., 2018). I arrested PIP-FUCCI cells using 1 μ M Palbociclib for 24h, then washed and released the cells into the cell cycle with or without Wee1i (Fig. 3.9.B). Using PIP-FUCCI degradation to quantify S-phase entry, I noticed this time, that cells were lacking S-phase entry delay. Both populations, DMSO and Wee1i, were entering S-phase at similar times (Fig. 3.9.C). As in my previous experiments a large fraction of cells were arrested in G0/G1 upon Wee1 inhibition (Fig. 3.9.D). 32% of G1 arrested cells with Wee1i compared to 12% for DMSO. I also noticed a higher population of cells arrested in G2 upon Wee1i, 24% for Wee1i compared to 0% for DMSO. These results hypothesised that the apparent delay in S-phase entry upon Wee1i perturbation at cell cycle re-entry could be due to a difficulty of distinguishing S-phase entry. However, the G1 arrest and G2 arrest phenotypes are a direct effect of Wee1 inhibition at cell cycle re-entry.

All the experiments have been realised for the moment in only one cell line. In order to strengthen my findings, I decided to repeat my experiments using other cell lines.

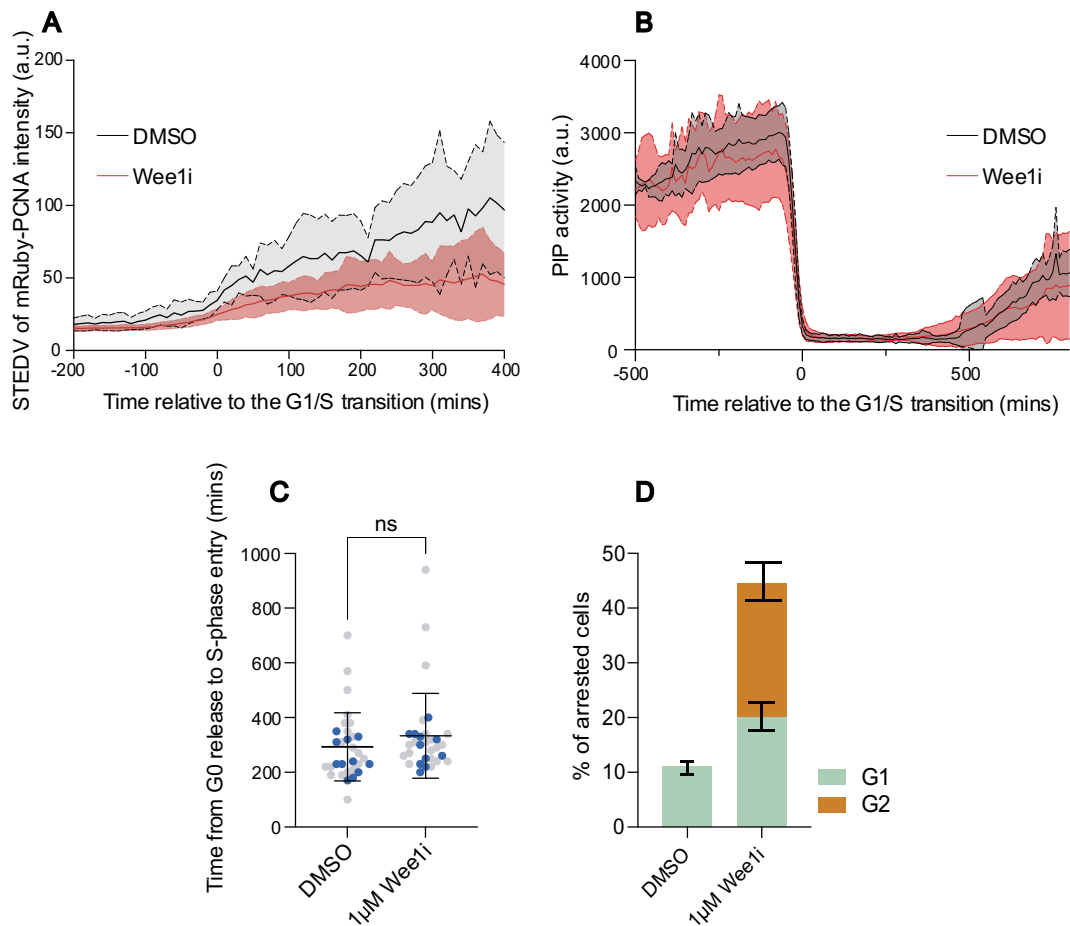


Figure 3-9. Perturbation of PCNA dynamics upon Wee1 inhibition.

(A) Quantification of PCNA STDEV at the G1/S transition upon Wee1 inhibition using live imaging data. PIP-FUCCI cells were arrested using 1 μ M Palbociclib for 24h and released for 48h with DMSO or Wee1i. Mean $\pm$ SD are shown (DMSO: n=20, Wee1i: n=20). **(B)** Quantification of PIP activity of PIP-FUCCI cells at the G1/S transition upon Wee1 inhibition using live imaging data. PIP-FUCCI cells were arrested using 1 μ M Palbociclib for 24h and released for 48h with DMSO or Wee1i. Mean $\pm$ SD are shown (DMSO: n=20, Wee1i: n=20). **(C)** Timing of S-phase entry of manually tracked PIP-FUCCI cells. Cells were arrested in G0/G1 using 1 μ M Palbociclib for 24h and released with DMSO or Wee1i for 48h. Single dots represents single cell data. Each colour represents one replicate (n=2). Unpaired T-test analysis was performed to calculate p values (ns=not significant). Mean $\pm$ SD are shown. **(D)** Percentage of PIP-FUCCI cells arrested in G1 and G2 (n=2). Mean $\pm$ SD are shown.

3.5 Cell cycle dynamics perturbation upon Wee1 inhibition is cell-line dependent.

To determine if the observed delay in S-phase entry with Wee1i is cell-line dependent, I repeated my experiments using other cell lines. In the next paragraph, experiments and analysis have been carried out by different students under my supervision. Xinwei Zou was a Masters student in Cancer Biology at Imperial College London. Emma Graham was a Bachelors student at University of Cambridge. Their names will be stated when needed.

In these paragraphs, design of experiments were carried out by me and Xinwei Zhou, and analysis was carried out by Xinwei Zhou.

To investigate if Wee1 inhibition affects cell cycle entry timing in other cell lines. We used non-small cell lung cancer (NSCLC) cell lines (NCI-H1944, A549,).

Both cell lines have been studied extensively and carry activating KRAS point mutation. These cell lines expressed wild type p53 which ensures that cell cycle progression is not perturbed by defective DNA damage repair pathways caused by p53 mutation (Ikediobi et al., 2006; Phelps et al., 1996). Moreover, it is interesting to test Wee1 inhibitors on cancer cell lines mimicking more trustfully the effect of Wee1i on patient tumours.

We first assessed if these cells could be efficiently arrested using Palbociclib. Cells were arrested using an increasing concentration of Palbociclib (2.5 and 5 μ M) for 24h. Cells were pulse labelled with EdU for 2h before fixation. Upon treatment and in both cell lines, Palbociclib decreased the percentage of EdU positive cells in a dose-dependent manner (Fig. 3.10.A, B). To visualise if these cell lines could recover from cell cycle arrest, we arrested cells with 5 μ M Palbociclib for 24 h before Palbociclib washing and released into the cell cycle. A 2h EdU pulse was applied 22 h post released, prior to fixation. In NCI-H1944 cells, the percentage of EdU positive cells increased. This indicates that a concentration of 5 μ M of Palbociclib can be used to transiently arrest NCI-H1944 (this concentration will be used in the rest of the experiments). In A549 cells, washout of Palbociclib led to slight decrease in the percentage of non-replicating cells. Lower concentrations and lower times treatment of Palbociclib could allow more A549 cells to be transiently arrested. We then evaluated the

inhibitory effect of Wee1i on these cell lines by measuring the level of p-Y15-CDK2 and CDK2. Cells were treated with Wee1i or Wee1i/Myt1i, for 1h before fixation. The results show a significant decrease in the pY15-CDK2/CDK2 ratio in Wee1i treated cells compared to vehicle-treated cells, in a dose-dependent manner (Fig. 3.10.C). To study the effect of Wee1 inhibition at cell cycle re-entry in NCI-H1944, we used PCNA to quantify cell cycle phases length. NCI-H1944 mRuby-PCNA cells were arrested in G0/G1 using 5 μ M Palbociclib for 24h and released into the cell cycle with or without Wee1i for 48h. Upon Wee1i treatment, no significant delay in S-phase entry was noticed. DMSO treated cell entered S-phase around 16.6h (+/-180 mins) compared to 14.5h (+/-300 mins) for Wee1i treated cells and 18.3h (+/- 240mins) for Wee1i treated cells (Fig.3. 10. D, E). Interestingly, only cells treated with Wee1i/Myt1i cells shown a significant increase in the number of arrested cells in G1 (Fig. 3.10.F). 9% of cells were arrested in G0 in DMSO treated cells compared to 13.5% for Wee1i and 53% for Wee1i/Myt1i. Additionally, in contrast with my previous data, using RPE1 cells, no cells upon Wee1 inhibition arrested in G2. Instead, cells able to progress through S phase would die from mitotic catastrophe.

Due to the slight increase in the percentage of arrested cells in G0/G1 upon Wee1 inhibition, we decided to perform a fixed cell assay to validate this phenotype. NCI-H1944 and A549 cells were synchronised using 5 μ M Palbociclib for 24h. We, then released the cells into the cell cycle with DMSO, Wee1i or Wee1i/Myt1i plus Edu for 24h. Cells were incubated 24h before fixation and stained for EdU. In both A549 and NCI-H1944 cells, Wee1i did not increase the percentage of arrested cells in G1 compared to DMSO (Fig. 3.10.G). As in the live imaging data, only cells treated with Wee1i/Myt1i shown an increase in the percentage of G1 arrested cells compared to DMSO.

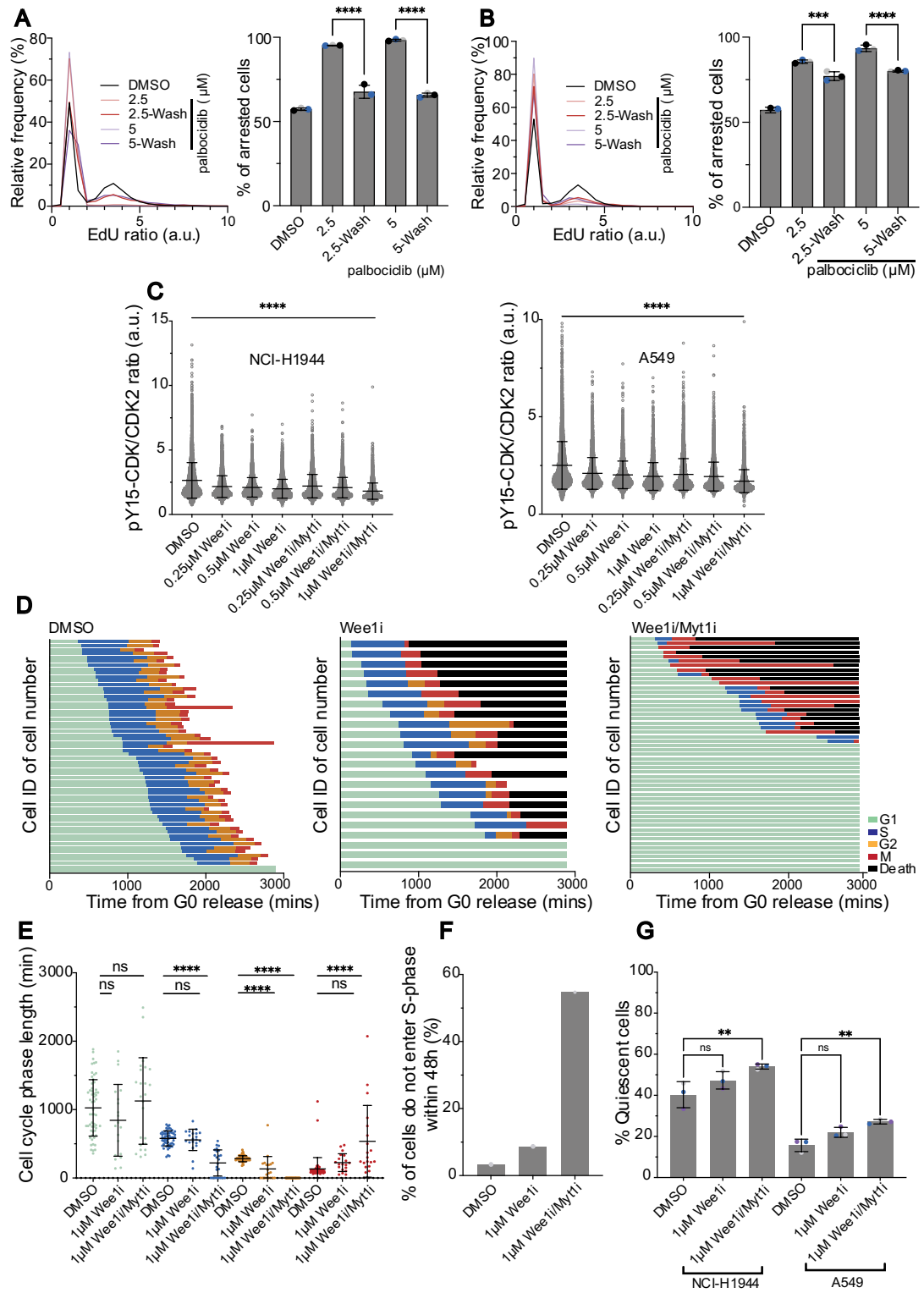


Figure 3-10. Perturbation of cell cycle dynamics upon Wee1 is cell line dependent.

(A) Quantification of the EdU ratio of NCI-H1944 cells by immunochemistry. Cells were stopped using an increasing concentration of Palbociclib for 24h. Cells were pulse labelled for 1h after 23h of treatment with 10 μ M EdU before fixation. EdU positive cells were quantified as cells with a nuclear:cytoplasmic ratio of EdU signal as greater than or equal to 1.2. Representative frequency distribution of measured intensities from one experimental repeat shown (left graph). Percentage of arrested cells in arrested and released population (right graph). Dots in plot represents replicate wells (3 wells per experiment). One-way ANOVA analysis was performed to calculate p-values (****= $p < 0.0001$). Mean $\pm$ SD are shown. **(B)** Quantification of the EdU ratio of A549 cells by immunochemistry. Cells were stopped using an increasing concentration of Palbociclib for 24h. Cells were pulse labelled for 1h after 23h of treatment with 10 μ M EdU before fixation. EdU positive cells were quantified as cells with a nuclear:cytoplasmic ratio of EdU signal as greater than or equal to 1.2. Representative frequency distribution of measured intensities from one experimental repeat shown (left graph). Percentage of arrested cell in arrested and released population (right graph). Dots in plot represents replicate wells (3 wells per experiment). One-way ANOVA analysis was performed to calculate p-values (****= $p < 0.0001$). Mean $\pm$ SD are shown. **(C)** Quantification of the ratio phosphoY15-CDK2/CDK2 in NCI-H1944 and A549 cells by immunochemistry. NCI-H1944 and A549 cells were treated with DMSO, Wee1i or Wee1i/Myt1i at various concentrations for 2h. Single dot represents single cell data. One-way ANOVA analysis was performed to calculate p-values (****= $p < 0.0001$). Mean $\pm$ SD are shown. **(D)** Manually tracked NCI-H1944 cells cell cycle progression upon G0 released. NCI-H1944 were arrested in G0 using 5 μ M Palbociclib for 24h and released for 48h with fresh media and DMSO, Wee1i or Wee1i/Myt1i. Each line represents one single cell (DMSO: n=50, Wee1i: n=23, Wee1i/Myt1i: n=50). Colours represents different phases of the cell cycle. **(E)** Quantification of phase length duration in synchronous NCI-H1944 cells upon DMSO or Wee1i treatment. Live imaging data from (D) is used. Single dots represent single cell data. One way ANOVA analysis was performed to calculate p-values (****= $p < 0.0001$, ns=not significant). Mean $\pm$ SD are shown. **(F)** Percentage of NCI-H1944 arrested cells in G1 after 48h upon DMSO, Wee1i or Wee1i/Myt1i. Live imaging data from (D) is used. **(G)** Percentage of Edu positive cells of NCI-H1944 and A549 cells by immunochemistry. NCI-H1944 and A549 cells were arrested using 5 μ M Palbociclib for 24h. Cells were washed and released into the cell cycle with fresh media plus DMSO, Wee1i or Wee1i/Myt1i and EdU for 24h. Dots in plot represents replicate wells (3 wells per experiment) colour coded by experimental repeats (n=3). One-way ANOVA analysis was performed to calculate p-values (****= $p < 0.0001$, *= $p < 0.05$, ns=not significant). Mean $\pm$ SD are shown.

In recent years, a number of preclinical studies have focused on understanding the functionality of Wee1 in breast cancer cells, particularly those with defective cell cycle regulation (Brunner et al., 2020; Pitts et al., 2020).

I then decided to investigate if Wee1 inhibition, during cell cycle re-entry, in breast cancer cell lines could exhibit the same phenotypes found in RPE1 cell lines. I decided to repeat my experiments using the breast cancer cell line MCF7.

In this paragraph, experimental design was carried out by me, experiments and analysis were carried out by Emma Graham and myself. In this work only Wee1i has been used.

We first assessed if these cells could be efficiently arrested using Palbociclib. Cells were arrested using an increasing concentration of Palbociclib (2.5, 5 and 10 μ M) for 24h. Cells were pulse labelled with EdU for 2h before fixation. Upon treatment and in both cell lines, Palbociclib decreased the percentage of EdU positive cells in a dose-dependent manner (Fig. 3.11.A). To visualise if these cell lines could recover from cell cycle arrest, arrested cells were washed and released in the cell cycle with fresh media. A 2h EdU pulse was applied 22 h post released, prior to fixation. Upon Palbociclib treatment MCF7 cells are able to be transiently arrested in G0/G1

We evaluated the inhibitory effect of Wee1 inhibition on this cell line by measuring the level of pY15-CDK2 and CDK2. An asynchronous population of MCF7 cells were treated with 1 μ M Wee1i for 1h before fixation. As expected, Wee1i treated cells show a significant decrease in the pY15-CDK2/CDK2 ratio (Fig. 3.11.B).

To study the effect of Wee1 inhibition at cell cycle re-entry in MCF7 cells, we used mRuby-PCNA to quantify cell cycle phase length. MCF7 mRuby-PCNA cells were arrested using 1 μ M Palbociclib for 24h and released into the cell cycle with DMSO or 1 μ M Wee1i (Fig. 3.11.C). Upon Wee1 inhibition no significant delay in S-phase entry could be noticed compared to DMSO. Cells entered S-phase around 15.6h (+/- 8h) with Wee1i treatment versus 16h(+/-7.8h) for DMSO (Fig. 3.11.C, D). Upon Wee1i treated cells an increased in G1 arrested cells could also be observed: 38% of cells were still arrested in Wee1i treated cells after 48h compared to 13.5 % in DMSO (Fig. 3.11.E). Furthermore, MCF7 cells treated with Wee1i were also able to arrest in G2. 62% of Wee1i treated cells were arrested in G2 compared to 2% in DMSO.

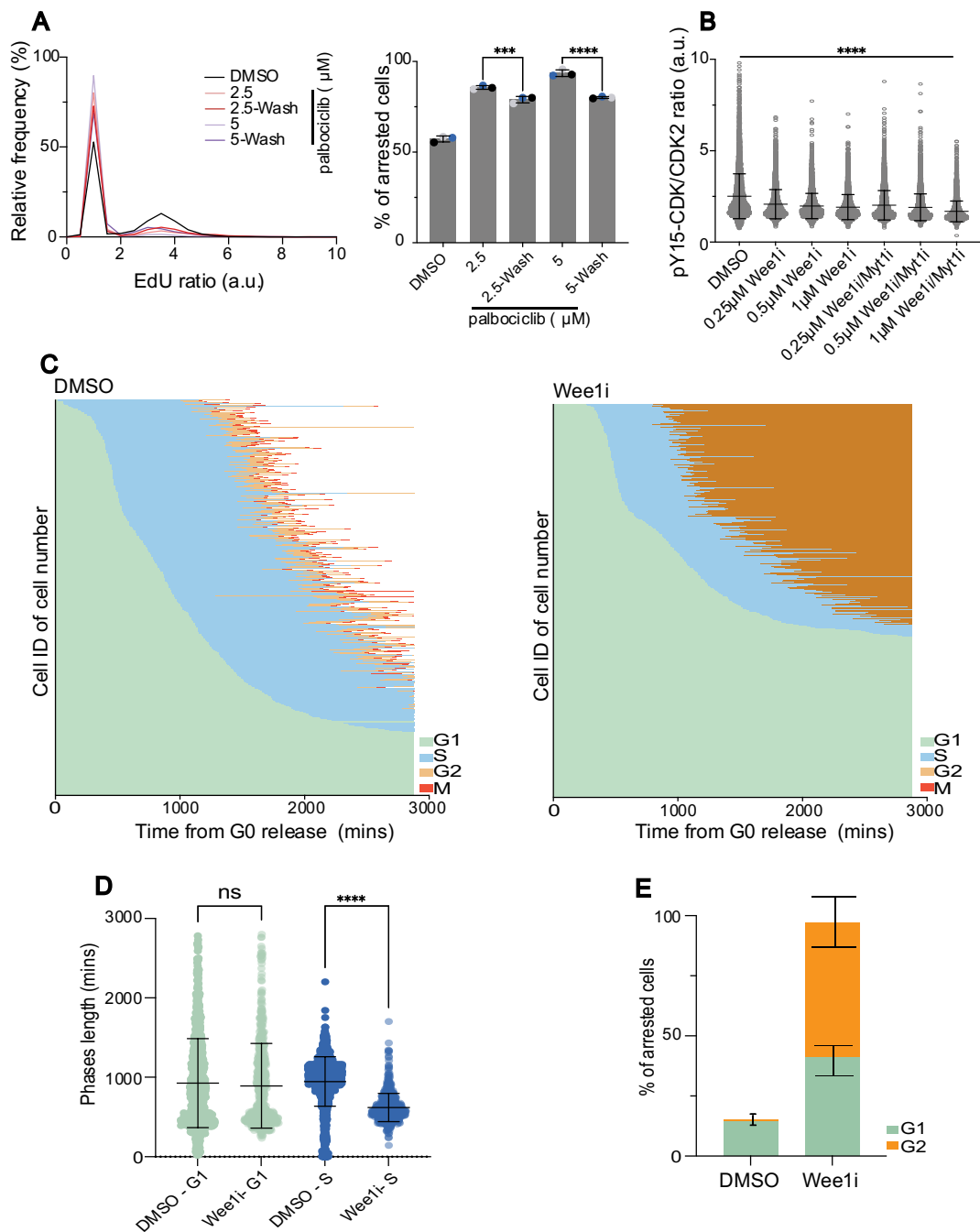


Figure 3-11. Wee1i perturbs cell cycle dynamics in breast cancer cell line.

(A) Quantification of the EdU ratio of MCF7 cells by immunocytochemistry. Cells were arrested using an increasing concentration of Palbociclib for 24h. Cells were pulse labelled for 1h after 23h of treatment with 10 μM EdU before fixation. EdU positive cells were quantified as cells with a nuclear:cytoplasmic ratio of EdU signal as greater than or equal to 1.2. Representative frequency distribution of measured intensities from one experimental repeat shown (left graph). Percentage of arrested cell in arrested and released population (right graph). Dots in plot represents replicate wells (3 wells per experiment). One way ANOVA analysis was performed to

calculate p-values (****= $p < 0.0001$, ***= $p < 0.001$). Mean $\pm$ SD are shown. **(B)** Quantification of the ratio phosphoY15-CDK2/CDK2 in MCF7 cells by immunocytochemistry. MCF7 cells were treated with DMSO, Wee1i or Wee1i/Myt1i at various concentrations for 2h. Single dot represents single cell data. One-way ANOVA analysis was performed to calculate p-values (****= $p < 0.0001$). Mean $\pm$ SD are shown. **(C)** Manually tracked MCF7 cells cell cycle progression. NCI-1944 were arrested in G0/G1 using 5 μ M Palbociclib for 24h and released for 48h with fresh media and DMSO, Wee1i or Wee1i/Myt1i. Each line represents one single cell (DMSO: n=978, Wee1i: n=983). Colours represents different phases of the cell cycle. **(D)** Quantification of phases length duration in MCF7 cells upon DMSO or Wee1i treatment. Single dots represent single cell data. One-way ANOVA analysis was performed to calculate p-values (****= $p < 0.0001$, ns=not significant). Mean $\pm$ SD are shown. **(E)** Percentage of MCF7 cells arrested in G1 and G2 (n=2). Mean $\pm$ SD are shown.

3.6 Summary

Previous studies have shown that inhibiting Wee1 during G2 accelerated mitosis entry and led to cell death due to mitotic catastrophe. In this chapter, I investigated the role of Wee1 during the G1/S transition. Previous study using CDK2 cell line where CDK2 cannot be phosphorylated on T14 and Y15 (CDK2AF), showed that constant CDK2 activation leads to an early S-phase entry and therefore a shorter G1 (Hughes et al., 2013a). Hence, in line with its known regulatory role on CDK inactivation and, I speculated that inhibiting Wee1 at cell cycle re-entry would lead to a quicker S-phase entry.

I was able to show that upon Wee1 inhibition, synchronised RPE1 cells did not rush into S-phase. Instead, the cells showed a delay in S-phase entry. In addition, I demonstrated that Wee1i-treated cells undergo an arrest in G0/G1 or G2 phase. These results point to a role for Wee1 during the G1/S transition, which is different from its role in G2. In order to account for potential off-target effects of Wee1i, I developed alternative approaches to perturb Wee1. These included the use of siRNAs targeting Wee1 and an auxin-inducible degron system. My results indicate that the effects of Wee1i upon the cell cycle re-entry were reproducible when downregulating Wee1 with siRNAs or the degron system. As seen before, S-phase entry was delayed, and a large number of cells arrested in G1. This shows that the Wee1i can be used as a reliable tool to inhibit Wee1 specifically and that the rate of off-target effects is minimal. Additionally, the observed delay in S-phase entry as well as the G0/G1 and G2 arrest is stronger upon Wee1 perturbations using siWee1 and the auxin-inducible degron system. The differing outcomes can be attributed to the distinct mechanisms of action employed by these perturbation methods. Wee1i specifically inhibits Wee1 molecules, but it's likely that not all molecules are targeted, potentially due to conformational changes or protein aggregations. In contrast, siRNA directly alters gene expression by degrading transcripts, leading to fewer Wee1 molecules within the cell. Previous studies have shown that siWee1 induces a more pronounced reduction in cell viability compared to Wee1i (Kim et al., 2015). Moreover, the strongest delay in S-phase entry and cell cycle arrest is observed when Wee1 is specifically degraded using the auxin-inducible degron system, resulting in cells predominantly arrested in G0/G1. Additionally, exploring the temporal dynamics of Wee1 inhibition reveals differences in cell cycle behaviour post-S-phase entry, suggesting potential

insights into cell cycle dynamics when Wee1 is specifically degraded. Overall, the variation in S-phase delay and G1/G0 arrest extent is attributed to the type of Wee1 perturbation employed, with the auxin-inducible degron system resulting in extremely low levels of Wee1 activity compared to siRNA and Wee1i, thus explaining its stronger phenotype. Further detailed explanations and analysis of these observed phenotypes will be provided in Section 7.8.

In my experiments, I was able to investigate how cells respond to different methods of arrest induction, notably CI and Palbociclib treatment.

I observed consistency in phenotypes between cells arrested through CI or Palbociclib upon Wee1 inhibition. However, I was able to demonstrate that, upon Wee1 inhibition, cells arrested with CI took a longer time to enter S-phase entry compared to Palbociclib arrested cells. This can be attributed to variations in the mechanisms underlying these two techniques. Whereas CI triggers growth arrest via membrane proteins and elevated p27 levels, preventing senescence by inhibiting the mTOR pathway, Palbociclib-mediated arrest operates independently of p21 or p27, with prolonged treatment leading to challenges in cell cycle re-entry due to increased p21 levels (Abercrombie, 1979; Leontieva et al., 2014; Pennycook & Barr, 2021; Serrano et al., 1996; Wieser et al., 1999).

Further detailed explanations and analysis of these observed phenotypes will be provided in Section 7.8.

Moreover, I showed that the timing of Wee1i treatment during G1 phase is pivotal for the effect on cell cycle dynamics. Inhibiting Wee1 during early G1 phase in synchronous population resulted in a delay in S-phase entry and an arrest in G1 in most of the cells but not when treated in late G1.

As a next step, I investigated whether the delay in S-phase entry and arrest in G1 or G2 only occurs in synchronised cells or if this is also the case in cycling cells. Despite the difficulties to monitor the first G1/S transition in an asynchronous population, I was able to observe that asynchronous cells treated with Wee1 inhibitors, also experience a delay in S-phase entry regardless from the time spent in G1 before drug addition. When testing the effect of Wee1i in an asynchronous cell population, I noticed that inhibiting Wee1 during early G1 does not lead to a G1 arrest, as observed when inhibiting Wee1 during cell cycle re-entry from G0, but that cells do still arrest in G2. However, when treating the cells in late G1, cells were more likely to progress through mitosis and arrest in the next G1 phase. These results indicate that

depending on the timing of Wee1 inhibition during G1 phase, the cells respond differently to Wee1i. The clear differences in the regulation of G1 in cells coming out of quiescence compared to continuously cycling cells rise interesting questions about how cells transition from a quiescent state to active proliferation and how this process is regulated differently in continuously cycling cells. It is possible that the heterogeneous cell cycle re-entry from quiescence serves two main purposes: ensuring cells to accumulate sufficient resources for a full cycle before exiting quiescence and regulating tissue replenishment (Coller et al., 2006; Everitts et al., 2013; Kieffer-Kwon et al., 2017; H.-J. Lee et al., 2017). Wee1 plays a crucial role in regulating these events, and its inhibition can disrupt S-phase entry. Changes in the expression of cell-cycle machinery, such as decreases in CDK inhibitors and increases in cyclins and CDKs, may contribute to the extended G1 phase upon quiescence exit. Understanding the regulatory mechanisms underlying cell proliferation from quiescence versus continuous cycling is essential for deciphering cellular proliferation. Recent research challenges the traditional concept of a G1 restriction point, suggesting that rapidly cycling cells can commit to the cycle in the G2 phase (Moser et al., 2018; Schwarz et al., 2018). The inherent molecular differences between synchronous and cycling cells likely account for the divergent phenotypic responses observed following Wee1 inhibition in these populations. Further detailed explanations and analysis of these observed phenotypes will be provided in Section 7.8.

Finally, I checked whether cells originating from different cancer cell types also show a delay in S-phase entry and a G1/G2 arrest upon Wee1i. Interestingly, I showed that the effects of Wee1i are cell line dependent. I revealed that the lung cancer cell lines, NCI-H1944 or A549 did not display any delay in S-phase entry or G1 or G2 arrest. Instead, they shown a tendency to progress through the cell cycle and die from mitotic catastrophe as described in (Do et al., 2015b; Hirai et al., 2009b; Schmidt et al., 2017). However, another cancer cell line from breast cancer, MCF7 displayed an arrest in G1 and G2 upon Wee1 inhibition at cell cycle re-entry, resembling the dynamics which were observed in RPE1 cells.

Employing three different cancer cell lines—two NSLC (A549, NCI-H1944) and one breast cancer (MCF7)—revealed variable responses. While MCF7 cells exhibited delays in S-phase entry and G0/G1 and G2 arrest compared to RPE1 cells, NCI-H1944 cells did not show such delays or increased arrest populations. However, across all cell lines, Wee1 inhibition during cell cycle re-entry resulted in a shortened

S phase, diminished G2 phase, and heightened mitotic cell death. Furthermore, treatment with dual Wee1/Myt1 inhibitors led to G0/G1 arrest and increased mitotic cell death. The differences observed among cell lines could stem from genetic and transcriptional heterogeneity, as shown in previous studies. Research by (Ben-David et al., 2018) highlighted significant transcriptomic variations across cancer cell lines, influencing their responses to anti-cancer drugs. Similarly, (Rødland et al., 2021) found divergent sensitivity to Wee1 inhibition among lung cancer cell lines, correlated with DNA damage levels induced by Wee1 inhibition. These findings align with observations in breast cancer cell lines by (Ghiasi et al., 2013) where responses to Wee1 inhibition varied, but dual Wee1/Myt1 inhibition increased treatment sensitivity. However, despite the variable responses, none of the cancer cell lines exhibited a shortened G1 phase upon Wee1 inhibition during G1, contrary to expectations. This suggests an unknown role of Wee1 during the G1 phase in cancer cell lines. Comparing cancer cell responses to Wee1 inhibition with those of healthy cells could unveil resistance mechanisms employed by cancer cells. Such insights could inform the optimisation of Wee1 therapies and their targeted application against cancer cells. Future studies should involve a broader range of cancer and normal cell lines, including those with p53 knockout status, to comprehensively understand Wee1 inhibition effects and potential therapeutic strategies. Further detailed explanations and analysis of these observed phenotypes will be provided in Section 7.8. Understanding the varied responses of the different cancer cell lines upon Wee1 inhibition during cell cycle re-entry holds promise for the development of innovative treatment approaches.

I have used PCNA as a readout for S-phase entry upon Wee1 inhibition. I realised that the emergence of the PCNA foci was perturbed upon Wee1i, compared to non-inhibited cells. This observation raises the question of whether the varied phenotypes observed are solely attributed to the disturbance in PCNA appearance and if they would be absent when using a different S-phase marker in alternative cell lines. To validate these phenotypes across different cell lines, I employed PIP-fucci cell lines, with PIP serving as an alternative marker for S-phase entry. Interestingly, while I observed G1 and G2 arrest upon Wee1 inhibition in this cell lines, the delay in S-phase entry seen in the RPE1 mRuby-PCNA cell line was absent. This discrepancy raises significant questions regarding the authenticity of the observed delay: is it a

genuine effect, or could it be due to limitations in accurately observing the G1/S transition using PCNA only?

It is noteworthy that the PIP cell line used in this experiments also exhibits overexpression of PCNA, which has been associated with a shorter G1 phase in previous studies (L. Leung et al., 2011). This underscores the critical importance of selecting appropriate cell lines for experiments. In future investigations, it will be crucial to utilise PIP-fucci cell lines with normal PCNA expression levels to confirm the potential delay in S-phase entry. Additionally, exploring other cell lines with different markers to distinguish S-phase entry, may be necessary for the comprehensive understanding of Wee1 inhibition on S-phase entry timing. Further detailed explanations and analysis of these observed phenotypes will be provided in Section 7.8.

Additionally, PCNA STDEV intensity also decreased during S-phase upon Wee1 inhibition potentially showing a perturbation of PCNA binding to the DNA. PCNA binding to DNA is a fundamental aspect of its function in various DNA-related processes (Dovrat et al., 2014; Strzalka & Ziemienowicz, 2011).

Other studies also described the important role of Wee1 to protect the genomic integrity by inactivating CDK2 (Hauge et al., 2019). For this reason, I speculated that inhibiting Wee1 at cell cycle re-entry interferes with DNA replication and could lead to one of my observed phenotypes described in this chapter which are S-phase entry delay, G1 and G2 arrest. This will be furthermore investigated in the next chapter.

Taken together, my results show that Wee1 plays a new role in G1/S transition. Upon Wee1 inhibition S-phase entry is delayed and the cells arrest in G1/G2. Interestingly, this effect is cell-line dependent, as numerous cancer types do not reveal the delay in S-phase entry, and the arrest in G0/G1 and G2 upon Wee1i. These mechanisms might give insights into potential resistance mechanisms emerging upon Wee1i.

Chapter 4. G2 arrest upon Wee1 inhibition during cell cycle re-entry is p21-dependent.

4.1 Introduction.

To guarantee equitable transmission of genetic material to offspring cells, the initiation of replication, is tightly regulated throughout the cell cycle (Bell & Dutta, 2002; Kunkel & Bebenek, 2000). The process of DNA replication is constantly challenged by intrinsic and extrinsic stresses throughout the genome (Técher et al., 2017; Zeman & Cimprich, 2014). Stresses interfering with DNA replication and posing a threat to genomic stability are termed replication stress (Lengauer et al., 1997; Negrini et al., 2010; Ubhi & Brown, 2019; Vogelstein et al., 2013). Replication stress is a recognised characteristic of cancer and is often triggered by oncogenic activities (Gaillard et al., 2015; Macheret & Halazonetis, 2015).

CDKs coordinate cell cycle checkpoints with DNA repair mechanisms that together maintain genome stability and promotes loading and activation of the replisome components (See introduction)(Neganova et al., 2011; Reuswig & Pfander, 2019; Zhu, 2004). In fact, origin licensing only occurs when cells experience low CDK activities (Diffley, 2004). Coherently, premature activation of the CDK2-Cylin E complex was shown to promote oncogenesis by impairing DNA replication, causing DNA replication stress (Koboldt et al., 2012). In addition, hyperactivation of CDK2-cyclin E complex directly interferes with DNA replication, leading to DSBs and genomic instability (Müller-Tidow et al., 2004). More recently it has been proposed that specific targeting of oncogenic CDK2-cyclin E might represent promising approach for new cancer therapies (Suski et al., 2021; Tadesse et al., 2020).

Wee1 is known to play an important role in protecting genomic integrity, as it inhibits CDKs, allowing replication to occur in the right conditions (Elbæk et al., 2022). Surprisingly, in my last chapter I described how Wee1 inhibition during cell cycle re-entry leads to a delay in S-phase entry and an arrest in G0/G1 and G2. To better understand the molecular mechanisms behind these phenotypes, I investigated whether Wee1 inhibition dysregulates DNA replication dynamics. It was previously described that loss of Wee1 leads to a CDK-driven increase in the initiation of replication and a subsequent disruption of the replication forks, leading to DNA damage (Beck et al.,

2012; Elbæk et al., 2022). In line with this, in the previous chapter, I noticed that in cells treated with Wee1i, the PCNA STDEV intensity signal was decreased, compared to DMSO treated cells. As PCNA intensity and localisation is used as a S-phase proxy in my experiments, this indicated that replication in the presence of Wee1i is perturbed. Hence, I hypothesised that the perturbation of the PCNA intensity is a direct readout of the increase in replication stress in Wee1i treated cells.

In this chapter I investigated how inhibition of Wee1 during cell cycle re-entry could affect DNA replication dynamics. I used a combination of bulk analysis and single cell live imaging to understand the molecular mechanisms causing a delay in S-phase entry and an arrest in G0/G1 and G2 upon Wee1 inhibition (Chapter 3).

First, I speculated that upon Wee1 inhibition, CDK2 activity could be perturbed. To test this, I first used single cell live imaging to follow CDK2 activity upon Wee1i at cell cycle re-entry. Then, I speculated that Wee1 inhibition during cell cycle re-entry leads to an increase in DNA damage. I carried out flow cytometry to monitor DNA distribution in cells upon Wee1i treatment. Moreover, I also used immunostaining to quantify DNA damage upon Wee1 inhibition. Finally, I used single cell live imaging to monitored proteins such as p21 and Cdc6. p21 is important in DNA damage response pathway and Cdc6 is essential for a “normal” progression through S-phase.

In this chapter I dissected whether the arrest in G0/G1, G2 and S-phase entry delay upon Wee1 inhibition at cell cycle re-entry occurs through a dysregulation of CDK2 activity and an accumulation of DNA damages.

4.2 G2 arrest upon Wee1 inhibition is p21 dependent.

Wee1 has been reported to inhibit CDK2 activity (Gu et al., 1992; Jeffrey et al., 1995; C. L. Wu et al., 2001). To test whether the S-phase entry delay or the G0/G1 and G2 arrest are due to a perturbation in CDK2 activity, I made use of a CDK2 activity sensor (Barr et al., 2016). This sensor allows us to monitor CDK2 activity upon Wee1 inhibition.

I arrested RPE1 mRuby-PCNA CDK2-PSLD-GFP cells in G0/G1 using 1 μ M Palbociclib for 24h and released with serum addition and either DMSO or Wee1i for 48h and followed CDK2 by timelapse imaging. Cells treated with Wee1i showed a higher level of CDK2 activation during G1 compared to DMSO but also a higher level of CDK2 activation at S-phase entry and across S-phase (Fig. 4.1.A). Moreover, using this cell line as oppose as the one carrying only mRuby-PCNA reporter, no significant difference was observed in the timing of S-phase entry upon Wee1 inhibition. Cells entered S-phase at 6.1h (+/- 93mins) upon Wee1 inhibition compared to 5.9h (+/- 104 mins) in DMSO treated cells (Fig. 4.1.B). Despite the lack of delay in S-phase entry upon Wee1 inhibition, I noticed an increase in the fraction of cells arrested in G0/G1 and in G2 in Wee1i treated cells. In DMSO 13% of cells were arrested in G0/G1 and 1% in G2 compared to Wee1i treated cells whereas 24% cells were arrested in G0/G1 and 43% in G2 (Fig. 4.1.C). These data indicates that the delay observed in S-phase entry and the arrest in G0/G1 upon Wee1 inhibition is p21 dependent. However, the absence of G2 arrested population upon Wee1 inhibition in p21 KO cell line indicate that this phenotype is p21 independent.

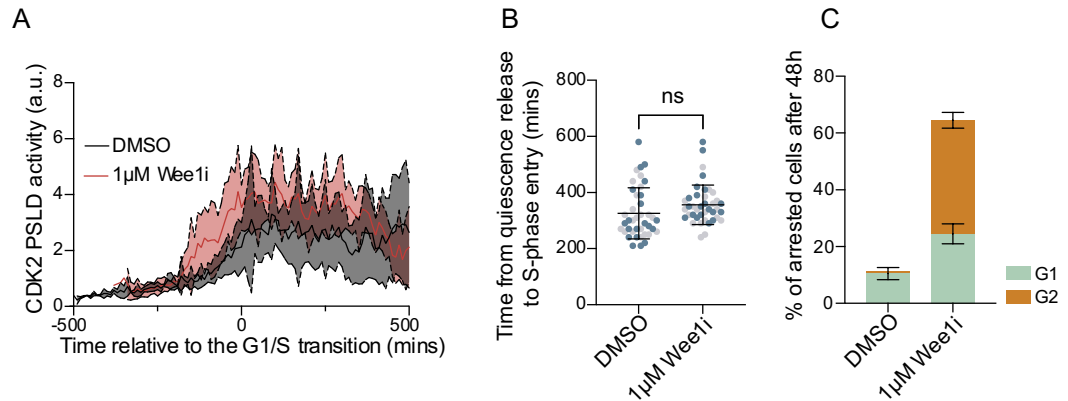


Figure 4-1. CDK2 activity increases during G1-S upon Wee1 inhibition.

(A) Quantification of CDK2 activity at the G1/S transition using live imaging data. RPE1 mRuby-PCNA CDK2-PSLD-GFP cells were arrested in G0/G1 using 1 μ M Pal-bociclib for 24h and released with serum addition and either DMSO or Wee1i for 48h. Mean intensity $\pm$ SD are shown (DMSO: $n=20$, Wee1i: $n=20$). **(B)** Timing of S-phase entry of manually tracked RPE1 mRuby-PCNA CDK2-PSLD-GFP cells. Live imaging data from (A) was used. Single dots represents single cell data with mean $\pm$ SD shown. Each colour represents one replicate ($n=2$). **(C)** Percentage of RPE1 mRuby-PCNA CDK2-PSLD-GFP arrested in G0/G1 and G2 upon Wee1 inhibition ($n=2$). Live imaging data from (A) was used.

4.3 Wee1 inhibition during cell cycle re-entry leads to an increase in replicative stress and DNA damage.

In chapter 3, I observed that upon Wee1 inhibition the fraction of cells entering S - phase displayed two different outcomes. A fraction of cells progressed through mitosis whereas another fraction of cells arrested in a G2 like state (see Chapter 3: Fig. 3.8.B). Additionally, cells that arrested in G2 had a shorter S-phase compared to DMSO treated cells (see Chapter 3: Fig. 3.7.A).

Since Wee1 is known to play a role in preventing the degradation of newly synthesised DNA at stalled replication forks (Elbæk et al., 2022), I hypothesised that cells arrested in a G2-like state did not properly replicate their DNA preventing them from enter mitosis whereas cells that were able to enter mitosis are those with properly replicated DNA. To study this, I analysed the DNA content of Wee1i treated cells using Flow cytometry. Using my data from (Chapter 3: Fig. 3.7.A, B), I knew that after 24h post-release the vast majority of my cells (treated with DMSO and Wee1i) already progressed through S and G2. To facilitate my experiments, I decided that releasing my cells for 24h instead of 48h would be sufficient to test my hypothesis. I carried out flow cytometry on Wee1 inhibited cells and control cells which were stained with propidium iodide (PI). This dye allows to stain the DNA content within the cell, revealing whether the cells experience perturbed replication dynamics.

I arrested RPE1 mRuby-PCNA cells in G0/G1 using 1 μ M Palbociclib for 24h and released with serum addition and either DMSO or Wee1i for 24h before fixation. Interestingly, cells that initiate S-phase in Wee1i treated cells display an abnormal DNA distribution compared to DMSO treated cells. Cells treated with DMSO display a clear population of 2N and 4N with a fraction of cells in G0/G1 at the time of the fixation (2N, G1) and a fraction of cells who completed DNA replication at the time of fixation (4N, G2). Cells between 2N and 4N in represent the fraction of cells that did not yet finish DNA replication. Upon Wee1i treatment the distribution of these 2N and 4N populations was disrupted. A fraction of cell was not able to progress through S and did not correctly replicate their DNA (Fig. 4.2.A). This suggests a perturbation on DNA replication upon Wee1 inhibition.

Since replication stress is correlated with DNA damage, I used immunostaining to monitor DNA damage upon Wee1 inhibition at cell cycle re-entry (Negrini et al., 2010b; Zeman & Cimprich, 2014). To quantify DNA damage, I monitored the level of

two known marker γ H2AX and p53 binding protein 1 (53BP1). γ H2AX takes place at the double strand break (DSB) site shortly after its formation, disappears at its resolution and is microscopically visible as discrete foci after antibody labelling (Paull et al., 2000; Rogakou et al., 1998). As for 53BP1, it localises rapidly to discrete foci within the nucleus of cells exposed to DNA DSB-inducing agents (Schultz et al., 2000). These foci represent sites of DSBs.

In the literature 53BP1 is often quantified as number of foci per nucleus whereas measurement of global γ H2AX intensity as a readout for DNA damage is preferred (Djuzenova et al., 2015). I arrested RPE1 mRuby-PCNA cells in G0/G1 using 1 μ M Palbociclib for 24h and released with serum addition and either DMSO or Wee1i for 24h before fixation. I observed an increase in γ H2AX and 53BP1 upon Wee1 inhibition compared to DMSO (Fig. 4.2.B).

Together, these data show that cells treated with Wee1i at cell cycle re-entry exhibit perturbation in DNA replication and an increase in DNA damage.

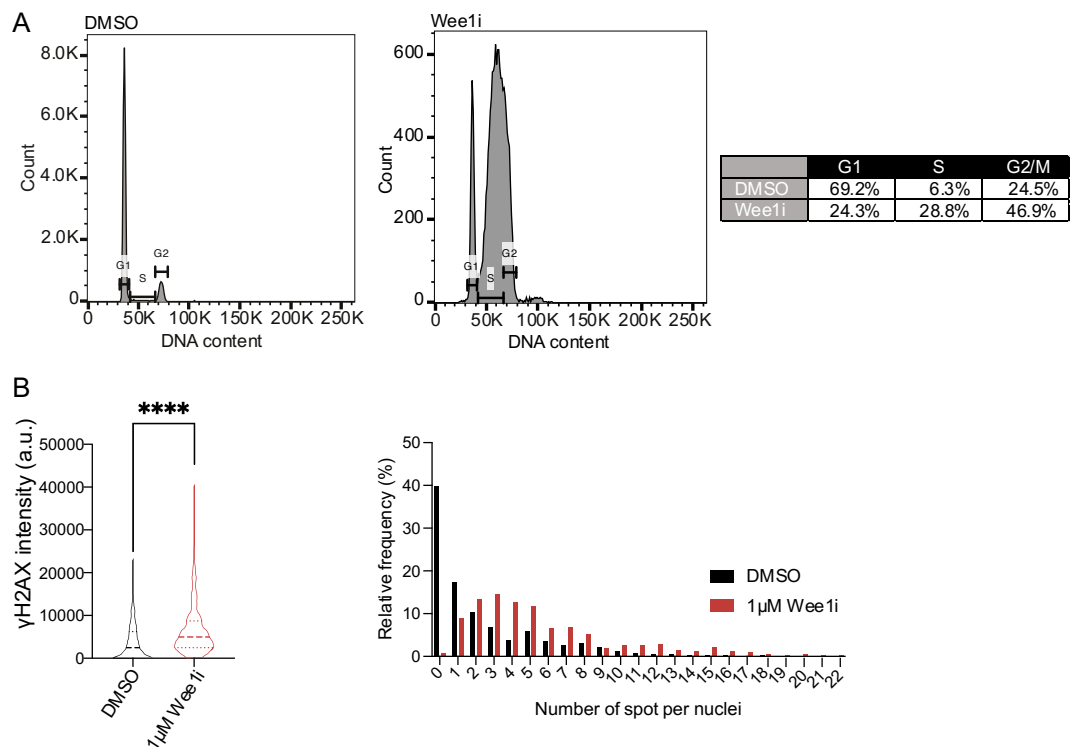


Figure 4-2. DNA replication perturbation and DNA damage upon Wee1 inhibition.

(A) DNA content analysis upon Wee1 Inhibition by flow cytometry. RPE1 mRuby-PCNA cells were arrested in G0/G1 using 1μM Palbociclib for 24h and released with serum addition and either DMSO or Wee1i for 24h. **(B)** Quantification of DNA damage upon Wee1 inhibition by immunostaining. RPE1 mRuby-PCNA cells were arrested in G0/G1 using 1μM Palbociclib for 24h and released with serum addition and either DMSO or Wee1i for 24h before fixation (left graph=γH2AX, right graph=53BP1). Unpaired t-test analysis was performed to calculate p-values (ns=not significant).

4.4 G2 arrest upon Wee1 inhibition is p21 dependent.

A high level of replication stress is often correlated with a high level of p21 (Barr et al., 2017; Galanos et al., 2016; Mansilla et al., 2020; Yoon et al., 2012). p21 can be upregulated in response to various forms of DNA damage, including replication stalling, DSBs or other lesions in the DNA. When cells experience severe level of replication stress or DNA damage, p53 can activate p21 and cause cells to exit the cell cycle before reaching mitosis in a p21 dependent manner (Deng et al., 1995; El-Deiry et al., 1993; El-Deiry et al., 1994; Warfel & El-Deiry, 2013). I speculated that upon Wee1 inhibition, cells exhibits high level of replication stress leading to an increase of p21 and an arrest in G0/G1 or G2. First, to test if Wee1 inhibition leads to higher p21 level, I used a cell line expressing p21-GFP (RPE1 mRuby-PCNA p21-GFP) (See material and method) (Barr et al., 2017).

I arrested RPE1 mRuby-PCNA p21GFP cells in G0/G1 using 1 μ M Palbociclib for 24h and released with serum addition and either DMSO or Wee1i for 48h and followed p21-GFP by timelapse imaging. In DMSO treated cells, p21-GFP displayed the expected heterogeneous expression during G1, followed by a sharp decrease in levels at the G1/S transition. During S-phase, p21-GFP levels remained low, followed by a gradual increase after exiting S-phase (see Materials and Methods).

Wee1i-treated cells have an increase in p21 at S-phase entry and throughout S-phase with the highest level of p21 in G2 (Fig. 4.3.A, B).

I then hypothesised that cells entering mitosis upon Wee1 inhibition would be cells with low level of p21 and cells arrested in G2 would be cells with higher level of p21. Using live imaging data from (Fig. 4.2.A, B), I compared p21 level across G1/S/G2 of cells entering mitosis or arrested in G2 upon Wee1 inhibition, I could not see a difference in p21 level in these two populations. However, it appears to be a noticeable trend that cells entering mitosis upon Wee1 inhibition have a slightly lower p21 level across S and G2 compared to cells arrested in G2.

To further test the hypothesis that p21 increases upon Wee1 inhibition, I used immunostaining at S-phase to quantify p21 levels upon Wee1 inhibition. Using live imaging data from (Fig. 4.2.A, B), I was able to approximatively time S-phase entry and thus determine at what time I needed to fix my cells to capture S-phase cells. I arrested RPE1 mRuby-PCNA cells in G0/G1 using 1 μ M Palbociclib for 24h and

released with serum addition and either DMSO or Wee1i for 10h. Cells were pulse labelled with 10 μ M EdU for 30 mins before fixation to quantify number of S-phase cells.

I observed that Edu positive cells that had entered S-phase had a higher p21 level in Wee1i treated cells compared to DMSO treated cells (Fig. 4.3.D).

To test if p21 not only increases in G2 but if it is required for the G2 arrest observed in my experiments, I used a RPE1 mRuby-PCNA p21KO cell line, I hypothesised that if Wee1 inhibition leads to p21 increase during S and G2, which ultimately lead to an exit of the cell cycle before mitosis, then using a cell line where p21 is KO, should rescue a normal G2 population (Barr et al., 2017) (See material and method). I arrested RPE1 mRuby-PCNA (p21WT), RPE1 mRuby-PCNA p21KO cells in G0/G1 using 1 μ M Palbociclib for 24h and released with serum addition and either DMSO or Wee1i for 48h and followed mRuby-PCNA by timelapse imaging. I noticed a delay in S-phase entry in the RPE1 mRuby-PCNA (p21WT) cells upon Wee1 inhibition. Cells entered S-phase around 12.5h (+/- 127 mins) with Wee1i, compared to 10h (+/- 89 mins) upon DMSO. In RPE1 mRuby-PCNA p21KO cells the same delay could be observed in Wee1i treated cells. Cells were entering S-phase approximately 10.3h (+/- 115 mins) upon Wee1 inhibition compared to 9.1h (+/- 101 mins) for DMSO treated cells (Fig. 4.3.E).

In RPE1 mRuby-PCNA (p21WT) cells, as previously described in my last chapter, Wee1i treated cells have a large fraction of cells arrested in G2. In the absence of p21, Wee1i treated cells did not arrest in G2 but rather progress to mitosis and arrest in the subsequent G1 or underwent cell death in G2 (Fig. 4.3.F). 26% of cells were arrested in G2 in p21WT cells compared to 3% in p21KO cells upon Wee1 inhibition. It is important to highlight that the fraction of arrested cells in G0/G1 remain the same upon Wee1 inhibition in p21WT and p21KO cells (Fig. 4.3.F). In p21-WT, upon Wee1 inhibition, 21% of cells remained arrested in G0/G1 compared to 23% in p21KO cells. Together these data suggest that the observed S-phase entry delay and G0/G1 arrest upon Wee1 inhibition is p21 independent whereas the G2 arrest is p21 dependent.

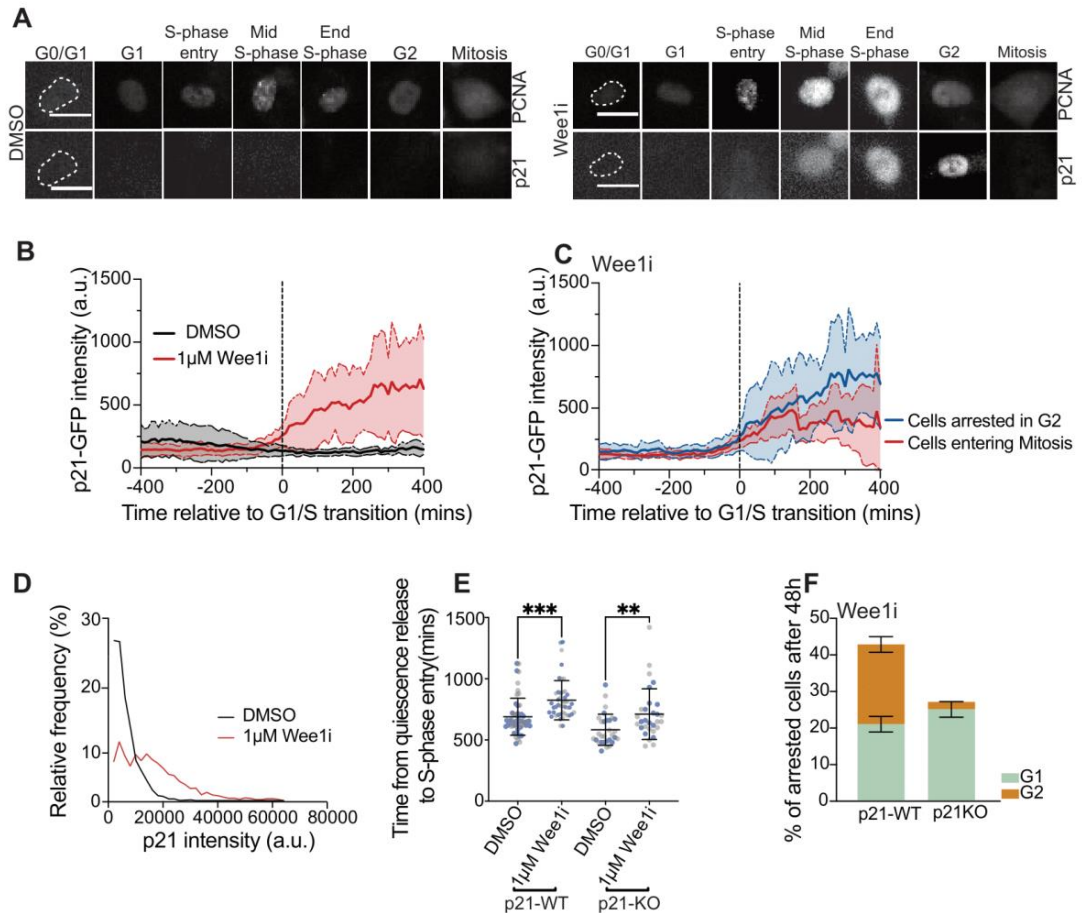


Figure 4-3. Wee1 inhibition in G0/G1 leads to an increase in p21 and a p21-dependent G2 arrest.

(A) Images of RPE1 mRuby-PCNA p21-GFP cells after Wee1 inhibition. RPE1 mRuby-PCNA p21-GFP cells were arrested in G0/G1 using 1 μ M Palbociclib for 24h and released with serum addition and either DMSO or Wee1i for 48h. Top panel shows PCNA intensity and localisation from a single cell at different times after cell cycle re-entry. Bottom panel show p21-GFP activity from the same cell. Phases were distinguish using PCNA sensor. Scale bar=20 μ m. **(B)** Quantification of p21-GFP intensity at the G1/S transition using live imaging data. RPE1 mRuby-PCNA p21-GFP cells were arrested in G0/G1 using 1 μ M Palbociclib for 24h and released with serum addition and either DMSO or Wee1i for 48h. Mean intensity $\pm$ SD are shown (DMSO: n=20, Wee1i: n=20). **(C)** Quantification of p21-GFP intensity at the G1/S transition upon Wee1 inhibition. RPE1 mRuby-PCNA p21-GFP cells were arrested in G0/G1 using 1 μ M Palbociclib for 24h and released with serum addition and DMSO or Wee1i for 48h. Mean intensity plus SD are shown. Cells arrested in G2 in blue, cells entering mitosis in red (Cells arrested in G2: n=10 Cells entering mitosis: n=8). **(D)** Representative frequency distribution of p21 intensity at S-phase entry by immunostaining. RPE1 mRuby-PCNA cells were arrested in G0/G1 using 1 μ M Palbociclib for 24h and released with serum addition and either DMSO or Wee1i for 10h before fixation. Cells were pulse labelled for 30 mins with 10 μ M EdU before fixation. EdU positive cells were quantified as cells with a nuclear:cytoplasmic ratio of EdU

signal as greater than or equal to 1.2. **(E)** Timing of S-phase entry of manually tracked p21WT and p21KO cells upon Wee1 inhibition. RPE1 mRuby-PCNA and RPE1 mRuby-PCNA p21KO cells were arrested using 1 μ M Palbociclib for 24h and released with serum addition and either DMSO or Wee1i for 48h. Single dots represents single cell data with mean and SD shown. Each colour represents one biological replicate (n=2). One way ANOVA analysis was performed to calculate p-values (****=p<0.0001, **=p<0.01). **(F)** Percentage of p21WT and p21KO cells arrested in G1 and G2 after 48h, treated with Wee1i (n=2). Live imaging data from (E) was used.

4.5 Wee1 inhibition during cell cycle re-entry perturbs replication origin licensing in G1.

It was previously shown that CDK2-cyclin E is required for accumulation of Cdc6, which is necessary for MCM loading (Chuang et al., 2009). Furthermore CDK2-cyclin E is also able to directly interact with the pre-RC, ORC1 and Cdc6 to promote the pre-RC assembly (Coverley et al., 2002; Furstenthal et al., 2001; Hossain et al., 2021; Hossain & Stillman, 2016). I speculated that the perturbation in DNA replication upon Wee1 inhibition might be due to a role of Wee1 in the formation of the pre-RC. Cdc6 plays a crucial role in facilitating MCM loading, and its accumulation represents one of the critical steps in the limited timeframe for MCM loading during the G1 phase. This timeframe concludes when the S phase commences. Consequently, the duration for which Cdc6 remains detectable in cell nuclei serves as an indicator of the available period for MCM loading during the G1 phase (Petersen et al., 1999; Tovar et al., 2006). The role of MCM is to prime chromatin for DNA replication by binding origins of DNA replication during the late M to early G1 phase of the cell cycle. Activated by S phase promoting protein kinases, the origin-bound MCM complexes unwind the double stranded DNA at the origins, recruit DNA polymerases and initiate DNA synthesis (Tye, 1999).

To investigate on this, I used live imaging using a Cdc6 sensor cell line and immunostaining to monitor MCM binding upon Wee1 inhibition (Matson et al., 2019). As explained in the beginning of this chapter, DNA origins are typically licensed during phases of low CDK activity. Wee1 inhibition disrupts DNA replication and increases CDK2 activity. I hypothesised that Wee1 inhibition during cell cycle re-entry could cause the window of time available for licensing DNA origins to be perturbed due to the high CDK2 activity and the observed delay in S-phase entry. To investigate this, I monitored the nuclear accumulation of Cdc6 following Wee1 inhibition. To test this, I used a Cdc6 sensor cell line (RPE1 Cdc6 mVenus PCNA mTurquoise2 DHB mCherry) to quantify the timing of licensing window (see materials and methods) (Mei et al., 2022). This cell line also allowed me to visualise CDK2 activity across my experiments. The CDK2 sensor in this cell line is different from the one used in the first figure of this chapter. In this cell line the sensor used is CDK2 - DHB from (Spencer et al., 2013). RPE1 Cdc6 mVenus PCNA mTurquoise2 DHB

mCherry cells were arrested in G0/G1 using 1 μ M Palbociclib for 24h and released with serum addition and either DMSO or Wee1i for 48h and followed by time lapse imaging. In this experiment, cells treated with Wee1i did not display any delay in S-phase entry (Fig. 4.4.A, C). Wee1 inhibited cells entered S-phase around 6.5h (+/- 111 mins) compared to 6.6h (+/- 84 min) for DMSO treated cells. However, cells entering S-phase upon Wee1 inhibition had a lower peak of Cdc6 intensity compared to DMSO treated cells (Fig. 4.4.A, B). The length of time Cdc6 was present in G1 is the time between nuclear Cdc6 rise and peak and represents the maximum “licensing window” for MCM loading in G1 (see Material and Methods). I observed a significant decrease in the licensing window upon Wee1 inhibition. DMSO treated cells have a licensing window of about 5h compared to Wee1i treated cells with a licensing window of 3h. Due to the possibility of following CDK2 activity in this cell line, I also quantified CDK2 activity with a distinct CDK2 sensor upon Wee1 inhibition (using live imaging data from Fig. 4.4.A). Wee1 inhibition leads to an increase in CDK2 activity during G1 and S-phase compared to DMSO treated cells (Fig. 4.4.E). Moreover, cells treated with Wee1i reached a significantly higher CDK2 activity at the time of peak Cdc6 and S-phase entry (Fig. 4.4.G). To continue exploring the potential origin licensing problem upon Wee1 inhibition, I used immunostaining to monitor MCM7 binding at different times after cell cycle re-entry upon Wee1i treatment to quantify chromatin bound MCM7. Using live imaging data from (Fig.4. A), I was able to determine that origin licensing occurs in the first 6h of my experiments. I arrested RPE1 mRuby-PCNA cells in G0 /G1 using 1 μ M Palbociclib for 24h and released with serum addition and either DMSO or Wee1i every 2h for 6h before fixation. I observed that at both 4h and 6h post-release, Cells treated with Wee1i had a significantly lower level of chromatin-bound MCM7 in G1 compared to DMSO-treated cells (Fig. 4.4.G). Together these data show a disruption of origin licensing upon Wee1 inhibitor at cell cycle re-entry and a decrease in MCM7 chromatin binding. This suggests that Wee1 is needed during G1 for normal origin licensing mechanism.

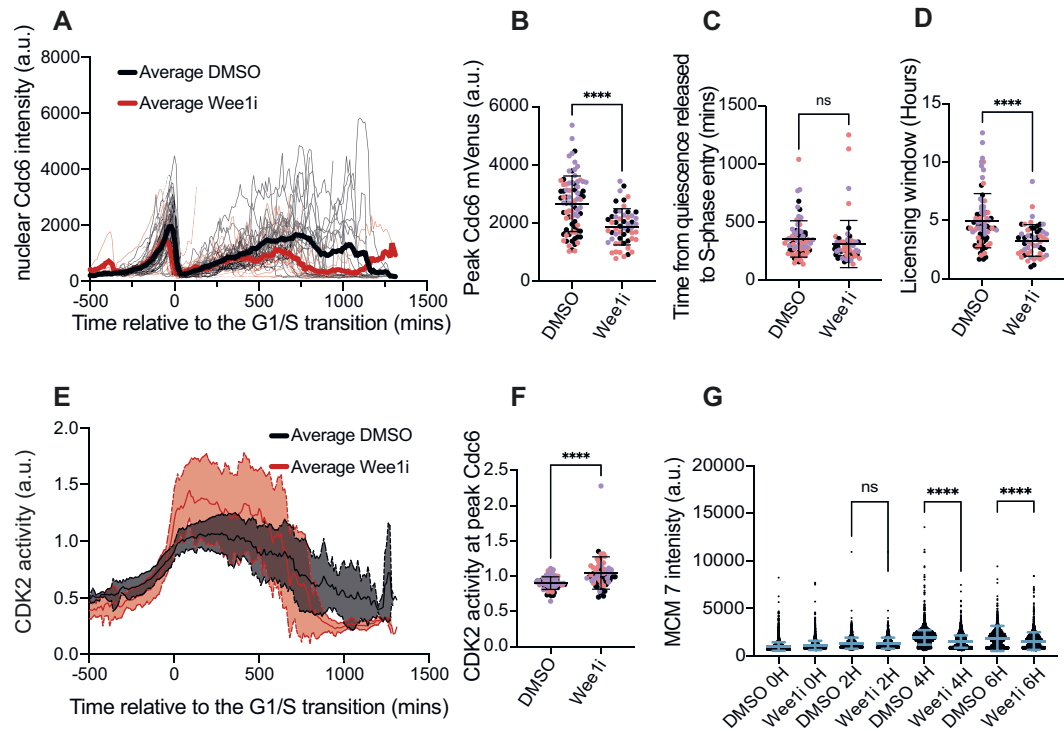


Figure 4-4. Wee1 inhibition perturbs DNA origin licensing.

(A) Quantification of Cdc6 intensity around the G1/S transition upon Wee1 inhibition. RPE1 Cdc6 mVenus PCNA mTurquoise2 DHB mCherry cells were arrested in G0/G1 using 1 μ M Palbociclib for 24h and released with serum addition and DMSO or Wee1i for 48h. Mean intensity plus SD are shown (DMSO n=20, Wee1i n=20). **(B)** Peak Cdc6 intensity upon Wee1 inhibition. RPE1 Cdc6 mVenus PCNA mTurquoise2 DHB mCherry cells were arrested in G0/G1 using 1 μ M Palbociclib for 24h and released with serum addition and DMSO or Wee1i for 48h. Single dots represents single cell data with mean and SD shown. Each colour represents one biological replicate (n=3). Unpaired T-test analysis was performed to calculate p-values (****=p<0.0001). **(C)** Timing of S-phase entry of manually tracked RPE1 Cdc6 mVenus PCNA mTurquoise2 DHB mCherry cells for cells imaged in (B). Each colour represents one biological replicate (n=3). Unpaired T-test analysis was performed to calculate p-values (ns=not significant). **(D)** Quantification of licensing window times (Cdc6 peak time minus Cdc6 rise time) for cells imaged in (B). Each colour represents one biological replicate (n=3). Unpaired T-test analysis was performed to calculate p-values (****=p<0.0001). **(E)** Quantification of CDK2 activity at the G1/S transition upon Wee1 inhibition from cells imaged in (A). Mean intensity plus SD shown (DMSO n=20, Wee1i n=20). **(F)** CDK2 activity at peak Cdc6 intensity in RPE1 Cdc6 mVenus PCNA mTurquoise2 DHB mCherry cells for cells imaged in (B). Each colour represents one biological replicate (n=3). Unpaired T-test analysis was performed to calculate p-values (****=p<0.0001). **(G)** Quantification of the chromatin-bound MCM7 upon Wee1 inhibition by immunostaining. RPE1 mRuby-PCNA cells were arrested in G0/G1 using 1 μ M Palbociclib for 24h and released every 2h for 6h with serum addition and either DMSO or Wee1i before fixation. Single dots represent single cell data with

mean and SD shown. One way ANOVA analysis was performed to calculate p-values (ns=not significant, ***= $p<0.0001$).

4.6 Summary.

Faithful DNA replication is critical for the maintenance of genomic integrity. Although DNA replication machinery is highly accurate, the process of DNA replication is constantly challenged by DNA damage and other intrinsic and extrinsic stresses throughout the genome. In the previous chapter, I observed three surprising phenotypes upon Wee1 inhibition at cell cycle re-entry. First, cells treated with Wee1i had a delay in S-phase entry. Second, abnormal PCNA emergence, which might be an indication that DNA replication is perturbed upon Wee1 inhibition. Lastly, a large fraction of cells is exiting the cell cycle before S-phase or before mitosis, by arresting in G0/G1 or G2.

In this chapter, I investigated the molecular mechanism behind the S-phase entry delay and G0/G1-G2 arrest upon Wee1 inhibition.

Firstly, due to the regulatory role of Wee1 on CDKs, I anticipated that the delayed S-phase entry in Wee1i treated cells is due to a dysregulated activation of CDK2 at the G1/S transition. I observed that cells treated with Wee1i had a higher level of CDK2 activity during G1, a higher level of CDK2 activity at the G1/S transition and sustained higher CDK2 activity during S-phase.

CDK2 regulates important facets of DNA replication and that excessive CDK2 activity during S-phase can be a source of replication stress (Elbæk et al., 2022; Reusswig & Pfander, 2019). Thus, I hypothesised that upon Wee1 inhibition at cell cycle re-entry, replication perturbation would appear. I was able to see that the duplication of the genetic material was perturbed upon Wee1 inhibition.

As a next step, I carried out immunostaining on γ H2AX and 51BP1. Wee1 was previously shown to have a role in protecting the genomic integrity of cells (Elbæk et al., 2022). Indeed, I was able to monitor that when upon Wee1i treatment, cells showed increased levels of γ H2AX and 53BP1. This confirms that Wee1i leads to higher levels of DNA damage within the cells.

Next, I checked whether the increase in replicative stress leads to an increase of p21 levels. As described before in (Barr et al., 2017), DNA damage during S-phase causes p53-dependent accumulation of p21 during G2 and can mediate cell cycle arrest through CDKs inactivation. I observed that the delay in S-phase entry remains persistent in both p21WT and p21KO cell lines upon Wee1 inhibition and thus p21-

independent. Interestingly, I showed that the G2 arrest upon Wee1 inhibition is p21-dependent. However, the fraction of cells arrested in G0/G1 did not change upon Wee1 inhibition. Thus, this shows that the G2 arrest is p21-dependent whereas both the delay in S-phase entry and the arrest in G0/G1 are p21-independent. I also show a surprising phenotype where I monitored an increase of p21 during S-phase. Lastly, I checked whether the increase in replication stress and DNA damage might be due to perturbed origin licensing. I speculated that hyper-activity of CDK2 upon Wee1 inhibition could lead to a decrease in licensing window. I showed that upon Wee1 inhibition, the cells had a decrease in origin licensing timing and a decrease in MCM7 binding.

The persistent delay in S-phase entry despite unaffected or increased CDK2-cyclin E activity following Wee1 inhibition raises intriguing questions. Studies, from (Koboldt et al., 2012) and (Elbæk et al., 2022) shed light on the intricate interplay between CDK2, Wee1, and DNA replication. Wee1 inhibition leads to hyper-activation of CDK2, resulting in excessive DNA2 activity and nucleolytic degradation, potentially disrupting DNA replication. Furthermore, observations from Chapter 4 revealed disturbances in PCNA foci appearance and localisation, suggesting a broader issue with DNA replication under Wee1 inhibition.

Drawing on findings from (Barr et al., 2017) which linked elevated DNA damage levels to p21 expression and CDK2 inhibition-induced G2 arrest, I hypothesised that Wee1 inhibition may induce aberrant DNA replication, activating p21 downstream of p53 and leading to G2 arrest.

Interestingly, while literature typically describes p21 protein levels as suppressed during S-phase by the PCNA-coupled CRL4Cdt2 degradation pathway, my findings indicated otherwise. To explore this discrepancy, I proposed a strategy involving Wee1 and CDK1 inhibition while monitoring p21 levels. Unfortunately, initial experiments combining Wee1 and CDK1 inhibition exhibited signs of cell distress, hindering further analysis. Nonetheless, further experiments with CDK1 inhibitor titration could elucidate the relationship between CDK1 activity, CRL4-Cdt2 degradation, and p21 accumulation during S-phase under Wee1 inhibition. Further detailed explanations and analysis of these observed phenotypes will be provided in Section 7.2.

Taken together, I was able to unravel the molecular mechanisms driving the G2 arrest upon Wee1 inhibition. I was able to show that Wee1i treated cells experience increased replicative perturbation and DNA damage, caused by a decrease in origin licensing. This is likely to increase p21 levels, which explain the G2 arrest upon Wee1 inhibition.

Considering the concept of dormant origins (see 1.8.3), it is plausible that cells activate these dormant origins as a compensatory response to Wee1 inhibition-induced replication stress. Activation of dormant origins may facilitate DNA replication progression under challenging conditions upon Wee1 inhibition, and thus allowing cells to progress through S and G2 and allow a small percentage of cells to enter mitosis.

Chapter 5. Wee1 inhibition during cell cycle re-entry leads to APC/C^{Cdh1} hyperactivation causing S-phase entry delay.

5.1 Introduction.

In this chapter my focus shifts to unravelling the mechanisms underlying the delay in S-phase entry and the arrest in G0/G1. Initially, considering the known role of Wee1 in regulating cell size in yeast, I explored the possibility of a similar influence in my context (Gould & Nurse, 1989; Nurse et al., 1976b). I hypothesised that inhibition of Wee1 at cell cycle re-entry perturbs cells growth and thus delayed S-phase entry to allow more time for cells to reach a critical size. However, measurements and analysis revealed that cell size variations did not contribute significantly to these cell cycle alterations (data not shown in this thesis) and thus will not be explored in the future (further information about cell size can be find in Chapter 7. Discussion). This led me to investigate other potential regulators. Interestingly, a new interaction between substrates other than CDKs and Wee1 has been described (Vassilopoulos et al., 2015). In this study, it is shown that in Mouse Embryonic Fibroblasts (MEFs) cells, Wee1 negatively regulates APC/C^{Cdh1} activity and that Wee1 perturbation leads to APC/C^{Cdh1} hyper-activation. First, they were able to show a direct interaction between Wee1 and two of the most important co-activators of APC/C: Cdh1 and Cdc20 (APC/C^{Cdh1} and APC/C^{Cdc20}) (see Chapter 1: Introduction). Furthermore, using MEFs lacking Wee1 (*Wee1*^{Co/-}; *Tm-Cre* MEFs) and shRNA-mediated knockdown (against Wee1) in 293T cells, the authors were able to show that the activity of APC/C^{Cdh1} increased, while ectopic overexpression of Wee1 decreased APC/C^{Cdh1} activity.

In mammalian cells, APC/C specifically targets cell cycle-related proteins for degradation exhibiting essential functions in the regulation of the eukaryotic cell cycle. Particularly, during G1, APC/C will bind to its co-activator Cdh1 and promote the degradation of cell cycle substrates (See Chapter 1: Introduction)(M. Li & Zhang, 2009; J. M. Peters, 2006; X. Yuan et al., 2014). Several of these substrates, including the CDK2-activator cyclin A and the ubiquitin ligase adaptor Skp2, play important regulatory roles during S-phase entry (See Chapter 1: Introduction). Moreover, APC/C^{Cdh1} indirectly mediates degradation of the CDK inhibitors p21 and p27, still further

amplifying the stability of G1. Depletion of Cdh1 by siRNA stabilises Skp2, resulting in p21 and p27 proteolysis in G1 and premature entry into S phase (Eguren et al., 2011). Furthermore, CDK2 and APC/C^{Cdh1} form a negative feedback loop to timely regulates S-phase entry. Hence, the inactivation of APC/C^{Cdh1} is needed for cells to enter S-phase and its inhibition marks the commitment point, a point of no return, where cells commit to enter a full cycle (see Chapter 1: Introduction) (Cappell et al., 2016; Pardee, 1974). Two mechanisms have been described for APC/C^{Cdh1} inactivation: Studies have shown that CDK2-cyclin A can phosphorylate Cdh1 and thereby promote its dissociation from the APC/C core complex (Huang et al., 2011; C. Lukas et al., 1999). Contradictory to that, other study highlighted the role of CDK2-cyclin E in APC/C^{Cdh1} inactivation (X. Yuan et al., 2014). The second mechanism is the binding of the inhibitor Emi1, blocking the binding between APC/C and Cdh1 (Cappell et al., 2018; Di Fiore & Pines, 2007; Machida & Dutta, 2007). Additionally, cyclin F, which is a component of the SCF^{cyclin F} an E3 ubiquitin ligases, also contributes to APC/C inactivation through degradation of Cdh1, providing an additional layer of regulation to the G1/S transition. cyclin F is targeted for degradation by APC/C in G1, and Cdh1 is targeted by SCF^{cyclin F} in S-phase (Choudhury et al., 2016a).

Interestingly, in addition to the study by (Vassilopoulos et al., 2015), interaction between Wee1 and components of the APC/C family (Components such as ANAPC7 and CDC27) have also been highlighted in a multiplexed kinase interactome profiling (Golkowski et al., 2023). Together, in this chapter, due to APC/C importance in the G1/S transition and to its newly unveiled interaction with Wee1, I hypothesised that APC/C^{Cdh1} is hyper-activated upon Wee1 inhibition during cell cycle re-entry and that this hyperactivation leads to an arrest in G0/G1 and a delay in S-phase entry. First given the potential negative regulation of Wee1 over APC/C^{Cdh1} I investigate if Wee1 inhibition leads to a higher Cdh1 level in my set-up. Second, I made use of different methods to perturb APC/C^{Cdh1} activity such as APC/C^{Cdh1} inhibitors (APC/C^{Cdh1}i) or siRNA. Finally, to decipher the impact of Wee1 inhibition on APC/C activity, I used single cell live imaging to monitor known APC/C substrate dynamics, such as Cdc25A, geminin, Cdc6 and cyclin A upon Wee1 and/or APC/C^{Cdh1} perturbation.

5.2 Wee1 inhibition leads to higher Cdh1 levels.

In (Vassilopoulos et al., 2015), Wee1 inhibition could lead to a hyperactivation of APC/C^{Cdh1}. To test if the same effect can be observed using Wee1i in my experimental setup, I first looked at Cdh1 protein levels. Based on timings from data collected in Chapter 3 and Chapter 4, I decided to collect samples 4h after cell cycle re-entry to be able to collect a large fraction of cells which are still in G1 in both control and Wee1i treated conditions. I also decided to treat my cells with increasing concentrations of Wee1i to monitor if higher concentrations of Wee1i lead to a greater change in APC/C^{Cdh1} levels. Unfortunately, I encountered difficulties in obtaining suitable western blot results to depict this phenotype. Nevertheless, despite encountering technical challenges resulting from poor image quality, I decided to proceed with the remainder of the experiments.

5.3 Available APC/Ci had little effect in perturbing APC/C^{Cdh1} activity in RPE1 cells.

As mentioned before, I hypothesised that a combination of Wee1 and APC/C perturbations should rescue a normal activity of APC/C^{Cdh1}. Currently two commercial inhibitors are available to perturb APC/C activity (proTAME and APCin) (Sackton et al., 2014; X. Zeng et al., 2010). It has been described that both treatments induce a spindle checkpoint dependent mitotic arrest as well as an increase in cell death (Richeson et al., 2020; Sackton et al., 2014). Moreover, proTAME perturbs both APC/C^{Cdc20} and APC/C^{Cdh1} whereas APCin has only been described to perturb APC/C^{Cdc20} activity. Additionally, due to the regulatory role of APC/C^{Cdh1} at the G1/S transition, perturbation of APC/C^{Cdh1} during cell cycle re-entry accelerate S-phase entry (Eguren et al., 2011). Hence, I hypothesised that using APC/C^{Cdh1}i during cell cycle re-entry perturbs cell cycle dynamics and thus confirming that I can use these inhibitors to target APC/C^{Cdh1}.

I decided to use APC/C^{Cdh1}i at 10 μ M, as this concentration was shown to be highly effective for perturbation of APC/C activity in mammalian cells (Richeson et al., 2020; X. Zeng et al., 2010). I first arrested RPE1 mRuby-PCNA cells in G0/G1 using 1 μ M Palbociclib for 24h and released them with serum addition and either DMSO, 10 μ M APCin, 10 μ M proTAME, or 1 μ M Wee1i for 48h. To assess S-phase entry, I quantified PCNA by time-lapse imaging. Cells treated with Wee1i show a delay in S-phase entry compared to DMSO treated cells. Wee1i-treated cells entered S-phase at 8.3h (+/- 57 mins) after released compared to DMSO treated cells, which entered S-phase 7.1h after released (Fig. 5.1.A). However, no significant difference was observed in the timing of S-phase entry upon APC/C^{Cdh1}i. Cells entered S-phase 6.7 h (+/- 57 mins) after released upon proTAME treatment and 6.85 h (+/- 63 mins) after released in APCin treated cells. The percentage of arrested in G0/G1 cells upon APCin and proTAME treatment did not show a significant difference compared to DMSO treated cells (Fig. 5.1.B). In DMSO treated cells 16% of the cells were arrested in G1 compared to 15% upon APCin and 13% upon proTAME. As already observed in other chapters, a large fraction of cells was arrested in G1 upon Wee1i. 32% of the cells were arrested upon Wee1 inhibition.

APCin and proTAME are known to also target Cdc20 (Sackton et al., 2014). To test if APCin and proTAME inhibitors were active in my experiments, I also monitored mitosis duration upon treatments. Once again, no significant difference was observed in the length of mitosis upon APCin and proTAME inhibitors compared to DMSO (Fig. 5.2.C). The mitosis length was 55 mins upon proTAME treatment and 65 mins upon APCin treatment, compared to 50 mins in DMSO treated cells. These results indicate that proTAME and APCin cannot be used at this concentration to fully inhibit the APC/C complex in RPE1 cells. To perturb APC/C^{Cdh1} without the use of commercial APC/C^{Cdh1}i, I decided to use siRNA targeting Cdh1 already in use in the lab (siCdh1).

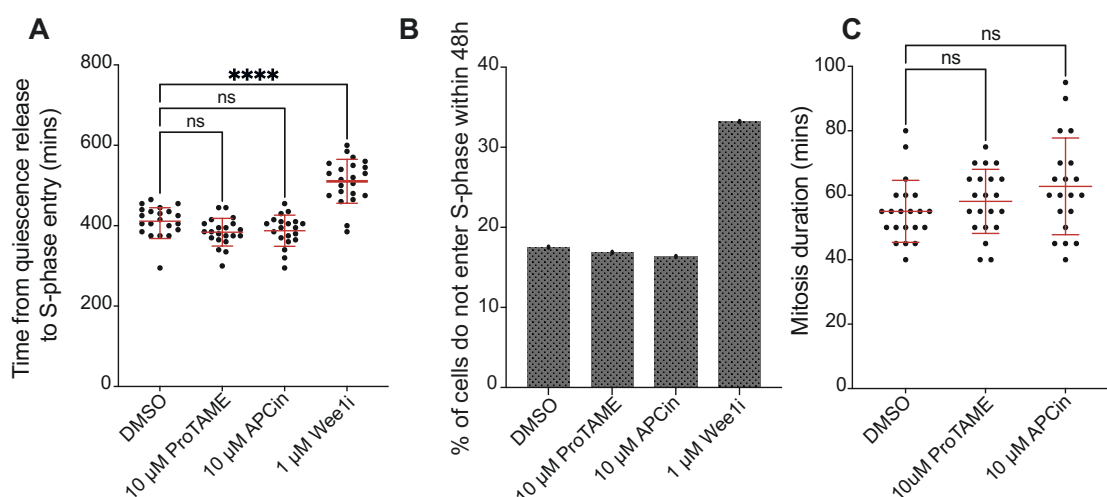


Figure 5-2. Absence of APC/C activity perturbations upon APC/Ci

(A) Timing of S-phase entry of manually tracked RPE1 mRuby PCNA cells upon APC/C^{Cdh1} and Wee1 inhibition. mRuby-PCNA cells were arrested in G0/G1 using 1 μM Palbociclib for 24h and released with serum addition and treated with DMSO, 10 μM ProTAME, 10 μM APCin or 1 μM Wee1i for 48h. Single dots represent single cell data. Mean +/- SD are shown (DMSO n=21, 10 μM ProTAME n=21, 10 μM APCin n=19, 1 μM Wee1i n=22). One way ANOVA analysis was performed to calculate p-values (ns=not significant, ****=p<0.0001). **(B)** Percentage of G1-arrested cells upon APC/C^{Cdh1} and Wee1 inhibition. Live-cell imaging data from (A) was used (n=1). **(C)** Duration of M-phase upon APC/C^{Cdh1} inhibition. Live-cell imaging data from (A) was used. Single dots represent single cell data. One way ANOVA analysis was performed to calculate p-values (ns=not significant). Mean +/- SD are shown (DMSO n=21, 10 μM ProTAME n=21, 10 μM APCin n=19).

5.4 Increase of APC/C^{Cdh1} activity upon Wee1 inhibition.

To continue addressing whether APC/C activity is increasing upon Wee1 inhibition at cell cycle re-entry I decided to quantify the expression of well-known APC/C^{Cdh1} G1 substrates upon Wee1i treatment. First, I decided to quantify the expression of Cdc25A, which is a G1 substrate of APC/C^{Cdh1} (M. Donzelli et al., 2002). Interestingly, previous data from the lab, showed that Cdc25A is expressed as a pulse during the G1/S transition in RPE1 mRuby-PCNA Cdc25A-GFP (unpublished). I hypothesised that Wee1 inhibition leads to higher levels of APC/C^{Cdh1}, causing increased APC/C^{Cdh1} activity and potentially a greater degradation of Cdc25A at the G1/S transition, and hence the loss of this pulse (Fig. 5.2.A). To test if Cdc25A-GFP sensor can be used as a direct read-out of APC/C^{Cdh1} activity, Cdc25A level has been monitored at the G1/S transition upon siCdh1 treatment (Fig 5.2.B). This experiment has been performed and analysed by Dr. Alexis Barr. To do that, asynchronous RPE1 mRuby-PCNA Cdc25A-GFP cells were transfected with NTC or siCdh1 for 6H before imaging. Then, Cdc25A intensity was followed by timelapse imaging for 10h. Cells treated with siCdh1 display higher levels of Cdc25A intensity across G1 and around the G1/S transition compared to DMSO-treated cells, confirming that Cdc25A is an APC/C^{Cdh1} substrate in G1. Interestingly, and confirming data obtained in the lab, Cdc25A level appears to be heterogeneous upon DMSO treatment, with cells expressing high level of Cdc25A and another population expressing a low level of Cdc25A (data not plotted). Significantly, I observed a decreased in Cdc25A-GFP expression upon Wee1i treatment (Fig. 5.2.C), suggesting that Wee1 does negatively regulate APC/C^{Cdh1} activity during G1. Knowing that Cdc25A can be used as a readout for APC/C^{Cdh1} activity, I hypothesised that a combination of Wee1i and siCdh1 should rescue a normal APC/C^{Cdh1} activity and thus allowing Cdc25A to be expressed as a pulse again (Fig. 5.2.D, E). To test that, I arrested RPE1 mRuby-PCNA Cdc25A-GFP cells in G0/G1 using 1 μ M Palbociclib for 48h and reverse-transfected during the last 24h of arrest with NTC or siCdh1. Then I released the cells with serum addition and 1 μ M Wee1i for 48h and followed Cdc25A by timelapse imaging. Cells treated with the combination of 1 μ M Wee1i and siCdh1 rescued Cdc25A expression to normal levels at the G1/S transition (for visualisation purpose, this graph has been simplified. All details can be found in the legend). To better understand the relation between Wee1 and APC/C^{Cdh1}, I also investigated the timing of APC/C^{Cdh1}

inactivation. I first looked at another substrate of APC/C^{Cdh1} that does not appear until APC/C^{Cdh1} activity is switched off. In Chapter 4.5, I used Cdc6 live sensor to investigate origin licensing timing upon Wee1 inhibition. It has been shown that Cdc6 is also targeted for ubiquitin-mediated proteolysis during G1 by APC/C and that APC/C^{Cdh1} is required and limiting for Cdc6 proteolysis (Petersen et al., 2000). Looking back at Cdc6 data in (Chapter 4: Fig 4.4.A, B), I observed that the timing of Cdc6 degradation did not change between Wee1i and DMSO treated cells. However, I could notice a diminution of peak Cdc6 intensity upon Wee1 inhibition compared to DMSO treatment around S-phase entry.

This suggests that APC/C^{Cdh1} is not only required but also limiting for Cdc6 proteolysis. Hyper-activation of APC/C^{Cdh1} upon Wee1 inhibition, contributes to the observed decrease in peak Cdc6 intensity compared to DMSO treatment around the G1/S transition. Furthermore, during S, G2 and M phases geminin binds to Cdt1 to prevents the loading of the MCM complex resulting in licensing inhibition (see Chapter 1: Introduction) (Nishitani et al., 2001; Tada et al., 2001; Wohlschlegel et al., 2000). During G1, APC/C^{Cdh1} degrades geminin to allow origin licensing program to occur (Clijsters et al., 2013). Hence, increase in geminin intensity has been correlated with S-phase entry and can be used as a readout for APC/C^{Cdh1} activity dynamics. In the previous chapter, I used a cell line with a PIP-fucci sensor as a S-phase entry marker (RPE1 PIP-fucci Geminin-mCherry mTurq2 PCNA) (see chapter 3.4). This cell line is also expressing a mCherry-Geminin sensor allowing the follow of geminin intensity across the cell cycle. I hypothesised that looking at geminin intensity upon Wee1 inhibition would give a better understanding on APC/C activity dynamics. Using live imaging data from Chapter 3.4, I noticed that geminin level increases at the same time around the G1/S transition upon Wee1 inhibition and DMSO. I could also observe a decreased in geminin intensity in G2 arrested cells upon Wee1 inhibition. I decided to continue looking into the dynamics of APC/C activity by focusing on a second substrate - cyclin A. As explained in Chapter 1: Introduction, cyclin A binds to CDK2 and regulates S-phase progression. It is then degraded early in pro-metaphase by APC/C. During G1, APC/C^{Cdh1} also degrades cyclin A and increase in cyclin A level is representative of APC/C^{Cdh1} inactivation. Hence cyclin A can be used as a readout of APC/C^{Cdh1} activity. In this experiment, cyclin A intensity has been monitored using a cyclin A live sensor (RPE1 cyclin A-mVenus mRuby-PCNA) (Fig 5.2.F). Dr Beth Pennycook from my lab performed and analysed these experiments. RPE1

cyclin A-mVenus mRuby-PCNA cells were arrested cells in G0/G1 using 1 μ M Palbociclib for 48h and released with serum addition and either DMSO or 1 μ M Wee1i for 48h (Fig 5.2.G). Once again in these experiments, no difference could be observed in the timing of cyclin A increase upon Wee1 inhibition compared to DMSO treated cells. Moreover, cyclin A intensity remained low in G2 arrested cells upon Wee1 inhibition. I have shown that upon Wee1 inhibition both Geminin and cyclin A started to increase coincidentally with the G1/S transition. This suggests that Wee1 does not affect the timing of APC/C^{Cdh1} switch off relative to the G1/S transition, only Cdh1 expression levels and its activity during G1. Further details about this phenotype will be described in the discussion of Chapter 7.

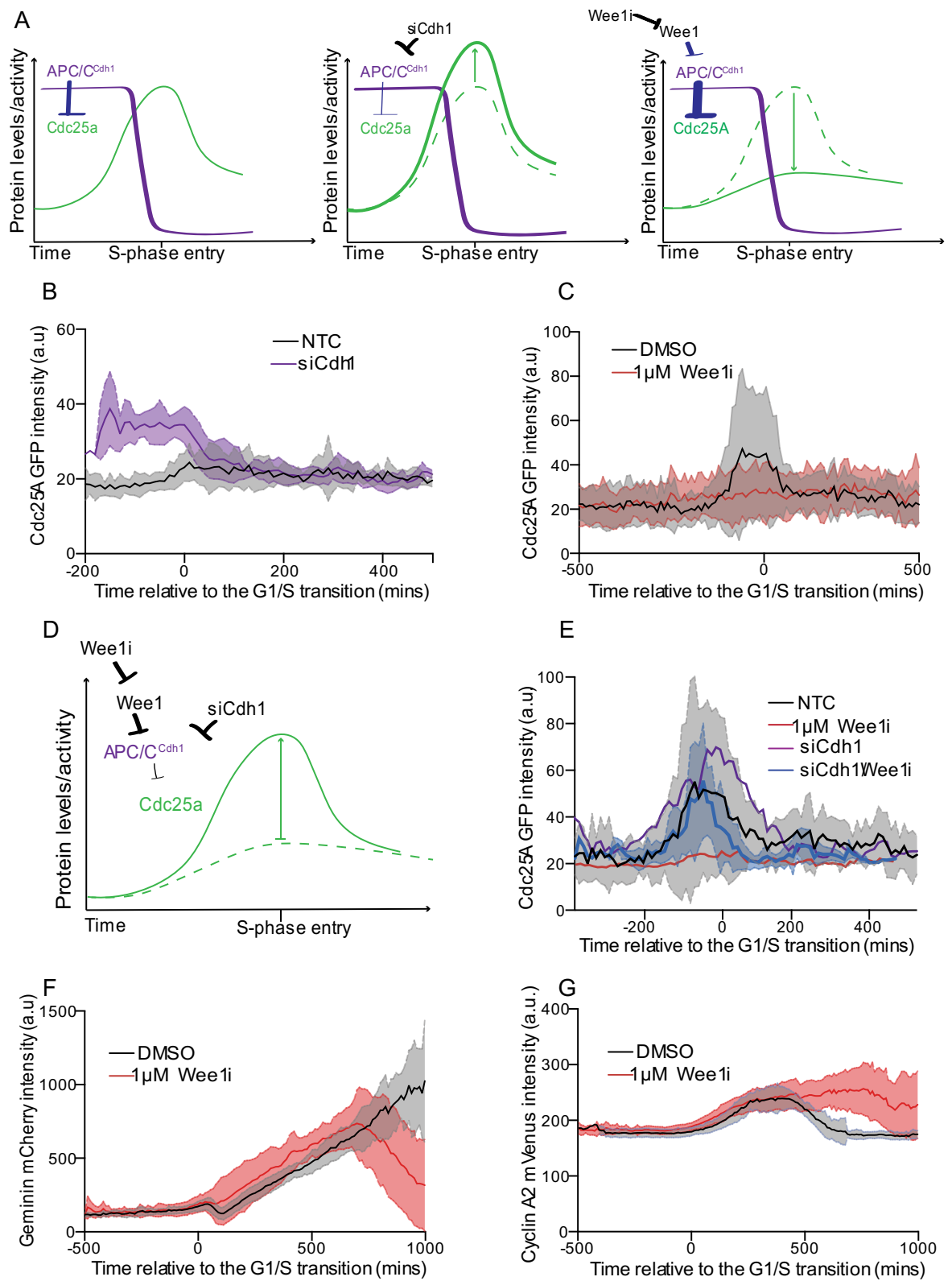


Figure 5-2. APC/C^{Cdh1} increase upon Wee1 inhibition.

(A) Schematic illustration of the relation between Cdc25A, APC/C^{Cdh1} and Wee1 around the G1/S transition. **(B)** Quantification of Cdc25A-GFP around the G1/S transition upon siCdh1. RPE1 mRuby-PCNA Cdc25A-GFP cells were arrested in G0/G1 using 1 μ M Palbociclib for 48h and reverse-transfected for 24h with NTC or siCdh1 before released with serum addition for 48h. Mean intensity $\pm$ SD are shown (DMSO in black n=20, siCdh1 in red n=20). **(C)** Quantification of Cdc25A-GFP around the G1/S transition upon Wee1 inhibition. RPE1 mRuby-PCNA Cdc25A-GFP cells were arrested in G0/G1 using 1 μ M Palbociclib for 24h and released with serum addition and either DMSO or 1 μ M Wee1i for 48h. Mean intensity $\pm$ SD are shown (DMSO in black n=20, Wee1i in red n=20). **(G)** Schematic illustration of Cdc25A levels around the G1/S transition upon siCdh1 and Wee1 inhibition. **(D)** Schematic illustration of the relation between Cdc25A, APC/C^{Cdh1}, Wee1 and Wee1i around the G1/S transition. **(E)** Quantification of Cdc25A-GFP around the G1/S transition upon siCdh1 and Wee1 inhibition. mRuby-PCNA Cdc25A-GFP cells were arrested in G0/G1 using 1 μ M Palbociclib for 48h and reverse-transfected 24h with NTC or siCdh1 for 24h before released with serum addition and 1 μ M Wee1i for 48h. Mean intensity $\pm$ SD are shown (NTC: n=15, siCdh1/Wee1i: n=15, siCdh1=10, Wee1i=10). **(F)** Quantification of mCherry-Geminin around the G1/S transition upon Wee1 inhibition. Live imaging data from Chapter 3.4. Mean intensity $\pm$ SD are shown (DMSO in black n=20, Wee1i in red n=20). **(G)** Quantification of cyclinA2-mVenus around the G1/S transition upon Wee1 inhibition. RPE1 cyclin A-mVenus mRuby-PCNA cells were arrested in G0/G1 using 1 μ M Palbociclib for 48h and released with serum addition and either DMSO or 1 μ M Wee1i for 48h. Mean intensity $\pm$ SD are shown (DMSO in black n=20, Wee1i in red n=20).

5.5 S-phase entry delay upon Wee1 inhibition is APC/C^{Cdh1} dependent.

I, then, tested if Wee1i leads to higher level of Cdh1 causing a delay in S-phase entry or a G1 arrest. I hypothesised if Wee1i inhibition leads to a delay in S-phase entry or a G1 arrest due to higher level of Cdh1, then a combination of Wee1i and siCdh1 should allow cells to progress with a normal timing into S-phase. I first arrested RPE1 mRuby-PCNA cells in G0/G1 using 1 μ M Palbociclib for 48h. 24h before released I reverse transfected my cells with NTC or siCdh1. This allows me to deplete Cdh1 while cells are still arrested in G0/G1. Then, I released the cells with serum addition and 1 μ M Wee1i for 48h. To assess S-phase entry, I quantified PCNA by time-lapse imaging. In cells treated with siCdh1, I observed an acceleration in S-phase entry timing compared to DMSO. Cells treated with siCdh1 entered S-phase around 5h ($\pm$ 64 mins) after release into the cycle compared to 8.5h ($\pm$ 88 mins) for NTC treated cells (Fig. 5.3.A). Once again cells treated with Wee1i display a delay in S-phase entry and entered S-phase 15.8h ($\pm$ 385 mins) post release. Cells treated with a combination of siCdh1 and Wee1i cells displayed the same S-phase entry dynamics as the DMSO treated cells. Cells entered S-phase around 8.1h ($\pm$ 47 mins) post release with the combination of siCdh1 and Wee1i.

In terms of percentage of arrested cells, I observed that 12 % of the cells were arrested in G1 and 2% of the cells were arrested in G2 when treated with siCdh1, compared to 13% of G1-arrested cells upon NTC treatment. Upon Wee1i, 33% of the cells were arrested in G1 and 41% were arrested in G2. In cells treated with a combination of Wee1i and siCdh1 21% of the cells were arrested in G1 and 47% of the cells were arrested in G2 (Fig 5.3.B). Additionally, the percentage of arrested cells in G2 remains the same upon Wee1 inhibition or combination of Wee1i and siCdh1.

These results indicate that Wee1 inhibition leads to higher levels of Cdh1 causing a delay in S-phase entry but not the arrest in G0/G1 or G2 arrest.

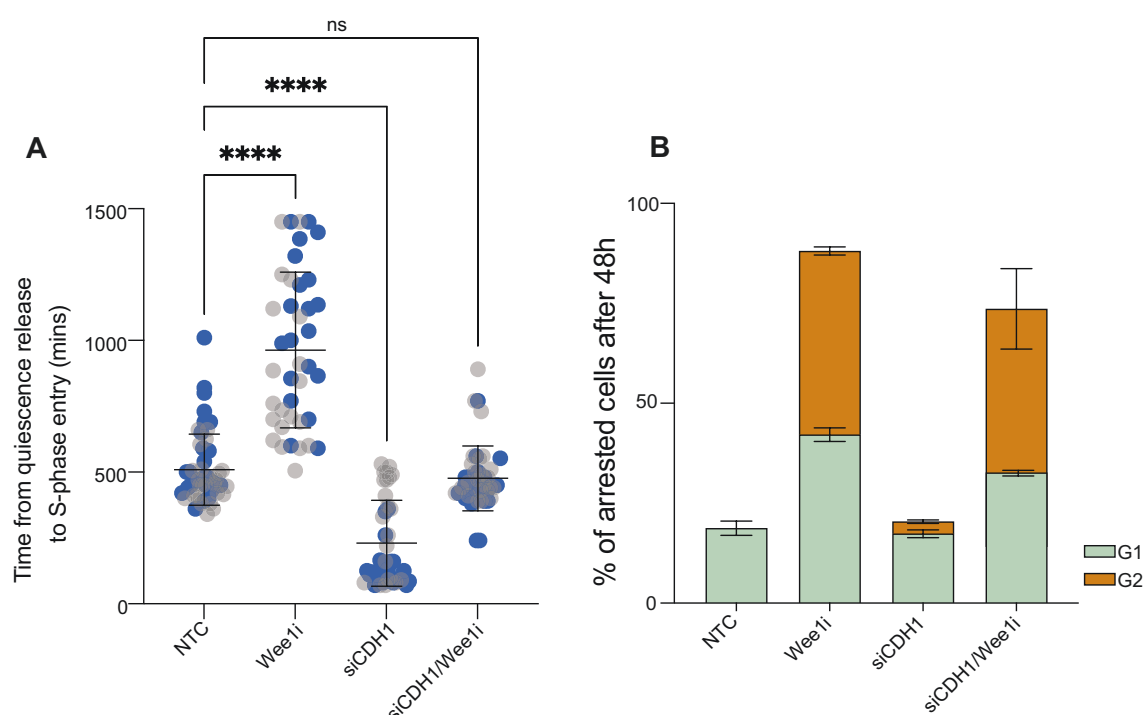


Figure 5-4. Perturbation of Wee1 and APC/C^{Cdh1} rescues a wild type S-phase entry timing.

(A) Timing of S-phase entry of manually tracked RPE1 mRuby-PCNA cells upon siCdh1 and Wee1 inhibition. RPE1 mRuby-PCNA cells were arrested in G0/G1 using 1 μ M Palbociclib for 48h and reverse-transfected for 24h with NTC or siCdh1. The cells were released with serum addition and treated with 1 μ M Wee1i for 48h. Single dots represent single cell data. Each colour represents one biological replicate (n=2). Mean intensity $\pm$ SD are shown. One way ANOVA analysis was performed to calculate p-values (ns=not significant, ****=p<0.0001). **(B)** Percentage of RPE1 mRuby-PCNA cells arrested in G1 and G2 after 48h upon siCdh1 and Wee1 inhibition. Live-cell imaging data from (A) was used. Mean $\pm$ SD are shown.

5.6 Summary.

Previous studies have shown that inhibiting Wee1 led to a hyper-activation of APC/C^{Cdh1} (Vassilopoulos et al., 2015). In this chapter I investigated if Wee1 inhibition at cell cycle re-entry leads to APC/C^{Cdh1} hyper-activation, potentially explaining the delay in S-phase entry and G1 arrest described in Chapter 3.

I hypothesised that this higher level of Cdh1 directly increase APC/C^{Cdh1} activity. To do that, I monitored proteins which are regulated by APC/C^{Cdh1} in G1 to use an alternative readout of APC/C^{Cdh1} activity. I showed that upon Wee1i treatment, APC/C^{Cdh1} activity increases (monitored by a decrease in Cdc25A-GFP and Cdc6-mVenus levels) whereas APC/C inactivation timing remains the same between Wee1i treated and untreated cells (monitored by cyclin A2-mVenus and mCherry-Geminin levels). Additionally, I also showed an increase in APC/C activity in the population of G2 arrested cells upon Wee1 inhibition.

Lastly, I speculated that if the S-phase entry delay and G1 arrest obtained after Wee1 inhibition was due to a hyper-activation of APC/C^{Cdh1}, then downregulation of Wee1 and APC/C^{Cdh1} at cell cycle re-entry should rescue a normal cell cycle dynamic. I showed that upon siCdh1 treatment, cells have a shorter G1-phase and that upon combination of Wee1i and siCdh1, cells rescued a normal timing into S-phase entry. However, it is worth noting that upon Wee1i and siCdh1 the population of arrested cells in G1 is still present.

In this chapter, I encountered challenges in proving the increased levels of Cdh1 upon Wee1 inhibition. To address this in future studies, it is suggested to employ western blot or proteomics analysis focused specifically on Cdh1 levels. Additionally, efforts to demonstrate a direct interaction between Wee1 and APC/C^{Cdh1} were inconclusive, possibly due to the rapid nature of this interaction. Conducting co-immunoprecipitation (Co-IP) experiments showed difficulties due to the precise timing required for sample collection. Exploring different time points in experiments may help identify the optimal window to observe this interaction. Alternatively, utilising the Wee1-GFP cell lines and employing a GFP-trap technology could facilitate the pull-down of Cdh1, potentially providing more robust results compared to conventional Co-IP methods. Further detailed explanations and analysis of these observed phenotypes will be provided in Section 7.3. Moreover, it is worth considering other

technological approaches to capture transient and weak protein interactions, such as crystallisation, cell-volume modulation techniques or Förster resonance energy transfer (FRET) technologies. These methods offer promising avenues for enhancing our understanding of complex protein interactions in future investigations. Further detailed explanations and analysis of these observed phenotypes will be provided in Section 7.3.

Additionally, Wee1 inhibition leads to increased expression of APC/C^{Cdh1} substrates Geminin and cyclin A coinciding with the G1/S transition. This implies that Wee1 primarily affects Cdh1 expression levels and activity during G1, rather than altering the timing of APC/C^{Cdh1} switch-off. Additionally, observations in Chapter 4 and Chapter 5 highlight changes in Cdc6, Geminin, and cyclin A levels in G2 arrested cells upon Wee1 inhibition, shedding further light on the intricate interplay between Wee1 and APC/C^{Cdh1} in cell cycle regulation. Further detailed explanations and analysis of these observed phenotypes will be provided in Section 7.3. Taken together, I was able to decipher the potential molecular mechanisms behind the delay in S-phase entry. I was able to show that the S-phase entry delay observed upon Wee1 inhibition at cell cycle re-entry is due to a hyper-activation of APC/C^{Cdh1}. However, this hyper-activation cannot explain the G1 arrest also observed upon Wee1 inhibition upon cell cycle re-entry.

Chapter 6. Wee1 phosphoproteomics and new substrates.

6.1 Introduction.

Tyrosine phosphorylation is a central regulatory mechanism in cell signalling (Hunter, 2000, 2002). Despite its importance, phosphotyrosine (pTyr) is often more challenging to characterise compared to phosphoserine (pSer) or phosphothreonine (pThr), due to its low levels in the proteome (Relative abundance of 1800:200:1 was estimated for pSer/pThr/pTyr) (Hunter, 2009; Lim & Pawson, 2010). Over half of the human tyrosine kinases have been reported as oncogenes. As a result, protein tyrosine kinases have become one of the major targets for many drug discovery programs (Shawver et al., 2002). In the previous chapter, I was able to show the importance of Wee1 in the regulation of the G1-S transition. As already explained in Chapter 1: Introduction, Wee1 is a tyrosine kinase involved in inactivation of CDKs through tyrosine inhibitory phosphorylation. In Chapter 3, I was able to show that contradictory to the supposed effect, Wee1 inhibition did not lead to an acceleration of S-phase entry but instead to a delay and an arrest in G0/G1 or G2. Additionally, as demonstrated in Chapter 4, even though Wee1 inhibition does lead to a delay into S-phase entry, CDK2 activity increase before S-phase entry and remains higher than untreated cells. This led me to think that Wee1 could have potential roles outside of its canonical regulation of CDKs. I hypothesised that Wee1 could phosphorylate other unknown proteins beside CDKs, to regulate cell cycle re-entry (see Chapter 1: Introduction for other known Wee1 substrates).

In order to address this research question, I decided to carry out phosphoproteomics experiments. This approach allows to identify and quantify changes within the phosphoproteome (Macek et al., 2009). Over the years, phosphoproteomics has been used to generate valuable proteome-wide phosphorylation data important for major advancements and insights into drug development, cancer resistance and for revealing specific metabolic components in certain cancer cell types (Bekker-Jensen et al., 2020; Carter et al., 2020). Given the fact that Wee1 is a tyrosine kinase, I decided to specifically target proteins with phosphorylated tyrosine residues (phospho-tyrosine

proteomics). Phospho-tyrosine proteomics is a specialised area of phosphoproteomics and has shown growing interest and allowed relevant insights in cancer research over the years (Noel et al., 2019; R. Zhang & Wong, 2017).

The main strategy of phospho proteomics-based mapping of phosphorylation events on a large-scale relies on mass spectrometry (MS), which allows a relatively unbiased approach. To do that, I worked closely with the Proteomics and Metabolomics Facility at the MRC LMS.

In this chapter, experimental design was performed by Dr. Alex Montoya, Dr. Pavel Shliaha and myself. Cell culturing and harvesting steps were carried out by myself. Protein extraction and MS experiment and analysis were carried out by Dr. Alex Montoya and Dr. Pavel Shliaha.

In order to investigate Wee1-dependent phosphorylation events during cell cycle re-entry, I decided to carry out a comparative analysis between pharmacological inhibition and specific protein degradation, shedding light on the potential overlap of the phosphorylation events, triggered under each condition.

The resulting phospho-proteome was then compared to mock treated cells, which served as a control. If there are changes in the pattern of tyrosine phosphorylation in proteins besides CDKs, this hints to a role of Wee1 in phosphorylating other proteins on their tyrosine residues during G1. These hits can then be used for further analysis to elucidate whether the phosphorylation of these proteins are responsible for the delay in S-phase entry and the arrest in G0/G1 or G2 upon Wee1 inhibition and degradation. I hypothesised that most likely these protein hits are involved in the cell cycle.

It is important to note that at the point of writing this thesis, the enrichment in tyrosine residues was not carried out yet. For this reason, I will only focus on global phosphosite enrichment analysis. This provides a broader perspective on the complex landscape of phosphorylation-mediated cellular processes which occur in a Wee1-dependent manner during cell cycle re-entry.

6.2 Wee1 inhibition leads to a change in the G0/G1 phospho-proteome.

To study whether Wee1 phosphorylates other proteins besides CDKs during cell cycle re-entry, it was important to decide at which time-point during G1 the cells need to be collected. For this, I made use of the results obtained in Chapter 3: Fig. 3.6.A, where I show that cells treated with Wee1i upon 4 to 6h upon release present a delay in S-phase and an arrest in G0/G1 or G2. I then supposed that I would be able to visualise change in phosphoproteomics during this time. For this reason, I decided to study changes in phospho-tyrosine residues upon Wee1 perturbation 4h post-release into the cell cycle. Due to the low levels of phosphorylated tyrosines in the proteome, one of the major challenges is to collect sufficient cells to obtain accurate protein levels for the subsequent analysis. Harvesting the cells merely 4 hours post-release does not allow adequate time for substantial protein production to occur, requiring the culturing of large amounts of cells. Furthermore, compared to other cell lines like HeLa cells, we have found that RPE1 cells yield lower protein amounts per cell upon lysis. These factors collectively highlight the difficulties encountered when carrying out this experiment. We decided to harvest 5mg of protein for each condition and we decided to use 5 replicates per conditions for this experiment.

I arrested RPE1 myc-OsTIR1 mRuby-PCNA Wee1-GFP-AID in G0/G1 using 1 μ M Palbociclib for 24h in order to have a confluency around 85 to 90 % the next day. RPE1 myc-OsTIR1 mRuby-PCNA Wee1-GFP-AID cells were released with serum addition and either DMSO, 1 μ M Wee1i, Doxycycline (as control for the degron system) or DOX+IAA (ID) for 4h before collection (Fig 6.C). Samples were then lysed and given to the proteomics facility for preparation, mass spectrometry and analysis (see Chapter 2: Materials and Methods).

To validate the effectiveness of Wee1 inhibition and Wee1 degradation in the phospho-proteomics experiments, I first analysed whether using Wee1i had the expected effect on CDK phosphorylation. I carried out, in parallel, an immunostaining monitoring pY15-CDK phosphorylation. A sub-population of RPE1 myc-OsTIR1 mRuby-PCNA Wee1-GFP-AID cells used for the phosphotyrosine experiments were plated in a 384 well plate and arrested in G0/G1 using 1 μ M Palbociclib. RPE1 myc-OsTIR1 mRuby-PCNA Wee1-GFP-AID cells were released at the same time as the one used

for the phosphotyrosine experiments with fresh media and the addition of DMSO or 1 μ M Wee1i for 4h before fixation. The results show a significant decrease in the pY15-CDK/CDK2 ratio in Wee1i treated cells compared to vehicle-treated cells (Fig 6.1.A). Next, I tested whether the ID treatment led to the degradation of Wee1 (Fig 6.1.B). For this I harvested a sub-population of RPE1 myc-OsTIR1 mRuby-PCNA Wee1-GFP-AID cells used in the phosphotyrosine experiments and carried out a western blot. To do that I arrested RPE1 myc-OsTIR1 mRuby-PCNA Wee1-GFP-AID cells in G0/G1 using 1 μ M Palbociclib. RPE1 myc-OsTIR1 mRuby-PCNA Wee1-GFP-AID cells were released at the same time as the one used for the phosphotyrosine experiments with fresh media and the addition of Dox or ID for 4h before sample collection. I was able to show that after 4h upon degron induction, Wee1 is degraded in my experiments.

We used Hierarchical Clustering to visualise the changes in protein phosphorylation between the different conditions (Fig. 6.1.D). I hypothesised that Wee1i treated cells and the specific degradation of Wee1 through the degron system should give me similar patterns of phosphorylation changes compared to DMSO and DOX control treated cells. However, a distinct pattern emerged in the dendrogram. Notably, three conditions: DMSO, DOX, ID clustered closely together, indicating a high degree of similarity in their phosphorylation profiles. In contrast, Wee1i treated cells formed a separated cluster, distinct from the other three. This isolation implies that Wee1i and specific degradation of Wee1 exhibits a different changes in phosphorylation. Additionally, even though Wee1 is a negative regulator, I could also observe an up-regulation of phosphorylation of certain proteins upon Wee1 inhibition.

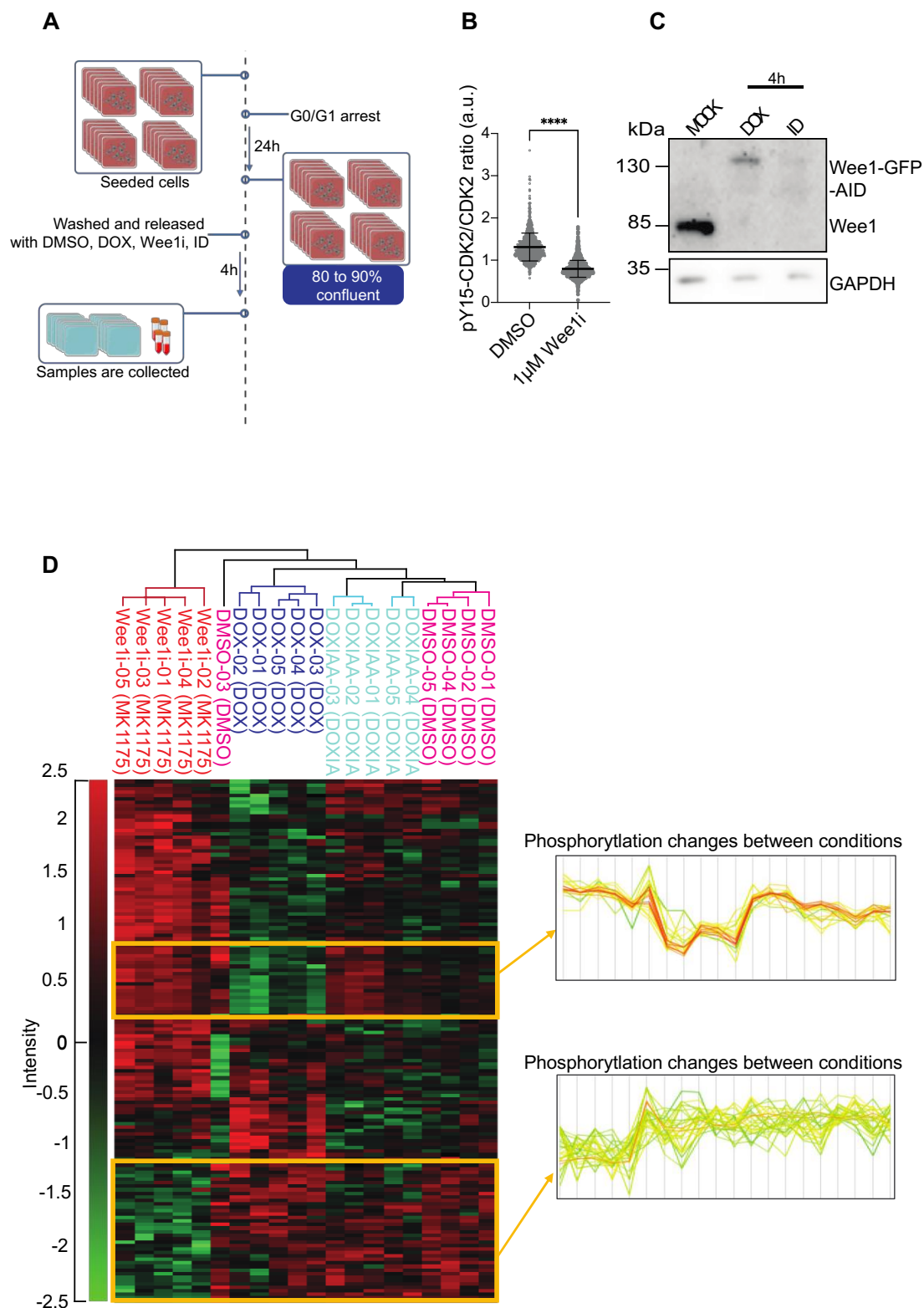


Figure 6-1. Wee1i upon cell cycle re-entry leads to a change in phospho-enrichment.

(A) Tyrosine-phospho proteomics experiments set-up. Schematic illustration of the phospho-tyrosine experiment. RPE1 myc-OsTIR1 mRuby-PCNA Wee1-GFP-AID arrested in G0 using 1 μ M of Palbociclib for 24h. RPE1 myc-OsTIR1 mRuby-PCNA Wee1-GFP-AID cells were released with serum addition and either DMSO, 1 μ M Wee1i, Doxycycline (as control for the degron system) or ID for 4h before collection.

(B) Quantification of the ratio pY15-CDK2/CDK2 in RPE1 myc-OsTIR1 mRuby-PCNA Wee1-GFP-AID cells by immunostaining. Cells were arrested in G0/G1 treated using 1 μ M Palbociclib for 24h. RPE1 myc-OsTIR1 mRuby-PCNA Wee1-GFP-AID cells were released with serum addition and either DMSO or 1 μ M Wee1i. Single dot represents single cell data. One way ANOVA analysis was performed to calculate p-values (****=p<0.0001). Mean $\pm$ SD are shown.

(C) Western blot showing Wee1-Aid degradation. myc-OsTIR1 mRuby-PCNA Wee1-GFP-Aid and myc-OsTIR1 mRuby-PCNA cells were arrested in G0/G1 treated using 1 μ M of Palbociclib for 24h. myc-OsTIR1 mRuby-PCNA Wee1-GFP-Aid and myc-OsTIR1 mRuby-PCNA cells were released with serum addition and either DOX or ID for 4h.

(D) Hits visualised with Hierarchical Clustering Analysis (HCA) (n=5). Top right graph represents significant upregulated phosphoprotein changes in both Wee1i and Wee1 specific degradation conditions. Bottom right graph represents significant downregulated phosphoprotein changes upon Wee1i conditions alone.

As I could only see a significant phosphorylation difference in the cells treated with Wee1i, I decided to focus on these samples and their mock treated control cells (DMSO). From my samples, 47 significant hits (see Chapter 2: material and methods) were determined between Wee1i and DMSO (Fig. 6.2.A). In the table, hits are ranked from most significant at the top to least significant at the bottom (Table 6.1). Because of the known role of Wee1 in CDKs tyrosine phosphorylation, I expected a decrease in pY15-CDK2 phosphorylation. Indeed, I could identify a decrease in phosphorylation of pY15 in CDK1, CDK2 and CDK3 (impossible to distinguish which specific CDKs present a phosphochanges in this experiment) in cells treated with Wee1i and thus validate my experimental process (Fig. 6.2.A, B). Other than CDKs, the only other hit with a change in a tyrosine phosphorylation was ACTBL2 (Table 6.1). ACTBL2 is not known to be involved in the cell cycle but is presumed to play a role in sustaining the cytoskeleton's integrity, essential for cell shape, division, and motility (Malek et al., 2021). Out of the 47 hits, few proteins are linked to the cell cycle progression such as MCM2 (Table 6.1). This protein is required for the initiation of DNA replication. The other hits represent phospho-changes within proteins, which cannot be linked to the cell cycle directly. However, most 15% of the significantly differently phosphorylated proteins are involved in cytoskeletal regulation mechanisms, such as ACTBL2 or Tensin-1 (TNS1) which are focal adhesion proteins linking the actin cytoskeleton to integrin receptor (H. Chen et al., 2000). This points to a role for Wee1 in the regulation of the cytoskeleton. To investigate further whether the proteins changing their phosphorylation status upon Wee1 inhibition are indirectly involved in the cell cycle, I made use of the STRING database to analyse the interactions among my identified target (Szklarczyk et al., 2015). This analysis allowed to test whether the protein hits show interactions with cell cycle regulators, and hence affect indirectly the cell cycle. Indeed, exploring the predicted interaction networks can suggest new directions for future experimental research and provide cross-species predictions for efficient interaction mapping. Despite the comprehensive analysis facilitated by STRING, the investigation yielded no significant findings or standout interactions to cell cycle regulators. Instead, the proteins identified by mass spectrometry which change their phosphorylation status upon Wee1 inhibition interacted with proteins involved in the actin-cytoskeleton pathway. This outcome suggests that Wee1 does not tyrosine-phosphorylate other unknown proteins of the cell cycle besides CDKs which could explain the observed phenotypes upon Wee1i. However, as

this experiments is not complete, more information could be obtained once the tyrosine enrichment will be finished. It indicates that Wee1 potentially plays a role in the cytoskeleton regulation (further description of hits would be discussed in Chapter 7: Discussion).

Protein name	Protein decription	Phospho-site
CDK1;CDK2;CDK3	Cyclin-dependent kinase 1;Cyclin-dependent kinase 2;Cyclin-dependent kinase 3	Y15
CDK1;CDK2;CDK3	Cyclin-dependent kinase 1;Cyclin-dependent kinase 2;Cyclin-dependent kinase 3	Y15
ACTBL2	Beta-actin-like protein 2	Y241
EGFR	Epidermal growth factor receptor	T693
EGFR	Epidermal growth factor receptor	T693
MYO9B	Unconventional myosin-IXb	T1346
NUMA1	Nuclear mitotic apparatus protein 1	T2000
RAVER1	Ribonucleoprotein PTB-binding 1	T463
TNKS1BP1	182 kDa tankyrase-1-binding protein	T131
TNS1	Tensin-1	T979
TGFB111	Transforming growth factor beta-1-induced transcript 1 protein	S194
PRPSAP2	Phosphoribosyl pyrophosphate synthase-associated protein 2	S227
KPNA6	Importin subunit alpha-7	S6
EGFR	Epidermal growth factor receptor	S695
STMN1	Stathmin	S25
UBA1	Ubiquitin-like modifier-activating enzyme 1	S4
MARCKS	Myristoylated alanine-rich C-kinase substrate	S63
MAP1B	Microtubule-associated protein 1B	S1797
MCM2	DNA replication licensing factor MCM2	S13
PDLIM4	PDZ and LIM domain protein 4	S135
HNRNPA3	Heterogeneous nuclear ribonucleoprotein A3	S358
AMPD2	AMP deaminase 2	S168
EIF4G1	Eukaryotic translation initiation factor 4 gamma 1	S1231
AHNAK	Neuroblast differentiation-associated protein AHNAK	S5731
AHNAK	Neuroblast differentiation-associated protein AHNAK	S559
AHNAK	Neuroblast differentiation-associated protein AHNAK	S511
TP53BP1	TP53-binding protein 1	S1028
SNTB2	Beta-2-syntrophin	S393
UBAP2L	Ubiquitin-associated protein 2-like	S609
SCRIB	Protein scribble homolog	S1348
ZNF638	Zinc finger protein 638	S605
SAMD4B	Protein Smaug homolog 2	S271
RAPH1	Ras-associated and pleckstrin homology domains-containing protein 1	S1154
DHX29	ATP-dependent RNA helicase DHX29	S200
PPP1R10	Serine/threonine-protein phosphatase 1 regulatory subunit 10	S313
NIBAN1	Protein Niban 1	S646
IRF2BPL	Probable E3 ubiquitin-protein ligase IRF2BPL	S659
GORASP2	Golgi reassembly-stacking protein 2	S451
PDLIM7	PDZ and LIM domain protein 7	S217
PPP4R2	Serine/threonine-protein phosphatase 4 regulatory subunit 2	S226
BCLAF1	Bcl-2-associated transcription factor 1	S177
EHD2	EH domain-containing protein 2	S468
EPS15L1	Epidermal growth factor receptor substrate 15-like 1	S229
CDC42EP3	Cdc42 effector protein 3	S100
ACIN1	Apoptotic chromatin condensation inducer in the nucleus	S710
AKAP2	A-kinase anchor protein 2	S720
NOC2L	Nucleolar complex protein 2 homolog	S56

Table 6-1. List of significant protein with phosphorylation changes upon Wee1i compared to DMSO treated cells.

Green lines represent significant down-regulated phosphoprotein changes and blue lines represents significant upregulated phosphoproteins changes, upon Wee1 inhibition.

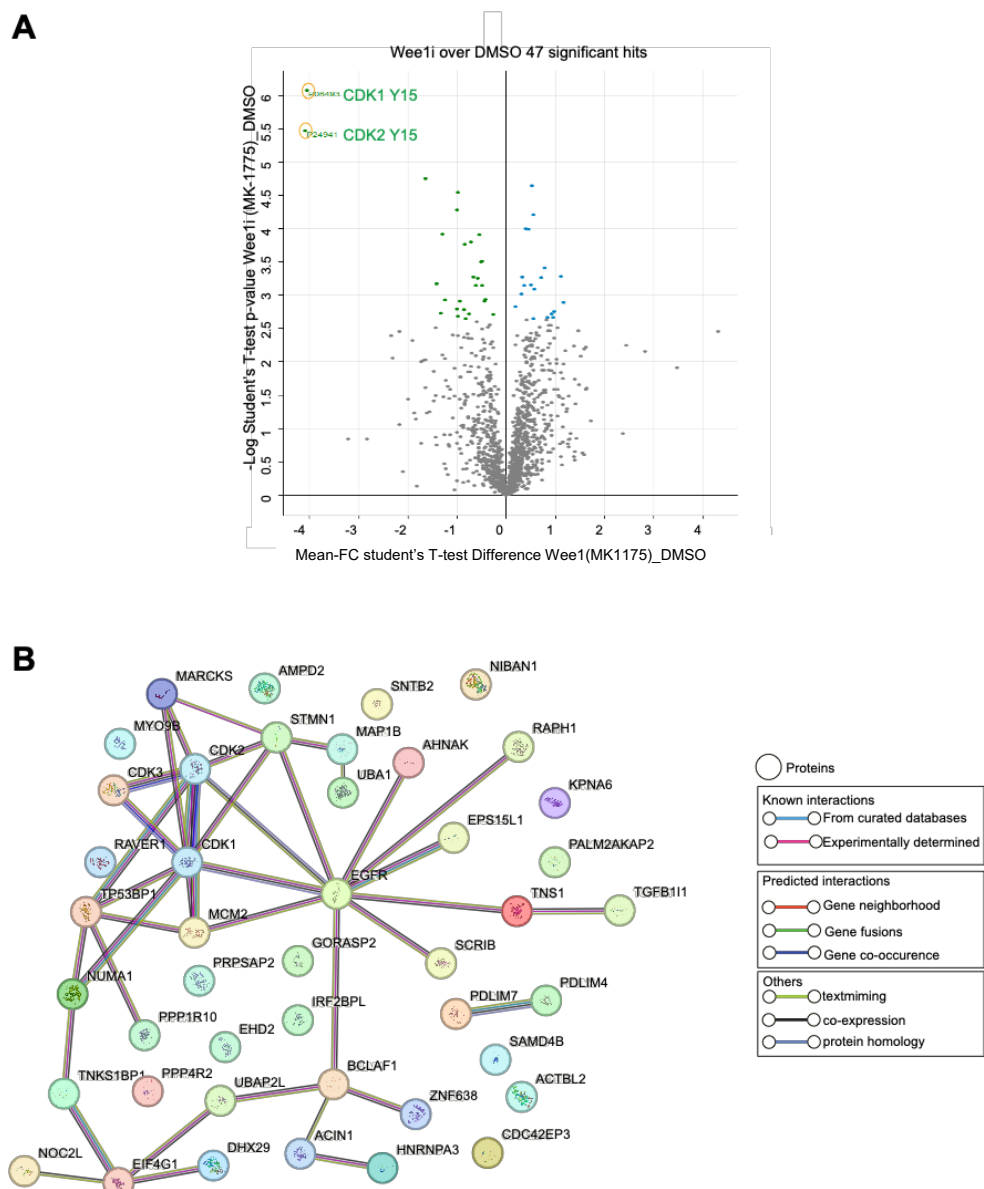


Figure 6-2. Significant hits in proteins phosphorylation changes upon Wee1 inhibition.

(A) Proteins hits upon Wee1i and DMSO treated cells. On the left: T-test significant hits protein from (Fig. 6.1.B) visualised with volcano plots. This graph has been plotted by Dr Alex Montoya. Green point represents significant downregulated phosphoprotein changes and blue point represents significant upregulated phosphoprotein changes upon Wee1i. **(B)** Interactions between protein hits. Data from (Fig. 6.3.B) is used.

6.3 Summary.

In this chapter, I aimed to identify potential new substrates of Wee1. To achieve this, I conducted phospho-tyrosine proteomics, where I compared mock treated cells with cells treated with Wee1i and cells where Wee1 is specifically targeted for degradation using the degron system. The primary objective was to understand whether Wee1 phosphorylates other regulators of the cell cycle besides CDKs. I hypothesised that these proteins might play a role in a delay into S-phase entry and G1 arrest upon Wee1 perturbation. The initial aim was to identify phosphorylation changes on tyrosine residues specifically. However, only the analyses for phosphorylation changes overall were completed. The results showed that only upon Wee1i treatment, and not upon Wee1 degradation, phosphorylation changes were occurring within the proteome. The discrepancy in the phospho-proteome following specific Wee1 degradation could be attributed to the rapid action of Wee1 inhibition compared to the potentially delayed degradation through ID induction. Wee1i quickly blocks Wee1 activity, whereas degradation via ID induction may take longer. Consequently, if CDK1/2 is phosphorylated during this period, it may remain so until Cdc25A activation, which occurs late in the G1 phase. Additionally, more cells are arrested in G0/G1 after Wee1 degradation, potentially resulting in lower CDK1/2 phosphorylation levels and fewer changes in the phospho-proteome. Further detailed explanations and analysis of these observed phenotypes will be provided in Section 7.6.

The protein hits did not represent any new cell cycle regulators beside CDKs. Moreover, when analysing the interactors of the protein hits, none of the proteins were involved in cell cycle regulation. This points to a unique role of Wee1 in phosphorylating CDKs during cell cycle re-entry and no other cell cycle regulators. However, proteins involved in cytoskeletal regulation were identified. This points to a role of Wee1 in the regulation of cytoskeletal dynamics during cell cycle re-entry. Whether these proteins are responsible for the delay in S-phase entry and the arrest in G0/G1 or G2 upon Wee1 perturbation remains to be deciphered. Further detailed explanations and analysis of these observed phenotypes will be provided in Section 7.6.

Chapter 7. Discussion.

7.1 Summary of findings.

Cell cycle progression is governed by CDK-cyclin, a regulatory complex pivotal in orchestrating the orderly transitions through each phase (Hartwell et al., 1970; Nurse et al., 1976a; Nurse & Bissett, 1980). Given its fundamental role, the activity of CDK-cyclin undergoes meticulous regulation to ensure the precise sequencing of each element within the cell cycle. Among the regulatory mechanisms, Wee1 kinase plays a crucial role. This kinase modulates CDK-cyclin by inhibiting it through phosphorylation, ensuring a timely and controlled regulation of the cell cycle (Nurse, 1990; P. Russell & Nurse, 1987).

In human cancer, research has unveiled the dependency of cancer cells on Wee1 for survival by inhibiting CDK-cyclin (Bridges et al., 2011a; Mir et al., 2010). The mechanism relied on the activation of the G2 checkpoint to allow cancer cells to undergo arrest, facilitating DNA repair and subsequent cell division without succumbing to cell death. Consequently, a recent innovative approach has surfaced: direct targeting of Wee1 (Bridges et al., 2011a; Squire et al., 2005; M. Zhang et al., 2017). The aim is to prevent cancer cells from arresting and, instead, drive them into mitosis with an accumulation of DNA damage. This ultimately induces mitotic death through mitotic catastrophe. Promisingly, Wee1i have advanced to clinical phase II trials, demonstrating effectiveness in disrupting cancer cell dynamics (Leijen, Van Geel, et al., 2016). Despite these advancements, the comprehensive role of Wee1 throughout the entire cell cycle remains to be fully elucidated. Further research is imperative to unravel the intricacies of Wee1 involvement, paving the way for a deeper understanding of its potential implications in cancer therapeutics. In this thesis, I focussed on the role of Wee1 during entry into the cell cycle from quiescence.

To investigate this, I monitored the impact of Wee1 inhibition during cell cycle re-entry from quiescence on cell cycle dynamics. My underlying hypothesis was that Wee1 inhibition during cell cycle re-entry would result in the hyperactivation of CDK2, leading to an accelerated entry into S-phase. Previous studies had hinted at a similar phenomenon and aligned with this hypothesis. For instance, when experiments were conducted using the non-phosphorylatable CDK1 (CDK1AF) mutant, an acceleration

of cell division was noted. Subsequent investigations using non-phosphorylatable CDK2 (CDK2AF) mutant further supported this trend, revealing an accelerated entry into S-phase. Consequently, I anticipated a similar outcome when I inhibited Wee1 based on these findings.

In the initial phase of my work, I demonstrated that Wee1 inhibition during cell cycle re-entry not only failed to accelerate entry into the S-phase but also induced cell cycle arrest in both G0/G1 and G2 phases (Fig. 3.3, 3.7). Moreover, this manifested within the first 4-6 hours post-cell cycle re-entry (Fig. 3.6). I was able to show that this effect is not attributable to off-target effects of Wee1i and was consistent with other perturbation methods. I generated a Wee1-GFP-AID cell line to observe Wee1 protein dynamics across the cell cycle. Coherent with previous reports, I have shown that Wee1 is mostly nuclear with a rapid switch to the cytoplasm during G2, just before mitotic entry (Fig. 3.5) (Baldin & Ducommun, 1995; Heald et al., 1993). Finally, using different cell lines, I observed that the obtained phenotypes (S-phase entry delay, G0/G1 and G2 arrest) upon Wee1 inhibition during cell cycle re-entry was cell line-dependent.

I decided to investigate the molecular mechanisms behind these phenotypes (S-phase entry delay, G0/G1 and G2 arrest). Outside the role of Wee1 in the regulation of the G2/M checkpoint, Wee1 is also known to have a role in protecting genomic integrity (Elbæk et al., 2022). I hypothesised that Wee1 inhibition dysregulates DNA replication dynamics causing the observed G2 arrest. To do that, I investigated changes in key elements in the DNA replication machinery upon Wee1 inhibition during cell cycle re-entry. I was able to show that Wee1 inhibition induces DNA damage resulting in increased p21 throughout S and G2 leading to the arrest in G2 (Fig. 4.2). Importantly, my findings indicate that the G0/G1 arrest and delayed entry into the S-phase are independent of p21 (Fig 4.3).

Finally, to continue to decipher the molecular mechanisms behind the G0/G1 arrest and S-phase entry delay upon Wee1i inhibition during cell cycle re-entry, I chose to explore potential novel Wee1 interactors. In recent years, Wee1 has demonstrated interactions beyond the conventional CDK1 and CDK2, regulating additional facets

of cellular mechanisms (Andrew V. Grasseti et al., 2021; D'Angiolella et al., 2012; Mahajan & Mahajan, 2013; Matsuo et al., 2003; Vassilopoulos et al., 2015).

First, work from (Vassilopoulos et al., 2015) showed that Wee1 inhibition leads to a hyper-activation of APC/C^{Cdh1}. They were able to show a direct interaction between Wee1 and APC/C^{Cdh1} (Further details about Wee1 and APC/C^{Cdh1} interaction will be discussed in Chapter 7.3). Consequently, with the known role of APC/C^{Cdh1} as a negative regulator of the cell cycle, I hypothesised that APC/C^{Cdh1} hyperactivation upon Wee1 inhibition could cause the S-phase entry delay and G0/G1 arrest (Cappell et al., 2016; Choudhury et al., 2016; Sigi et al., 2009). In my system, upon Wee1 inhibition, I observed a decrease in the levels of APC/C^{Cdh1} G1 substrates such as Cdc6 (Fig 4.4) and Cdc25A (Fig 5.2) suggesting an increased APC/C^{Cdh1} activity. By decreasing Cdh1 levels using siRNA, I was able to show that increased APC/C^{Cdh1} activity in G1 could cause the observed delay in S-phase entry after Wee1 inhibition, but not the G0/G1 arrest (Fig. 5.3).

Finally, to have a better overview of the different potential new Wee1 substrates, I decided to carry out a phosphotyrosine proteomics experiments. By comparing phospho-changes in the proteome following Wee1 inhibition, my hypothesis aimed to identify new Wee1 interactors. Although my experiments did not reveal any significant hits directly regulating the cell cycle to explain the G0/G1 arrest, they did uncover noteworthy alterations in proteins regulating the cytoskeleton (Table 6.1). This observation suggests a broader range of actions for Wee1 beyond its direct cell cycle regulatory role.

7.2 Wee1 is required to restrain CDK2 activity to allow proper origin licensing and efficient S-phase entry.

In this thesis, I demonstrated that inhibiting Wee1 during cell cycle re-entry not only resulted in a delay in S-phase entry but also led to G0/G1 and G2 arrest. These phenotypes observed in this study challenge the conventional understanding of Wee1's role in the inactivation of CDKs. CDK2 is a key regulator of the G1/S transition and its role in modulating G1 length has already been investigated. Indeed, it has been observed that a non-phosphorylatable mutant of CDK2, CDK2^{T14A Y15F} (CDK2AF), causes a shortened G1, rapid cyclin E degradation, abnormal DNA replication, and genome instability (Hughes et al., 2013). It is then even more surprising that Wee1 inhibition during G1 causes a longer G1 in my experiments. However, in the experiments in only the role of CDK2 is monitored and the role of Wee1 during G1 is not explored. Due to the unexpected phenotypes on S-phase entry after Wee1 inhibition, a question that arises in the beginning of this work is that perhaps Wee1 does not regulate CDK2 in G1. Early in my experiments, I used immunostaining to quantify the level of pY15-CDK2 upon Wee1i to assess whether my inhibitors were working (Fig 3.2.A). In these experiments, I showed that pY15-CDK2 decrease upon Wee1i and thus confirming that Wee1 does inhibit CDK2. However, in these experiments, I used asynchronous cells and an antibody which can also recognise the pY15 in CDK1/2/3 and 5. It is then possible to argue that in my experiments the observed decrease in pY15-CDK2/CDK2 ratio could be due to other CDKs and not CDK2. A previous report observed that Wee1 inhibition during G1 did not affect CDK2 activity (Daigh et al., 2018). In their work, they demonstrated, using a CDK2 sensor, that in unperturbed and asynchronous MCF10A, Wee1 inhibition during G1 did not affect CDK2 activity and that increases in CDK2 activity started only after S-phase entry. Thus, confirming that Wee1 potentially does not phosphorylate pY-15 in CDK2. Therefore, we looked specifically at pY15-CDK2 change upon Wee1 inhibition during G1 (Data not shown in this thesis). Dr. Beth Penycook was able to observe a decrease in the pY15-CDK2/CDK2 ratio upon Wee1i during G1. Thus, it seems that Wee1, similarly to the literature, regulates CDK2 through inhibitory phosphorylation. Other experiments that can be done to better understand if the observed phenotypes during G1 upon Wee1 inhibition is due to CDK2 would be to use the CDK2AF cell line. The idea behind this experiment would be to arrest cells in G0/G1

release them into the cell cycle with Wee1i and monitor cell cycle dynamics to observe if the S-phase entry delay as well as the G0/G1 and G2 arrest are still present. During my PhD, I did try to do this experiment, unfortunately I was unable to synchronise this cell line (HCT116-CDK2AF) in G0/G1. Further experiments on cell synchronisation using longer and increased concentration of Palbociclib could be done to try arresting this cell line.

To further understand the relation between CDK2 and Wee1 during G1, I followed CDK2 activity upon Wee1 inhibition during G1. Interestingly, I was able to show in Fig. 4.1.A and Fig. 4.4.E that CDK2 activity increases in G1 in Wee1i treated cells compared to untreated cells. It is to be noted that a difference in CDK2 activity upon Wee1i in these two experiments can be observed. In Fig 4.1.A, CDK2 activity seems to increase before S-phase entry compared to figure Fig. 4.4.E, where CDK2 activity increases at S-phase entry upon Wee1 inhibition. This variation could be due to the difference in CDK2 sensor used in both experiments. In Fig 4.4.E, I used the same sensor as used in (Spencer et al., 2013). This is a fluorescent sensor of CDK2 activity based on the C-terminal fragment of DNA helicase B. When CDK2 is activated, the CDK2 sensor leaves the nucleus. As explained in Chapter 4, by measuring the cytoplasmic to nuclear ratio, the authors were able to quantify CDK2 activity. The sensor used in Fig 4.1.A is used in (Barr et al., 2016). This sensor is a slightly longer version of the one in (Spencer et al., 2013).

Thus, the difference in the construction of these sensors can explain the slight difference in CDK2 activation timing upon Wee1 inhibition.

Further hypothesis about the role of CDK2 and Wee1 can be elucidated. In (Bártová et al., 2004), it has been shown that Y-15CDK2-cyclin E complexes are more stable than unphosphorylated one. This is because cyclin E promotes its own degradation when CDK2-cyclin E complexes are active (Clurman et al., 1996). Additionally, (Hughes et al., 2013), observed that Wee1 inhibition leads to high cyclin E turnover in G1. Thus, inhibiting Wee1 during G1 would be predicted to lead to destabilised CDK2-cyclin E complexes with a high cyclin E turn-over in G1 which could cause the delay in S-phase entry.

The fact that CDK2-cyclin E activity remains unaffected and even increases before S-phase entry following Wee1 inhibition and yet a delay in S-phase entry persists, is

intriguing. Interestingly, (Koboldt et al., 2012) have shown that CDK2-cyclin E premature activation promotes oncogenesis by impairing DNA replication. Elbaek and colleagues (Elbæk et al., 2022), further elucidated the intricate relationship between CDK2, Wee1, and DNA replication, revealing that Wee1 suppresses nuclear activities during S-phase. Specifically, Wee1 inhibits CDK2 to safeguard Hydroxyurea (HU)-stalled reversed replication forks against DNA2-mediated degradation. In the context of this pathway, Wee1 inhibition results in the hyper-activation of CDK2, leading to excessive DNA2 activity and nucleolytic degradation. Additionally, as described in Chapter 4, the appearance and localisation of PCNA foci were disturbed (Fig 4.3.A). All together it led me to think that Wee1 inhibition causes a problem in DNA replication potentially underpinning my observed phenotypes.

Building on findings in (Barr et al., 2017), where elevated levels of damaged DNA induce p21 expression and CDK2 inhibition, promoting G2 arrest, I proposed that Wee1 inhibition may induce aberrant DNA replication, activating p21 downstream of p53 and leading to G2 arrest. I was able to show Wee1i treated cells display an increase in p21 during S-phase and across G2 and that the G2 arrest is p21-dependent. At the moment, no literature reports an increase in p21 levels during S-phase under any perturbation. Interestingly (Barr et al., 2017) and (Heldt et al., 2018), described that p21 protein levels are usually suppressed during S-phase by the PCNA-coupled CRL4^{Cdt2} degradation pathway. CRL4^{Cdt2}-mediated degradation is then turned off at the S-G2 transition by CDK1, allowing p21 to reaccumulate in G2 (Nukina et al., 2018; Rizzardi et al., 2015). CDK1 activity blocks CRL4^{CDT2} by preventing chromatin recruitment of the substrate receptor, CDT2.

One possibility to explain how p21 can accumulate during S-phase would be to hypothesise that Wee1i may lead to premature or hyper-activation of CDK1 and promote the premature termination of CRL4^{Cdt2} degradation during S-phase and allow p21 to accumulate. In order to investigate this question, a strategy involving the combination of Wee1 inhibition and CDK1 inhibition while monitoring p21 levels could be employed. I would arrest RPE1 mRuby-PCNA p21-GFP cells in G0/G1 and release them into the cell cycle with Wee1i, CDK1i or a combination of both. If the observed increase in p21 during S-phase is indeed linked to CDK1-mediated inactivation of CRL4^{Cdt2}, the combined Wee1/CDK1 inhibition should rescue normal p21 levels during S-phase. During my PhD, I carried out such experiments, unfortunately cells treated with CDK1i and a combination of Wee1/CDK1i exhibited signs of distress or

compromised health and thus I was not able to analyse it. Further repeats using titration of CDK1i could be used.

As demonstrated in Chapter 4: Fig. 4.3.C, I could not see any difference in p21-GFP levels in cells that were able to enter mitosis versus cells that arrested in G2 after Wee1i treatment. However, the experiments from where this data is extracted was not thought to looked specifically at this phenotype. For example, in (Barr et al., 2017), the difference in p21 level during G2 in cells which are going to arrest in G2 or cells which are able to enter in mitosis and enter the next G1 is also minimal. Due to the small samples of quantified cells in my experiments, I'm not sure I have the statistical power to see these differences. The same experiment would need to be replicated with more cells to really distinguish if p21 level could explain why some cells can enter mitosis while others cannot.

Data from Chapter 4: Fig. 4.2 suggested a problem in DNA replication upon Wee1 inhibition in cells exiting from quiescence. Indeed, I have shown that cells display replicated DNA with an increase of a 3N population upon Wee1 inhibition. Furthermore, I have also shown an increase in DNA damage in the Wee1i-treated populations. Similar increases in DNA damage during S-phase upon Wee1 inhibition have already been observed in (Beck et al., 2010) and (Beck et al., 2012). In their experiments, immunoblotting revealed that Wee1 depletion led to an activated DNA damage response, as measured by increased phosphorylation of the ATR targets H2AX, RPA, and CHK1 (on Ser317). Furthermore, they linked the increase in DNA damaged to the activity of CDK1/2. By depleting Wee1 and either CDK1/2, they observed no visible increase in DNA damage suggesting the excess DNA damage after Wee1 depletion is mainly through deregulated CDK1 and -2 activity. This data is consistent with my hypothesis that the p21 increase during S-phase in Wee1i treated cells was due to an hyperactivation of CDK1/2. In addition to the previous suggested experiments to look at p21 level upon combination of Wee1 and CDK1/2 perturbation, the same experiments should be done in a fixed assay and DNA damage should be monitored to see if we can obtain a decrease in DNA damage upon Wee1 and CDK1,2 perturbations. In contrary to (Beck et al., 2012), my experiments were not suitable to officially call replication stress but more a perturbations in DNA replication, in order to properly suggest that Wee1i during cell cycle re-entry causes replication stress further experiments would need to be done. DNA fibre analysis is a central

technique to gain mechanistic insight into how genotoxic agents perturb replication fork dynamics genome-wide at single-molecule resolution (Bensimon et al., 1994; Halliwell et al., 2020; Jackson & Pombo, 1998; C. J. Merrick et al., 2004; Técher et al., 2017).

In this assay, progressing replication forks are sequentially labelled with two consecutive thymidine analogues: 5-iodo-2'-deoxyuridine (IdU) and 5-chloro-2'-deoxyuridine (CldU) (C. J. Merrick et al., 2004). Immunostaining of each of these analogues allows for microscopic visualisation of both tracts and to study how replication is perturbed genome wide by cumulative analysis of individual DNA molecules (Técher et al., 2017). This labelling scheme allows monitoring several key replication parameters including changes in replication fork progression, fork symmetry, and origin firing, as well as nucleolytic degradation of nascent DNA following replication stress induction (Vengrova & Dalgaard, 2009). Using DNA fibre assays upon Wee1 inhibition would allow me to monitor potential DNA fork progression impairment.

Additionally, my FACS experiments on DNA distribution upon Wee1 inhibition display a population of cells with under replicated DNA and a population of cells with fully replicated 4N population. I therefore hypothesised that cells able to enter mitosis are the population of cells with fully (4N) replicated DNA, whereas cells arrested in G2 upon Wee1 inhibition are the population of cells in this abnormal <4N population. An interesting experiment that could be done to distinguish the fates of these two populations would be to arrest cells in G0/G1, release them into the cell cycle with or without Wee1i plus nocodazole. Nocodazole blocks the self-assembly of tubulin and thus activates the assembly checkpoint and arrest cells in prometaphase. I would then stain my samples with PI (for DNA staining) and phospho-histone H3 which is a marker of mitotic entry. If my hypothesis were to be correct, I would expect that the population of 4N cells would be arrested in mitosis and thus would be positive to phospho-histone H3, <4N population of cells that I believed are the one arrested in G2 would not be positive to phospho-histone H3 confirming that only cells with fully replicated DNA are the one entering mitosis upon Wee1 inhibition.

From the data presented during Chapter 4, I also hypothesised that due to increased CDK2 activity in G1 after Wee1 inhibition, the period of time to license DNA origins would be reduced. Indeed in (Matson et al., 2019), the authors were able to show that the time that Cdc6 (essential MCM loading protein) is detectable in nuclei is a proxy for the length of time available for MCM loading and thus represents the time

where cells are able to license their origins of replication. Further work from (Mailand & Diffley, 2005), described that CDK2-cyclin E phosphorylates Cdc6 in late G1 to protect it from APC/C^{Cdh1} degradation. In Chapter 4: Fig. 4.A, B, D, I have shown that upon Wee1 inhibition, cells entering S-phase had a reduced licensing window and a lower Cdc6 level prior to S-phase entry.

Together, these data suggest that Wee1 is required to restrain CDK2 activity during G1 such that cells have sufficient time to licence DNA replication origins and initiate robust and efficient DNA replication.

7.3 Wee1 suppresses Cdh1 protein levels and APC/C^{Cdh1} activity to promote normal S-phase entry timing.

It was still confusing as to why Wee1i treated cells with high CDK2 activity in G1 did not enter S-phase earlier than control treated cells. Another significant event that occurs at S-phase entry is inhibition of APC/C^{Cdh1} activity. It has been suggested in (Vassilopoulos et al., 2015) that APC/C^{Cdh1} is overexpressed and hyperactive in Wee1 KO MEFs. The authors were able to confirm a direct interaction between Wee1 and Cdh1 and Cdc20 in cells using reciprocal immunoprecipitation and detect interaction of endogenous Wee1 with Cdc27, another major component of the APC/C complexes. Other components of the APC/C complexes are believed to interact with Wee1, such as APC3/6/7 (Biogrid data). In addition to the direct binding between Wee1 and Cdh1 they were able to highlight that Wee1 KO increases APC/C^{Cdh1} activity. APC/C was immunopurified from mitotic Wee1 KO MEFs and an in-vitro assay was performed using ubiquitination of cyclin B1 as a readout for APC/C activity. Thus, I hypothesised that in my experiments, Wee1 inhibition may lead to hyper-activation of APC/C^{Cdh1} causing G0/G1 arrest and a delay in S-phase entry. In my Chapter 5, I was able to show that Wee1 inhibition does in fact increase Cdh1 protein level in G1 cells and that the combination of Cdh1 and Wee1 perturbations do indeed rescue the timing in S-phase entry but not the G0/G1 arrest.

In addition to analysing the activity of APC/C^{Cdh1} during G1, I also wanted to determine when APC/C^{Cdh1} activity was turned off relative to the G1/S transition (as defined by mRuby-PCNA expression) in Wee1i treated cells. This is particularly important since I see advanced CDK2 activity relative to the G1/S transition and CDK2 activity and APC/C^{Cdh1} switch off are coupled under normal conditions (Cappell et al., 2016, 2018). Geminin and cyclin A2 are two APC/C^{Cdh1} substrates that are not expressed until after the G1/S transition. I have shown that upon Wee1 inhibition both Geminin and cyclin A2 started to increase coincidentally with the G1/S transition. This suggests that Wee1 does not affect the timing of APC/C^{Cdh1} switch off relative to the G1/S transition, only Cdh1 expression levels and its activity during G1.

Interestingly in Chapter 4: Fig. 4.4.A, I noticed that Cdc6 level does increase during G2 and continues to increase in G2 arrested cells (the distinction between Cdc6 level in G2 arrested cells and mitotic cells is not shown on the graph). Furthermore, in

Chapter 5: Fig 5.3.F and G, I could observe that geminin level decreases in G2 arrested cells and that cyclin A2 increases in G2 arrested cells upon Wee1 inhibition.

Due to the importance of the relationship between APC/C and Wee1, further experiments need to be done to better understand this new interactions. In my experiments, I did not show any direct interaction between APC/C^{Cdh1} and Wee1. Despite multiple attempts to observe an interaction using co-immunoprecipitation experiments, unfortunately no conclusive results were obtained. This may be due to several reasons. The first one would be the rapid and transient nature of the binding, emphasising the need to identify optimal sampling times for successful experimentation. Fortunately, recent new approaches to trap transient and weak interacting protein complexes such as crystallisation or cell volume modulation could be used (Chichili et al., 2013; Sukenik et al., 2017). Other technical approaches like FRET can also be used in my experiments. FRET is a non-radiative energy transfer that occurs by dipole–dipole coupling from an excited state donor (D) fluorophore to a ground state acceptor (A; another fluorophore or a quencher) when appropriate spectral overlap and proximity requirements are satisfied (Algar et al., 2019). This technique has been heavily used to observe protein interactions and would be helpful to observe any potential interaction between Cdh1 and Wee1 (Day & Davidson, 2012a, 2012b; Demarco et al., 2006; Okamoto & Sako, 2017).

Another reason that could explain my difficulties to observe Wee1 and APC/C^{Cdh1} interacting together would be simply due to the non-existence of binding between them. However, the latter seems unlikely based on published data and data from high-throughput IP-MS experiments (Biogrid).

Furthermore, in Chapter 5, I only looked at Cdh1 level using western blot analysis upon Wee1 inhibition, and I only used Cdc25A and Cdc6 levels as a direct readout of APC/C^{Cdh1} activity. Using a similar experience from (Vassilopoulos et al., 2015), I could monitor APC/C^{Cdh1} activity in G1 using an activity assay in G1 upon Wee1 inhibition.

Additionally, if my hypothesis is correct other protein could be used as a readout on increase APC/C^{Cdh1} activity. In Chapter 5, using Cdc25A-GFP (a substrate of APC/C^{Cdh1}), I observed that Wee1 inhibition caused a reduction in Cdc25A-GFP expression and that the combination of Wee1 and APC/C^{Cdh1} led to a rescue of Cdc25A-GFP levels at the G1/S transition. Previously used sensor such as Cdc6-

mVenus also helped me in observing change in APC/C^{Cdh1} activity. In Chapter 4: Fig. 4.4.A, I monitored Cdc6-mVenus following Wee1 inhibition. Despite the timing of Cdc6 degradation remaining unchanged upon Wee1 inhibition, I observed a reduction in the maximum level of Cdc6 at the G1/S transition in Wee1i-treated cells. This reduction could potentially be attributed to a hyper-activation of APC/C^{Cdh1}.

In my experimental approach, perturbation of APC/C^{Cdh1} was achieved through siFzr1 to target Cdh1. Exploring additional methods of perturbation could be investigated. Notably, a recent pre-print by (Mouery et al., 2023) used a novel cell line featuring inducible Emi1, an APC/Ci (Feringa et al., 2016). Leveraging this inducible Emi1 system, I could monitor S-phase entry delay in Wee1 and APC/C^{Cdh1} perturbations. Also, the rapid APC/C^{Cdh1} perturbations through Emi1 induction would afford me the opportunity to reiterate experiments, perturbing APC/C^{Cdh1} at various points during G1. This dynamic approach would enable a thorough exploration of the interplay between Wee1 and APC/C^{Cdh1} perturbations, potentially identifying a strategic window in G1 where the combination results in the restoration of wild-type S-phase entry timing. Such insights could prove instrumental in optimising conditions for my co-immunoprecipitation experiments.

Both Wee1 and APC/C^{Cdh1} are considered as negative regulators of the cell cycle progression. Therefore, it is intriguing to consider Wee1 as a negative regulator that counteracts another negative regulator, acting as a double negative regulator in the context of cell cycle regulation.

The molecular mechanisms on how Wee1 inhibits APC/C^{Cdh1} remains unclear. Interestingly using Biogrid (data not shown in this thesis) and recent work (Anna Höfler et al., 2023), further description on APC/C and Cdh1 interaction is available. Cdh1 helix interacts with the APC/C through non-polar interactions with a hydrophobic groove in one of the APC1 subunits via its N-terminal Tyr5, as well as another tyrosine residue Tyr91. According to PhosphositePlus, both Tyr5 and Tyr91 in Cdh1 can be phosphorylated. Therefore, due to its role in Tyr phosphorylation, I hypothesised that Wee1 phosphorylates Tyr5 and Tyr91 on Cdh1 to inhibit its interaction with APC/C. This can be tested *in vitro*. In addition, an interesting experiment would be to mutate these tyrosine sites to non-phosphorylatable residues to prevent potential inhibitory phosphorylation from Wee1. Then, I could look if the activity of APC/C^{Cdh1} is perturbed when mutated and treated with Wee1i.

All together, these data suggest that Wee1 normally acts during G1 to suppress Cdh1 levels and therefore APC/C^{Cdh1} activity to promote normal S-phase entry timing. In the absence of Wee1, APC/C^{Cdh1} switch off is still coordinated with the G1/S transition but CDK2 activity increase is advanced relative to G1/S, suggesting that Wee1 acts to couple events around the G1/S transition to promote normal S-phase entry.

7.4 Wee1 level is heterogeneous.

In this work, I developed a live biosensor to follow Wee1 intensity across the cell cycle. Previous data using fixed-cell assays, described that Wee1 is expressed in the nucleus throughout the cell cycle, before it shifts rapidly to the cytoplasm upon mitotic entry (Baldin & Ducommun, 1995). By using a Wee1-GFP live biosensor and single cell live imaging, I was also able to confirm that the Wee1 localisation is mostly nuclear across all phases before re-localising to the cytoplasm in late G2. It is likely that this spatial regulation is important to limit the activity of Wee1 within the cell. Wee1 is known to inactivate the CDK1-cyclin B complex. It is then possible to argue that Wee1 move to the cytoplasm late G2 to allow CDK1-B to rapidly translocate to the nucleus. Once in the nucleus and in the absence of Wee1 (present now in the cytoplasm), CDK1-cyclin B would promote its activation via double negative feedback loops and thus would be fully activated allowing a correct mitotic progression. To test this, it could be interesting to prevent Wee1 to leave the nucleus and observed if cells are still able to enter mitosis and also to monitor CDK1-cyclin B level. To do that, I could tether a nuclear localisation signals sequence (NLS) to Wee1. NLS are short peptides that act as a signal fragment that mediates the transport of proteins from the cytoplasm into the nucleus (J. Lu et al., 2021). When tether to a protein it will prevent it to move to the cytoplasm. I can hypothesise that if Wee1 is not able to re-localise into the cytoplasm because of the NLS sequence then, it will prevent CDK1-cyclin B activation and block entry or delay mitosis.

Based on the previous reports by (C. Li et al., 2010), CDK1/2-cyclin A is suggested to play an important role in the nuclear export of Wee1 kinase prior to mitotic entry. They identified that in G2 phase CDK1/2-cyclin A phosphorylates Wee1 on Thr239 and further showed that this may be required for the nuclear export of the Wee1 kinase CDK1/2-cyclin A (C. Li et al., 2010). It would be then interesting to monitor Wee1 localisation upon cyclin A perturbations and see if Wee1 re-localise to the cytoplasm even in this condition. Moreover, when I followed Wee1 levels in single cells, I was able to notice a heterogeneity of Wee1 intensity across cells, with cells expressing high Wee1 level and cells expressing lower level of Wee1 intensity (Data not shown in this thesis). It would be interesting to see if Wee1 intensity correlates with the timing into S-phase entry and the length of other phases. It would allow me

to understand if high Wee1 intensity correlates with the time spend into each phases of the cell cycle.

7.5 Interaction between Wee1 and Cdc25A during S-phase entry.

Since I observed that the phenotypes upon Wee1 inhibition was not the one expected, it is interesting to focus a bit more on his supposed analogue Cdc25. For the first time, my lab developed a Cdc25A live biosensor allowing us to monitor Cdc25A protein levels across the cell cycle in real-time (hTert-RPE1 mRuby-PCNA Cdc25A-GFP). As shown in Chapter 5: Fig. 5.2, perturbation of Wee1 led to a decrease in Cdc25A level at the G1/S transition. Furthermore, even in the control population I could observed a heterogeneous population of cells: a fraction of cells expressing high (visible) Cdc25A-GFP at the G1/S transition and another population of cells expressing low level (not visible) of Cdc25A-GFP (data not highlighted in my graph). This led to interesting questions such as how some cells can enter S-phase with a low level of Cdc25A. It will also be interesting to see if this heterogeneity in Cdc25A levels correlates with the heterogeneity in Wee1 level observed before. A double cell line expressing both Cdc25A and Wee1 biosensors could allow to monitor if cells expressing low Cdc25A are the one expressing high Wee1 and vice versa.

Similarly, it would be interesting to observe CDK2 activity upon Cdc25A perturbation and upon combination of Wee1 and Cdc25A perturbation. Of note, I was unable to observed Cdc25A-GFP expression during very early G1, in cells returning to the cell cycle from quiescence. This opens-up the question of whether Cdc25A does regulate CDK4/6 dephosphorylation under normal conditions. In (Terada et al., 1995c), they have shown that Cdc25A overexpression could overcome UV-mediated G1 arrest through CDK4/6 tyrosine dephosphorylation. However, my data suggest that Cdc25A may not be expressed at the right time to regulates CDK4/6 activities. As for the heterogeneous population of Cdc25A expression levels at the G1/S transition, it is possible that the low levels of Cdc25A in early G1 is only due to the limit in signal detection and to the fact that in this construct Cdc25A-GFP is heterozygously tagged.

7.6 Phosphoproteomics experiments upon Wee1 inhibition indicates a potential role for Wee1 in cytoskeleton regulation.

Wee1 has been primarily associated with its inhibitory action on CDKs across the cell cycle. The emergences of unexpected phenotypes upon Wee1 inhibition during cell cycle re-entry suggest a potential for Wee1 to have additional, undiscovered substrates that could play roles in governing cellular processes related to cell cycle progression.

In chapter 6, I conducted phospho-tyrosine experiments to identify potential new Wee1 substrates. The results showed that only upon Wee1i treatment and upon Wee1 degradation, phosphorylation changes were occurring within the proteome. However, in this experiments change in CDK1/2 phosphorylation was used as a control. No changes in phosphorylation on both CDKs could be observed upon Wee1 specific degradation, it has been then decided to focus only on the differences between Wee1i treated cells and DMSO treated cells. This intriguing results gives rise to an essential question on why specific degradation of Wee1 do not change the phospho-proteome. The question about why specific Wee1 degradation does not seem to change the phospho-proteome could be explained by how fast the methods work. Wee1i quickly blocks Wee1, while Wee1 degradation through ID induction could take longer. During this time, if CDK1/2 is phosphorylated, it might stay that way until Cdc25A becomes active, which only happens at the end of G1 phase. Also, more cells get stuck in G0/G1 after Wee1 degradation. These cells might have lower CDK1/2 phosphorylation, which could be the reason that there are not many changes in the phospho-proteome.

A key question would be understanding the timing of Wee1 degradation and how it relates to the phosphorylation of CDK1/2 in cells.

In recent study, (Petrosius et al., 2023), also conducted a phosphoproteomics experiments upon Wee1 inhibition. They showed that dysregulation of Wee1 impacted directly CDK1/2 phosphorylation and also indirectly the DNA damage pathway protein such as 53BP1 by blocking its association with chromatin.

Thus, in accordance with the role of the previously described role of Wee1 in protection genome integrity during S-phase, I can hypothesis that Wee1 may promote the protection of stalled forks from DNA2-driven degradation by specifically limiting CDK-driven phosphorylation of 53BP1. Similarly, to this study, my phosphoproteomics

experiments did not highlight any phosphorylation changes in cell cycle regulators, except CDK1/2.

Furthermore, and as described in Chapter 6, several key proteins playing a role in cytoskeleton regulation had a significant phospho-change upon Wee1 inhibition. For example, one of the hit proteins is MYO9B. Using Myo9B mice, (Hanley et al., 2010) have shown that Myo9B is necessary cell motility and cell shape. Indeed Myo9b^{-/-} macrophages migrated with a strikingly reduced velocity and impaired chemotaxis efficiency. Another protein in my hit list is NUMA1. NUMA1 has been identified as a key factor in the spindle assembly checkpoint and its loss causes cells to elongate their mitotic division time as well as an increase in cell death through mitotic catastrophe. Finally, the hit with the most significant phospho-changes was ACTBL2. It has been shown that ACTBL2 is the seventh actin isoform and is essential for cell motility (Malek et al., 2021). Loss of ACTBL2 or its over-expression causes dysregulated focal adhesion and dysregulated actin filament polymerisation ratio (Malek et al., 2021). Interestingly, nuclear actin plays a significant role in guiding cell cycle progression. Several mechanisms elucidate how the reorganization of the actin cytoskeleton influences cell fate. For instance, disruptions in the cytoskeleton can lead to cell cycle arrest, hindering the sustained activation of MAPK in response to mitogenic stimuli. Consequently, this prevents the induction of Cyclin D and the down-regulation of the cyclin-dependent kinase inhibitor p27KIP, pivotal events for advancing through the cell cycle (Hyrskyluoto & Vartiainen, 2020; Wollscheid & Ulrich, 2023). Thus, nuclear actin emerges as a crucial regulator in orchestrating the progression of the cell cycle by influencing key molecular pathways governing cell division.

Overall, my findings indicate yet undescribed roles of Wee1 regulatory roles on cytoskeleton regulation with potentially substantial impact on the clinical application of Wee1i. However, some questions arise from these experiments. It is not clear if Wee1 directly interact with these proteins to regulate their activity. Specially, Myo9b has been mostly observed in the cytoplasm and it seems unlikely that nucleus Wee1 directly interact with it. Due to its suggesting role in cytoskeleton regulation, it could be hypothesised that Wee1 inhibition disturbs cell motility in my experiments. At the moment, no strong changes in cell motility could be directly observed by looking at the live imaging data, however more specific experiments looking at specific cell mobility could be done. For example, a cell migration assay could be realised as well as monitoring cell velocity upon Wee1 inhibition.

It is important to note that in my experiment, a lot of phosphochanges were happening outside Tyr residues and happened at Ser or Thr residues. p-Tyr modifications represent direct substrate modifications by kinases, and alterations in p-Tyr levels could indeed reflect changes in Wee1 substrates. On the other hand, phosphorylation on Ser and Thr residues could represent downstream target and may result from the activity of other kinases, such as CDK2, triggered by Wee1 inhibition.

Additionally, in my phosphoproteomics experiments, the direction of phosphorylation could change, whether an increase or decrease, and it is a key aspect to consider. In the context of Wee1 inhibition, one would anticipate a decrease in phosphorylation status for substrates directly regulated by Wee1. Therefore, for substrates directly targeted by Wee1, the expectation is a decrease in phosphorylation levels upon Wee1 inhibition. However, downstream effects on other kinases, may lead to an increase in p-Ser/p-Thr due to altered kinase activity cascades.

Interpreting these changes in the context of the cell cycle and specific signaling pathways affected by Wee1 inhibition will be crucial for understanding the broader implications of Wee1i on cellular phosphorylation events. Consideration of these factors will contribute to a comprehensive and nuanced interpretation of your phospho-proteomics data.

Interestingly recent work on CDKs specificity highlighted the existence of CDK thresholds and the specificity of cyclin interactions. However, elucidating a comprehensive mechanism for encoding these thresholds has posed challenges due to the diverse structural and functional characteristics of CDK targets. It is then interesting to think about how CDKs achieve specificity and how altered CDKs function could impact cell cycle progression. CDKs orchestrate the timing and progression of various cell cycle events through phosphorylation of numerous target proteins. Despite extensive research, understanding how CDKs precisely control the temporal order of these events remains a challenge. This complexity arises from the diverse patterns of phosphorylation sites, docking motifs, and phosphorylation epitopes present in CDK target proteins. Traditionally, CDK specificity has been attributed to known consensus phosphorylation motifs, such as T/S-P+1-X-K/R+3, which contain a proline at position +1 and a basic residue at position +3 relative to the phosphorylated residue (Örd et al., 2019). However, studies have revealed phosphorylation of S/T residues by CDK1 even in the absence of a canonical P+1 residue, indicating the

existence of nonconventional CDK phosphorylation sites (Örd et al., 2019). Recent proteomic analyses have underscored the significance of these nonconventional sites in CDK signaling, suggesting that a substantial portion of CDK-mediated signaling may occur through such sites (Sugiyama et al., 2024; Valk et al., 2023). Altogether, these findings emphasize the need for a deeper understanding of how CDKs achieve specificity and how alterations in CDK function through kinase inhibitors could impact cellular processes and ultimately cell cycle progression.

As explained during Chapter 6, specific experiments to monitor the specific changes in Tyr-phosphorylation are on-going. These experiments would allow me to potentially give insight in the molecular mechanisms behind the arrest in G0/G1 upon Wee1 inhibition which is still unresolved. Further limitations of this experiments is the time to collect the samples, a better understanding of when Wee1 play a role during G1 would help me to find the correct time to collect my samples and do check at Tyr-phosphoproteomics changes.

7.7 Synergy upon Wee1 and Myt1 inhibition.

To understand the role of Wee1 during cell cycle re-entry, I first made use of two commercially available Wee1i: MK-1775 and PD-0166285. Both inhibitors demonstrated a dose-dependent reduction in the pY15-CDK/CDK2 ratio, indicative of their inhibition of Wee1. Notably, PD-0166285 exhibited a more pronounced decrease in the pY15-CDK/CDK2 ratio compared to MK-1775 (Fig. 3.2). This is likely to be caused by the lack of sensitivity of PD, as it inhibits not only Wee1 but also other tyrosine kinases such as CHK1, Myt1, c-SRC, EGFR, FGFR1, and PDGFR (Du et al., 2020; H. Wang et al., 2004). CHK1 and Wee1 act in a pathway upstream of CDK1 to inhibit cell cycle progression in response different signals such as DNA damages or replication stress. Inhibition of CHK1 increases the activity of the protein phosphatases Cdc25A/B/C, thereby reducing phosphorylation of Tyr15 and indirectly increasing CDK1/2 activity. Thus, combining Wee1 and CHK1 inhibitors, as it is the case in the PD-0166285 treatment, could explain the greater decrease in the pY15-CDK/CDK2 ratio upon PD-0166285 than compared to MK-1775. However, a previous study in Hela cells could not find the same effect upon drug combination (Guertin et al., 2012). In this study, they were unable to observe a substantial decrease in the

pY15-CDK/CDK2 upon the PD-0166285 treatment compared to MK-1775 alone, suggesting that CHK1 inhibition does not result in an additive inhibition of CDK1/2. However, the study demonstrated a synergistic relationship of Wee1 and CHK1 inhibition with regard to cell proliferation. Here, a continuous exposure to Wee1i or CHK1i for 48 hours did not significantly impact cell proliferation. However, when Wee1 and CHK1 inhibitors were combined, a notable increase of 45% in cell death was observed after 24 hours. This finding suggests that the combined inhibition of CHK1 and Wee1 elicits a synergistic effect on cell viability, highlighting a cooperative role between these two regulators in influencing cell survival dynamics.

This observed phenomenon has been previously documented by (Carrassa et al., 2012) and (K. D. Davies et al., 2011). Both studies showed that using siRNAs against Wee1 transcripts resulted in sensitising solid tumour cell lines to either CHK1 inhibition or CHK1 siRNA. (K. D. Davies et al., 2011) reported synergy between Wee1 inhibition and CHK1 inhibition in four different cell lines, three of which possessed wild-type p53. Similarly, (Carrassa et al., 2012) reported synergy in seven cell lines, irrespective of p53 status. Thus, my findings align with existing literature, indicating that the synergy between Wee1 and CHK1 inhibition is a widespread phenomenon with a ubiquitous underlying mechanism. To study the effect of Wee1, I decided to continue to use the MK-1775 treatment to target specifically Wee1 and no other tyrosine kinases.

7.8 Different methods of synchronisation arrest cells at different point in G0/G1.

To study the role of Wee1 during G1, I carried out live-cell imaging to obtain a population-wide dataset. In my research, I observed consistent phenotypes in Wee1-treated cells which were either arrested through CI or with Palbociclib. However, I found that the cells took a longer time to enter S-phase, even when untreated, when arrested by CI compared to Palbociclib. This disparity in arrest dynamics between the two techniques raises intriguing considerations and prompts further exploration into the underlying differences.

High cell density/confluency leads to CI arrest. This growth arrest is signalled by membrane proteins, and is mediated by elevated p27 (Abercrombie, 1979).

Additionally, p16 has also been suggested to mediate CI arrest, although its expression level does not change appreciably in human or mouse cultures (Serrano et al., 1996; Wieser et al., 1999). More recently it has been shown that cells arrested in quiescence by CI prevented the cells from entering a senescent state due to the inhibition of the mTOR pathway (Leontieva et al., 2014). This inhibition of mTOR suppressed p21-induced senescence and allowed cells to re-enter the cell cycle. In contrast, for Palbociclib (CDK4/6i), the work from (Pennycook & Barr, 2021) demonstrated that Palbociclib-mediated arrest is not dependent on p21 or p27 in RPE1 and MCF7 cells. In their experiments, using a p21-degradable cell line, they have shown that the absence of p21 did not significantly affect the percentage of EdU-positive cells, the percentage of G1 cells or the distribution of p-RB staining. They have thus established that cell cycle arrest in response to Palbociclib can both be initiated and maintained without p21 or p27.

Moreover, within the same study, it was noted that cells treated with Palbociclib for over 48 hours faced increased challenges in re-entering the cell cycle, with a higher proportion of cells failing to resume cycling. This phenomenon was attributed to the initiation of a rise in p21 levels following 48 hours of Palbociclib treatment.

Other studies looking at the effect of CDK4/6i-treated cells observed that arrested cells continue to accumulate biomass and increase in cell volume (Crozier et al., 2022, 2023; D. M. Davies et al., 2022; Nassar et al., 2021; Neurohr et al., 2019). It has been recently observed that this increase in biomass induced by CDK4/6i is sensed by cells and promotes widespread cellular proteome remodelling and p38-p53-p21-dependent cell cycle exit (Crozier et al., 2023).

It is noteworthy that both synchronisation techniques do not employ identical molecular mechanisms to induce cell arrest in G0/G1. It appears that the specific point at which cells are arrested may differ between these techniques. Previous research has introduced the concept of "quiescence depth," illustrating the progressive movement of cells into a deeper quiescent state accompanied by reduced sensitivity to growth signals. This decision to move deeper into quiescence is intricately regulated by a threshold within the Rb-E2F network switch, controlling quiescence depth as elucidated by (Kwon et al., 2017). Notably, cells in a deeper quiescent state exhibit a higher E2F switching threshold and experience a delayed RP passage. At any point during quiescence, cells can take the decision to re-enter the cell cycle. If needed, quiescent cells can also enter a senescence state where their arrest becomes

irreversible. The transition from quiescence to senescence has only recently been characterised, and it is believed to be under the control of C-Myc. Specifically, quiescent cells shift to senescence only after the degradation of C-Myc, as revealed by recent findings (Afifi et al., 2023). Additionally, cells arrested with Palbociclib have already produced cyclin D whereas CI arrested still lack cyclin D. This difference also explain the difference in S-phase entry timing between these two synchronisation methods, where CI arrested cells need more time to produce cyclin D and thus enter S-phase later on compared to Palbociclib arrested cells.

Altogether, this suggests that the cells arrested by CI in my experiments need more time to re-enter the cell cycle due to a “deeper” state of quiescence compared to Palbociclib arrested cells. It could be interesting to compared level of E2F in CI and Palbociclib arrested cells and potentially find in which depth in quiescence Palbociclib arrested cells are.

One intriguing outcome is that the observed delay in S-phase entry as well as the G0/G1 and G2 arrest not only occurs upon Wee1 inhibition but also present and even stronger upon Wee1 perturbations using siWee1 and the auxin-inducible degron system. The discrepancy in outcomes can be attributed to the differing mechanisms of action employed by these perturbation methods. Wee1i inhibits Wee1 molecules specifically, however it's likely that not all molecules are targeted. Different conformational changes and protein aggregations might block the binding of the inhibitor under different conditions. siRNA directly alter the expression of specific genes by degrading its transcripts, leading to less Wee1 molecules within the cell. This enhanced perturbation of Wee1 upon siWee1 compared to Wee1i was previously observed in (Kim et al., 2015). In their experiments, treatment with siWee1 demonstrated a more pronounced reduction in cell viability compared to cells treated with Wee1i. Additionally, the strongest delay in S-phase as well as the G0/G1 and G2 arrest were obtained when Wee1 was specifically degraded using the auxin-inducible degron system. As illustrated in (Fig 3.5.H, I.), Wee1 specific degradation resulted in cells mostly arrested in G0/G1 and barely any cells entering S-phase at all. The use of siRNAs reduces the transcript levels but does not degrade all of them. Hence it is likely that some Wee1 molecules will be produced and active within the cell. I was able to show that upon the auxin treatment Wee1 is completely degraded (Fig 3.5). The higher number of cells entering S-phase with Wee1i treatment compared to other forms of perturbations could be attributed to the time required for the

drug to target the Wee1 protein. Another factor that could explain this difference in number of cells entering S-phase in Wee1i treatment could include Wee1i concentration. During my PhD, I was able to monitor that upon increasing Wee1i concentration (2, 5, 10 μ M), the delay in S-phase entry and the percentage of arrested cells in G0/G1 increases (data not shown in this thesis).

Exploring the window of time necessary to target Wee1 could potentially explain why a significant number of cells still enter S-phase with Wee1i compared to specific degradation of Wee1 and siRNA treatments. For this reason, it would be interesting to test the phosphorylation state of downstream targets of Wee1 upon the different types of perturbation, to understand the temporal pattern of the inhibition of the Wee1 activity. Additionally, I did not track cells after S-phase upon siRNA and Wee1 specific degradation, due to the extreme low number of cells entering S-phase upon both perturbations. Thus, it would be interesting to track the cell cycle dynamics of these cells after S-phase entry to see if the cells are more likely to arrest in G2 or to enter mitosis. This might give insight into the cell cycle dynamics when Wee1 is specially degraded and no other substrates.

Taken together, the difference in the extend of S-phase delay and G1/G0 is due to the kind of Wee1 perturbation I carried out. It is likely that the auxin-inducible degron system results in extremely low levels of Wee1 activity, compared to siRNA and Wee1i, explaining its stronger phenotype.

To gain insights into what happens with the cells arrested in G0/G1, I investigated whether the cells are senescent or in a quiescent state. To answer that, I have carried out an experiment where I arrested cells in G0/G1 using Palbociclib and released my cells into the cell cycle with or without Wee1i (data not shown in this thesis). I then followed and imaged cells for 48h. At the end of this 48h, cells were washed, and fresh media was added. Cells where then imaged again for 24h. The hypothesis behind that was that upon drug washing and serum addition, cells arrested in G0/G1 could potentially re-enter the cell cycle, demonstrating that they were in a “quiescent” phase. Cells arrested in G0/G1 after 48h of Wee1i treatment were monitored and track for 24h after drug washing using live cell imaging. None of the cells arrested in G0/G1 did re-enter the cell cycle. Some limitations of this experiments need to be pointed out. I only imaged the cells for 24h post drug-washing, a time which may not be enough for the cells to re-enter the cell cycle. An increase in the length of imaging

post-washing could be done. Subsequently, the next possibility is to decipher if these cells are in a senescence stage. The Gil lab at the MRC LMS specialises in senescence and has developed a pipeline for determining whether a cell is senescent or not. For this, the lab developed a pipeline using the levels from different proliferative markers such as Rb, p21, p27, KI-67 as well as changes in cell morphology to define if a cell is senescent or not. A potential collaboration with them could provide valuable insights into the characteristics of the G0/G1 arrest observed in my experiments. I would arrest my cells in G0/G1 and release them with Wee1i and EdU (S-phase marker, to define the population of cells arrested in G0/G1 using Wee1i). After 10 to 15 days (period long enough to qualified cells has senescent if still arrested at that point), I would fix and stain with the different necessary marker needed for their pipeline. This would give me more information on the type of cell cycle arrest Wee1-treated cells undergo, whether this is quiescent or senescent.

7.9 Asynchronous and synchronous populations respond differently to Wee1 inhibition.

Across sections 7.3 to 7.3.2, I showed a difference in response between synchronous and asynchronous cell populations (Fig. 3.7 and 3.8). Palbociclib and Cl to synchronise cells in G0/G1, I observed a delay in S-phase entry and the G0/G1 and G2 arrest upon Wee1 inhibition at cell cycle re-entry. However, in asynchronous cycling cell populations, where Wee1 was inhibited in G1, G0/G1 arrest did not occur. Instead, the majority of cells were able to enter S-phase and arrest in G2 or in G1 of the next cell cycle. The probability of arrest in G2 depended on the timing of Wee1 inactivation. Indeed, when Wee1 is inhibited during early G1, cells were more likely to arrest in the first G2. On the other hand, when Wee1 is inhibited during late G1, more cells pass through G2 and M before arresting in G1 of the following cell cycle. The difference in phenotypes upon Wee1 inhibition during cell cycle re-entry in asynchronous populations and synchronous populations may be due to the intrinsic difference in cell cycle regulation between these two different populations. Indeed, there are clear differences in the regulation of G1 in cells coming out of quiescence compared to continuously cycling cells. For example, synchronous populations display a longer G1 before entering S-phase compared to asynchronous

populations. The delayed and heterogeneous cell-cycle re-entry into the cell cycle from quiescence can serve a dual purpose. Firstly, it helps ensure that the cell has gathered sufficient resources allowing the commitment to a full cycle before exiting the quiescent state. Secondly, this delay regulates the production of tissue replenishment. Indeed, this delay is necessary to let cells adequately prepare with the necessary resources before resuming division. This controlled process helps maintain the balance between tissue renewal and the preservation of cellular resources (Coller et al., 2006; Everitts et al., 2013; Kieffer-Kwon et al., 2017; H.-J. Lee et al., 2017). Upon cell cycle re-entry Wee1 can be necessary to help regulate these events. Thus, its inhibition could cause a dysregulation in S-phase entry. The requirement for significant changes in the expression of the cell-cycle machinery, including decreases in CDK inhibitors and increases in cyclins as well as the CDKs themselves, may also contribute to this longer G1 upon quiescence exit. Future work interrogating the differences between cycling cells and cell-cycle re-entry from quiescence will help determine how these regulatory mechanisms underlie cellular proliferation. Furthermore, while cells coming out of quiescence have a G1 restriction point, recent work using single-cell imaging has challenged whether this model is true in continuously cycling cells. Following mitosis, cells either enter a reversible, mitogen-sensitive state where CDK2 activity is low or immediately begin to increase CDK2 activity, entering G1 in a mitogen-insensitive state (Spencer et al., 2013). The cells born with high CDK2 activity have largely committed to the cell cycle when tested by mitogen removal. Thus, rather than a RP in G1, rapidly cycling cells can commit to the cell cycle in the G2 phase of mother cells (Moser et al., 2018; Schwarz et al., 2018). Although cells are no longer sensitive to mitogens past the RP, recent work challenged this idea (Cappell et al., 2016). In their report, the authors were able to show that cellular stress induction after mitogen RP could cause cell-cycle exit.

The authors used Neocarzinostatin (NCS), a commonly used drug to generate DBSs. When treated before APC/C^{Cdh1} inactivation in G1, 20% of the cells were re-entering quiescence compared to 5% in untreated conditions. However, cells exposed to the same reagents after APC/C^{Cdh1} inactivation committed to cell cycle division. APC/C^{Cdh1} inactivation is initiated shortly before the start of DNA replication by CDK2/Cyclin E and made irreversible by Emi1. Exposure to stress between Rb phosphorylation and APC/C^{Cdh1} inactivation, but not after APC/C^{Cdh1} inactivation, reverted cells to a mitogen-sensitive quiescent state, from which they can later re-enter the cell cycle. All

together this highlights the significance of APC/C^{Cdh1} as the key factor in cell cycle proliferation decision. Hence, they identified that the molecular basis for the point of no return for stress sensitivity is the rapid, bi-stable inactivation of APC/C^{Cdh1} (Barr et al., 2016; Cappell et al., 2016).

In chapter 4, I showed that the G2 arrest is p21-dependent. It will be interesting to decipher the reason of the next G1 arrest in the asynchronous population and see if it is also p21 dependent. To do that I could treat an asynchronous population of p21-GFP cell line and p21-KO cell line with Wee1i that I would follow for 48h by live-cell imaging. This would allow me to monitor the cell cycle dynamics and to observe if cells treated with Wee1i during G1 rely on p21 to arrest in the next G1.

Additionally in the asynchronous experiments, I noticed that cells display a delay in S-phase entry independent of the time spent in G1 before Wee1i treatment (Fig. 3.8.C). These results are in clear contradiction with the data obtained in (Fig 3.6.A) where I show that only cells treated with Wee1i 4-6h post release have a delay in S-phase entry. However, the limited number of tracked cells for each bracket of time spent in G1 before drug addition could be a part of the explanation. As these experiments have only been done once another repeat is required to confirm this phenotype. Indeed, it seems unlikely, for example, that cells which already spent 8-10h in G1 when treated with Wee1i would display a further delay in S-phase entry. More cells with smaller bracket of time would show whether the cells still display a delay in S-phase independent of the time spent in G1 before Wee1i treatment.

Another interesting phenotype in this part of my chapter is the decrease in S and G2 length (for cells able to enter mitosis) and the increase of mitosis length in both synchronous and asynchronous populations. The effect of MK-1775 and PD-0166285 (Wee1i and Wee1/Myt1i) on cell cycle dynamics have been investigated previously using fixed-cell assays (Araujo et al., 2016; Chang et al., 2016; Heijink et al., 2015; Krek and Nigg, 1991; Wang et al., 2001). Similarly, to my findings (Lockhead et al., 2020), shows that when cells are treated with Wee1i in S-phase or in G2, the cells display a shortened S and G2 phase in a gradual, concentration-dependent manner. Consistent with previous studies and my work they have also shown by using single cell live imaging that Wee1 inhibition during S or G2 results in long mitotic delays. This delay was only visible when using high concentrations of Wee1i and not when using low ones. However, cells that were treated with Wee1i late in G2 phase tended to enter mitosis more quickly than those treated early in G2 phase.

7.10 Changes in phenotypes upon Wee1 inhibition in different cell lines.

In chapter 4 and 5, I have shown that some cell lines such as RPE1 Cdc6 mVenus PCNA mTurquoise2 DHB mCherry or PIP fucci when treated with Wee1i display a loss of S-phase entry delay upon Wee1 inhibition during cell cycle re-entry. However, in this cell line PCNA is not endogenously tag but over-expressed. It has already been observed that PCNA over-expression led to a shorter G1 which could explain why I can observe a wild type S-phase entry timing in these cell lines (L. Leung et al., 2011).

Another information to take into account about my experiments is that the CRISPR-based knock ins strategy used in the lab led to a not so bright signal which can lead to phototoxicity while imaging at high intensity. In order to image for a cells for a long periods (over 2 days) a compromise needs to be made between the amount of light to which cells are exposed and how frequently the cells are imaged in order to perturb the cells as little as possible. If cells are exposed for long periods of time to a strong light, it may interfere with cell cycle dynamics and may lead to cell death. The amount of light used to monitor cell cycle dynamic in the cell line over-expressing PCNA (RPE1 Cdc6 mVenus PCNA mTurquoise2 DHB mCherry or PIP fucci) and the one expressing endogenously tagged (mRuby-PCNA) were then different. The changes in the amount of light used between my experiments could also explain the variation in S-phase entry timing between these cell lines.

7.11 Wee1 inhibition in cancer cell lines.

Wee1 inhibition is used for the development of new cancer therapies. For this reason, I decided to monitor the effect of Wee1 inhibition during G1 in cancer cell lines. Interestingly, I could observe that the S-phase entry delay as well as G0/G1 and G2 arrest upon Wee1 inhibition, occurs in a cell line-dependent manner. I made use of three different cancer cell lines: two NSLC cancer cell lines (A549, NCI-H1944) and one breast cancer cell line (MCF7). I was able to see a delay in S-phase entry as well as a G0/G1 and G2 arrest in MCF7 upon Wee1 inhibition, similar to that observed in RPE1 cells (Fig. 3.10 and 3.11). However, in the NCI-H1944 no delay in S-phase entry or increase in the arrested population in G0/G1 and G2 phases could

be noticed. However, in all the three cell lines, the cells displayed a shortened S phase and almost abrogate G2 phase as well as a high percentage of cell death in mitosis upon Wee1 inhibition during cell cycle re-entry. Furthermore, when treated with the dual Wee1/Myt1i a population of cells arrested in G0/G1 and again a population of cells died in mitosis.

The difference between these cell lines can be explained by different reasons. While cell lines are known to evolve in culture, the extent of the resultant genetic and transcriptional heterogeneity and its functional consequences remain understudied.

An interesting study from (Ben-David et al., 2018) was able to show the amount of difference in anti-drug treatment in different cancer cell lines. In their experiments they measured the transcriptomic variations across more than 30 cell lines and were able to show that 654 genes were differentially expressed by at least two-fold changes between cell lines. Strikingly they demonstrated that this change in gene transcription led to a disparate drug response where more than 75% of compounds could be completely inactive between the different cell lines. That the different cancer cell lines show different responses upon Wee1i was previously described by (Rødland et al., 2021). The authors explored the sensitivity of four cancer cell lines to Wee1i. First, they show that lung cancer cell lines like H460 and SW900 did responded differently to a Wee1i treatment. The H460 cell line was resistant upon Wee1i, whereas SW900 was highly sensitive and resulted in an increase of cell death upon Wee1i. They were able to show that the majority of the cell lines which are sensitive to Wee1i was dependent on the amount of DNA damage induced by Wee1i. By looking at replication fork dynamics and markers of DNA damage such as ATM, DNA-PK and RPA the cell lines that were most resistant showed unperturbed replication. Similarly, (Ghiasi et al., 2013) showed that in breast cancer cell lines different responses upon Wee1i treatment can be observed but, similarly to my data the Wee1/Myt1i treatment (PD-0166285) increased the sensitivity of the treatment.

The work presented in this thesis shows that different cancer cell lines respond differently to Wee1i when treated during G1. However, it can be noted that none of the cancer cell lines displayed a shortened G1 length, which is a phenotype which would be expected when Wee1 is inhibited during G1. This means that even though not all cell lines respond the same to Wee1i, the effect of Wee1i during G1 never gave the expected phenotypes highlighting an unknown role of Wee1 during G1 phase.

Comparing the cellular response to Wee1i during G1 in cancer cells compared to healthy cells, might give insights into resistance mechanisms which cancer cells employ to survive Wee1i therapy. Understanding how the cell cycle dynamics are affected in cancer cells, will give new ideas of how Wee1i therapies can be optimised and how they can be used to target cancer cells specifically. To further investigate on this, a large-scale analysis should be performed, including a wide pallet of cancer and other, outside RPE1, normal cell lines upon Wee1 inhibition during G1. Additionally, all experiments used in this thesis were done in cell lines with a wild-type p53 status. In human cancer, it is investigated that approximately 50% of cancer are p53 KO (Ozaki & Nakagawara, 2011). It is then necessary to repeat my experiments using a variety of cell lines exhibiting a p53 KO status to observed if Wee1 inhibition during G1 in these cell lines produce the same observed phenotypes (S-phase entry delay, G0/G1 and G2 arrest).

7.12 Wee1 and cell size.

Another hypothesis that emerges to explain my phenotypes upon Wee1 inhibition was linked to the importance of Wee1 in cell size control in yeast. During his work on fission yeast, (Nurse, 1975) was able to identify gene mutants in fission yeast displaying a dysregulated cell size where the cells were abnormally small upon mitosis entry. It led to the discovery of the Wee1 protein which is the main actor of this regulation. Fission yeast grows by linear extension at the cell tips with no change in cell width and then enter mitosis and divide at a threshold size caused by the regulated activation of CDK1 (Fantes & Nurse, 1977; Moreno et al., 1989).

More specifically, a system of protein kinases upstream of Wee1 has been proposed to contribute to size regulation. Pom1, which is a membrane protein, inhibits Cdr1 and Cdr2. Cdr1 and Cdr2 are both Wee1-kinase inhibitors. As cells grows, the concentration of Pom1, decreases. When its concentration drops below a certain threshold, its inhibition on Cdr1 and 2 weakens. Cdr1 and 2, in turn, inhibits Wee1. This inhibition of Wee1 activates CDK1, allowing the cell to enter mitosis. This inhibition ensures that the cell only enters mitosis once it attains a sufficient size, exemplifying the sophisticated control mechanisms that orchestrate proper cell growth and division in fission yeast (J. J. Turner et al., 2012). I then hypothesised that a similar

control could be happening in my experiments. I hypothesised that upon Wee1 inhibition during G1, the cells undergo a disrupted perception of cell size. Subsequently, an unidentified mechanism might detect that these undersized cells are unfit for S-phase entry and intervene by postponing it until they attain the appropriate size.

To investigate on this, I quantified the nuclear sizes of the cells as a proxy for cell size across G1 phases upon Wee1 inhibition. However, I was unable to observe any difference in cell size growth upon Wee1 inhibition (data not shown in this thesis). In addition, there was no difference in cell size at S-phase entry upon Wee1 inhibition (data not shown in this thesis). The fact that nucleus size correlates with cell size has already been demonstrated (Cantwell & Nurse, 2019). Recently, (Y. Wu et al., 2022) developed a more detail model where correlation between cell growth can be found in the change of nucleus size. Still, it can be interesting to specifically look at the entire cell volume change to notice any difference in cell size. First, I could directly measure the cytoplasm volume over time using cytoplasm live biosensor. Different biosensor could be used such as cell membranes or mitochondria dye to quantify cytoplasm volume changes. Furthermore, specific methods to quantify cell size changes can be used. For example, basic microfluidic cell culture chamber with narrow microchannels can be used to calculate the changes in morphology and cell volume on a single cell level. In this experiment cells are injected and pumped using high pressure or manual syringes inside tight channels with sizes a little bit smaller than the cells. Once cells are trapped, medium or drugs can be perfused at lower pressure through the microchannels. This technique was shown to accurately quantify changes in cell size (X. Li et al., 2018).

7.13 Future work.

Cancer cells may adapt to the drug while it is being administered, acquiring molecular changes that allow them to escape its effects. Tumour cells gradually develop resistance during kinase inhibitors therapy; however, the time frame for developing acquired drug resistance is not long. For instance, the effective time frame for treating NSCLC with first-generation epidermal growth factor receptor does not exceed one year. Kinase inhibitor resistance is a leading cause of recurrence, progression, treatment failure, low compliance, and mortality among cancer patients. The mechanisms of kinase inhibitors resistance are complex. Previous studies have affirmed the relationship between kinase inhibitors resistance and target-gene mutations. A growing body of evidence suggests that other factors, such as tumour microenvironment and epigenetics, are also involved in kinase inhibitors resistance. Such resistance to Wee1i have not been yet observed however, it is supposed that it's only a matter of time before such resistance appears. Thus, early investigation is necessary to better understand and improve future cancer therapies. During my PhD, I started investigating this question with the help of Jade-Ellen Brown, then a Master student at Imperial College London. In these experiments, MCF7 cells were cultivated for 4 weeks with the addition of Wee1i and fresh media every 3 days until colony formation. We hypothesised that this colony would be cells that mutated to survive the action of Wee1i. These cells would then be sent to sequencing to see changes in the genome that give the resistance to Wee1i. This experiment couldn't be finished and needs to be repeated. It is interesting to think that if cancer cells are treated with Wee1i while there are in G1 then the subsequent G0/G1 and G2 arrest could prevent cell death and thus led to a resistance mechanism. To further investigate this, a large-scale analysis should be performed, including a wide pallet of cancer cell lines.

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