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Cell cycle remodelling during human embryonic stem cell differentiation

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of Doctor of Philosophy

Declaration

I certify that the work presented in this thesis is my own, and that any work carried out by other has been properly acknowledged and appropriately referenced in the text.

Joseph Matthew Padgett

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Abstract

The cell cycle is a highly coordinated series of events that leads to the duplication and segregation of the cells' DNA into two daughter cells. While the molecular and mechanistic details of cell cycle regulation have been well studied in proliferating, somatic cells, the cell cycle is context-dependent on the specific cell type. How cell cycle networks adapt and change in different contexts is a fundamental, unanswered question. Human embryonic stem cells (hESCs) are a great system to address this question. In their pluripotent state, hESCs are highly proliferative and undergo rapid cell division cycles. The cell cycle of hESC is characterised by truncated gap phases, high activity of cyclin dependent kinases and low levels of CDK inhibitor proteins. Upon differentiation the cell cycle undergoes a remodelling process whereby fully formed gap phases are formed along with the establishment of precise checkpoint control. Here, we have combined advanced live cell imaging of hESC with cell cycle biosensors and computational analyses to monitor cell cycle remodelling at a single cell level during BMP4-driven differentiation of hESCs. By tracking single cells through several lineages, we have quantitatively characterised changes in cell cycle dynamics and the key role of CDK2 during this transition. We see that hESCs exhibit three levels of CDK2 levels: high, intermediate and low. During pluripotency, CDK2 is characterised by a unique, high activity dynamics and as cells undergo differentiation, CDK2 activity lowers to medium or low levels and becomes cyclic. We further show that maintenance of the CDK2 high state requires the synergistic activity of the ubiquitin ligase components Skp2 and Cdh1. The high CDK2 dynamics was shown to be essential to drive a short G1, bypass the G1/S checkpoint and ultimately help maintain the fast division cycles characteristic of hESC. Importantly, we see that high CDK2 activity is key to maintain pluripotency and cell identity. We envision that understanding cell cycle remodelling during differentiation will unravel conserved mechanisms of cell cycle control during early development and will have an impact on our understanding of the balance between health and disease states.

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Abbreviations

AA: Antibiotic-Antimycotic

Ag: Antigen

AI: Artificial Intelligence

AS: Analogue Sensitive

ATM: Ataxia-Telangiectasia

ATP: Adenosine Triphosphate

ATR: Ataxia-Telangiectasia and Rad3-Related Protein

BAX: Bcl-2-Associated X Protein

BER: Base Excision Repair

BMP4: Growth Factor Bone Morphogenic Protein 4

BSA: Bovine Serum Albumin

CAK: CDK Activating Kinase

CDKIs: CDK Inhibitors

CDK: Cyclin Dependent Kinase

CDT1: Chromatin Licensing and DNA Replication Factor

CIP/KIP: CDK Interacting Protein/Kinase Inhibitory Protein

CRISPR: Clustered Regularly Interspaced Short Palindromic Repeats

DAPI: 4',6-Diamidino-2-Phenylindole

DHB: DNA Helicase B

DMSO: Dimethylsulfoxide

dNTPs: Deoxynucleotide Triphosphate

DSBR: Double Strand Break Repair

DDN: Deep Neural Networks

DHFR: Dihydrofolate Reductase

DTT: Dithiothreitol

E6: Essential 6

FRET: Fluorescence Resonance Energy Transfer

FUCCI: Fluorescence Ubiquitin Cell Cycle Indicator

GFP: Green Fluorescent Protein

hESC: Human Embryonic Stem Cell

HRR: Homologous Recombination Repair
YH2AX: Gamma H2A Histone Family Member X
ICM: Inner Cell Mass
IF: Immunofluorescence
IP: Immunoprecipitation
IgG: Immunoglobulin G
IP: Immunoprecipitation
IVF: *In-vitro* Fertilisation
KS: Kolmogorov- Smirnov Test
MAD: Mean Absolute Deviation
MCC: Mitotic Checkpoint Complex
MCM: Minichromosome Maintenance Protein Complex
NCS: Neocarzinostatin
NER: Nucleotide Excision Repair
NES: Nuclear Export Sequence
NHEJ: Non-Homologous End Joining
NLS: Nuclear Localisation Sequence
NE: Neuroectoderm
NEBD: Nuclear Envelope Breakdown
mESC: Mouse Embryonic Stem Cell
PAM: Protospacer Adjacent Motif
PBS: Phosphate Buffer Saline
PCNA: Proliferating Cell Nuclear Antigen
PCR: Polymerase Chain Reaction
PEI: Polyethylenimine
Rb: Retinoblasoma Protein
RIPA: Radioimmunoprecipitation
RT-qPCR: Real-Time Quantitative PCR
RNAi: RNA Interference
ROCKi: Rho Kinase Inhibitor
SCF: Skp2, Cullin, F-box Containing Complex
TAE: Tris-Acetate EDTA
TBS: Tris-Buffered Saline

TMP: Trimethoprim

UV: Ultraviolet Light

YFP: Yellow Fluorescent Protein

Chapter 1. Introduction

1.1 The cell cycle – a historical overview

The cell is the basic structural and functional unit for all living organisms. The existence of cells was not made until the invention of the first microscope by Robert Hooke in 1665, who described them as ‘little boxes’ from cork tissue slices and did not fully understand the significance of what he had observed. It was only much later in the 1830s that German scientists Matthias Schleiden and Theodor Schwann proposed ‘cell theory’, and described these boxes as the basic unit of life which established the field of cell biology.

The cell cycle is a highly coordinated series of events that leads to the duplication and segregation of the hosts DNA into two daughter cells. The basic concept of the cell cycle was first proposed in the late 19th century through the discovery of cell division by Walther Flemming, who pioneered microscopy and staining methods to visualise salamander chromosomes in cells undergoing cell division (Paweletz, 2001). Flemming’s observations of chromosomes aligning and separating into two identical daughter cells which he referred to as ‘mitosis’ provided the first detailed description of the complex process of cell division. This laid the foundations for further studies on the cell cycle and its regulation.

Cell cycle research now encompasses the studies of the sequence of events that occur in the life cycle of a cell, including growth, DNA replication and cell division. The cell cycle is a fundamental process that is conserved across all single-cell and multicellular organisms and is at the heart of understanding the development and maintenance of all living organisms. Furthermore, abnormalities in cell cycle regulation underpin a variety of diseases such as cancer and research over the last 350 years has helped scientists identify potential targets for therapies. Overall, studying the cell cycle is imperative to aid our ability to diagnose and treat diseases, and understand the evolution of life on Earth.

1.2 The eukaryotic cell cycle – an overview

The cell cycle can be compartmentalised into a series of well-defined, ordered events. The majority of the cell cycle occurs during a phase called interphase, whereby the cell grows and replicates its genetic material in the preparation for cell division. Interphase consists of three main stages G1, S and G2. During G1 the cell grows and checks for internal and external environmental availability of growth factors and nutrients that are needed for cell cycle progression. Once these necessary requirements are fulfilled the cell progresses onto S (synthesis) phase, where DNA replication takes place, and the cell duplicates its genetic material. At the end of S phase two identical copies of each chromosome are made before cells progress into G2, where the cell has a final period of growth and preparation for cell division. These sequential events are interspersed with a series of checkpoints at the end of G1 and G2 where cells monitor the status of their genomic integrity before advancing onto the next stage of the cell cycle. When all checkpoint requirements are met and potential DNA damage is repaired, the cell then moves onto the physical separation of the cell into two daughter cells – termed mitosis. Mitosis itself is separated into five phases: prophase, prometaphase, metaphase, anaphase and telophase. During prophase, DNA condenses into visible chromosomes which is accompanied by nuclear envelope breakdown (NEBD) in prometaphase. These chromosomes then align along the equators of the cell during metaphase, allowing spindle fibres to attach to the kinetochores which pull apart the chromosomes to opposite sides of the cell during anaphase. Once chromosomes reach opposite poles of the cell a new nuclear envelope forms around each set of chromosomes and DNA begins to decondense in telophase. Finally, the cell physically splits in two during cytokinesis leaving two genetically identical daughter cells.

While these stages of the cell cycle are true for actively proliferating cells, cells can also enter a state of reversible exit from the cell cycle known as quiescence or G0 phase. If conditions are not favourable for growth or living organisms do not require an increase in cell number, cells can enter a state where they are metabolically active and carry out normal cell function, but do not replicate their DNA in preparation for mitosis. Once conditions are favourable or a cell needs to begin dividing again (such as in a response to injury or tissue regeneration), cells can re-enter the cell cycle and begin dividing again. Cells may also permanently exit the cell

cycle and not undergo further divisions, for example during terminal differentiation of neurons (Buttitta & Edgar, 2007).

The basic mechanisms that control cell cycle regulation are highly conserved across a wide range of organisms. While eukaryotic cells have evolved to have more complex regulation (for example checkpoint control and DNA replication), the core principles that govern cell cycle control have been conserved over billions of years of evolution. This suggests that the cell cycle is an essential and fundamental feature of life.

1.3 Somatic cell cycle regulation

Most of our knowledge of the mammalian cell cycle regulation has come from studying the behaviour of immortalised cell lines in vitro. This approach has been an invaluable tool to monitor how cells respond to different stimuli using elegant genetic, imaging and molecular biology techniques, allowing scientists to probe for the core principles of cell cycle control. While many of these discoveries can be translated to other systems, the dynamics and regulation of the cell cycle across various organisms is highly variable. Furthermore, even within the same species, cell cycle duration can differ drastically. As alluded to earlier, neurons are post-mitotic and do not undergo cellular divisions, while the first few cell divisions in the early embryo last approximately 10 hours, with cells toggling between DNA replication and mitosis without gap phases (Padgett & Santos, 2020). Embryonic stem cells have truncated gap phases resulting in a much shorter cell cycle at approximately 16 hours in comparison to the average proliferating somatic cell at 24 hours (Figure 1-1). Somatic cell cycles can also vary greatly in duration, with pancreatic β -cells having a cell cycle length of 27 hours (Hija et al., 2014), and skin fibroblasts up to 6 weeks (Alberts et al., 2017). This section will highlight the central themes regarding cell cycle regulation with a specific focus on how the cell cycle is orchestrated in somatic cells.

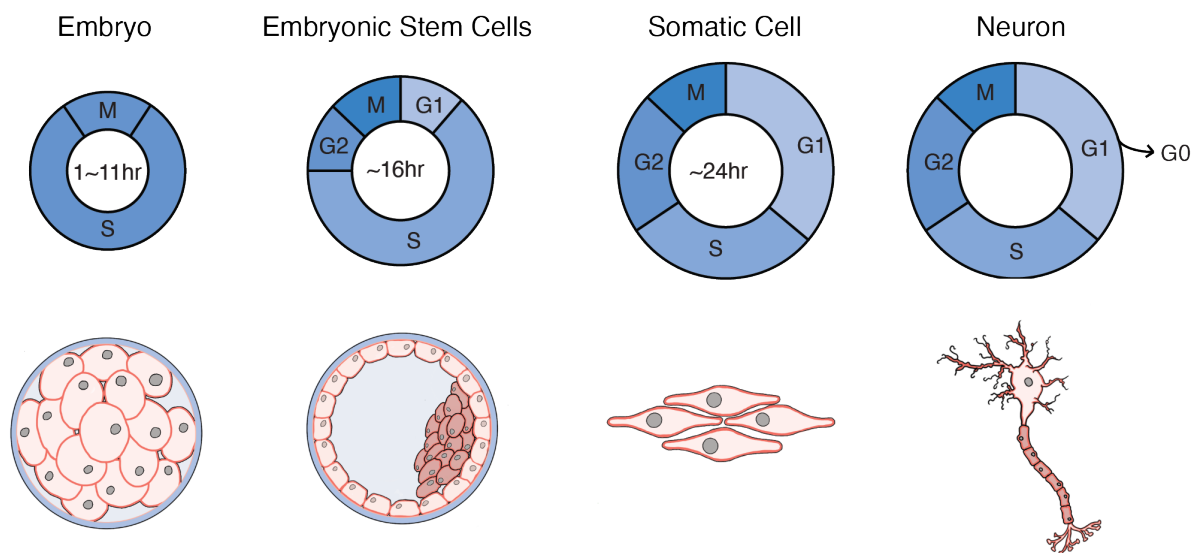


Figure 1-1- Cell cycle change during different stages of mammalian development – Schematic illustrating general cell cycle structure in different mammalian cell subtypes. Cells are not to scale. Adapted from Padgett & Santos 2020.

1.3.1 Cyclin-dependent kinases (CDKs) and cyclin specificity

The cell cycle is driven by cyclin dependent kinases (CDKs). CDKs expression is constant throughout the cell cycle and their activity is reliant on binding to their partner cyclin at different stages of the cell cycle. This triggers the phosphorylation of specific sets of downstream targets that drive the cell cycle forward. Cyclins were initially discovered in sea urchin embryos, where Tim Hunt and colleagues noticed that the levels of a certain proteins varied in a cyclical manner, peaking at specific times during the cell cycle (T. Evans et al., 1983). Further structural and functional studies have identified numerous cyclins which all bind through a conserved protein interaction domain known as the cyclin binding fold (CBF) (Wood & Endicott, 2018). It is now known that regulation of individual phases is tightly linked to specific CDK-Cyclin complexes (Figure 1-2). Furthermore, complexity in cell cycle regulation has been added depending on the organism and cell type, with 20 known CDKs having been discovered in mammalian cells (Malumbres et al., 2009). Only a subset of these CDKs are known to have cell cycle function which have been studied extensively to disentangle the role of each specific CDK-Cyclin complex. These include CDK1, CDK2, CDK4, CDK6 and CDK7. CDK1 is the major CDK involved in the control of the G2 to M transition of the cell cycle. CDK4 and CDK6 is involved in the regulation of G1 phase and CDK2 regulates the G1/S transition and G2. Finally, CDK7 (also known as CDK-activating kinase (CAK)) is required for the activation of other CDKs. This section will highlight how these CDKs are activated by specific cyclins, and their functional roles in the cell cycle.

D type cyclins are a subgroup of cyclins that are involved in regulating the cell cycle in G1 phase. In mammals there are three D-type cyclins, cyclin D1, D2 and D3. D-type cyclins bind to CDKs 4 and 6 forming CDK4/6-Cyclin D complexes (Sherr, 1995). One of the most significant roles of CDK4/6-Cyclin D is the phosphorylation and inactivation of retinoblastoma protein (Rb). Rb is a tumour suppressor that functions to inhibit the activity of E2F transcription factors that promote DNA replication. When Rb is bound to E2F, it prevents the expression of genes required for the cell to progress from G1 to S phase of the cell cycle. Growth factor signalling in G1 increases the expression of D type cyclins and hence CDK4/6 activity resulting in phosphorylation of Rb and partial relief on the inhibition of E2F transcription factors (Harbour & Dean, 2000).

An important role of the E2F transcription factor during the G1/S transition is the regulation of the CDK2 partner cyclins – Cyclin E1 and Cyclin E2 (Siu et al., 2012). E2F binds to Cyclin E promoter regions resulting in an increase in the expression of Cyclin E to form active CDK2-Cyclin E complexes (Ohtani et al., 1995). One of the downstream targets of active CDK2-Cyclin E is again Rb, which is further phosphorylated and inactivated. Rb itself has 14 phosphorylation sites and several models for Rb hyperphosphorylation have been proposed. Work from the Dowdy lab showed that Rb is initially monophosphorylated by CDK4/6-Cyclin, creating 14 isoforms that individually regulate early G1 functions, while CDK2-Cyclin E hyperphosphorylates Rb (Narasimha et al., 2014). This suggests that CDK2 activity alone is required for full inactivation of Rb. Despite this, recent single cell analysis studies have shown that CDK4/6 activity is continuously required to sustain Rb hyperphosphorylation in G1. Hyperphosphorylation by CDK4/6 in G1 was sufficient for Rb inactivation, whereas CDK2 activity was required to maintain Rb hyperphosphorylation after the onset of S phase (Chung et al., 2019; Yang et al., 2020). While there are many models for Rb phosphorylation, hyperphosphorylated Rb results in its full inactivation, leading to further transcription of Cyclin E genes. This positive feedback loop amplifies Cyclin E expression and drives cell cycle progression from G1/S phase. Activated CDK2-Cyclin E then regulates a wide range of cellular processes during S phase. In particular, CDK2-Cyclin E has an important role during DNA replication through the phosphorylation of licensing factors such as Cdc6 (Mailand & Diffley, 2005). This phosphorylation of Cdc6 prevents its degradation and allows pre-replication complexes to form. This assembly of pre-replication complexes prepares DNA for replication by initiating the assembly of replication machinery ensuring that replication occurs only once per cell cycle (Mailand & Diffley, 2005).

Hyperactive CDK2-Cyclin E can result in a variety of induced replication stress, leading to genomic instability (Spruck et al., 1999). It is therefore vital that the activity of CDK2-Cyclin E is tightly controlled during S phase after it has completed its function. The regulation of Cyclin E is predominantly controlled through proteolysis and is degraded after S phase initiation by both Cul3 and SCF ubiquitin ligases (Chu et al., 2021). CDK2 then partners with the S/G2 phase specific cyclins- Cyclin A1 and A2, which have a higher affinity for CDK2, driving S phase CDK2-Cyclin A activation (Merrick et al., 2008). Cyclin A has been shown to be transcriptionally regulated also by E2F (Schulze et al., 1995) and is involved in the regulation of DNA replication and cell cycle progression. Towards the latter stages of G2, Cyclin A-

CDK2 is also implicated in the stabilisation of the mitotic cyclin – Cyclin B1 and B2, by phosphorylating and inhibiting the APC/C ubiquitin ligase component Cdh1 (Lukas et al., 1999) which is involved in the ubiquitination and degradation of Cyclin B. Furthermore, Cyclin A-CDK2 localises to the centrosomes at the end of G2 to control the activation of Cyclin B1 and timing of mitotic entry (De Boer et al., 2008).

The increasing levels of Cyclin B1 in late G2 leads to the formation and activation of the mitosis specific CDK1-Cyclin B1. Cyclin A can also partner with CDK1, which primes cells for mitotic entry by phosphorylating Wee1, weakening the Wee1/Myt1 negative feedback loop that is required for CDK1-Cyclin B1 activation (Deibler & Kirschner, 2010). Cyclin A is then quickly degraded during prometaphase by the APC/C (den Elzen & Pines, 2001), allowing CDK1-Cyclin B1 to coordinate subsequent mitotic events. CDK1-Cyclin B1 initially prepares the cell for mitosis by regulating the condensation of chromatin before coordinating a plethora of mitotic events from nuclear envelope breakdown to spindle assembly. Finally, Cyclin B1-CDK1 is inactivated at the start of anaphase through degradation of Cyclin B1 triggered by the APC/C (Glutzer et al., 1991).

In addition to Cyclin binding, activation of CDKs require a phosphorylation event on the activation loop (also known as the T-loop) by Cyclin dependent kinase activating kinase (CAK). The mammalian CAK, CDK7 partners with Cyclin H and MAT1 and can phosphorylate CDK1, CDK2, CDK4 and CDK6 (Lolli & Johnson, 2005). Structural studies of CDK2-Cyclin A2 showed that phosphorylation by CAK on the T-loop facilitates the binding of protein substrates and arranges them for efficient transfer of phosphor groups (Russo, Jeffrey, & Pavletich, 1996). CDK7 can also be autophosphorylated, however this phosphorylation event is not required to initiate its kinase activity, as binding of MAT1 is sufficient to activate CDK7-Cyclin H complexes (Devault et al., 1995). CAK therefore provides an additional layer of control in activating CDKs. In contrast, inhibition of CDK activity is achieved through phosphorylation at two specific threonine and tyrosine residues (Thr14 and Tyr15) on CDKs by Wee1/Myt1 (Booher et al., 1997; McGowan & Russell, 1995), which can halt cell cycle progression. Cdc25 phosphatases on the other hand remove these inhibitory phosphorylations which can activate CDKs (Boutros et al., 2007). The balance between the activities of Wee1/Myt1 and Cdc25 therefore play a pivotal role in proper cell cycle regulation.

The discovery of this complex network of regulators demonstrates that cell cycle transitions can be ordered by specific CDK-Cyclin complexes and has been termed ‘the qualitative model’ of cell cycle progression (Figure 1.2). While this model sophisticatedly explains how substrate specific targets are phosphorylated at different stages of the cell cycle, there is a large degree of redundancy within the mammalian cell cycle. Numerous knockout studies have shown that while CDK1, Cyclin A2 and Cyclin B1 are essential for embryonic development in mice, most other CDK-Cyclin complexes are dispensable (Brandeis et al., 1998; Martín et al., 2005; Winston et al., 2000). Further studies have established in the absence of CDK2, CDK1 can bind to Cyclin E complexes to drive the G1/S transition (Aleem et al., 2005). This shows that within the mammalian cell cycle there are multiple compensatory mechanisms that enable adaptability to facilitate cell cycle progression. While the qualitative model for cell cycle ordering has provided extensive insight into cell cycle regulation, these studies raise further questions as to what the core principles of cell cycle control are.

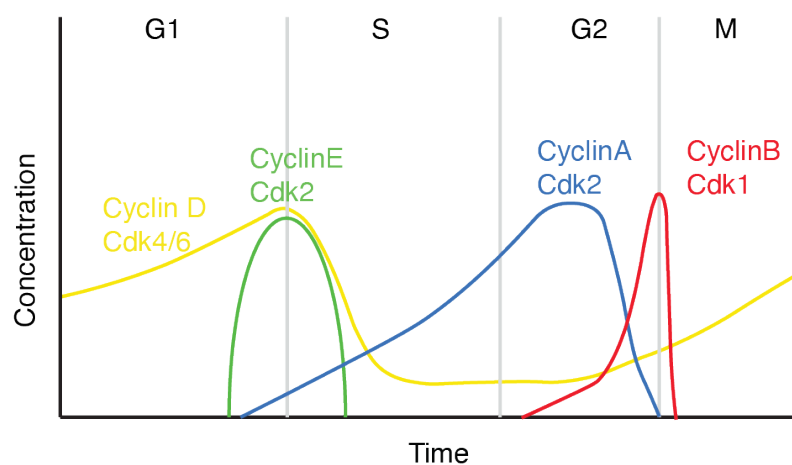


Figure 1-2- Somatic cell cycle regulation– Schematic illustrating the expression of different CDK-Cyclin complexes throughout the cell cycle. According to the qualitative cell cycle model the cyclic expression of different cyclins in different phases of the cell cycle can explain ordering and cell cycle progression. Adapted from Padgett and Santos 2020.

1.3.2 Checkpoint control

Checkpoint control refers to the surveillance mechanisms that monitor internal and external cellular conditions which halt cell cycle progression if criteria are not fulfilled (Hartwell &

Weinert, 1989). Checkpoints provide directionally within the cell cycle by ensuring one phase starts only after the full completion of the other. An activated checkpoint can result in cell cycle arrest to repair potential damage or commitment to programmed cell death. Checkpoints are critical for maintaining genomic stability, as they prevent cells from dividing with damaged DNA, replication stress or incomplete replication.

There are four main cell cycle checkpoints located at the end of G1, G2, mitosis and within S phase (Figure 1-3). The G1/S checkpoint ensures that the cell has adequate resources, and that the DNA is undamaged before entering S phase (Figure 1-3). The intra-S phase checkpoint monitors the progression of DNA replication during S phase of the cell cycle. The G2 checkpoint occurs near the end of G2 phases and checks if DNA replication has been completed effectively and whether the cell is ready to enter mitosis (Figure 1-3). The M checkpoint, known as the spindle assembly checkpoint (SAC) ensures that all chromosomes are correctly aligned on the spindle fibres before the cell proceeds to anaphase (Figure 1-3).

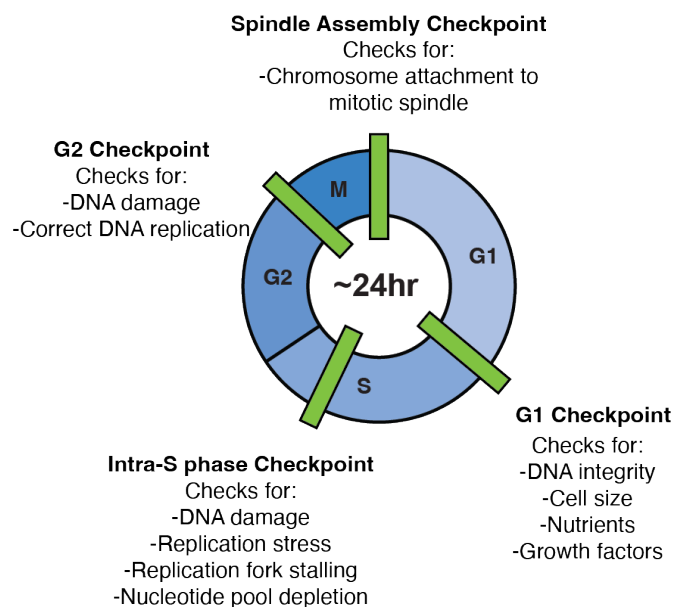


Figure 1-3- Cell cycle checkpoints – There are four major cell cycle checkpoints. The G1 checkpoint checks for DNA integrity, cell size and presence of nutrients and growth factors. The intra-S phase checkpoint monitors the progression of DNA replication during S phase of the cell cycle. The G2 checkpoint checks for DNA damage and correct DNA replication before progressing into M phase. The spindle assembly checkpoint checks for chromosome attachment to mitotic spindles before progressing onto anaphase. Adapted from Padgett and Santos 2020.

1.3.3 G1/S checkpoint control

As alluded to earlier, growth factor signalling plays an important role in the expression of D-type cyclins and activation of CDK4/6. This allows cells to relay information from their external environment to promote cell cycle progression. Studies using mammalian cells first observed that in conditions with low nutrient availability cells accumulated at the same point in G1 phase (Pardee, 1974). Further experiments showed that once there was sufficient growth factor stimulation, cells transitioned into S phase and became independent from growth factor signalling and extracellular queues (Zetterberg & Larsson, 1985). This point in the cell cycle was termed the ‘restriction point’, which refers to when cells are fully committed to entering the cell cycle (Pardee, 1974). Molecularly, it has been shown to depend on the Rb/E2F/Cyclin E positive feedback loop network (Rubin et al., 2020).

Negative regulation of CDKs to halt cell cycle progression at the G1/S checkpoint can also be achieved by the upregulation CDK inhibitors (CDKIs) proteins (Figure 1-4). The levels of these dynamic proteins are increased in response to a wide range of physiological queues such as DNA damage, growth factor signalling and cell-cell interactions. There are two main classes of CDK inhibitor proteins: the INK4 family and the Cip/Kip family (Figure 1-4). The INK4 family includes proteins such as p16, p15, p18 and p19, which specifically bind to CDK4 and CDK6 (Cánepa et al., 2007). The Cip/Kip family includes proteins such as p21, p27 and p57 which can inhibit multiple CDK-Cyclin complexes (Nakayama & Nakayama, 1999). Structurally, most CDKIs are intrinsically disordered and destabilise the interaction between the cyclin and CDK upon binding. In case of Cip/Kip family members, crystal structures of p27 bound to CDK2-Cyclin A revealed that p27 inserts into the catalytic cleft of CDK2, inhibiting the kinase activity of the CDK:Cyclin complex. (Russo, Jeffrey, Patten, et al., 1996). In contrast, INK4 family proteins inhibit CDK activity by binding to monomeric CDK4/6, preventing its interaction with Cyclin D (Ortega et al., 2002). CDKI proteins play a pivotal role in the DNA damage G1/S checkpoint to ensure that DNA damage is repaired before the cell progresses through the cell cycle. DNA damage is first sensed by ataxia telangiectasia mutated (ATM) kinase which leads to downstream phosphorylation and activation of checkpoint kinase 1 (CHK1) and checkpoint kinase 2 (CHK2) (Marechal & Zou, 2013). An immediate consequence of CHK1/2 activation is the rapid ubiquitination and degradation of CDK activating phosphatase Cdc25A (Mailand et al., 2000). CHK1/2 other function is to

stabilise the tumour suppressor protein p53, which is a transcriptional regulator of p21 (el-Deiry et al., 1994). This in turn leads to increased p21 levels which inhibits CDK activity. These two pathways synergistically combine to mediate cell cycle arrest by lowering CDK activity. p21 therefore plays a crucial role in maintaining genomic integrity. This is supported by the fact that p21KO mice have increased tumour susceptibility (Martín-Caballero et al., 2001).

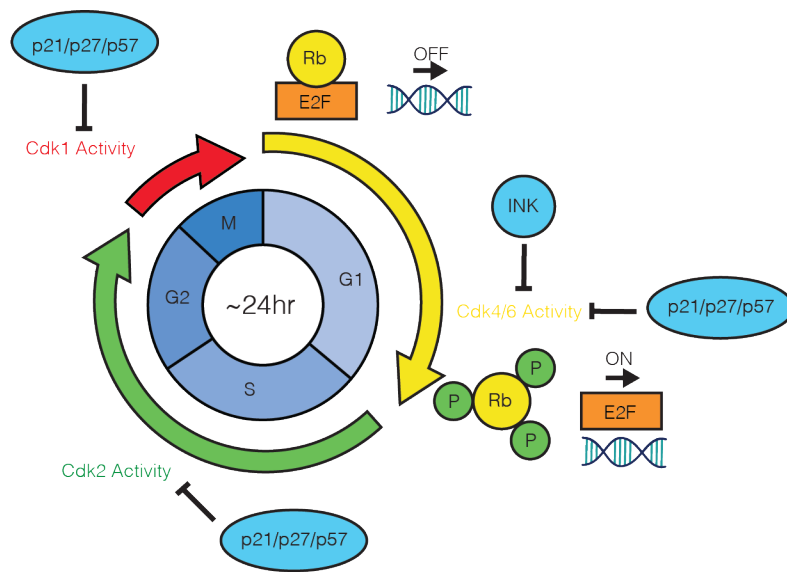


Figure 1-4- G1/S checkpoint control in somatic cells – Schematic illustrating how Rb and E2F transcription regulate G1/S progression. Levels of different CDK activities are shown throughout the cell cycle. CDK activity can be inhibited by upregulation of various CDK inhibitor proteins in response to stress to halt cell cycle progression. Adapted from Padgett and Santos 2020.

An ineffective G1/S checkpoint has been observed in multiple cases of oncogenesis, where bypassing surveillance mechanisms favour uncontrolled cell growth (Molinari, 2000; Paulovich et al., 1997). However even in the context of cells with fully functional checkpoint proteins, the efficiency of the G1/S checkpoint is limited (Chao et al., 2017). An explanation for the heterogeneity in cell fate outcomes in response to DNA damage was found by measuring the levels of CDK2 activity and p21 in response the radiometric drug Neocarzinostatin, which induces DNA double strand breaks (Nathans et al., 2021). Through careful single cell measurements, it was observed that if a cell is with 2 hours of S phase entry, persistent CDK2

activity would drive cell cycle progression onto S phase. This 2-hour delay was a consequence of the time interval between DNA damage and p21 induction, which was required to reach a threshold level before being able to inhibit CDK2. Furthermore, it was shown that rather than arresting at the G1/S transition, cells re-route back to G0 due to the presence of hypo phosphorylated Rb and low CDK2 activity. This challenges the model that checkpoints act as cell cycle ‘breaks’, but rather introduces the idea of switching cellular states to quiescence in response to DNA damage (Nathans et al., 2021).

Although the G1/S DNA damage checkpoint in response to exogenous agents in G1 phase has been reported to be inefficient, inheritance of naturally occurring DNA damage can mediate G1 arrest in daughter G1 cells (Barr et al., 2017). Heterogeneity in p21 expression was found to correlate with levels of endogenous DNA damage which could accumulate in both G1 and G2 phase of the cell cycle. Monitoring the levels of endogenous p21 in live, single cells revealed that p21 was not degraded during mitosis, allowing p21 levels to increase in G1 and mediate cell cycle arrest by inhibiting CDK2. Consistent with other reports (Nathans et al., 2021), it was shown that a threshold level of p21 had to be achieved to inhibit CDK2. At the onset of S phase the CRL4^{Skp2} and SCF^{Cdt2} ubiquitin ligases mediate degradation of p21 and create a bistable switch to promote cell cycle progression (Barr et al., 2017). Other studies have reported similar results and shown that endogenous replication stress in mother cells can influence the proliferation quiescence decision in daughter cells (Arora et al., 2017; Lezaja & Altmeyer, 2018; Yang et al., 2017). These highlight the importance of cellular memory in the inheritance of DNA damage so that cells can safeguard the genomic integrity in future generations.

While the prevailing model for cell cycle commitment at the G1/S transition involves the phosphorylation and inactivation of Rb by CDK4/6- Cyclin D and CDK2-Cyclin E, the molecular events underpinning this transition have come under contention in recent years. Furthermore, the exact point of irreversible commitment to the cell cycle where cells cannot return to quiescence has been an active area of research (Cappell et al., 2016, 2018; Nathans et al., 2021). While hyperphosphorylated Rb has classically been used as a marker for cell cycle commitment, new single cell studies have shed light into the role of APC/C^{Cdh1} in irreversibly committing to the cell cycle (Cappell et al., 2016). The APC/C^{Cdh1} ubiquitin ligase is involved in the degradation of many S phase specific regulators and is inactivated at the G1/S transition.

Using an APC/C^{Cdh1} degron reporter system, Cappell and colleagues showed that there is a large time delay between Rb phosphorylation and APC/C^{Cdh1} inactivation in single cells (Cappell et al., 2016). When combining this with stress perturbations, it was revealed that cells could reversibly return to quiescence in between Rb phosphorylation and APC/C^{Cdh1} inactivation, but not after APC/C^{Cdh1} inactivation (Cappell et al., 2016). The authors propose that a mechanism involving a dual negative feedback loop initiated by increasing CDK2 activity and the APC/C^{Cdh1} inhibitor Emi1, which creates a robust, bistable switch that inactivates the APC/C^{Cdh1} (Cappell et al., 2016, 2018). Hence, it was proposed that APC/C^{Cdh1} inactivation is the point of irreversible commitment to the cell cycle (Cappell et al., 2016, 2018).

1.3.4 Intra-S phase checkpoint

The intra-S phase checkpoint, also known as the S-phase checkpoint or DNA replication checkpoint, is a crucial cellular mechanism that monitors the progression of DNA replication during S phase of the cell cycle. This checkpoint ensures the accuracy and completeness of DNA replication and responds to various forms of DNA damage or replication stress, such as DNA lesions, replication fork stalling or nucleotide pool depletion (Iyer & Rhind, 2017). The intra-S checkpoint achieves this mainly by controlling origin firing and fork progression. DNA lesions during replication cause replicative polymerases to stall, resulting in the exposure of single stranded DNA (ssDNA). Replication protein A (RPA) binds to exposed ssDNA regions which serves as a signal for the activation of the ATR-Chk1 pathway (Iyer & Rhind, 2017). Upon activation of the intra-S phase checkpoint, replication forks are stalled and stabilised which provides the cell with time to repair damaged DNA or resolve replication problems. In addition to this, the ATR-Chk1 signalling pathway blocks cell cycle progression, decreases origin firing and initiates the restart of collapsed replication forks (Ciardo et al., 2019). The intra-S phase checkpoint is therefore a critical surveillance mechanism that ensures the fidelity of DNA replication.

1.3.5 G2/M checkpoint

The G2/M checkpoint serves to ensure that the cell has completed DNA replication and is properly prepared for mitotic division. If any errors in replication or DNA damage are detected,

the G2/M checkpoint halts the cell cycle and initiates DNA repair mechanisms before allowing the cell to enter mitosis. At the heart of the G2/M checkpoint is the regulation of the mitotic CDK-Cyclin complex, CDK1-Cyclin B1. Like the G1/S checkpoint, if double strand breaks are detected this leads to downstream activation of ATM/CHK1/CHK2 kinases (Furnari et al., 1997). This deactivates the phosphatase CDC25C which dephosphorylates and activates CDK1-Cyclin B1 (Furnari et al., 1997). In addition to this, the DNA damage response activates p53 which induces the expression of p21 and GADD45 (Vairapandi et al., 2002). Expression of p21 functions to inhibit the activity of CDK1-Cyclin B1 while GADD45 activates DNA repair mechanisms and simultaneously binds to CDK1, preventing its interaction with Cyclin B1 (Vairapandi et al., 2002). Together, these molecular events prevent cells from entering mitosis and safeguard genomic stability.

1.3.6 Spindle assembly checkpoint (SAC)

The spindle assembly checkpoint (SAC) ensures the accurate segregation of chromosomes during cell division. This regulatory system monitors the attachment of kinetochores to spindle microtubules and delays the onset of anaphase until all chromosomes are properly aligned and attached (Musacchio & Salmon, 2007). If any kinetochore is unattached, a signalling complex called the mitotic checkpoint complex (MCC) inhibits the APC/C cofactor Cdc20, preventing APC/C activation (Nilsson et al., 2008). The MCC is negatively regulated upon kinetochore attachment and spindle alignment, which allows Cdc20 to bind to the APC/C which promotes its activation. Two important substrates of the APC/C, securin and Cyclin B are then quickly ubiquitinated and degraded (Chang et al., 2003; Zachariae & Nasmyth, 1999). Securin acts as an inhibitor of separase, preventing it from cleaving cohesin proteins that hold the sister chromatids together. Destruction of securin therefore leads to the separation of sister chromatids and allows anaphase to progress. Cyclin B proteolysis is required to inactivate CDK1, which initiates mitotic exit and cytokinesis. The SAC therefore serves as a final safety apparatus to ensure faithful segregation and identical inheritance of hosts DNA into two daughter cells.

1.3.7 The quantitative model for cell cycle ordering

Due to the reported redundancy of CDK-Cyclin complexes, scientists have set out to identify the minimum set of components necessary to drive the cell cycle. In mouse embryos, removal of all interphase CDKS (CDK2, CDK4, CDK6) is still compatible with development up until midgestation, whereas removal of CDK1 results in embryos failing to develop to the morula and blastocyst stage (Santamaría et al., 2007). In the absence of interphase CDKs, CDK1 can bind to and form active complexes with all cyclins, which facilitates cell cycle progression (Santamaría et al., 2007). This shows that CDK1 alone is sufficient to drive the mammalian cell cycle (Santamaría et al., 2007). However, due to the complexity in cell cycle regulation in mammalian cells, it has been difficult to underpin the underlying principles of cell cycle control. Experiments employing simpler eukaryotic model organisms such as the fission yeast *Schizosaccharomyces pombe* have therefore been a useful tool to understand these minimum requirements. In fission yeast, a single CDK – Cdc2 drives the cell cycle and partners with three B-type cyclins, Cdc13, Cig1 and Cig2. Deletion of the interphase specific cyclins Cig1 and Cig2 results in impaired G1/S progression, however this is also mirrored by an increase in Cdc13 levels to compensate for the loss of interphase cyclins, which can also control entry into S phase (Martin-Castellanos et al., 1996). A new model was then proposed by Bodo Stern and Paul Nurse in 1996 that hypothesized that cell cycle progression was initiated by increasing levels of Cdc2 activity (Stern & Nurse, 1996). The framework of the model suggested that incremental threshold of CDK activity, rather than CDK-Cyclin specificity ordered cell cycle progression, where S phase is initiated when CDK activity increases from low to moderate activity, and a further increase in activity triggers mitosis (Figure 1-5).

Termed the quantitative model of cell cycle progression, this model was validated with experiments in fission yeast expression using a fusion protein of Cdc13-Cdc2 (termed M-CDK), while knocking out the endogenous cyclins, Cdc13 and Cig2 (Coudreuse & Nurse, 2010). This simplified cell cycle control network was able to sustain cell cycle progression utilising just one biochemical oscillator and provided fascinating insights into cell cycle ordering (Coudreuse & Nurse, 2010). By modulating Cdc13-Cdc2 activity, it was found that different thresholds of CDK activity were required for entry into S and M phases. Further phosphoproteomic studies revealed that the ordering of cell cycle substrates can be explained by a substrate specific activity threshold. Using the same Cdc13-Cdc2 fusion protein, it was

shown that early cell cycle substrates had a high sensitivity for CDK activity, whereas late substrates had a lower sensitivity (Swaffer et al., 2016).

In mammalian cells, it has been shown that increasing levels of CDK1-Cyclin B1 activity initiate different mitotic events using a CDK1-Cyclin B1 FRET activity sensor (Gavet & Pines, 2010). Despite this, as advanced eukaryotic organisms have become dependent on the additional cell cycle control machinery, it has been difficult to recapitulate the quantitative model in mammalian cells. Furthermore, when substituting the mitotic cyclin Cdc13 for the S phase specific cyclin Cig2 (termed S-CDK), fission yeast are unable to fully complete mitosis (Zarzov, 2002). This challenges the robustness of the quantitative model, however recent studies have shed light onto how both the quantitative and qualitative model can be reconciled (Basu et al., 2022). Using both monomeric S cyclins and M cyclins fusion CDKs, the authors were able to show that while M-CDK fusion proteins could fully complete mitosis, S-CDKs could only trigger mitosis but were unable to form spindles (Basu et al., 2022). Despite this, phosphoproteomic assays revealed that most of the S-CDK and M-CDK substrates are similar, supporting the quantitative model that different cyclins can be substituted for one another. Only 11 substrates were M-CDK specific, which included one of the main opposing phosphatases for CDK activity -PP1. The S-CDK phenotype could be rescued when PP1 was removed, which enabled cells to complete mitosis (Basu et al., 2022). Further genetic experiments revealed that a phosphorylation event on PP1 removes it from centrosomes. S-CDKs were able to fully complete mitosis when mutating the spindle pole adaptor protein Cut12 so that PP1 could no longer bind. While this study predominantly supports the quantitative model for cell cycle ordering due to the overwhelming overlap in substrate specificities between M and S-CDKs, there are small qualitative differences even in simple eukaryotes. This study also highlights the importance of the spatial regulation of CDK activity (Basu et al., 2022) (Santos et al., 2012). Altogether these studies underscore the importance of having a quantitative understanding of the activities of CDK-Cyclin to dissect principles of cell cycle control.

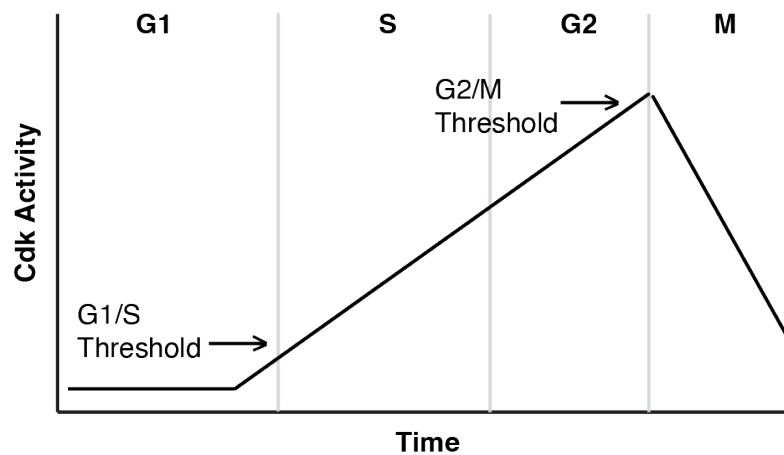


Figure 1-5- **Quantitative model for cell cycle ordering** – Cell cycle ordering is achieved via increasing levels of CDK activity. A low CDK activity threshold regulates the G1/S transition, and a higher CDK activity threshold regulates the G2/M transition.

1.3.8 Control of the cell cycle by degradation machinery

The periodic accumulation and removal of cell cycle regulators control key cell cycle transitions. This helps ensure that each phase of the cell cycle occurs in the correct order and at the appropriate time. E3 ubiquitin ligases are a family of enzymes that play a crucial role in determining the relative abundance of these regulators by mediating degradation of cell cycle machinery. A variety of ubiquitin ligases operate in the mammalian cell cycle within defined windows, but all follow the same molecular steps to achieve protein degradation. In the first step, a small 8 kDa protein named ubiquitin is activated by the ubiquitin-activating enzyme (E1) in an ATP dependent reaction before being transferred and covalently attached to an active cysteine residue on the ubiquitin-conjugating enzyme (E2). The ubiquitin loaded E2 enzyme then docks onto the ubiquitin ligase enzyme (E3) which catalyses the transfer of ubiquitin onto the target substrate. This process is repeated multiple times until the formation of a polyubiquitin chain on the substrate marks the target protein for degradation by the proteasome – a large multi-subunit protein complex that acts as the cells recycling centre (Dang et al., 2021).

Ubiquitin ligases play an important role in regulating the activity of CDKs, as substrates which include cyclins and CDKI proteins are degraded by these complexes. Degradation of cell cycle components are controlled predominantly via two sets E3 ubiquitin ligases, the APC/C and

SCF complexes which have different substrate specificities and cell cycle activities (Figure 1-6).

The APC/C is a multi-subunit complex whose activity is tightly regulated during the cell cycle by the binding of co-activators. APC/C complexes recognise substrates with a D-box (RxxLxxxxN) degron but can recognise additional motifs depending on which co-activator is bound (Dang et al., 2021). As previously mentioned, binding of the co-activator Cdc20 during mitosis activates the APC/C and mediates degradation of Cyclin B1 and securin, facilitating mitotic exit and has important roles in safeguarding the SAC. A consequence of Cyclin B1 proteolysis is the loss of CDK1 activity, which when active phosphorylates and inhibits another APC/C co-activator Cdh1 (Crasta et al., 2008). In yeast, degradation of securin relieves its inhibition on separase, promoting the separation of sister chromatids along with the activation of the phosphatase Cdc14. Cdc14 removes an inhibitory phosphorylation on Cdh1 (Sullivan & Uhlmann, 2003), allowing active APC/C^{Cdh1} complexes to quickly form in early G1. As well as D-box degrons, APC/C^{Cdh1} can target substrates with a KEN-box (KENxxxN) motif (Dang et al., 2021). APC/C^{Cdh1} activity then targets several mitotic regulators for degradation including Cyclin B1 and Cdc20, along with S phase regulators such as Skp2 and Cyclin A. Consistent with this, knocking down Cdh1 results in a shortened G1 phase (Peters, 2006). The switching of APC/C co-activators from mitosis to G1 therefore acts to remove phase specific regulators and provides unidirectionality during the cell cycle.

At the onset of S phase, APC/C^{Cdh1} needs to be inactivated to allow cell cycle progression. This is achieved through E2F dependent transcription of the APC/C^{Cdh1} inhibitor Emi1 (Hsu et al., 2002). Removal of Emi1 results in delayed APC/C^{Cdh1} inactivation and has been shown to be important in the bistability and hysteresis involved in APC/C^{Cdh1} inactivation (Cappell et al., 2016, 2018). This is achieved via a dual-negative feedback mechanism, where Emi1 acts as both a substrate and an inhibitor of the APC/C^{Cdh1} (Cappell et al., 2018). This sophisticated mechanism allows for an all or nothing bistable switch at the G1/S transition, and stabilises key proteins involved in S phase.

The deactivation of the APC/C^{Cdh1} at the end of G1 makes way for the activation of the S phase specific E3 ubiquitin ligase SCF^{Skp2}. SCF complexes contain three essential subunits, Cullin, Skp1, RBX1 and have a broad range of substrates due to the numerous F-Box proteins that can

bind to SCF (Dang et al., 2021). However only a subset of the F-box proteins have cell cycle functions, of which one of the most well characterised is Skp2. The co-activator for the SCF complex Skp2 is itself a target substrate for the APC/C^{Cdh1}, so its inhibition results in the accumulation of Skp2 (Wei et al., 2004). Furthermore, phosphorylation of Skp2 by CDK2-Cyclin E at the G1/S transition inhibits its proteasomal degradation by the APC/C^{Cdh1} which further stabilises Skp2 (Rodier et al., 2008). This results in the formation of active SCF^{Skp2} complexes that primarily target CDKI proteins p27, p21 and p57 for degradation. Prior to p27s degradation, CDK2-Cyclin E phosphorylates p27 which mediates substrate recognition and ubiquitination by SCF^{Skp2} (Gstaiger et al., 2001). The removal of CDKI proteins contributes to the positive feedback loop that fully activates CDK2-Cyclin E which promote cell cycle progression during S phase. Since SCF^{Skp2} targets tumour suppressor proteins for degradation, Skp2 functions as a positive regulator of the cell cycle. As such, overexpression of Skp2 has been noted in several cases of human cancers (Gstaiger et al., 2001).

The accumulation of Cyclin A during S phase due to APC/C^{Cdh1} inactivation brings about the formation of active CDK2-Cyclin A complexes to drive G2 phase. Furthermore, the efficient switching of Cyclin E to Cyclin A is regulated by SCF^{Skp2} mediated degradation of Cyclin E to assist sequential ordering of CDK2 activity complexes (Nakayama, 2000). An additional SCF complex, SCF^{Fbxw7} is also implicated in the degradation of Cyclin E which is initiated by multisite phosphorylation of Cyclin E by either CDK2 itself or glycogen synthase kinase 3 β (GSK3 β) (Hao et al., 2007; Minella et al., 2005). Several mechanisms lead to the deactivation of the SCF^{Skp2}. Transcriptionally, periodic expression of Skp2 is thought to be achieved via cell cycle dependent binding of GABP and E2F to Skp2 promoter regions (Imaki et al., 2003; L. Zhang & Wang, 2006). However, Skp2 levels are predominantly controlled post translationally by Cdh1, which binds to the Skp2 D box motif, leading to the ubiquitination and degradation by the APC/C^{Cdh1} to keep Skp2 levels low in G1 phase (Wei et al., 2004). In conclusion, there is a dynamic relationship between the ubiquitin ligases involved in cell cycle regulation. The multiple feedback mechanisms and complex interplay of E3 ubiquitin ligases ensure that protein degradation of cell cycle regulators is exquisitely controlled.

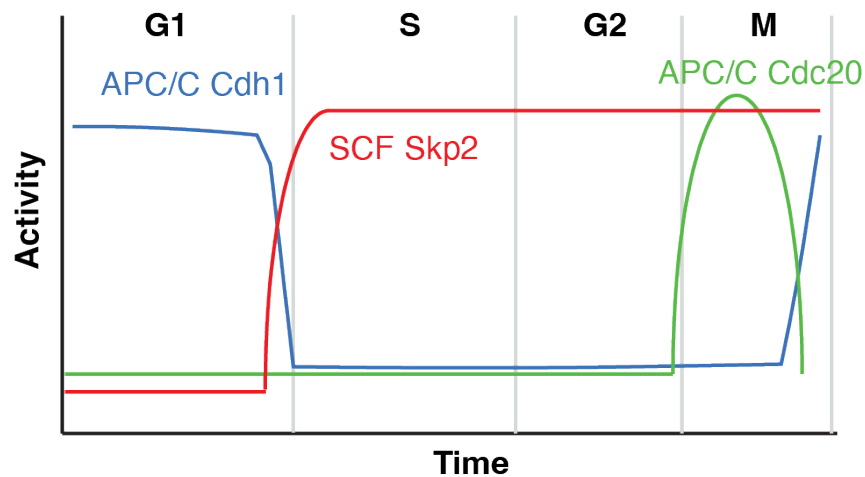


Figure 1-6- **Control of the cell cycle by E3 ubiquitin ligases** – Schematic illustrating the activities of different E3 ubiquitin ligases throughout the cell cycle. APC/C^{Cdh1} is active in late M phase and G1. SCF^{Skp2} is active in S/G2/M phases of the cell cycle. APC/C^{Cdc20} is active in mitosis. Adapted from Padgett and Santos 2020.

Organised waves of cyclin expression and degradation are a fundamental feature of eukaryotic cell cycle regulation. Although there is evolutionary conserved regulation of the cell cycle by CDKS, primitive organisms such as the archaea *Sulfolobus acidocaldarius* lack cyclins or CDKS (Tarrason Risa et al., 2020). Despite this, *Sulfolobus acidocaldarius* still display cell cycle dependent gene expression and proteasomal mediated degradation of key targets such as the ESCRT-III homologous protein CdvB to complete cell division (Tarrason Risa et al., 2020). CdvB assembles to form part of a contractile ring to initiate cytokinesis in *Sulfolobus acidocaldarius* and degradation of CdvB allows constriction of this ring to separate the cell into two daughter cells. Treatment of *Sulfolobus acidocaldarius* with the proteasomal inhibitor Bortezomib resulted in cell cycle arrest as the contractile ring can no longer constrict and disassemble properly. These evolutionary studies highlight the importance of protein degradation at the heart of cell cycle control.

1.4 The embryonic stem cell cycle

During early embryonic development, the first few cleavage cell divisions from a fertilised zygote result in a “ball” of cells called the morula (Figure 1.11). The cells of the morula are still totipotent and can give rise to all the cells in the developing organism. Cells of the morula then segregate to form two distinct cellular lineages, the inner cell mass (ICM) and the trophectoderm, which can differentiate into embryonic and extra embryonic lineages respectively, forming a structure known as the blastocyst (Thomson et al., 1998) (Figure 1.11).

Embryonic stem cells (ESs) are derived from the inner cell mass of a developing blastocyst and have the following characteristics. 1) Pluripotency: ES have the ability to differentiate into all the three primary germ layers which are formed during gastrulation (endoderm, ectoderm and mesoderm), meaning they can give rise to all cell types in the body, 2) Self-renewal: ES cells have the ability to divide and reproduce indefinitely while maintaining pluripotency and 3) *In-vitro* culture: ES cells can be maintained outside of the embryo in specific culture conditions that allow them to proliferate and maintain pluripotency. These unique features make ES cells a valuable tool for studying early development, as well as for potential therapeutic uses such as regenerative medicine (Amit et al., 2000; M. J. Evans & Kaufman, 1981; Thomson et al., 1998).

One of the most fascinating characteristics of embryonic stems is their high proliferation rate with short cell cycle lengths. Embryonic stem cells have on average a cell cycle length of approximately 10-16 hours depending on the species, in comparison to the average proliferating somatic cells which has an average cell cycle duration of approximately 24 hours (Becker et al., 2006; Fluckiger et al., 2006; Waisman et al., 2019). Furthermore, the cell cycle structure in ES cells is characterised by truncated gap phases, with G1 being only 1 hour in mouse ES (mES) and 2.5 hours in human ES (hES), comparatively to 8-10 hours in cycling somatic cells (Becker et al., 2006; White & Dalton, 2005). ES cell cycles are sustained by reorganisation of fundamental cell cycle control mechanisms which include: 1) Cell cycle independent cyclin expression, 2) Decreased expression of cell cycle inhibitors and 3) Modified checkpoint control (Figure 1-7 and Table 1-1). This section will highlight these adaptations that ensure rapid proliferation rates in ES cells.

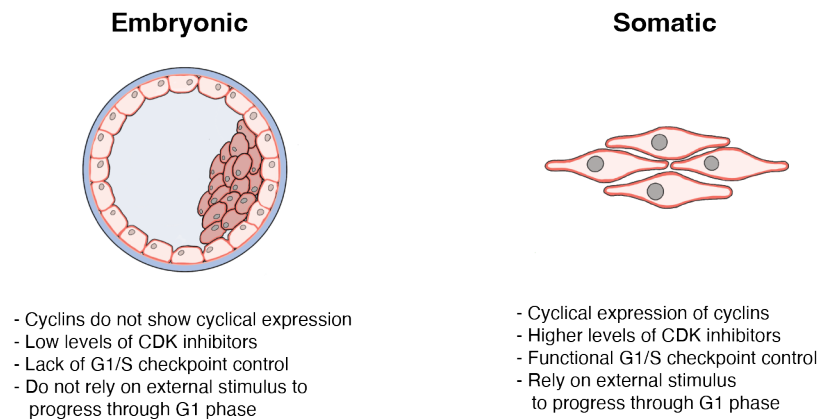


Figure 1-7- Differences in cell cycle regulation between ES and somatic cells – Schematic highlighting the key difference in cell cycle regulation between ES and somatic cells.

1.4.1 Embryonic stem cells have unique CDK-Cyclin activity dynamics

The first notable difference in the cell cycle of embryonic stem cells is the regulation of CDK activity. Differential CDK-Cyclin activities play a pivotal role in coordinating the cell cycle in somatic cells, but cyclin levels appear to be altered in the pluripotent state. In mES cells, the cyclical activity of CDK2 is thought to be largely absent via the constitutive expression of Cyclin E and A and remains active throughout the cell cycle (Fujii-Yamamoto et al., 2005; Stead et al., 2002; ter Huurne et al., 2017). The only CDK-Cyclin complex that has oscillatory activity dynamics is the mitotic CDK, CDK1- Cyclin B1, however ES cell still show increased levels of Cyclin B1 and higher CDK1 activity compared to somatic cells (Fujii-Yamamoto et al., 2005; Michowski et al., 2020; Stead et al., 2002; X. Q. Wang et al., 2017). Conversely, CDK4/6 appears to have minimal activity, due to low expression of all D-type cyclins (ter Huurne et al., 2017), though it is undetermined whether mESC display low CDK4/6 activity or this is a consequence of a reduced G1 phase. Elevated CDK2 activity is also seen in hES cells, however most cyclins exhibit periodic waves of expression, but at a reduced amplitude to their differentiated counterpart (Becker et al., 2006; Neganova et al., 2009). While we have some insight into how the activities of CDKs differ in ES cells, dynamic measurements in single cells to quantitatively measure how these activities differ in somatic cells are limited.

1.4.2 Embryonic stem cells have low levels of CDK inhibitor proteins

CDK inhibitor proteins are involved in binding to and inhibiting the activity of CDKs. While CDKIs play a significant role in preventing uncontrolled cell division and maintain proper regulation of the cell cycle in somatic cells, elevated CDK activity in ES cells is sustained by low expression of CDKI proteins. CIP/KIP CDK inhibitor proteins are expressed at low levels in ES cells. At the mRNA level, it has been reported that mES cells have low levels of p21 and p27 transcripts (Stead et al., 2002). This is also mirrored in hESC and upregulate both p21 and p27 upon induction of differentiation (Neganova et al., 2009b). Furthermore, p27 levels are regulated post-translationally by overexpression of the SCF co-activator Skp2 which triggers p27 for degradation, further enhancing CDK2 activity (Egozi et al., 2007). The INK4/Arf CDKI proteins p16 and p19 are also either expressed at low levels or undetectable in ES cells (H. Li et al., 2009; Neganova et al., 2009b). Although it was previously reported that mES cells do not display any CDK4/6 associated activity, additional studies have shown that mES cells down regulate Cyclin D3 upon ectoderm differentiation and exhibit high CDK6-Cyclin D3 activity (Stead et al., 2002), (Faast et al., 2004). Importantly, the activity of the CDK6-Cyclin D3 complex was not inhibited by overexpression of p16. Using in-vitro kinase assays, the authors showed that CDK6-Cyclin D1 complexes could be inhibited by p16, but CDK6-Cyclin D3 were insensitive to this inhibition. Further immunoprecipitation experiments revealed that while CDK4 could be found in stable complexes with p16, while CDK6 could be detected. These studies highlight how evasion from CDKI protein mediated inhibition can be controlled by activating specific CDK-Cyclin complexes to help maintain high CDK activity in ES cells.

1.4.3 Cell cycle adaptation in ES cells results in a truncated G1 phase

The retinoblastoma (Rb) and Rb-like proteins play a critical role in controlling cell growth and division by inhibiting the E2F transcription factor. A consequence of elevated CDK4/6 and CDK2 activity in combination with low levels of CDK inhibitor proteins is the hyperphosphorylation of Rb throughout the cell cycle in mESCs (Stead et al., 2002) (Figure 1.8 and Table 1). Therefore, inactivation of Rb is cell cycle independent and a fully functional restriction point is absent. This allows mES cells to activate E2F transcription factors immediately after mitosis and rapidly enter S phase. Consistent with this, knocking out all three Rb proteins (Rb^{p105}, Rb^{p107} and Rb^{p130}) has no observable phenotype on proliferation rates in

mES cells (J. Hsu et al., 2019). Although hypophosphorylated Rb populations have been observed in hESC, these studies have used synchronisation methods to enrich cell cycle populations to study the phosphorylation state of Rb (Sela et al., 2012). It is therefore possible that the presence of hypophosphorylated Rb in these experiments are a result of artificially re-setting Rb by treating cells with agents that arrest the cell cycle before release. Despite this, as hESC are thought to display some level of cell cycle regulated expression and degradation of cyclins (albeit at much lower amplitude comparatively to somatic cells), a more functional restriction point is thought to be present. Nevertheless, a clear mechanistic insight into how G1/S regulators orchestrate this transition has not been made, partly due to the limited number of studies using single cell analyses in asynchronously dividing hESC populations.

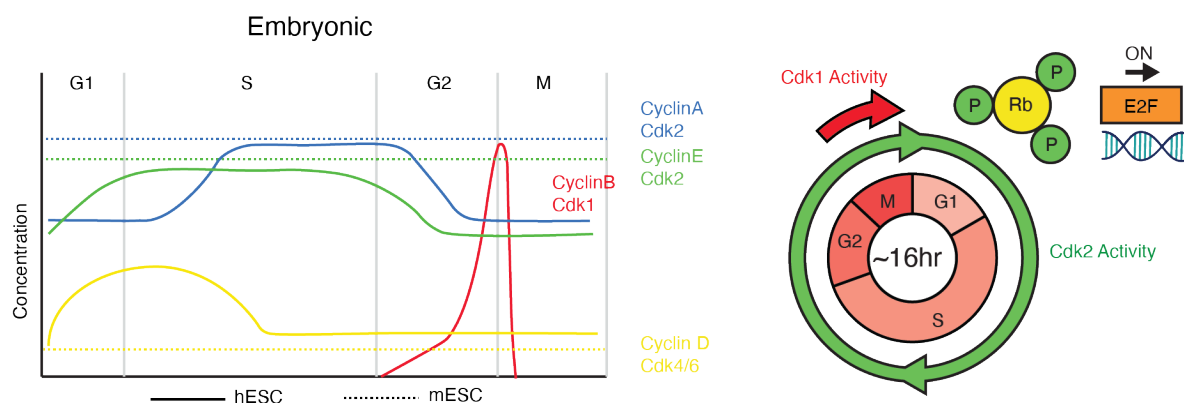


Figure 1-8- Embryonic stem cell cycle and structure – (left) Schematic illustrating the expression of different cyclins throughout the embryonic cell cycle. These cyclins then partner with CDKs to drive the cell cycle at different phases. ES cells have constitutively high CDK2 activity. Dashed line represents expression levels of cyclins in mESC and solid line represents hESCs. (right). Schematic illustrating hESC cell cycle structure. Due to elevated CDK activity, Rb is hyperphosphorylated and inactive throughout the cell cycle which results in cell cycle independent E2F transcription. Low levels of CDKI proteins in hESCs also increase CDK activity. Adapted from Padgett and Santos 2020.

1.4.4 Regulation of the ES cell cycle by degradation machinery

Differences in degradation machinery between somatic and embryonic stem cells is thought to contribute to differences in protein stability. For example, the difference in developmental tempo between mouse and human and embryonic stem cells has been associated to protein turnover, with mES cells having an approximately two-fold increase in protein stability (Rayon et al., 2020). This study sheds light onto how protein degradation can play a role in regulating

proliferation rates, and overall developmental tempo. Control of cell cycle degradation proteins is also altered in embryonic stem cells (Figure 1.9). APC/C^{Cdh1} activity is thought to be low in both mES and hES cells (Gharibi et al unpublished), which is partly attributed to the overall increased CDK activity in ES cells which results in an inhibitory phosphorylation Cdh1, but also due to increased levels of the APC/C inhibitor Emi1 (Ballabeni et al., 2011; Bar-On et al., 2010). This ensures that cyclin levels remain high and reinforces increased CDK activity. Reduced APC/C activity also results in the accumulation of the SCF component Skp2. Consequently, the SCF-Skp2 complex has high activity throughout the cell cycle and key targets such as the CDK inhibitor proteins p27 and p21 are targeted for degradation further supporting high CDK activity (Egozi et al., 2007). In summary, modifications in ubiquitin ligase activity help degrade regulators that act as cell cycle brakes and support the stability of cell cycle drivers.

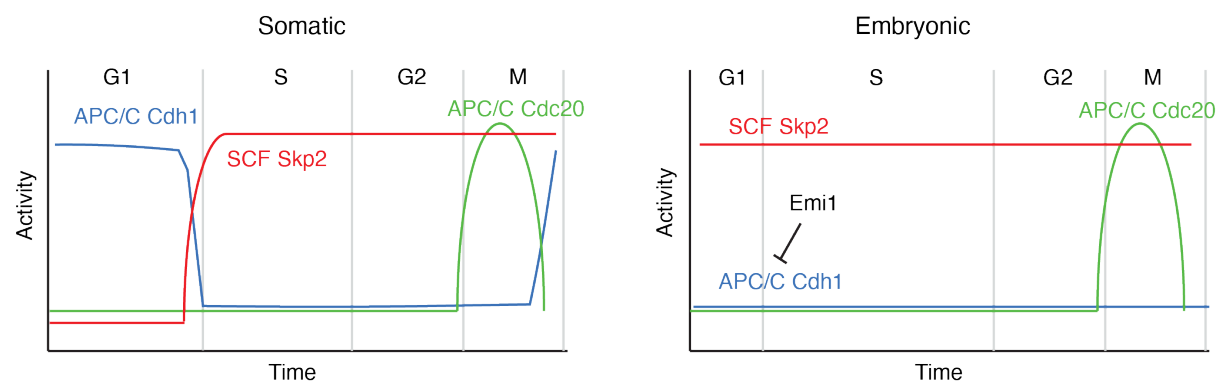


Figure 1-9- Comparison of E3 ubiquitin ligase activity between embryonic and somatic cells –ES cells have low APC/C^{Cdh1} activity due to high expression of the APC/C inhibitor Emi1. This results in elevated SCF^{Skp2} activity throughout the cell cycle. Adapted from Padgett and Santos 2020.

1.4.5 A modified G1/S checkpoint in embryonic stem cell cycles

The G1/S checkpoint ensures that all the necessary components for DNA replication are present before progressing into S phase, while safeguarding the genomic integrity of the cell by checking for internal DNA damage. Due to the partial functionality of the restriction point in ES cells, a classical G1/S checkpoint in ES cells is largely absent (Figure 1.8 and Figure 1.9). In line with this, ES cells do not arrest in G1 in response to exogenous DNA damaging agent (Fluckiger et al., 2006; Suvorova et al., 2016). Although one study has suggested that hESC are able to execute a G1/S arrest in response to ultraviolet radiation-C (UVC) radiation,

cells were again artificially synchronised cells in G1 before applying the cellular stress, making the results difficult to interpret (Bárta et al., 2010). This arrest was thought to be mediated via UV induced degradation of the CDK2 activating phosphatase Cdc25A rather than the classical p53/p21 G1/S checkpoint axis (Bárta et al., 2010). While it is unclear whether hESC can elicit a G1/S checkpoint response, this study highlights that hESC use an alternative mechanism to bring about a reduction in CDK2 activity that does not require the CDK inhibitor protein p21. In addition to this, p21 levels are dynamically controlled in response to stress by microRNAs (miRNAs). Treatment with UVC radiation in hESC stabilises p53 which induces p21 transcription, however p21 is undetectable at the protein level. Specifically, the MiR-302 family of miRNAs are upregulated in response to UVC treatment, which bind to and destabilise p21 mRNA transcripts resulting in reduced p21 translation (Dolezalova et al., 2012).

A repercussion of a G1/S checkpoint with limited functionality is that ES are particularly vulnerable to genomic instability. In line with this, hESCs acquire a higher frequency of DNA mutations and experience constitutive replication stress (Ahuja et al., 2016; Hyka-Nouspikel et al., 2012). To compensate for this deficiency, ES cells use a variety of adaptations to preserve the genomic integrity of the cell. Firstly, ES can repair DNA lesions more effectively from a variety of DNA damage sources (Maynard et al., 2008). Expression levels of multiple DNA repair genes involved in base excision repair (BER), nucleotide excision repair (NER), double-strand break repair (DSBR) and interstrand crosslink repair (ISL) are all expressed at elevated levels in hESCs (Maynard et al., 2008). ES cells also accumulate more ssDNA gaps during DNA replication, and as a result have more efficient replication coupled repair and fork reversal events to maintain genomic integrity (Ahuja et al., 2016). Finally, rather than trying to repair accumulated DNA damage, ES cells may preferentially commit to apoptosis in response to DNA damage (J. C. Liu et al., 2013; Momcilovic et al., 2010). A strong apoptotic response in ES cells has been reported after treatment with DNA damage (J. C. Liu et al., 2013; Momcilovic et al., 2010). It was found that a rapid p53 response triggers apoptosis in hESC, as shown by the presence of the pro-apoptotic marker cleaved PARP (c-PARP), while in differentiated cells had no detectable levels of c-PARP despite also upregulating p53 (J. C. Liu et al., 2013). The transcriptional targets of p53 were similar between hESC and differentiated cells, but the apoptotic response in hESC was thought to be achieved via cytoplasmic localisation of p53, which primes mitochondria for a pro-apoptotic response (J. C. Liu et al., 2013). Furthermore, it has been shown that the apoptosis regulator BAX is constitutively active

at the Golgi in hESC and shuttles to the mitochondria via a p53 dependent mechanism after treatment with DNA damage which primes hESC for apoptosis (Dumitru et al., 2012). BAX is lost upon induction of differentiation, which reduces the sensitivity of differentiated cells towards apoptosis. These mechanisms that promote apoptosis rather than trying to repair the vast amounts of DNA damage that ES cells accumulate prevent the spread of mutations which could potentially lead to disease. As DNA mutations acquired in embryonic stem cells will be propagated to the whole organism during development, the elimination of these cells therefore safeguards genomic integrity.

1.4.6 G2/M and SAC Checkpoint control in ES cells

In contrast to the G1/S checkpoint, the G2/M checkpoint appears to have some functionality in ES cells (Figure 1.10). Furthermore, due to the lack of surveillance mechanisms in G1, ES cells can efficiently repair DNA damage at the G2/M checkpoint to compensate for this. Activation of ATM in response to DNA damage can effectively stabilise p53 in G2 phase in mES cells, resulting in the formation of γ H2AX foci on DNA, suggesting that the DNA damage response in G2 is intact (Chuykin et al., 2008). While somatic cells use a combination of nonhomologous end joining (NHEJ) and homologous recombination repair (HRR), ES cells preferentially use HRR to repair double strand breaks which has a higher fidelity in comparison to NHEJ (Tichy et al., 2010). As such, Rad51, which is involved in the recognition of DNA breaks and catalysis of strand invasion used in HRR, is constitutively expressed in mESCs (Yoon et al., 2014). ES therefore gain an extra level of genomic stability by favouring the use of HRR instead of NHEJ.

Although there are limited studies on the spindle assembly checkpoint (SAC) in ES cells, the SAC appears to be operational. Treatment with the microtubule disrupting agent nocodazole leads to cell cycle arrest, suggesting the ES cells can sense spindle assembly formation (Mantel et al., 2007). Despite this, activation of the SAC does not trigger an apoptotic response as it does in differentiated cells, which results in more frequent polyploidy events in daughter cells (Mantel et al., 2007). The fast cell cycle displayed in ES cells may therefore function to promote rapid growth before reaching a critical mass of cells that supports survival of the expanding embryo. Elimination of polyploid cells thereafter may function to balance the requirements for both genomic stability and growth during early development.

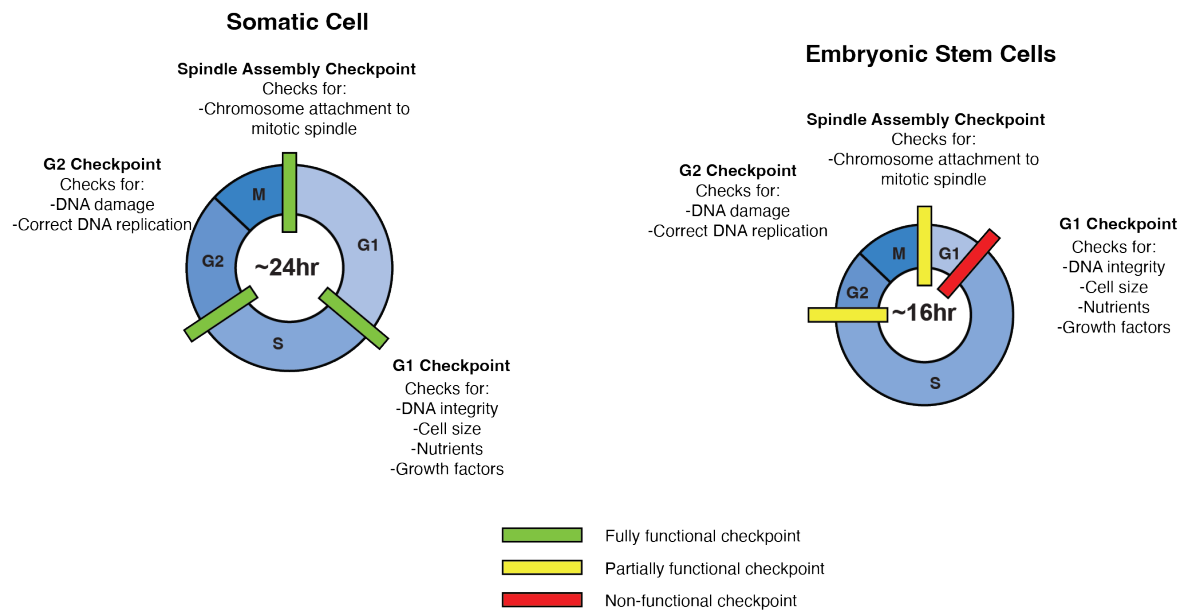


Figure 1-10- Checkpoint control in embryonic stem cell cycles – Schematic illustrating active checkpoints throughout the cell cycle in embryonic and somatic cells. Green bars indicate fully functional checkpoint, yellow indicates partially functional and red indicates non-functional checkpoint. Adapted from Padgett and Santos 2020.

1.5 Cell cycle changes during developmental transitions

When ES cells commit to differentiation, this is accompanied by cell cycle changes as cells transition to a somatic state. This includes lengthening of G1 phase, cyclical activity of CDKs and up regulation of CDK inhibitor proteins (White & Dalton, 2005). In mESC, differentiation triggers the introduction of cell cycle regulated CDK2 activity which in turn leads to the activation of the Rb/E2F pathway and a functional restriction point (White et al., 2005). The lowering and periodic oscillation of CDK2 activity is achieved partly by down regulation of cyclins, and cell cycle transcriptional control of E2F targets including Cyclin E (Filipczyk et al., 2007; Savatier et al., 1996; White et al., 2005). Expression of Cyclin E and A is also decreased during hESC differentiation, which leads to an overall decrease in CDK2 activity and increased pRb activation (Stead et al., 2002). The restriction point is established during differentiation. Proper checkpoint control at the G1/S transition is achieved via upregulation of CDKIs such as p16, p21 and p27 (Egozi et al., 2007; Faast et al., 2004; Neganova et al., 2009a), which serves not only promote cell cycle arrest in response to intrinsic and extrinsic stress but also to lower CDK2 activity.

Despite our current understanding of both somatic and embryonic cell cycle networks, what regulates the transition between these two states is poorly understood. Our current understanding of this process has been accomplished mostly via population-based assays in fixed cells between the embryonic and somatic state. Consequently, valuable information about the dynamics of cell cycle remodelling in single cells is lacking.

Cell cycle regulator	ES cells 	Somatic cells 	References
Cyclins			
Cyclin A	High and non-(M) or oscillatory (H) expression	Lower expression; cell cycle regulated	Savatier 1996, Stead 2002, Faast 2004, Fujii-Yamamoto 2005, Ghule 2007, Neganova 2009, Pauklin and Vallier 2013, Wang 2017
Cyclin B	High expression (M)(H)	Lower expression; cell cycle regulated	
Cyclin D	Low (M) or intermediate (H) expression	High expression; cell cycle regulated	
Cyclin E	High, non-(M) or oscillatory (H) expression	Lower expression; cell cycle regulated	
CDKs			
Cdk1	High expression, oscillatory activity (M)(H)	Cell cycle regulated activity	Stead 2002, Faast 2004, White 2005, Fujii-Yamamoto 2005, Ghule 2007, Neganova 2009, Bar-On 2010
Cdk2	High expression, constant (M) or oscillatory activity (H)	Cell cycle regulated activity	
Cdk4	Low (M) or intermediate (H) activity	Cell cycle regulated activity	
Cdk6	Low, oscillatory activity (M)	Cell cycle regulated activity	
CDK Inhibitors			
p21/p27/57 p16/18/19	Low or absent expression (M)(H) Low or absent expression (M)(H)	Higher, cell cycle regulated expression Higher, cell cycle regulated expression	Stead 2002, Fujii-Yamamoto 2005, Egozi 2007, Neganova 2009, Li 2012,
G1-phase			
RB	Hyperphosphorylated (M)(H), Hypophosphorylated (H)	Hypo/hyperphosphorylated	Savatier 1994, Stead 2002, Fujii-Yamamoto 2005, White 2005 Conkin and Sage 2009, Bar-On 2010, Ballabeni 2011, Yang 2012, Matson 2017
E2F	De-repressed form, cell cycle independent transcription (M)	Cell cycle dependent transcription	
APC/C Cdh1	Low activity (M)(H)	Cell cycle regulated activity	
p53	Does not induce G1-arrest in response to damage (M)(H)	p53-depedent arrest in response to damage	
Cdt1	High expression (M)(H)	Cell cycle regulated expression	
Emi1	High expression (M)(H)	Cell cycle regulated expression	
S-phase			
Cdc25A	High expression (H)	Cell cycle regulated expression	Fujii-Yamamoto H 2005, Zhang 2009, Ballabeni 2011, Matson 2017
MCM complex	Fast loading (M)(H)	Slower, cell cycle regulated loading	
Cdc6	High expression (M)(H)	Lower expression; cell cycle regulated	
Geminin	High expression (M)(H)	Lower expression; cell cycle regulated	
G2/M-phase			
Plk1	High activity (H)	Cell cycle regulated activity	Egozi 2007, Bar-on 2010, Ballabeni 2011, Yang 2011, Yang 2012, Zhang 2012, Gonzales 2015
Skp2	High levels throughout the cell cycle (H)	Lower expression, no longer present in G1	
ATM/ATR-CHEK2	Expressed and active G2-checkpoint (M)(H)	Expressed and active G2-checkpoint	
APC/C-Cdc20	Lower actity (M)	Higher activity, cell cycle regulated	

Table 1-1- Molecular changes in the cell cycle during the embryonic to somatic transition. (M) indicates mESC and (H) indicates hESCs. Adapted from Padgett and Santos 2020.

1.6 Interplay between cell cycle control and pluripotency

Embryonic stem cells have a unique property called self-renewal, which allows them to maintain pluripotency. Pluripotency is regulated by a complex network of genes and signalling pathways, largely driven by the transcription factors Oct4, Sox2 and Nanog which are essential for maintaining pluripotency in ES cells (Yilmaz & Benvenisty, 2019). As demonstrated by Shinya Yamanaka and colleagues, somatic cells can be reprogrammed back to the pluripotency state by overexpressing Oct4, Sox2, Klf4 and c-Myc, which became known as the Yamanaka factors (Takahashi & Yamanaka, 2006). Pluripotency is also regulated by various signalling pathways including Wnt, BMP and FGF signalling pathways which can repress gene expression and influence cell fate decisions. In addition to this, epigenetic modifications such as DNA methylation and histone modifications can activate or repress gene expression (Yilmaz & Benvenisty, 2019). The discovery of factors that influence the pluripotent state have helped deepen our understanding of the processes that are essential for maintaining embryonic stem cells.

The cell cycle is thought to play an important role in maintaining pluripotency in embryonic stem cells. Removal of several G1/S regulators including CDK2, Cyclin E and D-type cyclins can lead to loss of pluripotency (Gonnot et al., 2019; L. Liu et al., 2017; Neganova et al., 2011), while overexpression of Cyclin E can stabilise the pluripotent state (Gonnot et al., 2019). Furthermore, isolation of hESC cells that displayed hypophosphorylated Rb were shown to have higher differentiation potential (Sela et al., 2012). These studies have led to the view that a short G1 phase helps maintain pluripotency. Moreover, G1 is thought to be a window of opportunity for differentiation in ES cells (Pauklin & Vallier, 2013), thus having a reduced G1 phase decreases exposure to differentiation cues. Using the FUCCI cell cycle reporter, cells from different cell cycle phases were isolated and placed in defined differentiation conditions. It was found that hESC can only differentiate into mesoderm/endoderm in early G1, whereas late G1 favours neuroectoderm differentiation. Conversely, differentiation into all three germ layer cell types was significantly reduced outside of G1. Mechanistically, this is believed to be regulated by CDK4/6-Cyclin D, which phosphorylates and inactivates the endoderm signalling regulator Smad2/3 in late G1 (Pauklin & Vallier, 2013). In addition to this, G1 cyclin complexes have been shown to phosphorylate Nanog, Sox2 and Oct4, which stabilises these pluripotency factors by inhibiting proteasomal mediated degradation by polyubiquitination (L.

Liu et al., 2017). Together, G1/S regulators play a pivotal role in both maintaining pluripotency and repressing differentiation.

While there is mounting evidence that G1/S regulators play an important role in the maintenance of pluripotency, there is contradictory data on the role of overall G1 length on pluripotency. For example, pluripotency is still retained when overexpressing p21 and p27 in mES cells to increase G1 length (V. C. Li et al., 2012), and mES cells growth culture conditions where ERK is inhibited have an extended G1 phase but still maintain pluripotency (ter Huurne et al., 2017). Both lines of evidence suggest that lengthening G1 might not be sufficient to lose cell identity.

Links between pluripotency and the cell cycle have also been observed outside of G1. A high throughput RNAi screen in hESCs identified that downregulation of G2/M transition genes including ATM/ATR and Cyclin B1 resulted in quicker loss of Nanog upon withdrawal on self-renewal signals (Pauklin & Vallier, 2013). The authors propose that S/G2 regulators therefore might have an essential role in pluripotency maintenance. In line with this, an additional study noted that downregulation of CDK1 resulted in loss of pluripotency and upregulation of differentiation genes (Neganova et al., 2014). The mechanism by which CDK1 promotes pluripotency has now been revealed by utilising an analogue sensitive (AS) mutant of CDK1 (Michowski et al., 2020). The authors show that in embryonic stem cells, CDK1 targets genes involved in epigenetic regulation including the H3K79 methyltransferase Dot1l. Phosphorylation of Dot1l negatively regulates its activity by changing its cellular localisation from the nucleus to cytoplasm. This was shown to be important in maintaining the epigenetic landscape that regulates pluripotency in mES cells (Michowski et al., 2020).

Disentangling the hierarchy for either cell cycle regulation or pluripotency has proven difficult due to the reciprocal influence of both networks on each other. Despite this, the transcription factor Myc may act as a master regulator of both networks, as it is involved in both cell cycle regulation and pluripotency maintenance. Myc is one of the four Yamanaka factors and has diverse roles including epigenetic regulation and modulating signalling pathways that are essential in the maintenance of pluripotency (Cartwright et al., 2005). In the context of cell cycle regulation, Myc is involved in the transcriptional control of genes involved in the G1/S transition such as Cyclin D and Cyclin E, and negatively regulates cell cycle repressors

including p27 and p21 (Smith & Dalton, 2010). However, the exact mechanism by which Myc regulators both the cell cycle and pluripotency are complex, and likely involve interactions with other proteins and pathways. Further research is needed to fully understand the roles of Myc in these processes.

1.7 Using hESCs as a tool to understand cell cycle remodelling during differentiation

hESC are a highly tractable system for studying early human development and modelling disease mechanisms *in vitro*. hESC can be maintained and grown in 2D culture and can be differentiated to specific cell subtypes upon the induction with different cues (Figure 1.11). Furthermore, advances in genome editing/gene silencing technologies have enabled precise genetic modifications in hESC, allowing researchers to study the function of specific genes and their roles in development and cell cycle control. In combination with this, the behaviour of single cells can be tracked using live cell imaging, allowing scientists to study the heterogeneity within hESC populations.

The work presented in this thesis predominantly uses bone morphogenetic protein 4 (BMP4) to induce differentiation in hESCs. BMP4 is a growth factor that plays a critical role in embryonic development, particularly in the formation of mesoderm and the differentiation of mesodermal lineages such as muscle, bone and blood vessels (Xu et al., 2002). BMP4 treatment of hESC *in vitro* initiates a signalling cascade that leads to the activation of SMAD proteins, which is essential in expression of mesodermal markers such as Brachyury and the loss of pluripotency markers such as Oct4, Sox2 and Nanog within 3 days (Gunne-Braden et al., 2020; Yu et al., 2011). BMP4 treatment is therefore a powerful tool for directing hESC differentiation due to its simplicity and its rapid induction of cell fate markers. Importantly, BMP4-driven differentiation of hESC results in remodelling of the cell cycle within 3 days. This makes this model system an incredible useful one to study in single cells how the cell cycle changes during cellular differentiation.

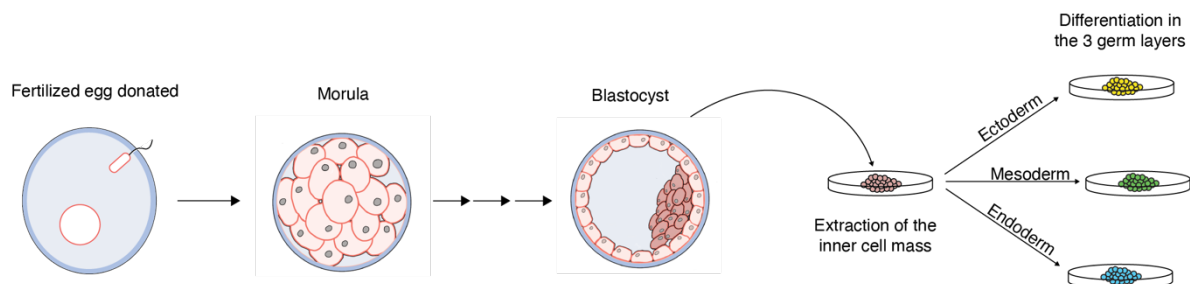


Figure 1-11- Isolation of hESCs – In-vitro fertilisation (IVF) donors are cultured in vitro and undergo embryonic cleavage divisions before forming a pre-implantation blastocyst. hESCs are isolated from the inner cell mass of this blastocyst and are maintained in vitro. hESC can be differentiated into specific cell subtypes upon the induction of various differentiation cocktails.

1.8 Live, single cell approaches for cell cycle studies

In the 1950s, advances in cell culture techniques allowed researchers to study the cell cycle *in vitro*. Using microscopy and cell synchronisation methods, researchers were able to study the morphological changes and DNA replications patterns that occur during the cell cycle. In the 1960s and 1970s, biochemical assays were developed to study the genetic and molecular mechanisms that underpin cell cycle control. These assays measured the average behaviour of a population of cells, typically in artificially synchronised cellular populations in static states. These classical methods laid the foundation for our understanding of the cell cycle and its regulation and continue to be important tools in modern research. However, cells within a population display significant heterogeneity with variations in gene expression, proteins levels and other cellular properties.

It is becoming more evident that single cell studies are essential to investigate, explain and understand the inherent heterogeneity within cellular populations. The cell cycle is no exception to this, and many studies have already highlighted this importance to explain why some cells have longer G1 phases than others (Spencer et al., 2013). More importantly, the need to study the cell cycle in live, asynchronous populations is vital to explore the dynamics of cell cycle regulation where potential artefacts from stress-related synchronisation are eliminated. This approach is essential to understand how the cell cycle changes during the embryonic to somatic transition. For example, it is not known exactly when and how the cell cycle remodels, whether it is a graded or a switch-like process or whether individual cells follow the same dynamics.

1.8.1 Biosensors for cell cycle dynamics

The development of novel cell cycle biosensors is now allowing us to examine quantitatively the dynamics of individual cell cycle phases and of key cell cycle regulators. One of the most prolific examples of this is the FUCCI sensor developed by the Miyawaki Lab (Sakaue-Sawano et al., 2008) which employs a truncated fragment of the proteins Cdt1 and Geminin fused to fluorescent proteins. These proteins have opposing roles in the DNA replication control system, where Cdt1 licenses DNA replication in G1 phase and Geminin inhibits this process in S/G2/M. These proteins oscillate during the cycle due to the APC/C^{Cdh1} mediated degradation of Geminin

in G1, and SCF^{Skp2} mediated degradation of Cdt1 in S/G2/M. The fluorescent readout of these proteins can therefore be used to quantify different stages of the cell cycle (Sakaue-Sawano et al., 2008).

A newer FUCCI cell cycle biosensor, named FUCCI(CA) can now quantify all four phases of the cell cycle (See methods 2.9.4 for details). FUCCI(CA) also employs truncated fragments of Cdt1 and Geminin but has reengineered the Cdt1 to be specifically degraded in S phase by the ubiquitin ligase CUL4^{Dbp1} (Sakaue-Sawano et al., 2017). This means all four phases can be reliably tracked and quantified in single cells.

Proliferating cell nuclear antigen (PCNA) can also quantify all four phases of the cell cycle (Zerjatke et al., 2017) (See methods 2.9.4 for details). PCNA is processivity factor for DNA polymerase and localizes to sites of active replication during S phase. When visualising fluorescent PCNA, it initially localises to the nucleus in G1 and upon S phase entry forms speckles as it is loaded onto DNA. These speckles then disappear at the start of G2.

1.8.2 Biosensors for cyclin dependent kinases

CDK activity biosensors have also been developed, pioneered by work in somatic cells using a CDK1-Cyclin B1 FRET biosensor to study mitotic entry (Gavet & Pines, 2010). Additional CDK1 biosensors have also been developed, based on the translocation of Cyclin B1 to study how mitosis is kept short and constant (Santos et al., 2012). The most recognised CDK2 activity biosensor is also based on translocation (Spencer et al., 2013). The sensor uses the amino acids 994-1087 of human DNA helicase B (DHB) fused to a fluorescent protein, four CDK phosphorylation sites, a nuclear localization signal and a nuclear export signal. When CDK2 activity is low, DHB remain nuclear but as CDK2 activity increases, DHB gets phosphorylated and translocated out of the nucleus. Interestingly, using this sensor in somatic cells revealed that two distinct behaviours of CDK2 activity. After mitosis the activity of CDK2 ‘bifurcates’, with some cells having high CDK2 activity, and the others having low activity. Cells with low activity were shown to enter quiescence with prolonged G1 phases whereas intermediate CDK2 activity cell proliferated.

To measure CDK4/6 activity another established translocation-based sensor has been developed (Yang et al., 2020). This sensor utilises a CDK4/6 specific docking site of Rb (aa 886-928) fused to an NLS and NES with two CDK phosphorylation sites and mCherry. When CDK4/6 activity is low, the sensor remains in the nucleus and as CDK4/6 activity increases and the sensor gets phosphorylated and moves into the cytoplasm. By measuring the cytoplasmic to nuclear ratio can therefore give a direct readout of CDK4/6 activity. Although this fragment of Rb (aa 886-928) has been shown to be specific for CDK4/6-Cyclin D binding and phosphorylation and not CDK2-Cyclin E (Topacio et al., 2019) (Wallace & Ball, 2004), the authors of this sensor noticed that approximately 35% of the sensors' activity is contributed by CDK2. To compensate for this, the sensor is used in combination with the CDK2 sensor and 0.35 of the measured CDK2 activity is subtracted away from the CDK4/6 reporter to measure the corrected CDK4/6 activity. Together these sensors allow us to monitor changes in important master regulators in cell cycle control.

1.9 Aims of thesis

By combining advanced live cell imaging of hESCs with cell cycle biosensors and computational analysis, we aim to monitor cell cycle remodelling at the single cell level during hESC differentiation. Bone Morphogenic Protein 4 (BMP4) driven hESC differentiation, an established 2D model for early human gastrulation, will be used as a model system. By tracking single cells through lineages and using perturbation approaches, we aim to quantitatively characterise cell cycle and CDK2 activity dynamics during differentiation, and dissect the molecular mechanisms underpinning cell cycle remodelling during this transition. Finally, we aim to understand how cell cycle regulation impacts on stem cell identity.

Specific Aims:

- 1) Develop single cell approaches to monitor cell cycle remodelling in hESC.
- 2) Quantitatively characterise how the cell cycle changes during BMP4-driven hESC differentiation in live, single cells.
- 3) Measure how CDK2 activity changes during hESC differentiation.
- 4) Unravel the molecular mechanisms underlying cell cycle remodelling and changes in CDK2 activities.
- 5) Address the functional importance of the unique cell cycle displayed in hESCs, and how it impacts on stem cell identity

Chapter 2. Methods

2.1 hESC Culture

H1 human embryonic stem cell (hESC) lines were used in the majority of the experiments presented in this thesis. H9 human embryonic stem cell lines were used for additional key experiments. Both H1 and H9 lines are derived from the inner cell mass (ICM) of a pre-implantation blastocyst from in-vitro fertilization (IVF) donors. These cells are maintained in 2D culture system and have the propensity to differentiation to all three germ layers. H1 lines have a XY karyotype and H9 lines have a XX karyotype. Cells were routinely (every 3 weeks) tested for mycoplasma and karyotyped every 100th passage. A list of reagents used for maintenance of hESC can be found in Table 2-1.

Table 2-1- List of reagents used in hESC culture

Reagent	Source	Catalogue Number
H1, H9 wild-type hES	WiCell	WA01, WA09
BMP4	ThermoFisher (Life Technologies)	PHC9534
ROCK Inhibitor (Y-27632)	Stem Cell Technologies	72302
Polybrene	Millipore Ltd	TR-1003-G
Polyethylenamine (PEI)	Sigma	764965-1G
Puromycin	ThermoFisher (Life Technologies)	A1113803
mTeSR1	Stem Cell Technologies	85870
DMEM:F12	ThermoFisher (Life Technologies)	11320033
OptiMEM	ThermoFisher (Life Technologies)	51985026
mFreSR	Stem Cell Technologies	5855
PBS	Sigma	D8537
Gentle Dissociation Buffer	Stem Cell Technologies	7174
Matrigel	Corning	354230
Lipofectamine RNAiMAX Transfection Reagent	ThermoFisher (Life Technologies)	13778075
Doxycycline	Sigma	D9891
Trimethoprim (TMP)	Sigma	T7883
AA	ThermoFisher (Life Technologies)	15240062
CloneR	Stem Cell Technologies	5888
Cell Lifters	Corning	3008

2.1.1 Maintaining and passaging hESC

H1 and H9 cell lines were cultured at 37°C and 5% CO₂ on Matrigel (Corning) coated 6 well plates. Plates were prepared by diluting Matrigel to a final concentration of 100µg/mL in DMEM:F12 and allowed to set at room temperature for 1 hour. Additional DMEM:F12 was added after this incubation to avoid plates from drying out and kept at 4°C for a maximum of 2 weeks. Cells were fed daily with mTeSR1 (StemCell Technologies) without antibiotics and split 1:3 every 2-3 days. For passaging, cells were washed with PBS and incubated with gentle dissociation buffer (Stem Cell Technologies) for 3 minutes at 37°C before aspiration and replacement with fresh mTeSR1. Cell lifters were used to detach cells from the plate before transferring into a new well.

2.1.2 Differentiating hESC

Growth factor bone morphogenic protein 4 (BMP4) was added to media at a final concentration of 50ng/mL to drive differentiation.

2.1.3 Freezing and thawing hESC

On the day of freezing, mFreSR (StemCell Technologies) was removed from -20°C storage and allowed to thaw on ice. hESCs were grown to 80-100% confluency on 6 well plates before washing twice with PBS, followed by the addition of 1mL of mTeSR1 per well. Cell lifters were used to scrape cells of the plates and transferred to 15mL falcon tubes to centrifuge at 250 x g for 5 minutes. After removal of supernatant, the cell pellet was resuspended with 1mL of ice cold mFreSR per well of hESC and broken up gently to make smaller, homogenous fragments of hESC colonies. The resuspended cells were aliquoted into cryotubes (1mL per tube) and transferred into freshly prepared isopropanol freezing containers at -80°C. After 24 hours, cells were moved into liquid nitrogen tanks for long term storage.

hESC were thawed by placing liquid nitrogen stored cryotubes into a 37°C water bath. Immediately after the suspension was thawed, cells were transferred to 15mL falcon tubes, followed by the addition of 2mL mTeSR1 drop-wise. Cells were centrifuged at 250 x g for 5 minutes and after the removal of supernatant, the cell pellet was resuspended gently in 1mL of mTeSR1 containing 10µM of ROCK inhibitor to assist with cell adhesion. Cells were plated

onto a 12-well Matrigel coated plates and after 24 hours, cells were washed once with PBS and replaced with fresh mTeSR.

2.1.4 Generation of human gastruloids

Human gastruloids were generated as in (Moris et al., 2020). Briefly, hESC at 40% confluency were pretreated with 1:1 Nutristem (Corning):mTeSR for 1 day. On day 2, media was replaced with full Nutristem. On day 3, hESC were treated with Nutristem supplemented with 3 μ M CHIR99021 (Tocris Biosciences, 4423) for 24 hours before undergoing single cell dissociation (see 2.2.5 for details) and aggregation in E6 media (StemCell Technologies) + 10 μ M ROCK Inhibitor (Y-27632) + 0.5 μ M CHIR99021 . Cells were counted to achieve a final concentration of 10 cells/ μ L and plated onto U-bottom ultra-low-adherence 96 well plates (CellStar, 650970). Plates were then centrifuged at 250 x g for 2 minutes at room temperature before returning to incubator. After 24 hrs, media was replaced with fresh E6 and exchanged daily for 3 days.

2.2 Generation of stable cell lines

2.2.1 Constructs used to make stable cell lines

A variety of cell cycle biosensors, over-expression and perturbation systems were used in this study to monitor cell cycle dynamics and control levels of key cell cycle regulators. All constructs were fully sequenced before proceeding for experiments. A list of constructs used in this thesis can be found in Table 2-2.

Table 2-2- Constructs used in this thesis

Biosensor	Vector	Template/Gene	Label/Tag	Source
FUCCI(CA)2	pCSII-EF1 α	Cdt1(aa30-120) Geminin(aa1-100)	mCherry & Venus	Sakaue-Sawano et al., 2017
FUCCI(CA)2.2	pCSII-EF1 α	Cdt1(aa30-120) Geminin(aa1-100)	mCherry & mTurquoise	Sakaue-Sawano et al., 2017
PCNA-YFP	pCSII-EF1 α	PCNA	YFP	QSCB Lab
PCNA-Cerulean	pCSII-EF1 α	PCNA	Cerulean	QSCB Lab
PCNA-RFP	pCSII-EF1 α	PCNA	RFP	QSCB Lab
Cdt1-YFP	pCSII-EF1 α	Cdt1(aa30-120)	YFP	QSCB Lab
CDK2 Sensor mCherry	pCSII-EF1 α	DHB (aa994-1087)	mCherry	Spencer et al., 2013
CDK2 Sensor Venus	pCSII-EF1 α	DHB (aa994-1087)	Venus	Spencer et al., 2013
CDK2 Sensor Cerulean	pCSII-EF1 α	DHB (aa994-1087)	Cerulean	Spencer et al., 2013
iRFP-H2B	pCSII-EF1 α	H2B	iRFP	QSCB Lab
NLS-mCherry	pCSII-EF1 α	3xNLS + FKBP	mCherry	QSCB Lab
NLS-YFP	pCSII-EF1 α	3xNLS + FKBP	YFP	QSCB Lab
DHFR-YFP-p21	pLVX EF1 α	p21	DHFR/YFP	This thesis
DHFR-YFP-hCDH1	pLVX EF1 α	CDH1	DHFR/YFP	This thesis
TetOne-p21	pLVX-TetOne	p21	-	QSCB Lab
YFP-CDK2-AS- F80G+L32V	pLNT	CDK2- AS(F80G+L32V)	YFP	QSCB Lab

2.2.2 Lines made for this study

A variety of cell lines were generated to monitor cell cycle dynamics and control levels of key cell cycle regulators. A list of stable lines generated in this thesis can be found in Table 2-3.

Table 2-3- Lines made in this study

Cell Line	Biosensor
H1 hESC	FUCCI(CA)2
H1 hESC	Cdt1-YFP + NLS-mCherry
H1 hESC	PCNA-YFP + NLS-mCherry
H1 hESC	CDK2 Sensor mCherry + NLS YFP
H1 hESC	CDK2 Sensor mCherry + Cdt1-YFP
H1 hESC	CDK2 Sensor mCherry + PCNA-YFP
H1 hESC	DHFR-EYFP-P21
H1 hESC	DHFR-EYFP-P21 + PCNA-Cerulean + CDK2 Sensor mCherry
H1 hESC	DHFR-YFP-hCDH1
H1 hESC	DHFR-YFP-hCDH1 + PCNA-Cerulean + CDK2 Sensor mCherry
H1 hESC	TetOne-p21
H1 hESC	TetOne-p21 + CDK2 Sensor mCherry + Cdt1-YFP
H1 CDK2 KO	YFP-CDK2-AS-F80G+L32V
H1 CDK2 KO	YFP-CDK2-AS-F80G+L32V + FUCCI(CA)2.2
H1 CDK2 KO	YFP-CDK2-AS-F80G+L32V + CDK2 Sensor mCherry + iRFP-H2B
H9 hESC	FUCCI(CA)2
H9 hESC	CDK2 Sensor mCherry + NLS YFP

2.2.3 Lentiviral production for hESC infection

HEK 293T cells were used for lentiviral production. Cells were seeded at 50% confluency in DMEM (Gibco) and 10% FBS in a 6cm dish. On the day of transfection, media was replaced with 3mL mTeSR1. A transfection mixture containing lentiviral assembly vectors (2µg Pax2, 1µg VSVG), the expression vector of interest (5µg) and Polyethylenimine (PEI) (30µg) were diluted in Optimem up to 300uL and incubated at room temperature for 30 minutes. The transfection mixture was then added to HEK 293T cells and incubated for either 48 or 72 hours before harvesting virus containing media. Concentrated virus was produced by adding

harvested virus to Vivaspin 6mL columns and spun for 20 minutes at 2800 x g. Virus was either used immediately or 1mL vials were flash frozen in liquid nitrogen and stored at -80°C.

2.2.4 Lentiviral infection of hESC

hESC were infected overnight with 1mL of virus containing mTeSR1 and 12µg/mL polybrene for at least 12 hours. After 12 hours, cells were washed with PBS and replaced with fresh mTeSR1. For selection, constructs with puromycin resistance were selected with 250-500ng/mL of puromycin to select for cells bearing the expression vector of interest. Otherwise, Fluorescence Activated Cell Sorting (FACS) was used to select for low to medium expression levels of cell cycle reporters to prevent potential artefacts of overexpression (see 2.2.5 for details)

2.2.5 Fluorescence activating cell sorting (FACS)

A single cell suspension was prepared by washing cells in PBS followed by an incubation with gentle dissociation buffer for 10 minutes at 37°C. Cells were fully detached from the plate by the addition of mTeSR1 and transferred to a 15mL falcon tube to centrifuge at 250 x g for 5 minutes. After removal of supernatant, the pellet was resuspended in PBS + 1%BSA + antibiotic-antimycotic (AA) and passed through a 70µm cell strainer before sorting. Cells were collected in fresh mTeSR1 + AA and following sorting were spun down at 250 x g for 5 minutes. After removal of supernatant, cells were resuspended in fresh mTeSR1 + AA + 10µM ROCK inhibitor to assist with cell adhesion before plating in an appropriate size well depending on the number of positive cells collected. The next day media was replaced with fresh mTeSR1 + AA.

2.2.6 Generation of CDK2 analogue sensitive (AS) line

To generate the CDK2 AS mutant, two mutations were made in human CDK2 tagged with YFP by site directed mutagenesis. 1) mutation of the bulky gatekeeper residue of CDK2 from F80G (Bishop et al., 2000) and 2) A suppressor of the gatekeeper mutation to maintain normal CDK2 activity from L32V (C. Zhang et al., 2005). The AS-CDK2-YFP mutant was then cloned into a LentiGuide expression vector, to knockout endogenous CDK2 and substitute with an

overexpression of the AS (Campeau et al., 2009). A gRNA was designed to target exon3 of endogenous CDK2 and added to the LentiGuide construct. Mutation in the PAM sequence was not required since the F80G mutation in the AS CDK2 mutates this gRNA (sequence in Table 2-4). A doxycycline inducible Cas9 hESC line was then infected with lentivirus produced from this construct with the addition of 10µg/mL of doxycycline for 12 hours later. Lentivirus was removed and replaced with mTeSR1 supplemented with 10µg/mL of doxycycline for an additional 24 hours. Cells were then prepared for FACS as in 2.2.5 to undergo single cell sorting. Matrigel coated 384-well plates were prepared on the day of sorting before adding 25uL of mTeSR1 + AA + 1xCloneR per well. YFP positive cells were then sorted, and a single cell was seeded per well to isolate individual clones. Cells were left to incubate for 2 days before replacing with fresh mTeSR1 + AA + 1xCloneR. On day 4 an additional 6.25µL of mTeSR1 + AA + 1xCloneR was added and on day 5 1xCloneR was removed from the media. Cells then underwent daily media changes with mTeSR + AA until surviving clones had grown enough to be expanded to larger wells (approximately 2 weeks). Surviving clones were subsequently screened by western blot to identify clones that had successfully knocked out endogenous CDK2 and express the heavier, YFP tagged AS-CDK2. Unfortunately, we were unable to knockout endogenous CDK2, however the expression levels of the AS mutant CDK2 were much higher than WT CDK2 (Figure 2-1). This cell line therefore functions as a dominant negative.

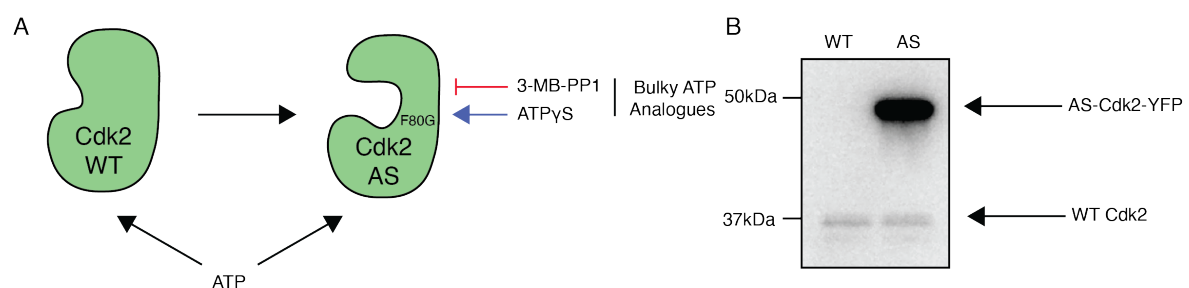


Figure 2-1- CDK2-AS development A. Schematic of CDK2-AS mutant, F80G mutation increases ATP binding pocket size which can be occupied by bulk ATP analogues. B. Western blot of total CDK2 expression levels for WT and CDK2-AS mutant in hESC lines.

Table 2-4- CDK2 gRNA sequence

Gene	Exon Target	gRNA sequence (5'-3')
CDK2	Exon3	CACCGGTTTTTGAATTTCTGCACC

2.3 Perturbation Approaches

2.3.1 siRNA used in this study

To knockdown gene of interest, siRNA mediated knockdown was used. All siRNAs used in this study were purchased from Dharmacon using the ON-TARGETplus pooled siRNAs sets. A list of all siRNAs used in this thesis can be found in Table 2-5.

Table 2-5- List of siRNAs used in this study

Target Gene	Catalogue Number
CCND1	L-003210-00-0005
CCND2	L-003211-00-0005
CCND3	L-003212-00-0005
CCNE1	L-003213-00-0005
CCNE2	L-003214-00-0005
CCNA2	J-003205-11-0005
CCNB1	L-003206-00-0005
CCNH	L-003218-00-0005
CDK1	L-003224-00-0005
CDK2	L-003236-00-0005
CDK4	L-003238-00-0005
CDK6	L-003240-00-0005
CDK7	L-003241-00-0005
CDC25A	L-003225-00-0005
CDC25B	L-003227-00-0005
CDC25C	L-003228-00-0005
SKP2	L-003324-00-0005
WEE1	L-005050-00-0005
FBXO5	L-012434-00-0005

2.3.2 siRNA transfections

A mixture of the diluted siRNA (5-50nM according to the manufacturer instructions dependent on individual genes) and lipofectamine RNAiMAX (1:50) was made in OptiMEM. The concentration of diluted siRNA was determined by performing a dose response for individual target genes to find the lowest concentration that achieved maximum knockdown efficiency. The volume of this mixture was 20% of the final volume of each well (e.g. for a 96 well 20 μ L) and was plated onto Matrigel coated dishes for 20 minutes to allow RNA/Lipofectamine complexes to form. A single cell suspension of hESCs was then made described in 2.2.5 and cells were counted to seed the appropriate number (typically 200,000 cells/mL for a 3-day experiment). Cell number was scaled appropriately depending on length of experiment and severity of knockdown on cell survival. Cells were then seeded in fresh mTeSR1 + 10 μ M ROCK inhibitor to assist with cell adhesion. The volume of cells added was 80% of the final volume of each well (e.g. for a 96 well 80 μ L). The following day, cells were washed once with PBS and replaced with fresh mTeSR1. Knockdown was confirmed either by immunofluorescence (IF) 48 hours after transfection or real-time quantitative PCR (RT-qPCR) 24 hours after transfection.

2.3.3 Doxycycline inducible overexpression

A TetONE inducible overexpression system was used to overexpress p21 and Cdh1 (Gossen et al., 1995). The TetOne inducible system induces gene expression in the presence of doxycycline (Figure 2-2). p21 and Cdh1 were cloned into the pLvX-TetONE-puro construct and used for lentiviral production and infection of hESC. Cells that had successfully integrated the pLvX-TetONE-puro construct were selected with 500ng/mL puromycin. 1-10 μ g/mL doxycycline was used to induce expression of p21 or Cdh1 for 24 hours prior to the start of experiment. Overexpression was confirmed either by immunofluorescence (IF) or real-time quantitative PCR (RT-qPCR).

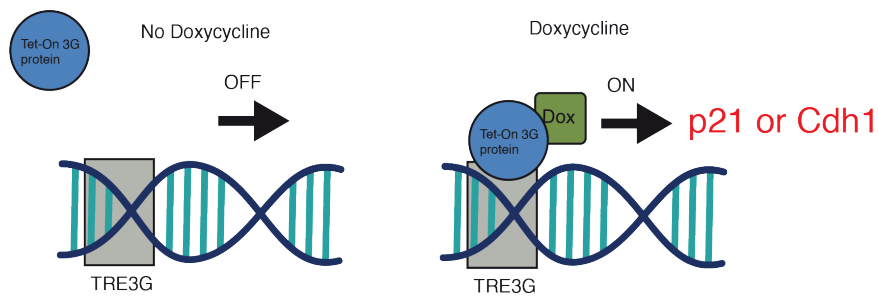


Figure 2-2- **Doxycycline inducible overexpression** See 2.3.3 description for details.

2.3.4 Dihydrofolate reductase (DHFR)-Trimethoprim (TMP) protein stabilization system

The DHFR-TMP protein stabilization system was used to rapidly increase levels of p21 and Cdh1 (Iwamoto et al., 2010). This system utilises a DHFR fusion protein tagged onto Cdh1 which under basal conditions is continuously degraded. The addition of TMP stabilises the DHFR degron, increasing the protein levels of Cdh1 rapidly (Figure 2-3). YFP tagged p21 and Cdh1 were fused with the DHFR domain from *E.coli* and cloned into a pLVX lentiviral expression vector by Gibson assembly. After lentiviral production and infection of hESC, cells that had successfully integrated the pLVX-DHFR construct were selected with 500ng/mL puromycin. 1-10 μ M of TMP was used to stabilise fusion protein to increase levels within the cell. Increased levels of p21 or Cdh1 were confirmed by the presence of YFP signal.

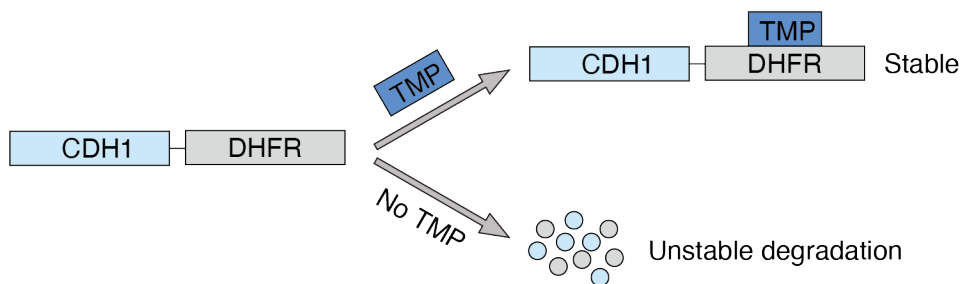


Figure 2-3- **Dihydrofolate reductase (DHFR)-Trimethoprim (TMP) protein stabilization system** See 2.3.4 for details.

2.3.5 Small molecule inhibitors

A variety of small molecules and inhibitors were used in this study to perturb key cell cycle regulators, induce cellular stress, and study cell cycle dynamics during differentiation. A list of all the small molecules and inhibitors used in this study is shown in Table 2-6.

Table 2-6- Small molecules used in this study.

Name	Source	Catalogue Number	Use	Concentration
BMP4	Life Technologies	PHC9534	Differentiation of hESC	50ng/mL
Doxycycline	Sigma	D9891	Induction of overexpression constructs	1µg/mL
Trimethoprim (TMP)	Sigma	T7883	Stabilisation of DHFR fused proteins	1-10µM
Puromycin	Life Technologies	A1113803	Selection of transduced cells	500ng/mL
Wee1 Inhibitor	Sigma	PD166285	Inhibition of Wee1 and Myt1	1µM
3-MB-PP1	Sigma	529582-5MG	Inhibition of CDK2-AS mutant	10nM-100µM
R0-3306	Bio-Techne	4181/10	Inhibition of CDK1	20µM
Palbociclib	Selleckchem	S1116	Inhibition of CDK4/6	1µM
Neocarzinostatin	Sigma	N9162	Induce double strand DNA breaks	50-200ng/mL
Aphidicolin	Tocris	5736	Inhibition of DNA replication	0.3µM

2.4 Cloning

2.4.1 Polymerase chain reaction (PCR)

PCR was used to specifically amplify DNA fragments. A mastermix containing template DNA and specific primers was made in PCR tubes containing:

- 5 μ L 10X Pfu High Fidelity Buffer (final concentration 1X)
- 2.5U Pfu DNA Polymerase
- 1 μ L of 10mM dNTPs
- 100ng template DNA
- 25pmol Forward Primer
- 25pmol Reverse Primer
- Top to 50uL with dH₂O

Samples then underwent a typical PCR cycle consisting of the following:

- 1) 2 minutes at 95°C to denature the sample
- 2) 30 cycles of the following –
 - 30 seconds denaturation at 95°C
 - 45 seconds annealing at optimal primer annealing temperature
 - 60 seconds elongation at 68°C
- 3) Final extension for 5 minutes at 68°C

5uL of the PCR samples was run on an agarose gel to confirm amplification at the right size. PCR product was then purified using Qiagen PCR purification kit.

Table 2-7 List of primers used for cloning in this study

Fragment	Forward Primer (5'-3')	Reverse Primer (5'-3')	Details
p21	GACTATCCGTACGACGTACC AGACTACGCAATGTCAGAAC CGGCTGGGG	TATCGATAAGCTTGATC CCTCGATGTAACTCTA GATTAGGGCTTCCTCTT GGAGAAGA	Amplify p21 gene to clone into pCSII-EF1 α DHFR-YFP plasmid by Gibson Assembly
FZR1 (Cdh1)	CCGCCGGGATCACTCTCGGC ATGGACGAGCTGTACAAGCA TATGGTTAACATGGACCAGG ACTATGAGCGG	CTCCCTACCCGGGTAGA ATTATCTAGAGTCGCGG CCGCTTACCGGATCCTG GTGAAGAGGT	Amplify Cdh1 gene to clone into pCSII-EF1 α DHFR-YFP plasmid by Gibson Assembly

2.4.2 Restriction digestion of plasmid DNA

Restriction digest of plasmid DNA was utilised to open vector DNA to either clone in PCR amplified products or purify insert of interest. A typical digest would consist of the following:

- 5µg Plasmid DNA
- 10U Restriction Enzyme 1
- 10U Restriction Enzyme 2
- 2.5µL of 10X buffer (Either CutSmart, NEBuffer or NEBuffer 3.1) (final concentration 1X)
- ddH₂O up to 25µL

Samples were incubated at 37°C for 1 hour before heat inactivation at 65°C for 10 minutes. Shrimp alkaline phosphatase was added for an additional 1 hour at 37°C before heat inactivation for plasmid only reactions to prevent re-ligation.

2.4.3 Purification of DNA bands from agarose gel

1% agarose gel was prepared by dissolving agarose powder in 1x Tris Acetate EDTA (TAE) buffer using a microwave. After cooling, 1X RedSafe DNA stain (Chembio) was added, and Agarose was poured into a gel support to set. Once solidified, the gel was transferred to a tank containing 1X TAE and samples were loaded into individual wells in 1X DNA loading buffer (New England Biolabs) alongside a 10kb ladder (New England Biolabs) before running at 120V for 45 minutes. Bands of the correct size were excised from the gel using a scalpel and purified using Qiagen Gel Extraction Kit according to manufacturer's instructions.

2.4.4 Ligation of insert and plasmid

To ligate purified vector and insert, a ratio of 1:3 to 1:12 of vector:insert was used depending on insert size. This mixture was then used for a ligation reaction that would consist of the following:

- 1µL T4 DNA ligase (New England Biolabs)
- 4µL 5X T4 DNA Ligase buffer (Invitrogen) (final concentration 1X)
- ddH₂O up to 20µL

Reactions were incubated overnight at 16°C.

2.4.5 Gibson isothermal assembly

For multiple fragment-based cloning, Gibson Isothermal Assembly was used (Gibson et al., 2009). Primers were designed to contain a 30bp overlapping sequence with the backbone before amplification and purification of the desired PCR product. After digestion of the plasmid (see 2.4.2 for details) a 5µL mixture was made containing equimolar amounts of each DNA fragment (based on the base pair length of each fragment and concentration measured). This mixture was then added to a Gibson Isothermal Assembly reaction consisting of the following:

- 5µL mixed DNA fragments
- 0.08U of T5 Exonuclease
- 0.5U of Phusion High Fidelity DNA Polymerase
- 80U of Taq DNA Ligase
- 4µL of 5X Reaction Buffer (final concentration 1X)

5X reaction buffer contained the following:

- 500nM Tris HCl pH7.5
- 50nM MgCl₂
- 1mM dGTP
- 1mM dATP
- 1mM dTTP
- 1mM dCTP
- 50mM DTT
- 5mM NAD⁺
- 250mg/mL PEG-800

Sample was incubated for 1 hour at 50°C and purified using Qiagen QIA quick PCR purification Kit according to manufacturer instructions.

2.4.6 Transformation of competent bacteria

DNA constructs were transformed into *E.coli* Dh5 α chemically competent bacteria (Sigma). Bacteria were stored at -80°C and 50 μ L aliquots were thawed on ice for 30 minutes before adding 0.5 μ g of plasmid. After an incubation on ice for 20 minutes, cells were transformed by heat shock at 42°C for 30 seconds and placed on ice for a further 2 minutes. 250 μ L of S.O.C media was then added to cells before incubating at 37°C while shaking to allow expression of antibiotic resistance gene. Bacteria were spread onto LB agar plates containing the correct antibiotic (either 100 μ g/mL ampicillin or 50 μ g/mL kanamycin) and left to incubate overnight at 37°C.

2.4.7 Isolation of plasmid DNA

Individual bacterial colonies were picked and grown in LB media with the appropriate antibiotic overnight at 37°C. For minipreps, the volumes of LB media was 5mL. For midi and maxipreps, 250 μ L of this initial overnight culture was added to 25mL or 100ml LB media respectively. Plasmid DNA was extracted using QIAGEN Plasmid Mini, Midi and Maxi Kits according to the manufacturer instructions. Purified DNA plasmids were then sent to Plasmidsaurus for full length sequencing.

2.5 Quantitative Reverse Transcription PCR (RT-qPCR)

2.5.1 RNA extraction

1mL of TRI reagent (Life technologies) was added per well in a 6 well plate to isolate RNA. MaXtract High Density Tubes (Qiagen) were subsequently used to purify RNA according to the manufacturer instructions. Concentration of RNA was determined using nanodrop.

2.5.2 cDNA conversion

Purified RNA was prepared for cDNA conversion using Qiagen QuantiTect RT kit. DNA contaminants were initially removed from 1µg of RNA by setting up the following reaction:

- 2µL DNA Wipeout Buffer
- 1µg RNA
- RNase-free water up to 14µL

Samples were incubated for 2 minutes at 42°C then placed immediately on ice. An RT master mix was prepared consisting of the following and added to the template RNA:

- 1µL RT
- 1µL RT buffer (5X) (final concentration 1X)
- 1µL Primer Mix
- 14µL RNA from previous DNA elimination reaction step

Samples were incubated for 20 minutes at 42°C followed by 3 mins at 95°C. The RT reaction was diluted 1:10 in DNase-RNase free water.

2.5.3 qPCR

cDNA stocks were prepared for qPCR using iTaq SYBR Green qPCR master mix (Bio-Rad). The following reaction was set up for each individual gene of interest in a 384 well PCR plate:

- 10µL 2X iTaq Universal SYBR green supermix (final concentration 1X)

- 1 μ L Forward Primer
- 1 μ L Reverse Primer
- 4 μ L cDNA stock
- 4 μ L ddH₂O

qRT-QPCR was performed using a QuantStudio 12k Flex Real-Time PCR System. PCR conditions consisted of the following:

- 1) 1 cycle 95°C for 3 minutes
- 2) 40 cycles:
 - 95°C for 5 seconds
 - 60°C for 30 seconds

GUS β primer sets were used as a housekeeping gene for individual samples. CT values were calculated by QuantStudio 12k Flex Real-Time PCR System. The Livak method (Livak & Schmittgen, 2001) was used to calculate $2^{-\Delta\Delta CT}$ fold change in expression. Primer sets used in this study are listed in Table 2-8.

Table 2-8- *Primer sets used in this study for qPCR.*

Gene	Forward Primer (5'-3')	Reverse Primer (5'-3')
GUSB	CTCATTGGAATTTTGCCGATT	CCGAGTGAAGATCCCCTTTTTA
CDK1	AGCCTAGCATCCCATGTCAA	TGCCATTTTGCCAGAAATTCG
CDK2	GGCCCGGCAAGATTTTAGTA	CGAAATCCGCTTGTTAGGG
CDK4	CGGTGGTACCTGAGATGGAG	AGTGCTGCAGAGCTCGAAA
CDK6	TCGATGAACTAGGCAAAGACC	GGTCCTGGAAGTATGGGTGA
CDK7	AGGAAAAGAACAGAGGCCTTAGA	TTTTGGCTATTTCCCTCAGTAGT
CCNA1	AATTGTGCCTTGCTGAGTG	GAGGTGAAGTTCTGGAAGTGC
CCNA2	AGTGTCTCTGGTGGGTGAG	ACCTACCTCAAAGCACCACA
CCNB1	TCTTCCAGTTATGCAGCACC	TTAGCATGCTTCGATGTGGC
CCND1	TGTCCTACTACCGCCTCACA	TGACTCCAGCAGGGCTTC
CCNE2	GCCTTGTGCCATTTTACCTC	TGACAAAAGGTACCATCCAATCT
CDKN1A	CTGGAGACTCTCAGGGTCGAA	CGGATTAGGGCTTCCTCTTG
CDKN1B	GAGCAATGCGCAGGAATAAG	GGGGAACCGTCTGAAACAT
CDKN1C	CCGTGTCCCTCTCCAAGC	CCCTTCCAACGTCCGCTG
CDKN2A	CTGAGGAGCTGGGCCATC	GACCTTCCGCGGCATCTAT
CDKN2B	ACTAGTGGAGAAGGTGCGAC	GCGCTGCCCATCATCATG
CDKN2C	TGCACAAAATGGATTTGGAA	CAAATCGGGATTAGCACCTC
CDC20	ATGGCTTTGCACAGAACCAG	CCTCAGGGTCTCATCTGCTG
CDH1	TACCCCTCCCTGACCCAG	TCCAGCACCAGTGACGATG
SKP2	TGCCCCAATCTTGTCCATCT	GAGTGATAGGTGTTGGAGGTAGT
DTL	GAATGCCGTCTTTGACCTGG	TCACCAGCTTTTACGTCCCA
FBOXO6	TGGGAGGAGCTACTAGACACA	CAGCTGCACTTTGAGTTGGT
CHEK1	TTTGTGGAAGACTGGGACTTG	CACTGCGACTGCTTCTTCAG
CHEK2	ACCGAACATACAGCAAGAAACA	AAGGTTCCATTGCCACTGTG
ATR	AATCCTCCCCACTTATGTTTGT	CAATCCGCAGAAGTCTCGTT
ATM	ACATGATAGAGCTACAGAACGAA	GGAATCTGAATGCCGATCTGA
WEE1	ATTGGCGGGCTCTGTTGAT	CCCACGCAGAGAAATATCGA
PKMYT1	TACTTCCGCCACGCAGAA	GCTGATGGGAATGCTGCC
PLK1	CTACCTACGGACCTGGTTCC	GGAGACTCAGGCGGTATGTG
MASTL	CCACAGCAGTCCCAAATGG	TTGCGCATAACTTTTCAACTGC
AURKB	TGCATCACACAACGAGACCT	TGAGCAGTTTGAGATGAGGT
BUB1B	AGGTCTTCTGGGATGGGTC	GGCATTGAGAATCCGCACAA
GADD45A	AGGAATTCTCGGCTGGAGAG	AGTGATCGTGCGCTGACTC
PPP2R2D	CATCCGCCTGTGTGACATG	TCTGAGAAGAAGGACCTACTGC
PP1A	ACCACCCTGACACATAAA	AGACAAGGTCCCAAAGAC

PPP1CA	CTTCAACTGCCTGCCCATC	CGCCGAATCTGCTCCATAGA
CDC25A	ATACGAGGGAGGCCACATC	CGCTTGCCATCAGTAGGTACA
CDC25B	GCCCTGTAGCCTGGACAAG	GGTCTCGTTCCCTGATGAAA
CDC25C	TCTGGGAGGACACATCCAG	CTCTTCTGGGTGTCCAAAGG
ENSA	GGATCTGCCCCAGAGAAAGT	GGGAAGCGTCTCAGGATCT
RB1	TCTTCCTCATGCTGTTTCAGG	TCTCTGCATGAAGACCGAGT
E2F1	CCGACAGCTGCAGGAGAG	CTCAGGAGGGGCTTTGATCA
FOXMI	GGACCAAACCTGCAGCTAG	CCTGCTGCCTCACCATCT
TP53	TTGGAActCAAGGATGCCC	TTATGGCGGGAGGTAGACTG
CDT1	CCCGCAACCTGATTTACCC	CAGTGTGGCCTGGGAGA
GEMININ	AGTCACCCAGGAGTCATTTGA	GCTTCATACAGCGCCTTTCT
PCNA	ATGGGCGTGAACCTCACC	GCCAAGGTATCCGCGTTAT
ORC1	GTCGATCAGGACTGGAGGAA	CTCTGACATGGTGGGGTACG
TFAP2A	CCCCTGGAGCTGTCAAGTAA	CTCGCAGTCCTCGTACTTGA
ISL1	CAGCAGCCCAATGACAAAAC	TTTCCAAGGTGGCTGGTAAC
CDX2	CAGGACGAAAGACAAATATC	ATGTAGCGACTGTAGTGA
NEUROD1	CTGCCTGCAACTCAATCCTC	TAGGGGTGTACAGGGAAGGA
OLIG3	AAAGAGAGAGAGAGCGCGAG	ACTGCGTCGAAAGGAAAACC
PAX6	TGCAGACACACATGAACAGTC	CTTCCGGGAACCTGAACTGG
LHX5	AGTGCAAAACCAACCTCTCG	TGCCAAAGCGCCTGAAAAAG
FOXA2	CACTCGGCTTCCAGTATG	TGCATAGTAGCTGCTCCA
SOX17	TTTCATGGTGTGGGCTAA	CAGCATCTTGCTCAACTC
GATA2	TACTACAAGCTGCACAAT	CTTGCTCTTCTTGGACTT
GATA3	AAGGCATCCAGACCAGAA	AAGTCCTCCAGTGAGTCAT
FOXC1	TAGCTGTCAAATGGCCTTCC	CTTTTCCTGCTTTGGGGTTC
HAND1	CTACATCGCCTACCTGAT	TTCTTGAGTTCAGCCTTG
MESP1	CCTCCTGCTTGCCTCAAAGTGTC	TCGAAGTGGTTCCTTGGCAGAC
MESP2	CCAGCAACTTTGGAGCTG	GCAGTCTCTGGCATGATGG
APLNR	AGCAGAGAGAAGCCTGGC	TGGATGCTTTGAGGACAGCT
PRDM1	TGTCAGACTTGCAACAAGGG	TGCTGGTGTCTGCTAAATCTC
TFAP2C	ACGAAATGAGATGGCAGCTAGG	GCAGTTCTGTATGTTCTGTCTCC
PDPN	GGAGACACACAGACAACAGTTG	TTGCACCAATGAAGCCGATG
NANOS3	TGACCTGTGGACAGATTACCTG	TGGTTTCAGGACCCTCTTTCC
POU5F1/OCT4	ACACTCGGACCACATCCTTC	AGTGAGAGGGCAACCTGGAGA
SOX2	CCATGCAGGTTGACACCGTTG	TCGGCAGACTGATTCAAATAATACAG
NANOG	TCTTCTGCTGAGATGCCTCAC	TGCAGAAGTGGGTTGTTTGC
BRY/TBXT	GATCACTTCTTTCTTTGCATCAAG	TGCTTCCCTGAGACCCAGTT
GATA6	GGTAATAGCAATAATTCATTCC	GCTGTAGGTTGTGTTGTG

MIXL1	ATTATCCTCAACCACTGT	AGTTCACATCTACCTCAA
GCS	AGGGAAGAGGAAGGTAAA	CGAGTTAGGTAAGTAATACG
EOMES	TGCCCCACTACAATGTGTTCG	TGCCCTGCATGTTATTGTTCG
BMP4	ACGAAGAACATCTGGAGAA	TGCTGCTGAGGTTAAAGA
WNT5A	ATCCTCATGAACCTGCACAAC	CTACATGAGCCGGACACCC
GDF3	TGTACTTCGCTTTCTCCCAGAC	TGGGCCAATGTCAACTGTTC
WNT3	TGTTAGTGTCCAGGGAGTTCG	TGCATTTGAGGTGCATGTGG
FGF8	ACCTTTGGAAGCAGAGTTCG	AGCGCTGTGTAGTTGTTCTC
WNT6	GCAACTGCACAACAACGAG	CGAAATGGAGGCAGCTTCTG
CER1	GGGTCATCTTGCCCATCAAAAG	CGCATTTCCCAAAGCAAAGG
NOG	TTCAGTAAGCGCTCGTGCTC	ACTCGGAAATGATGGGGTACTG
BMP2	GCAGCTTCCACCATGAAGAATC	CTGTTCTCGGAAAACCTGAAGC
LEFTY1	TGGACAAATGCTCTGTGCTC	TCCAGTGGCCAAAGATTCTC
LEFTY2	TGGACAGGGAATTGGGATAC	GAGGAAATGACGAGCCAAAG
NODAL	TGTTGGGGAGGAGTTTCATC	TCCACATACAGCATGCTCAG
FGF4	CCTTCTTCACCGATGAGT	ACTTGTAGGACTCGTAGG
FOXH1	ATCATCCGTCAGGTCCAG	AAAGGTTGTGGCGAATGG
LEF1	ACAAGCACAAACCTCTCAGG	TTGTTGTAGAGGCCTCCATCTG
TLE1	GGAATGTGAGAACTGGCAAGTG	GGGATGACTTGTGCACAAATCG
FGF17	ATCAAAGGGGCTGAGAGTGAG	CCGTATAGTTGTTCTCCAGCAC
SP5	TCGCTGCAGGCCTTTCTC	GGCACCTGCAGGAAGTCC

2.6 Protein-based techniques

2.6.1 SDS-PAGE and western blotting

Cell lysates were prepared by washing cells once in PBS followed by the addition of ice cold radioimmunoprecipitation (RIPA) buffer (50mM Tris HCl, 150mM NaCl, 1.0% (v/v) NP-40, 0.5% (w/v) Sodium Deoxycholate, 1.0mM EDTA, 0.1% (w/v) SDS and 0.01% (w/v) sodium azide at pH 7.4) supplemented with protease and phosphatase inhibitors to lyse cells. Cell lysates were transferred to a microcentrifuge tube and kept at constant agitation for 30 minutes at 4°C before centrifugation at 18000 x g for 20 minutes at 4°C. Supernatant was taken, and protein concentration measured using BCA kit (Pierce) according to manufacturer instructions. Between 10-40µg of protein was mixed with 1X Laemmli sample buffer (BioRad) + fresh dithiothreitol (DTT) and boiled at 100°C for 5 minutes. Samples were loaded on to 10% Criterion TGX pre-cast polyacrylamide gels (BioRad) along with a molecular weight marker and run at 100V for 1-2 hours to fractionate samples. Gels were transferred to a PVDF membrane using the Trans blot Turbo System (BioRad) before blocking membrane in Tris-Buffered Saline (TBS) + 0.1% Tween-20 (TBST) + 5% Milk for 1h at room temperature. Primary antibody was added at in TBS + 5% BSA and incubated overnight at 4°C. Membrane was washed 3X in TBST (5 minutes each) before incubation with secondary antibody in TBST + 5% Milk for 1-2 hours. Membranes were washed 3X in TBST (5 minutes each) and horseradish peroxidase (HRP) conjugated secondary antibodies were developed using Clarity Western ECL Substrate (BioRad). HRP conjugated antibodies were imaged using Amersham Imager 600 and Alexa-680 conjugated antibodies were imaged using Odyssey Infrared Imaging System (LI-Cor biosystems).

2.6.2 Immunoprecipitation (IP)

Cell lysates were prepared by adding ice cold non-denaturing lysis buffer which contained the following:

- 20mM Tris HCl pH 8
- 137mM NaCl
- 1% Nonidet P-40 (NP-40)
- 2mM EDTA

Fresh protease and phosphatase inhibitor were added to the lysis buffer on the day of cell lysate preparation. Cell lysates were transferred to a microcentrifuge tube and kept at constant agitation for 30 minutes at 4°C before centrifugation at 18000 x g for 20 minutes at 4°C. Supernatant was taken and an appropriate dilution of antibody (as recommended in the product datasheet) was added to the cell lysate. The cell lysate was then incubated overnight with gentle rocking at 4°C. Protein G Dynabeads (Thermofisher) were prepared by placing 50µL of resuspended beads into an Eppendorf tube and onto a magnet to separate beads. Beads were washed and separated twice in PBS before addition of sample to Dynabeads followed by an incubation with rotation for 1 hour at 4°C to allow antigen (Ag) to bind to the Dynabeads. The Dynabead-antibody-Ag complex was then washed by separating on the magnet followed by the addition of diluted non-denaturing lysis buffer (1:3) three times. Sample was mixed with 1X Laemmli sample buffer (BioRad) + fresh dithiothreitol (DTT) and boiled at 100°C for 10 minutes. Western blot protocol was then followed as in 2.6.1. A list of all antibodies used for western blot are displayed in Table 2-9.

Table 2-9- List of antibodies used for western blots and IPs

Name	Source	Catalogue Number	Species	Dilution	RRID
CDK2	Abcam	ab32147	Rabbit	1:1000 for WB/ 1:40 for IP	AB_726775
Cyclin E2	Cell Signalling Technology	4132	Rabbit	1:1000	AB_2071197
Alpha Tubulin	Sigma	T5168	Mouse	1:1000	AB_477579
Anti-rabbit IgG HRP Conjugate	Cayman	10004301	Goat	1:1000	AB_328089
Alexa 680 goat anti-mouse IgG	LICOR	A21057	Goat	1:1000	AB_141436

2.7 Mass spectrometry

2.7.1 On-Bead tryptic digestion

Immunoprecipitation samples were initially prepared as in 2.6.2. Samples in 50mM NH₄HCO₃ were prepared using on-bead tryptic digestion protocol prior to Mass Spectrometry. Sample was reduced with 5mM DTT for 1 h at 37°C in a thermomixer. Proteins were then alkylated with 10 mM IAA for 30 min in the dark at room temperature. After reduction/alkylation, Beads were trypsinised in order to digest the baits and the interacting proteins (0.4ug/ul trypsin in 50mM NH₄HCO₃). Digestion performed overnight at 37°C in a thermomixer, at 450 rpm shaking. Then, the reaction was stopped with 10% formic acid (FA). Peptides were recovered and the beads were discarded by using magnetic rack. Finally, a C18 clean-up was performed using EV2018 EVOTIP PURE. The desalting was done according to manufacturer's instructions protocol. Briefly, the Evotips tips were conditioned with 0.1% Formic acid in acetonitrile; equilibrated with 0.1% Formic acid in water and approximately 1 µg of each digested sample was loaded onto Evotips. Peptides were eluted from the evotips with 50% acetonitrile into a vial and vacuum dried by SpeedVac to remove any traces of organic solvents and the dried peptides were re-suspended in 30 µL 0.1% formic acid.

2.7.2 Mass spectrometry analysis

The resulting peptides were analysed by nano-scale capillary LC-MS/MS using an Ultimate U3000 HPLC (ThermoScientific Dionex, San Jose, USA) to deliver a flow of approximately 300 nL/min. A C18 Acclaim PepMap100 5 µm, 75 µm x 20 mm nanoViper (ThermoScientific Dionex, San Jose, USA), trapped the peptides prior to separation on an EASY-Spray PepMap RSLC 2 µm, 100 Å, 75 µm x 500 mm nanoViper column (ThermoScientific Dionex, San Jose, USA). Peptides were eluted with a 90 min gradient of acetonitrile (2%v/v to 80%v/v). The analytical column outlet was directly interfaced, via a nano-flow electrospray ionisation source, to a hybrid quadrupole orbitrap mass spectrometer (Lumos Tribrid Orbitrap mass spectrometer, ThermoScientific, San Jose, USA). Data dependent analysis (DDA) was carried out, using a resolution of 120,000 for the full MS spectrum, followed by MS/MS spectra acquisition in the linear ion trap using "TopS" mode. MS spectra were collected over a m/z range of 300–1800. MS/MS scans were collected using a threshold energy of 32% for collision-induced dissociation.

2.7.3 Mass spectrometry data analysis

LC-MS/MS raw files were processed in MaxQuant (version 2.0.3.1). The LFQ algorithm and match between runs settings were selected. Data were searched against the reviewed UniProt human proteome using the Andromeda search engine embedded in MaxQuant. Trypsin was set as the digestion enzyme (cleavage at the C-terminal side of lysine and arginine amino acid residues unless proline is present on the carboxyl side of the cleavage site) and a maximum of two missed cleavages were allowed. Cysteine carbamidomethylation was set as a fixed modification, while oxidation of methionine and acetylation of protein N-termini were set as variable modifications. The “match between runs” feature was used with a matching time window of 0.7 min and an alignment time window of 20 min. Label-free quantification was performed using the MaxLFQ feature included in MaxQuant according to default LFQ parameters. Minimum peptide length was set at 7 amino acid units. Target decoy analysis was performed by searching a reverse database with an overall FDR of 1% at the protein and peptide levels. The MaxQuant protein groups file was imported into Perseus software (version 1.4.0.2) for further statistical analysis and data visualization. Contaminant and reverse protein hits were removed. LFQ intensities were log₂ transformed. Missing values (NaN) were imputed from a normal distribution with default values. For each sample, the triplicate was grouped. Data were filtered for at least two out of three replicates LFQ intensity values in at least one group. Protein LFQ intensities were normalised, and missing values were imputed by values simulating noise around the detection limit using the default parameters. A protein was considered significantly differentially expressed when $FDR < 0.05$.

2.8 Microscopy

2.8.1 Immunofluorescence (IF)

Cells were fixed with 4% paraformaldehyde in PBS for 15 minutes at room temperature and permeabilized using 0.3% Triton X-100 in PBS for 5 minutes at room temperature. Cells were then blocked for 1 hour in blocking buffer containing 10% FBS and 3% BSA in PBS at room temperature. Primary staining was performed by incubating cells overnight at 4°C with primary antibodies diluted in primary antibody dilution buffer (1X PBS + 5%BSA + 0.3% Triton X-100). Cells were washed 3x in PBS between successive steps. The next morning, cells were washed 3x with 0.3% Tween in PBS. Secondary staining was performed incubating the cells with secondary antibody diluted in blocking buffer for 1 hour at room temperature. For nuclei visualization, cells were stained using DAPI (0.5 µg/mL). All primary antibodies used in this study are listed in table Table 2-10, and Table 2-11 for secondary antibodies. Imaging was performed on ScanR (Olympus) microscope (see 2.8.2 for details).

Table 2-10- List of primary antibodies for immunofluorescence used in this study.

Name	Source	Catalogue Number	Species	Dilution	RRID
Phospho Cyclin B1 (pS126)	abcam	ab55184	Rabbit	1:100	AB_879764
Wee1	NOVUS	H00007465-B01P	Mouse	1:50	AB_10719496
Phospho Cdk2 (T14)	abcam	ab68265	Rabbit	1:50	AB_1139872
Phospho Cdc6 (S54)	abcam	ab75809	Rabbit	1:250	AB_1310068
Phospho Rb (Ser807/811)	Cell Signaling Technology	8516	Rabbit	1:1000	AB_11178658
ATR	abcam	ab2905	Rabbit	1:500	AB_303400
p21	Cell Signaling Technology	2947	Rabbit	1:200	AB_823586
p27	Cell Signaling Technology	3686	Rabbit	1:1600	AB_2077850
FZR1(Cdh1)	abcam	ab89535	Mouse	1:100	AB_2040662
Emi1	Santa Cruz	sc-365212	Mouse	1:50	AB_10707690
Cyclin B1	Santa Cruz	sc-245	Mouse	1:100	AB_627338
Cyclin D1	Santa Cruz	sc-20044	Mouse	1:50	AB_627346
Cyclin E1	Santa Cruz	sc-247	Mouse	1:50	AB_627357
Cdk2	abcam	ab32147	Rabbit	1:200	AB_726775
Skp2	Cell Signaling Technology	2652T	Rabbit	1:200	AB_11178941
Phospho Rb (T373)	abcam	ab52975	Rabbit	1:100	AB_2177344
Phospho Rb (S608)	Cell Signaling Technology	2181	Rabbit	1:200	AB_331517
Nanog	Cell Signaling Technology	4893	Rabbit	1:1000	AB_10548762
Oct4	R&D Systems	AF1759	Goat	1:200	AB_354975
Lhx5	R&D Systems	AF6290	Goat	1:200	AB_10973257
γ H2AX	Cell Signaling Technology	9718	Rabbit	1:200	AB_2118009
p53	Cell Signaling Technology	2524	Mouse	1:2000	AB_331743
pChk2	Cell Signaling Technology	2661	Rabbit	1:50	AB_331479
Sox2	Cell Signaling Technology	3579	Rabbit	1:400	AB_2195767
TFAP2C	abcam	ab110635	Mouse	1:250	AB_10858471
Sox17	Cell Signaling Technology	81778	Rabbit	1:3000	AB_2650582
TBXT	R&D Systems	AF2085	Goat	1:1000	AB_2200235
Cyclin A	Santa Cruz	sc-271682	Mouse	1:200	AB_10709300
Cdt1	abcam	ab109421	Rabbit	1:100	AB_10866437
p15	abcam	ab88304	Mouse	1:100	AB_2042535
Cdc25A	Cell Signaling Technology	3652	Rabbit	1:200	AB_2275795
Pax6	abcam	ab195045	Rabbit	1:350	AB_2750924

Table 2-11- List of secondary antibodies for immunofluorescence used in this study.

Name	Source	Dye	Catalogue Number	Dilution	RRID
Donkey anti-mouse IgG	Life Technologies	Alexa 488	A21202	1:200	AB_141607
Donkey anti-mouse IgG	Life Technologies	Alexa 594	A21203	1:200	AB_141633
Donkey anti-mouse IgG	Life Technologies	Alexa 647	A31571	1:200	AB_162542
Donkey anti-rabbit IgG	Life Technologies	Alexa 488	A21206	1:200	AB_2535792
Donkey anti-rabbit IgG	Life Technologies	Alexa 594	A21207	1:200	AB_141637
Donkey anti-rabbit IgG	Life Technologies	Alexa 647	A31573	1:200	AB_2536183
Donkey anti-goat IgG	Life Technologies	Alexa 488	A11055	1:200	AB_2534102
Donkey anti-goat IgG	Life Technologies	Alexa 594	A11058	1:200	AB_142540
Donkey anti-goat IgG	Life Technologies	Alexa 647	A32849	1:200	AB_2762840

2.8.2 Live cell imaging

Cells were split 1:3 to 1:24 depending on length of experiment and plated on 96 well Matrigel coated plates (Greiner Bio-OneLtd). Cells were allowed to attach for at least 8 hours cells, then washed with PBS and replaced with fresh media. Live cell imaging was performed on ScanR (Olympus) microscope. The ScanR is a fully automated, high-content screening inverted epifluorescent microscope system and was used for all experiments presented in this thesis. The system is equipped with temperature, humidity and CO₂ controlled incubators. 10x or 20x plan (UCPLFLN) fluorescence objective (NA0.7) and a sCMOS camera (Orca Flash 4.0, Hamamatsu) were used. LED based illumination was used for excitation. Excitation and emission filters were as follows: DAPI [ex:391/20nm, em:440/521/607/700]; YFP [ex:509/22nm, em:515-560nm]; mCherry [ex:554/23nm, em:440/521/607/700nm]; Alexa-647 [ex:650/13nm, em:690/50nm]. For long term live cell imaging, an acquisition frequency of 15 minutes was used.

2.8.3 Light-sheet microscopy of gastruloids

Gastruloid aggregates were loaded in multi-well FEP holders and imaged every 1h using a LS1 Live Light sheet microscope from Viventis equipped with two opposing Nikon Plan Fluor 10X objectives, a Nikon 25X W NA 1.1 detection lens and Hamamatsu ORCA-Fusion Camera. Image volumes were acquired using the following combination of laser lines and filters: Laser 488nm, 60mW diode laser, Semrock FF03-525/50 or Semrock FF01-523/610 or Chroma ZET405/488/561/640mv2 and Laser 561nm, 50mW DPSS laser, Semrock FF01-523/610 or

Chroma ZET405/488/561/640mv2. Z-step between planes was set to 2 μ m with a thickness of the light-sheet set at 3.3 and the final magnification at 18.7x. Images were processed using FIJI software.

2.9 Image Analysis

2.9.1 Cellular segmentation

For image analysis of live cell imaging experiments, nuclei were segmented using either NLS, H2B, PCNA or summed intensities from both FUCCI(CA) fluorescent reporters. Deep neural networks (DDN) were trained using these masks to generate probability maps of cell nuclei using the Artificial Intelligence (AI) module in the ScanR software (Olympus). Manual annotations of cells from a variety of fluorescent intensities from different time points were given to train the DDN to have precise segmentation for particularly difficult cell cycle transitions where there are large fluctuations in fluorescent intensities (for example during the G1/S transition in the FUCCI(CA) reporter). Segmentation was then performed by applying an intensity threshold on the AI probability map (Figure 2-4). This image analysis pipeline was published in collaboration with Olympus and can be viewed in Appendix B (Padgett et al., 2021).

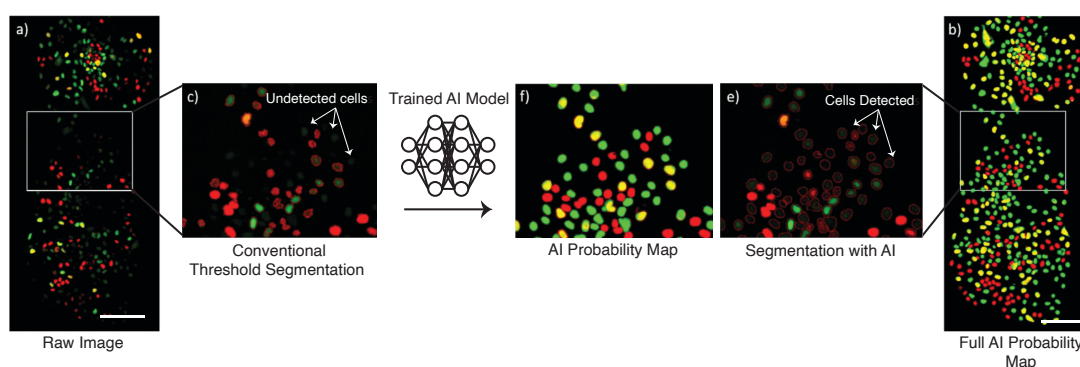


Figure 2-4 - **Schematic of ScanR AI segmentation** See 2.9.1 for details. Scale bar = 100 μ M.

For IF experiments, nuclei were stained using DAPI and segmented with StarDist (Schmidt et al., 2018) using the 2D versatile fluorescent pretrained model.

2.9.2 Quantification of cellular parameters

Fluorescent signals were quantified in live cell imaging experiments using ScanR software (Olympus). Background subtraction was applied before measurement of fluorescent intensity. For IF images, custom python scripts were used to quantify cellular fluorescence using skikit-

image (van der Walt et al., 2014). A rolling ball filter was applied to remove background before quantification. For cytoplasmic measurements, a ring region around the nuclear mask was made in both ScanR and skikit-image based analyses. Overlapping ring regions where cells were in close contact with each other were removed from quantification. Mean average intensities were calculated from segmented regions.

2.9.3 Single cell tracking

Automatic, single cell tracking was performed predominately with the kinetic module in the ScanR software with a radius of 50 pixels between two frames to detect cells. This tracking pipeline was recently published in collaboration with Olympus and can be viewed in Appendix B (Padgett et al., 2021). Lineage analysis was performed using the Tracking Tool (Hilsenbeck et al., 2016). For both tracking softwares, individual tracks were manually inspected and corrected before exporting quantification of cellular fluorescence.

2.9.4 Analysis of cell cycle biosensors

A variety of cell cycle biosensors were used in this thesis to quantitatively measure the dynamics of individual cell cycle phases during hESC differentiation including the FUCCI sensor developed by the Miyawaki Lab (Sakaue-Sawano et al., 2008), which employs a truncated fragment of the proteins Cdt1 and Geminin fused to fluorescent proteins. These proteins have opposing roles in the DNA replication control system, where Cdt1 licenses DNA replication in G1 phase and Geminin inhibits this process in S/G2/M. These proteins oscillate during the cycle due to the APC/C^{Cdh1} mediated degradation of Geminin in G1, and SCF^{Skp2} mediated degradation of Cdt1 in S/G2/M. The fluorescent readout of these proteins can therefore be used to quantify different stages of the cell cycle. This system can be simplified even further to investigate G1 dynamics individually just by using the Cdt1 moiety (Figure 2-5).

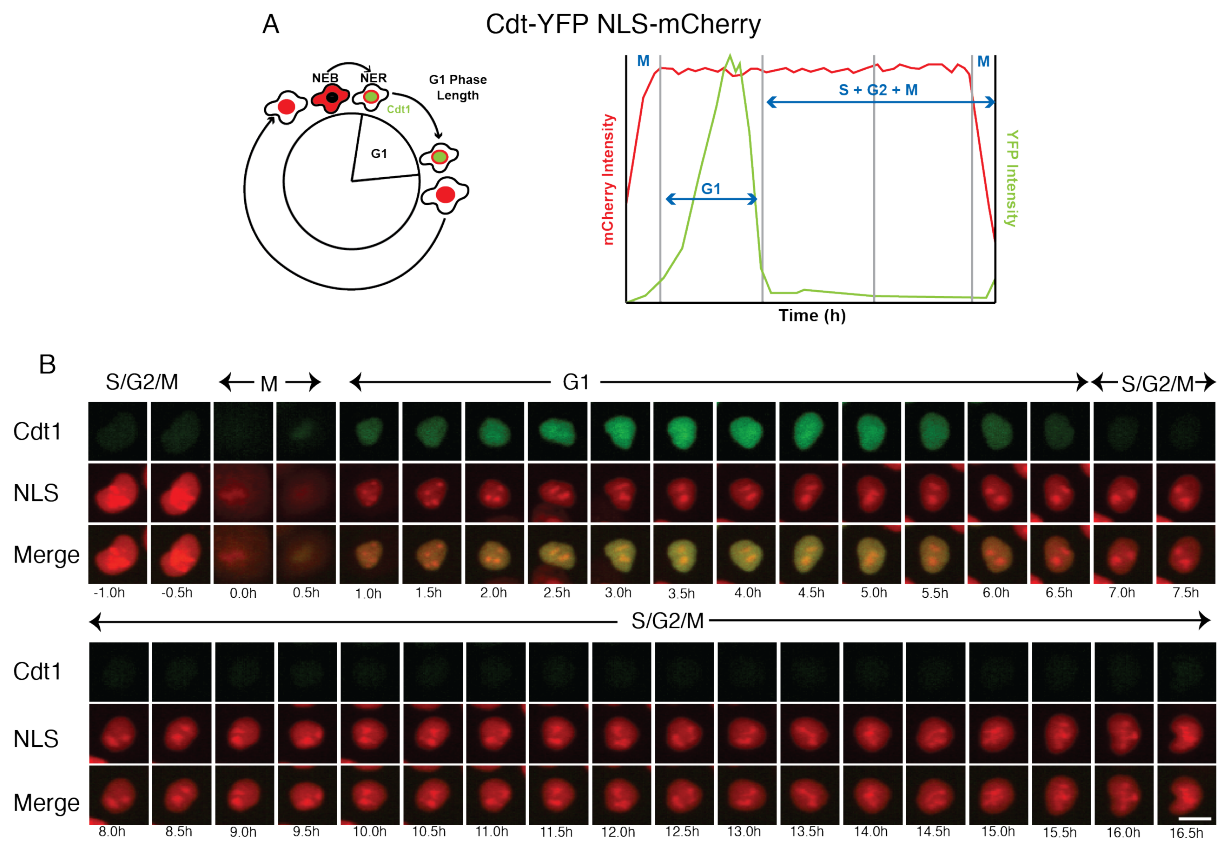
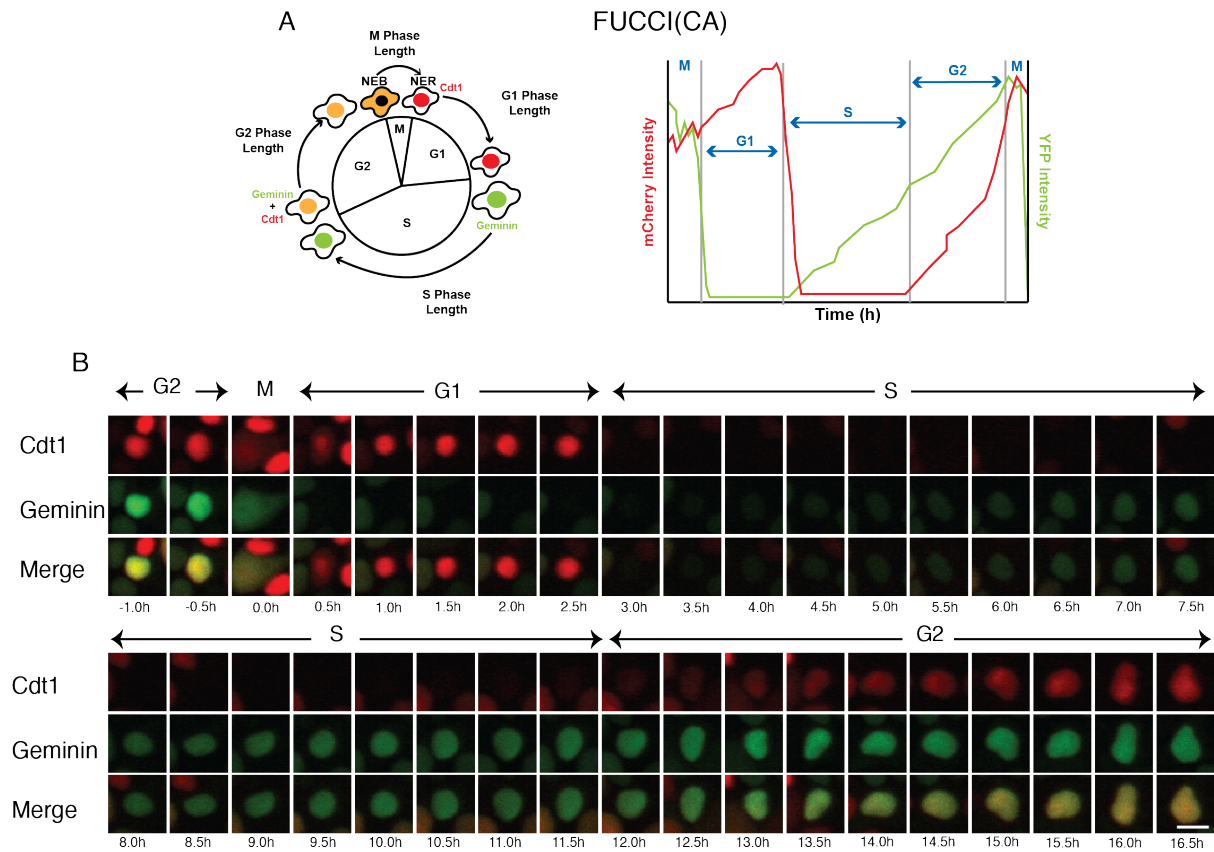


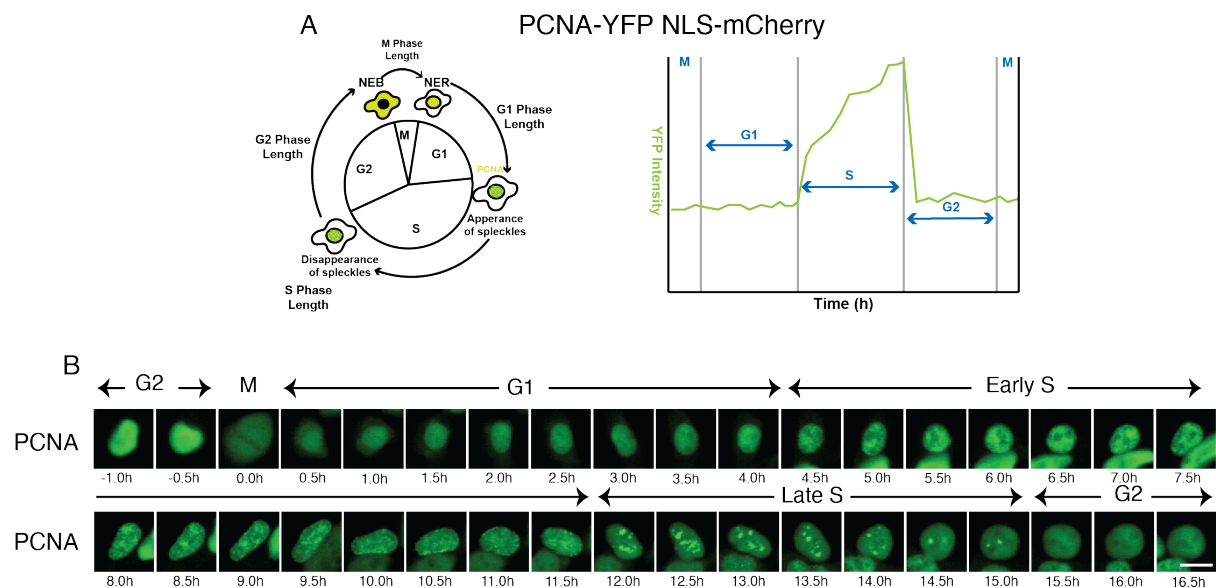
Figure 2-5- G1 biosensor *A. Schematic of changes in localisation and quantification expected for G1 sensor. See 2.9.4 description for details. B. Representative cell showing employing the G1 biosensor. Phase durations are highlighted with arrows. Scale bar = 10 μ M.*

To measure all four phases of the cell cycle in the same cell, the FUCCI(CA) reporter was used. FUCCI(CA) employs truncated fragments of Cdt1 and Geminin fused to fluorescent proteins but has reengineered the Cdt1 to be specifically degraded in S phase by the ubiquitin ligase CUL4^{Dbd1} (Sakaue-Sawano et al., 2017). In comparison to the older FUCCI sensor which can only distinguish G1 and S/G2/M (Sakaue-Sawano et al., 2008), FUCCI(CA) can distinguish all four phases of the cell cycle reliably (Figure 2-6).



*Figure 2-6- **FUCCI(CA)** biosensor A. Schematic of changes in localisation and quantification expected for FUCCI(CA) sensor. See 2.9.4 description for details. B. Representative cell employing the FUCCI(CA) sensor. Phase durations are highlighted with arrows. Scale bar = 10 μ M.*

Proliferating cell nuclear antigen (PCNA) can also quantify all four phases of the cell cycle (Zerjatke et al., 2017). PCNA is processivity factor for DNA polymerase and localizes to sites of active replication during S phase. When visualising fluorescent PCNA, it initially localises to the nucleus in G1 and upon S phase entry forms speckles as its loaded onto DNA. These speckles then disappear at the start of G2 (Figure 2-7).



*Figure 2-7- **PCNA biosensor** Schematic of changes in PCNA foci and quantification expected for PCNA sensor. See 2.9.4 description for details. B. Representative cell employing the PCNA sensor. Phase durations are highlighted with arrows. Scale bar = 10 μ M.*

2.9.5 Analysis of CDK activity biosensors

An established CDK2 activity biosensor based on translocation was used in this thesis (Spencer et al., 2013a). The sensor uses the amino acids 994-1087 of human DNA helicase B (DHB) fused to a fluorescent protein, four CDK phosphorylation sites, a nuclear localization signal and a nuclear export signal. When CDK2 activity is low, DHB remain nuclear but as CDK2 activity increases, DHB gets phosphorylated and translocated out of the nucleus. Measuring the cytoplasmic to nuclear ratio can therefore give a direct readout of CDK2 activity. An H2B/NLS nuclear marker was utilised to track and segment cells (Figure 2-8).

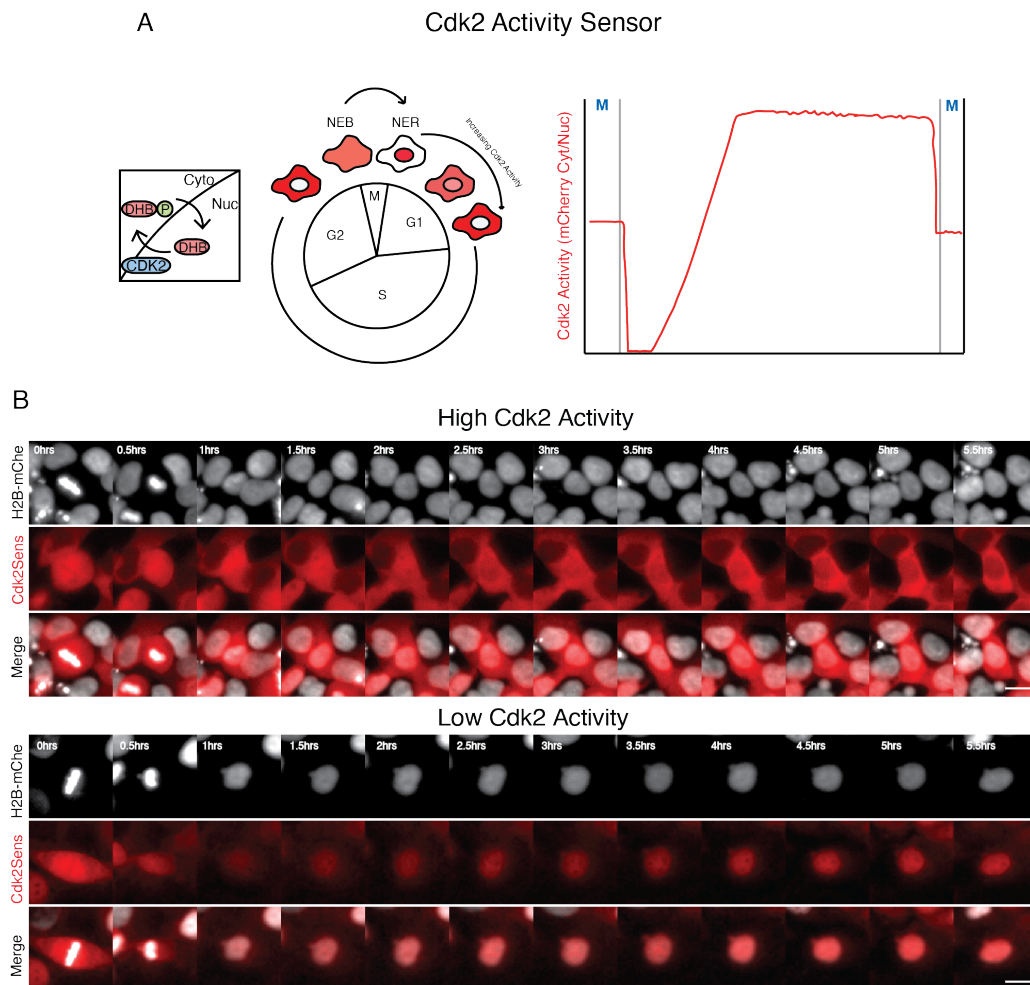


Figure 2-8- CDK2 activity biosensor A. Schematic of changes in CDK2 sensor localisation and quantification expected for CDK2 sensor. See 2.9.5 for details. B. Representative cell employing the CDK2 sensor. Top panel shows localisation of sensor when there is high CDK2 activity, and bottom panel shows localisation of sensor when there is low CDK2 activity. Scale bar = 10µM.

Chapter 3. Results 1: Cell cycle characterisation during the embryonic to somatic transition

3.1 Aims of chapter

The first aim of the work presented in this chapter is to quantitatively characterise how the cell cycle changes during BMP4-driven differentiation of hESCs. We set out to establish single cell methodologies in hESCs to monitor the dynamics of the cell cycle during the embryonic to somatic transition. In addition to this, we aim to unravel the molecular basis for remodelling and precisely measure the activities of regulators underpinning this transition.

3.2 Cell cycle dynamic changes during hESC differentiation

3.2.1 Using BMP4 as a model differentiation system

To monitor cell cycle dynamic changes during hESC differentiation, H1 hESCs cells were stimulated using BMP4. *In vivo*, BMP4 drives the initiation of gastrulation in the post implantation blastocyst (Jones et al., 1996). *In vitro*, it has been shown that within 3 days of BMP4 treatment, hESC lose pluripotency markers NANOG, SOX2 and OCT4 and start upregulating mesendoderm and primitive streak markers such as TBXT, TFAP2C, and SOX17 (Gunne-Braden et al., 2020; Xu et al., 2002). We therefore used this protocol and stained for these markers 72 hours after BMP4 stimulation and found that we could robustly recapitulate these staining patterns in our cultures (Figure 3-1). We found that cells in the centre of the colony expressed the pluripotency markers OCT4, SOX2 and NANOG (Figure 3-1A,B,C), while the cells away from the centre of the colony expressed the lineage specific markers TFAP2C, SOX17 and TBXT in various patterns depending on their proximity to the centre of the colony (Figure 3-1D,E,F). TFAP2C is a marker for trophoblast and was found to be expressed furthest away from the colony (Figure 3-1D). TBXT, a marker for mesoderm and SOX17, a marker for endoderm in contrast were expressed closer the colony centre (Figure 3-1E,F). Furthermore, we found that the cells away from the colony centre changed in cellular

morphology and flattened out, while cells in the centre of the colony remained tightly packed. This shows how BMP4 stimulation can initiate the differentiation of a variety of cell types that are found in early human gastrulation, while maintaining a core of pluripotency cells in the centre of the colony.

This differentiation protocol is highly tractable as hESC can be imaged and manipulated live, making it a great system for live cell imaging. Furthermore, this differentiation protocol is only 72 hours, reducing potential phototoxic affects from long term live cell imaging. We therefore decided to use this protocol as a model system to understand how cell cycle dynamics change during differentiation. (Figure 3-1).

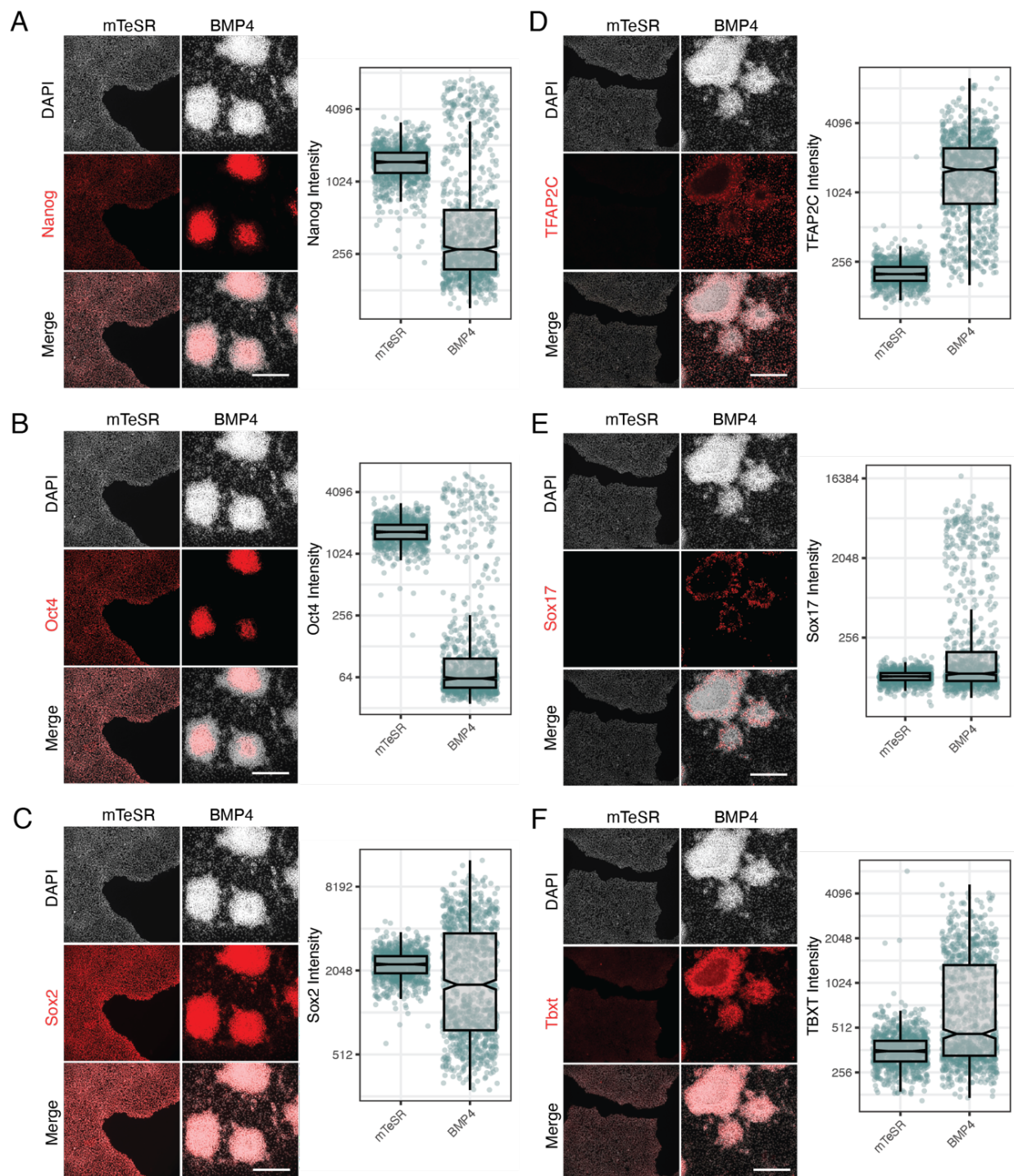


Figure 3-1- BMP4 drives hESC differentiation A-F (left) Representative images of immunostaining for hESC and BMP4-treated cells for 3 days. DAPI images are shown in gray scale and staining in red. (right) Single cell (nuclei) quantification of immunostaining for hESC and BMP4-treated cells for 3 days. $n=2000$ for each staining. Scale bar = $300\mu\text{M}$.

3.2.2 Comparison of cell cycle dynamics between hESC and BMP4-treated cells

To quantitatively measure how individual cell cycle phases change during differentiation, we generated a H1 hESC line stably expressing the FUCCI(CA) biosensor (described in (Sakaue-Sawano et al., 2017), Figure 3-2A). In order to compare the cell cycle of pluripotent and differentiated cells, hESC were either kept in pluripotency media (mTeSR) or 50ng/mL of BMP4 for 3 days before live cell imaging for 48h with an acquisition frequency of 15 minutes per frame. We then tracked single cells and measured total cell cycle length and individual phase lengths between the two conditions. While H1 hESCs kept in mTeSR had an overall cell cycle length of approximately 16 hours, BMP4-treated cells increased their cell cycle to an average of approximately 24 hours (Figure 3-2B). When deciphering the cause for this increase in total cell cycle length, we found that G1 increased the most extensively during differentiation, from 1.9 hours to 4.1 hours, with some cells having extremely long G1s (> 30 hours) (Figure 3-2B). While S and M phases lengths remained constant, we saw a mild decrease in G2 duration from 7.0 hours to 5.9 hours (Figure 3-2B). Of note, the variation in G1 lengths greatly increased in BMP4-treated cells, comparatively to the other cell cycle phases as measured by mean absolute deviations (MAD=3.50). hESC in contrast had G1s that were always short and constant (MAD=0.46). These changes in cell cycle dynamics can also be visualised using stacked bar plots for individual cells, which clearly illustrate how most of the variation in cell cycle length is contributed by G1 length for BMP4-treated cells (Figure 3-2C). G2 phase in contrast decreases mildly, and S phase remains constant. Altogether these data show that during BMP4-driven differentiation of hESC, G1 is the phase that is remodelled the most extensively, increasing not only in duration but variability.

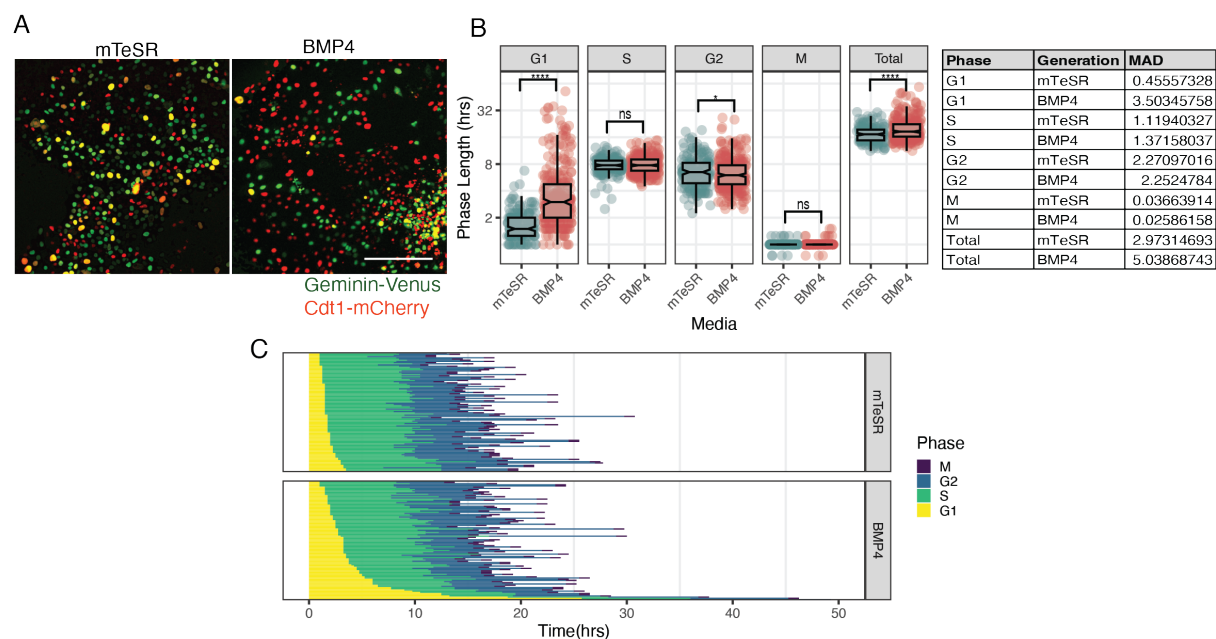


Figure 3-2- Comparison of hESC and BMP4 cell cycle dynamics *A*, Representative images of stable H1 hESC lines expressing FUCCI(CA) kept in mTeSR and with BMP4 for 3 days. Scale bar = 300µM *B*, Quantification of individual phase lengths using the FUCCI(CA) reporter for hESC treated with either mTeSR or BMP4 for 3 days. Boxplots are overlaid to indicate minimum, maximum, first quartile, third quartile and median values. KS tests were performed to calculate *p*-values (*ns*= not significant, *****p* < 0.0001, ****p* < 0.001, ***p* < 0.01, **p* < 0.05) (*n*=400, *N*=2 independent experiments). Table with mean absolute deviations (MAD) for each condition is shown on the right. *C*, Stacked bar plots from data in *B* showing individual phase lengths for single cells.

3.2.3 Correlation of cell cycle phases between hESC and BMP4-treated cells

To quantitatively analyse the relative contribution of individual phases to the overall cell cycle length, correlation analysis was performed on hESC and differentiated cells. Understanding whether different cell cycle phases are correlated and if this correlation changes during differentiation might pinpoint to potential regulatory mechanisms that underpin cell cycle structures and if there is any coupling between cell cycle phases. G1, S and M phase lengths correlated poorly ($R^2=0.29$, $R^2=0.29$, $R^2=0.005$ respectively) with total cell cycle length in hESCs, while G2 length correlated very strongly ($R^2=0.81$) (Figure 3-3). In BMP4-treated cells, S, G2 and M phases also correlated poorly with total cell cycle length ($R^2=0.09$, $R^2=0.31$ and $R^2=0.0036$ respectively) (Figure 3-3). Strikingly, in these cells the correlation between G1 phase and total cell length increased to $R^2=0.69$ (Figure 3-3). These data further highlight how most of the variation in BMP4-treated cells is contributed by G1 length, while in hESC most of the variation is contributed by G2 phase. This data is consistent with the fact that G1 duration increases during differentiation.

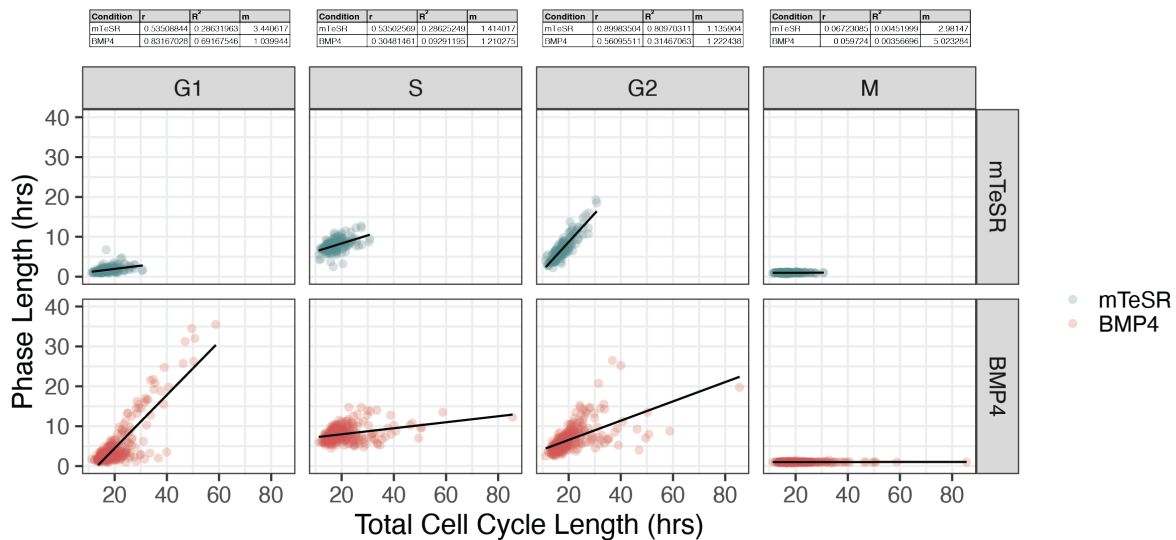
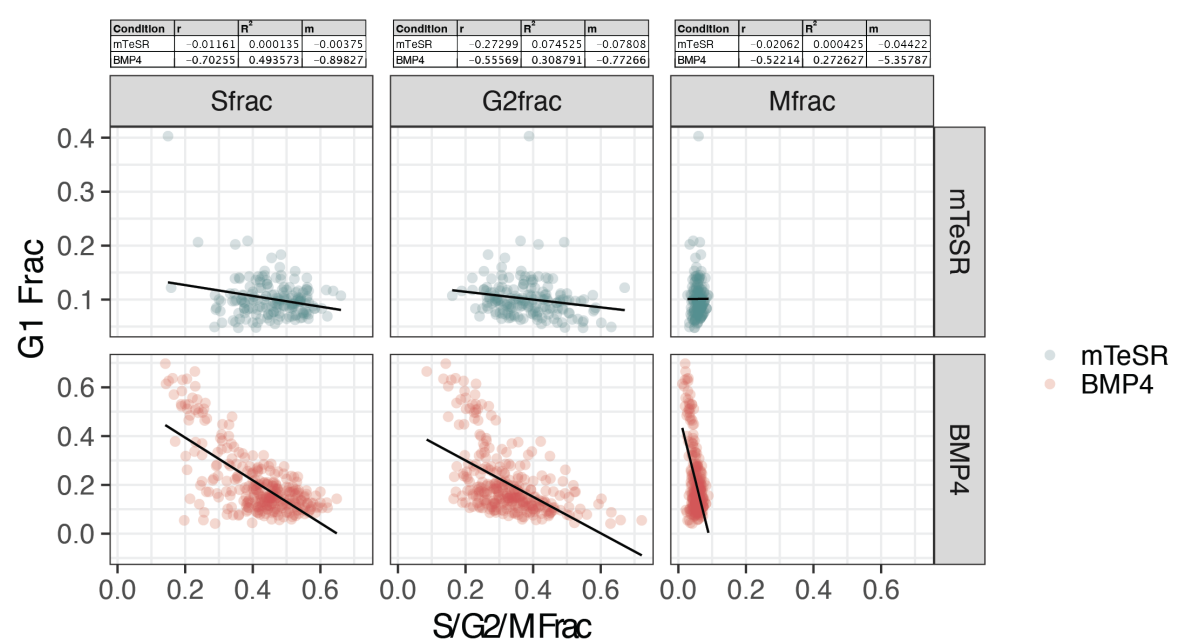


Figure 3-3- Correlating cell cycle phase between hESC and BMP4-treated cells Individual phase lengths correlated with total cell cycle length using the H1 FUCCI (CA) cell cycle biosensor for hESC treated with mTeSR or BMP4-treated hESCs for 3 days. Pearson correlation coefficient (r), coefficient of determination (R^2) and slope of trend line (m) are shown in the above table for each correlation ($n=400$, $N=2$ independent experiments).

As phase duration and total cell cycle length varies greatly between the undifferentiated and differentiated state, the time that each phase spent as a percentage of total cell cycle duration was plotted (Figure 3-4). This showed that in differentiated cells, an inverse correlation between G1 with the other phases of the cell cycle is established, in comparison to hESC, where there was no correlation. These results hypothesizes that the coupling between different cell cycle phases change during differentiation, further suggesting regulatory changes in cell cycle structure and control.



3.2.4 Cell cycle dynamics across lineages during hESC differentiation

After characterising the fundamental differences in cell cycle structures between hESC and BMP4-treated cells, we next wanted to address exactly when cell cycle structure changed during differentiation. Analysing cells as they transit from pluripotent to differentiated allowed us to quantitatively measure exactly how many divisions it took for individual phases to remodel. This would also let us observe if this transition happened in a graded or switch like manner, allowing us to make predictions on the potential regulatory mechanism behind this transition. For this we performed long-term live cell imaging of BMP4-driven differentiation of H1 hESC and tracked single cells over multiple generations. Stimulating pluripotent H1 cells with 50ng/ml of BMP4 for 5 days gave rise to the expected changes in morphology and to changes in FUCCI(CA) sensor (Figure 3-5A,B). As expected from the previous analysis, S, G2 and M phase lengths remained relatively constant throughout subsequent divisions (Figure 3-5C). Although there was no significant increase in G1 length after 1 division (F0 to F1), there was a considerable increase in G1 phase length and G1 heterogeneity after 2 divisions ($p < 0.0001$, F1 MAD = 0.56, F2 MAD = 2.86). This increase was seen to an even greater extent after 3 divisions ($p < 0.001$, F3 MAD=4.79) (Figure 3-5C). Similar results were seen for H9 hESCs (Figure 3-6).

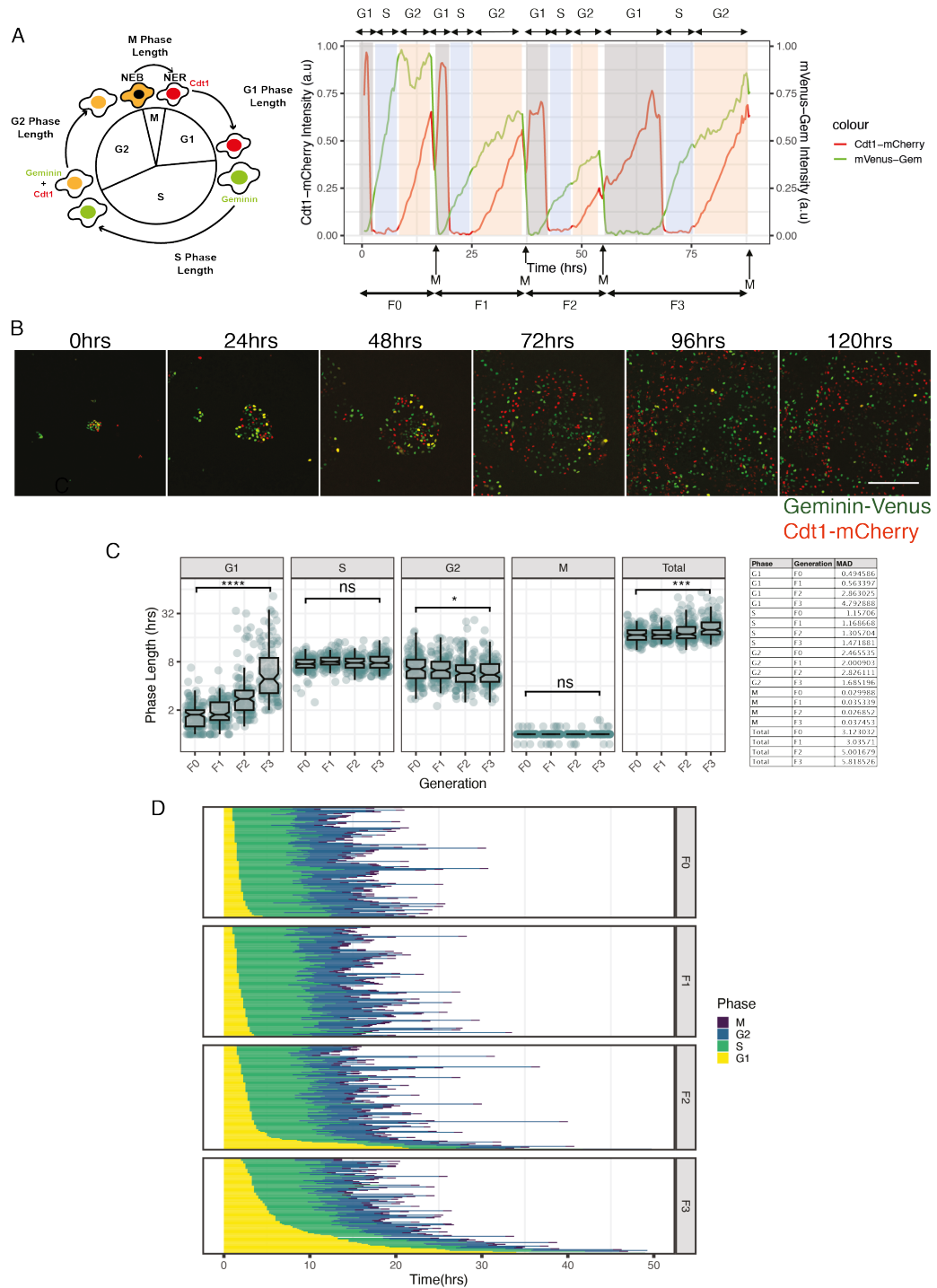


Figure 3-5- Cell cycle dynamics during differentiation in lineages A.(left) Schematic for FUCCI(CA) biosensor (right) Quantification of Cdt1-mCherry and mVenus-Geminin for a single cell after BMP4 treatment. B. Representative images of stable H1 hESC lines expressing FUCCI(CA) construct in the presence of 50ng/ml of BMP4 over incremental time points. Scale bar = 300µm C. Quantification of individual phase lengths using the FUCCI(CA) reporter for individual generations after treatment with BMP4. Boxplots are overlaid to indicate minimum, maximum, first quartile, third quartile and median values. KS tests were performed to calculate p-values (.ns= not significant, **** $p < 0.0001$, *** $p < 0.001$, ** $p < 0.01$, * $p < 0.05$) ($n = 400$, $N = 2$ independent experiments). Table with mean absolute deviations (MAD) for each condition is shown on the right. D. Stacked bar plots from data in C showing individual phase lengths for single cells in each generation.

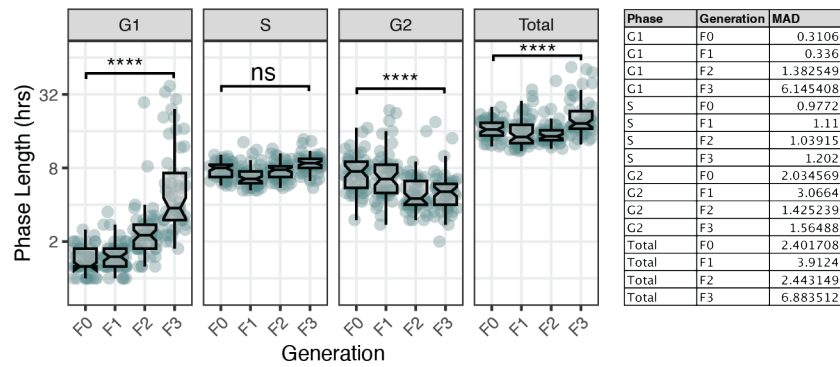


Figure 3-6- Cell cycle dynamic changes during differentiation using an independent cell line Quantification of individual phase lengths using the Fucci(CA) reporter in H9 hESC for individual generations after treatment with BMP4. Boxplots are overlaid to indicate minimum, maximum, first quartile, third quartile and median values KS tests were performed to calculate p-values (ns= not significant, **** $p < 0.0001$, *** $p < 0.001$, ** $p < 0.01$, * $p < 0.05$) ($n=200$). Table with mean absolute deviations (MAD) for each condition is shown on the right.

To investigate the exact timing to when G1 length was increasing, birthtime for individual cells in each generation after treatment with BMP4 was plotted. From this we could then see whether cells that had elongated G1 after just 2 divisions were further ahead in differentiation time than cells that did not. Although a few cells in the F2 generation increased their G1 before 48hrs, the majority of cells that had increased their G1 in generation F2 were exposed to BMP4 for at least 2 days (Figure 3-7A,B). Additionally, all the cells in the F3 generation had been treated with BMP4 for at least 2 days (Figure 3-7A,B). These data reveal that although it takes at least 2 divisions for cell cycle remodelling to occur, cells also need to be exposed to BMP4 for at least 48 hours.

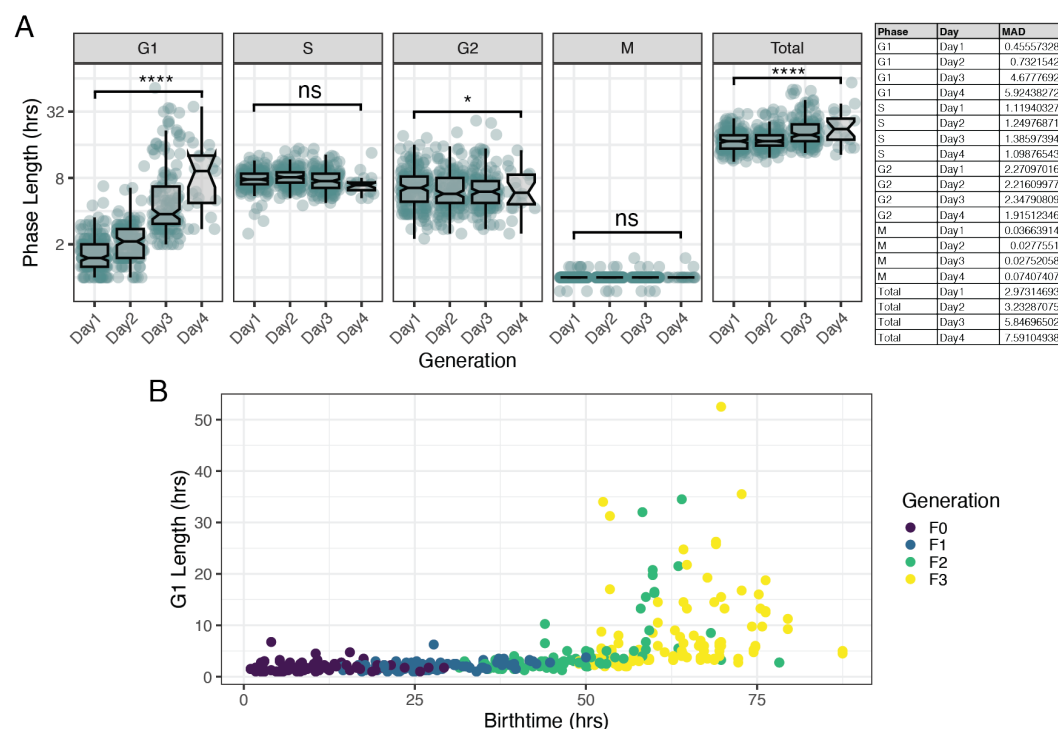


Figure 3-7- Cell cycle dynamics during differentiation in time Quantification of individual phase lengths using the FUCCI(CA) reporter in H1 hESC for individual days after treatment with BMP4. Boxplots are overlaid to indicate minimum, maximum, first quartile, third quartile and median values KS tests were performed to calculate p-values (.ns= not significant, **** $p < 0.0001$, *** $p < 0.001$, ** $p < 0.01$, * $p < 0.05$) ($n=400$, $N=2$ independent experiments). Table with mean absolute deviations (MAD) for each condition is shown on the right B. Scatter plot from data in A illustrating relative birthtimes for individual cells after BMP4-treatment and their G1 phase durations. Colours indicate distinct generations.

3.2.5 Cell cycle dynamic during differentiation are conserved with multiple biosensors

To validate cell cycle measurements and to understand whether cell cycle remodelling after BMP4-differentiation is conserved in other cell lines, we monitored cell cycle dynamics using additional cell cycle biosensors. Stable H1 hESC lines expressing Cdt1-YFP (described in (Sakaue-Sawano et al., 2008)) and PCNA-YFP biosensors (described in (Zerjatke et al., 2017)) were made. All lines were subjected to long-term live cell imaging after BMP4-stimulation, and single cells were tracked over multiple generations (Figure 3-8). To identify any discrepancies between cell lines, total cell cycle length for individual generations was measured. This revealed that regardless of the cell line used, all displayed an increase in cell cycle length between the first generation and last (Figure 3-8). The first generation showed a cell cycle length of between 16-18 hours, which increased to between 21-26 hours for the last

generation. hESCs infected with the PCNA sensor showed a slightly higher division rate comparatively at the start of imaging compared to the other two sensors. This discrepancy is likely to be a result of overexpressing PCNA.

In order to understand which phases of the cell cycle displayed the greatest amount of change during differentiation, individual phases were quantified. As seen with the FUCCI(CA) sensor, while S phase remained relatively constant during differentiation, there was a dramatic increase in G1 phase length alongside an increase in its variability after 2 divisions with all the sensors tested. Furthermore, G1 lengthening increased to a greater extent after 3 divisions. This was true for the two different hESC cell lines tested using the different sensors: H1 PCNA (G1 increased from 2.2 to 4.8 hours, p-value <0.001, F0 MAD = 0.056, F3 MAD = 5.79), H1 Cdt1 (G1 increased from 4.7 to 7.2 hours, p-value<0.0001, F0 MAD = 0.76, F3 MAD = 6.62) (Figure 3-8).

For FUCCI (CA) and PCNA lines, measurements of G2/M length were also possible. These measurements showed a slight decrease in G2 length during differentiation, which took between 1-3 division depending on the cell line. In H1 FUCCI this dropped from 7.0 to 5.9 hours (p-value < 0.05) in H9 FUCCI from 7.7 to 5.4 hours (p-value < 0.0001) and in PCNA from 5.4 to 4.4 hours (p-value < 0.01) between the first and last generation. Although there was no decrease in S/G2/M measurements in the Cdt1 cell line between the first and last generation, there was a vast decrease in the variation in S/G2/M measurements (F0 MAD = 3.24, F3 MAD = 1.31) (Figure 3-8).

Altogether, and consistently with the initial measurements with the FUCCI(CA) sensor, we observed that despite a slight decrease in G2 length as cells differentiate, remodelling of the cell cycle occurs predominantly during G1 phase.

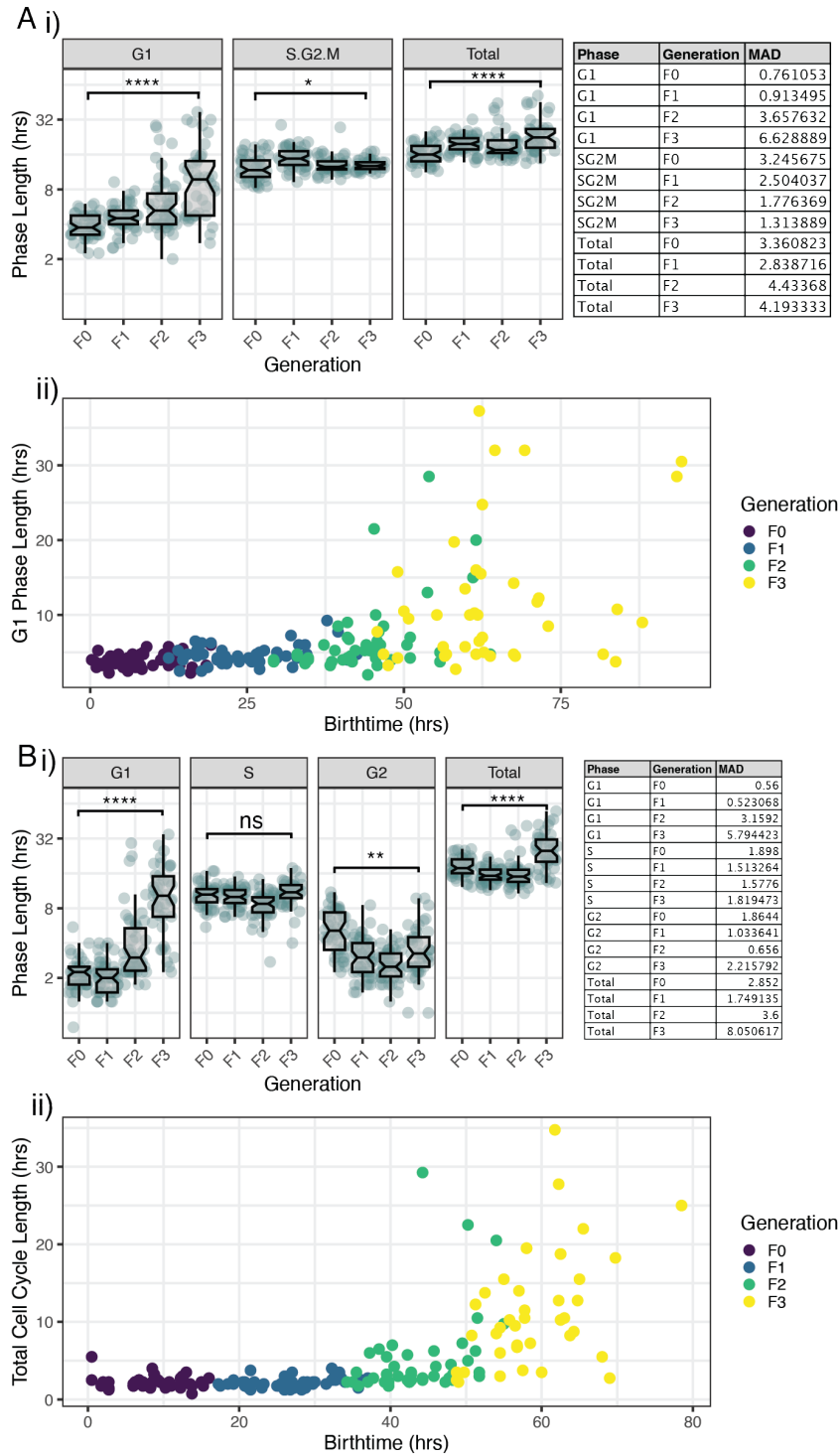


Figure 3-8- Cell cycle dynamic changes during differentiation using additional cell cycle biosensors. i) Quantification of individual phase lengths using the A. *Cdt1*-YFP biosensor B. *PCNA*-YFP biosensor in H1 hESC for individual generations after treatment with BMP4. Boxplots are overlaid to indicate minimum, maximum, first quartile, third quartile and median values KS tests were performed to calculate *p*-values (.ns= not significant, *****p* < 0.0001, ****p* < 0.001, ***p* < 0.01, **p* < 0.05) (*n*=200 for each biosensor) Table with mean absolute deviations (MAD) for each condition is shown on the right. ii) Scatter plot from data in A. *Cdt1*-YFP biosensor B. *PCNA*-YFP biosensor illustrating relative birthtimes after BMP4-treatment for individual cells and their G1 phase durations. Colours indicate distinct generations.

3.2.6 Sister cells have correlated cell cycle dynamics during differentiation

A degree of correlation between sister cell cycles has been reported in several studies (Sandler et al., 2015), (Barr et al., 2017), (Waisman et al., 2019) suggesting inherited factors play an important role in determining cell cycle dynamics of daughter cells. To further assess how cell cycle structures adapt during differentiation, we tracked daughter H1 cells from both undifferentiated and BMP4-treated hESC to see if lineage correlations were evident and/or preserved during differentiation. While both hESC and BMP4-treated cells had statistically significant correlations for cell cycle length between daughter cells, this correlation was much stronger in BMP4-treated cells (Figure 3-9). This trend was also seen when extending this analysis to individual phases, with G1 phases correlated the most strongly in comparison to S and G2 phases (Figure 3-9). Despite this, all phases of the cell cycle correlated stronger in BMP4-treated cells suggesting a higher degree of cell cycle coupling in differentiated cells. Interestingly this might mean that inheritance of factors that determine cell cycle dynamics may be more prevalent in differentiated cells. Altogether this analysis suggests that G1 is the phase of the cell cycle that is correlated the most in daughter cells, and potentially points at inherited factors being important in determining the length of G1.

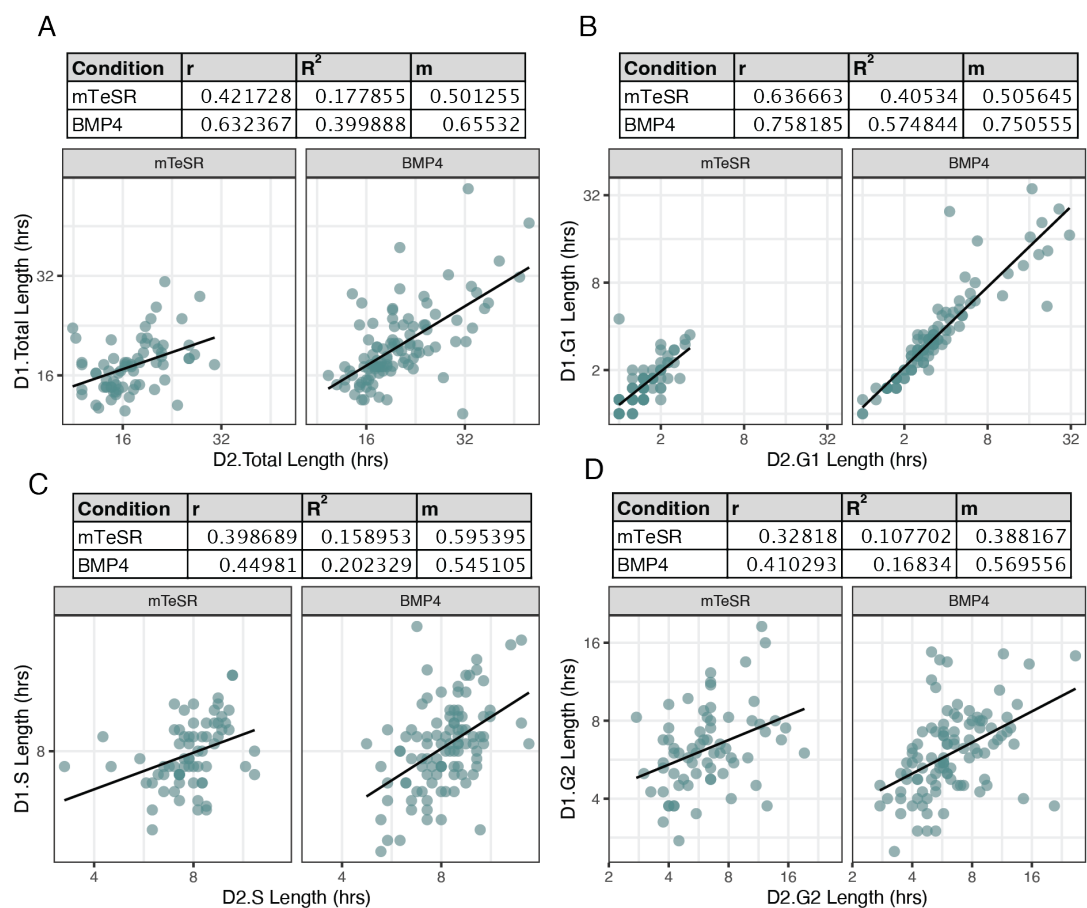


Figure 3-9- Sister cells have correlated cell cycle dynamics A-D. Correlation between daughter cells for hESC treated with either mTeSR or BMP4 for 3 days. A. Total Cell Cycle Length. B. G1 length, C. S length, D. G2 length. Pearson correlation coefficient (r), coefficient of determination (R^2) and slope of trend line (m) are shown in the above table for each correlation ($n=200$, $N=2$ independent experiments).

3.3 Molecular basis of cell cycle remodelling

3.3.1 RNAseq reveals that only a subset of cell cycle regulators change at the gene expression level

We next sought to address the molecular basis for G1 remodelling during differentiation. To identify differences in gene expression of cell cycle regulators between pluripotent and differentiated cells, we made use of bulk RNAseq data from our lab. RNAseq was performed on G1 and G2/M synchronized H1 hESC and BMP4-treated cells (Gharibi et al unpublished) (Figure 3-10A). Samples were synchronised by addition of the CDK1 inhibitor RO-3306 (Tocris) at 25 μ M for 8 hours followed by release for 16 hours and addition of RO-3306 for a further 10 hours. G2/M synchronised samples were released from RO-3306 for 30 minutes and G1 samples were released for 3 hours. As G1 was the phase that was remodelled the most extensively, we focussed our analysis on key G1/S regulators. Surprisingly, very few G1/S regulators were differentially regulated at the mRNA level in hESC when comparing them to BMP4-treated cells in both G1 and G2/M synchronised samples. The only statistically significant gene that was upregulated during differentiation in G1 was the CDK4/6 inhibitor protein CDKN2B (p15). CDKN2B was also upregulated in the G2/M synchronised sample, along with another CDK inhibitor protein CDKN1A (p21) and the CDK2 cyclin partner CCNE2 (Cyclin E2). In both G1 and G2/M synchronised samples, the only gene that was down regulated was the CDK2 binding partner CCNA1 (Cyclin A1) (Figure 3-10B,C). These results show that despite vast changes in cell cycle dynamics during differentiation, very few alterations are made at the gene expression level for key cell cycle regulators. Importantly, most of the notable changes appear to influence CDK activity, in particular CDK2 and CDK4/6.

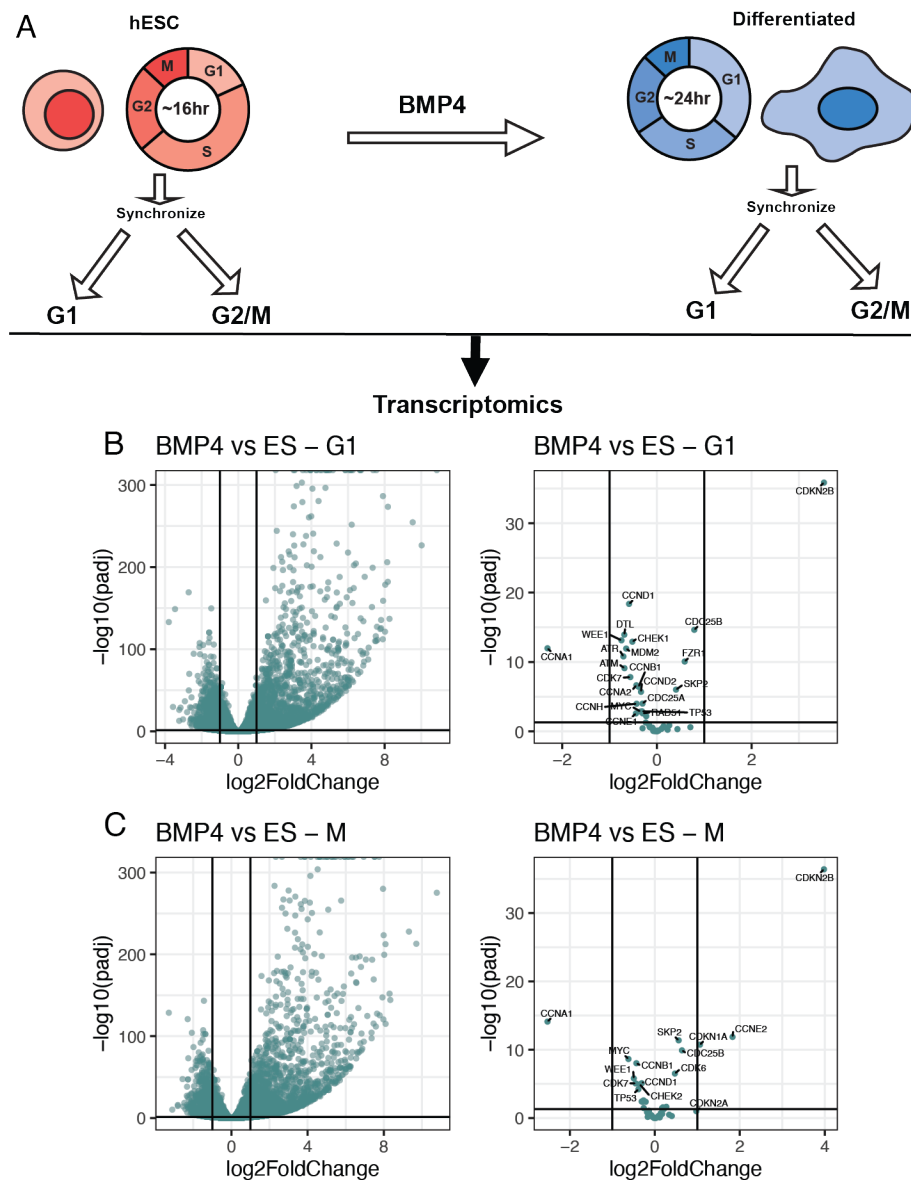


Figure 3-10- RNAseq reveals limited changes at the gene expression level for cell cycle regulators Schematic illustrating how synchronised hESC and BMP4-treated cells were subjected to bulk RNAseq analysis. B-C Volcano plots of RNAseq transcriptomic data comparing BMP4-treated hESCs for 3 days vs hESC for B. G1 synchronised samples C. G2/M synchronised samples. Left panels show all differentially expressed genes and right panels highlight specific G1/S cell cycle regulators. Solid black lines on x axis represent boundaries for up or down regulated genes. Solid line on y axis represents significantly differentially expressed genes with a p -adjusted value < 0.05 . Analysis results from $n=3$ replicates per condition.

3.3.2 Immunofluorescent screen identifies changes in protein abundance for many major cell cycle regulators during differentiation

After noting minimal changes at the RNA level for cell cycle regulators, we speculated that cell cycle dynamics were being regulated at the protein level. Furthermore, we wanted to identify exactly when in time alterations to cell cycle regulators happened following BMP4-stimulation. To do this, we used an unpublished dataset from our lab (Gharibi et al unpublished) which performed a time course with hESC treated with BMP4 and stained for a variety of known cell cycle regulators. For our analysis, we gated cells based on size and DAPI intensity as an estimate for cell cycle phase before quantification of cellular signal (Figure 3-11A). This would allow us to detect differences caused by changes in cell cycle regulators as opposed to just consequential differences caused by changes in cell cycle structure. To validate our gating, we stained for Cdt1, which is known to be present in G1 exclusively, and Cyclin A, whose expression increases throughout the cell cycle. As expected, Cdt1 was present at much higher levels in the G1 gating, and Cyclin A increased in levels as cells progressed through the cell cycle. We therefore used this gating on all other proteins (Figure 3-11B,C).

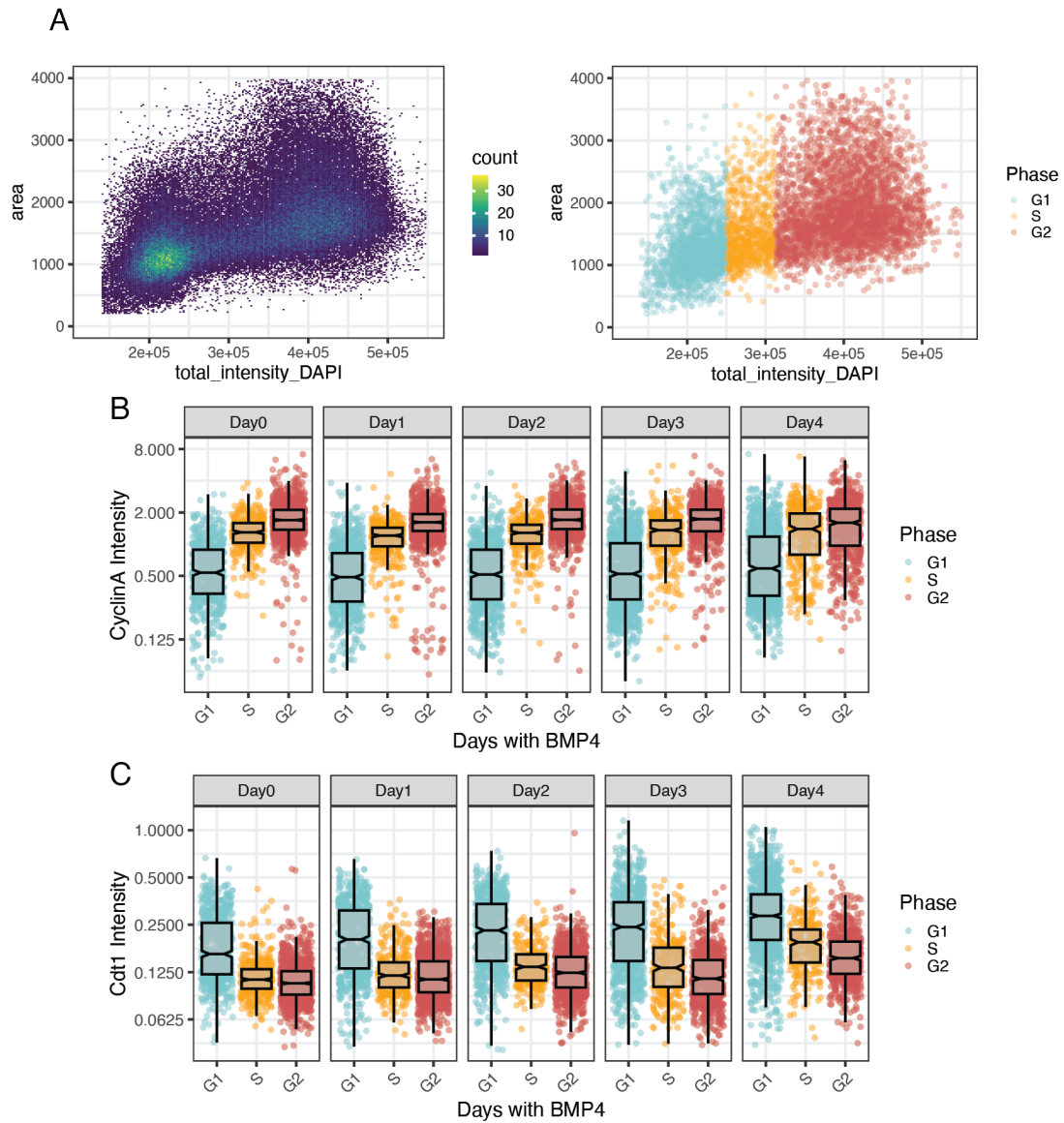


Figure 3-11- Cell cycle gating for immunofluorescent quantification A. (left) 2D density plot comparing total DAPI intensity with cellular area showing 3 distinct cellular populations. (right) Single cell scatter plot comparing total nuclei DAPI intensity and nuclei area, with cell cycle gates used for analysis colour coded. B-C. Single cell (nuclei) quantification of immunostaining for hESC treated with BMP4 over a 4-day time course. Cell cycle gating in A is used to quantify nuclei intensity for each cell cycle phase. A. Cdt1 staining B. Cyclin A intensity. $n=15,000$ for each staining. Boxplots are overlaid to indicate minimum, maximum, first quartile, third quartile and median values.

We first stained for hyper phosphorylated (Ser807/811) retinoblastoma (Rb), to see if hESCs had the ability to enter quiescence. Cells that have hyperphosphorylated Rb are relieved from E2F dependent transcription allowing DNA replication to occur and are thought to be committed to the cell cycle. Somatic cells are normally born with unphosphorylated Rb in G1,

and as cells commit to the cell cycle phosphorylate Rb and enter S phase. Surprisingly, we found that on Day 0 before BMP4-stimulation, all cells had hyperphosphorylated Rb, even in G1. 3 days after BMP4-stimulation we found that a fraction of cells in G1 started to have hypo, mono or unphosphorylated Rb, which increase further after 4 days (Figure 3-12). This data suggests that hESC are born committed to the cell cycle, having cell cycle independent regulation of Rb. Furthermore, the absence of an hypophosphorylated Rb population may suggest a lack of G1/S checkpoint control.

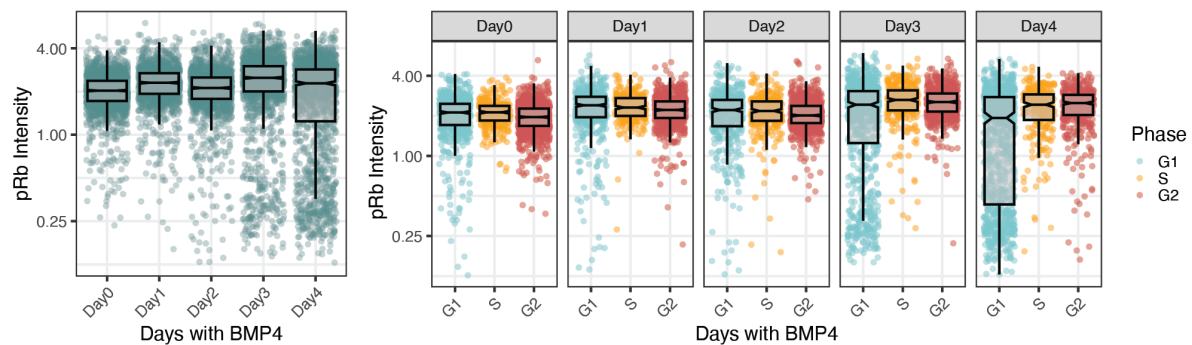


Figure 3-12- Rb is hyperphosphorylated in hESC (left) Single cell (nuclei) quantification of pRb (S807/811) immunostaining for hESC treated with BMP4 over a 4-day time course (right) Quantification of immunostaining of pRb (S807/811) with cell cycle gating over a 4-day time course with BMP4. $n=15000$. Boxplots are overlaid to indicate minimum, maximum, first quartile, third quartile and median values.

To investigate this further, we stained for a variety of CDK inhibitor proteins including p21 and p15 which were also hits from the RNAseq data set. With both CDK inhibitors we found that hESC have much lower levels of these proteins. p15 appeared to increase gradually following BMP4-stimulation over successive days, whereas p21 had a sharp increase in levels on day 3. When looking at the cell cycle gating, differentiated cells appeared to have elevated levels of both CDK inhibitor proteins in G1 in comparison to the other phases of the cell cycle (Figure 3-13). Consistent with the RNAseq dataset, hESC had lower levels of CDK inhibitor proteins which are often upregulated in response to DNA damage and stress, further indicating an absence of checkpoint control. The diminished levels of CDK inhibitor proteins may also be a mechanism by which higher CDK activity is achieved.

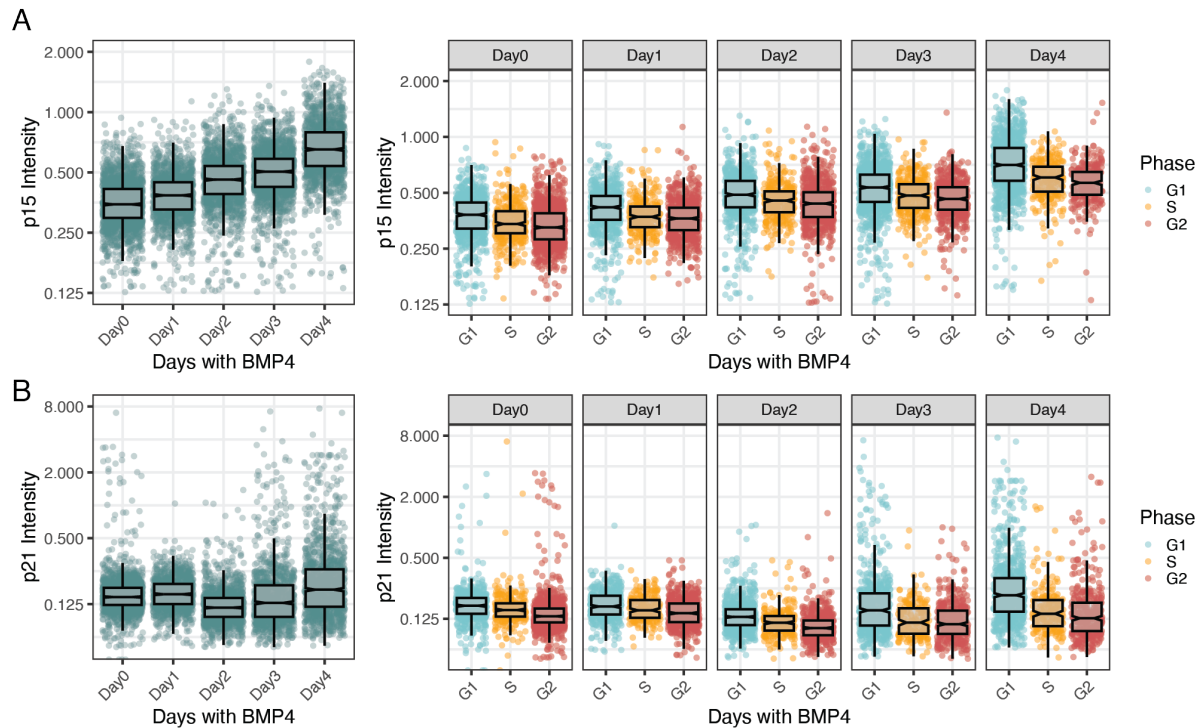


Figure 3-13- Levels of CDK inhibitor proteins increase during differentiation A-B. (left) Single cell (nuclei) quantification of immunostaining for hESC treated with BMP4 over a 4-day time course (right) Quantification of immunostaining with cell cycle gating over a 4-day time course with BMP4. $n=15000$ for each staining. A. p15 staining B. p21 staining. Boxplots are overlaid to indicate minimum, maximum, first quartile, third quartile and median values.

As we had noted several changes at the protein level for cell cycle regulators, we next wanted to see if there were any differences in the levels of regulators involved in protein degradation. We first looked at the anaphase-promoting complex (APC/C) which degrades a plethora of cell cycle regulators including cyclins. To do this, we stained for the APC/C co activator Cdh1 as a proxy for APC/C activity. hESC had very low levels of Cdh1 which increased during differentiation. Furthermore, levels remained low throughout the cell cycle whereas 3 days after BMP4-stimulation, Cdh1 became cell cycle dependent, having much higher levels in G1 (Figure 3-14A). We next wanted to look at the SCF ubiquitin ligase complex, which is involved in degrading cell cycle inhibitor proteins. For this we stained for an important component of the SCF ubiquitin ligase, the S-phase kinase-associated protein 2 (Skp2). Interestingly, we found that in hESC, Skp2 was at elevated levels across the whole cell cycle. After 3 days of BMP4-stimulation, we found that Skp2 levels had reduced and become cell cycle regulated, having much lower levels in G1 (Figure 3-14B). Taken together, this data suggests that hESC have different ubiquitin ligase activity in comparison to differentiated cells. As both ubiquitin ligases have a diverse selection of known cell cycle targets and we see a lack change at the

gene expression level, protein degradation may be at the heart of rewiring existing cell cycle networks during differentiation.

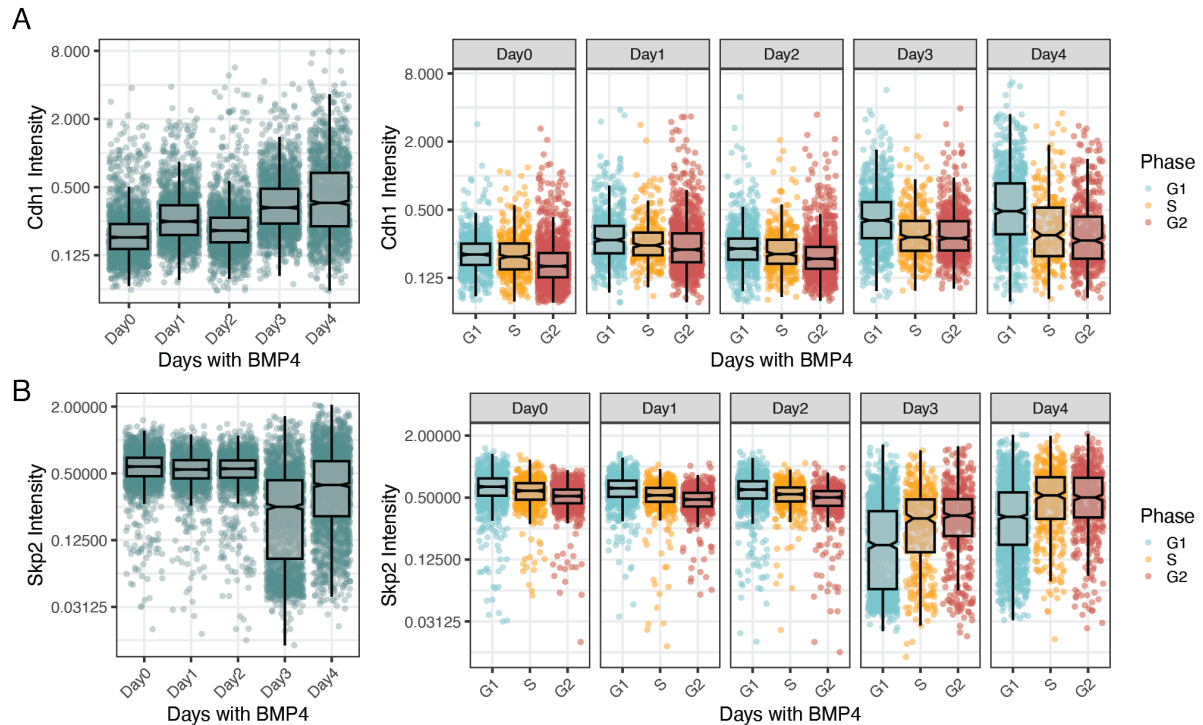


Figure 3-14- hESC display differential ubiquitin ligase activity during differentiation A-B. (left) Single cell (nuclei) quantification of immunostaining for hESC treated with BMP4 over a 4-day time course (right) Quantification of immunostaining with cell cycle gating over a 4-day time course with BMP4. n=15000 for each staining. Boxplots are overlaid to indicate minimum, maximum, first quartile, third quartile and median values. A. Cdh1 staining B. Skp2 staining,

3.3.3 Phosphoproteomics reveals changes in CDK activity during differentiation

As minimal changes were observed at the gene expression level, we analysed how the activity of G1 regulators changed during hESC differentiation by analysing changes in phosphorylation as measured by phospho-proteomics (Gharibi et al unpublished, Ochoa et al., 2016). As protein phosphorylation is a fundamental mechanism that regulates cell cycle control, we reason that this could be a valuable data source to identify differential kinase activities. As with the RNAseq sample preparation (Figure 3-10), cells were initially synchronised in G1 and G2/M phases of the cell cycle before undergoing phosphoproteomics to measure kinase activities (Gharibi et al unpublished, Ochoa et al., 2016). In the G1 synchronised samples, CDK1, CDK6

and CDK2 all showed significantly higher kinase activities in pluripotent cells (Figure 3-15). Conversely, ATR and CHEK2 which are involved in the DNA damage response and checkpoint control showed lower activities in pluripotent cells (Figure 3-15). Like G1 synchronised cells, the G2/M populations, CDK1, CDK6 and CDK2 all showed increased kinase activities (Figure 3-15). The checkpoint proteins CHEK2 and ATM also showed decrease kinase activities.

Taken together, these results show that the 3 major CDKs that drive the mammalian cell cycle are all at elevated activities in pluripotent cells while kinases involved in checkpoint control and DNA damage response have lower activities. Increasing the activities of kinases that promote cell cycle progression while reducing the activities of kinases that halt cell cycle progression may therefore be a mechanism that brings about the faster cell cycle displayed in hESC.

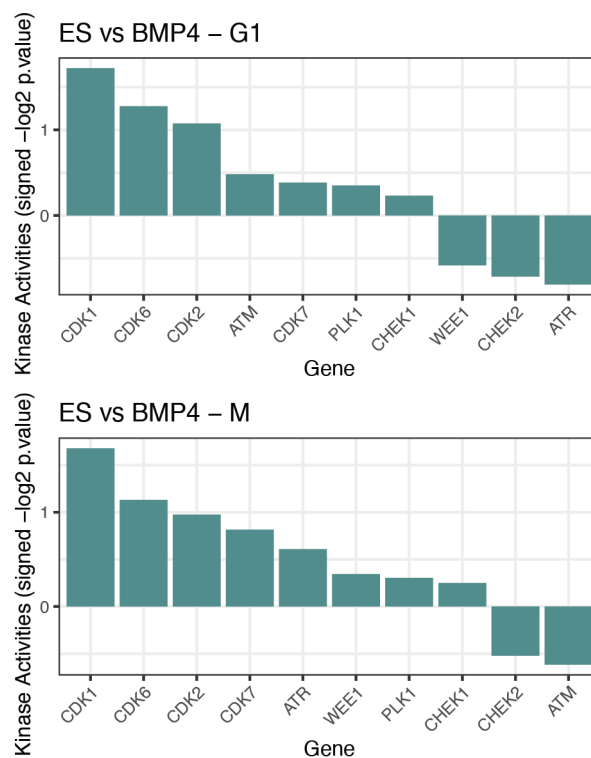


Figure 3-15- Phosphoproteomics reveals changes in kinases activities during differentiation Kinase set enrichment analysis (KSEA) was used to predict differential kinase activities comparing hESC and BMP4-treated hESC for 3 days as done in (Ochoa et al., 2016) for A. G1 synchronised samples B. G2/M synchronised samples. Analysis results from n=3 replicates per condition.

3.3.4 CDK activity biomarkers reveal CDK activity decrease during differentiation

We next wanted to confirm the results from the phosphoproteomics showing changes in CDK activity during hESC differentiation. To do this, we performed immunofluorescence to stain for known endogenous CDK phopho-substrates comparing hESCs and BMP4-treated cells. As a proxy for CDK1 activity, we used Cyclin B1 phosphophorylated at S126, which has previously been reported to be important in CDK1 activation (Santos et al., 2012). A known CDK2 substrate, phosphorylated Cdc6 at S54 was used as it was previously shown to be a readout for CDK2 activity (Spencer et al., 2013). Finally, to measure CDK4/6 activity, we stained for S780 phosphorylation of Retinoblastoma protein (Rb), which has been shown to be important for the initial Rb inactivation by CDK4/6 (Kim et al., 2022). In all 3 readouts, hESC displayed higher intensities of these phosphosubstrates, indicative of higher CDK activity comparatively to BMP4-treated cells (Figure 3-16). This confirms the phospho-proteomics analysis and suggest that hESC have increased CDK1, CDK2 and CDK4/6 activities.

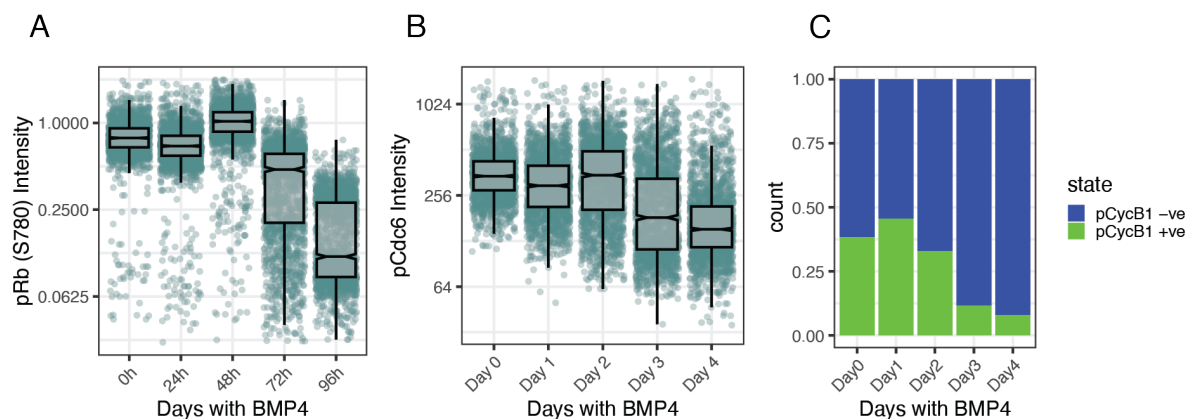


Figure 3-16- Endogenous CDK activity changes during hESC differentiation Single cell (nuclei) quantification of immunostaining for hESC treated with BMP4 over a 4-day time course. A. pRb S780 staining B. pCdc6 staining. $n=5000$ for each staining. Boxplots are overlaid to indicate minimum, maximum, first quartile, third quartile and median values. C Stacked bar plot illustrating percentage of cells with positive and negative Phospho (S126) Cyclin B1 Staining. $n=5000$.

3.4 CDK2 activity dynamics during differentiation

3.4.1 hESC have high CDK2 activity

Since CDK2 is the major regulator of the G1/S transition we set off to quantitatively explore how CDK2 activity dynamics changed during differentiation at the single cell level during BMP4-driven differentiation. We made use of an established CDK2 activity biosensor based on translocation (Spencer et al., 2013). The sensor uses the amino acids 994-1087 of human DNA helicase B (DHB) fused to a fluorescent protein, four CDK phosphorylation sites, a nuclear localization signal and a nuclear export signal. When CDK2 activity is low, DHB remain nuclear but as CDK2 activity increases, DHB gets phosphorylated and translocated out of the nucleus (Figure 3-17A).

We first set out to measure how this sensor behaves in both hESC and differentiated conditions to establish the key differences in CDK2 activity dynamics before looking at this transition in real time. In BMP4-treated cells, the sensor behaved similarly to previous reports in proliferating somatic cells (Figure 3-17B,C). We observed an initial drop in CDK2 activity after mitosis, with a variable delay in this low state before a steady increase in activity for the remainder of the cell cycle. In hESC, the sensor exhibited very different characteristics. Firstly, CDK2 activity increased immediately after mitosis, without the drop in activity displayed in differentiated cells (Figure 3-17B,C). These data illustrate how CDK2, an essential driver the G1/S transition, displays altered dynamics in the hESC.

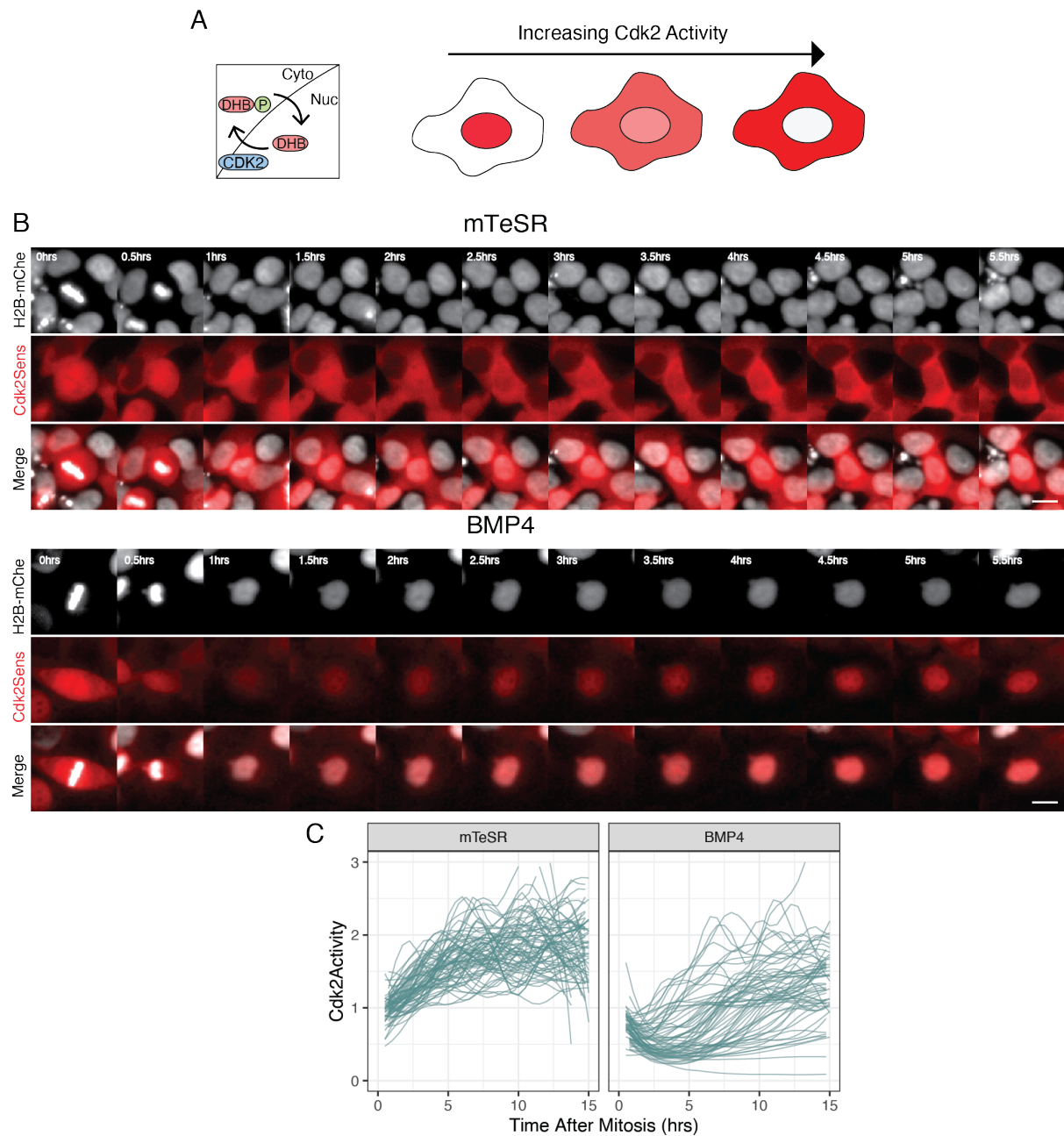


Figure 3-17- CDK2 activity is high in hESC *A. Schematic illustrating how the CDK2 activity biosensor works. DHB-mCherry is phosphorylated by CDK2 and gets translocated out of the nucleus. Increasing cytoplasmic to nuclear fluorescent ratios indicate increasing CDK2 activity. B. Fluorescent images of hESCs bearing the CDK2 activity sensor in pluripotency media (mTeSR) (top) and after treatment with BMP4 for 3 days (bottom). H2B images are shown in gray scale and CDK2 sensor in red. Scale bar = 10 μ M. Figure is repeated from Figure 2-8- **CDK2 activity biosensor** C. Quantification of individual CDK2 activity traces aligned from anaphase onset from hESCs in either pluripotency (mTeSR) medium (left) (n=40) or after treatment with BMP4 for 3 days (right) (n=100, N=2 independent experiments).*

3.4.2 Distinct three-tier CDK activity states

To further characterise different CDK2 activity dynamics in live, single cells, we measured changes in CDK2 activity as cells progressed through differentiation. We sub-grouped individual pooled traces from both hESC and differentiated conditions based on their CDK2 dynamics. We performed similar analysis as in (Spencer et al., 2013), where traces are divided into two groups depending on their activity 2.5 hours after anaphase. Consistent with their analysis, we found a sub population of cells that increased their activity from an intermediate level (0.5-1.0) (designated CDK2 Int) (Figure 3-18A,B). We also found a population of traces whose CDK2 activity dropped below 0.5 and had a much more variable time in this low state (designated CDK2 Low). The CDK2 low state could later re-enter the proliferative state but had a much longer cell cycle duration in comparison to CDK Int cells, consistent with previous reports (Spencer et al., 2013) (Figure 3-18A,B). Strikingly, we also found a population of cells with CDK2 activity that had never been previously reported. These cells did not drop their CDK2 activity after anaphase onset, and had an activity higher than 1.0, 2.5 hours after mitosis. We designated this “CDK2 High” (Figure 3-18A,B). We performed an unbiased, independent clustering of our single cell traces and compared this to our manual threshold-based grouping. We found that our threshold-based grouping had a high degree of overlap in comparison to the clustered traces (Figure 3-18C,D). In summary, these analyses show three distinct CDK2 activity dynamics in traces pooled from both the ES and differentiated state, allowing us to explore how proportions of these states changes during differentiation.

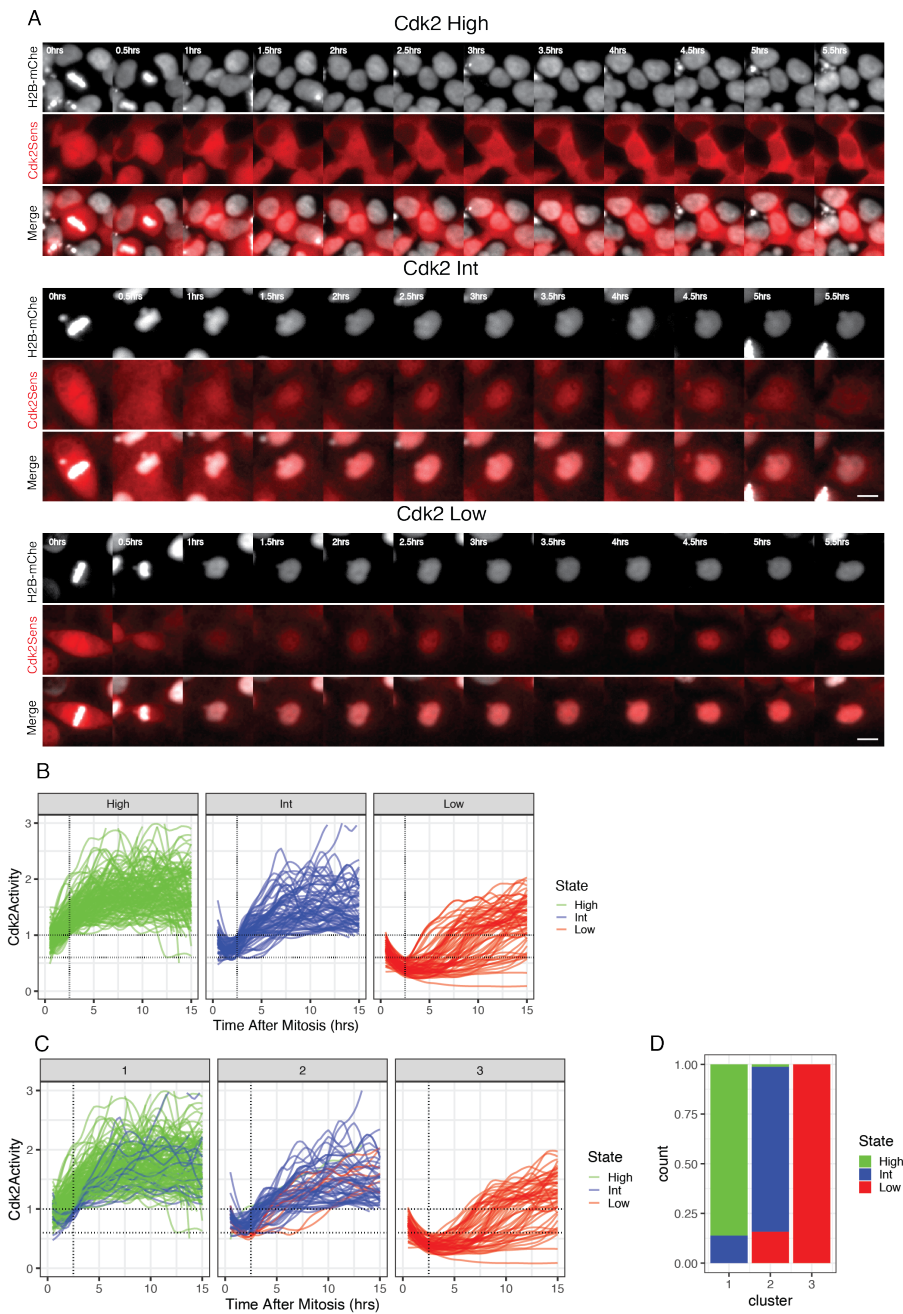


Figure 3-18- Distinct three-tier CDK2 States *A.* Fluorescent images of representative CDK2 activity states bearing the CDK2 activity sensor. H2B images are shown in gray scale and CDK2 sensor in red. Scale bar = 10 μ M. Figure is repeated from Figure 2-8. *B.* Pooled CDK2 activity traces aligned from the start of anaphase onset from both hESC and differentiated conditions. Dashed line on x axis represents 2.5 hours after mitosis. Dashed lines on y axis represent different thresholds of CDK2 activity used to classify the 3 states, 2.5 hours after mitosis. For CDK2 high state (green, n=124), activity is above 1.00 2 hours after mitosis. For CDK2 Int state (blue, n=66), activity is between 0.50 and 1.00 2 hours after mitosis. For CDK2 Low state (red, n=85), CDK2 activity is below 0.50 2 hours after mitosis. *C.* (left) Independent clustering of pooled CDK2 activity traces aligned from the start of anaphase onset from both hESC and differentiated conditions. Traces are colour coordinated depending on which threshold group they are in to allow comparison between threshold based grouping and independent clustering. Cluster 1 n=158, Cluster 2 n= 60, Cluster 3 n = 57. (right) Stacked bar plot illustrating the proportion of each threshold based group in individual clusters.

3.4.3 Changes of CDK2 activity through generations

Having observed striking differences in CDK2 activity dynamics between the ES and differentiated state, we next sought to investigate when this transition happens in real time. For this, we tracked single cells through lineages, and measured CDK2 activity traces for individual generations (Figure 3-19). For each generation, we then sub-grouped traces depending on whether their behaviour fitted into the CDK2 High, Int or Low states. In the first generation (F0) we found that the majority of traces (91%) displayed CDK2 high behaviour, with the remaining 9% showing CDK2 Int behaviour. After 1 division (F1), we found that approximately 29% of the population shifted into the CDK2 Int state, while the remaining 71% continued in the CDK2 high state. After 2 divisions (F2) there was a significant reduction in the high CDK2 state (22%), and the CDK2 low state was introduced with approximately 42% of cells displaying this behaviour. Finally, after 3 divisions, the proportion of cells in the CDK2 low state increased to 72%, and only 3% of cells were left in the high state (Figure 3-19). These results illustrate how the three different behaviours of CDK2 activity shift during differentiation, with each division bringing about changes in the proportions of the states. Furthermore, changes in CDK2 activity dynamics happens surprisingly early after just 1-2 division. The introduction of the CDK2 low state is only apparent after 2 divisions. This correlates well with the cell cycle dynamics measurements in (Figure 3-5) which show that changes in G1 length and variability happen after 2 divisions.

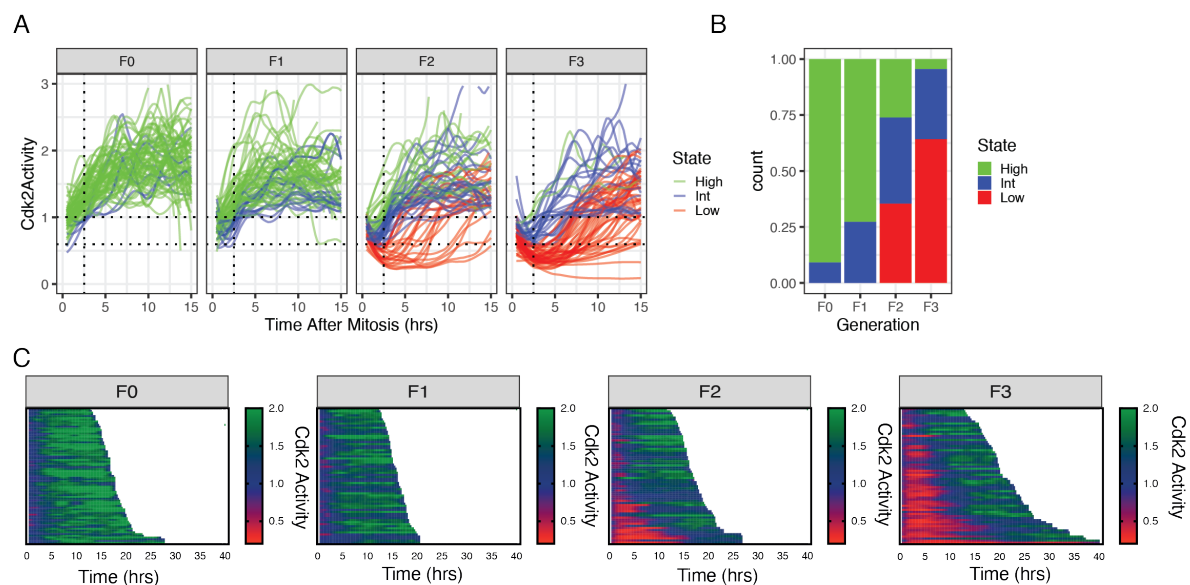


Figure 3-19- Changes in CDK2 activity in lineages during BMP4-driven differentiation A. CDK2 activity traces aligned to time after anaphase onset for generations F0-F3 after BMP4 treatment. Colour for individual traces represent either CDK2 high (green), Int (blue) or Low (red) behaviour. $n=275$ (right) B. Stacked bar plot illustration how the proportion of the 3 CDK2 activity states changes in successive generations after BMP4 stimulation. C. Heatmap of CDK2 for individual generations. Each row represents a trace of a single cell after mitosis.

3.4.4 Changes in CDK2 activity correlate with changes in G1 length during differentiation

To investigate how CDK2 activity influences G1 lengthening during differentiation in hESC, we built a cell line combining both the CDK2 activity sensor and a G1 sensor (Cdt1-YFP). With this, we could correlate in live, single cells exactly how changes in CDK2 activity state affected G1 length, and whether these traces could explain the heterogeneity in G1 length seen in the differentiated state. From the Cdt1 traces, as expected, we saw an increase in G1 length after 2 divisions, which was further increased after 3 divisions (Figure 3-20A). Importantly, the CDK2 activity traces also followed beautifully similar dynamics through differentiation (Figure 3-20A). Overlaying individual traces, we noticed that cells with an elongated G1, either in the 2nd or 3rd division exhibited a vast drop in CDK2 activity after mitosis, similar to those cells that showed CDK2 low behaviour (Figure 3-20A). To confirm this, we sub-grouped individual traces into the 3 CDK2 activity states and measured G1 length (Figure 3-20C). This showed that the CDK2 low state had a significantly higher G1 length in comparison to the CDK2 Int and High states (Figure 3-20C). These data is also supported by the fact that there is a negative correlation between time spent in G1 and CDK2 activity (Figure 3-20B). In

summary, these data suggest that there is a correlation between changes in CDK2 activity and G1 lengthening during differentiation. These two events correlate well, and the introduction of the CDK2 low state can explain the heterogeneity in G1 length displayed in the 2nd and 3rd divisions after treatment with BMP4.

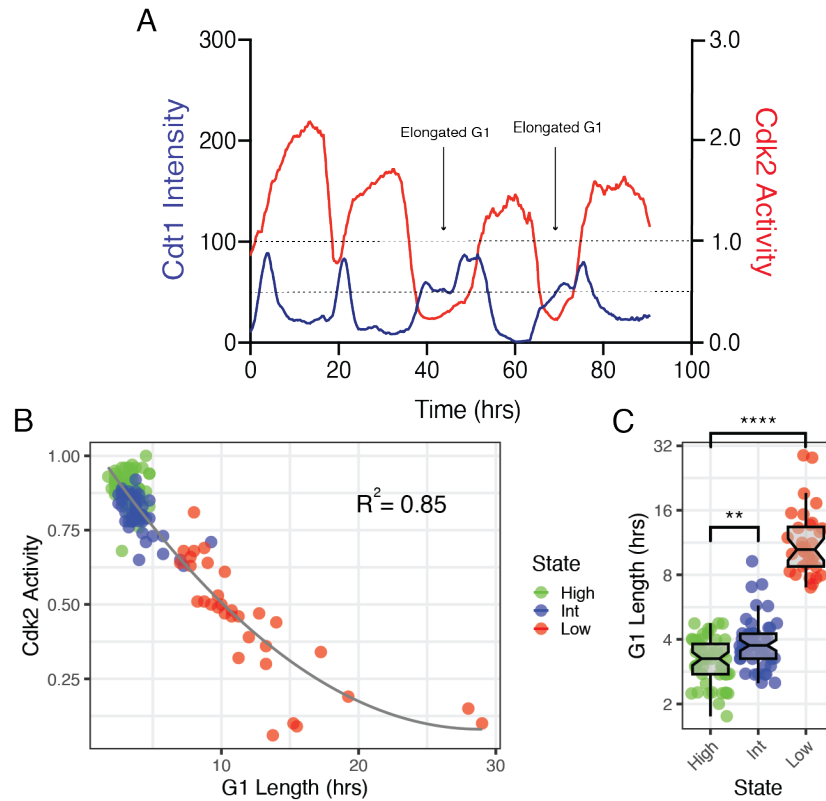


Figure 3-20- Changes in CDK2 activity correlate with G1 lengthening. A. Example trace of CDK2 Activity (red) and Cdt1-YFP (blue) in single cells after treatment with BMP4. Cells with an elongated G1 display low levels of CDK2 activity after mitosis. This happens 2-3 divisions after treatment with BMP4. Dotted lines represent different levels of CDK2 activity 1 and 0.5 B. Anti-correlation between G1 length and CDK2 activity ($n=137$). One-phase decay curve is fitted with R^2 value to indicate goodness of fit. C. Quantification of G1 lengths pooled from F0-F3 generations. Individual cells were then divided into 1 of 3 groups depending on their CDK2 activity state (High, Int or Low). Boxplots are overlaid to indicate minimum, maximum, first quartile, third quartile and median values. KS tests were performed to calculate p -values. (ns= not significant, **** $p < 0.0001$, *** $p < 0.001$, ** $p < 0.01$, * $p < 0.05$). $n=137$.

3.4.5 Development of an CDK2 analogue sensitive mutant

To confirm whether changes in CDK2 activity were directly responsible for the lengthening in G1 length during differentiation, we needed to specifically inhibit CDK2 and see how this may affect G1 duration in hESC. Although there are many commercially available pharmacological inhibitors that target CDK2, most of them are non-specific due to the conservation of the ATP binding pocket and the similarity between different cyclin dependent kinases, making interpretation of results difficult. To circumvent this problem, we set out to develop an analogue sensitive (AS) CDK2 mutant in hESC (see methods 2.2.6 for details) (Bishop et al., 2000). These mutants have an enlarged ATP binding pocket, which can be occupied by very specific bulky inhibitors such as 3-MB-PP1 (Figure 3-21A). This inhibitor has been shown not to inhibit any other kinases in the mammalian kinome (Bishop et al., 2000b). We also tried to knockout endogenous CDK2 in parallel with overexpressing the CDK2-AS mutant however we were unsuccessful in our attempts. However, the expression levels of the AS mutant CDK2 were much higher than WT CDK2, therefore this cell line therefore functions as a dominant negative (Figure 3-21B). To test if hESC stably expressing CDK2-AS mutant can be specifically inhibited by 3-MB-PP1, we treated CDK2-AS cells with the compound and observed a reduction in cell proliferation in CDK2-AS hESC (Figure 3-21C). As a control, we also treated WT cells with 3-MB-PP1 and observed no change in cell proliferation (Figure 3-21C) suggesting that this approach allows us to specifically inhibit CDK2 in hESCs.

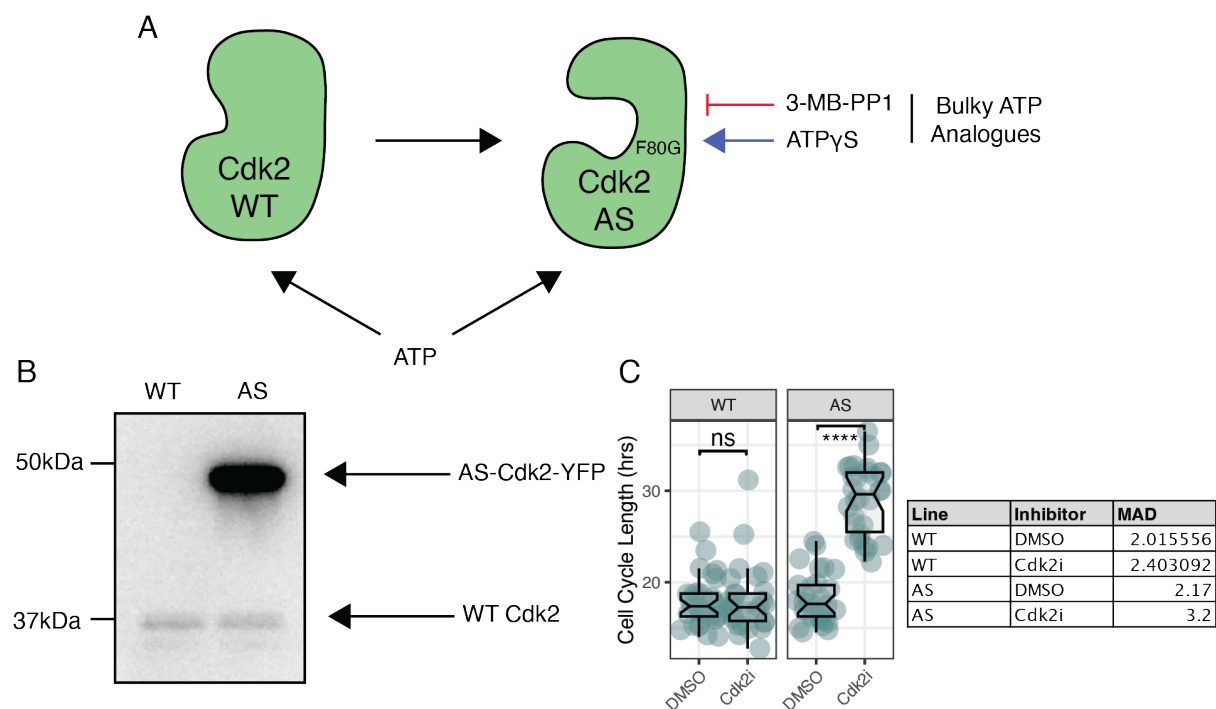


Figure 3-21- Development of CDK2 analogue sensitive mutant *A. Schematic of CDK2-AS mutant, F80G mutation increases ATP binding pocket size which can be occupied by bulk ATP analogues. B. Western blot of total CDK2 expression levels for WT and CDK2-AS mutant hESC lines. C. Quantification of cell cycle length for WT and CDK2-AS mutant hESC lines treated with either DMSO or 20uM 3-MB-PP1. Measurements were taken 30 minutes after addition of either DMSO or 3-MB-PP1. KS tests were performed to calculate p-values (.ns= not significant, **** $p < 0.0001$, *** $p < 0.001$, ** $p < 0.01$, * $p < 0.05$). Table with mean absolute deviations (MAD) for each condition is shown on the right.*

3.4.6 Inhibiting CDK2 increases G1 length in a dose-dependent manner

After developing the CDK2-AS mutant hESC line, we generated further H1 hESC stable cell lines that expressed both the CDK2 activity sensor and cell cycle sensors. With this we could test that the CDK2-AS mutant specifically inhibited CDK2 activity, and whether this influenced G1 lengthening. We initially performed a dose-response with varying concentrations of 3-MB-PP1 and found that increasing 3-MB-PP1 concentration did indeed lower the CDK2 sensor's activity (Figure 3-22A,B). Interestingly, we could add specific concentrations that would either exclusively lower CDK2 activity to the intermediate state (1 μ M), the low state (100 μ M) or a mixture of both as seen in somatic cells (20 μ M) (Figure 3-22A,B). This result shows that using the CDK2-AS mutant is a robust approach to specifically inhibit CDK2 and fine tune the activity of this kinase in a precise manner.

We next added the FUCCI(CA) biosensor into the CDK2-AS mutant line to be able to measure changes in G1 duration after modulating CDK2 activity. We performed a dose response to see how lowering CDK2 activity influenced G1 length (Figure 3-22C). Strikingly, we found that increasing the concentration of 3-MB-PP1 increased G1 length in a dose dependent manner (Figure 3-22C). Altogether, these data suggest that the correlation between CDK2 activity and G1 length is causal, and that changes in CDK2 activity during differentiation are partly responsible for G1 lengthening during differentiation.

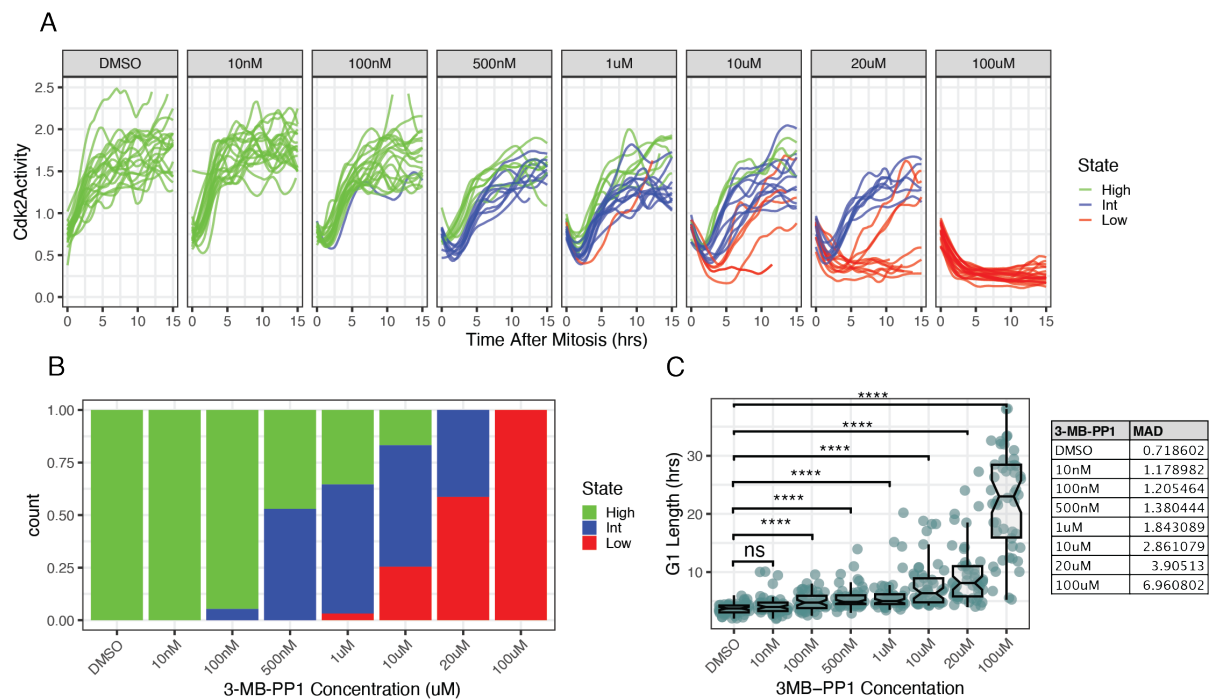
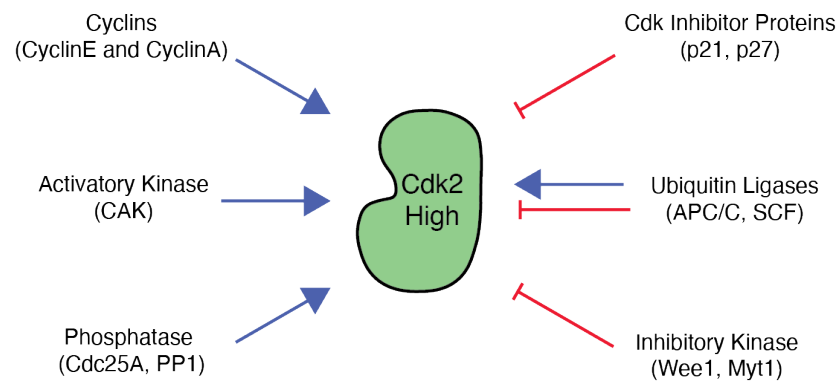


Figure 3-22- CDK2-AS dose response measuring CDK2 activity and G1 length A. CDK2 activity traces aligned to time after anaphase onset for hESC treated with different doses of 3-MB-PP1 in the CDK2-AS mutant line. Colour for individual traces represent either CDK2 high (green), Int (blue) or Low (red) dynamics. $n=120$. Measurements were taken 30 minutes after addition of either DMSO or 3-MB-PP1. B. Stacked bar plot indicating the proportion of states for different doses of 3-MB-PP1. C. Quantification of G1 length using the FUCCI(CA) biosensor with different doses of 3-MB-PP1 in the CDK2 AS mutant line. Measurements were taken 30 minutes after addition of either DMSO or 3-MB-PP1. Boxplots are overlaid to indicate minimum, maximum, first quartile, third quartile and median values. $n=400$. KS tests were performed to calculate p -values. (ns= not significant, **** $p < 0.0001$, *** $p < 0.001$, ** $p < 0.01$, * $p < 0.05$). Table with mean absolute deviations (MAD) for each condition is shown on the right.

3.5 What regulates high CDK2 activity in hESCs?

3.5.1 Perturbing Cyclins A and E reduces CDK2 activity and increase G1 length

We showed that CDK2 activity directly regulates G1 length during hESC differentiation. The next question was thus: what regulates the hyperactivated CDK2? We set out to find which CDK2 regulators were critical in regulating high CDK2 activity and, ultimately, G1 duration. CDK2 has a plethora of known regulators shown in (Figure 3-23) which can both increase and decrease the kinase's activity.



*Figure 3-23 - **What regulates high CDK2 Activity?** Schematic illustrating known regulators for CDK2. Blue arrows indicate positive regulators, red lines indicate negative regulators.*

CDK2 activity primarily depends on binding of regulatory cyclin proteins (Cyclin E and Cyclin A). We had seen that Cyclin A1 and Cyclin E1 expression changed from pluripotent to differentiated both at the protein and RNA level (Figure 3-10 and Figure 3-11). We therefore set out to perturb cyclin expression and monitor the effect on CDK2 activity and G1 length. To down regulate cyclins, we used an siRNA approach and found that we could knockdown cyclins at an efficiency of approximately 90% (Figure 3-24). We initially knocked down Cyclin E1, which is required for the G1/S transition. When perturbing Cyclin E1, CDK2 activity was reduced, with around half the cells shifting towards the intermediate state (Figure 3-24A). Importantly, we found an increase in G1 length of approximately 2 hours (Figure 3-24A). We next knocked down Cyclin A2, which normally binds to CDK2 later in the cell cycle during S phase. Although minimal changes were observed in terms of the CDK2 activity states, we found a decrease in the overall amplitude of CDK2 activity an increase in G1 length of

approximately 2 hours (Figure 3-24B). Finally, as one of the main hits from the RNAseq dataset was Cyclin A1 which had higher expression in hESC, we made use of a Cyclin A1 Knockout (KO) H1 hESC line from our lab (Perez et al unpublished) and measured CDK2 activity and G1 length. Like Cyclin A2, we saw a small increment of cells showing intermediate levels of CDK2 which led to an increase in G1 duration (Figure 3-24C). Taken together, these data show that elevated cyclin expression is vital for maintaining high CDK2 activity and a short G1 in hESC. Perturbing Cyclin E1 had more striking effects on early CDK2 activation, while perturbing Cyclin A2 decreased the overall amplitude of CDK2 activity, likely due to its role later in the cell cycle. Although the phenotype of the Cyclin A1 KO was more subtle, this could be due to potential compensatory mechanisms that allow the cell cycle of pluripotent cells to adapt in the absence of Cyclin A1. Importantly, all the perturbations that affected CDK2 activity resulted in changes in G1 length confirming that changing CDK2 to lower levels affects G1 duration.

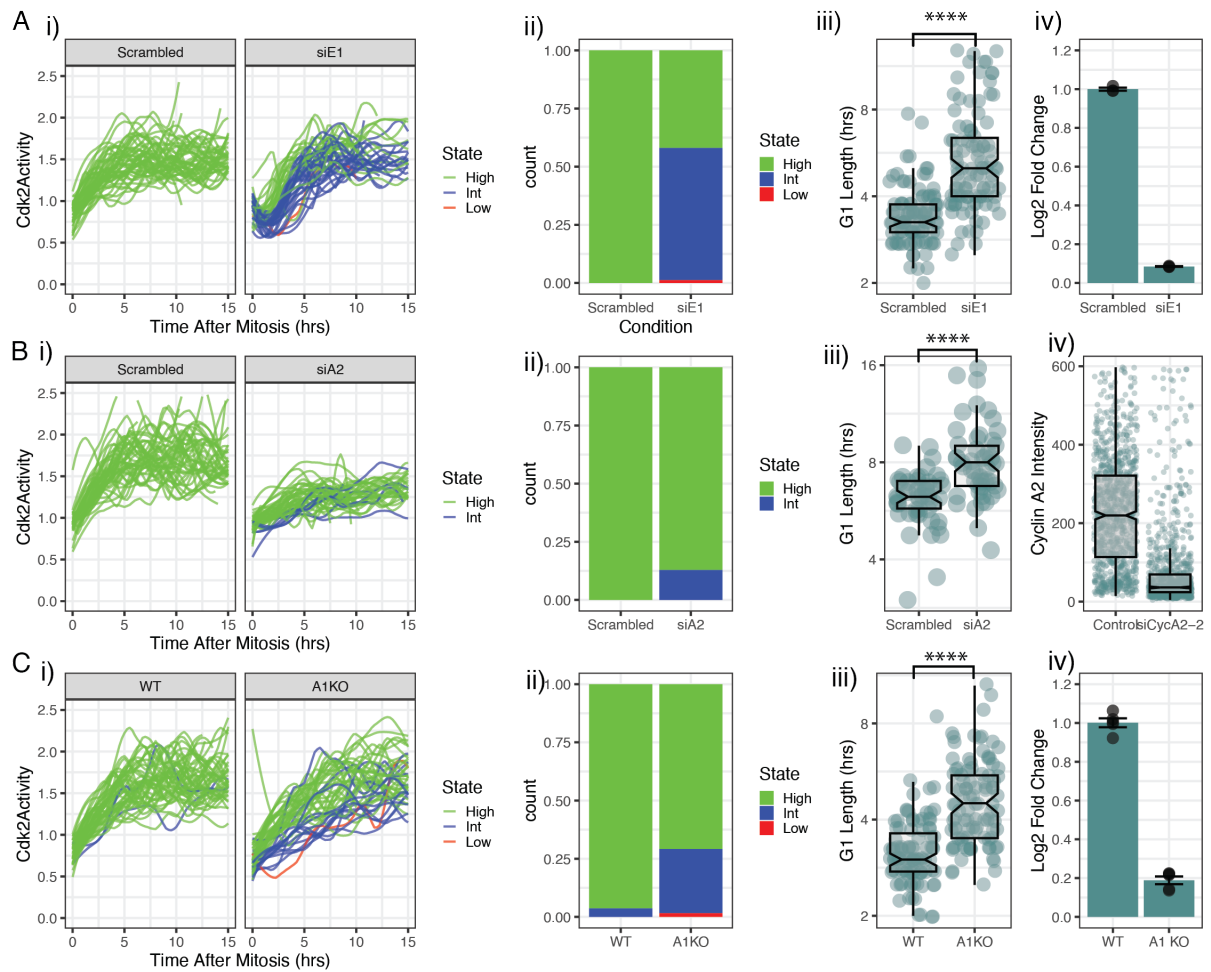


Figure 3-24- Perturbing G1 cyclins reduces CDK2 activity and increases G1 length A-C (i) CDK2 activity traces aligned to time after anaphase onset for both control and perturbed hESCs. $n=100$ for each perturbation (ii) Stacked bar plot illustrating how the proportion of the 3 CDK2 activity states changes in control and perturbed conditions (iii) Quantification of G1 length in control and perturbed conditions. Boxplots are overlaid to indicate minimum, maximum, first quartile, third quartile and median values. KS tests were performed to calculate p -values. (ns= not significant, **** $p < 0.0001$, *** $p < 0.001$, ** $p < 0.01$, * $p < 0.05$) $n=200$ for each perturbation (iv) Quantification of knockdown/knockout using either qPCR fold change expression values or immunofluorescent single cell nuclei staining. $n=3$ for qPCR measurements. (A) siCyclin E1 knockdown (B) siCyclin A2 knockdown (C) Cyclin A1 KO. Measurements were made 3 days after siRNA transfections.

3.5.2 Overexpressing p21 reduces CDK2 activity

Further to our observation that hESCs had low levels of CDK inhibitor proteins, we set to ectopically express in hESC one of the key CDK2 inhibitor proteins, p21, and test the effect on CDK2 activity and G1 length. We generated a stable, p21 doxycycline inducible overexpression H1 hESC line (Figure 3-25A). It is worth noting that this system shows a degree of “leakiness” i.e. even in the absence of doxycycline, CDK2 activity was decreased compared

to WT hESC. Despite this, after treatment with 1 μ g/mL doxycycline for one day, we found that CDK2 activity decreased even further, with a significant amount of cells entering the intermediate and low state (Figure 3-25B,C). This was also accompanied by an increase in G1 length of approximately 3 hours (Figure 3-25D). This data shows that low levels of p21 in hESC are important in maintaining high CDK2 activity, in particular the absence of the CDK2 low state. This data is consistent with previous reports showing that p21 regulates the initial bifurcation in CDK2 activity in somatic cell lines (Spencer et al., 2013).

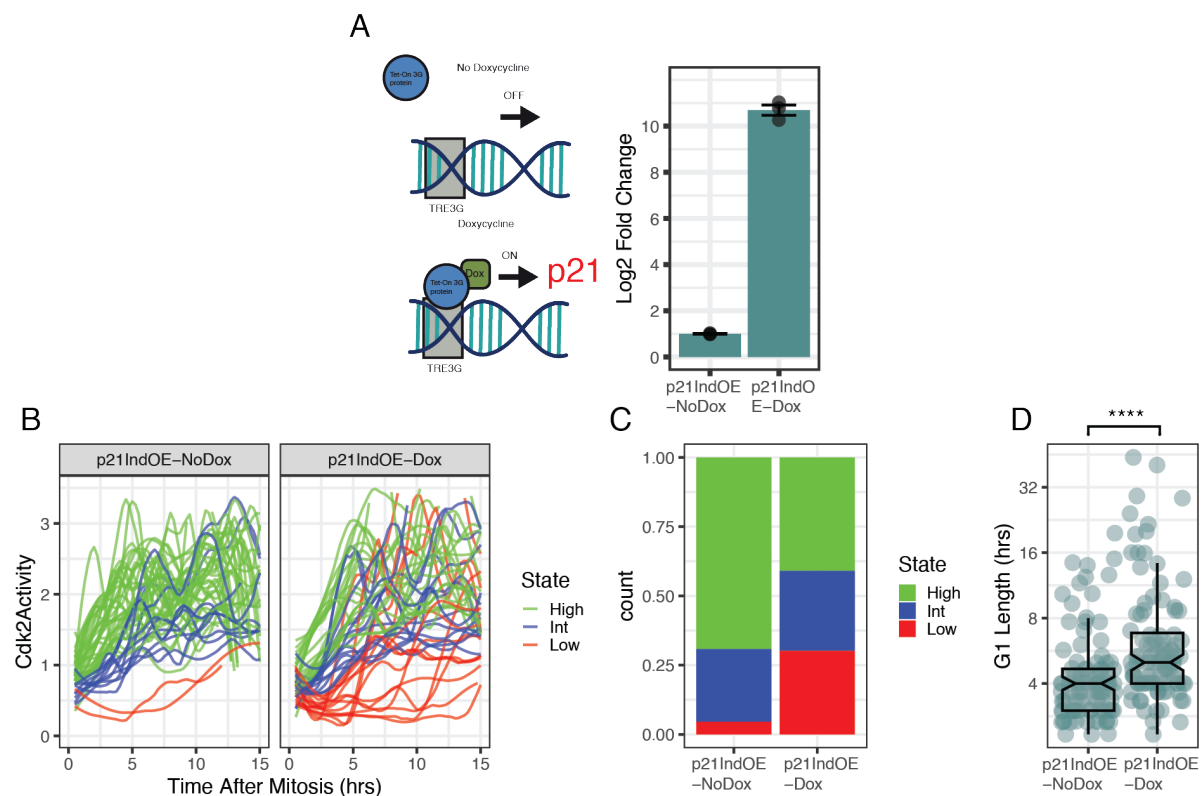


Figure 3-25- Overexpressing p21 reduces CDK2 activity A. (left) Schematic illustrating doxycycline inducible p21 system (right) Quantification of overexpression using qPCR fold change expression values. $n=3$. B. CDK2 activity traces aligned to time after anaphase onset for both control and perturbed hESCs. $n=100$ C. Stacked bar plot illustrating how the proportion of the 3 CDK2 activity states changes in control and perturbed conditions D. Quantification of G1 length in control and perturbed conditions. Boxplots are overlaid to indicate minimum, maximum, first quartile, third quartile and median values. KS tests were performed to calculate p-values (.ns= not significant, **** $p < 0.0001$, *** $p < 0.001$, ** $p < 0.01$, * $p < 0.05$) $n=200$. All measurements were made 24 hours after doxycycline induction.

3.5.3 Ubiquitin ligases play an important role in maintaining high CDK2 activity.

Since we observed that hESC potentially had differential ubiquitin ligase activity, we set out to see how perturbing protein degradation would affect CDK2 activity and G1 length. As we found that hESC had high levels of the SCF complex component Skp2 throughout the cell cycle (Figure 3-14), we sought to perturb Skp2 using an RNA interference approach. Knocking down Skp2 reduced CDK2 activity and shifted over half of the cells to the CDK2 intermediate state, which was mirrored by an increase in G1 length (Figure 3-26A,B,C,D). We then decided to overexpress the APC/C complex component Cdh1, which was found to be at low levels in hESC (Figure 3-14). To do this, we generated a stable, Cdh1 doxycycline inducible overexpression line (Figure 3-26E). After treatment with 5 μ g/mL doxycycline for one day, we found that CDK2 activity decreased, with 35% of the cells presenting the CDK2 intermediate state. Again, this was mirrored by an increase in G1 length (Figure 3-26F,G,H)

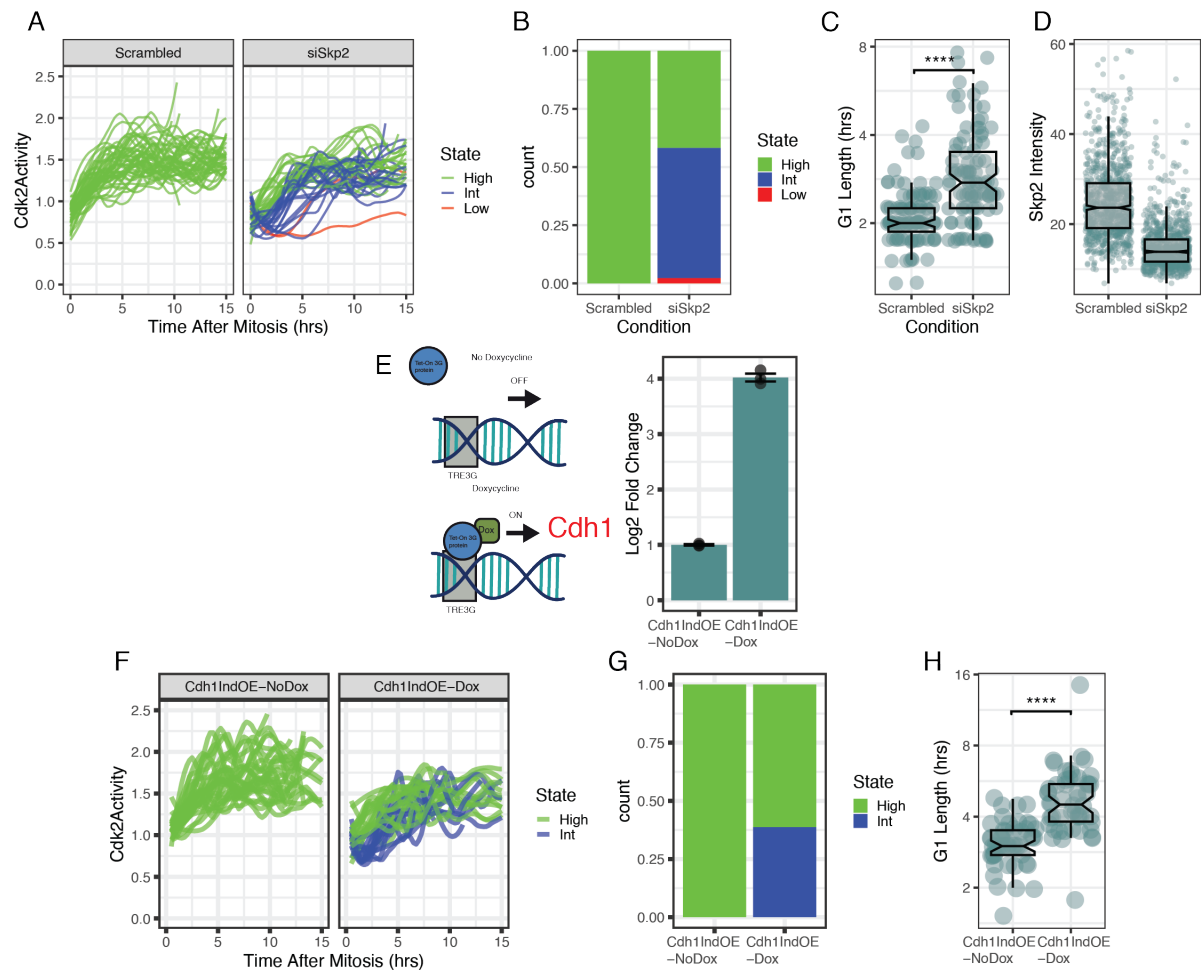


Figure 3-26- Ubiquitin ligases play an important role in maintaining high CDK2 activity A. CDK2 activity traces aligned to time after anaphase onset for both Scrambled and siSkp2 hESCs. $n=100$. B. Stacked bar plot illustrating how the proportion of the 3 CDK2 activity states changes for both Scrambled and siSkp2 hESCs C. Quantification of G1 length for both Scrambled and siSkp2 hESCs. Boxplots are overlayed to indicate minimum, maximum, first quartile, third quartile and median values. KS tests were performed to calculate p-values (.ns= not significant, **** $p < 0.0001$, *** $p < 0.001$, ** $p < 0.01$, * $p < 0.05$) $n=200$. D. Quantification of knockdown using immunofluorescent nuclei staining. $n=2000$. E. (left) Schematic illustrating doxycycline inducible Cdh1 system (right) Quantification of overexpression using qPCR fold change expression values. $n=3$. F. CDK2 activity traces aligned to time after anaphase onset for Cdh1IndOE hESC treated with and without doxycycline. G. Stacked bar plot illustrating how the proportion of the 3 CDK2 activity states changes for Cdh1IndOE hESC treated with and without doxycycline. H. Quantification of G1 length for Cdh1IndOE hESC treated with and without doxycycline. Boxplots are overlayed to indicate minimum, maximum, first quartile, third quartile and median values. KS tests were performed to calculate p-values (.ns= not significant, **** $p < 0.0001$, *** $p < 0.001$, ** $p < 0.01$, * $p < 0.05$) $n=200$. For A-D measurements were made 3 days after siRNA transfection. For F-H, measurements were made 24 after doxycycline induction.

As we had difficulties with “leakiness” in the doxycycline inducible overexpression system, we repeated this experiment using the dihydrofolate reductase (DHFR)-trimethoprim (TMP) protein stabilisation system (Iwamoto et al., 2010) to inducibly increase protein levels of Cdh1. This system utilises a DHFR fusion protein tagged onto Cdh1 which under basal conditions is continuously degraded. The addition of TMP stabilises the DHFR degron, increasing the protein levels of Cdh1 rapidly (Figure 3-27A). Consistent with the previous data, overexpressing Cdh1 shifted cells towards the intermediate state of CDK2 activity. Finally, we combined both perturbations where we overexpress Cdh1 and knocked down Skp2 and strikingly found that CDK2 activity lowered dramatically, with cells shifting towards the intermediate and low state, and, consequently, increased G1 duration (Figure 3-27B,C,D). In conclusion, this data shows that just by modifying the activity of ubiquitin ligases, one can manipulate CDK2 activity to a level that is seen in differentiated cells. It is therefore interesting to speculate that protein degradation may be an important regulatory mechanism at the heart of cell cycle remodelling during hESC differentiation.

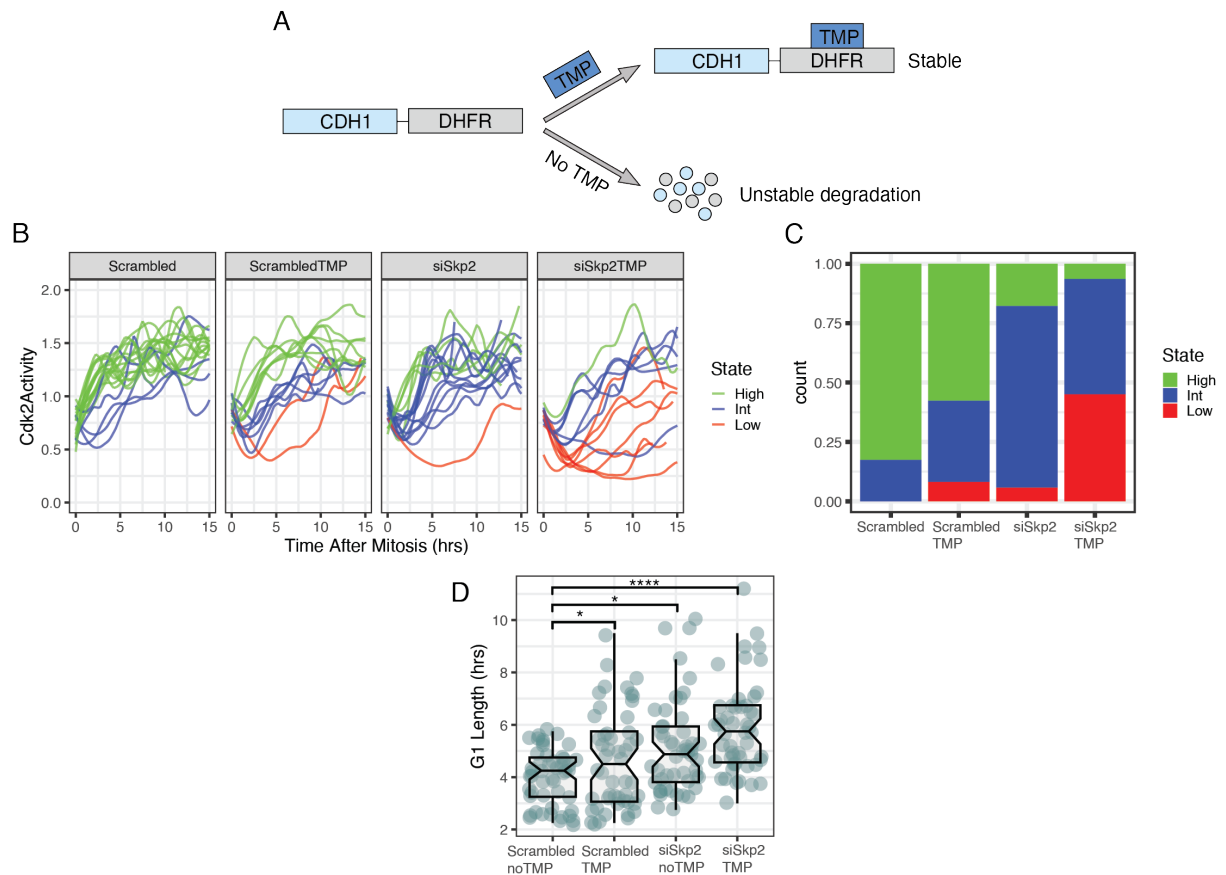


Figure 3-27- Overexpressing Cdh1 in combination with Skp2 knockdown reduces CDK2 activity to somatic levels. Schematic illustrating DHFR-TMP protein stabilisation system. Cdh1-TMP is continuously degraded in normal condition but upon addition of TMP is stabilised leading to upregulation of Cdh1. B. CDK2 activity traces aligned to time after anaphase onset for control, TMP stabilised Cdh1, siSkp2 and both TMP stabilised Cdh1 in combination with siSkp2. n=80. C. Stacked bar plot illustrating how the proportion of the 3 CDK2 activity states changes onset for onset for control, TMP stabilised Cdh1, siSkp2 and both TMP stabilised Cdh1 in combination with siSkp2. D. Quantification of G1 length for control, TMP stabilised Cdh1, siSkp2 and both TMP stabilised Cdh1 in combination with siSkp2. Boxplots are overlayed to indicate minimum, maximum, first quartile, third quartile and median values. KS tests were performed to calculate p-values. (ns= not significant, **** $p < 0.0001$, *** $p < 0.001$, ** $p < 0.01$, * $p < 0.05$) n=200. All measurements were made 3 days after siRNA transfection and 24 hours post TMP induction.

3.5.4 Perturbing Cdc25A has no influence on CDK2 activity in hESCs

Phosphatase play a critical role in the regulation of the cell cycle. In particular, Cdc25A regulates CDK2 activity by dephosphorylating threonine 14 and tyrosine 15 (T14/Y15) which activate CDK2 allowing it to phosphorylate downstream targets. To assess the influence of Cdc25A on high CDK2 activity in hESCs, we down regulated Cdc25A using an siRNA approach (Figure 3-28A). To our surprise, downregulation of Cdc25A had no influence on CDK2 activity (Figure 3-28B,C) suggesting that expression of Cdc25A is not an important factoring in regulating high CDK2 activity in hESCs. We also repeated this for Cdc25B and Cdc25C and found similar results (data not shown).

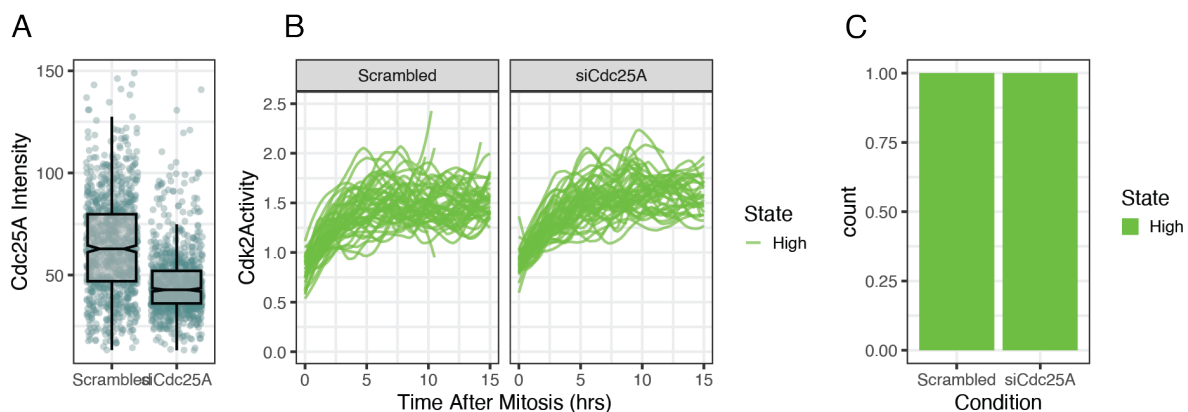


Figure 3-28- Perturbing Cdc25A has no influence of CDK2 activity in hESCs Quantification of knockdown using Immunofluorescent nuclei staining. n=2000. B. CDK2 activity traces aligned to time after anaphase onset for both Scrambled and siCdc25A hESCs. n=100. C. Stacked bar plot illustrating how the proportion of the 3 CDK2 activity states changes for both Scrambled and siCdc25A hESCs. All measurements were made 3 days after siRNA transfection.

3.5.5 The influence of other cell cycle kinases on CDK2 activity and G1 length.

The next question we wanted to address was whether CDK2 activity exclusively was important in driving G1 lengthening, or whether other cell cycle kinases played a role. To test this, we treated hESC with pharmacological inhibitors against the two main other CDKs that drive the mammalian cell cycle, CDK1 and CDK4/6. Using the CDK1 specific inhibitor RO-3306, we found minimal change in CDK2 activity and no change in G1 length (Figure 3-29A). In contrast, treatment with the CDK4/6 inhibitor Palbociclib increased G1 length by approximately 7.5 hours. In addition to this, inhibition of CDK4/6 decreased CDK2 activity,

with a higher fraction of cells entering the intermediate state. Furthermore, the activation of CDK2 after mitosis was also perturbed, with many cells displaying a sustained level of CDK2 activity before progressing into S phase (Figure 3-29B). Taken together this data suggests that CDK4/6 activity also plays a role in regulating G1 length in hESCs. As inhibiting CDK4/6 also perturbs CDK2, CDK4/6 seems to be critical in activating and maintaining high CDK2 activity.

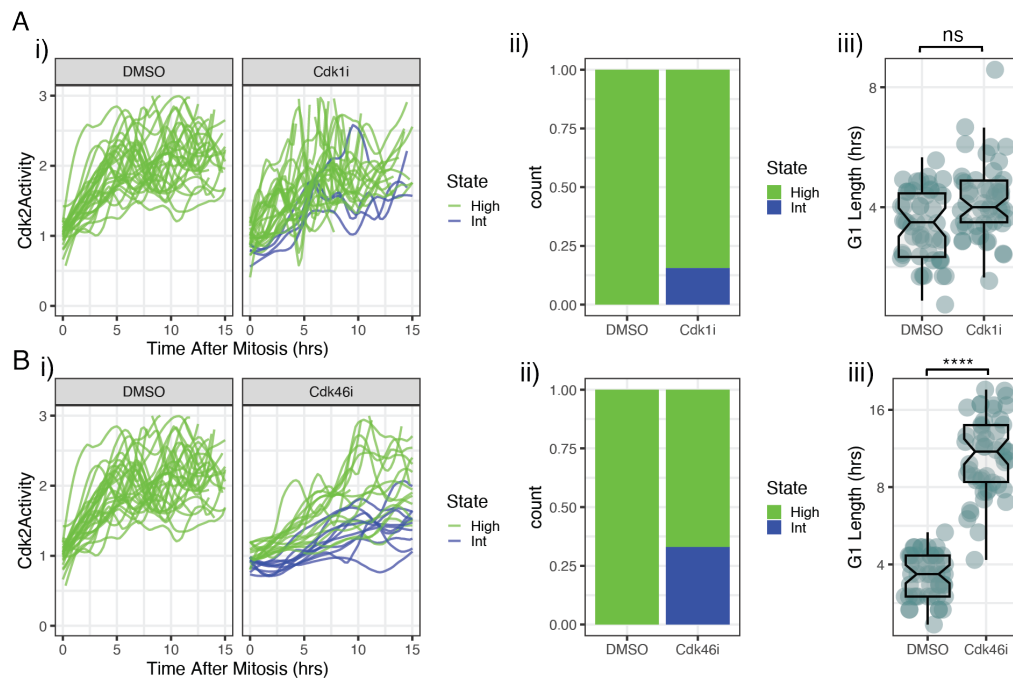


Figure 3-29- The influence of other cell cycle kinases on CDK2 activity A-B (i) CDK2 activity traces aligned to time after anaphase onset for both control and perturbed hESCs. $n=60$ for each perturbation (ii) Stacked bar plot illustrating how the proportion of the 3 CDK2 activity states changes in control and perturbed conditions (iii) Quantification of G1 length in control and perturbed conditions. Boxplots are overlaid to indicate minimum, maximum, first quartile, third quartile and median values. KS tests were performed to calculate p -values. (ns= not significant, **** $p < 0.0001$, *** $p < 0.001$, ** $p < 0.01$, * $p < 0.05$) $n=200$ for each perturbation (A) $20\mu\text{M}$ RO-3306 (CDK1i) (B) $1\mu\text{M}$ Palbociclib (CDK4/6i). All measurements were made 30 minutes after drug addition.

Finally, we perturbed the CDK inhibitory kinase Wee1 using the pharmacological inhibitor PD166285 and measured CDK2 activity and G1 length. To our surprise we found that inhibiting Wee1 increased G1 length and shifted CDK2 activity to both intermediate and low states (Figure 3-30). Wee1 is involved in the positive feedback networks that underlie CDK activation, and inhibiting Wee1 has been shown to be important in the rapid activation of CDK1 and short mitosis (Araujo et al., 2016). We therefore speculate that perturbing positive feedback is also important in the sharp activation of CDK2 and maintenance of a short G1. Further experiments are required however to confirm this hypothesis.

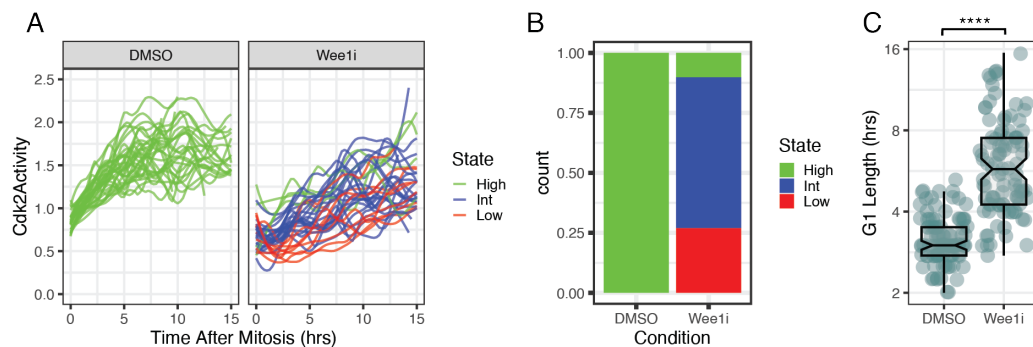


Figure 3-30- Inhibition of Wee1 decreases CDK2 activity A. CDK2 activity traces aligned to time after anaphase onset for both control and perturbed hESCs. $n=60$ for each perturbation B. Stacked bar plot illustrating how the proportion of the 3 CDK2 activity states changes in control and perturbed conditions C. Quantification of G1 length in control and perturbed conditions. Boxplots are overlayed to indicate minimum, maximum, first quartile, third quartile and median values. KS tests were performed to calculate p -values. (ns= not significant, **** $p < 0.0001$, *** $p < 0.001$, ** $p < 0.01$, * $p < 0.05$). All measurements were made 30 minutes after drug addition.

3.6 Highlights from chapter

In this chapter we have established several biosensors in hESCs to monitor cell cycle dynamics at the single cell level during BMP4-driven differentiation. Using quantitative, live cell imaging, we found that G1 is the phase that is remodelled the most extensively during differentiation, which increased in both length and variability. We tracked single hESCs through lineages which revealed that remodelling of G1 takes approximately 2 divisions after treatment with BMP4. To identify the molecular basis for G1 lengthening during differentiation, a variety of screening approaches were used which identified CDK2 as a potential master regulator. We precisely measured the activity of CDK2 in live, single cells during differentiation using an established CDK2 activity biosensor (Spencer et al., 2013). We see that hESCs exhibit three states of CDK2 levels: high, intermediate and low. During pluripotency, CDK2 is characterised by a unique, high activity dynamics and, as cells undergo differentiation, CDK2 activity lowers to medium or low levels and becomes cyclic. Finally, we found that the maintenance of high CDK2 activity is contributed by high cyclin expression, low expression of CDK2 inhibitors (namely p21) and atypical expression of the ubiquitin ligase components Cdh1 and Skp2. Interesting CDK2 activity could be lowered to somatic level by modulating the expression of these ubiquitin ligases. This work demonstrates the power of using single cell approaches to investigate cell cycle control principles during the embryonic to somatic transition.

Chapter 4. Results 2: Unravelling the functional importance of high Cdk2 activity dynamics in hESCs

4.1 Aims of chapter

The aim of the work presented in this chapter is to understand the functional importance of a short G1 and high CDK2 activity in stem cell identity. As we found in the previous chapter that hESC exhibit unique CDK2 activity dynamics, we wanted to investigate the molecular ties between CDK2 and the unique features displayed in ES cells. We decided to focus on two essential features of stem cell identity, atypical checkpoint control and pluripotency. We hypothesise that acyclic, high CDK2 activity dynamics is essential for bypassing the G1/S checkpoint and for maintenance of the pluripotent state. By testing this, we aim to address how the unique features of the pluripotent cell cycle impact on stem cell identity.

4.2 CDK2 activity and the establishment of G1/S checkpoint control

4.2.1 hESC and differentiated cells respond differently to double strand breaks

We reasoned that in order to understand the importance of high CDK2 for the absence of a classical G1/S checkpoint, we could challenge cells with stress inducing agents while modulating CDK2 activity to intermediate and/or low levels (thereby mimicking CDK2 activity of differentiated cells). It has been well established that in response to double strand breaks, somatic cells activate a DNA damage response pathway and can arrest cells in G1 (Di Leonardo et al., 1994). This allows a pause in proliferation to repair DNA damage before commitment to the cell cycle, safeguarding the genomic integrity of the cell (Paulovich & Hartwell, 1995; Shaltiel et al., 2015). Neocarzinostatin (NCS) is a radiomimetic agent that induces DNA double-strand breaks has been used frequently in previous studies to monitor the DNA damage response in single cells (Cappell et al., 2016; Chao et al., 2017; Nathans et al., 2021). In somatic cells, treatment with NCS activates a DNA damage response, causing the accumulation of p21, a decrease in CDK2 activity resulting in an increase in G1 length.

Furthermore, the proportion of cells that have hypophosphorylated Rb increases after treatment with NCS (Nathans et al., 2021). With this in mind, we first set out to characterise if and how hESC and differentiated cells activated the G1/S checkpoint in response to NCS treatment.

hESC and BMP4-stimulated hESC were initially treated for 2 hours with NCS before fixing and staining for G1/S checkpoint markers 6 hours later (Figure 4-1). Both hESC and BMP4-treated hESC upregulated the DNA damage marker γ H2AX (Figure 4-1A). Although both hESC and BMP4-treated cells upregulated p21 in response to NCS, p21 levels were much lower in hESC (Figure 4-1B), suggesting that hESCs have a reduced capacity to activate the G1/S checkpoint in response to DNA damage. In agreement with this result, hESC had lower levels of the p21 transcriptional activator p53 (Figure 4-1C). We next stained for pRb and found in BMP4-treated cells, cells increased the proportion of cells with hypophosphorylated Rb in response to NCS (Figure 4-1D), suggesting fewer cells were committed to the cell cycle. Furthermore, when correlating CDK2 activity versus p21 intensity, BMP4-treated cells with low CDK2 activity had high p21 levels. In contrast, even hESC with higher p21 levels had high CDK2 activity (Figure 4-1E,F,G). Interestingly, when comparing the scatter plots between both hESC and differentiated cells, it was observed that a threshold of p21 was required to decrease CDK2 activity. This threshold has been noted in previous studies (Nathans et al., 2021), and explained the heterogeneity seen in the response to NCS treatment in somatic cells. In striking contrast, all hESC had hyperphosphorylated Rb, even in response to NCS treatment suggesting induction of the DNA damage response pathway had no effect on cell cycle commitment. Taken together these data show that after treatment with NCS, hESC have a reduced capacity to activate key checkpoint proteins in response to DNA damage, suggesting altered fate outcomes between hESC and differentiated cells in response to cellular stress.

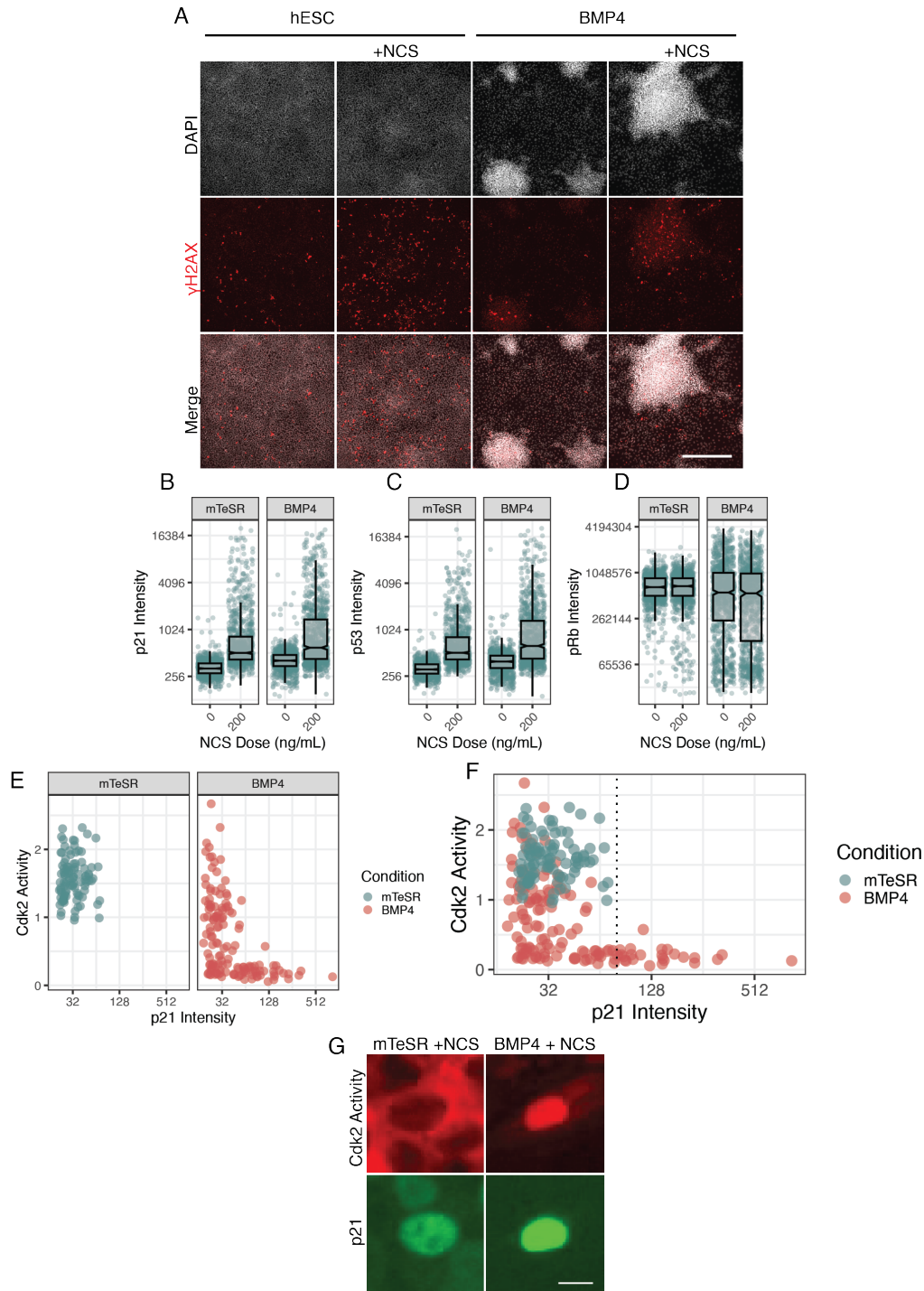


Figure 4-1- hESCs and differentiated cells respond differently to NCS *A.* Representative images of γ H2AX immunostaining for hESC and BMP4-treated cells for 3 days with and without addition of NCS. DAPI images are represented in gray scale and γ H2AX images are represented in red. Scale bar = 100 μ M. *B-D.* Single cell (nuclei) quantification of immunostaining for hESC treated with and without 200ng/mL of NCS for *B.* p21 *C.* p53 *D.* pRb 807/811. *n*=2000 for each condition. Boxplots are overlaid to indicate minimum, maximum, first quartile, third quartile and median values. *E.* Scatterplot of nuclear p21 levels vs CDK2 activity for hESC and BMP4-treated cells with 200ng/mL of NCS. *n*=200 *F.* Scatterplot overlaying data from *E.* *n*=200. *G.* Representative image example of p21 staining and CDK2 activity sensor with of hESCs and BMP4-stimulated cells treated with 200ng/mL of NCS. Scale bar = 10 μ M. All measurements were made 6 hours after addition of NCS.

4.2.2 hESC do not elicit a G1/S arrest in response to NCS

With the observation that hESC exhibited different levels of G1/S checkpoint proteins in response to stress, we next set out to monitor how NCS affected both hESC and differentiated cell cycle progression in live, single cells. To do this, we used the PCNA cell cycle biosensor and treated asynchronously cycling hESC and BMP4-treated hESC with NCS and measured the effect on G1 length. After treatment with NCS, BMP4-treated cells increased their G1 duration from 10.6hrs to 21hrs suggesting a functional G1/S checkpoint was present. Conversely, hESC displayed no difference in G1 length after treatment with NCS, suggesting a lack of a functional G1/S checkpoint (Figure 4-2C). We next extended our analysis to measure how NCS treatment influenced CDK2 activity. Previous studies have shown that somatic cell lines increase their proportion of CDK2 low cells in response to NCS (Nathans et al., 2021), and consistent with this we found that BMP4-treated cells increased the proportion of cells in the CDK2 low state in response to NCS (Figure 4-2A,B). Treatment of hESC with NCS in contrast had no effect on CDK2 activity, with cells remaining in the CDK2 high state. Furthermore, G1 length was unaffected by NCS treatment in hESCs, while in BMP4-treated cells this resulted in an increase in G1 length. This dynamic data together with the molecular characterisation in Figure 4-1 demonstrates that while differentiated cells can elicit a proper G1/S checkpoint in response to NCS, hESC are able to bypass this checkpoint and continue cycling through the cell cycle at the G1/S transition, even in the presence of double stranded breaks.

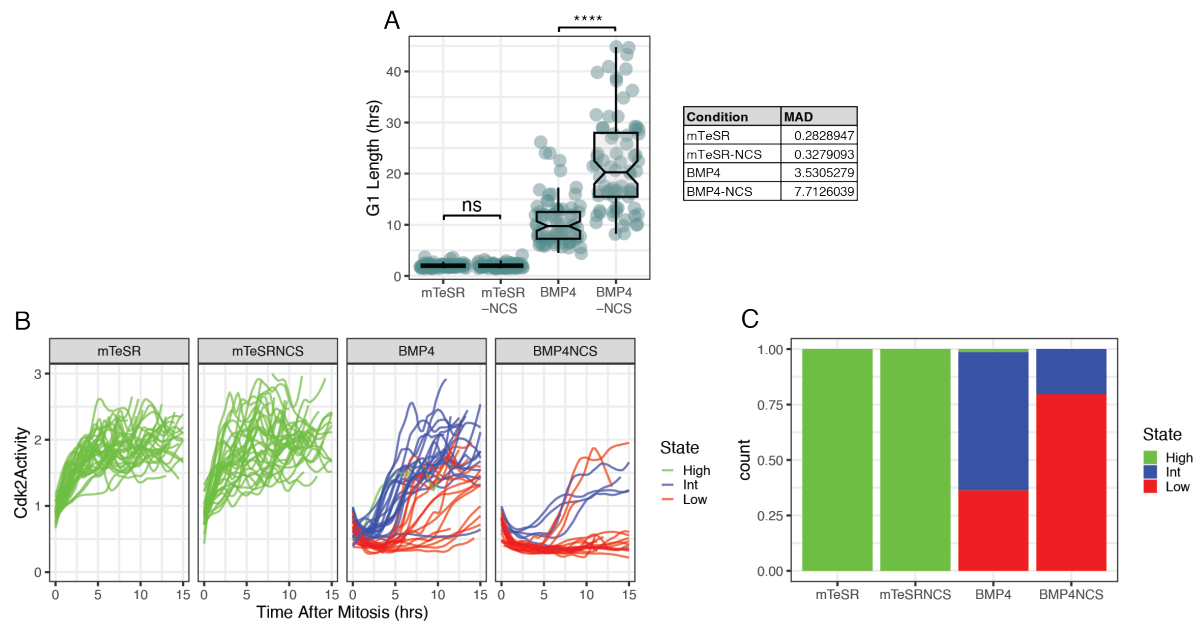


Figure 4-2- hESC do not elicit a G1/S arrest in response to NCS A. Quantification of G1 length after treatment with NCS for hESC and BMP4-treated cells for 3 days. Boxplots are overlaid to indicate minimum, maximum, first quartile, third quartile and median values. $n=100$ for each condition. KS tests were performed to calculate p-values. (ns= not significant, **** $p < 0.0001$, *** $p < 0.001$, ** $p < 0.01$, * $p < 0.05$). Table with MAD values is shown on the right. B. CDK2 activity traces aligned to time after anaphase onset after treatment with NCS for hESC and BMP4-treated cells. $n=40$ for each condition. C. Stacked bar plot illustrating how the proportion of the 3 CDK2 activity states changes in after treatment with NCS for hESC and BMP4-treated cells. All measurements were made 2 hours after addition of NCS.

4.2.3 hESCs are more sensitive to NCS treatment

Although hESC showed greater proliferative potential after treatment with NCS, a consequence of bypassing the G1/S checkpoint could be the propagation of DNA mutations accumulated from DNA damage. We therefore wanted to address the long-term fate of hESC after treatment with NCS and whether cells that had experience DNA damage were able to survive long term. For this we treated hESC and BMP4 cells with different doses of NCS and measured cell number after 1 day of exposure. We then compared total cell number with the untreated conditions of hESC and BMP4 cells. Both conditions decreased cell number in a dose-dependent manner (Figure 4-3). However, hESC appeared to have increased sensitivity in response to NCS, with almost all cells dying at high doses of NCS treatment (Figure 4-3). This demonstrates that while hESCs can bypass the G1/S checkpoint, hESC may preferentially commit to apoptosis in response to DNA damage to avoid inheritance of DNA mutations to future generations.

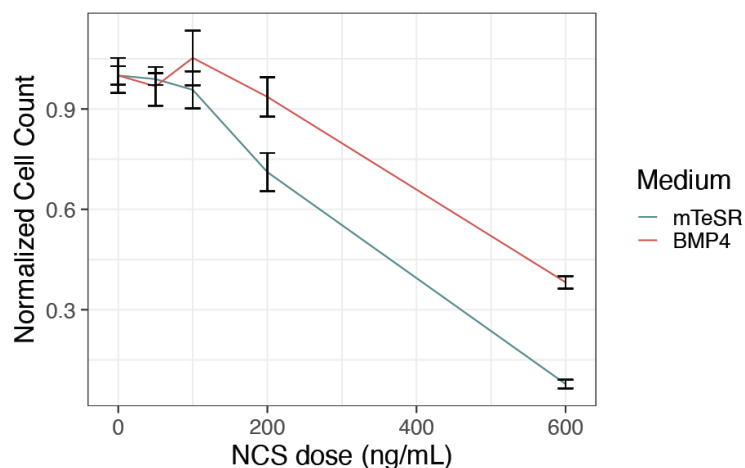


Figure 4-3- hESC are more sensitive to NCS treatment Quantification of cell count relative to either hESC or BMP4-stimulated cells (for 3 days) untreated with NCS. Error bars represent standard error. $n=3$. Measurements were made 24 hours after addition of NCS.

4.2.4 Modulating CDK2 activity allows hESC to respond to NCS treatment.

Based on the observation that hESC lacked a fully functional G1/S checkpoint, we subsequently sought to determine whether we could re-establish a G1/S checkpoint in hESC. In line with our earlier discovery that hESC have hyperactive CDK2 dynamics, we wondered whether we could re-establish the G1/S checkpoint by modulating CDK2 activity to mimic its levels in differentiated cells. To do this we used the CDK2-AS mutant in the CDK2 activity sensor cell line to decrease CDK2 activity to somatic levels prior to treatment with NCS. We then treated these cells with NCS for two hours and then performed live cell imaging for 48 hours. This allowed us to measure how the proportion of the CDK2 activity states changed in response to NCS treatment. As expected, control (DMSO) treated hESC remained in the CDK2 high state, even after exposure to NCS (Figure 4-4A,B). In contrast, when inhibiting CDK2 activity to somatic levels, a higher fraction of cells entered the CDK2 low state after exposure to NCS (Figure 4-4A,B).

We then extended this analysis further and used the FUCCI(CA) cell cycle biosensor to measure G1 length and found that in hESC without CDK2 inhibition, G1 remained short and constant at 3 hours in duration in response to NCS (MAD = 0.47 and 0.53 respectively) (Figure 4-4C). However, after treatment with the CDK2i (3-MB-PP1) in the Cdk2-AS system, both the length of G1 length and its variability increased (from 3 to 5 hours respectively, MAD 0.47 to 1.35) (Figure 4-4C). This increased to an even greater extent after treatment with NCS (from 5 to 7 hours), further suggesting the establishment of a functional G1/S checkpoint (Figure 4-4C). To see if the levels of G1/S checkpoint associated proteins were also re-established, we stained for p21 and found that inhibiting CDK2 increased the p21 levels in response to NCS (Figure 4-4D). Furthermore, we examined the phosphorylation status of Rb and found that inhibiting CDK2 introduced a population of cells with hypophosphorylated Rb in response to NCS (Figure 4-4E). Taken collectively, these data show that hyperactive CDK2 is important in bypassing the G1/S checkpoint in hESCs, and that reducing CDK2 activity to somatic levels can re-establish the G1/S checkpoint. This suggests that CDK2 plays a critical role in regulating the response to DNA damage and G1/S checkpoint regulation.

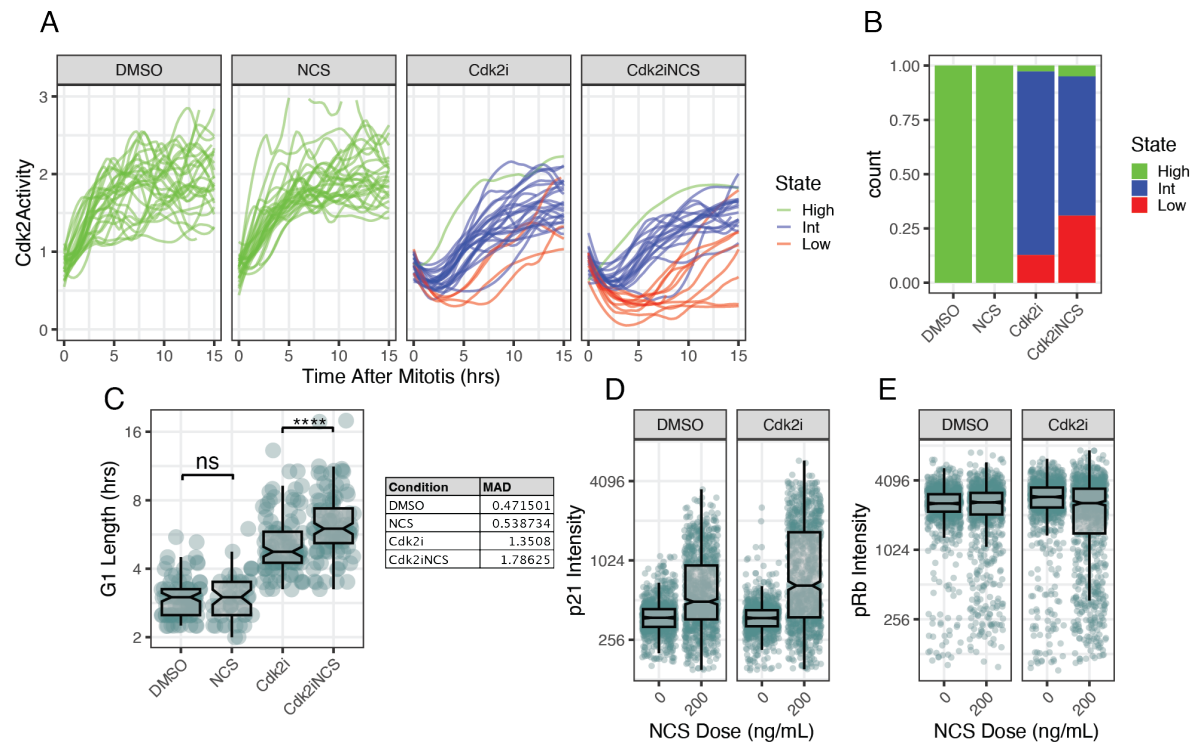


Figure 4-4- Modulating CDK2 activity allows for hESCs to respond to NCS treatment A. CDK2 activity traces aligned to time after anaphase onset after treatment with NCS for DMSO and CDK2i (3-MB-PP1) cells in the CDK2-AS system. $n=30$ for each condition. Measurements were made 2 hours after addition of both CDK2i (3-MB-PP1) and NCS. B. Stacked bar plot illustrating how the proportion of the 3 CDK2 activity states changes after treatment with NCS for DMSO and CDK2i (3-MB-PP1) cells in the CDK2-AS system. C. Quantification of G1 length after treatment with NCS for DMSO and CDK2i (3-MB-PP1) cells in the CDK2-AS system. Boxplots are overlaid to indicate minimum, maximum, first quartile, third quartile and median values. $n=100$ for each condition. Measurements were made 2 hours after addition of both CDK2i (3-MB-PP1) and NCS in the CDK2-AS system. KS tests were performed to calculate p -values. (ns= not significant, **** $p < 0.0001$, *** $p < 0.001$, ** $p < 0.01$, * $p < 0.05$). D-E. Single cell (nuclei) quantification of immunostaining for hESC treated with NCS for DMSO and CDK2i (3-MB-PP1) cells in the CDK2-AS system. D. p21 staining E. pRb 807/811. $n=2000$ for each condition. Boxplots are overlaid to indicate minimum, maximum, first quartile, third quartile and median values. Measurements were made 6 hours after addition of both CDK2i (3-MB-PP1) and NCS in the CDK2-AS system.

4.2.5 Perturbing Skp2 in hESC increases p21 levels in response to NCS treatment

As modulating CDK2 activity was enough to reintroduce a G1/S checkpoint, we next sought to address the molecular mechanism underpinning this. p21 plays an important role in G1/S checkpoint control by binding to and inhibiting CDK2 during the DNA damage response, thereby preventing phosphorylation of key substrates that initiate DNA replication and entry into S phase. Since threshold levels of p21 must be reached to inhibit CDK2 and hESC have lower levels of p21 (Figure 4-1), we focused our attention onto p21 regulation. In the previous chapter we showed that hESC had elevated levels of SCF complex component Skp2 throughout the cell cycle (Figure 3-14). Since Skp2 is involved in the ubiquitination and degradation of p21, we rationalised that the absence of a G1/S checkpoint could be due to a lack of an accumulation of p21 in response to DNA damage.

To test this, we knockdown Skp2 using RNAi (Figure 4-5A). We then treated hESC with NCS for 2 hours, fixed cells 6 hours later and stained for p21. Under basal conditions, knocking down Skp2 already increased levels of p21 in hESC (Figure 4-5BC). Importantly, after treatment with NCS, both the levels and the percentage of cells expressing p21 increased dramatically in hESC with perturbed Skp2 expression levels in comparison to control cells treated with NCS (Figure 4-5B,C). This data shows Skp2-mediated degradation plays a vital role in keeping the levels of p21 low under conditions of cellular stress in hESCs. It also sheds light as to why hESCs continue cell cycle progression, even in the presence of DNA damage.

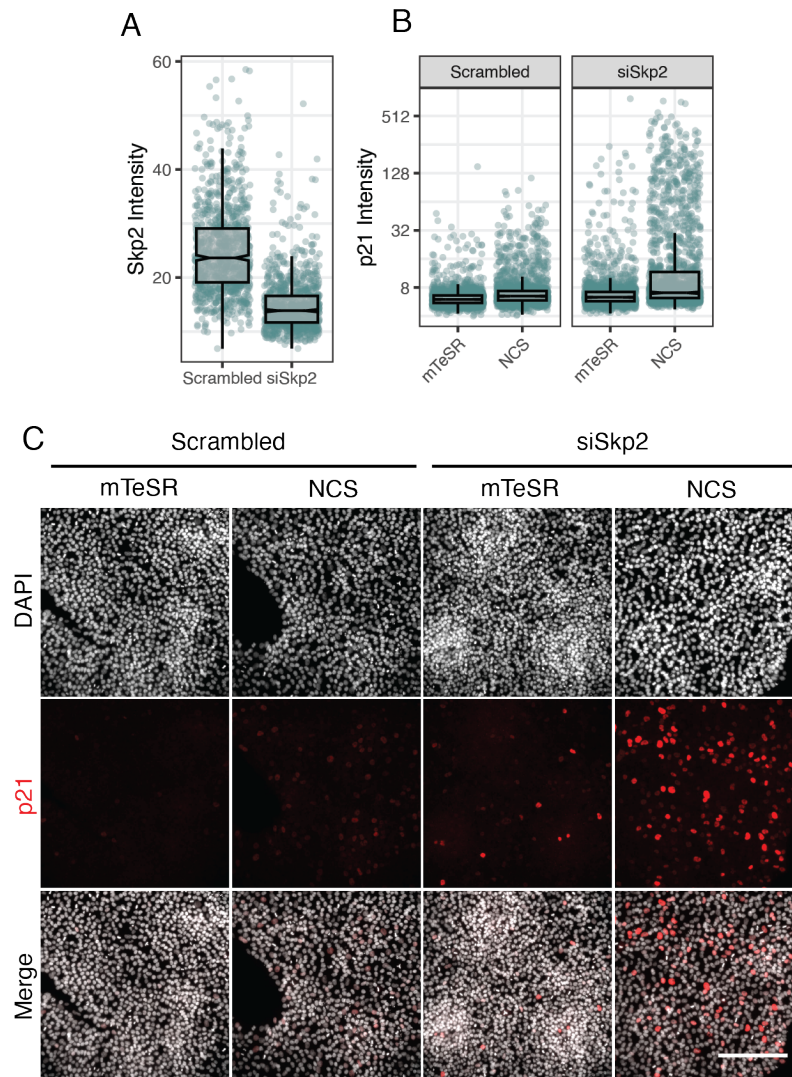


Figure 4-5- Perturbing Skp2 in hESCs increase p21 levels in response to NCS treatment *A. Single cell (nuclei) quantification of Skp2 immunostaining for Scrambled and siSkp2 cells. n=2000 for each condition. Figure is repeated from Figure 3-26- Ubiquitin ligases play an important role in maintaining high CDK2 activity* *D B. Single cell (nuclei) quantification of p21 immunostaining for hESC treated with NCS for Scrambled and siSkp2 cells. n=2000 for each condition. Boxplots are overlaid to indicate minimum, maximum, first quartile, third quartile and median values. C. Representative images of p21 immunostaining for hESC treated with NCS for Scrambled and siSkp2 cells. DAPI images are represented in gray scale and p21 images are represented in red. Scale bar = 100 μ M. N=2 independent experiments. All measurements were made 3 days after siRNA transfection and 6 hours post NCS treatment.*

4.2.6 Perturbing Skp2 allows hESCs to arrest in G2 in response to NCS

We next sought to address cell cycle dynamic changes in hESC in response to NCS after Skp2 down-regulation, and whether increased p21 levels in response to NCS were accompanied by a decrease in CDK2 activity and an increase in G1 length. To do this we used a cell line with both the CDK2 activity biosensor and PCNA cell cycle biosensor to measure how perturbing Skp2 influenced cell cycle dynamics and CDK2 activity in response to NCS treatment. For our experiments we treated cells with NCS for two hours and then performed live cell imaging for 48 hours. Consistent with our previous results, treatment with NCS alone had no impact on cell cycle dynamics and CDK2 activity (Figure 4-6B,C,D). Surprisingly, knocking down Skp2 had no influence on G1 length in response to NCS, and showed only a subtle change in the proportion of CDK2 activity states, with a small increase in the proportion of cells in the CDK2 intermediate state (Figure 4-6B,C,D). However, while there was no change in S-phase duration (Figure 4-6E), we found a significant increase in G2 length (from 5hrs to 6.25 hours) in response to NCS treatment (Figure 4-6F). It is well established that p21 is also involved in the G2 checkpoint through inhibition of CDK1-Cyclin A/B, resulting in cell cycle arrest in G2/M (Bunz et al., 1998). Although knocking down Skp2 and increasing p21 levels appears to not influence the duration of G1 hESC in response to NCS, the increased p21 levels displayed when perturbing Skp2 results in altered G2 dynamics in response to NCS. Our results postulate that hESCs preferentially deal with DNA damage during G2 and suggest that perturbing Skp2 may play a specific role in regulating the G2/M checkpoint in response to DNA damage, potentially through p21 mediated CDK1-Cyclin A/B inhibition.

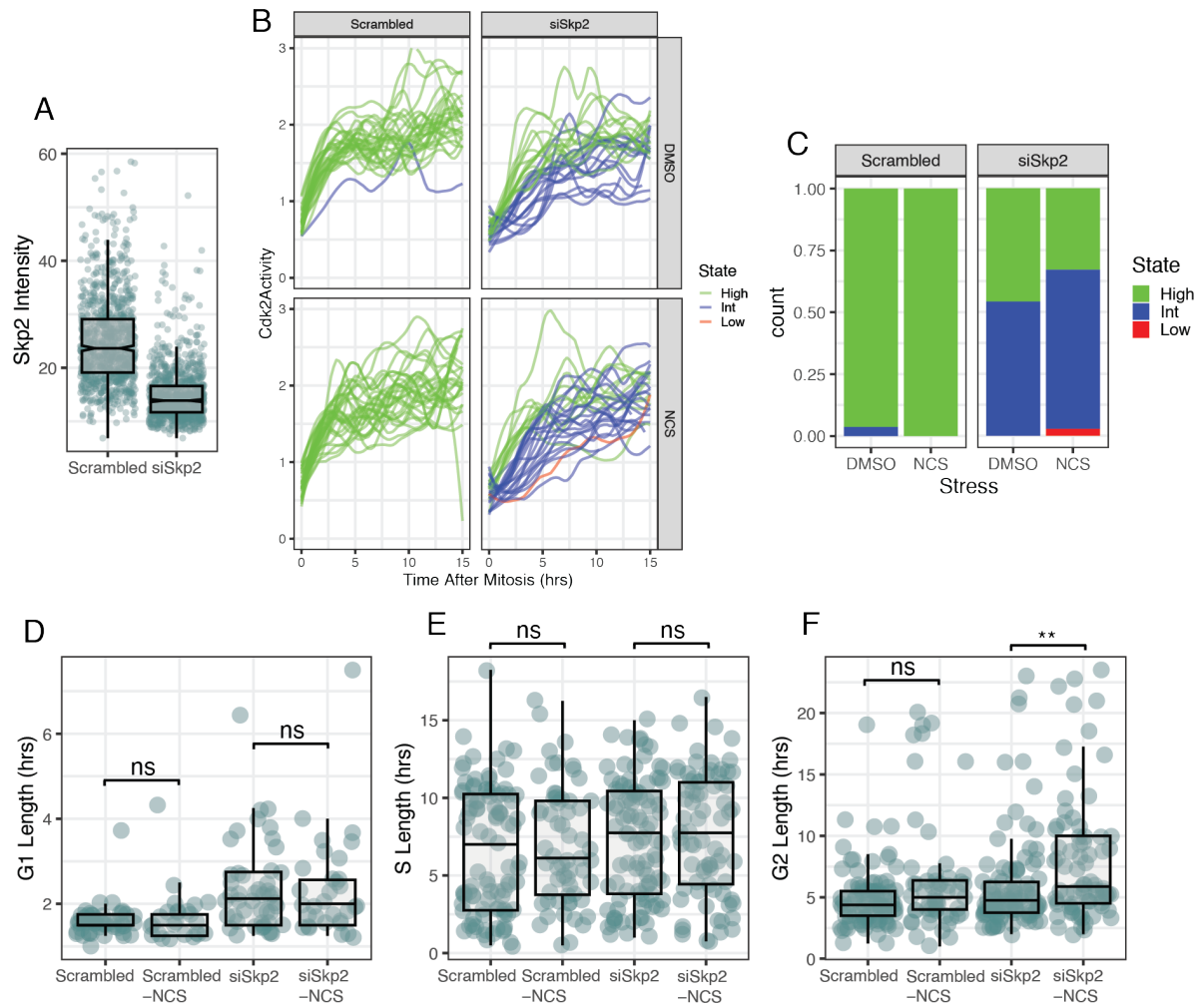


Figure 4-6- Perturbing Skp2 allows hESCs to arrest in G2 in response to NCS *A*. Single cell (nuclei) quantification of Skp2 immunostaining for Scrambled and siSkp2 cells. $n=2000$ for each condition. Figure is repeated from *Figure 3-26- Ubiquitin ligases play an important role in maintaining high CDK2 activity* *D*. *B*. CDK2 activity traces aligned to time after anaphase onset after treatment with NCS for Scrambled and siSkp2 cells. $n=30$ for each condition. *C*. Stacked bar plot illustrating how the proportion of the 3 CDK2 activity states changes in after treatment with NCS for Scrambled and siSkp2 cells. *D-F*. Quantification of phase lengths for hESCs treated with NCS for Scrambled and siSkp2 cells. $n=50$ for each condition. *D*. G1 phase lengths *E*. S phase lengths *F*. G2 phase lengths. KS tests were performed to calculate p -values. (ns= not significant, **** $p < 0.0001$, *** $p < 0.001$, ** $p < 0.01$, * $p < 0.05$). *B-F* measurements were made 3 days after siRNA transfection and 2 hours post NCS treatment.

4.2.7 Perturbing both Cyclin D and Skp2 restores the G1/S checkpoint in hESC

A question remained though: Why increasing p21 levels by knocking down Skp2 in hESC does not activate G1/S checkpoint in response to NCS? One possible explanation could be that due to hyperphosphorylation of Rb, cells are past the cell cycle commitment point. If true, even high levels of p21 would be insufficient to inhibit CDK2 to arrest cells in G1. Furthermore, the levels of p21 after Skp2 down-regulation might not have been sufficient to pass the threshold necessary for CDK2 inactivation. One study has shown that many cells fail to properly activate the G1/S checkpoint due to persistence in CDK2 activity resulting from a 2-hour delay between DNA damage and p21 induction above a threshold (Nathans et al., 2021). Furthermore, as APC/C^{Cdh1} inactivation marks irreversible cell cycle commitment and hESC have already low APC/C^{Cdh1} activity (Cappell et al., 2016), failure to activate the G1/S checkpoint may be a result of premature cell cycle commitment regardless of p21 levels. With this in mind, we set out to perturb both Skp2 and increase APC/C^{Cdh1} activity to investigate whether we could reintroduce the G1/S checkpoint by allowing threshold levels of p21 to increase before APC/C^{Cdh1} inactivation.

Our first approach was to knock down Skp2 to increase p21 levels while also knocking down cyclins D1, D2, D3 (Figure 4-7A) to increase G1 length and therefore the window before cell cycle commitment. We then measured CDK2 activity to see if the proportion of cells entering the CDK2 low state increased in response to NCS. While neither Cyclin D1D2D3 knockdown or Skp2 knockdown alone increased the proportion of cells entering the CDK2 low state in response to NCS, combining the two perturbations did, with more cells entering the low state in response to NCS (Figure 4-7B,C). To see if the levels of G1/S associated checkpoint proteins had increased, we stained for p21 and found that unsurprisingly, the perturbations involving Skp2 had higher levels of p21 in response to NCS (Figure 4-7D). Importantly, when staining for hyperphosphorylated Rb, knocking down Cyclins D1D2D3 alone showed a population of cells with hypophosphorylated Rb, however when treating cells with NCS this population reduced by 8% (Figure 4-7E). It was only when we combined the Skp2 and Cyclin D1D2D3 perturbations that cells increased their fraction of hypophosphorylated Rb by 17% in response to NCS (Figure 4-7E).

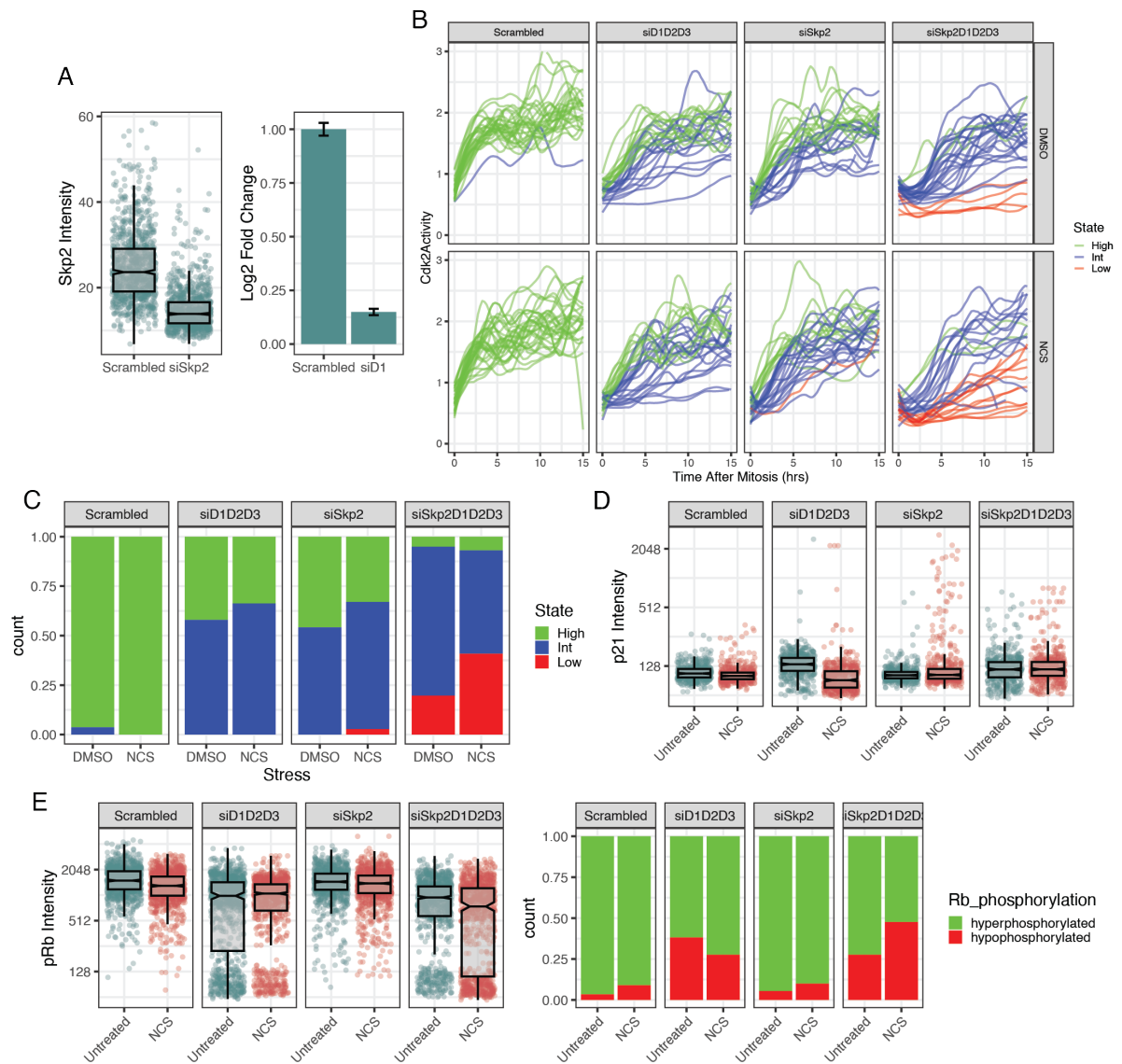


Figure 4-7- Perturbing both Cyclin D and Skp2 restores G1/S checkpoint in hESCs *A.* Quantification of knockdown efficiency for (left) siSkp2 using Skp2 nuclei immunostaining and (right) siCyclin D1 using qPCR fold change expression values. Measurements are made 3 days post siRNA transfection. Left figure is repeated from *Figure 3-26- Ubiquitin ligases play an important role in maintaining high CDK2 activity*. *B.* CDK2 activity traces aligned to time after anaphase onset after treatment with NCS for Scrambled, siCyclin D1D2D3, siSkp2, siSkp2D1D2D3 cells. $n=30$ for each condition. *C.* Stacked bar plot illustrating how the proportion of the 3 CDK2 activity states changes after treatment with NCS for Scrambled, siCyclin D1D2D3, siSkp2, siSkp2D1D2D3 cells. *D-E.* Single cell (nuclei) quantification of immunostaining for hESC treated with NCS for Scrambled, siCyclin D1D2D3, siSkp2, siSkp2D1D2D3 cells *D.* p21 staining *E.* pRb 807/811. $n=2000$ for each condition. Boxplots are overlaid to indicate minimum, maximum, first quartile, third quartile and median values. For *D.* Stacked bar plots are shown to illustrate how the proportion of cells with hypo and hyperphosphorylated Rb changes after treatment with NCS. $N=2$ independent experiments. *B-C* measurements are made 72 hours post siRNA transfections and 2 hours after addition of NCS. *D-E* measurements are made 72 hours post siRNA transfections and 6 hours after addition of NCS.

To investigate whether the observed response to DNA damage was specific to NCS, we treated cells with additional stresses including the DNA replication inhibitor Aphidicolin and UV light to induce DNA damage. Aphidicolin has previously been shown to induce p21 expression, decreasing CDK2 activity resulting in an accumulation in cells arresting in G1 and G2 (Barr et al., 2017). UV light exposure also activates p21, which can subsequently inhibit the activity of CDK2 (Poon et al., 1996). We therefore examined the effect of Skp2 and Cyclin D1D2D3 knockdown on Rb phosphorylation in response to Aphidicolin and UV treatment for 6 hours. Consistent with our previous finding with NCS treatment, we see that only the combination of Skp2 and Cyclin D1D2D3 knockdown, and not the single perturbations, resulted in an increase in hypophosphorylation of Rb after Aphidicolin and after UV light (Figure 4-8A,B). Aphidicolin increased this proportion by 60% (Figure 4-8A) and UV by 46% (Figure 4-8B). These results suggest that the effects of the dual (Cyclin D1D2D3 and Skp2) perturbation and consequent DNA damage response is not specific to NCS but is also induced by other DNA damaging agents such as Aphidicolin and UV light. Together our findings suggest expression of Skp2 and Cyclin D1D2D3 act synergistically in regulating the response to DNA damage response in hESCs to bypass the G1/S checkpoint.

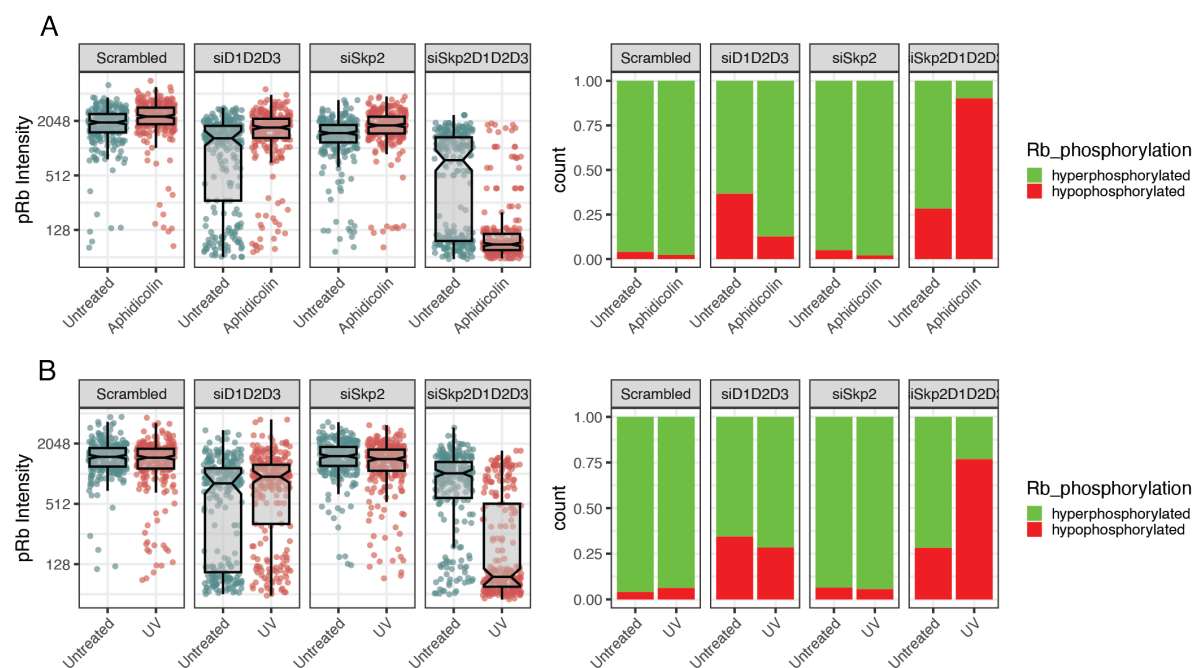


Figure 4-8- Perturbing both Cyclin D and Skp2 restores G1/S checkpoint with other stresses A-B. Single cell (nuclei) quantification of pRb 807/811 immunostaining for hESC treated with different stresses for Scrambled, siCyclin D1D2D3, siSkp2, siSkp2D1D2D3. A. 0.3 μ M Aphidicolin treatment B. 20 J/m² treatment of UV light. n=500 for each condition. Boxplots are overlaid to indicate minimum, maximum, first quartile, third quartile and median values. Stacked bar plots are shown to illustrate how the proportion of cells with hypo and hyperphosphorylated Rb changes after treatment with NCS. All measurements are made 72 hours post siRNA transfections and 6 hours after addition of Aphidicolin/UV.

Our second approach was to see if cells would express G1/S checkpoint markers in response to NCS by upregulating Cdh1 to increase (and prolong) APC/C^{Cdh1} activity while modulating Skp2 expression. To do this, we used the dihydrofolate reductase (DHFR)-trimethoprim (TMP) protein stabilisation system (Iwamoto et al., 2010) to induce levels of Cdh1. This system uses a DHFR fusion protein tagged onto Cdh1, which under basal conditions is continuously degraded (Figure 4-9A). The addition of TMP stabilises the DHFR degron, increasing Cdh1 protein levels rapidly within 2 hours. We knocked down Skp2 and pre-treated cells with TMP for 24 hours which increased Cdh1 levels 3-fold (Figure 4-9A) before stressing cells with NCS 2 hours and fixing 6 hours later. We found that the presence of TMP did not alter the expression of p21 in response to NCS (Figure 4-9B). We did however see an increase in the hypophosphorylated (active) form of RB after stabilising Cdh1, suggesting that stabilisation of Cdh1 alone results in some Rb reactivation (Figure 4-9C). Despite this, we still observed a response to NCS in the absence of Cdh1 stabilisation, with an increase in the population of cells with hypophosphorylated Rb by 27% (Figure 4-9C). Furthermore, knocking down Skp2 alone without the addition of TMP was sufficient to increase the population of cells with hypophosphorylated Rb in response to NCS (Figure 4-9C). When looking at the fluorescent images of Cdh1-DHFR-YFP, we found that the DHFR-TMP protein stabilisation system was “very leaky” for Cdh1 expression under basal conditions which could explain why we see a population of hypophosphorylated Rb in the absence of TMP. Despite this, these data show that overexpression of Cdh1 alone can increase levels of G1/S checkpoint markers in response to NCS in hESCs. Studies have shown that Cdh1 is responsible for degrading Skp2 (Bashir et al., 2004), which could explain why Cdh1 overexpression alone is sufficient to upregulate G1/S associated markers. This is consistent with our own results showing that treatment with NCS decreased levels of Skp2 in the presence or absence of TMP (Figure 4-9D). This confirms that Cdh1 activity plays a role in regulating Skp2 expression in response to DNA damage, and that high levels of Skp2 might result from the decreased Cdh1 activity in hESCs. While the TMP protein stabilisation system might not be the best tool to induce Cdh1 expression, our results highlight that protein degradation plays an important role in regulating the DNA damage response in hESCs. Current efforts are on-going in the lab to use CRISPR-mediated activation of Cdh1 to validate these conclusions.

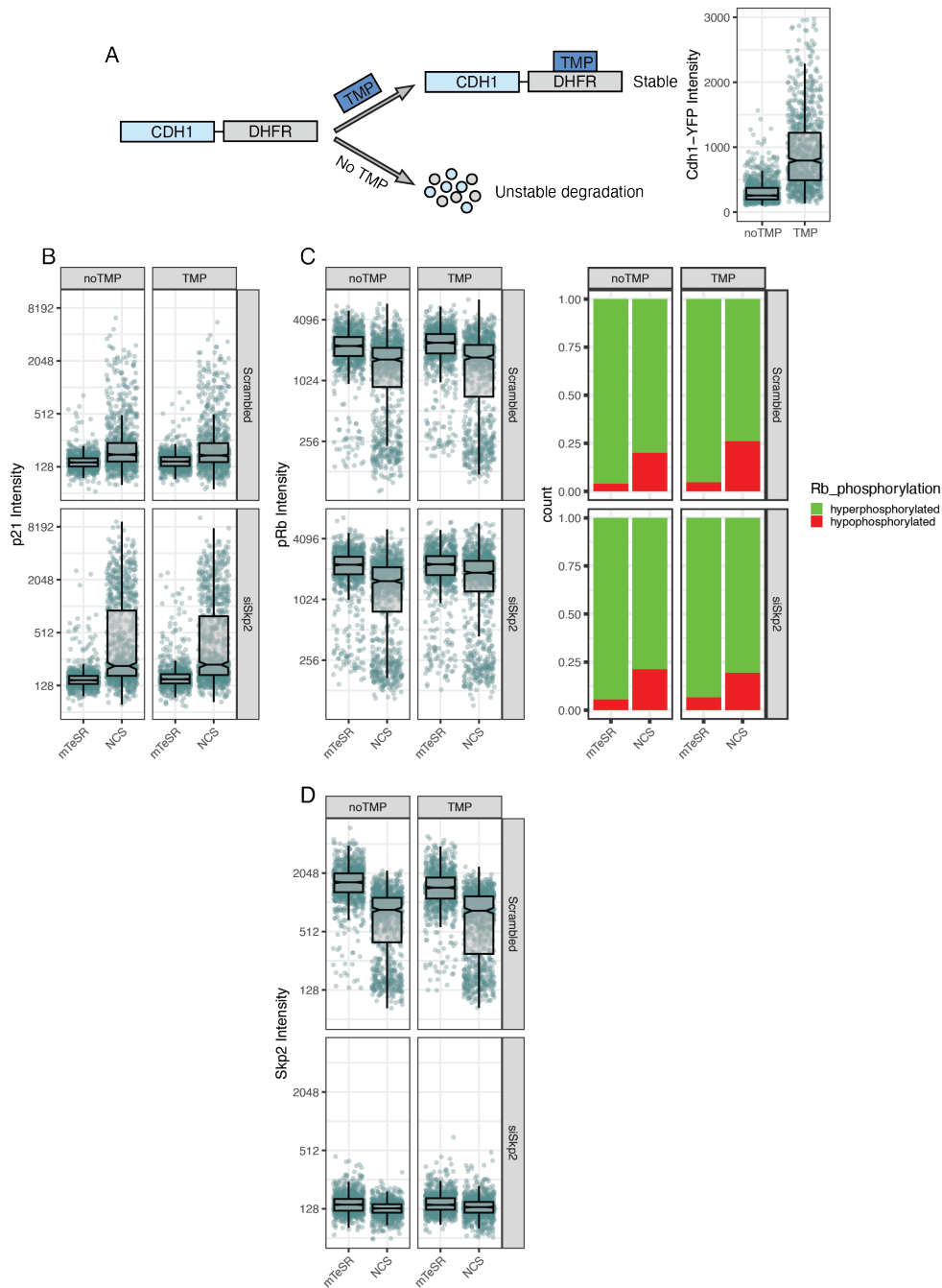


Figure 4-9- Overexpressing Cdh1 increases the proportion of cells with hypophosphorylated Rb in response to NCS A. (left) Schematic illustrating how the DHFR-TMP protein stabilisation system works. Under basal condition DHFR tagged Cdh1 is continuously degraded but upon TMP addition stabilises the protein. (right) Quantification of nuclei Cdh1-DHFR-YFP signal with and with addition of TMP for 24hours. B-D Single cell (nuclei) quantification of immunostaining for hESC treated with NCS for Scrambled, siSkp2 cells with and without TMP. B. p21 staining C. pRb 807/811 staining D. Skp2 staining. n=2000 for each condition. Boxplots are overlaid to indicate minimum, maximum, first quartile, third quartile and median values. For C. Stacked bar plots are shown to illustrate how the proportion of cells with hypo and hyperphosphorylated Rb changes after treatment with NCS. B-D measurements are made 24 hours after addition of TMP and 6 hours after addition of NCS.

4.3 CDK2 activity and the maintenance of cell identity

4.3.1 Inhibiting CDK2 results in rapid loss of Nanog

The maintenance of Nanog, Oct4 and Sox2 expression is crucial for pluripotency and self-renewal of hESCs (Yilmaz & Benvenisty, 2019). After determining that CDK2 activity was important in G1/S checkpoint control, we next wanted to address if high CDK2 activity was important in the maintenance of the pluripotent identity. To test this, we first used the CDK2 AS hESC line to specifically inhibit CDK2 at different doses for 1 day before measuring the levels of the three core pluripotency markers Oct4, Sox2 and Nanog by immunofluorescence. Surprisingly, while Oct4 and Sox2 levels remained the same after treatment with the CDK2i (3-MB-PP1) in the CDK2-AS system, we observed a dose dependent loss of Nanog, with levels almost completely abolished at the highest concentration (Figure 4-10A,B). This data strongly suggests that CDK2 activity might play a vital role in maintaining pluripotency in hESCs.

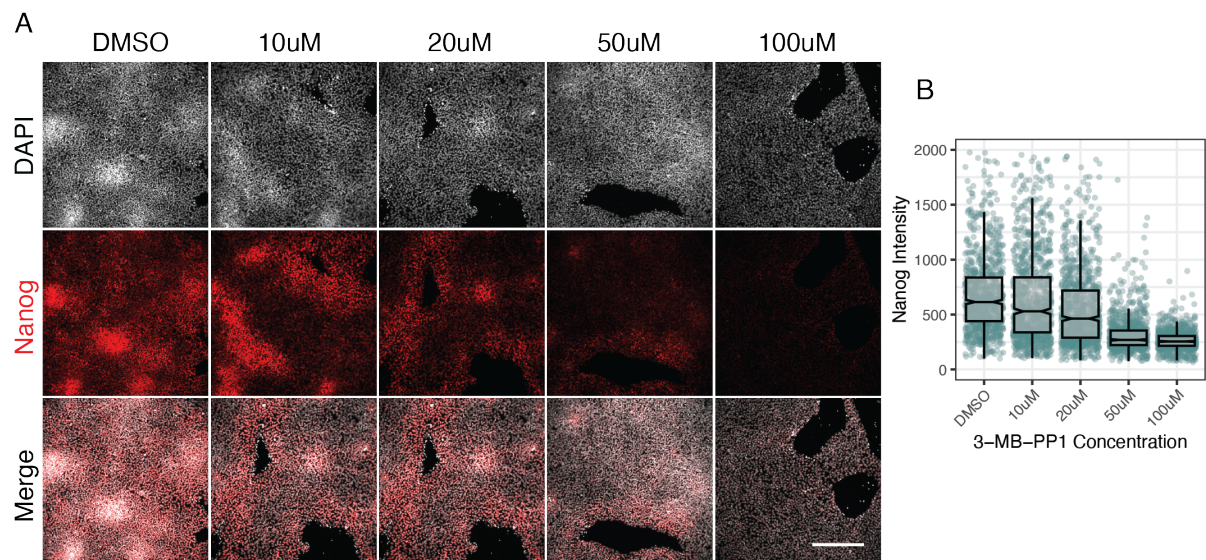


Figure 4-10- Inhibiting CDK2 results in rapid loss of Nanog A. Representative images of Nanog immunostaining for hESC treated with different doses of CDK2i for 1 day (3-MB-PP1) in the CDK2-AS System. DAPI images are represented in gray scale and Nanog images are represented in red. Scale bar = 100 μ M B. Single cell (nuclei) quantification of Nanog immunostaining for hESC treated with different doses of CDK2i (3-MB-PP1) in the CDK2-AS system. n=2000 for each condition. Boxplots are overlaid to indicate minimum, maximum, first quartile, third quartile and median values.

4.3.2 Knocking down regulators of CDK activity reduce Nanog levels in hESC

Since we had identified multiple regulators that were involved in regulating high CDK2 activity, we next sought to address whether other perturbations could recapitulate the effects of CDK2 activity in maintaining Nanog levels in hESCs. Furthermore, we wanted to understand whether specifically, CDK2 activity, or alternatively, global CDK activity, was important in regulating Nanog levels and thereby, promoting hESC pluripotency. To do this we knockdown several CDK2 regulators using RNAi and stained for Nanog 3 days later to ascertain if individual regulators were required to maintain high Nanog levels. Our results showed that most of the tested CDK2 activity regulators had no significant effect on Nanog expression in hESCs. However, we observed a significant reduction in Nanog levels upon knocking down Cyclin A2, suggesting CDK2-Cyclin A2 activity may play a key role in maintaining pluripotency in hESCs (Figure 4-11). Additionally, knocking down Cyclin A2 changed the morphology of the cells, further suggesting loss of stem cell identity (Figure 4-11). These data suggest that regulation of Nanog by CDK2 in hESC is potentially mediated by CDK2-Cyclin A2 although we cannot rule out that CDK1-Cyclin B1 complexes are not involved. In support of this, perturbing Wee1, a common inhibitor of CDK1 and CDK2, decreases Nanog expression (Figure 4-11). In addition, we also observed a significant reduction in Nanog levels after knocking down Cyclin D1D2D3 and Cyclin B1 (Figure 4-11). This supports a model whereby CDK4/6 and CDK1 activities may also play a role in regulating Nanog in hESCs. Interestingly, we see that the perturbations that shifted CDK2 activity to the low state all reduced Nanog levels (e.g. siD1D2D3Sklp2 and Wee1i), while perturbations that affected the intermediate state (siSklp2 and siE1E2) did not (Figure 4-11). Altogether, these results suggest that global CDK activity is important to keep Nanog expression levels high and promote pluripotency identity.

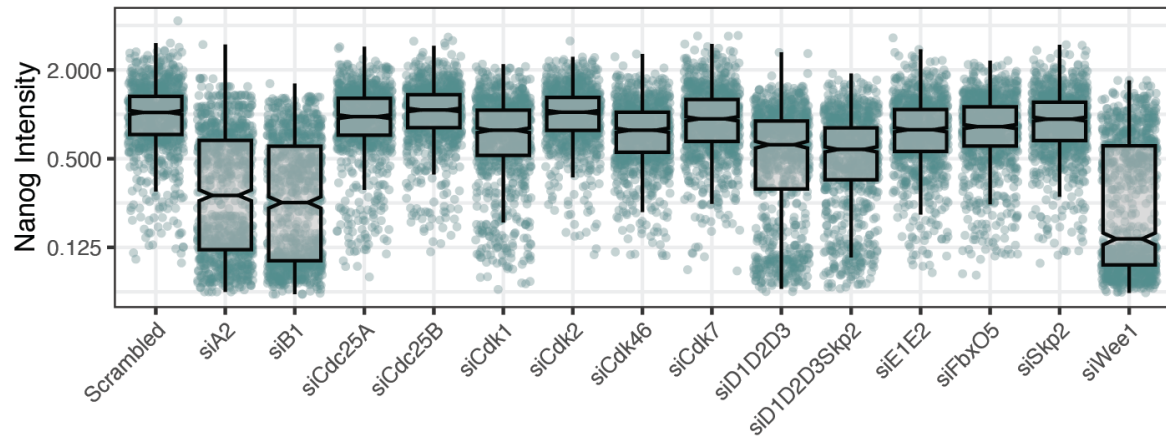


Figure 4-11- Knocking down CDK activity regulators reduces Nanog levels in hESCs Single cell (nuclei) quantification of Nanog immunostaining for hESC treated with different siRNAs against CDK activity regulators. $n=2000$ for each condition. Boxplots are overlayed to indicate minimum, maximum, first quartile, third quartile and median values. Measurements are made 72 hours post siRNA transfection.

4.3.3 Loss of Nanog is reversible

With the knowledge that CDK2 played an important role in the maintenance of pluripotency in hESCs, we wanted to explore whether loss of Nanog led to irreversible loss of pluripotency. To test this, we made use of the dynamic nature of the CDK2-AS mutant hESC line. We incubated cells with the CDK2 inhibitor (3-MB-PP1) for different periods of time before washing away the inhibitor and returning cells back to pluripotent media (Figure 4-12A). We tested irreversibility with three different concentrations of CDK2 inhibitor where 1) cells were predominantly in the CDK2 intermediate activity state (1 μ M), 2) cells had a mix of the intermediate and low activity states (20 μ M) and 3) cells were predominantly in the low CDK2 activity state (100 μ M) (Figure 4-12B,C). At the lowest concentration, we found no change on Nanog levels over a period of 1 day (Figure 4-12B). This data shows that shifting cells towards the CDK2 intermediate state only is not sufficient to induce loss of Nanog in hESCs (Figure 4-12B). However, at higher CDK2 inhibitor concentrations, with both intermediate and low states of CDK2 activity, we found that inhibition of CDK2 resulted in reduction to Nanog. Surprisingly, washing away the CDK2 inhibitor and switching cells back to pluripotency media showed full recovery of Nanog levels, showing that CDK2 effect on Nanog is reversible (Figure 4-12B). This was also mirrored by a reversible increase in the proportion of cells with hyperphosphorylated Rb, indicating that CDK2 inhibition is also reversible for a period of 1 day (Figure 4-12C).

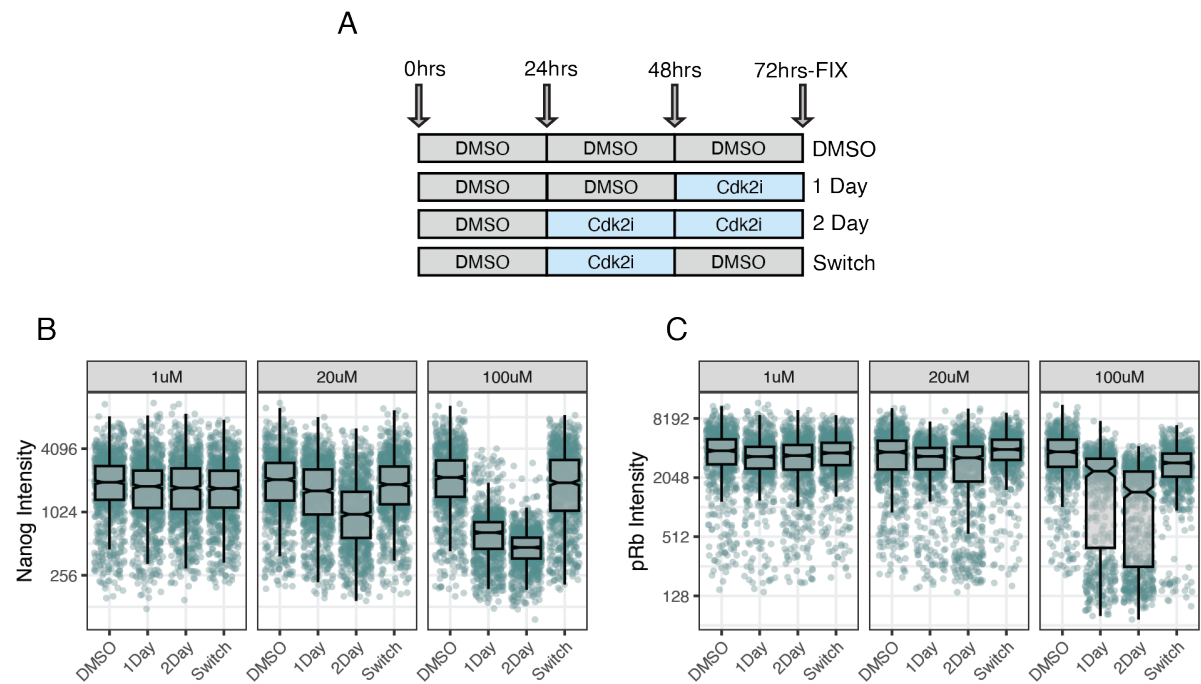


Figure 4-12- Loss of Nanog is reversible *A. Schematic illustrating inhibitor switching conditions. B-C. Single cell (nuclei) quantification of immunostaining for hESC treated with CDK2i (3-MB-PP1) in the CDK2-AS system switching conditions at different concentrations. B. Nanog staining C. pRb 807/811 staining. n=2000 for each condition. Boxplots are overlaid to indicate minimum, maximum, first quartile, third quartile and median values.*

4.3.4 Long term CDK2 inhibition results in neuroectoderm differentiation

What happens after long-term CDK2 inhibition? Although we observed no changes in the other pluripotency markers Oct4 and Sox2 after 1 day of CDK2 inhibition, some studies have shown that knocking down Nanog in hESCs results in complete loss in pluripotency and differentiation to neuroectoderm (NE) lineages (Vallier et al., 2009; Wang et al., 2012). To test the long-term effects of CDK2 inhibition, we inhibited CDK2 in the CDK2-AS system so that cells had a mixture of intermediate and low CDK2 activity states, similar to those in differentiated cells, for a period of 5 days and measured the levels of pluripotency markers. In agreement with our previous results, Nanog was depleted after a period of 5 days (Figure 4-13A). We also noted a loss in the pluripotency marker Oct4 and an increase in Sox2 (Figure 4-13B,C). Interestingly, this expression signature of pluripotency factors where cells are Nanog (-)/Oct4 (-)/ Sox2(+) has been shown to be compatible with NE differentiation (S. Zhang, 2014). We therefore stained for the NE specific markers Pax6 and Lhx5 and found an upregulation in CDK2 inhibited cells (Figure 4-13D,E). To further assess the degree of similarity between CDK2 inhibited cells and NE cells, we performed quantitative PCR (qPCR) to analyse the levels of 43 genes involved in lineage specification and compared them to hESC directly differentiated to NE cells, using dual SMAD inhibition. Strikingly, we see that the gene expression profile of hESC after 5 days of CDK2 inhibition was very similar to that of NE differentiated cells (Figure 4-13F). Our results suggest that prolonged inhibition of CDK2 can lead to loss of pluripotency and a shift towards NE differentiation in hESCs, showing that the cell cycle impacts on cell identity and fate determination. This data is also consistent with the idea that Nanog is specific repressor of NE differentiation (Z. Wang et al., 2012).

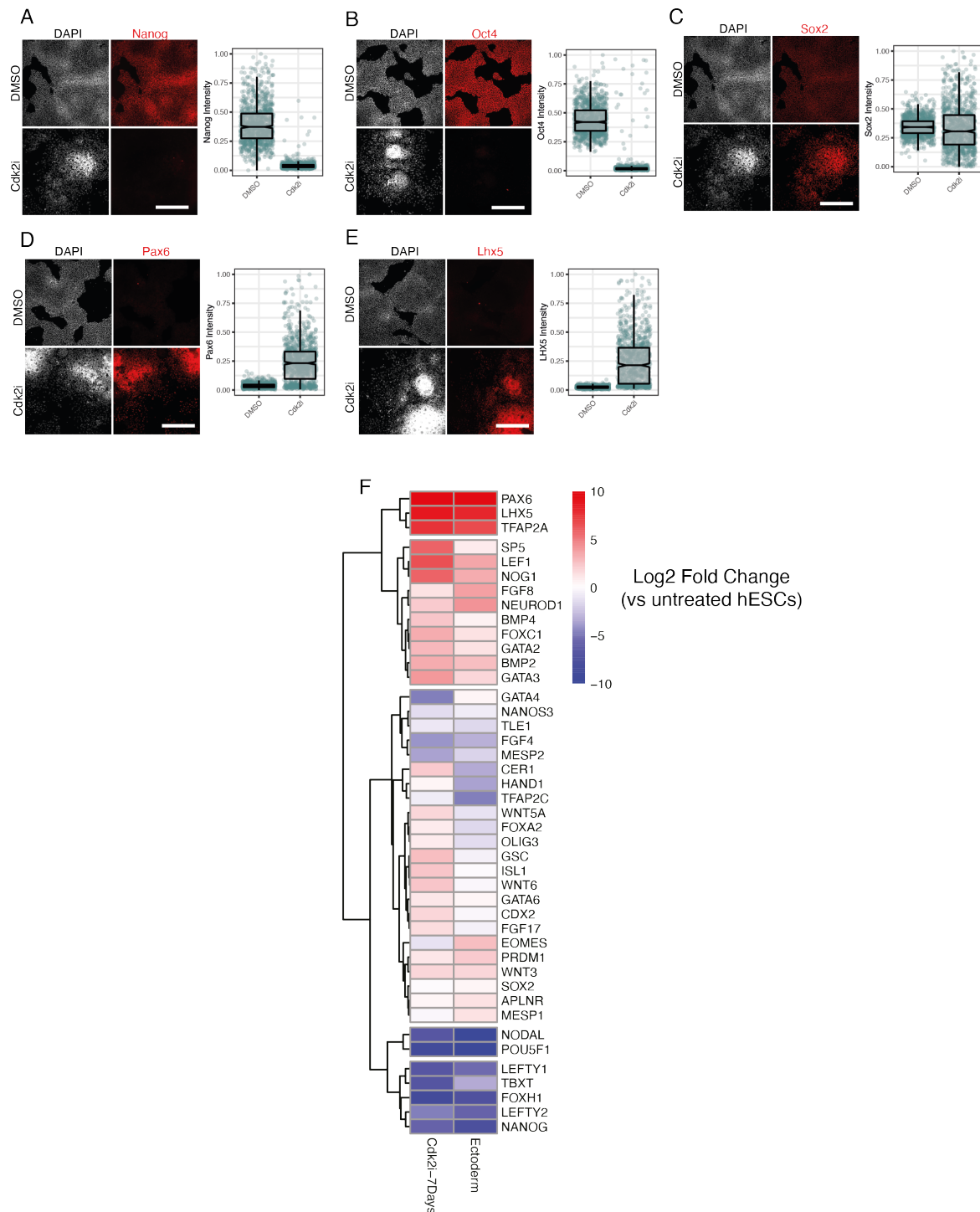


Figure 4-13- Long term CDK2 inhibition leads to neuroectoderm differentiation A-E. (left) Representative images of immunostaining for hESC treated with 20 μ M CDK2i (3-MB-PP1) for 5 days in the CDK2-AS system. DAPI images are shown in gray scale and staining in red. (right) Single cell (nuclei) quantification of immunostaining for hESC treated with 20 μ M CDK2i (3-MB-PP1) in the CDK2-AS system for 5 days. Scale bar = 200 μ M. A. Nanog staining B. Oct4 staining C. Sox2 staining D. Pax6 staining E. Lhx5 staining. F. Heatmap comparing the expression levels by qPCR of 43 fate markers involved in pluripotency and differentiation for cells treated with 20 μ M CDK2i (3-MB-PP1) in the CDK2-AS system for 5 days and directly differentiated ectoderm cells. Expression levels are compared to untreated hESCs. Housekeeping gene GUSB was used for normalisation. N=3 replicates.

4.3.5 CDK2 activity changes in 3D models that recapitulate early human gastrulation

As hESCs are a highly tractable system, amenable to a variety of single cell imaging approaches, the use of hESCs in 2D culture systems has been an invaluable tool to study the regulation of early human development and the complex molecular and cellular events that underlie it. Despite these advantages, 2D culture systems do not provide the same physiological cues and environmental conditions that are found *in vivo*, which can limit the ability of ESCs to accurately model early human development. Furthermore, due to the complex ethical, legal, and technical restriction of embryo research, it is very challenging to conduct *in vivo* research to confirm finding from 2D hESC culture systems. BMP4-driven differentiation and the beginning of gastrulation happen post-implantation, a time that is inaccessible to experimentation in human. But is cell cycle remodelling and CDK2 high *in vivo*? To address this, we set out to see whether CDK2 activity changed in a 3D model for early human gastrulation (gastruloids) (Figure 4-14A). These 3D structures are derived from hESCs and designed to mimic one of the earliest stages of human development, gastrulation where the embryo begins to form distinct embryonic layers of mesoderm, endoderm and ectoderm which will eventually give rise to all the different tissues and organs in the body. We used hESCs harbouring the CDK2 activity sensor, combined with a H2B nuclear marker, and used light-sheet microscopy to observe the localisation of the fluorescent reporter at different stages of gastruloid formation as a readout of CDK2 activity. While at early stages of gastruloid formation (24hrs), the signal from the CDK2 activity sensor was mainly cytoplasmic indicating high CDK2 activity, at later stages (from 48hrs onwards) cells started to display nuclear localisation indicating low CDK2 activity (Figure 4-14B). This data demonstrates that the activity of CDK2 also changes in a more physiologically relevant 3D environment, confirming our findings in 2D hESC culture systems. Although the light-sheet microscopy images only provide a snapshot of CDK2 activity in single cells, our results show that the use of gastruloids as a model system is an exciting approach to study cell cycle dynamics during early human development.

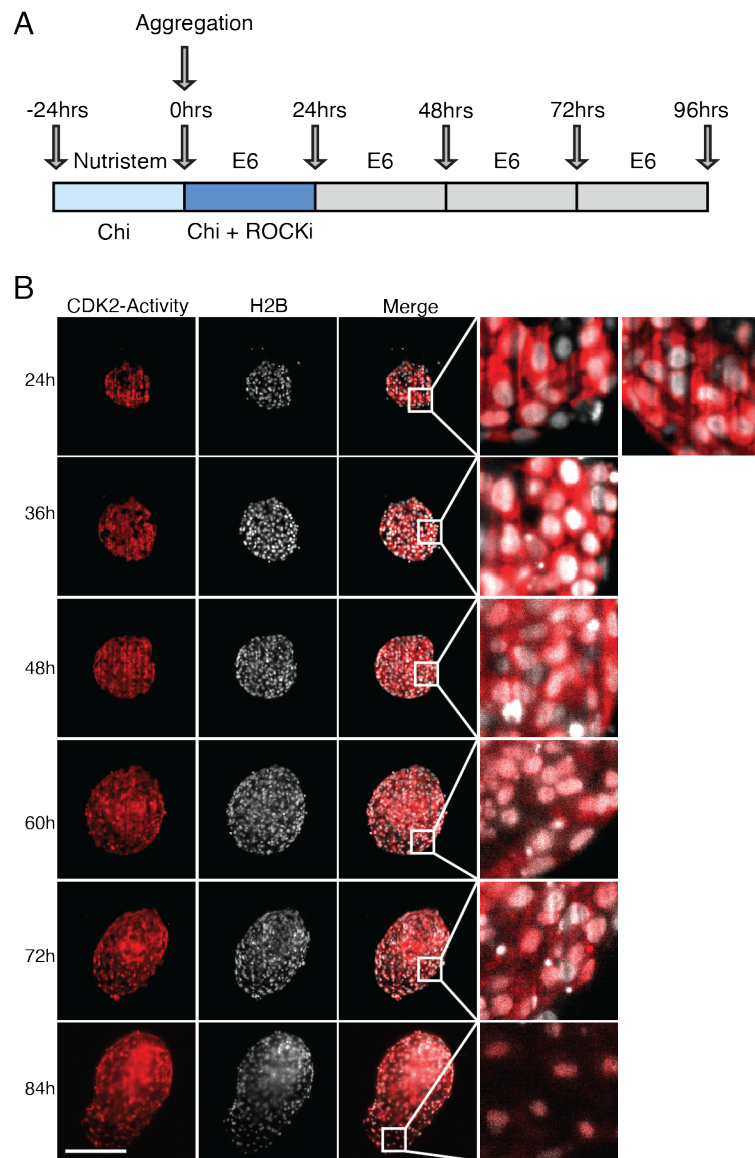


Figure 4-14- CDK2 activity changes in 3d models of early human gastrulation *A.* Schematic illustrating the human gastruloid protocol with culture conditions. Chi=Chiron (CHIR99021) E6= Essential 6 medium ROCKi = Rock inhibitor. *B.* Representative light sheet images of human gastruloids at different stages of formation. CDK2 activity is shown in red and H2B is gray scale images. n=12 gastruloids. Scale bar = 200 μ M.

4.4 Highlights from Chapter

The main aim of this chapter was to investigate the functional importance of having high CDK2 activity in hESCs. To address this, we tested what would happen to stem cell function when CDK2 activity was perturbed. We show for the first time in live, single cells in asynchronously dividing populations that hESCs are unable to elicit a G1/S checkpoint response to the radiomimetic drug NCS in comparison to BMP4-treated hESCs. We found that high CDK2 activity was vital in bypassing G1/S checkpoint control in hESCs, and that we could reintroduce a checkpoint by modulating CDK2 activity. Molecularly, this is achieved by having high Skp2 levels in hESCs, which functions to keep p21 levels low in response to NCS. Furthermore, low APC/C^{Cdh1} activity functions to decrease the window before irreversible cell cycle commitment and maintain high levels of Skp2. Perturbing both these regulators synergistically was sufficient to reintroduce a G1/S checkpoint in hESCs. In addition to this, we show that high CDK2 activity is important in maintaining pluripotency. Perturbing CDK2 activity reduced Nanog levels in a dose dependent manner after just 24 hours, and loss of Nanog was reversible for a period of up to 3 days. Prolonged CDK2 inhibition resulted in NE differentiation after 5 days, as shown by the upregulation of the NE lineage specific markers Pax6 and Lhx5. Finally, we show that CDK2 activity also changes in a more physiologically relevant 3D environment using human gastruloids, a model for early human gastrulation, confirming our findings from 2D culture systems. This work provides evidence that the unique high CDK2 activity dynamics displayed in hESC are an essential feature of hESC function.

Chapter 5. Discussion

5.1 New insights into cell cycle remodelling using single cell approaches

The first aim of the work presented in this thesis was to establish new single cell approaches to quantitatively measure cell cycle dynamics during hESC differentiation. We established several stable hESC lines employing a variety of cell cycle biosensors that could monitor and measure the duration of individual phase length in live, single cells during this transition (Figure 3-5). Due to the limited number of studies using single cell methodologies in live, asynchronously dividing hESCs, the development of these tools allowed us to observe the inherent heterogeneity within stem cell populations and make precise measurements on individual phase lengths. Furthermore, due to the crowded and compact nature of hESC colonies, performing live cell imaging in the past was a particular obstacle due to inaccurate tracking of individual cells. We circumvented this problem by developing custom neural networks to accurately segment our cells (See methods 2.9.1, Appendix B, and also published in (Padgett J et al., 2021)), which vastly improved tracking accuracy when combined with commercially available tracking algorithms. Although we could generate numerous long-term live cell imaging tracks following hESCs through several divisions using our custom image analysis pipeline, these tracks still needed to be individual (manually) inspected and curate, as tracking is still partially incorrect. There has been a surge in machine learning based tracking algorithms in recent years, and utilising our cell segmentation pipeline in parallel with these new tracking tools which have been specifically trained on our datasets could increase tracking accuracy further. This would improve the automation for our analysis, which would result in increased sample sizes and more efficient data processing. Nevertheless, the development of tools presented in this thesis provided exciting opportunities to investigate cell cycle dynamics using modern, single cell approaches.

Using BMP4 as a model for hESC differentiation, we found that upon induction of differentiation G1 length increased not only in duration but variability (Figure 3-5). This was consistent with all the cell cycle biosensors that we tested (FUCCI(CA), PCNA and Cdt1) and with another independent hESC stem cell line. In comparison, S phase remained constant, and we observed a mild yet significant decrease in G2 during differentiation. This indicates that G1

is the phase that is remodelled the most extensively during hESC differentiation (Figure 3-5). These results are the first precise measurements of individual phase lengths at the single cell level during the embryonic to somatic transition in hESCs. Although there was a slight discrepancy between individual phase lengths depending on the cell cycle biosensor, all measurements showed the same trend of G1 lengthening during differentiation. The discrepancy is probably attributed to the way these biosensors measure cell cycle lengths, with the FUCCI(CA) system based on the activity of ubiquitin ligases and PCNA on the localisation onto active replication complexes. Moreover, contrary to the FUCCI(CA) and Cdt1 sensors, which are truncated proteins, overexpression of (full length) PCNA could induce some degree of cell cycle changes. This sensor has been widely used for quantification of cell cycle dynamics (Chao et al., 2019; Moris et al., 2020; Zerjatke et al., 2017) and we purposely FACS sorted cells with moderate levels of PCNA expression, but ideally a PCNA knock-in would be the ideal tool to corroborate our findings. In addition to this, some phase transitions are easier to quantify than others. While the FUCCI(CA) biosensor has a sharp loss in Cdt1 signal during the G1/S transition, the S/G2 transition is based on the accumulation Cdt1 and Geminin, which is more difficult to detect. In contrast the S/G2 transition is easier to detect in the PCNA biosensor in comparison to the G1/S transition. Using other PCNA-like sensors such as DNA ligase and/or DNA Dnmt1 (Easwaran et al., 2005) as knock-ins would help make reliable measurements. The data presented in this thesis therefore underscores the importance of using multiple cell cycle biosensors to validate quantitative measurements in live cells.

While most of the variability in cell cycle dynamics in ES cells was contributed from G2 phase, in differentiated cells G1 length contributed the most to the overall variability in cell cycle duration (Figure 3-3, Figure 3-4). In somatic cells, G1 is thought to be the period of the cell cycle where the cell senses internal and external environmental queues, which results in vast heterogeneity in G1 duration. This is believed to be due to many aspects of G1 regulation including G1/S checkpoint activation. In contrast, G1 is short and constant in the embryonic state (Figure 3-2), which suggests that ES cells are less responsive to G1/S checkpoint activation in the presence of either endogenous or exogenous stress. Furthermore, we observed a slight decrease in G2 length during differentiation (Figure 3-2). A longer G2 length in the embryonic state may therefore serve to compensate for the lack checkpoint activation in G1, resulting in an increase in G2 length to repair potential DNA damage or activation of apoptotic pathways (J. C. Liu et al., 2013; Momcilovic et al., 2010).

With the observation that G1 changed the most during differentiation, we next wanted to address exactly when in time this occurred and what are the underlying molecular mechanism regulating it. For this we performed lineage analysis and found that at the population level it took at least 2 divisions after BMP4 induction for G1 to remodel (Figure 3-5) i.e. G1 durations in the first two divisions are not statistically significantly different. In addition to this, cells needed to be exposed to BMP4 signal for 48-72h hours before changes in G1 length were apparent (Figure 3-7). This change happened in a switch like fashion rather than a graded increase in G1 duration. Interestingly, at the single cell level we see that G1 increases with each new division cycle, suggesting that at the single cell level, G1 remodelling is in fact a graded process. Mechanistically, this suggests that potentially some rewiring of regulatory networks and activities that orchestrate G1/S transition happens as cells enter their 2/3rd division cycle. Studies in our lab have shown that commitment to differentiation happens surprisingly early after BMP4 treatment, demonstrated by the fact that just a 30-minute pulse treatment of BMP4 is sufficient to induce differentiation in hESCs (Gunne-Braden et al., 2020). Furthermore, while induction of a specific set of early commitment genes such as GATA3 are detected after just 2 hours, loss of pluripotency factors and upregulation of lineage specific markers are observed after 48-72h. This would argue that changes in cell identity are concomitant with remodelling of the cell cycle and that the initial induction by BMP4 may directly regulate cell cycle targets. In support of this, both p21 and Cdh1 are upregulated after BMP4 stimulation (Gunne-Braden et al., 2020). Furthermore, GATA3 Chip-Seq data from our lab shows peaks near a few cell cycle genes such as Cyclin D3 and CDK6 (Sullivan et al, unpublished), suggesting direct regulation on cell cycle genes from BMP4 signalling. In addition to this, loss of pluripotency itself may regulate the cell cycle, as both Oct4 and Sox2 directly regulate the miR-302, which negatively regulates Cyclin D expression (Greer Card et al., 2008), suggesting that the two processes are intrinsically connected. Finally, As BMP4 is a relatively quick differentiation protocol, it will be interesting to see if this trend is conserved with longer cell fate inductions, or whether different fates show distinct cell cycle trajectories.

Another advantage of single cell analysis is the ability to compare the dynamics of cell cycle phases between cells across the same lineage. We observed that the correlation of cell cycle duration and individual phases between daughter cells increased during differentiation (Figure 3-9). In particular, G1 phase correlations increased drastically during differentiation (Figure

3-9). This raises the exciting possibility that the presence of inherited factors plays a more prevalent role in determining G1 length in the differentiated state. Numerous studies have observed a strong correlation between daughter cell G1 lengths and inherited factors, such as p21 and Cyclin D1 in somatic cells (Barr et al., 2017; Nathans et al., 2021; Waisman et al., 2019a; Yang et al., 2017). Of note, endogenous DNA damage accumulated in mother G2 phases causes an increase in p21 levels which can be inherited by daughter cells resulting in cell cycle arrest in G1 phases (Barr et al., 2017). The lack of G1 variability in hESCs suggests that this mechanism may not be used in the pluripotent state, which further highlights that hESC are unable to activate a classic G1/S checkpoint. Precise, single cell measurements of endogenous p21 would allow us to observe when p21 would become inherited in hESCs, and potentially probe for the mechanistic details for the absence of G1 variability.

5.2 Activities of CDKs are at the heart of cell cycle remodelling

Using previously obtained RNAseq and phosphoproteomics data in the lab, we found that very few cell cycle regulators changed at the gene expression level (Gharibi et al unpublished) (Figure 3-10). For example, only three G1/S regulators displayed differential regulation between the embryonic and somatic state, which included the CDK2 activity regulators, p21, Cyclin A1 and Cyclin E2. In line with this, phosphoproteomic data revealed that CDK2 activity decreased during differentiation (Gharibi et al unpublished) (Figure 3-15). Given the drastic changes in cell cycle dynamics during differentiation, it was surprising that there were limited hits from the RNAseq data. We therefore hypothesized that G1 dynamics was being primarily regulated at the protein level. A single cell immunofluorescence screen for cell cycle regulators showed that this was indeed the case (Gharibi et al, unpublished), and, in particular, we observed huge differences in the protein levels of key G1/S regulators (Figure 3-13, Figure 3-14). A particularly intriguing result was that of the E3 ubiquitin ligase components Cdh1 and Skp2. Skp2 was at elevated levels throughout the cell cycle, while Cdh1 was at low levels throughout the cell cycle of pluripotent hESCs (Figure 3-14). Upon differentiation, Cdh1 became up regulated and Skp2 expression decreased (Figure 3-14). The expression of both became cell cycle dependent (Figure 3-14). As these proteins are involved in the degradation of a diverse range of cell cycle regulators, this would explain the differences between the hits obtained from the RNAseq data and immunofluorescent screen. An interesting hypothesis that arose from these observations was that the rewiring of cell cycle networks during cellular differentiation could be achieved by modulating the cells degradation machinery. As the cell cycle is constantly changing depending on multiple intrinsic and extrinsic factors, altering a limited set of (degradation) regulators would have a profound impact on a variety of other cell cycle regulators. Together these data also highlight the importance of studying cell fate transitions and both the transcript and protein level.

In terms of how Cdh1 is regulated itself, direct phosphorylation of Cdh1 by both CDK1 and CDK2 inhibits the formation of active APC/C^{Cdh1} complexes (Zachariae et al., 1998). Phosphorylated Cdh1 then gets translocated out of the nucleus where it undergoes proteasomal degradation (Benmaamar & Pagano, 2005). The hyperactive CDK2 activity dynamics displayed in hESCs may therefore directly regulate Cdh1. As low APC/C^{Cdh1} activity then results in high SCF^{Skp2} activity, these two ubiquitin ligases could therefore work in conjunction

with each other to bring about atypical degradation dynamics in hESCs. This is supported by the fact that in our Cdh1 overexpression experiments, we noted a reduction in Skp2 levels (Figure 4-9). Interestingly, Cdh1 overexpression is required for neuronal differentiation (Almeida, 2012). In this context, high APC/C^{Cdh1} activity functions to degrade Cyclin B1 to keep neurons in the post-mitotic state. As such, knocking down Cdh1 triggers apoptosis in neurons (Almeida et al., 2005), illustrating how high APC/C^{Cdh1} activity is an integral part of neuronal function. It is therefore fascinating that in other systems, changing the activity of ubiquitin ligases plays an important role in determining cell cycle structure and identity.

To probe for the molecular mechanism for G1 changes during differentiation, we decided to investigate how the activity of the master regulator of the G1/S transition, CDK2, changed during this transition. As mentioned above, at the molecular level, a large number of cell cycle regulators that displayed differential RNA or protein abundance were regulators of CDK2 activity. To study CDK2 activity in live, single cells, we made use of a powerful established CDK2 activity sensor in hESCs to look at CDK2 dynamics (Spencer et al., 2013). Strikingly, this revealed that hESCs display a new level of CDK2 activity state behaviour that we termed ‘CDK High’, where the activity increased immediately after mitosis, rather than dropping to either an intermediate or low level, as seen in somatic cells (Figure 3-18, Figure 3-19). This is consistent with previous findings that (mouse) ES cells have high CDK2 activity (Stead et al., 2002), although our results show the dynamics of CDK2 activity in unperturbed, single cells for the first time. There are some important differences between our results and previous bulk CDK2 activity measurements. Firstly, although CDK2 activity is predominantly high in hESCs, we do see oscillatory behaviour in CDK2 activity. These oscillations in activity however rarely fall below 1.0 and the maximum amplitude of CDK2 activity is much higher immediately after mitosis in comparison to differentiated cells. In addition to this, there is very little heterogeneity in CDK2 activity within the whole population of hESCs, with >91% of cells displaying high CDK2 activity state (Figure 3-19). In somatic cells, it has been shown the CDK2 activity ‘bifurcates’ immediately after mitosis, which regulates whether cells proliferate or enter quiescence (Spencer et al., 2013). The absence of this bifurcation in hESCs could therefore explain the lack of heterogeneity in G1 lengths observed in ES cells and is likely a consequence of how quickly the cell cycle runs through G1 (due to hyperactivation of CDK2).

During differentiation, the behaviour of CDK2 activity changes dramatically (Figure 3-19). After 1 division, approximately half of the population shifts to a CDK2 intermediate state, before changes in cell cycle dynamics are evident. After 2 divisions, most of the cells had transitioned out of the CDK2 high state, and cells are now able to display CDK2 low behaviour. This suggests that changes in CDK2 activity happens with each division and that it takes 2 divisions before cells can enter a quiescent state. This is indeed supported by the fact that both a hypophosphorylated population of Rb and high p21 levels are apparent 3 days after differentiation (Figure 3-12). While the initial decrease in CDK2 activity happens surprisingly early, it is not enough to bring about changes in cell cycle dynamics. In fact, very few CDK2 activity regulators change at the protein level early on in differentiation (Figure 3-13, Figure 3-14). This may stem from the fact that the changes in CDK2 activity early on in differentiation are subtle and only measurable at the single cell level. Using immunofluorescence to probe for small changes in CDK2 activity dynamics may therefore be difficult, as our single cell dynamic measurements show that CDK2 activity only transiently drops to an intermediate state early on in differentiation.

Our results suggested that G1 length and its variability could be direct causes of CDK2 activity. We combined the CDK2 sensor with a G1 sensor and observed that cells with CDK2 low dynamics had a much longer G1 in comparison to the CDK2 High and Int states (Figure 3-20). Furthermore, there was a strong positive correlation between time spent where CDK2 activity was below 1.00 and G1 length. One of the biggest limitations in studying CDK2 activity function was the lack of tools available to specifically inhibit this kinase. Pharmacological inhibitors of CDK2 tend not to show specificity and inhibit other CDKs. We therefore set out to develop an analogue sensitive CDK2 mutant in hESCs which could be specifically inhibited using bulky ATP analogues such as 3-MB-PP1 and thereby specifically inhibited. Co-expression of the CDK2-AS mutant with the CDK2 activity sensor showed that we were able to nicely modulate CDK2 activity with increasing levels of 3-MB-PP1 (Figure 3-22). Although we were unable to generate a hESCs line where endogenous CDK2 was knocked out, due to the higher expression levels of the CDK2 AS mutant in comparison to WT CDK2 (Figure 3-21), this mutant behaved as a dominant negative, potentially by sequestering cyclin partners. Since the CDK2-AS mutant was used in combination with the CDK2 activity sensor and the latter responds to the activity of both endogenous and exogenous CDK2 activity, it still allowed us to study the function of high CDK2 in hESCs. We found that the correlation between G1

length and CDK2 activity was causative. While the development of this tool was predominantly used to investigate the role of CDK2 activity on G1 length, the application of the CDK2-AS line can be extended further to identify global CDK2 activity substrates. This would facilitate in the detection of additional roles of CDK2 in hESCs outside the context of G1/S regulation. Finally, although the generation of the CDK2-AS hESC line is a powerful method for studying the function of CDK2 activity, it will be important work in the future to optimise the CDK2-AS tool and to replace endogenous CDK2 to support these conclusions. This will require initial optimisation and testing of different gRNAs to show that they cut efficiently as well as screening for more clones. Transfecting two gRNAs instead of one with RNPs instead of expressing the gRNAs from the same plasmid where we express CDK2-AS might prove to be a more efficient strategy to achieve a knockout.

CDK2 is known to be regulated by a variety of factors including other CDKs, small inhibitors (e.g. p21), phosphatases and ubiquitin ligases. Of note, only some of these regulators were found to impact on the CDK2 high state. We demonstrated that Cyclin A2 was important to sustain the high amplitude of CDK2 activity while other regulators such as Cyclin E1, Skp2 and Cdh1 were key regulators of the intermediate state (Figure 3-24, Figure 3-27, Figure 3-29). In addition to this, we could introduce the low CDK2 activity state by overexpressing the CDK inhibitor protein p21. This result was consistent with what is known to happen in somatic cells, where p21 is known to have a key role in regulating CDK2 activity (Barr et al., 2017; Spencer et al., 2013). However, our data still illustrates how low p21 levels in hESCs plays an important role in regulating high CDK2 activity.

Interestingly, our data suggests that in hESC, regulation of CDK2 activity is likely to require the synergy of different regulators. When we combined perturbations of two regulators, we saw that we could recapitulate the same levels of CDK2 expression that we see in somatic, differentiated cells. For example, combining perturbations of the dual ubiquitin ligase by overexpressing Cdh1 and knocking down Skp2, we were able to decrease CDK2 activity to what is seen in somatic cells (Figure 3-27). We were also able to introduce somatic levels of CDK2 activity by knocking down both D type cyclins and Skp2 (Figure 4-7). Overexpressing Cdh1 may function to increase degradation of mitotic cyclins and the window before APC/C inactivation and hence G1/S progression, while removal of Skp2 would allow the accumulation of CDK inhibitor proteins to inhibit CDK2 and enter the low state. Knocking down D type

cyclins could also function to increase the window before APC/C inactivation. Together this shows that by targeting CDK2 activity from multiple angles, we could recapitulate somatic levels of CDK2 activity. As high CDK2 activity is a fundamental feature of the fast hESC cycle, having multiple regulators to maintain this high CDK2 activity would therefore be advantageous to safeguard high proliferative rates.

Although we did not observe any differences in CDK2 activity when the CDK2 activating phosphatase Cdc25A was perturbed (Figure 3-28) the ratio between kinases and phosphatases plays a pivotal role in determining phosphorylation levels in cells. Studies in mESCs have demonstrated that in response to DNA damage, the CDK2 activating phosphatase Cdc25A is degraded, however this has no influence on CDK2 activity and G1/S progression (Koledova et al., 2010). The authors suggest that CDK2 activity is sheltered from the regulation of Cdc25A due to CDK2 subcellular localisation, with many CDK2 molecules being localised in the cytoplasm and centrosomes whereas Cdc25A is nuclear. Our results are consistent with this phenotype, however it is yet to be determined exactly why CDK2 is irresponsive to Cdc25A depletion. Although the localisation of specific CDK2 activity regulators could play a role, due to the high expression/activity of positive regulators of CDK2 activity present in hESCs, removal of Cdc25A may not be sufficient to override this. Alternatively, additional phosphatases could compensate for the loss of Cdc25A to maintain high CDK2 activity.

The binding of specific partner cyclins to CDKs plays an important role in coordinating individual cell cycle phases. It has been well established that CDK4/6 binds to Cyclin Ds, CDK2 can partner with both E and A type cyclins, while CDK1 can partner with A and B type cyclins. It is believed that, at least in mammalian cells, these different CDK-Cyclin complexes phosphorylate a specific, likely non-redundant, set of substrates which are vital for regulating phase specific events. This might not be a universal principle, though. In fission yeast, for example, a single CDK1 is sufficient to drive the cell cycle (Coudreuse & Nurse, 2010). Furthermore, CDK1 is the only essential CDK in the mammalian cell cycle (Santamaría et al., 2007) and numerous CDK knockout studies have demonstrated that there is indeed redundancy within the mammalian cell cycle (Brandeis et al., 1998; Martín et al., 2005; Winston et al., 2000). We find that a high degree of redundancy might also happens in hESC (Figure 5-1). An immunoprecipitation (IP) of Cyclin B1 followed by mass spectroscopy identified, amongst other proteins, CDK2 as a binding partner in hESCs (Figure 5-1) (Cannucciari et al

unpublished). Importantly this interaction was also confirmed by co-immunoprecipitation (Figure 5-1) (Cannucciari et al unpublished). This raises the interesting possibility that high CDK2 activity in hESC could be brought about by the ability of this kinase to bind and be activated by several cyclin partners. Given that Cyclin B1 rapidly re-accumulates in early G1 in hESCs due to the absence of Cdh1, CDK2-Cyclin B1 complexes could contribute to high CDK2 activity described in this thesis. Understanding how CDK2 activity might change after Cyclin B1 perturbations will be an interesting avenue to follow in future research. While we did not see binding of Cyclin D proteins to CDK2, neither by mass spectrometry nor by co-immunoprecipitation experiments (data not shown), our data is insufficient to rule out that CDK2 does not partner with Cyclin D.

Our results are in line with *in vitro* studies which have shown that the mitotic Cyclin B1 can form stable complexes with CDK2 and generate an active kinase (Brown et al., 2007). Recently, it has been shown that in perturbed conditions (in the absence of CDK1), CDK2 can form complexes with Cyclin B1 to assume the role of CDK1-Cyclin B1 which facilitates mitotic entry in cancer lines (Lau et al., 2021). Our data shows for the first time that in a physiological context CDK2 can interact with Cyclin B1, further highlighting differential regulatory mechanisms that are used by hESCs. Further experiments are needed to investigate if functional CDK2-Cyclin B1 complexes contribute to CDK2 kinase function, and to characterise the roles of different CDK-Cyclins regulating the hESC cell division cycle.

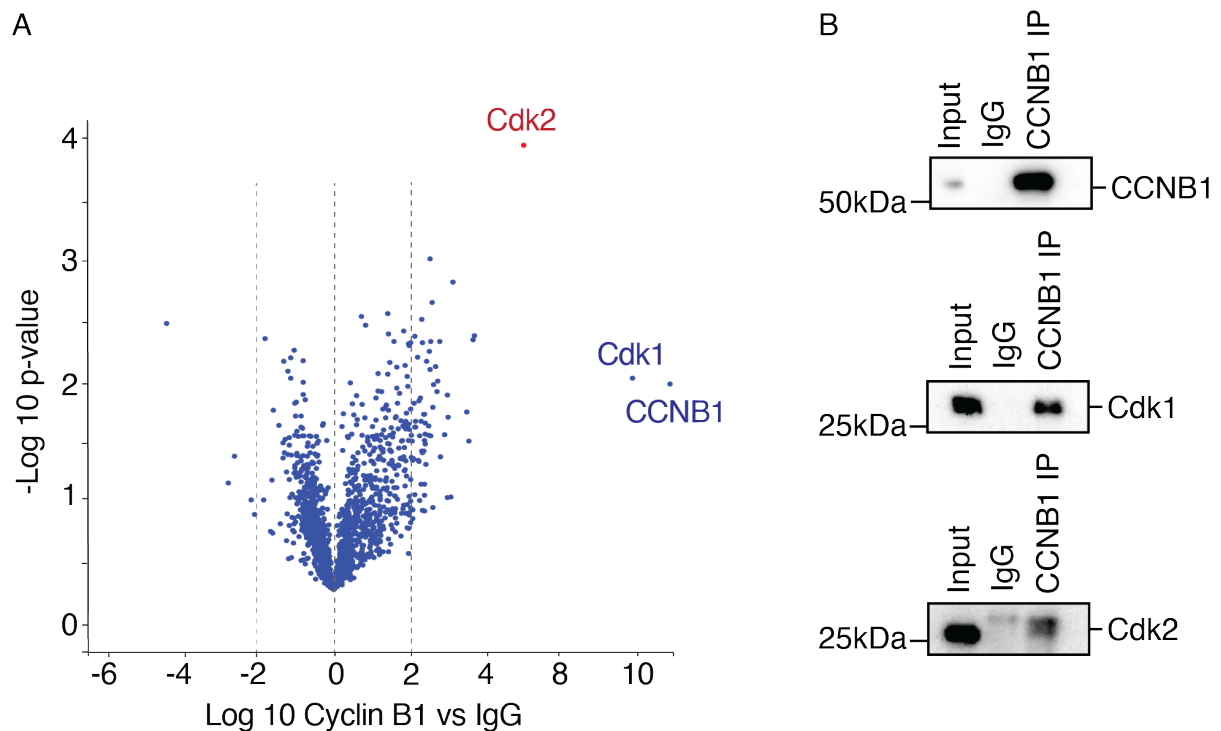


Figure 5-1- CDK2 forms complexes with Cyclin B1 A. Mass spectrometry data of Cyclin B1 immunoprecipitation from hESC and BMP4-treated cell lysates showing Label free quantification (LFQ) intensity normalised to Immunoglobulin G (IgG) control for Cyclin B1. B. Western blot of Cyclin B1 immunoprecipitation from hESC cell lysates stained with Cyclin B1, CDK1 and CDK2. IgG was used as a control for unspecific binding.

Finally, constitutively high CDK2 activity raises multiple questions regarding cell cycle progression in hESCs. For example, how is the quantitative model for cell cycle ordering still compatible with the embryonic state? i.e. if there are no oscillation in CDK activity then how are different thresholds met for proper ordering of the cell cycle? Work in somatic cells has shown that a precise CDK activity threshold determines the passage through the restriction point (Schwarz et al., 2018). Whether this threshold is prevalent in the embryonic state is yet to be determined, and what the adaptations are that ought to be made in hESCs for these thresholds to happen despite the high CDK activity is unknown. In addition to this, hyperactive CDK2 activity in somatic cells is associated with high replication stress and genomic instability (Spruck et al., 1999). As such, CDK2 is often overactivated in cancer (Tadesse et al., 2020). Down regulation of CDK2 during the cell cycle is therefore vital in the maintenance of proper karyotype, and how ES cells adapt to these conditions remains an exciting and open question for future investigation.

5.3 CDK activity is important in maintaining stem cell identity

Pluripotency is generally defined as the ability to self-renew and to differentiate and give rise to the three germ layers (endoderm, mesoderm and ectoderm) (Yilmaz & Benvenisty, 2019). Hallmarks of hESC identity include expression of pluripotency markers, an open epigenetic landscape, self-renewal, lack of classical checkpoint control and the ability to differentiate into any cell in the embryo. As we found that high CDK2 activity was a fundamental feature in hESCs, we wanted to investigate what would happen to stem cell identity if by modulating CDK2 activity we would change the pluripotent cell cycle. To probe for this, we made use of the CDK2-AS line to specifically inhibit CDK2 and probe for how this affected stem cell function. We decided to focus on two essential hESC characteristics, pluripotency and absence of G1/S checkpoint control. In doing this we could address the functional importance of having high CDK2 activity in hESCs.

hESC accumulate high levels of DNA damage in comparison to differentiated cells. Is this a consequence of high levels of CDK2 activity? We hypothesised that since CDK2 high activity gave rise to the short, less variable duration of G1, it could also contribute to the lack of a fully functional G1/S checkpoint. This would result in these increased levels of DNA damage. In agreement with this, treatment with the radiomimetic agent NCS had no influence on G1 length in hESC, while in somatic cells this resulted in an increase in G1 length (Figure 4-2). Furthermore, somatic cells displayed higher levels of the G1/S checkpoint markers p21 and hypophosphorylated Rb (Figure 3-12, Figure 4-1). Although the absence of a G1/S checkpoint may not be favourable in terms of genomic stability, we see that hESCs are more sensitive to DNA damage (Figure 4-3). Elimination of damaged cells rather than trying to repair DNA damage may therefore be the mechanism used by hESCs to maintain genomic stability. In the early days of development, it is crucial to divide fast in order to increase cell numbers to start the process of cellular differentiation (specification). Consequently, during these early divisions, cells seem to tolerate high levels of damage. Later, when the cell cycle remodels during gastrulation, cells acquire classical G1/S checkpoint control and DNA repair takes over apoptosis to deal with “faulty” cells. This early strategy might limit the propagation of DNA mutations acquired at the early stages of development to the whole organism and prevent potential disease.

Modulating CDK2 activity to somatic levels was sufficient to re-introduces the G1/S checkpoint in response to NCS (Figure 4-4). This was demonstrated by the increased proportion of CDK2 low state cells, higher p21 levels, increased G1 duration and hypophosphorylated Rb in response to NCS (Figure 4-4). This shows that hyperactive CDK2 activity dynamics play a vital role in bypassing the G1/S checkpoint. In somatic cells, it was shown that the G1/S checkpoint is surprisingly inefficient due to persistent CDK2 activity (Nathans et al., 2021). A 2-hour window between DNA damage and induction of p21 is required for sufficient levels of p21 to accumulate and inhibit CDK2 (Nathans et al., 2021). The absence of a G1/S checkpoint could therefore be explained two-fold. Firstly, as Skp2 levels are elevated throughout the cell cycle, hESCs might fail to accumulate threshold levels of p21 to adequately inhibit CDK2. In support of this, we demonstrated that in response to NCS, hESCs have much lower levels of p21 in comparison to differentiated cells and CDK2 activity is decoupled from p21 expression. In other words, CDK2 activity remains high despite the increase in p21 expression (Figure 4-1). Secondly, as G1 phase is extremely short in hESC, the window before G1/S progression is too short that even if p21 levels were allowed to accumulate, the molecular events that underpin irreversible G1/S progression are already in full motion. For example, hyperphosphorylation of Rb due to high CDK2 (and likely CDK4/6) activity is likely to contribute to a permissive S-phase entry due to release of E2F inhibition. To test this, we increased G1 duration by removing D-type cyclins (which resulted in decreasing CDK4/6 activity) and increased p21 levels by down regulating Skp2. This dual perturbation was sufficient to reintroduce the G1/S checkpoint and supported the hypothesis that the absence of checkpoint control is due to low CDK activities and low p21 expression, both causing a short G1-phase (Figure 4-7).

It would be interesting in the future to see how re-establishing a G1/S checkpoint influences early development. For example, if cells prioritise repairing DNA damage rather than favouring proliferation, what implications does this have in the developing embryo? One could imagine that if proliferation in the early embryo is compromised in any way, this may affect early development, implantation of the blastocyst and the proportions of cells formed during gastrulation. Alternatively, having an additional layer of checkpoint control could favour embryo survival, as there would be fewer instances of DNA mutations and the genomic integrity of individual cells could be greater. It would be interesting to understand whether congenital disorders could be avoided by having tighter cell cycle control during early

development. All these questions are fascinating avenues for future research and could assist in increasing the *in-vitro* fertilisation rates and prevent congenital disorders.

What impact does lower CDK2 activity have on cell identity? We demonstrated that high CDK2 activity was important in maintaining the pluripotent state (Figure 4-10). Inhibition of CDK2 to somatic levels resulted in rapid loss of the pluripotency factor Nanog in a dose-dependent manner. While the molecular details regarding the loss of Nanog are still being investigated, it has been shown that there are 11-SP phosphorylation sites on human Nanog (Brumbaugh et al., 2014). Furthermore, phosphorylation of Nanog has been shown to be important in maintaining the protein stability of Nanog (Brumbaugh et al., 2014; Moretto-Zita et al., 2010). Some of these sites have been predicted to be CDK1 sites (Phosphosite), raising the possibility that they might also be *in vivo* CDK2 sites. If CDK2 targets any of these sites, it is possible that the stability of Nanog could be regulated through direct phosphorylation by CDK2. The use of the CDK-AS line developed in this thesis to detect global CDK2 substrates would be a powerful approach to identify if Nanog is a direct target of CDK2. Alternatively, CDK2 could regulate additional proteins that are involved in the regulation of Nanog. For example, it has been shown *in vitro* that CDK1 and Erk can target these phosphosites on Nanog (Brumbaugh et al., 2014). When we inhibited CDK2, we also found that this influenced the activity of CDK1 and CDK4/6 as read by the endogenous phosphorylation of Cyclin B1 and the CDK4/6 phosphospecific site on Rb S608 and T373 (Figure 5-2). Furthermore, perturbation of the CDK1 and CDK2 regulators including Cyclin B1, Wee1 and Cyclin A2 all resulted in decreased levels of Nanog. As Nanog is an essential transcription factor integral to the maintenance of pluripotency, the regulation of its stability is likely to be mediated through multiple mechanisms.

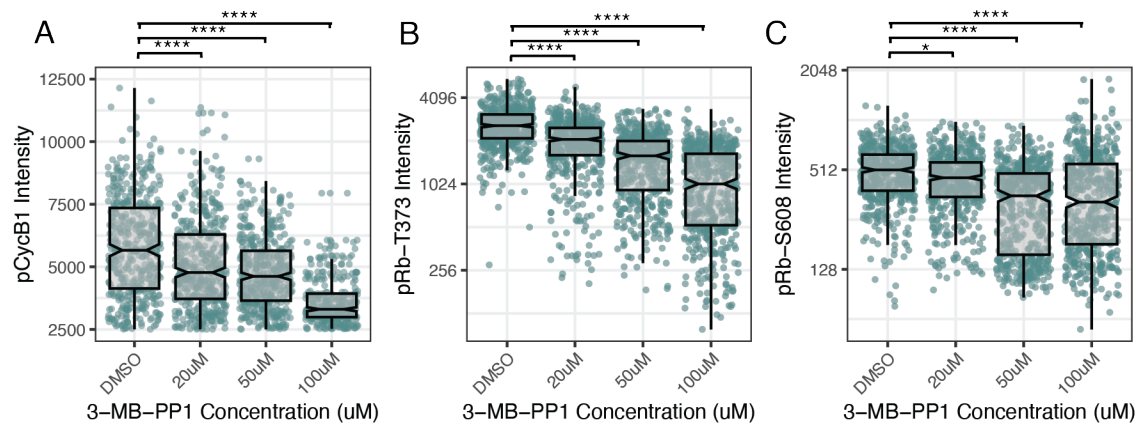


Figure 5-2- Inhibiting CDK2 influences the activity of other CDKs A-C. Single cell (nuclei) quantification of immunostaining for hESC treated with different dose of 3-MB-PP1 for A. pCycB1(S126) staining B. pRb -T373 staining C. pRb-S608 staining. $n=4000$ for each staining. Boxplots are overlaid to indicate minimum, maximum, first quartile, third quartile and median values.

Interestingly, we find that loss of Nanog and cell identity by modulating cell cycle dynamics is reversible for about 48h (Figure 4-12). There is a window of temporary loss in pluripotency of approximately 2 days, in which, after removal of CDK2 inhibitor, cells can return to expressing normal Nanog levels. This is consistent with CDK2 potentially affecting Nanog stability. However, treatment with CDK2 inhibitor for >3 days resulted in an irreversible loss of Nanog. Limited studies have investigated the effects of transient Nanog downregulation, however transcriptional changes upon Nanog removal within the first 24 hours are limited in murine embryonic stem cells (MacArthur et al., 2012). Using a doxycycline inducible shRNA system, the authors showed that while they could efficiently downregulate Nanog after 24 hours, Oct4 levels decreased only 3 days after downregulation. This indicates that the pluripotency network is stable upon initially downregulation of Nanog, however sustained Nanog downregulations has a large impact on stem cell identity. This could potentially explain why inhibition of CDK2 for 2 days resulted in a reversible return to normal Nanog levels, as immediate loss of Nanog seems to have no impact on the pluripotent state (MacArthur et al., 2012). Prolonged inhibition of CDK2 is therefore required in order for cells to irreversibly exit the pluripotent state.

Interestingly, Nanog overexpression is not required to reprogram specialised cells into iPSCs, however the induction of Nanog during this process is required to establish pluripotency. The

additional pluripotency factors Oct4 and Sox2 transcriptionally control Nanog expression which leads to the stabilisation of the pluripotency gene regulatory network (Rodda et al., 2005). Nanog therefore functions lower down in this regulatory hierarchy, which could explain why the initial loss of Nanog has no impact on the immediate pluripotency state. Finally, the removal of Nanog probably functions more as a non-inductive differentiation signal, as we are just removing one factor that impacts pluripotency. In contrast, BMP4 acts as an inductive differentiation signal, activating a plethora of cell fate genes as early as 24 hours after stimulation (Gunne-Braden et al., 2020, Sullivan et al. Unpublished). As removal of Nanog is not directly activating any lineage specific genes, this would also explain why the response to Nanog removal is slower in comparison to inductive differentiation signals.

We found that long term CDK2 inhibition resulted in differentiation into neuroectoderm (NE) lineages (Figure 4-13). This is consistent with the fact that the loss of Nanog has been shown to result in NE differentiation (Z. Wang et al., 2012). It is also possible that CDK2 activity could directly repress NE differentiation. As recent studies have shown that CDK1 regulates multiple epigenetic regulators (Michowski et al., 2020), it is feasible that CDK2 could have broader substrates that are involved in the regulation of both pluripotency and lineage specification. Most NE differentiation protocols involve dual SMAD inhibition, which represses both BMP and TGF β signalling pathways (Chambers et al., 2009). This results in the upregulation of NE markers such as Pax6 and removal of Oct4 typically 6-10 days after induction of differentiation. These lengthy differentiation protocols are often a rate limiting step for researchers and there has been a demand for developing accelerated methods for generating NE cells. Strikingly, we observe homogenous cultures of NE cells after just 5 days of CDK2 inhibition. When we compared the gene expression signatures for 43 lineage specification genes between CDK2 inhibited cells and a 7-day directed NE differentiation protocol, we observed a large degree of similarity (Figure 4-13). This suggests that changing cell cycle dynamics promotes ectoderm differentiation. It would be interesting to see if inhibition of CDK2 results in decreased levels SMAD signalling, or if this phenotype is being achieved through an alternative mechanism. Either way, CDK2 inhibition could be a useful tool in directing or improving the efficiency of NE differentiation.

The work presented in this thesis used hESC as a model system to understand remodelling of the cell cycle during the embryonic to somatic transition. While hESCs have been an in

invaluable tool to study the regulation of early human development, there is still debate on how well hESC recapitulate the physiological and environmental conditions *in vivo*. We used 3D organoid models of human development (gastruloids) as a proxy for human gastrulation. We found that CDK2 activity also decreases during gastruloid development (Figure 4-14). Although we only used fixed time points for gastruloid development, these data demonstrates that CDK2 activity changes are still observed in a more physiologically relevant 3D environment. Furthermore, from these images, it appears as not all cells within the gastruloids have the same levels of CDK2 activity. This raises the interesting possibility that potentially different embryonic cell types have different cell cycle dynamics. If true, this will have profound implications in developing better protocols for directed differentiation and reprogramming.

Chapter 6. Final Conclusions

The work presented in this thesis demonstrates the power of using single cell approaches to investigate cell cycle control principles. We set up to quantitatively measure changes in dynamics of cell division during the embryonic to somatic transition, in the process of cellular differentiation. We used 2D cultures of unperturbed, asynchronously dividing hESC. Cellular differentiation was triggered by BMP4, an important developmental cue and a trigger of early gastrulation. We combined live, single cell imaging of hESC expressing cell cycle biosensors, with perturbation approaches and state-of-the art image analysis to understand how the cell cycle remodels during hESC differentiation and its impact on cell identity.

We see that during BMP4-driven differentiation the cell cycle remodels primarily by lengthening G1-phase. At the single cell level, we see that this is a graded process in which G1 duration increases with each consecutive division.

We find that at the heart of G1 control is the cyclin dependent kinase CDK2. We show that in hESC, CDK2 exhibits three-tier activation: high, intermediate and low. We find that the high CDK2 activity dynamics is an essential feature of hESCs and that it drives the short G1-phase durations, characteristic of pluripotent cells. As well as regulating the short division cycles of hESCs, high CDK2 activity was shown to be essential for bypassing the G1/S checkpoint. As the absence of checkpoint control is a hallmark of cancer, modulating CDK2 activity may be a mechanism by which cancer cells increase genomic instability and malignant transformation. Specific inhibition of CDK2 could therefore prove to be an effective therapeutic option for the treatment of cancer.

Finally, we observe that high CDK2 activity is important in the maintenance of hESC identity. This study sheds light onto the molecular ties between cell cycle and pluripotency networks, which illustrates how this fast cell cycle sustains the unique characteristics of embryonic stem cells. Nanog has additional roles outside the context of pluripotency, for example during epithelial to mesenchymal transition, metastasis, and apoptosis (Gawlik-Rzemieniewska & Bednarek, 2016). Exploring how CDK2 activity influences these additional cell fate decisions would therefore be interesting avenues for future research.

Our work highlights the advantage of using hESC as a model system to understand how cell cycle networks rewire during changes in cell identity. It is fascinating that one genome can produce a diverse array of cell cycle phenotypes. Our work shows that modifications in cell cycle structure can be achieved just by modulating the activities of key cell cycle regulators, which can be mediated through modifications in degradation machinery. We envision that the principles learnt from this work could be applied to other contexts where the cell cycle remodels, for example during malignancy, terminal differentiation, or when viral infections hijack cell cycle machinery. In this context, modulating protein degradation could be a simple, regulatory motif underlying these important cellular transitions.

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Appendix A

From clocks to dominoes: lessons on cell cycle remodelling from embryonic stem cells

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REVIEW ARTICLE

From clocks to dominoes: lessons on cell cycle remodelling from embryonic stem cells

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Cell division is a fundamental cellular process and the evolutionarily conserved networks that control cell division cycles adapt during development, tissue regeneration, cell de-differentiation and reprogramming, and a variety of pathological conditions. Embryonic development is a prime example of such versatility: fast, clock-like divisions hallmarking embryonic cells at early developmental stages become slower and controlled during cellular differentiation and lineage specification. In this review, we compare and contrast the unique cell cycle of mouse and human embryonic stem cells with that of early embryonic cells and of differentiated cells. We propose that embryonic stem cells provide an extraordinarily useful model system to understand cell cycle remodelling during embryonic-to-somatic transitions. We discuss how cell cycle networks help sustain embryonic stem cell pluripotency and self-renewal and how they safeguard cell identity and proper cell number in differentiated cells. Finally, we highlight the incredible diversity in cell cycle regulation within mammals and discuss the implications of studying cell cycle remodelling for understanding healthy and disease states.

Keywords: cell cycle remodelling; embryonic stem cells; embryonic-to-somatic transition

On clocks: the early embryonic cell cycle

Cell division is a fundamental cellular process, and the correct partitioning of chromosomes between two daughter cells requires tight regulation of the cell cycle machinery. In essence, cell division is dependent on the activation and de-activation of cyclin-dependent kinases (CDKs) and the oscillatory expression of cyclin proteins, which regulate CDK activities in all cells.

Biochemical oscillations of Cdk–cyclin activity are at the heart of early embryonic division cycles and

allow the embryonic cell cycle to function as an oscillator that toggles rapidly between DNA duplication and chromosome segregation.

Work in classical embryonic cell cycle models, such as the frog *Xenopus laevis* [1,2], zebrafish *Danio rerio* [3,4] and the fruit fly *Drosophila melanogaster* [5], has shown that early embryonic divisions are fast, due to the absence of gap phases, and often show a degree of synchrony. These synchronous cell division cycles, due to their regulation by the abovementioned biochemical

Abbreviations

APC/C, Anaphase-promoting complex; ATM, Ataxia telangiectasia-mutated; ATR, Ataxia telangiectasia and Rad3-related protein; CDK, Cyclin-dependent kinase; CdkI, Cdk inhibitor; CHK2, Checkpoint kinase 2; CIP/KIP, CDK-interacting protein/kinase inhibitory protein; EpiSCs, Mouse postimplantation epiblast cells; ES, Embryonic stem; G1, Gap phase 1; G2, Gap phase 2; H2AX, H2A histone family X; hES, Human embryonic stem; ICM, Inner cell mass; M, Mitosis; MAPK, Mitogen-activated protein kinase; MCM, Minichromosome maintenance; mES, Mouse embryonic stem; miRNAs, MicroRNAs; ORC, Origin recognition complex; PCNA, Proliferating cell nuclear antigen; Plk1, Polo-like kinase 1; pre-RC, Prereplication complex; RB, Retinoblastoma; SAC, Spindle assembly checkpoint; SCF, Skp2-Cullin-F-box; Skp2, S-phase kinase-associated protein 2; UNG, Uracil-DNA glycosylase; UVC, Ultraviolet radiation C.

oscillator, were termed ‘clock-like’ by Murray and Kirschner [1].

The cell cycle has evolved from this simple oscillatory system to include more complex regulatory features that are necessary to maintain genomic stability through generations in specialised, somatic cells.

Speed and regulation by the cyclin–CDK biochemical oscillator are features that seem to be conserved in mammals. Indeed, cell division in early-stage mammalian embryos closely resembles these classical models. Embryonic mammalian cells progress rapidly through cell division with truncated or absent gap (G1 and G2) phases [6,7] (Fig. 1). Mouse blastomeres are believed to undergo cell division every 4.4 to 10 h from embryonic days E4.5 to E7, expanding from 20 cells to 4000 [7,8]. Cell cycle duration in early human embryos, as measured by time between mitoses, is 11 h (between the first mitosis and the second mitosis) and 1 h (between the second mitosis and the third mitosis) [8].

Molecularly, knockout studies have demonstrated that while Cdk1 and cyclins A and B are essential for early mouse development, the majority of Cdk–cyclin proteins are fairly redundant [9–11]. The Cdk–cyclin oscillator is cell autonomous and therefore insensitive to serum deprivation; as such, it does not require mitogenic or developmental signals in order to progress through the G1 phase.

Furthermore, like frog or zebrafish embryos, early divisions in the pre-implantation mammalian embryo are independent of restriction point – the point at which cells become ‘committed’ to the cell cycle and no longer require extracellular proliferative cues [12] – and are typically not (or only very loosely) subjected to checkpoint control [13–17].

Key to this seems to be the fact that early embryonic divisions are independent of the retinoblastoma (RB) protein, which, in adult, somatic cells, plays a crucial role in controlling the G0/G1-phase and the G1/S-phase transitions in somatic cells. This gatekeeping role of RB results from negatively regulating the

activity of E2F transcription factors, which are master regulators of G1–S transcription and, thereby, of the transition from G1 into S phase [18].

While RB null mice die at day E15, this is at least in part due to placental insufficiency [19]. In fact, loss of RB has little effects on cell division and differentiation prior to implantation [20], supporting the idea that early embryonic divisions might be independent of RB.

Does this mean that early embryonic divisions are totally devoid of gatekeepers? The answer seems to be no: although an apoptotic response does not take place in early divisions in the mouse embryo and it has been reported that both the cell cycle arrest and the apoptotic functions of p53 are not active, irradiation of 1-cell mouse embryos in G2-phase delays mitotic entry, suggesting the existence of some sort of checkpoint control mechanisms, at least in G2 [21–23].

Lastly, early embryonic divisions can occur before activation of zygotic transcription, by relying on maternal mRNA [2,4]. Early embryonic cells (blastomeres) have a typical morphology with a high nuclear-to-cytoplasmic ratio and, for the most part, do not undergo any cytoplasmic growth. In these early cells, embryonic divisions seem to serve the purpose of increasing cell number in order to prepare for lineage specification. Each blastomere is totipotent and can, in principle, give rise to a new whole organism.

From clocks to dominoes: cell cycle adaptations of embryonic stem cells

The first specification of the totipotent embryo gives rise to two distinct cellular populations during early mammalian development: the trophoblast and the inner cell mass (ICM), which will in turn give rise to the extra-embryonic lineages and the embryo, respectively [24].

Embryonic stem (ES) cells originate from the ICM and have the following unique characteristics: they derive from the pre-implantation embryo; have

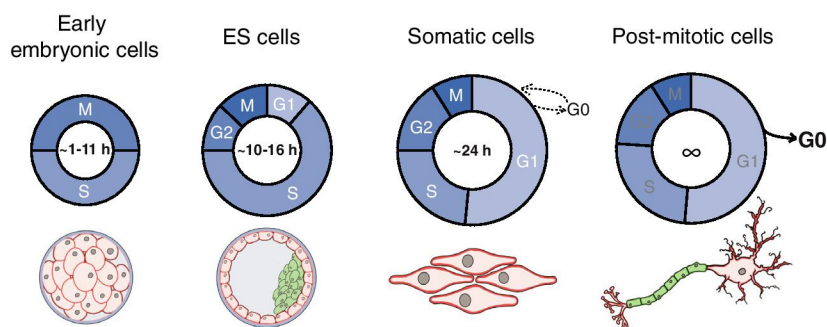


Fig. 1. Cell cycle remodelling during cellular differentiation. Schematic showing differences in cell cycle structure of early embryonic divisions, embryonic stem (ES), differentiated (somatic) and postmitotic cells. Average cell cycle duration is shown in hours. Cells are not to scale.

Table 1. Molecular differences between cell cycle regulators in somatic and embryonic stem cell division cycles. (M) and (H) indicate mouse and human embryonic stem cells, respectively

Cell cycle regulator	ES cells 	Somatic cells 	References
Cyclins			
Cyclin A	High and non-(M) or oscillatory (H) expression	Lower expression; cell cycle regulated	Savatier 1996, Stead 2002, Faast 2004, Fujii-Yamamoto 2005, Ghule 2007, Neganova 2009, Pauklin and Vallier 2013, Wang 2017
Cyclin B	High expression (M)(H)	Lower expression; cell cycle regulated	
Cyclin D	Low (M) or intermediate (H) expression	High expression; cell cycle regulated	
Cyclin E	High, non-(M) or oscillatory (H) expression	Lower expression; cell cycle regulated	
CDKs			
Cdk1	High expression, oscillatory activity (M)(H)	Cell cycle regulated activity	Stead 2002, Faast 2004, White 2005, Fujii-Yamamoto 2005, Ghule 2007, Neganova 2009, Bar-On 2010
Cdk2	High expression, constant (M) or oscillatory activity (H)	Cell cycle regulated activity	
Cdk4	Low (M) or intermediate (H) activity	Cell cycle regulated activity	
Cdk6	Low, oscillatory activity (M)	Cell cycle regulated activity	
CDK inhibitors			
p21/p27/57	Low or absent expression (M)(H)	Higher, cell cycle regulated expression	Stead 2002, Fujii-Yamamoto 2005, Egozi 2007, Neganova 2009, Li 2012,
p16/18/19	Low or absent expression (M)(H)	Higher, cell cycle regulated expression	
G1-phase			
RB	Hyperphosphorylated (M)(H), Hypophosphorylated (H)	Hypo/hyperphosphorylated	Savatier 1994, Stead 2002, Fujii-Yamamoto 2005, White 2005 Conkin and Sage 2009, Bar-On 2010, Ballabeni 2011, Yang 2012, Matson 2017
E2F	De-repressed form, cell cycle independent transcription (M)	Cell cycle dependent transcription	
APC/C Cdh1	Low activity (M)(H)	Cell cycle regulated activity	
p53	Does not induce G1-arrest in response to damage (M)(H)	p53-depedent arrest in response to damage	
Cdt1	High expression (M)(H)	Cell cycle regulated expression	
Emi1	High expression (M)(H)	Cell cycle regulated expression	
S-phase			
Cdc25A	High expression (H)	Cell cycle regulated expression	Fujii-Yamamoto H 2005, Zhang 2009, Ballabeni 2011, Matson 2017
MCM complex	Fast loading (M)(H)	Slower, cell cycle regulated loading	
Cdc6	High expression (M)(H)	Lower expression; cell cycle regulated	
Geminin	High expression (M)(H)	Lower expression; cell cycle regulated	
G2/M-phase			
Plk1	High activity (H)	Cell cycle regulated activity	Egozi 2007, Bar-on 2010, Ballabeni 2011, Yang 2011, Yang 2012, Zhang 2012, Gonzales 2015
Skp2	High levels throughout the cell cycle (H)	Lower expression, no longer present in G1	
ATM/ATR-CHEK2	Expressed and active G2-checkpoint (M)(H)	Expressed and active G2-checkpoint	
APC/C-Cdc20	Lower activity (M)	Higher activity, cell cycle regulated	

prolonged undifferentiated proliferation without undergoing senescence or quiescence; and have stable developmental potential to form derivatives of all three embryonic germ layers. Under specific conditions, much like early embryonic cells, these cells can remain proliferative, and maintain self-renewal capacity and pluripotency, thanks to a unique transcriptional profile [25–27]. The ability to grow them *in vitro* makes them an incredible trackable system to study cell division.

Pluripotency is sustained by rapid divisions cycles [28–34], with mouse ES (mES) cells having an average cell cycle duration of approximately 10 h and human ES (hES) cells of 15–16 h (Fig. 1). ES cells from other primates also divide on average every 15 h [35]. Shorter cell cycle durations in ES cells are coupled with a cell cycle structure that is not too dissimilar to that of early embryonic divisions: the pluripotent cell cycle is characterised by abbreviated G1 and G2 phases and on average over 50% of ES cells are found in S phase [35,36] (Fig. 1).

Embryonic stem cells display other characteristics of early embryonic divisions most notably the ability to self-renew without undergoing quiescence or senescence. ES cells are refractory to contact inhibition and are independent (or less dependent) of extracellular signals, such as mitogen signals, to divide. For example, inhibiting mitogen-activated protein kinase (MAPK) activation (a potent mitogenic cue) has no effect on embryonic cell cycle progression in ES cells [35,37].

Unique Cdk–cyclin activation patterns in embryonic stem cells

Four cell cycle regulatory mechanisms are at the heart of pluripotent cell division cycles (summarised in Table 1): (a) non-oscillatory activation dynamics of cyclin-dependent kinases, Cdks (Fig. 2), (b) overexpression of cyclin proteins (Fig. 2), (c) reduced activity of cell cycle inhibitors (Fig. 2) and (d) atypical (or impaired) checkpoint control (Figs 3 and 4).

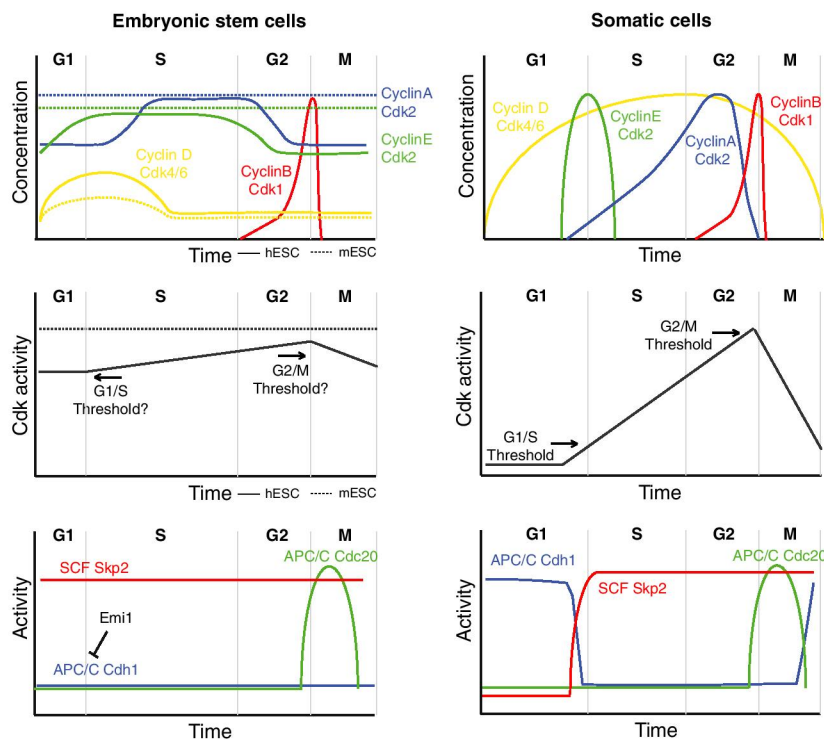


Fig. 2. Comparison of expression levels of cell cycle regulators in somatic and embryonic division cycles. Top: Expression levels of Cdk–cyclin complexes in embryonic (left) and somatic stem cells (right). Solid lines indicate human embryonic stem (hES) cells, and dashed lines indicate mouse embryonic stem (mES) cells. Middle: oscillations of CDK activity in embryonic (left) and somatic stem cells (right). Threshold levels for G1/S and G2/M transition are shown or only hypothesised (question marks) for somatic cells and for embryonic stem cells, respectively. Bottom: Expression levels of APC–Cdh1, APC–Cdc20 and SCF Skp2 in embryonic (left) and somatic stem cells (right).

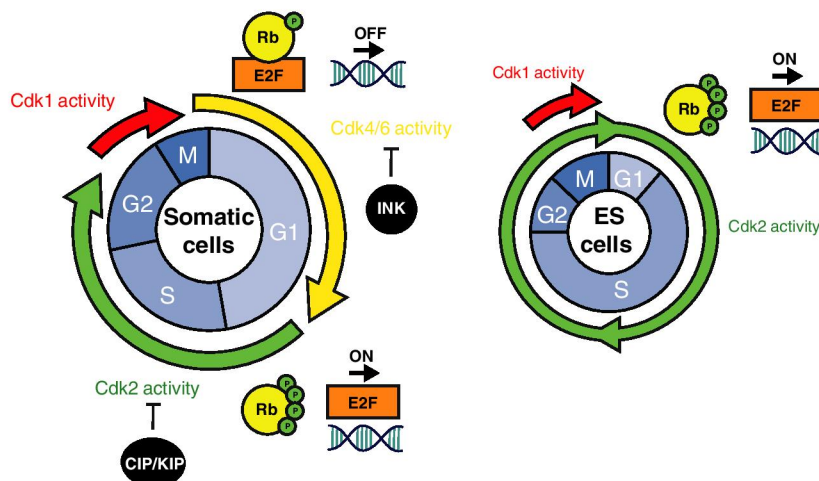


Fig. 3. G1/S checkpoint is compromised in embryonic stem cells. Schematic highlighting differences between G1/S checkpoint control in somatic and embryonic stem cells. E2F transcription and RB phosphorylation are cell cycle-regulated in somatic divisions and constitutive in embryonic stem cell divisions. Levels of CDK activities are shown for somatic and embryonic stem cell division cycles.

At the core of pluripotent cycles is a unique Cdk–cyclin pattern [38–41]. Cdk1, the kinase responsible for the onset of and progression through mitosis, is the only Cdk to show oscillatory activity in both mES and hES cells, despite the high expression of its regulatory partner cyclin B [42,43]. While Cdk4/6 shows minimal activity due to low expression of cyclins D1, D2 and D3, the G1/S master regulator, Cdk2, is constitutively active throughout the embryonic stem cell cycle due to the constitutive expression of its binding partner cyclins E and A [35,42,44–46].

These unusual activation patterns of cyclin-dependent kinases have been proposed to be important to maintain pluripotency in embryonic stem cells [38,41,47]. Mitotic cyclins, cyclins A and B, are essential in cell cycle progression in ES cells, whereas G1 cyclins have been shown to be only important for the maintenance of pluripotency [47]. Indeed, cyclins D and E, have been shown to phosphorylate and thereby prevent degradation of pluripotency factors Nanog, Oct4 and Sox2 [47].

Due to the aberrant activation pattern of Cdk2, the G1/S gatekeeper RB remains hyperphosphorylated

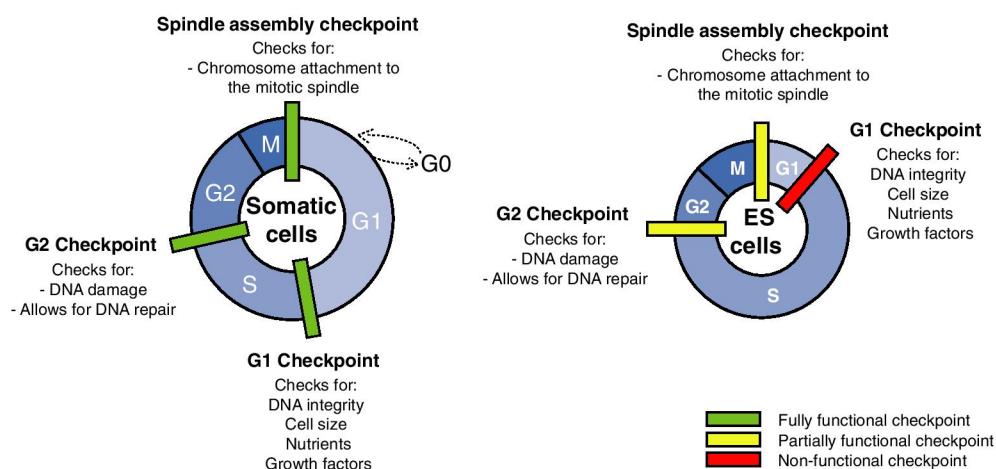


Fig. 4. Checkpoint control in somatic and embryonic stem cell divisions. Schematic showing the checkpoints at play in somatic and embryonic stem cells. Green, yellow and red bars indicate a fully functional, a partially functional and a nonfunctional checkpoint regulation, respectively.

and inactive throughout the cell cycle [48] (Fig. 3). As a result, embryonic stem cells rapidly enter S phase, following a fairly short G1 phase of approximately 1 h in mES cells and 2–3 h in hES cells [36], showing that truncated gap phases and absence of a restriction point are likely to be conserved features of mammalian pluripotent [42]. However, while elevated levels of Cdk2 activity are also seen in hES cells, most cyclins display relative cell cycle-dependent expression in these cells [33,36] (Fig. 2). In addition, Cdk4/6 shows cell cycle-regulated dynamics in hES cells with maximum activity, albeit timid, during G1 phase. As a consequence of Cdk4/6 and Cdk2 activity, slightly reduced levels RB hyperphosphorylation have been reported in G1 for hES cells [49,50].

The differences between mES and hES cells suggest that a more functional restriction point might be potentially present in hES cells. This idea is supported by the fact that knockout of all three RB (Rb^{p105}, RbL1^{p107} and RbL2^{p130}) protein family, also known as pocket proteins, having an effect only on hES cell proliferation rate but showing no effect on mES cells [50–53]. A clear mechanistic insight into how G1/S regulators orchestrate this transition in hES cells remain, nevertheless, to be established. These differences in pluripotent divisions between mES and hES cells are likely to be a consequence of the different developmental stages of the two cell types: mES cells are thought to be in an earlier, ‘naïve’ pluripotent state, as opposed to hES cells, which are thought to be ‘primed’ pluripotent cells. In other words, hES cells might represent a slightly later

developmental stage, equivalent to mouse postimplantation epiblast cells (EpiSCs) [54].

At the heart of the nonoscillatory cyclin expression and the unusually high CDK activation are the low expression levels of CDK inhibitors in ES cells (Fig. 2, Table 1). The CDK-interacting protein/kinase inhibitory protein CIP/KIP and INK Cdk inhibitor (CdkI) proteins, for example, play important roles in cell cycle arrest by binding to and inhibiting Cdks. These include p21^{CIP} and p27^{Kip1}, which bind to and antagonise the activity of Cdk1 and Cdk2 and p15, p16 and p18, which inhibit Cdk4 and Cdk6 and all show low or no expression in pluripotent embryonic cell cycles [55,56,57].

In addition to CdkIs, regulation of cell division in ES cells depends on other important cell cycle inhibitors. In particular, the anaphase-promoting complex (APC/C), for example, targets a plethora of cell cycle regulators for degradation, including cyclins. In ES cells, the activity of the APC/C is predominantly low due to elevated levels of the APC/C inhibitor Emi1 [58,59]. The attenuation of APC by Emi1 in ES cells is thought to help increase the expression of cyclins and thereby elevate Cdk activity in ES cells. In addition to APC/C, other proteins that regulate protein degradation during the cell cycle contribute to the atypical expression patterns of cell cycle regulators in ES cells. One example is S-phase kinase-associated protein 2 (Skp2) which targets p27 for degradation. In ES cells, Skp2 is expressed at high levels throughout the cell cycle, as opposed to its restricted expression to S/G2/M in somatic divisions [57].

Together, these special features of high, nonoscillatory Cdk activity, high expression of cyclins and reduced CDK inhibition in ES cell cycles give rise to an atypical Cdk activity and promote a speedy entry into S phase. Interestingly, in this scenario, how Cdk activity is counteracted by phosphatases and how cell cycle events are ordered in ES cells is largely unanswered. For example, whether a quantitative model of Cdk activity is still relevant in embryonic division cycles when nonoscillatory levels of Cdk activity prevails remains to be understood [60,61] (Fig. 2).

Checkpoint control and maintenance of genomic integrity in embryonic stem cells

Maintenance of genome integrity is a demanding task for rapidly proliferating cells. Since ES cells retain the potential to produce any cell type in the adult body, mutations in ES cells can potentially compromise multiple cell lineages and subsequent generations. The question is, thus, how is the fast cycling through cell division in ES cells compatible with preservation of genomic integrity? One adaptation from early embryonic to pluripotent divisions is the acquisition of checkpoint control mechanisms that help with surveillance of genomic integrity (Fig. 4).

ES cells have different mechanisms to protect integrity of their genomes: (a) they overexpress genes important for repair pathways, (b) they show hypersensitivity to DNA damage and, importantly, (c) they show a lower threshold towards programmed cell death, apoptosis.

The modified G1/S checkpoint in ES cell cycles

The G1/S checkpoint serves to protect genomic integrity and to prevent cells with damaged DNA from entering S phase. However, as a consequence of hyperactivation of Cdk2, low level of CdkI expression and misregulation of RB, ES cells lack a fully functional G1/S checkpoint [16,35,62]. Accordingly, monkey ES cells do not arrest in G1 after gamma radiation, reflecting the absence of a classical G1 checkpoint [35].

Although the lack of a functional G1/S checkpoint facilitates speedy entry into S phase and shortened G1 phase, ES cells are particularly vulnerable to DNA damage and show constitutive replication stress [63,64]. Due to this vulnerability, ES cells have adapted to accommodate their dynamic cycling. In fact, ES cells have the capacity to repair a variety of DNA lesions by various agents more efficiently than somatic cells [65]. In addition, ES cells employ an effective replication-coupled repair mechanism to

protect genome integrity, characterised by slow replication fork speed and frequent fork reversal [64].

While ES cells are able to upregulate p53 mRNA in response to DNA damage, p53 fails to translocate to the nucleus and activate cell cycle arrest [64]. In addition, after DNA damage, while p21, an important p53 target gene during cell cycle arrest, is elevated at the mRNA level, it is not detectable at a protein level [66]. In line with this, the mir-302 family of microRNAs (miRNAs), which negatively regulates p21 protein expression, is upregulated in ES cells in response to double-strand breaks [66].

Although the DNA damage response does not rely on cell cycle arrest in G1 by the p53-p21 axis in ES cells, an increased apoptotic response to damaged cells has been reported [16]. In line with this, the pro-apoptotic BAX protein has been shown to be constitutively active in ES cells [67]. Elimination of damaged cells may represent for these cells a safer alternative to cell cycle arrest.

Recently, it has been suggested that hES cells might be capable of partially executing the G1/S checkpoint activation after ultraviolet radiation C (UVC) light treatment [68]. After exposure, hES cells show lower levels of Cdk2 activity and undergo G1 arrest. This lowering of Cdk2 activity is not achieved via the classical ATM-CHK2-p53-p21 inactivation but instead by downregulation of the Cdk2-activating phosphatase Cdc25A. In contrast, downregulation of Cdk2 in hES cells triggers the G1 checkpoint through the activation of the ATM-CHK2-p53-p21 pathway, while also promoting differentiation and loss of pluripotency, highlighting the causality between Cdk2 hyperactivation and lack of a fully functional G1/S checkpoint [69].

Checkpoint control at G2/M in ES cell cycles

The absence of a fully operational G1/S checkpoint means that ES cells are prone to replication stress and DNA damage [64]. Therefore, alternative DNA repair mechanisms are necessary to be in place to maintain genomic integrity. This control is achieved during G2 phase.

DNA repair during G2 phase is surprisingly efficient in hES cells. This is partly due to increased expression levels of genes involved in base excision repair and involved in double-strand break repair such as uracil-DNA glycosylase (UNG), BRCA1 and ATM/ATR-dependent phosphorylation of H2A histone family X (H2AX) [65,70]. Moreover, in order to repair double-strand breaks, ES cells favour homologous recombination [16,71]. Rad51, which is critical for homologous recombination, is constitutively expressed throughout

the cell cycle in mES cells [72]. Rad51 suppression causes cells to accumulate in G2, suggesting a role for Rad51 in G2/M progression, while also contributing to maintenance of genome integrity.

Together, these mechanisms help protect genomic integrity at the earliest stages of mammalian development.

The spindle assembly checkpoint in ES cell cycles

The spindle assembly checkpoint (SAC) helps maintain chromosomal integrity during mitosis. While the SAC is active in ES cell cycle activation of SAC and apoptosis are not coupled, the SAC does not activate apoptosis as it does in differentiated cells after mitotic abnormalities [16,73]. This potentially can lead to an increased tolerance to changes in ploidy and karyotype abnormalities in embryonic stem cells [73]. In fact, following treatment with a microtubule poison, mES cells transiently activate the SAC but nevertheless exit mitosis and enter G1 with a 4C DNA content, without undergoing apoptosis [73], a process known as mitotic slippage. Upon differentiation, coupling between apoptosis and spindle assembly checkpoint control is activated and any deviations in ploidy are eliminated [73].

S-phase control in ES cells

To sustain their high proliferative requirements, ES cells must be primed to initiate DNA replication in an efficient manner.

Before DNA replication can start, replication origins need to be licensed so that helicases can unwind DNA and synthesis begin. Licensing factors include the six subunit origin recognition complex (ORC), which surrounds the replication origin and further recruits Cdt1 and Cdc6 in G1 phase. ORC, Cdt1 and Cdc6 then load the minichromosome maintenance (MCM) helicase complex to complete the fully licensed replication origin (i.e. prereplication complex, pre-RC). This DNA/protein complex is now primed for activation and thousands of these origins initiate DNA synthesis throughout S phase [74].

As licensing occurs exclusively in G1 (to prevent re-replication), ES cells have elevated levels of Cdt1 and Cdc6 [43,59,75] to compensate for the abbreviated G1 phase. This has been shown to mediate rapid MCM loading in pluripotent cells in comparison with their differentiated counterpart [75]. Slowing down MCM loading resulted in elongation of G1 and promoted differentiation [75], highlighting that efficient replication origin licensing is an intrinsic feature of embryonic cell cycles and is vital for pluripotency.

Histone biosynthesis during the G1/S transition is an essential process to package newly synthesised DNA in S phase [76]. Histone biosynthesis is in part regulated by Cdk2-dependent phosphorylation of p220 (NPAT), which localises to Cajal bodies and promotes histone H4 gene expression during S phase [77]. The hyperactivation of Cdk2 results in phosphorylation of p220(NPAT) and its premature accumulation in foci at Cajal bodies during G1, preceding the onset of DNA synthesis [78].

Regulation of transcription in ES cells

Cell cycle-regulated transcription is a vital mechanism to bring about changes in gene expression and coordinate progression through the cell cycle. As these dynamic changes in gene expression are brought about by changes in Cdk activities, a consequence of modified Cdk activities in ES cells means that the vast majority of cell cycle regulators have an elevated transcriptional activity. Interestingly, this is often coupled to histone acetylation of their promoter region [44].

In particular, since RB is predominantly inactive in ES cells, E2F transcription is cell cycle-independent. In other words, there is no cyclic expression of E2F targets and E2F targets are instead actively transcribed throughout the cell cycle. As a result, ES cells are primed to transcribe S-phase-specific genes immediately after mitosis and traverse G1 phase. How the E2F transcriptional program functions in hES cells is not fully established.

Control of G2/M phases in ES cells

The regulatory network that drives G2/M transition is conserved in early embryonic and in ES cells and relies on the activation of Cdk1–cyclin B complexes. The transcription factor B-MYB, a target of E2F, is thought to regulate Cdk activity by upregulating many Cdk1 regulatory proteins such as cyclin B1 and Plk1 [79,80].

Since Cdk1–cyclin B1 expression levels are high in ES cells, in order to prevent premature mitotic entry, oscillatory activity of Cdk1 is thought to be maintained by interaction with Oct4, one of the core pluripotent transcription factors, which forms a complex with and inhibits Cdk1 activation in ESC prior to mitosis [81]. Consistent with this, overexpression of Oct4 leads to delayed mitotic entry, whereas downregulations lead to a decrease in G2 duration [81].

Cdk1 is thought to be at the heart of a multitude of events in ES cells including self-renewal, pluripotency, genome maintenance, apoptosis and G2/M checkpoint

activation [32,34,41]. Knockdown of Cdk1 in hES cells leads to loss of pluripotency, spontaneous differentiation, gross genome instability and an inability to execute apoptosis or G2/M checkpoint [34].

In cooperation with Cdk1, mitotic kinases also serve to regulate multiple M phase events such as spindle assembly, APC activation and cytokinesis [82]. Aurora A is one such kinase and localises to centrosomes and spindle microtubules during mitosis and is vital for cell cycle progression, spindle pole orientation and microtubule stability in proliferation cells [83]. In mESC, Aurora A also displays roles outside of mitosis and is important in self-renewal [84]. Interestingly, a recent study has proposed that in hES cells, S/G2 regulators such as cyclin B1 and ATM/ATR-CHEK2 are important for maintenance of pluripotency or what they term 'pluripotency dissolution' outside of G1 phase [31], linking S/G2 cell cycle regulators to self-renewing and maintenance of pluripotency.

The interplay between cycle regulation and pluripotency is an emerging question in stem cell biology.

The interplay between cell cycle control and pluripotency

It has been hypothesised that short division cycles and, in particular, a rapid progression through G1 phase are important to sustain pluripotency, as an abbreviated G1 phase potentially minimises exposure to differentiation signals [28,49,85,86]. Mounting evidence has underscored the importance of cell cycle regulators to maintain pluripotency in ES cells.

Cdk4/6 bound to G1 cyclins partners are known to directly phosphorylate and stabilise the pluripotency factors Nanog, Oct4 and Sox2 [47]. In addition, studies showed that downregulation of key cell cycle regulators such as Cdk1, Cdk2, cyclin E and cyclin B resulted in loss of pluripotency and triggered spontaneous differentiation [29–34,87]. On the other hand, ectopic expression of cyclin E and cyclin B promotes self-renewal in ES cells [31]. In line with this, studies in hES cells have shown that artificially increasing Cdk4/6 activity during G1 or lowering RB hyperphosphorylation can lead to an increase in G1 duration and an enhanced differentiation potential [49,88]. It has been further proposed that Cdk4/6–cyclin D regulates the activation of the transcription factor Smad2/3, an important transducer of pluripotency [86].

Despite these observations, there is conflicting evidence on the importance of G1 length in maintaining pluripotency in ES cells. For example, elongating G1

in mES cells by overexpressing p21/p27 or knocking down CDK2/cyclin A is still compatible with the pluripotent state and does not induce differentiation [89]. In addition, mES cells grown in naïve pluripotency conditions (2i/LIF) have an extended G1 but are nevertheless more pluripotent than primed mES cells [45]. In hES cells, while p27 overexpression leads to the upregulation of Brachyury and Twist, two important factors in differentiation, it has no effect on core pluripotency factors [90]. While there is compelling evidence that the activity of key cell cycle regulators influences the pluripotent state in ES cells, the requirement for an abbreviated G1 phase may be less crucial.

Finally, an important shared regulator of both pluripotency and cell cycle control in ES cells is the transcription factor MYC [91]. MYC is essential for the maintenance of pluripotency [92] but also upregulates the transcription of CdkIs and cyclins, including cyclins D, E and A, while simultaneously negatively regulates CdkIs, p21 and p27 [93]. Furthermore, Myc also targets the miRNA cluster mir-17-92, which negatively regulates translation of p21, E2F1, RB and cyclin D [94,95]. Altogether, these observations put this transcription factor at the heart of the interplay between pluripotency and cell cycle regulation.

On dominoes: the cell cycle of somatic cells

Somatic cells have requirements that are very different from those of embryonic cells. With cellular specification and the acquisition of restricted patterns of gene expression during embryogenesis arises, the need to maintain cellular identity and cell number within tissues. In addition, somatic cells lose self-renewing capacity and acquire the ability to reversibly exit the cell cycle, a process known as quiescence. The cell cycle has a crucial role in these properties of differentiated cells, and these somatic cells have adapted cell cycle control to fulfil these important requirements.

Differentiation of embryonic stem cells is accompanied by extensive gene expression changes, upregulation of lineage-specific programs and epigenetic modifications that maintain those programs. Cell morphology changes dramatically during differentiation: cells become larger and elongated and the ratio of nuclear-to-cytoplasmic volume inverts, due to extensive cytoplasmic growth. The cell cycle is also actively remodelled through differentiation. Somatic divisions are long (typically 24 h), asynchronous and have long gap phases, most notably G1 phase, and can have adopted mechanisms to maintain genome integrity and cellular identity [28,30,35,37,38] (Fig. 1).

Concomitantly with the lengthening of G1, differentiated (somatic) cells become dependent on external cues such as mitogenic factors to undergo cell division and develop sensitivity to serum deprivation. In other words, a restriction point is established. Mitogenic-dependent progression through G1 phase is mediated by induction of the cyclin D family of proteins, which, in somatic cells, is typically rapidly degraded but becomes stabilised in the presence of mitogenic signals. In fact, protein stabilisation and degradation play an important role in cell cycle control in somatic cells. The expression levels of other key cell cycle regulators such as p53, p21, p27, cyclin E and E2F are all regulated by degradation.

In somatic cells, G1 phase can be seen as a critical point in the cell cycle where decisions on whether to proliferate, enter a quiescent state or exit the cell cycle completely take place (Fig. 1). If a cell passes the so-called 'restriction point' at the end of G1 phase, it is irreversibly committed to progress through the cell cycle, undergo DNA replication and segregate chromosomes during mitosis.

Introduction of cell cycle-dependent activities of cell cycle regulators in somatic divisions

Degradation gives rise to one of the most fundamental control features of the transition from a pluripotent to somatic division cycle: the appearance of oscillatory activation and expression of cell cycle regulators (Fig. 2). Somatic cells downregulate cyclins and upregulate Cdk inhibitor proteins, including increasing the activation patterns of APC/C (Fig. 2). Important somatic cycle regulators are the CIP/KIP family (p21, p27, p57) inhibitors which control cell cycle progression by inhibiting Cdk1 and Cdk2 complexes [96], and the INK4 family (p15, p16, p18 and p19) proteins, which display specificity for regulating Cdk4 and Cdk6 [96]. CdkIs provide an effective way of slowing down the cell cycle by arresting cell division – CdkIs bind to and inhibit Cdk–cyclin complexes by steric inhibition, thereby blocking access to substrates or ATP and hence reducing specificity to cyclin proteins and destabilising Cdk–cyclin interactions.

In somatic cells, Cdk expression levels are downregulated relative to embryonic cells and remain constant throughout the cell cycle, while cyclins levels start to oscillate in a cell cycle-dependent way. Cyclin proteins are unstable in nature and their levels are regulated both at a transcriptional level and at the level of protein stability in a cell cycle-dependent manner in somatic cell divisions. The oscillatory expression of cyclin proteins during somatic divisions is critical for

normal somatic cell cycle progression. Failure in regulating cyclin levels can trap cells in a particular cell cycle phase. Progression through each cell cycle phase requires the activation of at least one phase-specific Cdk–cyclin complex. Each of these promotes activation of the next complex ensuring that the cell cycle progresses in an ordered fashion.

The combination of the new waves of cyclin oscillations with the appearance of CdkIs results in an overall decrease in Cdk activity. Importantly, the activation dynamics of each Cdk–cyclin complex becomes cell cycle-dependent (Fig. 2, Tables 1 and 2).

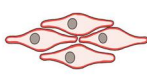
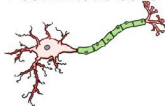
Introduction of a restriction point and checkpoint control mechanisms in somatic divisions

Sophisticated control mechanisms involved in the regulation of cell division in somatic cells couple extracellular signals to the cell cycle machinery. These not only provide directionality in cell cycle progression but also have strategically placed restriction points that provide safeguards for genomic integrity.

Somatic cells have intact checkpoint mechanisms in place to detect a plethora of DNA damage events in order to activate cell cycle arrest and repair the damage both during G1 and G2 phases [97]. Mutation frequency in somatic cells is two orders of magnitude that of embryonic stem cells ($\approx 10^{-4}$) [98]. Failure to repair DNA damage typically results in apoptosis. Checkpoint control mechanisms are fundamental to keep the four cell cycle phases of each round of cell division in the correct temporal order, and to ensure that chromosomal DNA is faithfully replicated and then distributed equally to each daughter cell. This ensures the maintenance of genomic integrity through generations of daughter cells (Fig. 4). Checkpoint control allows the cell cycle of a somatic cell to behave like the pieces in a falling line of dominoes: one phase only starts after full completion of the previous phase [1].

Checkpoints can be divided into two groups: (1) cell-intrinsic checkpoints that depend on intrinsic signals, for example, indicating the integrity of the genome; and (2) cell-extrinsic checkpoints that depend on mitogenic signals such as growth factors. One such extrinsic checkpoint controls the passage from early to late G1, or the restriction point. The appearance of the restriction point is one of the key features of somatic cell division cycles. The restriction point marks the commitment point after which a cell no longer needs external signals to undergo a new cell division cycle. Control of the restriction point relies

Table 2. Differences in cell cycle control amongst early embryonic, embryonic stem cell, somatic and terminally differentiated cells

Early embryonic cells	ES cells	Somatic cells	Post-mitotic cells
 - Autonomous oscillations of active CDK cyclin complexes - Truncated or non-existent GAP phases - Absent or reduced checkpoints - Do not rely on external stimulus to progress through G1 phase - Independent of restriction point - Insensitive to serum deprivation - Divisions can occur without zygotic transcription	 - Non cyclical, high expression of cyclins - Non cyclical, constitively active CDKs - Low levels of CDK inhibitors - Lack of G1/S checkpoint control - Do not rely on external stimulus to progress through G1 phase - Independent of restriction point - Insensitive to serum deprivation - Non cyclical expression of E2F targets - Non cyclical Rb hyperphosphorylation - Acetylation of histones at promoter regions of cell cycle genes	 - Cyclical expression of cyclins - Cell cycle regulated CDK activity - Cyclic levels of CDK inhibitors - Functional G1/S, G2 and SAC checkpoints - Rely on mitogenic and differentiation cues to progress through G1 phase - Dependent of restriction point - Sensitive to serum deprivation - Cyclic expression of E2F targets - Cyclic hyperphosphorylation of Rb - Deacetylation of histones at promoter regions of cell cycle genes	 - Down-regulation of G1 cyclins - Down-regulation of CDK Activity - High levels of CDK inhibitors - Permanent Rb/E2F repression of genes - Silencing of cell cycle genes by methylation of histones at promoter regions

heavily on control of Cdk4/6-cyclin D complexes and RB (Fig. 3).

The acquired oscillatory activity of Cdk4/6 and Cdk2 is key in regulating the restriction point via the phosphorylation of RB. Under normal conditions in early G1, RB is in a hypophosphorylated, active state and binds to and suppresses the E2F transcription factor, thereby inhibiting transcription of S phase-specific genes, such as cyclin E or Cdc25A, and those involved in DNA replication such as thymidine kinase or DNA polymerase- α and proliferating cell nuclear antigen (PCNA). After mitogenic signalling, RB is hyperphosphorylated by Cdk4/6 and releases E2F inhibition, thereby promoting E2F-dependent transcription (Fig. 3). One such target of E2F is cyclin E, which forms a complex with and activates Cdk2 and goes on to promote further phosphorylation and inhibition of RB and progression through G1/S. This positive feedback loop marks a point of no return in cell cycle entry [99].

E2F regulation in somatic cell cycles leads to a transient repression of E2F-regulated transcription during G1, and so expression of E2F target genes becomes cell cycle-dependent in somatic cells. An overall decrease in the expression of cell cycle-related genes is further achieved by deacetylation of histones at promoter regions of cell cycle regulators [100].

The establishment of this G1/S checkpoint is one of the hallmarks of the somatic cell cycle. It restricts premature entry into S phase and allows for tuning of the speed of proliferation (Fig. 4). The main targets of the G1/S checkpoint are Cdk-cyclin complexes that drive the transition from G1 to S phase [18]. Following the activation of the checkpoint sensors, ATM and Cdh2, Cdc25A phosphatase becomes inactivated by

phosphorylation, rendering Cdk2 inactive. This results in the rapid initiation of G1 arrest until Cdk2 activity is allowed to rise. Cdk2 inactivation and G1 arrest are actively maintained by p21 upregulation.

Later in the cycle, the G2/M checkpoint prevents cells containing damaged DNA from entering mitosis (M). During G2 phase, cyclin B is expressed and activates Cdk1 so that the complex can drive nuclear envelope breakdown and entry into M phase. Cdk1-cyclin B1 is activated until G2/M, when Cdk1 inhibitor Wee1 causes inactivation by phosphorylation of threonine 14 and tyrosine 15 residues. These inhibitory phosphorylations are counteracted by the phosphatase Cdc25C, a target for the checkpoint protein Chk2. Together with Chk2, the p53 targets p21 and GADD45 aid with maintenance of a G2 arrest and prevent somatic cells from entering into mitosis.

The last piece of the falling domino: the cell cycle of terminally differentiated cells

Terminal differentiation is characterised by permanent exit from the cell cycle and is the most common cellular state of fully differentiated adult cells. Its most prominent feature is a prolonged cell cycle arrest with a G1 (i.e. 1C) DNA content, that is G0 (Fig. 1).

Indeed, one of the hallmarks of postmitotic cells is that they become refractory to mitogen or other proliferative signals that promote cell cycle reactivation. A permanent withdrawal from the cell cycle causes irreversible loss of proliferative potential (Table 2).

Molecularly, cell cycle arrest is brought about by downregulation of G1 cyclins [47], high expression of

Cdk inhibitors and the presence of inactive, hypophosphorylated RB. Together, these give rise to downregulation of Cdk activity and a permanent inhibition of E2F-dependent transcription [100] (Table 2).

Cell cycle-driven gene expression is further silenced by methylation of histones at the promoters of genes encoding on cell cycle regulators. RB plays an important role in this, both by inhibiting E2F and by recruiting histone deacetylases and methyltransferases to help repress E2F target genes. This suggests that terminal differentiation might not be an easily reversible cellular state [101], but the very last piece of a falling domino.

In line with this, protein degradation machinery also plays a key role in mitotic exit [102]. While both APC/C and Skp2-Cullin-Fbox (SCF) ubiquitin ligases seem to promote quiescence, RB and APC/C have been shown to physically interact and induce degradation of Skp2, which normally targets the Cdk inhibitors p21 and p27 for degradation. The inhibition of Skp2 results in accumulation of the inhibitors and permanent cell cycle arrest.

Concluding remarks

Our current knowledge of the differences between embryonic and somatic division cycles mainly stems from research on established model systems (xenopus, zebrafish, drosophila model organisms, and yeast and mammalian cells). While these studies have paved the way to our understanding of cell division, they have perhaps fallen short of showing how the biochemical machinery regulating cell division cycles changes during developmental and cellular transitions (Figs 2–4 and Table 1). Embryonic stem cells can be instrumental in filling this gap and provide clues as to how the cell cycle changes while organisms develop and grow.

The pluripotent cell cycle has characteristics of both embryonic and somatic divisions, and the differences in cell cycle regulation between embryonic stem cells and their differentiated counterparts (summarised in Table 2) likely result from their specific functions. ES cells self-renew and retain the potential to produce all cell types in an adult organism and thereby can transmit any errors resulting from cell division to progeny during cell specification. On the contrary, genome integrity in somatic cells is important to maintain cellular identity and to protect these cells from malignant transformation. Cell cycle regulation in cells with different functions has therefore adapted to fulfil these specific requirements. Remarkably, this highlights how the diversity of cell cycle regulation within mammalian

species can differ more than those of distantly related species.

Embryonic stem cells can be utilised to understand how one genome can produce such diverse phenotypic outcomes by monitoring developmental cell cycle changes in real time. Understanding the molecular mechanisms underlying these adaptations will not only shed light into embryonic-to-somatic transition during development but, importantly, it will also shed light on how rapid cell divisions boosting cell proliferation adapt to become slow and controlled division cycles. This has great implications for targeting cancer and other pathological conditions hallmarked by a failure to arrest proliferation. The fact that embryonic cell cycles rely on unusual activation dynamics of oncogenes, but yet do not undergo oncogenic transformation, opens new avenues of research with translational potential.

Finally, understanding how proper cell cycle progression is achieved when oscillations of key cell cycle regulators are lost in embryonic stem cells and how fidelity of genome replication and segregation are ensured in the absence of a functional G1-checkpoint control are open, fascinating and important questions.

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Appendix B

Monitoring Cell Cycle Dynamics During Stem Cell Differentiation Using the scanR System's TruAI™ Deep-Learning Technology

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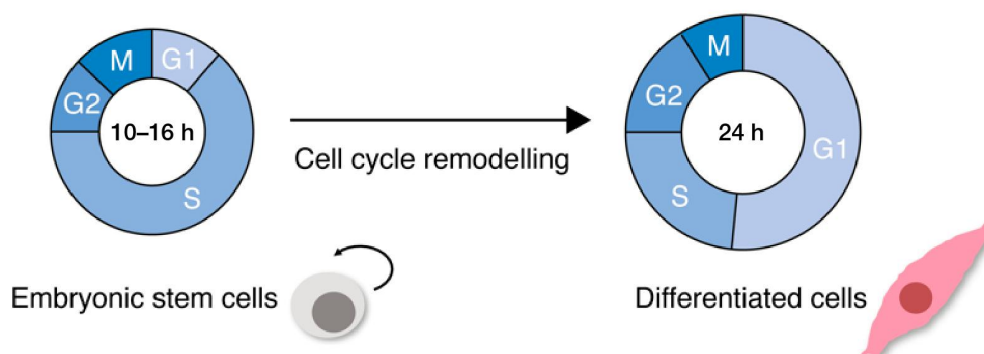
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Monitoring Cell Cycle Dynamics During Stem Cell Differentiation Using the scanR System's TruAI™ Deep-Learning Technology

Introduction

During early mammalian development, embryonic stem cells (ESC) undergo cellular specification and give rise to all the embryonic germ layers, which ultimately generate the multitude of cell types in the adult organism. ESCs are characterized by their incredible ability to self-renew and proliferate, having short division cycles due to truncated GAP phases (G1 and G2). During cellular differentiation, hallmark cellular events happen: cells change their morphology, growing about ten times their size, changing their nucleus-to-cytoplasmic ratio and becoming flat and elongated; fate-specific gene expression programs are activated and global chromatin modifications occur. Importantly, the cell cycle slows down. Differentiated, somatic cells have long G1- and G2-phases, tight checkpoint control, and well-regulated division cycles (Padgett and Santos 2020) (Figure 1).



Cell cycle remodeling is not just a phenomenon that happens in ESC differentiation. Changes in cell cycle dynamics happen frequently in biology; for example, when cells undergo regeneration after viral infections or during malignancy. Studying how cell cycle dynamics change in the context of cellular differentiation is therefore important in understanding potentially conserved mechanisms of cell cycle control and will have an impact on our understanding of the balance between health and disease states.

The changes during differentiation can be monitored in single cells by measuring the activity of genetically encoded proteins involved at different stages of the cell cycle (Araujo et al. 2016; Sakaue-Sawano et al. 2008). One such sensor is the fluorescent ubiquitination-based cell cycle indicator FUCCI(CA), developed by Miyawaki and colleagues (Sakaue-Sawano et al. 2017), which monitors the degradation of Cdt1 and Geminin, two important cell cycle proteins, producing a sharp triple color-distinct separation of the G1, S, and G2 phases (red, green, and yellow) that can be observed in the YFP and mCherry channels of a microscope (Figure 2).

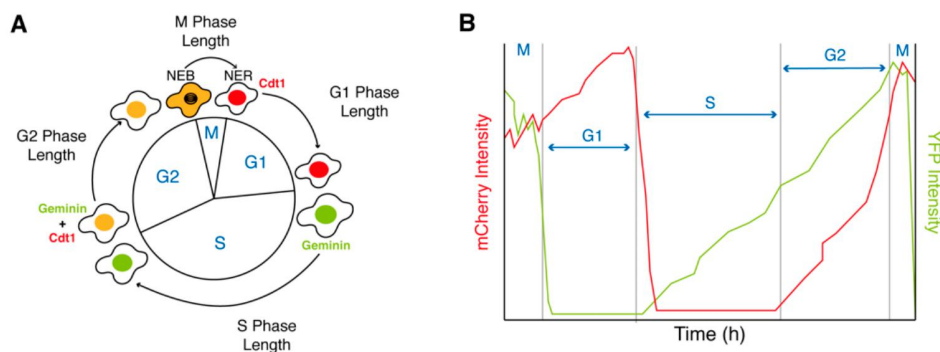


Figure 2. Measuring G1, S, G2 and M-phases of the cell cycle using the FUCCI(CA) sensor. (A) Schematic of the FUCCI(CA) sensor. (B) Example traces of G1, S, G2 and M-phase duration based on expression levels of Cdt1 (mCherry channel) and Geminin (YFP channel).

However, monitoring cell cycle dynamics during cell differentiation presents both imaging and data-analysis challenges. Long-term live cell imaging over days requires a stable temperature and CO₂ control, gentle illumination to minimize phototoxicity, and robust autofocus routines to ensure good-quality images during the whole time-lapse process.

For the analysis, the biggest challenge is the segmentation and subsequent tracking of single cells at low fluorescence intensities and high-confluency conditions while the ESCs are moving, dividing, and dramatically changing their shape during differentiation. All these challenges can be overcome with the Olympus high-content epifluorescence scanR microscope, equipped with a cellVivo incubator, Z-drift compensation (ZDC) hardware for autofocusing, TruAI™ deep-learning technology for segmentation, and a kinetic module for tracking.

Objectives

In this application note, we show that applying TruAI technology can significantly improve detection and tracking of single ESCs through long-term imaging based on the FUCCI(CA) sensor. This is particularly powerful with cells that do not have a nuclear marker for tracking, avoiding unnecessary illumination and phototoxicity.

To achieve this, TruAI technology is used to generate a deep neural network (DNN) model that can robustly identify the position of pluripotent ESCs and differentiated cells at all time points, even when the fluorescence intensity is very weak. This enables robust monitoring of G1, S, and G2 phases of cell populations over time. Subsequently, by applying the scanR kinetic module, we can track thousands of cells, evaluate their traces for long periods of time, and gain quantitative dynamic information on cell cycle changes at the single cell level.

To demonstrate the versatility of the scanR system as a research tool, all the data shown, including graphs, scatter plots, object galleries, tracks, and kinetic traces, are generated using only the scanR software.

Experimental Setup

H1 human embryonic stem (hESC) cells expressing the FUCCI(CA) sensor are treated with 50 ng/ml of bone morphogenic protein 4 (BMP4) to drive differentiation into the mesendoderm lineage. These H1 cells are then imaged using the scanR microscope with a 10X UPLANSAPO lens in the mCherry and YFP channels every 15 minutes for 6 days.

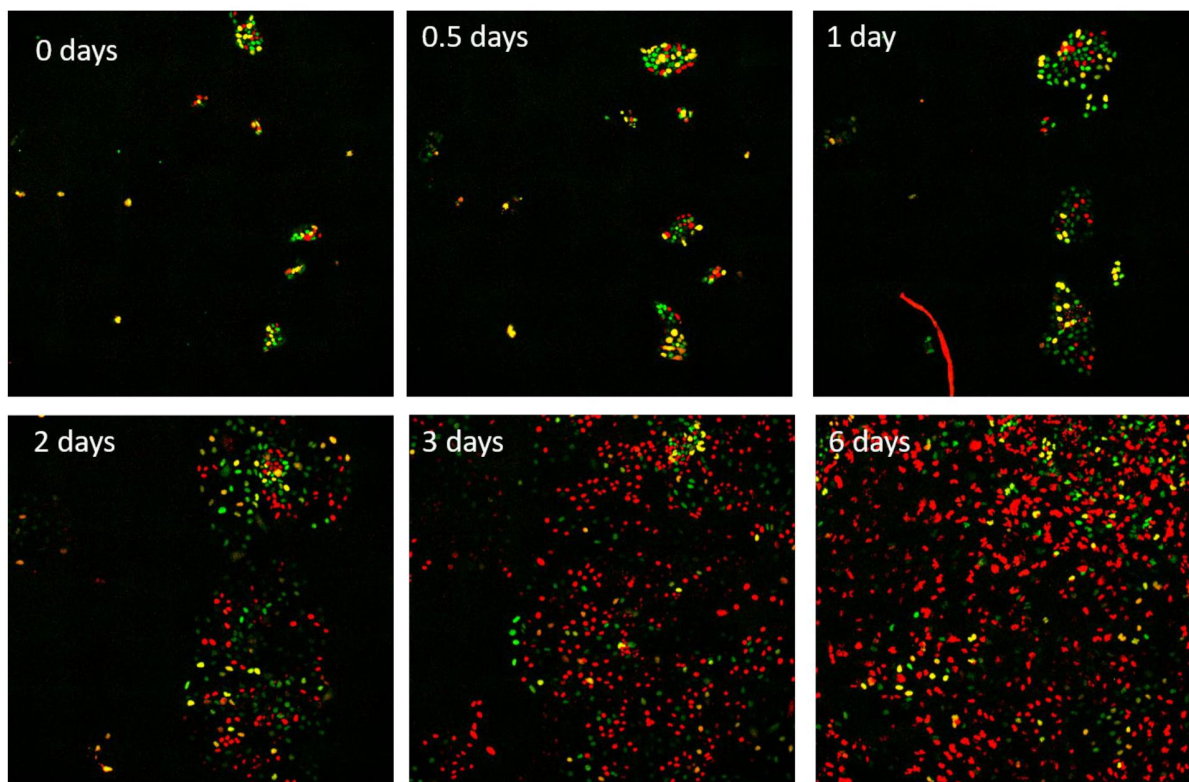


Figure 3. Time series over 6 days of hESC H1 cells expressing the FUCCI(CA) sensor, which were stimulated with BMP4 at the start of imaging. Red, green, and yellow correspond to the G1, S, and G2 phases respectively.

Developing a DNN to Detect Cells for Life Cell Imaging Conditions

A single cell expressing the FUCCI(CA) sensor may exhibit strong variations in fluorescence intensity when monitored over several days. In particular, the total intensity can be very low in the transition from G1 to the S phase (Figure 2b). This can cause tracking algorithms to fail as tracks break at the time points in which the cells are difficult to be detected.

To achieve cell tracking over several days for the largest number of cells possible, a DNN model capable of detecting cells at very weak intensities needs to be developed. In a previous white paper, we showed that this is possible by using pairs of images of long exposure and intense illumination of fluorescent cells versus short exposure and weak illumination (Woerdemann 2020). Briefly, the long exposure images are used for segmentation of cells and a mask is generated. This mask is used as ground truth to train the DNN model to detect cells in the low exposure levels.

Alternatively, the DNN model can be trained by performing manual annotations in the dataset to be analyzed. For this task, Olympus' cellSens™ software can be used. DNN models developed in cellSens software can be imported into the scanR software. In this application note, we used the latter manual method and provided in the order of hundreds of annotations of cells of varying intensities at different time points to train the DNN model.

Segmentation: Comparison of TruAI Deep-Learning Technology vs Classical Methods

Once developed, the DNN model is applied to the individual mCherry and YFP fluorescence channels. This creates a pixel AI probability map for each channel. The higher the AI probability in a pixel, the higher the confidence that the pixel belongs to a cell. Subsequently, the AI probability maps of both channels are summed, and segmentation is performed by applying an intensity threshold to this summation (Figure 4e and 4f).

To compare the results with classical methods, the mCherry and YFP fluorescence channels are background corrected using a rolling ball algorithm, summed, and the summation is segmented using an intensity threshold (Figure 4c) or an edge detection method (Figure 4d). All the image processing steps described above are conducted on scanR analysis software.

The statistical tools of the scanR system are used to create Table 1, in which results are referred to one single field of view over a period of 6 days.

Table 1. Comparison of number of cells segmented per method

Time points	Segmentation methods		
	Intensity threshold	Edge detector	TruAI technology
0 h	78	103	93
24 h	209	188	240
48 h	421	281	475
72 h	847	654	986
96 h	1480	1330	1590
160 h	1340	1460	1610
Summation of all frames 0–160 h	504131	573179	659199

A simple comparison of the fluorescence image against the AI probability image (Figure 4a and 4b) reveals that the TruAI approach has a higher sensitivity while detecting all types of cells. This is supported by the results summarized in Table 1 along with the whole time lapse. The classical intensity threshold method fails to detect weak fluorescent cells and to determine their correct shape (Figure 4c). The edge detection method improves the contour of the detected cells, but still misses many dim cells (Figure 4d). TruAI deep-learning technology reliably detects dim cells, and their boundaries are well defined (Figures 4e and 4f).

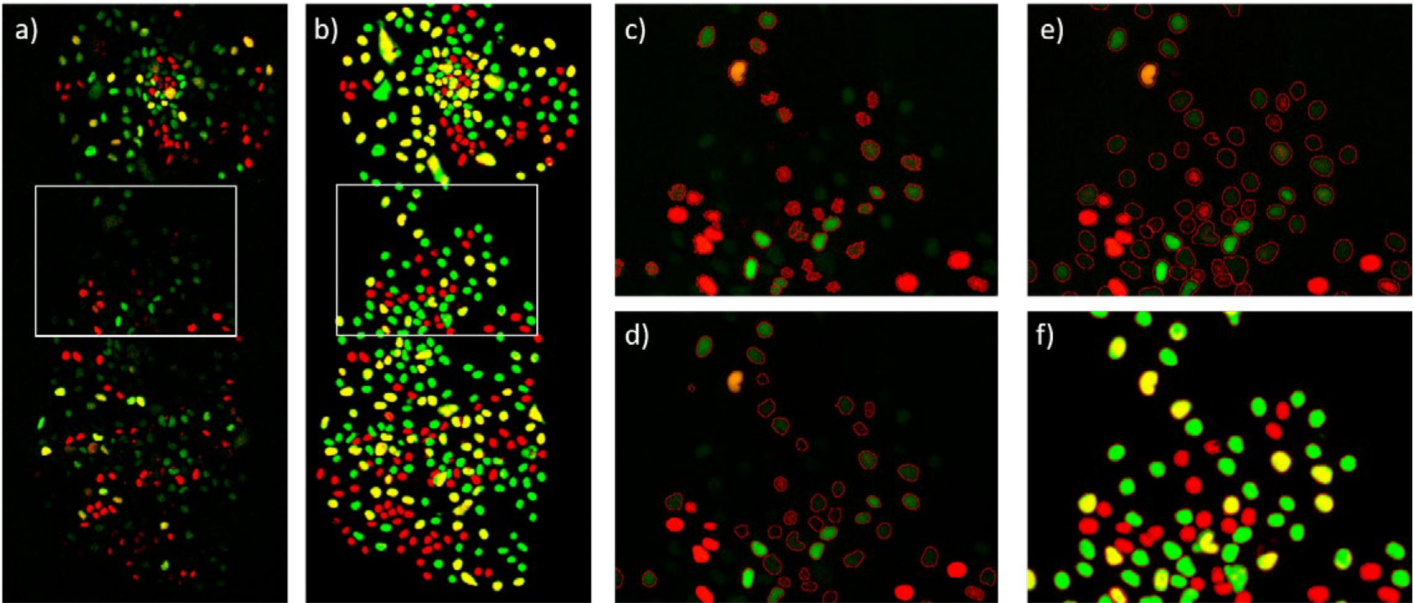


Figure 4: ESC cell colony at time point 48 h. a) mCherry (red, G1 phase) and YFP (green, S phase) fluorescence channels. The G2 phase is seen as a combination of mCherry and YFP (yellow). b) TruAI probability in the mCherry (red) and YFP (green). Cells with high AI probability in both channels are displayed in yellow. c) Segmentation using an intensity threshold in the summation of the mCherry and YFP fluorescence channels d) Segmentation using an edge detector in the summation of the mCherry and YFP fluorescence channels e) Segmentation in the summation of the TruAI probability intensities, overlaid with the fluorescence image f) same as e) but overlaid with the TruAI probability.

Analysis of Cell Populations Over Time

The mCherry and YFP intensities of segmented cells using TruAI technology are displayed in a scatter plot at different time intervals (Figure 5). Cells only having mCherry intensity correspond to the G1 phase, cells exhibiting only YFP intensity correspond to the S phase, and cells having intensities in both channels corresponds to cells in the G2 state. Figure 5 shows that at early times most of cells are in the S state, whereas at later times most of the cells are in the G1 state. This population shift matches with the cell cycle profiles expected for pluripotent and differentiated cells, respectively (Figure 1).

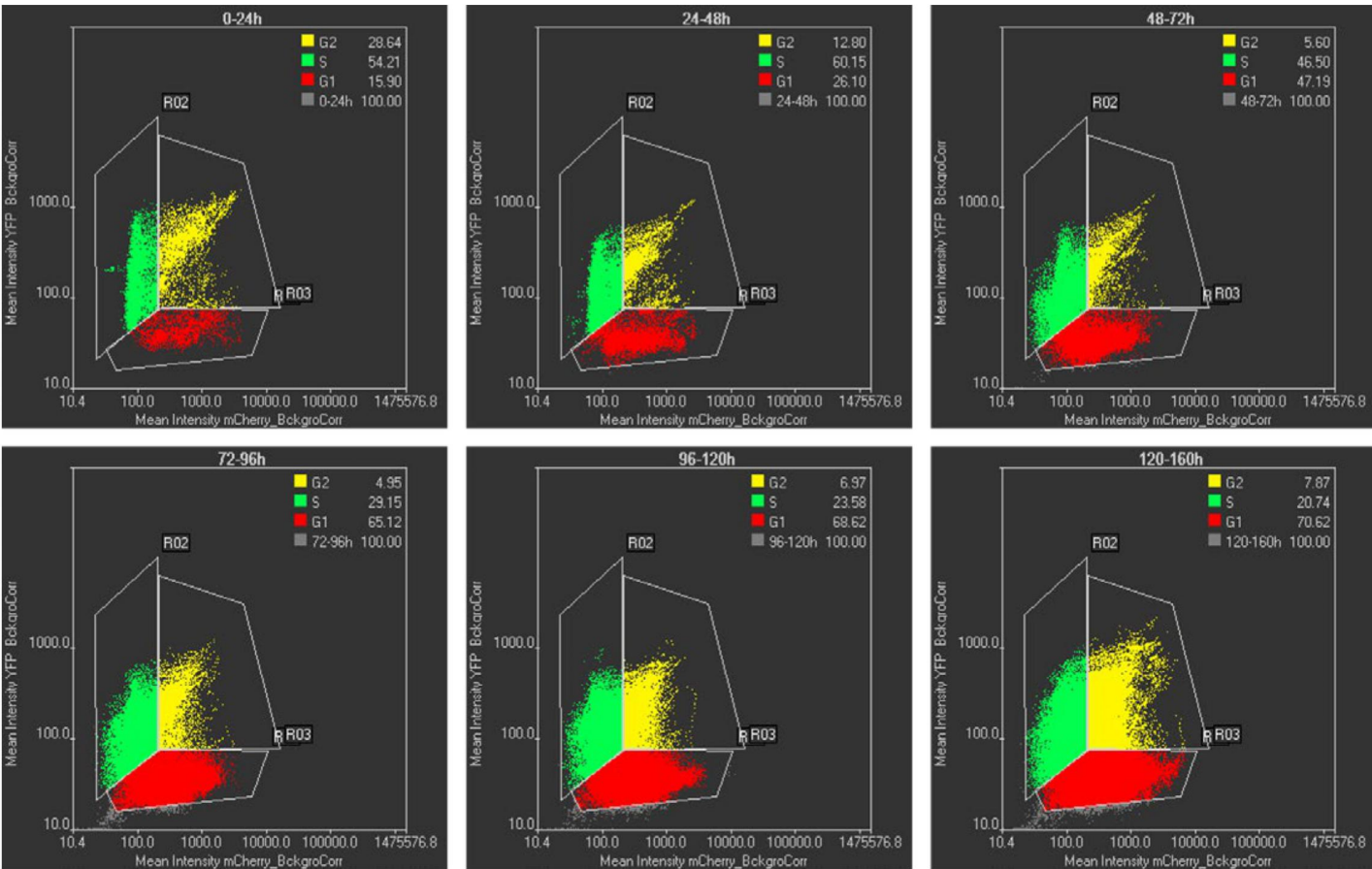


Figure 5: Scatterplots of mCherry vs YFP intensities at different time intervals. Each dot in the scatterplot represents a segmented cell by TruAI technology. G1, S, and G2 cells are represented in red, green, and yellow respectively. The % of the G1-S-G2 populations are shown in the inserts. The time intervals are shown at the top of each graph.

Cell Tracking: Comparison of TruAI Technology vs Classical Methods

Cells segmented with the three methods (intensity threshold, edge detector, and TruAI technology) were tracked using the same tracking settings of the scanR kinetic module. The statistical tools of the scanR system are used to create Table 2, in which results are referred to one single field of view over a period of 6 days.

Table 2. Comparison of number of cells tracked per method

Time points	Number of cells tracked		
	Intensity threshold	Edge detector	TruAI technology
24 h or more	302	1030	1965
48 h or more	16	126	426
72 h or more	0	27	72
96 h or more	0	0	5

The table shows that using TruAI technology for segmentation clearly improves the subsequent tracking results. For this data set, considering an average number of 200 cells per field of view at time 24 h (Table 1), more than 70 cells can be tracked over 3 days and it is even possible to track cells for 4 days or longer. The ability to track this high number of cells for such long periods of time is important because it enables the study of cell cycle dynamics over many cell divisions in a statistically meaningful manner.

Analysis of Cellular Tracks

For each track generated by the scanR system, a wide range of parameters can be extracted.

- To identify the longest tracks, the parameter “lifetime” can be plotted in a histogram, and a gate for tracks over 24 h can be created (Figure 6a).
- To filter the tracks further, the parameters “First (time)” and “Last (time)” can be represented in a scatter plot (Figure 6b). This can help us identify the cells only tracked during the first days (ESC cells with short cycle times), the cells tracked only in the last days (differentiated cells with long cell cycle times) and the cells that have been tracked from the beginning till the end of the time lapse (cells in which the differentiation process is monitored). Once these tracks are identified the intensities of mCherry and YFP can be plotted over time to gain quantitative information on the cell cycle dynamics (Figure 6 c, d, e).

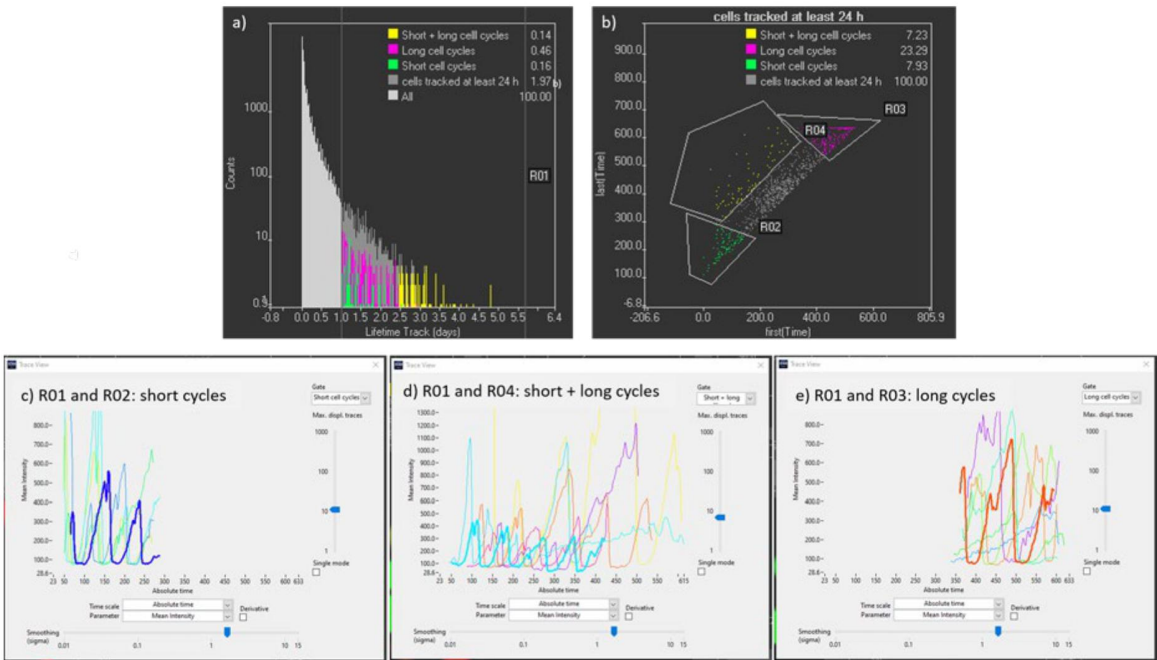


Figure 6. a) Track lifetime histogram used to filter long tracks (R01). b) Scatter plot with initial and final time point of the tracks to identify cells tracked from the beginning till the end of the time lapse (R01 and R04). c), d), and e) Kinetic traces of mCherry cells tracked more than 24 h with early starting and ending points, with early starting points and late ending points, and with late starting and ending points respectively. Time points are spaced 15 minutes apart.

From Figure 6c to 6e, it is observed that mCherry (G1 state) signal duration increases with time, from approximately 2 hours for ESC cells to 24 h once the cells have differentiated. For the in-depth analysis of the cell cycle dynamics, it is possible to select tracks of region R01 and R04, in which the transformation of ESC to differentiated cells is monitored over four or more cell divisions (Figure 6d).

Figure 7 represents the monitoring of one ESC cell undergoing differentiation over five cell cycles. In Figure 7a, the position of the ESC in its colony is shown at time point 52 (13 h), along with its track up to time point 422 (105 h). In Figure 7b, a snapshot at time 280 (70 h) is shown. Note the intensity of this cell is extremely weak and it went undetected using the classical methods. In Figure 7c, the same snapshot is shown, but with the TruAI probability image. The probability intensity of the tracked cell is as high as for the rest of the cells. Figures 7d and 7e represent a gallery of the tracked cell at time points every 15 minutes for fluorescence and TruAI probability respectively. Note that when finishing the G1 phase (red color), the total intensity is always very weak. Figure 7f represents the mCherry (top) and YFP (bottom) fluorescence intensity oscillations from which the cell cycle periods of G1, S, G2, and M states can be extracted.

From these data, we observe that this particular cell spends increased time in the G1 state and reduced time in S and G2 phases as time progresses. The M state is always very short; its exact position appears like a dip in the mCherry signal and as a drastic decay in the YFP signal.

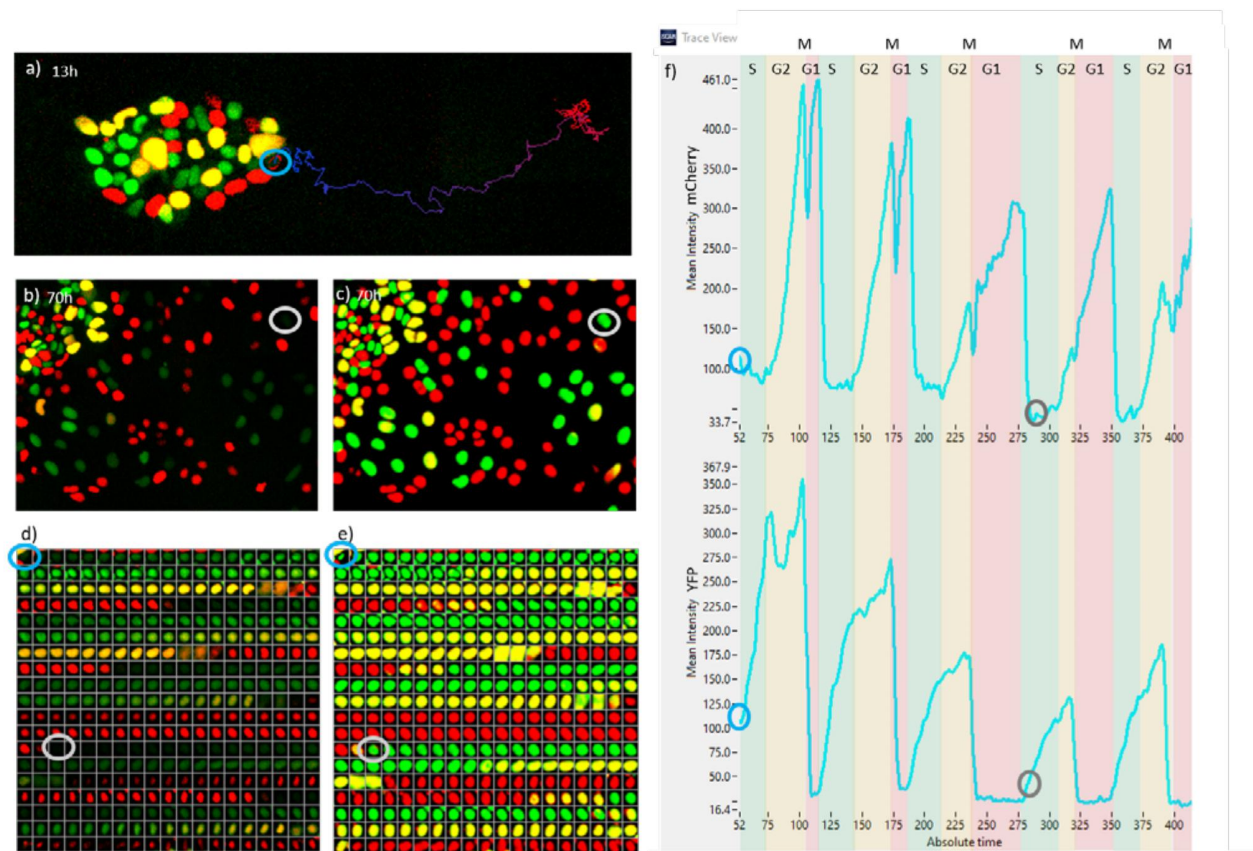


Figure 7. a) Fluorescence snapshot at the 13 h time point. b) Fluorescence snapshot at the 70 h time point. c) TruAI snapshot at 70 h. d) Fluorescence time-lapse sequence of the tracked cell at 15-minute intervals. e) TruAI time-lapse sequence of the tracked cell at 15-minute intervals. f) Fluorescence traces of mCherry and YFP of the tracked cell indicating the periods of the different cell cycle phases. Time steps correspond to 15 min. Blue and gray callouts in a–f indicate the same cell at 13 h or 70 h, respectively.

Conclusion

This novel deep-learning approach of analyzing cell cycle dynamics during ESC differentiation combined with the kinetic module of the scanR high-content screening system enables the acquisition of highly reproducible, quantitative, and statistically meaningful data on how cell cycle dynamics change in single cells during differentiation. Thousands of cells changing in shape and morphology can be recognized and tracked over days of differentiation using fluorescence measurements. This is a powerful tool to study cell cycle dynamics in early development and reprogramming, as well as in cells undergoing cellular transformation (malignancy) where cell cycle regulation takes central stage.

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