

Investigating the role of CDK1 in governing the transcriptional landscape in cancer cells



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A thesis submitted for the degree of Doctor of Philosophy

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October 2024

Declaration

This thesis presents research conducted at the Sir William Dunn School of Pathology, University of Oxford, between September 2020 and October 2024. Contributions from others have been clearly acknowledged where applicable; otherwise, the work presented is my own. No portion of this dissertation has been, nor will be, submitted for any degree, diploma, or other professional qualification at the University of Oxford or any other academic or professional institution.

The results discussed in **sections 4.5.-4.5.2. and 5.5.-5.5.2.** were created in collaboration with Dr Callum Henfrey, who performed the bioinformatics analysis of my RNA-seq data using University of Oxford's Advanced Research Computing (ARC) cluster and generated **Figures 4.4.-4.10. and 5.7.-5.11.**

The introduction chapter (**Chapter 1.**) of this thesis is adapted from a review I authored (Pluta et al., 2023), with contributions from Dr Cécilia Studniarek, Prof Shona Murphy, and Prof Chris Norbury, from whom I obtained permission to include the review in this work (attached below).

Aleksandra Joanna Pluta

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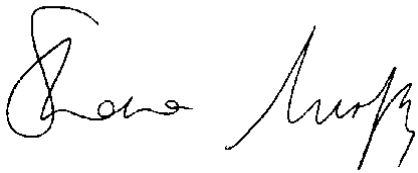
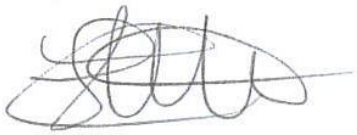
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to be used as the introduction to Aleksandra Pluta's thesis.

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Acknowledgements

I would like to express my gratitude to the many individuals who have offered me their continuous support and encouragement over the past four years.

I am incredibly privileged to have worked under the supervision of Prof Shona Murphy and Prof Chris Norbury, who have provided me with invaluable guidance, expertise, and motivation throughout my research journey. My sincere thanks are also extended to Prof Nicholas Proudfoot, whose external insights and feedback were highly constructive to the progress of this project. I would also like to thank all the members of the Murphy lab for their unwavering support and advice: Mathias Fagbemi, Dr Bella Maudlin, Dr Cécilia Studniarek, Dr Michael Tellier, Dr Gilbert Ansa, Dr Callum Henfrey, and Steph Barker.

I am incredibly appreciative of the numerous members of the Dunn School labs and facilities, who I could always turn to for help and assistance. I would also like to gratefully acknowledge the Oxford EPA Scholarship for funding my doctoral studies. Finally, my deepest thanks go to my family, who was my constant source of strength throughout this PhD.

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List of abbreviations

%	percentage
°C	degrees Celsius
AS	analogue-sensitive
ATP	adenosine triphosphate
bp	base pair
CAK	CDK-activating kinase
Cdc2/28	Cell division control protein 2/28
CDK	cyclin dependent kinase
cDNA	complementary deoxyribonucleic acid
ChIP	chromatin immunoprecipitation
chrRNA	chromatin-associated ribonucleic acid
CMV	cytomegalovirus
CRISPR	Clustered regularly interspaced short palindromic repeats
CTD	carboxy-terminal domain
DMSO	dimethyl sulfoxide
DNA	deoxyribonucleic acid
DNase	deoxyribonuclease
dNTP	deoxynucleotide triphosphate
DTB	double thymidine block
DTT	dithiothreitol
EtOH	ethanol
EU	5-ethynyl uridine
g	grams
<i>g</i>	relative centrifugal force
G1	Gap 1
G2	Gap 2
gDNA	genomic DNA
gRNA	guide RNA
h	hour
H3	histone 3
H3S10(p)	(phosphorylated) histone 3 serine 10
HDR	homology-directed repair
HIV	human immunodeficiency virus
IgG	immunoglobulin G
kDa	kilodalton
KO	knockout

M	molar
mESC(s)	mouse embryonic stem cell(s)
mg	milligram(s)
min	minute(s)
ml	millilitre(s)
mM	millimolar
mRNA	messenger RNA
PCR	polymerase chain reaction
PI	propidium iodide
pol	polymerase
poly(A)	polyadenylated
qRT-PCR	quantitative reverse-transcription polymerase chain reaction
RNA	ribonucleic acid
RNAi	ribonucleic acid interference
RNase	ribonuclease
rpm	revolutions per minute
rRNA	ribosomal RNA
s2/5/7(p)	(phosphorylated) serine 2/5/7
SDS	sodium dodecyl sulphate
SEM	standard error of the mean
siRNA	small interfering ribonucleic acid
snoRNA	small nucleolar ribonucleic acid
snRNA	small nuclear ribonucleic acid
ssDNA	single-stranded deoxyribonucleic acid
T4	threonine 4
TBP	TATA-binding protein
TBS	Tris Buffered Saline
tCDK	transcriptional cyclin-dependent kinase
tRNA	transfer RNA
TSS	transcription start site
V	volts
WT	wild-type
μl	microliters
μM	micromolar

Abstract

The family of cyclin-dependent protein kinases (CDKs) drives many aspects of eukaryotic cell function. The literature suggests a binary distinction between cell-cycle CDKs and transcriptional CDKs (tCDKs), but increasing evidence points to the functional overlap between these groups. Considering the need for temporal coordination of the cell cycle and the transcription cycle, it can be speculated that the CDKs might play a critical role in linking the two processes. Cyclin-dependent kinase 1 (CDK1) is a highly conserved essential regulator of mitosis, with recognised transcriptional functions revolving around the mitotic repression of gene expression. Recently, CDK1 was shown to act as a transcriptional repressor in early mouse embryos, where its heightened activity counteracts the differentiation of stem cells. CDK1 overexpression is also observed in several types of cancer. The work described in this thesis aimed to test the hypothesis that CDK1 dysregulation contributes to transcriptional re-programming during tumorigenesis by imposing a stem cell-like gene expression programme. An elegant method of examining this role is through the use of cell lines expressing an ATP analogue-sensitive CDK1 (AS-CDK1), which allows for rapid and specific inhibition of the kinase. This project details the characterization of the HeLa AS-CDK1 experimental model, and the development of two new cell lines for studying CDK1 function, HCT116 CDK1^{WT/AS} and HEK293 AS-CDK1. A novel method of positive selection for AS-CDK1 clones is also described. HeLa AS-CDK1 was used for the initial analysis of the transcriptional effects of CDK1 inhibition in cancer cells. Using EU staining and chromatin-associated RNA-seq approaches, it was shown that the inhibition of HeLa AS-CDK1 cells with the ATP analogue 1-NM-PP1 results in the upregulation of gene expression. The examination of the two most dysregulated genes from the analysis revealed that their regulation also changes in unmutated HeLa cells, exposing previously unexplored off-target effects of 1-

NM-PP1. The regulation of these genes by 1-NM-PP1 was then tested in the new cell lines, and through the use of other CDK1 inhibitors. Treatment of HEK293 AS-CDK1 cells with 1-NM-PP1 resulted in a global decrease in transcription but an upregulation of several dozen protein-coding genes. Subsequent analysis of the upregulated genes identified several transcription factors as candidates for involvement in mediating the transcriptional effects of CDK1 across different cell types, after their binding motifs were found to be enriched upstream of the transcription start site of the dysregulated genes. Together, the data collected in this study explored the potential of AS kinase technology in investigating CDK1 function in transcription, and provided crucial insight into the off-target effects of 1-NM-PP1, underlining the necessity of having wild-type controls while studying AS kinases. Although the extent of gene dysregulation following short CDK1 inhibition was limited, the gathered evidence pointed to previously unknown ways in which CDK1 could govern transcription. The newly identified motifs and their respective transcription factors indicate novel mediators of CDK1's transcriptional functions, with potentially significant biological effects. The elucidation of the molecular mechanisms behind CDK1 regulation of these transcription factors will surely remain an intriguing avenue of research.

1.INTRODUCTION

1.1. Cyclin-dependent kinases

Cyclin-dependent kinases (CDKs) are a conserved eukaryotic family of heterodimeric serine/threonine protein kinases, whose catalytic activity is entirely dependent on association with a specialized regulatory cyclin subunit and the phosphorylation status of the CDK activating domain, the T-loop (Brown et al., 1999; Malumbres, 2014; Pines, 1995; Pluta et al., 2023; Wood & Endicott, 2018). CDKs have been implicated in numerous processes in the cell, the most prominent being the cell cycle and the transcriptional cycle (Lim & Kaldis, 2013; Palmer & Kaldis, 2020) (**Figures 1.1., 1.2.**). These kinases function through dynamic and generally reversible phosphorylation of a wide range of targets, and 20 proteins belonging to this family (CDK1 to CDK20) have been identified in metazoans (Kalra et al., 2017; Malumbres et al., 2009; Marak et al., 2020; Roskoski, 2019) (**Tables 1.1., 1.4., 1.7.**). They can be further subdivided based on amino acid sequence and function into eight sub-families (Cao et al., 2014; J. Liu & Kipreos, 2000; Malumbres, 2014) (**Figure 1.3.**).

CDKs in general are relatively long-lived proteins and their levels do not markedly fluctuate through the cell cycle. However, the levels of their cyclin partners can vary over a wide range (Evans et al., 1983; Malumbres, 2014; Pines, 1995). Due to the crucial role of the CDK-cyclin complexes in governing cell behaviour, dysregulation of their activity often contributes to disease. As CDKs are involved in guarding both uncontrolled proliferation and genomic instability, they have become increasingly appealing as therapeutic targets in anticancer treatment (Malumbres & Barbacid, 2009).

1.2. CDKs drive the cell cycle

1.2.1. Overview of the mammalian cell cycle

The cell cycle is the name given to the series of organised events that lead to the duplication of cell's genetic material and division into two daughter cells (Alberts et al., 2002). The individual processes occurring in the cell define the distinctive phases of the cell cycle: Gap 1 (G1), S phase, Gap 2 (G2), and M phase (**Figure 1.1.**). The complete duplication of the cell's DNA ensues during the S phase, named S for synthesis. The M phase, named after mitosis, is characterised by the condensation of chromosomes, segregation of the genetic material during nuclear division (mitosis), and finally cytoplasmic division (cytokinesis), to form two genetically-identical daughter cells. The two remaining phases are interspaced between the aforementioned ones, with G1 occurring before S and G2 before M. During G1 and G2, the cell has the time to monitor its internal and external environment before it commits to the events of S or M phases. Additionally, the cell contents get copied and the cell continues to grow to ensure that enough protein mass can get distributed to its daughter cells. If the conditions for division are not met, the cell can exit G1 into a quiescent G0 (G zero) phase, where it can remain indefinitely, sometimes for many years. Collectively, the G1, S, and G2 phases are known as the “interphase”.

The timing of the cell cycle can vary between different cell types. In many mammalian cells, the cell cycle lasts approximately 24h, with interphase occupying 23h, and 1h left for the events of mitosis. In budding yeast, the cell cycle may be completed within 90min. The duration of the cell cycle can vary even within a single organism, with early mammalian embryo cell divisions shortening to less than 30min, achieved by omitting the G1 and G2 phases and massively accelerating S phase. Despite these differences in

cell cycle duration, the fundamental cell cycle control system remains relatively conserved throughout eukaryotic evolution (D. O. Morgan, 2007). The correct progression through the cell cycle is guarded by a series of checkpoints, which can stop the advancement of the next cell cycle phase if errors occur or all the necessary steps from the previous stage have not yet been completed (**Figure 1.1.**). Continuous unregulated proliferation can lead to the cells acquiring a cancerous phenotype, or to the accumulation of errors resulting in reduced cell viability and cell death (Hanahan & Weinberg, 2011; H. Wang et al., 2015).

1.2.2. Cell-cycle CDKs

The orderly replication and segregation of chromosomes during the cell cycle is governed by the sequential activation and inactivation of distinct CDK-cyclin complexes. Three CDK-dependent cell cycle transitions have been identified: the initiation of chromosomal DNA replication, the initiation of mitosis, and cell cycle commitment in the G1 phase, which in mammals is termed the restriction (R) point (**Figure 1.1.**).

The orchestration of mitosis by CDK1 together with cyclin B is an essential aspect of eukaryotic cell cycle control conserved from fungi and plants to vertebrates. The universality of this function is underscored by the fact that human CDK1 was first identified through its capacity to complement loss of Cdc2 activity in fission yeast (M. G. Lee & Nurse, 1987). Interestingly, genetic inactivation of CDK1 in yeast and mammals not only blocks entry into mitosis but also de-represses DNA replication, leading to endoreduplication of nuclear DNA (Hayles et al., 1994; Itzhaki et al., 1997). Germ-line deletion of CDK1 or cyclin B in mice is associated with very early embryonic lethality (Santamaría et al., 2007), whereas tissue-specific deletion of CDK1 in hepatocytes results in mitotic arrest, endoreduplication and cellular hypertrophy (Diril et al., 2012). In

mammals, the essential role of CDK1 is supplemented by three ‘interphase’ CDKs – CDK2, CDK4, CDK6 (**Figure 1.1.**). These four kinases can bind to ten individual cyclins, which in turn belong to four classes (A-, B-, D- and E-cyclins) (Malumbres & Barbacid, 2009). Progression through the R point, beyond which cells have reduced dependency on mitogenic signalling, is attributed to activation of CDK4/6-cyclin D complexes, while CDK2-cyclin A/E activity is important for initiation of DNA replication (Malumbres, 2014). Each of the cell cycle CDKs is susceptible to inhibition by proteins of the Cip/Kip and/or Ink4 families, providing an additional level of regulation (Jeffrey et al., 2000; Kiyokawa & Koff, 1997; Lim & Kaldis, 2013; Pavletich, 1999; Sherr & Roberts, 1999). Further, all CDKs share two inhibitory phosphorylation sites in their ATP-binding pocket, which are targeted by negative regulator kinases, and an activating T-loop motif (Gould & Nurse, 1989; Lim & Kaldis, 2013; Loyer et al., 2005; Norbury et al., 1991).

Yeast CDK1 (originally named Cdc28 in budding yeast and Cdc2 in fission yeast) sequentially partners different cyclin proteins and can support all cell cycle functions (Nurse, 1990). Similarly, mouse development can proceed until mid-gestation with CDK1 alone when CDKs 2, 4 and 6 are absent (Malumbres & Barbacid, 2009; Santamaría et al., 2007; Satyanarayana et al., 2008; Satyanarayana & Kaldis, 2009). The core cell cycle machinery is therefore conserved throughout eukaryotic evolution, despite the appearance of additional cell-cycle CDKs in more recent evolutionary time (**Table 1.1.**).

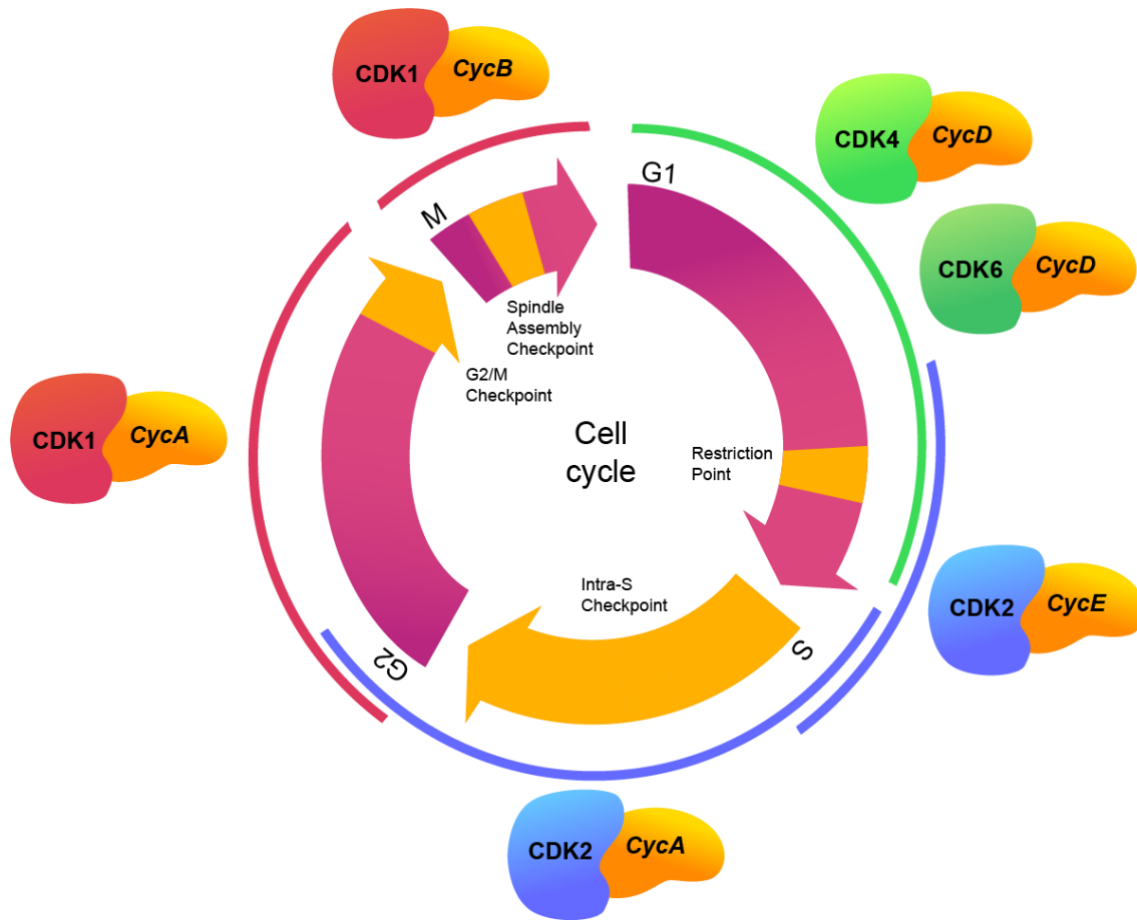


Figure 1.1. CDKs in the mammalian cell cycle. Different CDK-cyclin complexes drive the individual phases of the cell cycle. Chromosomal DNA is replicated during the synthesis (S) phase; replicated chromosomes are segregated to daughter nuclei in M phase (mitosis). Gap phases G1 and G2 of variable duration may separate M from S and S from M, respectively. Mitogen-dependent cells become committed to a new cycle and escape the requirement for mitogen signalling at the restriction (R) point in G1. In addition, progression through the cell cycle may be delayed by the presence of abnormal DNA structures at the point of DNA replication origin firing (the intra-S checkpoint) or at the G2/M transition (the G2/M checkpoint), or by unattached chromosomes at the spindle attachment checkpoint in mitosis.

1.3. Transcriptional CDKs drive the transcription cycle

1.3.1. Overview of mammalian transcription of protein-coding genes

The process of transcription is a tightly-regulated mechanism at the beginning of gene expression, during which genetic information embedded in DNA gets rewritten into RNA molecules. It is governed by the RNA polymerase enzymes, and in eukaryotes there exist 3 distinct RNA polymerases that transcribe different classes of genes (Roeder & Rutter, 1969). RNA polymerase I (pol I) transcribes 18S and 28S ribosomal RNA, RNA polymerase II (pol II) – mRNA, snRNA, snoRNA, and microRNA, and RNA polymerase III (pol III) - 5S rRNA and tRNA (Thomas & Chiang, 2006). Pol II is perhaps the most well-defined of the three, as it regulates the transcription of protein-coding genes.

In eukaryotes, the transcription of protein-coding genes by RNA polymerase II can be characterised as a cycle of initiation, pausing, elongation, and termination (**Figure 1.2.**) (Shandilya & Roberts, 2012). In short, transcription initiation starts by the recruitment of the preinitiaton complex (PIC), which includes a hypo-phosphorylated pol II with an assembly of general transcription factors, to the promoter region of the gene. After interaction with the Mediator complex, PIC transforms into an open complex and the removal of the Mediator allows the escape of pol II from the promoter. Promoter escape marks the first step of transcription elongation, and is followed by promoter-proximal pausing (Saunders et al., 2006). The pause occurs in the vicinity of the 5' end of the gene and acts as a rate-limiting checkpoint before productive elongation. After the release of the pause, pol II continues to synthesise the RNA transcript, while interacting with additional elongation factors that improve its processivity or that regulate co-transcriptional RNA processing events (F. X. Chen et al., 2018). Finally, transcription termination occurs when pol II encounters a DNA sequence signalling the 3' end cleavage

(Kuehner et al., 2011). The newly-synthesised RNA transcript is released for maturation and further processing, and gets exported into the cytoplasm for translation. The transcription complex with pol II also dissociates from the DNA, after which the enzyme is recycled and can be used for new subsequent cycles of transcription (Saunders et al., 2006).

Unsurprisingly, each of the transcriptional phases has to be accurately-timed and requires a different set of transcriptional machinery-associated factors (Buratowski, 2009; Shandilya & Roberts, 2012; Svejstrup, 2004; Thomas & Chiang, 2006; Zaborowska et al., 2016). The regulation of transcription can be performed by modifying pol II directly, acting on the individual transcription factors, or by altering the chromatin environment (Saunders et al., 2006). Additionally, the regulatory factors can act in a general fashion, or their function might be tied only to a specific subset of genes.

1.3.2. Transcriptional CDKs

The family of CDKs is also involved in governing the individual steps of transcription. The best-characterized transcriptional (t)CDKs to date are CDK7, CDK8, CDK9, CDK12, CDK13, and the CDK8 paralogue CDK19 (**Table 1.4.**). These CDKs not only coordinate the transcription cycle, but also regulate co-transcriptional processes, such as 5' end capping, splicing, 3' end cleavage and polyadenylation, termination and regulation of the chromatin landscape (Chou et al., 2020; Fisher, 2017; Svejstrup, 2004; Zaborowska et al., 2016). One well-studied target of the tCDKs is the carboxy-terminal domain (CTD) of the largest pol II subunit, RPB1. In humans, the CTD comprises 52 heptad repeats with the consensus motif Tyr₁Ser₂Pro₃Thr₄Ser₅Pro₆Ser₇, with the serine and threonine residues subject to dynamic and reversible phosphorylation by the tCDKs (Bartkowiak & Greenleaf, 2011; Galbraith et al., 2019; Zaborowska et al., 2016) to produce patterns of

phosphorylation termed the CTD code (Buratowski, 2003). CTD phosphorylation plays a major role in the recruitment of transcription and RNA processing factors at the right point of the transcription cycle (Harlen & Churchman, 2017; Meinhart et al., 2005; Zaborowska et al., 2016). However, each tCDK has a range of other targets, which are in the process of being fully characterized (Fan et al., 2020; Krajewska et al., 2019; Larochelle et al., 2012; Rimel et al., 2020; Sansó et al., 2016; Tellier et al., 2020, 2022).

In contrast to cell cycle CDKs, tCDKs usually have a single cyclin partner and are recruited to the transcriptional machinery as part of larger protein complexes (Galbraith et al., 2019) (**Table 1.4.**). For example, CDK7 associates with cyclin H and MAT1 to form the ternary kinase module of the TFIIF complex, a general transcription factor (TF) required for early steps of RNA pol II transcription (Glover-Cutter et al., 2009; Maldonado & Reinberg, 1995; Rimel & Taatjes, 2018; Roy et al., 1994). CDK8, together with its paralogue CDK19, form the mediator complex kinase module through binding to cyclin C, MED12 and MED13 (Luyties & Taatjes, 2022). Together, this complex can regulate pol II activity through direct interaction with the transcriptional machinery, with activating or repressing functions depending on the context (Dannappel et al., 2019; Fant & Taatjes, 2019; Luyties & Taatjes, 2022; Parua & Fisher, 2020; D. Wu et al., 2021). Further, CDK9 and cyclin T make up positive transcription elongation factor b (P-TEFb), which associates with a variety of transcription factors and coactivators, and forms part of the Super Elongation Complex (Bacon & D'Orso, 2019; Egloff, 2021; Luo et al., 2012). P-TEFb kinase activity releases pol II from a promoter-proximal pause into productive elongation by phosphorylating negative elongation factors associated with pol II (Jonkers & Lis, 2015; Parua et al., 2018; Parua & Fisher, 2020; Zaborowska et al., 2014). Finally, the most recent kinases described as *bona fide* tCDKs are CDK12 and CDK13. CDK12 is required for efficient elongation and pre-mRNA 3' end formation

through recruitment of elongation and polyadenylation factors (Bösken et al., 2014; S. H. Choi et al., 2020; Greenleaf, 2019; Lui et al., 2018; Tellier et al., 2020). Although both bind to cyclin K and are redundant for some functions, such as the regulation of DNA damage response pathway (Fan et al., 2020; Greenleaf, 2019; Krajewska et al., 2019), the literature on the role of CDK13 in transcription is less extensive. As its individual role is being gradually characterised, it is becoming clearer that despite their structural similarities, the function of CDK13 is distinct from CDK12 and they can affect different sets of genes (Fan et al., 2020; Greifenberg et al., 2016; K. Liang, Gao, et al., 2015). However, both CDK12 and CDK13 have been shown to regulate splicing and are able to phosphorylate the CTD on Ser2 and Ser5 (H. H. Chen et al., 2007; H.-H. Chen et al., 2006; Galbraith et al., 2019; Panzeri et al., 2013; Zaborowska et al., 2016).

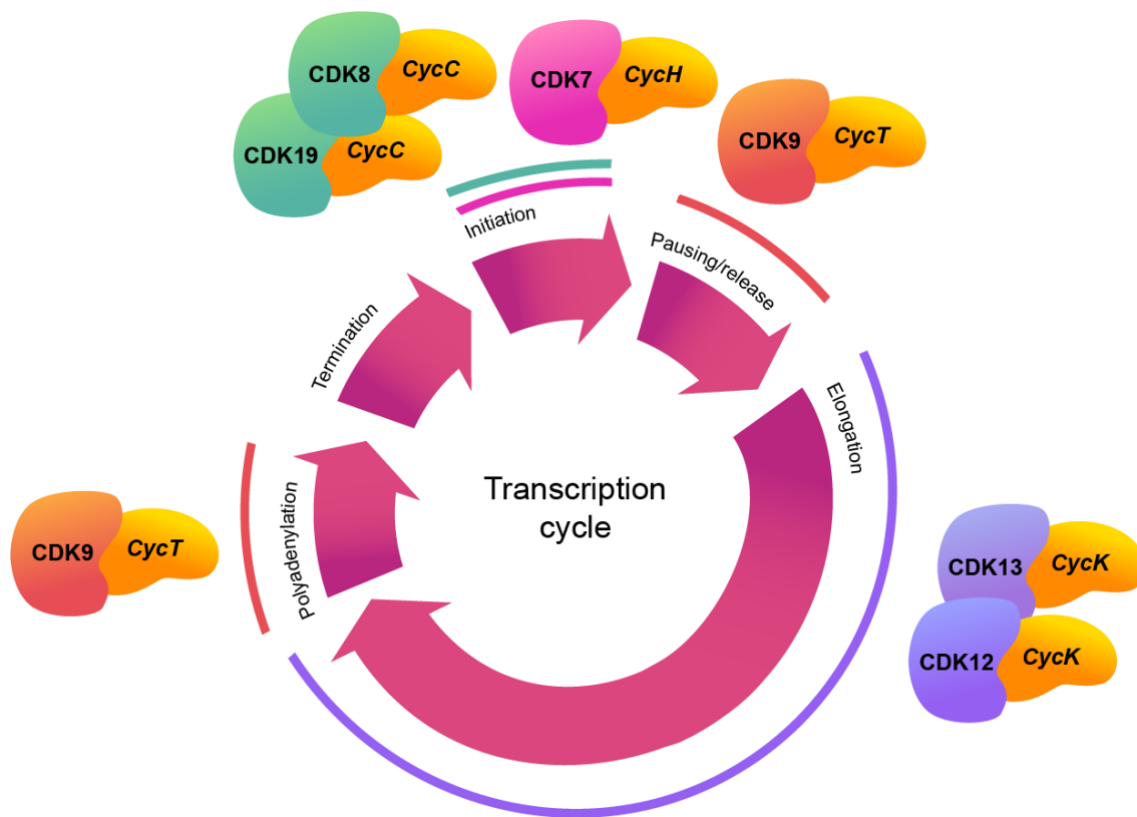


Figure 1.2. CDKs in transcription of mammalian protein-coding genes. Individual CDK-cyclin complexes are implicated in driving the sequential stages of transcription – initiation, pausing and release, elongation, polyadenylation and termination. The replication-dependent histone genes have a specialized RNA 3' processing signal rather than a poly(A) site but the cycle is thought to be similar (Marzluff & Koreski, 2017). The transcriptional CDKs influence transcription by phosphorylating the carboxy-terminal domain of RNA polymerase II (pol II) and other members of the elongation complex. Additionally, CDK8/CDK19 form part of the mediator complex, which can promote or repress transcription.

1.4. CDKs have roles outside the cell cycle and transcription

CDKs are implicated in a variety of cellular processes in addition to the cell or transcription cycles. This is true both for metazoans and yeast, with CDK1 alone implicated in DNA replication, cell morphogenesis and polarity, genome stability, telomere maintenance, pheromone signalling, mitochondrial bioenergetics, and positive regulation of mRNA translation (Enserink & Kolodner, 2010; Haneke et al., 2020; Hochegger et al., 2007; Xie et al., 2019). CDKs have also been recently found to mediate inflammatory responses in mammals (Sundar et al., 2021). They also govern parts of the DNA damage response pathways, including DNA repair and damage checkpoint signalling (Palmer & Kaldis, 2020). Interestingly, CDKs may even perform functions independent of their kinase activity: CDK6 was demonstrated to upregulate the expression of p16INK4a and VEGF-A, while CDK1 has been suggested to act as an adaptor protein during the recruitment of the proteasome (Kollmann et al., 2013; V. P. C. C. Yu et al., 2005).

A novel and intriguing area of research focuses on the roles of CDKs in stem cell biology (Jirawatnotai et al., 2020), which may also uncover new ways in which CDKs are implicated in cancer. For example, there are similarities between undifferentiated stem cells and malignant progenitor cells which are relatively undifferentiated but which give rise to more differentiated progeny within the tumour. CDK8 is implicated in cancer cell de-differentiation and helps to maintain stem cell pluripotency due to regulation of the Myc proto-oncogene (Adler et al., 2012; Peyressatre et al., 2015). Similarly, CDK2 seems to play a role in maintaining a pluripotent neural progenitor cell pool (Caillava et al., 2011; Chi et al., 2020). Relevant to this, CDK2 knockout mice are viable, but their neural progenitor cells display impaired proliferation in adults (Satyanarayana & Kaldis, 2009).

1.5. Less-studied CDKs

Additional CDKs have less well-understood functions (**Table 1.7.**). However, it is worth noting that most of them have reported roles in both cell cycle and transcription (Chou et al., 2020; Kasten & Giordano, 2001; Lim & Kaldis, 2013; Loyer & Trembley, 2020; Malumbres & Barbacid, 2005, 2009; Trembley et al., 2003; D. Zheng et al., 2008) (**Table 1.7.**). For instance, apart from its involvement in transcription and splicing (Chohan et al., 2018; Dickinson et al., 2002; Hu et al., 2003; Loyer & Trembley, 2020; Malumbres & Barbacid, 2009; Trembley et al., 2003), CDK11 is required for transcription of replication-dependent histone genes during S phase, and depletion of this kinase induces accumulation of cells in G1 (Gajdušková et al., 2020). A separate CDK11p58 isoform functions at the G2/M transition (Hu et al., 2007; Petretti et al., 2006). The roles of these kinases only emphasise that CDKs are versatile and not always easily pigeonholed.

1.6. The structural complexity of CDKs and cyclins

Throughout evolution, the CDK family has maintained some important features among its members. A protein kinase can be classified as a CDK based on its structural similarities with the canonical CDKs, including the presence of a PSTAIRE-like cyclin-binding element in the catalytic domain (Lim & Kaldis, 2013; Loyer & Trembley, 2020; Malumbres, 2014; Malumbres et al., 2009; Pines, 1995). Cyclins, by comparison, share more limited amino acid sequence similarity (Wood & Endicott, 2018). They were first identified by and named after their marked cell cycle-dependent degradation on exit from mitosis and subsequent resynthesis (Evans, 1983). It is now clear that this cyclical behaviour is a feature of a minority of cyclins; members of the family are more generally identified through one or two repeats of a ‘cyclin box’ motif which assumes a fold comprising five alpha helices (Pines, 1995; Wood & Endicott, 2018).

The structural characterisation of the CDK family began with studies on the CDK2-cyclin A complex in various activation states and was used as an overarching model for CDK activation and regulation (Echalier et al., 2010; Wood & Endicott, 2018). It has now become apparent that the CDK-cyclin complexes are highly variable in their 3D organisation, and that this original standard is not applicable to many of the family members (Peissert et al., 2020). In addition, the size of the CDKs can range from just 297 amino acid residues in CDK1, to 1512 residues in the case of CDK13 (Malumbres, 2014; Malumbres & Barbacid, 2005; Marqués et al., 2000).

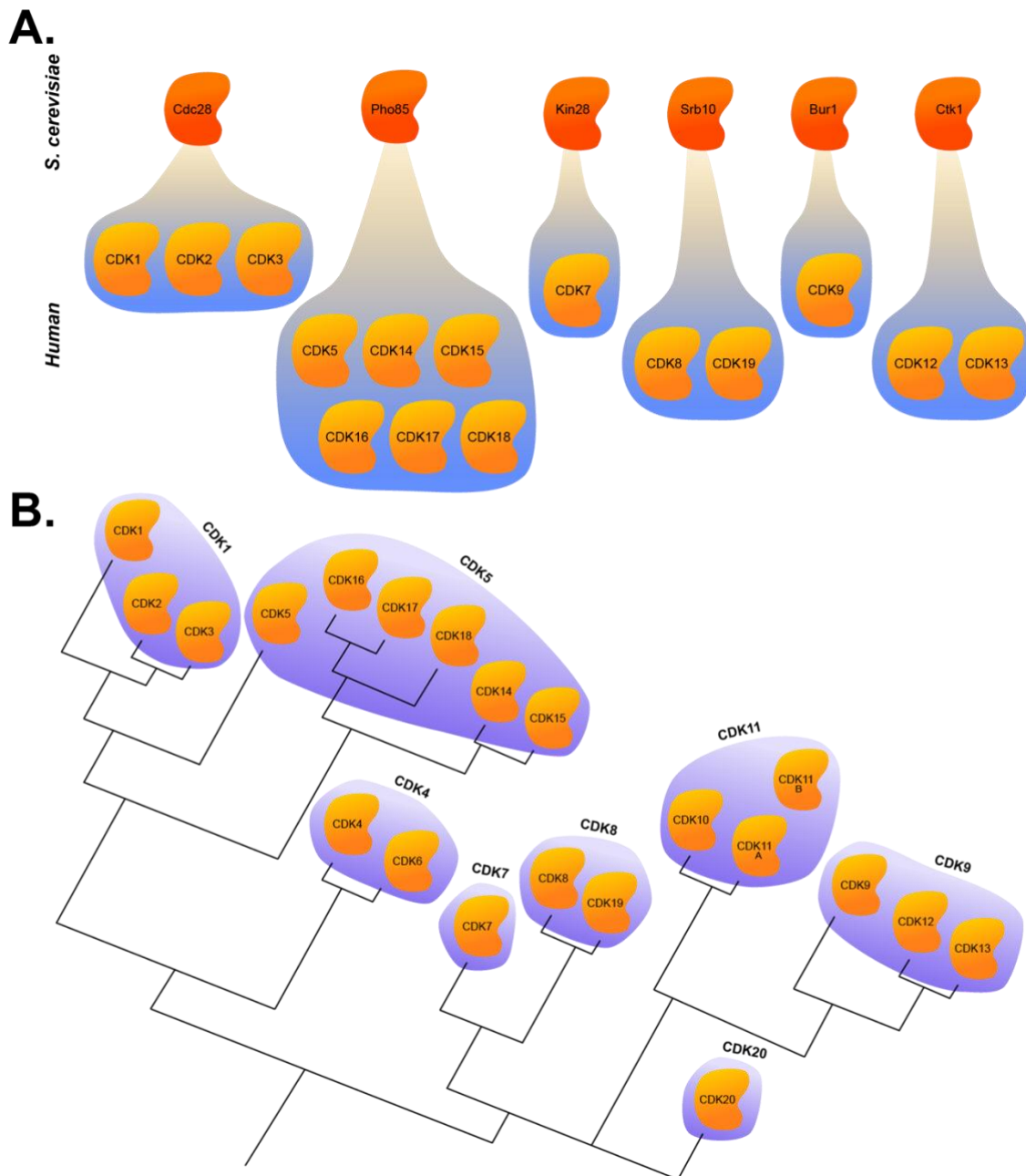


Figure 1.3. Evolution of CDKs. **A.** *Saccharomyces cerevisiae* possesses the archetypal cell-cycle CDK Cdc28, as well as Pho85, and four transcriptional CDKs – Kin28, Srb10, Bur1, Ctk1. These kinases have 15 human CDK homologues. It is important to note that not all of the human CDKs have budding yeast homologues. **B.** The relationships between human CDKs, based on their amino acid sequences, identify eight subfamilies.

1.7. The cell cycle versus the transcription cycle: a false CDK dichotomy?

1.7.1. Half a century of CDKs

The archetypal CDKs were discovered in the 1970s and 1980s, through genetic analysis of the yeast cell cycle (Hartwell et al., 1973; D. O Morgan, 1995; Nurse et al., 1976), while cyclins were first identified through investigations of cell cycle-regulated protein synthesis in sea urchin embryos (Evans et al., 1983). With the realisation that the mitosis-promoting activity of CDK1 depends on its association with cyclins, the term ‘cyclin-dependent kinase’ was first coined in 1991, at which point it was widely assumed that other enzymes of this class might also be involved in cell cycle regulation (Draetta et al., 1989; Labbe et al., 1989; Malumbres & Barbacid, 2005; Nurse, 1990). With the ever-increasing availability of cDNA and ultimately whole-genome sequences, it quickly became apparent that there is a multiplicity of CDKs, even in single-celled eukaryotes. Budding yeast have six CDKs and 23 cyclins, while mammals have at least 20, along with a complement of approximately 30 distinct cyclins (Cao et al., 2014; Ercan et al., 2021; D. Huang et al., 2007; King et al., 1996; J. Liu & Kipreos, 2000; Malumbres, 2014) (**Tables 1.1.-1.9.**).

Parallel but independent lines of investigation led to the identification of CDK-cyclin complexes involved in transcriptional regulation, notably CDK7-cyclin H in TFIIF (Akoultchev et al., 1995; Drapkin & Reinberg, 1994; Shiekhata et al., 1995) and CDK9-cyclin in T/P-TEFb (Graña et al., 1994; Marshall et al., 1996; J. Peng et al., 1998; Peterlin & Price, 2006). These distinct routes to the identification of cell-cycle CDKs on the one hand and tCDKs on the other inevitably suggested a binary distinction between CDKs

based on these broad biological functions, but it is becoming increasingly clear that such a classification is overly simplistic, as discussed below.

1.7.2. Regulation of transcription through the cell cycle

During the cell cycle, specific transcriptional programmes ensure its directionality and proper timing, creating an oscillating pattern of gene expression (Gottesfeld & Forbes, 1997; Johnson & Holland, 1965; Ramos-Alonso et al., 2023; Segil et al., 1996; Zaret, 2014). A significant change occurs at the onset of M phase, when transcription is largely silenced. This mitotic repression of all RNA polymerases is a well-documented phenomenon (Gottesfeld & Forbes, 1997; Johnson & Holland, 1965; Parsons & Spencer, 1997). The main driving force behind mitotic silencing is the loss of chromatin accessibility, which is achieved through chromatin condensation during prophase, and/or through a range of histone modifications (Gottesfeld & Forbes, 1997; Ramos-Alonso et al., 2023). The second essential feature of transcriptional repression during mitosis is the displacement of transcription factors from chromatin, including even the actively engaged pol II (Gottesfeld & Forbes, 1997; K. Liang, Woodfin, et al., 2015; Timmers & Verrijzer, 2017).

Mitotic silencing requires the hyperphosphorylation of pol II, and release of the general factors TFIID and TFIIH from core promoters (Loyer et al., 2005; Loyer & Trembley, 2020; Segil et al., 1996). Thus, the phosphorylation status of pol II not only changes through the transcriptional cycle, but with the cell cycle as well. A low-level transcriptional programme remains active during mitosis, keeping a subset of genes ready for re-entry into interphase (Palozola et al., 2017; Timmers & Verrijzer, 2017; Zaret, 2014). Thus, some general transcription factors, including TFIID, can be retained on some genes, in a process known as mitotic ‘bookmarking’ (Y. Liu et al., 2017; Teves et al.,

2016). General transcription is subsequently re-established in telophase (Gottesfeld & Forbes, 1997). With the clear need for communication between the cell and transcription cycles to ensure they occur in the right order, the emerging evidence of interconnected functions of the cell-cycle and tCDKs could point to their key role in coupling these processes.

1.7.3. Cell-cycle CDKs with direct transcriptional roles – CDK1 as a tCDK?

Progression through the consecutive stages of cell division requires activation of distinct transcriptional programmes; factors expressed at a particular stage of the cell cycle will activate processes and proteins important for the next phase, thus creating a one-way movement. The ‘cell-cycle’ CDKs play therefore important roles in transcriptional control. For example, in yeast the archetypal cell-cycle kinase CDK1 activates transcriptional programmes required for maintaining the necessary unidirectionality of the cell cycle (Chymkowitch et al., 2012; Cosma et al., 2001; Enserink & Kolodner, 2010). Depending on the cell-cycle stage, budding yeast Cdc28 communicates with distinct complexes and proteins to perform its function. Although the direct transcriptional effects of Cdc28 still need to be fully characterised, it is known that, for instance, in G2 Cdc28 stimulates the expression of CLB2 cluster of genes through phosphorylation of forkhead transcription factor Fkh2 and rate-limiting transcriptional transactivator Ndd1 (Cho et al., 2001; Darieva et al., 2003; Pic-Taylor et al., 2004; Reynolds et al., 2003; Wittenberg & Reed, 2005), while in M phase it regulates the MCM, SIC1, MAT, and PHO gene clusters by phosphorylating transcription factors such as Swi5, Ace2 and Pho2 (Archambault et al., 2004; Cho et al., 2001; Jans et al., 1995; C. Liu et al., 2000; Moll et al., 1991; O’Conalláin et al., 1999). However, the best characterised impact of Cdc28 on transcription is during G1; here Cdc28 acts on genes

bound by the transcription factors Mlu1-box binding factor (MBF) and Swi4/6-dependent box-binding factor (SBF), which encode proteins involved in DNA repair and cell cycle progression (Enserink & Kolodner, 2010). Cdc28 recruits pol II, TFIIB, and TFIIH to promoter regions of these genes through phosphorylation of Whi5, which then dissociates from SBF leading to activation of genes responsible for cell cycle entry (Cosma et al., 2001; De Bruin et al., 2004; Enserink & Kolodner, 2010). Notably, Cdc28 is able to directly phosphorylate the pol II CTD at S5 at the SBF target genes, through a positive feedback loop with Kin28 (TFIIH) (Enserink & Chymkowitch, 2022; Kõivomägi et al., 2021; Ubersax et al., 2003). However, in mutant yeast cells which do not express S phase or mitotic cyclins, almost 70% of cell cycle-regulated genes are still activated on time, suggesting an independent transcriptional oscillator functions alongside Cdc28 (Haase & Reed, 1999; Orlando et al., 2008; Simmons Kovacs et al., 2008).

Apart from regulation of the cell cycle-dictated transcriptional programmes, there is growing evidence of the involvement of Cdc28 in governing basal transcription. In *S. cerevisiae*, Cdc28 activity regulates a number of highly-expressed housekeeping genes, such as *PMA1* (Chymkowitch et al., 2012; Embo et al., 1982; Enserink & Chymkowitch, 2022) by phosphorylating the S5 residue of pol II to upregulate transcription and promote the recruitment of capping factors. Once again, it is able to perform this role through a mutual priming system with Kin28 (Chymkowitch et al., 2012; Cosma et al., 2001; Enserink & Chymkowitch, 2022; Kõivomägi et al., 2011). Cdc28 may phosphorylate the CTD not only at the S5 residue, but also at T4 and S2 *in vivo* (Nemec et al., 2019). Additionally, Cdc28 can regulate transcriptional processes in a more indirect manner; for instance, it phosphorylates the NuA4 chromatin modifier leading to increased Lys14 acetylation on histone Htz1 and increased transcription (Enserink & Kolodner, 2010; Ficarro et al., 2002; Fiedler et al., 2009).

Intriguingly, there appears to be a marked difference in the effect of Cdc28 on the transcriptional machinery in budding yeast and the effect of CDK1 on transcription in metazoans. In *S. cerevisiae*, the onset of M-phase does not induce a dramatic shutdown of transcription; this may reflect the limited extent of mitotic chromatin condensation in this organism, which is also unusual by comparison with many other eukaryotes in that the key mitotic event of microtubular spindle assembly overlaps with S phase. Cdc28 usually serves to upregulate transcription, whilst CDK1 has a seemingly opposite function in human cells. The reasons behind this are not yet fully understood, but a possible explanation is that so far CDK1 studies were focused mainly on the transcriptional silencing that occurs during the M phase of the mammalian cell cycle, while the transcriptional actions of CDK1 in interphase have been largely overlooked (Enserink & Chymkowitch, 2022).

It is clear that in human cells, as in yeast, CDK1 engages primarily in the control of cell-cycle transcriptional programmes to regulate cell division. For instance, during interphase, the CDK1-cyclin A complex phosphorylates E2F to induce transcription of S-phase genes, and its inhibition leads to E2F transcription factor-dependent cell death (Shapiro, 2006). Although the CDK1-cyclin B complex is thought to be mostly cytoplasmic during interphase, effects on transcription may be carried out by CDK1 partnered with a different cyclin, or simply indirectly (Gavet & Pines, 2010b, 2010a; Maryu & Yang, 2022). However, the best characterised transcriptional role of CDK1 is that of transcriptional silencing during M phase of the metazoan cell cycle. Unlike in budding yeast, where mutual phosphorylation between Cdc28 and Kin28 upregulates transcription, in human cells, CDK1 inhibits CDK7 through phosphorylation of the Ser164 residue in its T-loop, therefore suppressing TFIIH-dependent phosphorylation of the CTD (Akoulitchev & Reinberg, 1998; Cisek & Corden, 1989; Guo & Stiller, 2004;

Kobor & Greenblatt, 2002; Long et al., 1998; Loyer & Trembley, 2020). Other direct mitotic phosphorylation targets of CDK1 include several general transcription factors, including TBP and TBP-associated factors (Enserink & Chymkowitch, 2022; Long et al., 1998). Notably, CDK1 has also been shown *in vitro* to directly phosphorylate the CTD itself, on the S2 and S5 residues (Enserink & Chymkowitch, 2022; Gebara et al., 1997; Y. X. Xu et al., 2003; Y. X. Xu & Manley, 2004; J. Zhang & Corden, 1991b, 1991a). This phosphorylation is speculated to inhibit recruitment of pol II to genes as part of the preinitiation complex (PIC). It is worth mentioning that the very first kinase demonstrated to phosphorylate the CTD was murine CDK1, although *in vitro* studies of CTD phosphorylations often cannot be replicated in other systems (Chymkowitch & Enserink, 2013; Cisek & Corden, 1989; Pines, 1995).

Intriguingly, an unexpectedly high level of CDK1-cyclin A activity in mouse embryonic stem cells phosphorylates multiple chromatin-associated proteins to maintain the stem cell epigenetic landscape, and its inhibition leads to stem cell differentiation (Michowski et al., 2020). It remains to be seen if hyperactive CDK1 is similarly involved in the maintenance of cancer stem cell populations, but this discovery clearly underlines that cell-cycle CDKs can regulate functions previously attributed purely to tCDKs (Sánchez-Martínez et al., 2019). It also serves as further example of the involvement of CDKs in stem cell biology (see Section 4). In prostate cancer, ABCC5-bound CDK1 has been found to directly phosphorylate the AR transcription factor to stimulate its activity, further highlighting that CDK1 might possess non-mitotic transcriptional roles in different cellular contexts (Ji et al., 2021). CDK1 may also carry out cell cycle-dependent phosphorylation of splicing factors and the polyadenylation machinery (Colgan et al., 1998; Okamoto et al., 1998). It is even conceivable that the key role of ancestral CDK1 lay in transcriptional regulation, and that functions of CDK1 in the more direct regulation

of the mitotic apparatus evolved more recently (Chymkowitch & Enserink, 2013; Enserink & Chymkowitch, 2022).

1.7.4. The relationship of interphase CDKs to transcription

The transcriptional roles of interphase CDKs in metazoans are also becoming increasingly evident. Progression through the R point requires CDK4/6 to phosphorylate the retinoblastoma protein (Rb), which is a broad-specificity transcriptional repressor, and Rb-related pocket proteins p107 and p130 (Dymlacht, 1997). In addition, substrates of CDK4/6 include other transcription factors, such as Smads (Anders et al., 2011; Cobrinik, 2005; Goel et al., 2018; Malumbres & Barbacid, 2001). CDK4/6-dependent phosphorylation maintains activity of the Forkhead Box M1 (FOXO1) transcription factor, thereby preventing cells from entering senescence (Anders et al., 2011). CDK4 was further found to phosphorylate c-Jun to form active AP-1 transcription complexes in non-dividing immune cells (Vanden Bush & Bishop, 2011). CDK6 is also implicated in influencing transcription during angiogenesis by upregulating expression of pro-angiogenic VEGF-A; and in pro-inflammatory signalling where it phosphorylates and so activates the p65 subunit of NF- κ B (Handschiek et al., 2014; Kollmann et al., 2013). Even further, CDK6 was found to phosphorylate and inactivate transcription factors which drive cell differentiation, for example in osteoblast and osteoclast cells or during neurogenesis (Grossel & Hinds, 2006; Pauklin et al., 2016; Urbach & Witte, 2019).

The CDK2-cyclin E complex has several transcriptional targets including Rb and the transcription factor ELK4. Activation of ELK4 through phosphorylation leads to an increase in c-fos expression, which facilitates malignant transformation in, for example, melanoma development (C. Peng et al., 2016). CDK2 also promotes cell survival in response to DNA damage through phosphorylation of the pro-apoptotic TF FOXO1,

which then relocates to the cytoplasm (H. Huang et al., 2006). Intriguingly, it has been found that CDK2 is recruited during interphase to stimulate transcription during human immunodeficiency virus 1 (HIV-1) infection (Agbottah et al., 2006; Nekhai et al., 2002; Rice, 2018). To sustain HIV-1 transcriptional elongation, CDK2 binds and phosphorylates the viral transactivator protein Tat, which in turn activates CDK7 leading to pol II clearance of the HIV-1 proviral promoter. Tat also stimulates the recruitment of CDK9, and together these kinases phosphorylate the pol II CTD, which allows for the cell cycle-dependent expression of HIV-1 (Deng et al., 2002; Nekhai et al., 2002). CDK2 may also be involved in phosphorylation of Hepadnavirus core protein C-terminal domain during human hepatitis B infection (Ludgate et al., 2012). Although CDK2 can phosphorylate the pol II CTD *in vitro*, it is unclear if it phosphorylates the pol II CTD *in vivo* outside of HIV-1 infection (Guo & Stiller, 2004; Malumbres, 2014; Palancade & Bensaude, 2003). A recent study used the analogue-sensitive kinase (AS) approach, which utilises bulky ATP analogues carrying a transferable thiophosphate (Larochelle et al., 2006, 2007; Schachter & Fisher, 2013), to identify nuclear targets of CDK2 (Chi et al., 2020). Notably, several identified substrates were chromatin-modifying proteins, such as the histone demethylase LSD1 and the histone methyltransferase DOT1L, in addition to several transcription factors, like the general TF GTF2I, or BCL11A and AF9, which are linked to cancer. This study implicates CDK2 directly in transcription regulation, which may provide an additional link to cell cycle control.

1.7.5. tCDKs with cell cycle roles

As the cell-cycle CDKs clearly have a profound influence on transcription, it is logical to consider the reciprocal effect of tCDKs on the cell cycle. A good example of a CDK with dual roles is CDK7 (**Table 1.4.**), which despite being traditionally classified as a tCDK,

was initially identified as the metazoan CDK-activating kinase (CAK), required for activation of both CDK1 and CDK2 by phosphorylating the 'T-loop' region in a way that is essential for their activity (Fisher, 2005). More recently, CDK7 was also shown to activate CDK4/6 (Schachter & Fisher, 2013) and tCDKs CDK9, 12 and 13 (Fisher, 2012; Larochelle et al., 2012; Rimel et al., 2020). Interestingly, when its central role in transcriptional regulation was first uncovered, its status as a CAK was questioned (Fisher, 2005; Harper et al., 1998). This may be due to the fact that its budding yeast ortholog, Kin28, does not perform this function (Fisher, 2005, 2019; Malumbres, 2014). Not only do *S. cerevisiae* cells possess a specialised CAK responsible for activating CDK1 (and Kin28), they also have a separate CAK which activates the tCDKs including the CDK9 ortholog Bur1 (Espinoza et al., 1998; Fisher, 2019; Ostapenko & Solomon, 2005; Yao & Prelich, 2002). In multicellular organisms, however, CDK7 is indispensable for cell proliferation and development. For example, CDK7 activity is required for cell division in *Drosophila* and *C. elegans* (Larochelle et al., 1998; Wallenfang & Seydoux, 2002). In addition, CDK7 deficiency in mice leads to early-embryonic lethality and premature aging of adult tissues with high proliferative ability, such as skin or intestinal epithelium (Ganuza et al., 2012). In human cells expressing the AS version of CDK7, CDK7 was shown to be essential for G1 phase progression, DNA replication and mitotic entry through activation of CDK4/6, CDK2 and CDK1, respectively (Bisteau et al., 2013; Larochelle et al., 2007; Schachter & Fisher, 2013). Similarly, inhibition of CDK7 with the selective inhibitor YKL-5-124 increased the number of cells in G1 and G2, with a concomitant decrease of cells in S phase (Olson et al., 2019). While CDK7 is considered constitutively active and stably expressed during the cell cycle, several mechanisms explain the timely and specific activation of the cell-cycle CDKs. Firstly, mitogenic signals can trigger CDK7 T-loop phosphorylation leading to an increase activity towards

CDK4, which in turn phosphorylates Rb to promote R point transition (Schachter & Fisher, 2013). In addition, CDK7 has a preference for cyclin-associated CDK1 but for the monomer of CDK2, which is likely to mediate the sequential activation of CDK2 followed by CDK1 (Larochelle et al., 2007; Merrick et al., 2008, 2011). Other factors, such as post-translational modifications of cyclin H or MAT1, as well as association with its partners in TFIIH, are likely to account for CDK7 substrate specificity during the cell cycle (Akoulitchiev et al., 2000; Rimel et al., 2020; Schneider et al., 2002).

Further, CDK8-cyclin C can inhibit CAK activity through phosphorylation of cyclin H *in vitro* (Malumbres & Barbacid, 2005; Szilagyi & Gustafsson, 2013). CDK8 also targets p21, a broad-specificity CDK inhibitor protein that represses cell cycle progression following exposure to a variety of stresses, including activation of the p53 tumour suppressor (Donner et al., 2007; Szilagyi & Gustafsson, 2013). CDK8 acts as a co-activator of *WAF1*, the gene encoding p21, promoting cell-cycle arrest at the R point and p21, in turn, directly stimulates the activity of CDK8, creating a potential positive feedback loop (Porter et al., 2012). Conversely, as a part of the mediator complex, CDK8 activates the β -catenin transcriptional programme, which promotes cell cycle commitment (Niehrs & Acebron, 2012; Szilagyi & Gustafsson, 2013). Although there is no evidence that CDK8/19 can influence the G2/M transition in metazoans, the CDK8 ortholog in fission yeast phosphorylates Fkh2, which controls a cluster of genes expressed at the onset of mitosis (Buck et al., 2004; Szilagyi et al., 2012). CDK8 has also been implicated in G1 cell cycle commitment in budding yeast, where the mediator complex is recruited to the SBF-controlled genes by Swi5 transcription factor, before they are acted upon by the Cdc28 (Bhoite et al., 2001; Cosma et al., 2001; Kishi et al., 2008; Szilagyi & Gustafsson, 2013). The implication of CDK8 in the restriction point is further highlighted by its role in maintaining quiescence of vulval precursor cells in *C. elegans*, which start

performing superfluous cell divisions after loss of the mediator complex (Clayton et al., 2008; Szilagyi & Gustafsson, 2013).

Despite CDK12/13 being typically classified as tCDKS, similarly to CDK7, they were first identified during cDNA screens for cell-cycle regulators (Ko et al., 2001; Kohoutek & Blazek, 2012; Marqués et al., 2000). Similarly, the gene encoding their cyclin partner, cyclin K, was first identified through its ability to complement G1 cyclin gene deletion mutants in *S. cerevisiae* (Edwards et al., 1998). The pattern of Cyclin K expression also correlates positively with proliferative capacity (Dai et al., 2012; Lei et al., 2018; Xiang et al., 2014). More recently, the knockdown of either CDK12 or cyclin K in different human cell lines was shown to induce cell cycle arrest in G1 by preventing assembly of the pre-replicative complex (Lei et al., 2018). In particular, CDK12-cyclin K complex was found to phosphorylate cyclin E1 in G1 to restrict its ability to interact with CDK2, thereby favouring formation of the pre-replicative complex. A peak of CDK12 expression in early G1 adds further weight to this discovery (Bertoli et al., 2013; Paul et al., 2019). Whether CDK12/cyclin K implication in G1 progression is direct or transcriptional is, however, still under debate. A chemical genetic approach identified CDK12 activity as critical for G1 to S progression in HCT116 cells, but this was shown to depend on its function in activating RNA pol II processivity on key DNA replication genes (Paul et al., 2019). Interestingly, depletion of CDK12 in human cells also causes a G2/M arrest, but this may reflect a specific requirement for CDK12 activity for effective transcription of long DNA-damage response genes (Blazek et al., 2011; Dubbury et al., 2018; Geng et al., 2019; Krajewska et al., 2019; S. Liang et al., 2020; Schecher et al., 2017; Tellier et al., 2020). CDK12 was also shown to control translation of mitotic regulatory gene mRNAs in human U2OS cells (S. H. Choi et al., 2019). Specifically, CDK12 directly phosphorylates the translation repressor 4E-BP1, in cooperation with the mTORC1

kinase, to promote its dissociation from the 5' end of target mRNAs. In comparison, the involvement of CDK13 in the cell cycle is not as well-understood. However, in gastric cancer cells, which experience increased cell proliferation due to overexpression of the HMGA2 protein, Gene Ontology analysis indicated CDK13 as a cell-cycle related target of HMGA2 (Z. Wu et al., 2021). The mechanism of rapid cell proliferation in these cells is thought to rely on shortening of the S phase and quicker progression through the G2/M transition, and it was suggested that joint inhibition of CDK13 and HMGA2 could act as antiproliferative treatment. Intriguingly, in breast cancer cells, upregulation of genes involved in S and G2/M progression was caused by inhibiting CDK12/13, further highlighting the role of these kinases in these stages of the cell cycle, and indicating such effects may be cell type-dependent (Quereda et al., 2019).

Does CDK9 perform cell-cycle roles? Its expression and that of cyclin T is relatively constant throughout the cell cycle, which may suggest such a role is unlikely (Garriga et al., 2003). However, in certain cell types, such as T cells, signals which induce cell cycle entry can also upregulate cyclin T expression (Garriga et al., 1998; Herrmann et al., 1998), suggesting that it is not the cell cycle but cell activation state that regulates the CDK9-cyclin T complex (H. Liu & Herrmann, 2005). However, depletion of CDK9 in non-small cell lung, and head and neck squamous cell carcinoma cell lines induced cell cycle delay with an accumulation of cells in G1 and a corresponding decrease in S phase cells (Cai et al., 2006; Storch & Cordes, 2016). In addition, RNAi-mediated knockdown of CDK9 in *Drosophila* cells led to cell cycle arrest in G1 (Anshabo et al., 2021; Yang et al., 2008). Further, CDK9 phosphorylates the Rb protein *in vitro* and *in vivo*, which could be linked to the decrease of D-type cyclins and increase in E-type cyclins observed after downregulation of this kinase (Graña et al., 1994; Simone et al., 2002; Storch & Cordes, 2016). CDK9 activity was also found necessary for cell cycle recovery after replication

stress (D. S. Yu et al., 2010). Recent phosphoproteomic analysis also identified phosphorylation targets of CDK9 that are implicated in the cell cycle, such as FAM122A (PABIR1), which is involved in blocking the G2/M transition, and PCNP, which promotes proliferation (Tellier et al., 2022). Although not as direct as the effects of cell-cycle CDKs on transcription, tCDKs can also play a role in influencing the cell cycle, the extent of which is still being uncovered.

1.8. CDKs and disease

With CDKs implicated in virtually all cellular processes, it is unsurprising that mutation or dysregulation of these kinases can cause a wide range of diseases. Their involvement extends from defects in proliferation, through ischaemia, to rare congenital disorders (Colas, 2020; Łukasik et al., 2021). For example, CDK4 is thought to play a role in the development of some neurodegenerative diseases (Greene et al., 2007; Icreverzi et al., 2015; Łukasik et al., 2021; Mcshea et al., 1997; Sanphui et al., 2013), while CDK5 is specifically implicated in Alzheimer's disease, cardiovascular disorders, and diabetes (Arif, 2012; Cicenias & Valius, 2011; Łukasik et al., 2021; Malhotra et al., 2021). As noted above, CDKs can also be co-opted to help with the replication of viral genomes (Yan et al., 2022). Accordingly, a range of specific and potent CDK inhibitors have been developed, both for clinical and research purposes. Several dozen small-molecule CDK inhibitors are now being used or tested as treatments for a range of diseases (Cicenias & Valius, 2011).

1.8.1. Cell-cycle CDKs and cancer

The unscheduled cell proliferation that characterises tumorigenesis is in many cases attributable to decreased requirement for authentic mitogen signalling and so loss of stringent R point control. The first indications that dysregulation of interphase CDK-

cyclin complexes might contribute to this aspect of tumour development came from observations of chromosomal translocations involving the *CCND1* gene, which encodes cyclin D1, in a human parathyroid carcinoma and, most notably, B cell lymphomas (Hsi et al., 1996; Lesage et al., 2005; H. Liu et al., 2004; Shane, 2001; Thomázy et al., 2002; Vasef et al., 1999; L. Zhao et al., 2014). A causative role for the resulting cyclin D1 overexpression in tumorigenesis was supported by findings from mouse models in which tissue-specific promoter-driven transgenic expression of cyclin D1 led to a high frequency of tumour formation in the corresponding tissue (Fantl et al., 1995; Sicinski et al., 1995). Furthermore, tumorigenesis driven by oncogenes such as Erb-B2 in transgenic mice was blocked by the simultaneous genetic knock-out of CDK4 or cyclin D1, or by expression of a mis-sense mutant form of cyclin D1 that prevents activation of the bound CDK (Landis et al., 2006; Q. Yu et al., 2001, 2006). In familial human melanoma, where the predisposing alleles include loss-of-function mutations in *CDKN2A*, which encodes the p16 CDK inhibitor, and mis-sense mutations in *CDK4* which result in failure of p16 inhibition of the mutant kinase (Zuo et al., 1996). Thus, cyclin D1, normally expressed in a stringently mitogen-dependent manner, can be activated pathologically as a result of oncogenic mutations in components of mitogen signalling pathways, and constitutive CDK4/6-cyclin D1 activity can drive constitutive cell-cycle commitment as tumours develop. The most widely-mutated genes across all tumour types include *CDKN2A* and *RB1*, most frequently loss-of-function, and *CCND1*, which usually increases in copy number (Aaltonen et al., 2020). As cyclin D1 ablation attenuates tumour growth and activation of cellular senescence, therapeutic targeting of cyclin D1-associated CDK activity may be therapeutically useful in human cancers (Y. J. Choi et al., 2012).

The process of developing successful CDK inhibitors has been challenging, and many have not progressed beyond early-stage clinical trials (Asghar et al., 2015; Chohan et al.,

2018) largely because they act as competitive inhibitors of ATP binding to CDKs, which are just one sub-group of some 518 protein kinases in the human proteome. The multiplicity of protein kinases and the limited availability of active, pure preparations of these enzymes has meant that the specificity of even those CDK inhibitors that have been approved for use in the clinic is unclear (see, for example, Fry et al., 2004). These small-molecule inhibitors may therefore have biologically significant impacts on multiple protein kinases, and perhaps other ATP-dependent processes, in patients. There is therefore an urgent need for allosteric CDK inhibitors with increased specificity (Ghafouri-Fard et al., 2022; Marak et al., 2020; Sánchez-Martínez et al., 2019).

Despite these caveats, the ATP-competitive inhibitors palbociclib, ribociclib and abemaciclib show some specificity for CDK4/CDK6 inhibition *in vitro* and inhibit the proliferation of cancer cell lines, inducing G1 arrest in an Rb-dependent manner (Finn et al., 2009). These inhibitors were approved by the U.S. Food and Drug Administration as a therapy against hormone receptor-positive advanced breast cancer, in conjunction with endocrine agents (Finn et al., 2016; Sánchez-Martínez et al., 2019). Ribociclib and abemaciclib confer measurable overall survival benefits (Im et al., 2019; Sledge et al., 2020) and they are now being tested in combination with other drugs, immuno- and chemotherapy, as well as against other types of cancer (Bonelli et al., 2019; Lynce et al., 2018; Spring et al., 2019). However, evidence is lacking that the clinical responses seen were due to inhibition of CDK4/6 in patients' tumours.

Although CDK2 is not required for proliferation of brain or connective tissue, its inhibition in glioblastoma and osteosarcoma cell lines causes decrease in proliferation of transformed cells (Malumbres & Barbacid, 2009). In this context, it is notable that amplification of the gene encoding the CDK2 partner cyclin E1, is even more common

than *CCND1* amplification across numerous human tumour types (Aaltonen et al., 2020). Overexpressed cyclin E1 may be redundant with cyclin D1 in driving R point transit and may contribute to genomic instability by promoting re-replication of chromosomal DNA. Because of its role in ensuring developmental viability, and its nonredundant role in the cell cycle, CDK1 is one of the least frequently mutated CDKs in cancer (Asghar et al., 2015; Otto & Sicinski, 2017; Peyressatre et al., 2015). In tumours, CDK1 activity is often dysregulated through indirect alterations in DNA damage response pathways, or loss of CDK inhibitors (Asghar et al., 2015). However, there is growing evidence that CDK1 can act as a driver of cancer development and progression (Otto & Sicinski, 2017; Sofi et al., 2022). Overexpression of CDK1 has been found to be a biomarker in a number of different types of cancer, including lung, pancreas and sarcomas (M. Li et al., 2020; Q. Li et al., 2019; Piao et al., 2019; Yamamura et al., 2020). Similarly, overexpression of its partner cyclin B is often associated with poor prognosis in, for instance, breast cancer patients (Agarwal et al., 2010; Winters et al., 2001). Inhibition of CDK1 can induce apoptosis in several types of malignancy (Ongkeko et al., 1995; Otto & Sicinski, 2017) and could prove effective against cancer stem cells, particularly in gliomas and pancreatic cancers (Sánchez-Martínez et al., 2019). In the absence of evidence to the contrary, it is even possible that clinical responses to CDK inhibition are due, in part at least, to off-target inhibition of CDK1.

1.8.2. tCDKs and cancer

As malignant cells generally rely on a higher transcriptional output than healthy cells, often relying on oncogenic transcription factors or super-enhancers, it is not surprising that dysregulation of key tCDKs is implicated in a range of cancers. As a result, tCDKs are currently seen as important pharmaceutical targets and biomarkers of cancer, with

several tCDK inhibitors currently in clinical trials for cancer treatment (Franco & Kraus, 2015; Galbraith et al., 2019; Parua & Fisher, 2020; Sánchez-Martínez et al., 2019).

The dual actions of the highly related kinases CDK8/CDK19 (**Table 1.4.**) have led them to be implicated in cancer development as both drivers or suppressors of tumorigenesis. Inhibition of both kinases, whose catalytic domains share 94% amino acid sequence identity, by cortistatin A leads to decreased proliferation of malignant cells (Chou et al., 2020; Pelish et al., 2015). In addition, overexpression of CDK8 can contribute to the development of melanoma, as well as colorectal, gastric and breast cancers (Chohan et al., 2018; Peyressatre et al., 2015). In prostate cancer cells, inhibition of CDK8/19 leads to premature G1/S transition and cell death (Nakamura et al., 2018). However, CDK8 can also inhibit tumour growth, for instance in the case of endometrial cancer. It was shown that ectopic expression of CDK8 inhibited proliferation in KLE cancer cell line, and that it could even block growth of a mouse tumour model *in vivo* (Gu et al., 2013). In addition to cortistatin A, a number of CDK8/CDK19 inhibitors are currently under development but none has yet reached the clinic (Galbraith et al., 2019).

Interest in designing a specific CDK9 inhibitor began when it was discovered that pan-CDK inhibitors, for instance flavopiridol, potently inhibit this kinase. CDK9 is hyperactivated in a number of cancers, particularly haematological malignancies (Chohan et al., 2018; Galbraith et al., 2019). The involvement of CDK9 in lymphoma development is also connected to its role in maintaining the expression of short-lived anti-apoptotic mRNAs, such as *MCL1*. It is further recruited to the chromatin by various cancer-dependent transcription factors and super-enhancers (Galbraith et al., 2019). Because of its central role in transcription elongation, CDK9 remains a high-priority target, with a number of inhibitors currently undergoing clinical trials (Galbraith et al., 2019).

Like CDK8/CDK19, CDK12 and CDK13 have been shown to have both tumour-permissive and suppressive functions. CDK12 is recognised as a significant player in genomic instability of cancers and its loss-of-function mutations have been implicated in advanced ovarian and breast malignancies (Cheng et al., 2022; Chohan et al., 2018; Galbraith et al., 2019; H. Liu et al., 2021). On the other hand, its overexpression is associated with HER2-positive breast cancers and increased levels of ERBB2 (Quereda et al., 2019). Importantly, depletion of CDK12 preferentially decreases the expression of DNA damage response (DDR) genes (Blazek et al., 2011; S. H. Choi et al., 2020; Dubbury et al., 2018; Krajewska et al., 2019; Manavalan et al., 2019). This allows for sensitisation of the tumour to synthetic-lethal therapy, for instance with poly-ADP ribose polymerase (PARP) inhibitors (Chou et al., 2020; Parua & Fisher, 2020). Despite being less studied, CDK13 has also been found to be amplified in some primary liver and colon cancers (Galbraith et al., 2019). Although a clinical inhibitor for CDK12 or CDK13 is still in the works, there currently exists a range of research-grade tools used to inhibit these kinases. Further, as seen with the example of PARP inhibitors, CDK12/CDK13 inhibitors can synergise with other therapeutic agents (Galbraith et al., 2019; Niu et al., 2022; Quereda et al., 2019; Tadesse et al., 2020). Preliminary data suggest triple-negative breast cancer as a potential therapeutic target for CDK12/13 inhibition, which would depend on suppressing the DNA damage response pathway and leading to sensitisation of malignant cells to apoptosis (Hopkins & Zou, 2019; Quereda et al., 2019; Tadesse et al., 2020).

Perhaps the biggest success in therapeutic targeting of tCDKs is seen in the example of CDK7, with four selective inhibitors currently in Phase I/II clinical trials (ICEC0942/CT7001, SY-1365, SY-5609 and LY3405105) (Sava et al., 2020). The dual actions of CDK7, which participates in both regulation of transcription and the cell cycle,

have made it an important player in tumorigenesis. Its overactivation is associated with gastric, breast, ovarian, and liver cancers, and it has been suggested that the malignant cells show greater dependency on elevated CDK7 levels than healthy cells (Galbraith et al., 2019; Sava et al., 2020; Y. Wang et al., 2015). THZ1 is a covalent CDK inhibitor, and although it primarily targets CDK7, it also has some anti-CDK12/CDK13 activity (Sánchez-Martínez et al., 2019). Encouragingly, treating cells advanced breast cancer cells with THZ1 leads to apoptotic cell death (Franco & Kraus, 2015; Y. Wang et al., 2015). Several other groups also showed that selective inhibition of CDK7 leads to cell cycle arrest, apoptosis, and decreased transcription levels, especially of genes associated with super-enhancers (Galbraith et al., 2019; Sava et al., 2020). Importantly, inhibition of CDK7 shows promise in treating cancers with heightened transcription, even without the presence of any apparent oncogenic driver mutations (Y. Wang et al., 2015). Additionally, CDK7 inhibitors show promise of synergistic treatment, for example with pro-apoptotic agents (L. He et al., 2022; Kalan et al., 2017). In the same manner, colorectal cancer cells treated with the antimetabolite 5-fluorouracil became sensitised to reversible or covalent CDK7 inhibitors, and showed increased rates of apoptotic cell death (Kalan et al., 2017).

1.9. Aims of this project

The picture emerging from the field of CDK biology is one of great complexity. The multi-tasking CDKs are a versatile group of kinases, which help to receive, interpret, and act on a variety of endogenous and exogenous signals. In cancer, tumours acquire dependencies on CDK-guarded processes, which may become rate-limiting for tumour growth and progression. It is also increasingly apparent that a binary distinction between cell-cycle and transcriptional CDKs is inappropriate. Rather, the involvement of several CDKs in both aspects of biology suggests an evolutionarily ancient role in the co-ordination of cell proliferation and gene expression.

It is becoming more evident that CDK1 functions outside of its archetypal role in the control of mitosis, and that its tasks might extend to interphase as well. Of particular interest to this project was the influence of CDK1 activity on transcription, as indicated in previous literature (**Section 1.7.3.**). It was clear, however, that the transcriptional roles of CDK1 might be heavily cell type-dependent and that, so far, no universal mechanism for CDK1 function in transcription is apparent. The experiments described here focused on the effects of CDK1 on transcription in cancer cells, as CDK1 overexpression is a hallmark of some types of tumours (M. Li et al., 2020; Q. Li et al., 2019; Piao et al., 2019; Yamamura et al., 2020).

The aim of this project was to test the hypothesis that CDK1 dysregulation contributes to transcriptional re-programming during tumorigenesis, and if so, whether it is possible to inhibit tumour-associated CDK1 activity to promote cancer cell differentiation, while sparing the essential proliferative CDK1 activity in healthy tissues. The work described in this thesis outlines the characterisation of the experimental model and the creation of two new cell lines to study CDK1 function (**Chapter 3**); followed by the investigation of

transcriptional effects of CDK1 inhibition in cancer cells (**Chapter 4**); and how these results compare between different cell lines and inhibition methods (**Chapter 5**).

Table 1.1. Cell-cycle CDKs in metazoans.				
<i>Cyclin-dependent kinase</i>	<i>Binding partner</i>	<i>Roles in cell cycle</i>	<i>Roles in transcription</i>	<i>Selected additional roles</i>
CDK1	Cyclin A, B		Phosphorylates factors in cell-cycle transcriptional programmes <i>(Akoulitchev & Reinberg, 1998; Chymkowitch & Enserink, 2013; Cisek & Corden, 1989; Enserink & Chymkowitch, 2022; Gavet & Pines, 2010a, 2010b; Kobor & Greenblatt, 2002)</i>	Regulates mitochondrial bioenergetics
		Orchestrates mitosis and determines its timing <i>(Maryu & Yang, 2022; Santamaría et al., 2007; Satyanarayana et al., 2008; Satyanarayana & Kaldis, 2009b)</i>	Maintains pluripotency of embryonic stem cells (mouse) <i>(Michowski et al., 2020)</i>	<i>(B. Xie et al., 2019)</i> Upregulates mRNA translation <i>(Haneke et al., 2020)</i>
			Inhibits neuronal differentiation by phosphorylating Ngn2 <i>(Ali et al., 2011)</i>	Functions in DNA damage response and DNA repair <i>(Palmer & Kaldis, 2020)</i>
			Phosphorylates of splicing and polyadenylation factors (in cell cycle-dependent manner) <i>(Colgan et al., 1998; Okamoto et al., 1998)</i>	
CDK2	Cyclin A, E	Participates in G1/S transition <i>(Satyanarayana et al., 2008; Satyanarayana & Kaldis, 2009b)</i>	Targets transcriptional repressor Rb <i>(Dynlacht, 1997; Hydring et al., 2016)</i>	Maintains pluripotent neural progenitor cell pool
			Inhibits FOXO1 to promote cell survival <i>(H. Huang et al., 2006)</i>	<i>(Caillaeva et al., 2011)</i>
		Orchestrates S phase events, such as initiation of replication <i>(Coverley et al., 2000)</i>	Activates ELK4 to stimulate cell transformation <i>(Peng et al., 2016)</i>	Functions in DNA damage response and DNA repair <i>(Satyanarayana & Kaldis, 2009a)</i>
			Stimulates	

		transcription during HIV-1 infection (Agbottah et al., 2006; Deng et al., 2002; Nekhai et al., 2002; Rice, 2018) Targets chromatin-modifying proteins and general transcription factors (e.g., DOT1L, GTF2I) (Chi et al., 2020) Inhibits neuronal differentiation by phosphorylating Ngn2 (Ali et al., 2011)
CDK4	Cyclin D	Governs progression through R-point (Malumbres & Barbacid, 2005; Satyanarayana & Kaldis, 2009b) Targets transcriptional repressors (Rb, p107, p130) and Smad transcription factors (Cobrinik, 2005; Dynlacht, 1997; Ezhevsky et al., 1997; Goel et al., 2018; Hydbring et al., 2016) Activates FOXM1 to protect against senescence (Anders et al., 2011) Activates c-Jun to form AP-1 transcription complexes (Vanden Bush & Bishop, 2011)
CDK6	Cyclin D	Governs progression through R-point (Malumbres & Barbacid, 2005; Satyanarayana & Kaldis, 2009b) Targets transcriptional repressors (Rb, p107, p130) and Smad transcription factors (Cobrinik, 2005; Dynlacht, 1997; Ezhevsky et al., 1997; Goel et al., 2018; Hydbring et al., 2016) Activates FOXM1 to protect against senescence (Anders et al., 2011) Acts as sensitiser for apoptosis (MacKeigan et al., 2005)

		Negatively regulates differentiation (Grossel & Hinds, 2006; Matushansky et al., 2000; Urbach & Witte, 2019) Activates NF-κB (Buss et al., 2012; Handschick et al., 2014) Upregulates expression of pro-angiogenic VEGF-A (Kollmann et al., 2013)

Table 1.2. Cell-cycle CDKs in <i>S. cerevisiae</i> .				
Cyclin-dependent kinase	Binding partner	Roles in cell cycle	Roles in transcription	Selected additional roles
Cdc28	Cln1-3, Clb1-6	Orchestrates mitosis and determines its timing (Enserink & Kolodner, 2010; Hartwell et al., 1973; Mendenhall & Hodge, 1998)	Phosphorylates factors in cell-cycle transcriptional programmes (Archambault et al., 2004; R. J. Cho et al., 2001; Cosma et al., 2001; Darieva et al., 2003; De Bruin et al., 2004; Enserink & Chymkowitch, 2022; Jans et al., 1995; Kõivomägi et al., 2011; Moll et al., 1991; O'Conalláin et al., 1999; Pic-Taylor et al., 2004; Reynolds et al., 2003; Ubersax et al., 2003; Wittenberg & Reed, 2005) CTD kinase (T4, S5) (Kõivomägi et al., 2021; Nemec et al., 2019) Maintains transcription of highly expressed housekeeping genes (e.g., PMA1) (Chymkowitch et al., 2012) Increases transcription through activating NuA4 leading to	Implicated in bud morphogenesis (Enserink & Kolodner, 2010; Lew & Reed, 1993) Responsible for pheromone signalling in G1 (Enserink & Kolodner, 2010) Governs genome stability and DNA repair pathways (Enserink & Kolodner, 2010)

	increased Lys14 acetylation on Htz1 (Fiedler et al., 2009)

Table 1.3. Cell-cycle CDKs in <i>S. pombe</i> .				
Cyclin-dependent kinase	Binding partner	Roles in cell cycle	Roles in transcription	Selected additional roles
Cdc2	Cdc13, Cig1, Cig2, Puc1, Pas1, Pch1, Rem1	Governs cell cycle commitment at START in G1; orchestrates mitosis and determines its timing (Beach et al., 1982; Coudreuse & Nurse, 2010; Gutiérrez-Escribano & Nurse, 2015; Fisher & Nurse, 1996; Martín-Castellanos et al., 1996, 2000; Nurse, 1975; Nurse & Bissett, 1981; Reymond et al., 1993)	Required for Cdc10/Sct1 transcription complex formation (Connolly et al., 1997) Inhibits Ste11 (Kjærulff et al., 2007)	

Table 1.4. Transcriptional CDKs in metazoans.				
Cyclin-dependent kinase	Binding partner	Roles in cell cycle	Roles in transcription	Selected additional roles
CDK7	Cyclin H	CAK activity (for CDK1/2/4/6) (Bisteau et al., 2013; Fisher, 2005, 2012; Larochelle et al., 1998, 2007; Merrick et al., 2008; Schachter & Fisher, 2013) Required for cell cycle progression (Ganuza et al., 2012; Larochelle et al., 1998, 2007; Olson et al., 2019; Wallenfang & Seydoux, 2002)	CAK activity (for CDK9/12/13) (Fisher, 2012; Larochelle et al., 2012; Rimel et al., 2020) Part of TFIID complex, important for early steps of pol II transcription (Fisher, 2005; Glover-Cutter et al., 2009; Larochelle et al., 2012; Maldonado & Reinberg, 1995; Rimel & Taatjes, 2018; Roy et al., 1994) CTD kinase (S5, S7) (Akhtar et al., 2009; Fisher, 2005; Glover-Cutter et al., 2009; Pinhero et al., 2004; Roy et al., 1994; Wallenfang & Seydoux, 2002)	Implicated in DNA damage and repair pathways (Rimel & Taatjes, 2018)
CDK8	Cyclin C	Inhibits CAK activity (Akoulitchev et al., 2000; Szilagyi & Gustafsson, 2013) Promotes cell-cycle commitment through β-catenin pathway (Firestein et al., 2008; Szilagyi & Gustafsson, 2013) Promotes G2/M transition (Philip et al., 2018; Xu et al., 2015) Promotes cell cycle arrest at R point through p21 activation (Donner et al., 2007; Porter et al., 2012; Szilagyi & Gustafsson, 2013)	Part of mediator complex (Dannappel et al., 2019; Fant & Taatjes, 2019; Galbraith et al., 2010; Luyties & Taatjes, 2022; Poss et al., 2013; D. Wu et al., 2021) CTD kinase (S5) (Pinhero et al., 2004; Rickert et al., 1999) Can upregulate and downregulate transcription (Galbraith et al., 2010) Inhibits TFIID to downregulate transcription (Akoulitchev et al., 2000)	Acts as sensitiser for apoptosis (MacKeigan et al., 2005) Regulates Myc to maintain stem cell pluripotency (Adler et al., 2012) Essential for early embryogenesis (mouse) (Westerling et al., 2007) Implicated in DNA damage and repair pathways (Poss et al., 2016)

		Maintains quiescence of VPC in <i>C. elegans</i> <i>(Clayton et al., 2008)</i>	Promotes expression of hypoxia-inducible genes after recruitment by HIF1A <i>(Galbraith et al., 2013)</i>	
CDK19	Cyclin C		Part of mediator complex <i>(Dannappel et al., 2019; Fant & Taatjes, 2019; Luyties & Taatjes, 2022)</i>	Implicated in DNA damage and repair pathways <i>(Poss et al., 2016)</i>
CDK9	Cyclin T	Necessary for cell cycle recovery after replication stress <i>(D. S. Yu et al., 2010)</i> Upregulates cell proliferation through PABIR1 and PCNP <i>(Tellier et al., 2022)</i> Gets recruited to chromatin to promote progression through G1 <i>(Anshabo et al., 2021; Cai et al., 2006; Storch & Cordes, 2016; Yang et al., 2008)</i>	Part of P-TEFb complex, important for pol II release from the promoter-proximal pause and elongation <i>(Anshabo et al., 2021; Bacon & D'Orso, 2019; Egloff, 2021; Jonkers & Lis, 2015; Luo et al., 2012; Parua et al., 2018)</i> CTD kinase (S2, T4, S5, S7) <i>(Eick & Geyer, 2013; Heidemann et al., 2013; Pinhero et al., 2004; Zaborowska et al., 2016)</i> Targets transcriptional repressor Rb <i>(Graña et al., 1994; Simone et al., 2002; Storch & Cordes, 2016)</i>	Implicated in DNA damage and repair pathways <i>(Anshabo et al., 2021)</i>
CDK12	Cyclin K	Phosphorylates cyclin E1 to stimulate formation of pre-replicative complex, therefore controlling G1/S progression <i>(Lei et al., 2018; S. Liang et al., 2020; Manavalan et al., 2019)</i> Controls translation of mitotic-regulator gene mRNAs <i>(Choi et al., 2019)</i> Promotes G2/M transition <i>(Blazek et al., 2011; Geng et al., 2019; H. R. Chen et al., 2017; Schecher et al., 2017)</i>	Required for efficient elongation and pre-mRNA 3' end formation <i>(Bösken et al., 2014; Choi et al., 2020; Eiffler et al., 2015; Fan et al., 2020; Lui et al., 2018; Quereda et al., 2019; Tellier et al., 2020)</i> CTD kinase (S2, S5) <i>(Bartkowiak et al., 2010; Blazek et al., 2011; Bösken et al., 2014; Cheng et al., 2012; Tellier et al., 2020)</i> Implicated in regulation of splicing <i>(H. H. Chen et al., 2006; K. Liang, Gao et al., 2015; S. Liang et al., 2020; Panzeri et al.,</i>	Implicated in DNA damage response <i>(Blazek et al., 2011; H. R. Chen et al., 2017; Choi et al., 2020; Dubbury et al., 2018; Fan et al., 2020; Juan et al., 2015; Krajewska et al., 2019; K. Liang, Gao et al., 2015; S. Liang et al., 2020; Manavalan et al., 2019; Quereda et al., 2019; Tellier et al., 2020)</i> Promotes neurogenesis <i>(H. R. Chen et al., 2017)</i>

		Downregulates G2/M transition in breast cancer cells (Quereda et al., 2019)	2013) Inhibition severely affects expression of long DNA Damage Response (DDR) genes (Blazek et al., 2011; Choi et al., 2020; Dubbury et al., 2018; Krajewska et al., 2019) Negatively regulates differentiation (Dai et al., 2012)	Essential for early embryogenesis (mouse) (Juan et al., 2015)
CDK13	Cyclin K	Promotes G2/M transition in gastric cancer cells (Z. Wu et al., 2021) Downregulates G2/M transition in breast cancer cells (Quereda et al., 2019)	Required for efficient elongation and pre-mRNA 3' end formation (Fan et al., 2020; K. Liang, Gao et al., 2015; Quereda et al., 2019) CTD kinase (S2, S5) (Greifenberg et al., 2016) Regulates expression of snoRNA genes (K. Liang, Gao et al., 2015) Implicated in regulation of splicing (H. H. Chen et al., 2007; K. Liang, Gao et al., 2015; Panzeri et al., 2013) Activates RNA surveillance pathway (Insko et al., 2023) Negatively regulates differentiation (Dai et al., 2012)	Implicated in DNA damage response (Fan et al., 2020) Implicated in growth signalling pathways (Fan et al., 2020; Greifenberg et al., 2016) Promotes cell survival in breast cancer cells (Quereda et al., 2019) Promotes neurogenesis (H. R. Chen et al., 2017)

	Increases splicing of HIV-1 mRNA (Berro et al., 2008)

Table 1.5. Transcriptional CDKs in *S. cerevisiae*.

Cyclin-dependent kinase	Binding partner	Roles in cell cycle	Roles in transcription	Selected additional roles
Kin28	Ccl1	Essential for proliferation (Simon et al., 1986) Binds to SBF promoters and can rescue cell cycle defects caused by Cln3 depletion (Kõivomägi et al., 2021) Creates autoregulatory loop with Srb10 to govern meiosis (Ohkuni & Yamashita, 2000)	Part of TFIIF complex, important for early steps of pol II transcription (Cismowski et al., 1995; Feaver et al., 1994; Svejstrup et al., 1996) Upregulates transcription (Hengartner et al., 1998; Valay et al., 1995) CTD kinase (S5, S7) (Akhtar et al., 2009; Hengartner et al., 1998; Komarnitsky et al., 2000) Important for recruitment of mRNA processing machinery (e.g., for 5' capping) (Komarnitsky et al., 2000; Rodriguez et al., 2000; Schroeder et al., 2000) Primes CTD for Bur1 recruitment (Qiu et al., 2009, 2012) Stimulates mediator disassociation from preinitiation complex leading to promoter escape (Y. Liu et al., 2004; Wong et al., 2014)	Participates in nucleotide excision repair (Bhatia et al., 1996)

Srb10	Srb11		Part of mediator complex (<i>Carlson, 1997</i>)	
			Downregulates transcription, e.g. through interaction with Gcn4 (<i>Carlson, 1997; Chi et al., 2001; Hengartner et al., 1998; Holstege et al., 1998; Kuchin & Carlson, 1998; Rosonina et al., 2012</i>)	
		Represses meiotic genes in response to glucose by decreasing their mRNA stability (<i>Surosky et al., 1994</i>)	Upregulates transcription	Participates in glucose repression
		Creates autoregulatory loop with Kin28 to govern meiosis (<i>Ohkuni & Yamashita, 2000</i>)	(<i>Andrau et al., 2006; Galbraith et al., 2010; Y. Liu et al., 2004; X. Zhu et al., 2006</i>)	(<i>Kuchin et al., 1995</i>)
			CTD kinase (S5) (<i>Hengartner et al., 1998; Liao et al., 1995</i>)	Coordinates nutrient starvation response (<i>Holstege et al., 1998</i>)
		Coordinates entry into stationary phase (<i>Chang et al., 2001</i>)	Phosphorylates and decreases stability of Ste12 in normal nitrogen conditions (<i>Nelson et al., 2003</i>)	
			Represses α -specific genes in α cells (<i>Wahi & Johnson, 1995</i>)	
			Activates Sip4 in nonfermentable carbon sources (<i>Vincent et al., 2001</i>)	

Bur1	Bur2	Required for activation of Sch9 leading to progression through G1 (Jin et al., 2022)	Required for efficient elongation (Keogh et al., 2003; Murray et al., 2001; Wood et al., 2005; Wood & Shilatifard, 2006; Yao et al., 2000)	
			CTD kinase (S2, S5, S7) (Y. Liu et al., 2009; Murray et al., 2001; Qiu et al., 2009; Tietjen et al., 2010)	Governs DNA damage and replication stress pathways (Clausing et al., 2010)
			Phosphorylates Spt5 to recruit PAF1 (Y. Liu et al., 2009; Qiu et al., 2012)	Implicated in lengthening of telomeres (Connelly et al., 2022)
			Represses SUC2 basal promoter (Prelich & Winston, 1993)	Essential for cell growth (Irie et al., 1991; Winzeler et al., 1999)
			Regulates epigenetic histone modifications, e.g. through PAF recruitment (Chu et al., 2006, 2007; Larabee et al., 2005; Y. Liu et al., 2009; Wood et al., 2005)	Suppresses mating pheromone hyperadaptivity (Irie et al., 1991)
			Modulates co-transcriptional splicing (Maudlin & Beggs, 2021)	
Ctk1	Ctk2, Ctk3	Role unclear – deletion results in cell cycle defects (Chymkowitch et al., 2012)	Required for efficient elongation (Jona et al., 2001; J. M. Lee & Greenleaf, 1997; Murray et al., 2001)	Implicated in cell growth (J. M. Lee & Greenleaf, 1991)
			Regulates co-transcriptional processes such as mRNA 3'-end processing, polyadenylation, and nuclear export of mRNA (Ahn et al., 2004; Hurt et al., 2004; Skaar & Greenleaf, 2002)	Required for translation initiation and elongation (Coordes et al., 2015; Röther & Sträßer, 2007)
			Promotes release of basal transcription	Implicated in transcription by RNA pol I and synthesis of rRNA (Bouchoux et al., 2004; Grenetier et al., 2006)

		factors from pol II (Ahn et al., 2009) CTD kinase (S2) (E. J. Cho et al., 2001; J. M. Lee & Greenleaf, 1989, 1991) Downregulates CTD phosphorylation during logarithmic phase growth, but upregulates during diauxic phase (Patturajan et al., 1999) Important for transcription termination of small non-coding RNAs (Lenstra et al., 2013) Upregulates H3K36me3 by interaction with Set2 and Spt6 (Dronamraju & Strahl, 2014; Xiao et al., 2003; Youdell et al., 2008) Implicated in glucose-dependent transcriptional regulation (Van Driessche et al., 2005)	Implicated in DNA damage response (Ostapenko & Solomon, 2003)

Table 1.6. Transcriptional CDKs in *S. pombe*.

Cyclin-dependent kinase	Binding partner	Roles in cell cycle	Roles in transcription	Selected additional roles
Mc6	Mcs2	CAK activity (for Cdc2) (Buck et al., 1995; Damagnez et al., 1995; Lee et al., 1999; Saiz & Fisher, 2002) Inhibition impairs cytokinesis	Part of TFIIH complex, important for early steps of pol II transcription (Booth et al., 2018; Spåhr et al., 2003; Viladevall et al., 2009) CTD kinase (S5, S7)	Essential for growth (Damagnez et al., 1995; Molz et al., 1989; Saiz & Fisher, 2002)

Srb10	Srb11	(Buck et al., 1995; Saiz & Fisher, 2002; Viladevall et al., 2009) (Amour et al., 2012; Booth et al., 2018; Viladevall et al., 2009) Recruits P-TEFb to pol II by priming the CTD, leading to mRNA capping (Amour et al., 2012; Viladevall et al., 2009) Controls transcription of cell-cycle periodic genes through interactions with Sep1 (Lee et al., 2005)		
		Part of mediator complex (Borggreffe et al., 2002; Samuelsen et al., 2003; Spåhr et al., 2003) Downregulates transcription <i>in vitro</i> (Spåhr et al., 2003) Controls entry into mitosis by phosphorylating Fkh2 (Banyai et al., 2014; Szilagyi et al., 2012) CTD kinase <i>in vitro</i> (S2, S5) (Borggreffe et al., 2002) Downregulates expression of adhesins (Linder et al., 2008; Samuelsen et al., 2003) Blocks mediator-pol II interactions (Elmlund et al., 2006; Samuelsen et al., 2003) Acts as a global regulator of mitotic transcription (Banyai et al., 2014)		

SpCDK9	Pch1	Inhibition impairs cytokinesis (Viladevall et al., 2009)	Part of P-TEFb complex, important for efficient elongation (Amour et al., 2012; Bartkowiak & Greenleaf, 2011; Guiguen et al., 2007; Parua et al., 2018; Viladevall et al., 2009)	
			Regulates an early elongation checkpoint (Booth et al., 2018; Guiguen et al., 2007; Pei et al., 2003; Viladevall et al., 2009)	
Lsk1	Lsc1	Upregulates the Septation Initiation Network to promote cytokinesis (Karagiannis et al., 2005; Karagiannis & Balasubramanian, 2007)	CTD kinase (S2, S5, marginally S7) (Amour et al., 2012; Guiguen et al., 2007; Pei & Shuman, 2003)	Essential for growth (Bimbó et al., 2005; Guiguen et al., 2007; Pei & Shuman, 2003)
			Phosphorylates Spt5 elongation factor (Amour et al., 2012; Booth et al., 2018; Parua et al., 2018; Pei & Shuman, 2003)	Implicated in DNA damage response (Gerber et al., 2008)
Lsk1	Lsc1	Upregulates the Septation Initiation Network to promote cytokinesis (Karagiannis et al., 2005; Karagiannis & Balasubramanian, 2007)	Couples transcription to mRNA capping through its association with Pct1 and Pcm1 (Amour et al., 2012; Guiguen et al., 2007; Pei et al., 2003, 2006; Viladevall et al., 2009)	
			Downregulates PP1 isoform Dis2 to create a termination switch (Parua et al., 2018)	
Lsk1	Lsc1	Upregulates the Septation Initiation Network to promote cytokinesis (Karagiannis et al., 2005; Karagiannis & Balasubramanian, 2007)	Upregulates histone H2B mono-ubiquitylation (Sansó et al., 2012)	
			Deletion causes minimal changes in transcription (Booth et al., 2018)	Downregulates sexual differentiation through interaction with Ste11 (Coudreuse et al., 2010; Sukegawa et al., 2011)
Lsk1	Lsc1	Upregulates the Septation Initiation Network to promote cytokinesis (Karagiannis et al., 2005; Karagiannis & Balasubramanian, 2007)	CTD kinase (S2) (Booth et al., 2018; Karagiannis &	

	<i>Balasubramanian, 2007; Viladevall et al., 2009)</i> Upregulates meiosis via stress-responsive MAPK pathway <i>(Coudreuse et al., 2010; Sukegawa et al., 2011)</i>

Table 1.7. Additional CDKs in metazoans.				
Cyclin-dependent kinase	Binding partner	Roles in cell cycle	Roles in transcription	Selected additional roles
CDK3	Cyclin A, C, E	Promotes G0/G1 and G1/S transitions <i>(Ren & Rollins, 2004; Sage, 2004; Satyanarayana & Kaldis, 2009b; Teo et al., 2022; Van Den Heuvel & Harlow, 1993; Ye et al., 2001)</i>	Targets transcriptional repressor Rb <i>(Hofmann & Livingston, 1996; Ren & Rollins, 2004)</i> Phosphorylates c-Jun and ATF1 to simulate cell transformation <i>(Cho et al., 2009; Zheng et al., 2008)</i>	Promotes apoptosis <i>(Meikrantz & Schlegel, 1996)</i> Promotes EMT <i>(Lu et al., 2016)</i>
CDK5	p35, p38	Blocks cell cycle in postmitotic neurons <i>(Cicero & Herrup, 2005; Zhang et al., 2008)</i> Promotes proliferation in neuroendocrine thyroid cancer <i>(Pozo et al., 2013)</i>	Implicated in transcriptional programmes for neuronal differentiation <i>(Cicero & Herrup, 2005)</i> Activates STAT3 <i>(A. K. Y. Fu et al., 2004)</i> Inhibits MEF2 to promote apoptosis <i>(Gong et al., 2003)</i> Activates mSds3 to promote histone acetylation <i>(Z. Li et al., 2004)</i> Activates p53 and Rb <i>(Lee et al., 1997; Pozo et al., 2013; Zhang et al., 2002)</i>	Implicated in a wide range of neuronal processes <i>(Chae et al., 1997; Cicero & Herrup, 2005; Cruz & Tsai, 2004; Maestre et al., 2008; Nikolic et al., 1996; Ohshima et al., 1996; Pao & Tsai, 2021; Tanaka et al., 2001)</i> Regulates circadian rhythm through phosphorylation of CLOCK and PER2 <i>(Brenna et al., 2019; Kwak et al., 2013; Pao & Tsai, 2021)</i> Protects against mitochondrial dysfunction and oxidative stress <i>(Pao & Tsai, 2021; Qu et al., 2007; K. H. Sun et al., 2008)</i> Implicated in DNA damage response <i>(Kim et al., 2008; W. Liu et al., 2017)</i>
CDK10	Cyclin M	Promotes G2/M transition <i>(S. Li et al., 1995)</i>	Phosphorylates ETS2, leading to silencing of the MAPK pathway <i>(Guen et al., 2013; Kasten & Giordano, 2001)</i>	Regulates cilium biogenesis and degradation <i>(Guen et al., 2016, 2018; Windpassinger et al., 2017)</i>

			Promotes transcription of 20E-inducible genes (<i>W. Liu et al., 2014</i>)	Regulates the cytoskeleton and actin dynamics (<i>Guen et al., 2016, 2018</i>)
CDK11	Cyclin L	Participates in G2/M transition and cytokinesis (primarily CDK11p58 isoform) (<i>An et al., 2020; Barna et al., 2008; Hu et al., 2007; Loyer & Trembley, 2020; Petretti et al., 2006; Wilker et al., 2007; Yokoyama et al., 2008</i>) Required for transcription of replication-dependent histone genes during S phase (<i>Gajdušková et al., 2020</i>)	Associates with multiple transcription elongation factors (<i>Loyer & Trembley, 2020; Trembley et al., 2002, 2003</i>) Links transcription with RNA processing events (<i>Loyer & Trembley, 2020; Trembley et al., 2002, 2003</i>) Implicated in regulation of splicing (<i>Hluchý et al., 2022; Hu et al., 2003; Loyer et al., 2005; Loyer & Trembley, 2020; Shin & Manley, 2004</i>) CTD kinase <i>in vitro</i> (S2) for replication-dependent histone genes (<i>Gajdušková et al., 2020</i>) Involved in processing and polyadenylation of HIV-1 transcripts (<i>Pak et al., 2015; Rice, 2018</i>)	Part of autophagy machinery (<i>Wilkinson et al., 2011</i>) Represses oestrogen and vitamin D receptor pathways (<i>Chi et al., 2009; Wang et al., 2009</i>) Promotes apoptosis (<i>Ariza et al., 1999; Beyaert et al., 1997; Lahti et al., 1995; J. Shi et al., 2003, 2009; L. Shi et al., 1994; Tang et al., 1998</i>) Essential for early embryogenesis (mouse) (<i>T. Li et al., 2004</i>)
CDK14	Cyclin B, Y	Stimulates Wnt/β-catenin signalling pathway to drive cell cycle progression (<i>Gu et al., 2015; Niehrs & Acebron, 2012; Ou-Yang et al., 2017; T. Sun et al., 2014</i>)	Upregulates phosphorylation of Rb to deactivate it (<i>L. Chen et al., 2019</i>)	Implicated in tumour cell migration and EMT (<i>L. Chen et al., 2019; Gu et al., 2015; Ou-Yang et al., 2017; Pang et al., 2007; Zhu et al., 2016</i>) Promotes axon regeneration (<i>Hisamoto et al., 2021</i>)

			Implicated in glucose homeostasis (Tang et al., 2006)
CDK15	Cyclin Y	Promotes cell proliferation through β-catenin/ MEK-ERK pathway in colorectal cancer (C. Huang et al., 2022)	Protects against tumour cell migration (S. Li et al., 2019) Downregulates apoptosis (Park et al., 2014)
CDK16	Cyclin Y, p35	Promotes cell proliferation (Gillani et al., 2022; X. Li et al., 2022; Yanagi et al., 2014)	Deactivates p27 and p53 tumour suppressors (J. Xie et al., 2018; Yanagi et al., 2014) Involved in terminal differentiation in spermatogenesis (Mikolcevic, Rainer, et al., 2012; Mikolcevic, Sigl, et al., 2012) Implicated in neural outgrowth (Graeser et al., 2002; Mokalled et al., 2010) Functions in vesicular trafficking (Y. Liu et al., 2006; Ou et al., 2010; Palmer et al., 2005; Shehata et al., 2019) Implicated in brain development (Cole, 2009; Fu et al., 2011; Le Bouffant et al., 2000; Mokalled et al., 2010; Shehata et al., 2015, 2019) Suppresses apoptosis (Gillani et al., 2022; Yanagi & Matsuzawa, 2015) Implicated in glucose homeostasis (X. Y. Chen et al., 2012; Tang et al., 2006) Promotes myogenesis

				(Shimizu et al., 2014)
CDK17	Cyclin Y		Phosphorylates Histone 1 (Hirose et al., 1997)	Promotes Alzheimer's pathology (Chaput et al., 2016) Implicated in neuronal development (Hirose et al., 2000; Yamochi et al., 2001) Downregulates autophagy (Leonardi et al., 2019)
CDK18	Cyclin A	Induces cell cycle arrest in glioblastoma cells (Naumann et al., 2005) Required for S phase progression (Barone et al., 2016)		Promotes Alzheimer's pathology, promotes phosphorylation of tau (Chaput et al., 2016; Herskovits & Davies, 2006) Downregulates autophagy (Leonardi et al., 2019) Promotes ATR-dependent homologous recombination (Ning et al., 2019) Prevents accumulation of DNA damage and genome instability (Barone et al., 2016, 2018) Involved in reorganisation of the actin cytoskeleton (Matsuda et al., 2014)
CDK20	Cyclin H	Possible CAK activity (for CDK2) (X. An et al., 2010; Y. Liu et al., 2004; Tian et al., 2012; questioned by Wohlbold et al., 2006; Wu et al., 2009)	Upregulates EZH2 and H3K27me3 (Feng et al., 2015)	Phosphorylates MAK-related kinase/intestinal cell kinase (MRK/ICK) to suppress apoptosis (Z. Fu et al., 2023) Regulates ciliogenesis, and Hedgehog signalling downstream

	Promotes G1/S transition, and to a lesser extent G2/M	(Snouffer et al., 2017; Y. Yang et al., 2013)
	(Mok et al., 2018)	Promotes cell growth and survival in different contexts
	Promotes progression through the cell cycle by cyclin D upregulation	(Lai et al., 2020)
	(Wu et al., 2009)	Promotes the establishment of an immunosuppressive tumour microenvironment
	and cyclin E	
	(X. An et al., 2010)	
	Stimulates Wnt/ β -catenin/TCF signalling pathway to drive cell cycle progression	(Mok et al., 2018; Zhou et al., 2018)
	(Feng et al., 2011)	

Table 1.8. Additional CDKs in *S. cerevisiae*.

Cyclin-dependent kinase	Binding partner	Roles in cell cycle	Roles in transcription	Selected additional roles
Pho85	Pcl1, Pcl2, Pho80, Clg1, Pcl5–10	Required for G1 progression and cell cycle commitment in the absence of Cln1 and Cln2	Deactivates Pho4 in high phosphate conditions	Downregulates Gsy2 glycogen synthase
		(Espinoza et al., 1994; Measday et al., 1994, 1997)	(Huang et al., 2007; Lenburg & O'Shea, 1996)	(Carroll & O'Shea, 2002; Huang et al., 1998, 2007)
		Phosphorylates Sic1 leading to its degradation and G1/S transition	Phosphorylates Gcn4p to target it for degradation in high amino acid conditions	Implicated in bud morphogenesis
		(Nishizawa et al., 1998)	(Carroll & O'Shea, 2002; Huang et al., 2007; Meimoun et al., 2000; Shemer et al., 2002)	(Carroll & O'Shea, 2002; Huang et al., 2007; Moffat & Andrews, 2003)
		Phosphorylates Rim15 to prevent cells from entering G0	Phosphorylates Rim101 for export from the nucleus to regulate alkali stress response	Downregulates autophagy
		(Huang et al., 2007)		(Wang et al., 2001)
		Establishes mitotic spindle in M and interacts with spindle assembly checkpoint	(Nishizawa et al., 2010)	Regulates the actin cytoskeleton
			Phosphorylates Crz1	(J. Lee et al., 1998)

	genes MAD1 and BUB3 (Daniel et al., 2006)	in high calcium conditions (Huang et al., 2007; Sopko et al., 2006)

Table 1.9. Additional CDKs in *S. pombe*.

Cyclin-dependent kinase	Binding partner	Roles in cell cycle	Roles in transcription	Selected additional roles
Pef1	Pas1, Clg1, Psl2	Promotes pre-meiotic DNA replication (Matsuda, Kikkawa, & Nakashima, 2021) Upregulates cohesin binding during the cell cycle (Biot et al., 2020)	Activates Res2p-Cdc10p on MCB genes (Tanaka & Okayama, 2000)	Regulates sexual differentiation through upregulation of Ste11 and balancing the TORC1 pathway and autophagy (Matsuda, Kikkawa, Uda, et al., 2020) Downregulates cellular lifespan (Chen et al., 2013)
CDK11	Lcp1		Upregulates assembly of the mediator complex (Drogat et al., 2012)	

2.MATERIALS AND METHODS

2.1. Experimental methods

2.1.1. Cell culture

Human cell lines used in the study were grown at 37°C, 5% CO₂ in GS Biotech 170L CO₂ Incubator. *Drosophila* S2 cells were cultured in Shields and Sang M3 Insect Medium (Sigma-Aldrich, #S3652) and were kept at room temperature. Passaging of cells was completed in a category 2 laminar flow hood. For experiments, the cells were grown to 60-80% confluency and were treated with drugs at concentrations and durations specified in the text. Typically, CDK1 was inhibited by addition of 7.5µM of the ATP analogue 1-NM-PP1 (Cayman Chemical; #13330) for 0.5h or 2h. Control samples were incubated with DMSO (Sigma-Aldrich; #D8418), the resuspension vehicle for all drugs used in this study.

2.1.1.1. *HeLa*

HeLa cells were obtained from American Type Culture Collection (ATCC® CCL-2™). The line was cultured in Dulbecco's Modified Eagle Medium (DMEM; Sigma Aldrich; #D6429), supplemented with 10% foetal bovine serum, 2mM L-glutamine, 100U/ml penicillin, and 100µg/ml streptomycin.

The HeLa AS-CDK1 cell line was a gift from Prof Helfrid Hochegger (Rata et al., 2018). The line was created by delivering a pcDNA3.1 vector with stably expressed Cdk1-F80G-myc protein into HeLa MKK1 cells, and further deleting the endogenous CDK1 (Rata et al., 2018). Both HeLa and AS-CDK1 HeLa cell lines were cultured in the same conditions.

2.1.1.2. HEK293

HEK293 cells were obtained from American Type Culture Collection (ATCC® CRL-157™). The line was cultured in Dulbecco's Modified Eagle Medium (DMEM; Sigma Aldrich; #D6429), supplemented with 10% foetal bovine serum, 2mM L-glutamine, 100U/ml penicillin, and 100µg/ml streptomycin.

HEK293 AS-CDK1 cells were created during the course of this study, by introducing a single point mutation in a wild-type HEK293 cell line using CRISPR-Cas9 system. Remaining AS-CDK HEK293 cell lines (AS-CDK7, AS-CDK8, AS-CDK9, AS-CDK10, AS-CDK12, AS-CDK13, AS-CDK12/13) were generated previously in the lab, also with the use of CRISPR methods. All HEK293 cell lines were cultured in the same conditions.

2.1.1.3. HCT116

HCT116 cells were acquired from Horizon Discovery (#HD PAR-073). HCT116 cells were grown in McCoy's 5A (Modified) Medium (Gibco; #16600082) supplemented with 10% foetal bovine serum, 100U/ml penicillin, and 100µg/ml streptomycin.

HCT116^{WT/AS-CDK1} cell line was created during the course of this study, by introducing a single point mutation in wild-type HCT116 cells using CRISPR-Cas9 system. Both HCT116 cell lines were cultured in the same conditions.

2.1.2. CRISPR methods

To create AS-CDK1 cell lines, a single amino acid substitution of phenylalanine 80 to glycine needs to be introduced in the wild-type CDK1 sequence in order to enlarge its ATP-binding pocket. In addition, methionine 32 can be exchanged to valine to boost kinase activity, although this substitution does not change its specificity (Bishop et al., 2000; Lopez et al., 2014). Additional silent mutations need to be added to each base

change to introduce new restriction sites and facilitate screening of transformed mutants. The desired sequence changes for CDK1 F80G and M32V mutations are outlined in the table below.

Mutation	F80G	M32V
Base change	TTT > GGC	ATG > GTG
Overall sequence changes	TTTGAGTTT > GGCGAATTC	AGCCATG > GGCCGTG
Restriction site introduced	G ^v AATTC	Y ^v GGCCR
Restriction enzyme used for screening	<i>EcoRI</i>	<i>EaeI</i>

2.1.2.1. Design of CRISPR constructs

In this project, the editing of DNA with CRISPR was achieved through homology-directed repair, and as such required custom constructs with guide RNAs (gRNAs) and repair templates. A range of paired and single gRNAs used in this study was devised using the Benchling software, while the homology arms of the repair templates were designed manually. The homology arms flanked the mutation locus approximately 500bp on each side. The primers used for the creation of all of the constructs can be found in **section 2.2.5.1.**

2.1.2.2. Molecular cloning

Molecular cloning of gRNAs and repair templates was conducted according to standard procedures. For gRNA construct preparation, the forward and reverse primers of a single gRNA were first ligated with the *BbsI* sites of a cut pSpCas9n(BB)-2A-Puro (PX462) V2.0 plasmid (Cong et al., 2013; Ran et al., 2013) carrying a nickase mutant, in 20µl of

final reaction volume. Subsequently, 10µl of the ligation was transformed into 100µl of competent *E. coli* cells (NM541 strain) and the bacteria were grown on LB agar plates with 100µg/ml ampicillin overnight. Following bacterial transformation, single colonies were picked up and placed in 200µl 2xYT broth with 100µg/ml ampicillin for overnight inoculation at 37°C. The following day, plasmid DNA was purified using the QIAprep Spin Miniprep Kit (Qiagen, #27106) according to the enclosed protocol. The DNA was eluted in filter-sterilised distilled water and sent for sequencing with the SourceBioscience Sanger Sequencing Service in Oxford to confirm the correct sequences of the constructs.

Once the sequences of the clones were checked, the appropriate colonies were picked out again and this time inoculated overnight at 37°C in 100ml of 2xYT broth with 100µg/ml ampicillin. The DNA was then extracted from the bacteria with the use of QIAGEN Plasmid Maxi Kit (Qiagen; #12163). The protocol of the kit was followed up to and including the step of precipitation of the eluted DNA with isopropanol. After the centrifugation of the precipitated DNA, the supernatant was carefully poured out and the pellet was resuspended in 1ml of isopropanol. The samples were then moved to 1.5ml tubes and centrifuged twice at 15800 x *g* for 2min at room temperature to remove the supernatant. The pellets were resuspended in 180µl of Nuclease-Free Water (Invitrogen; #AM9937) and 20µl of 4M ammonium acetate was added. The samples were then left at 4°C to dissolve. Once ready, they were sent for sequencing with the SourceBioscience Sanger Sequencing Service to further confirm the construct sequences.

After the sequences were validated, the extracted DNA of the chosen constructs was filtered by phenol/chloroform extraction. The samples were first mixed with 200µl of phenol. The tubes were vortexed and centrifuged at 15800 x *g* for 5min. The top aqueous

layer was then moved to a new tube, and 500µl of 100% EtOH was added. The samples were vortexed and centrifuged twice at room temperature at 15800 x *g* for 5min and then 1min to remove the ethanol. The individual gRNAs were then dissolved in 100µl of Nuclease-Free Water. The DNA quantity of the samples was determined using the NanoDrop™ One Spectrophotometer (Thermo Scientific, #ND-ONE-W). Finally, the constructs were diluted to 1µg/µl to ready them for cell transfections.

In order to prepare the repair template construct, firstly the forward primer of the second homology arm was phosphorylated in 20µl of reaction volume using the T4 Polynucleotide Kinase (Thermo Scientific; #EK0031) according to the manufacturer's instructions. The homology arm templates were then amplified by setting up two 50µl PCR reactions with the HyperPFU™ high-fidelity DNA polymerase (APExBio; #K1031) following the producer's guidelines. The PCR products were run on a 2% agarose gel with 0.001% ethidium bromide to confirm the correct amplification of the homology arms, and were subsequently purified by phenol/chloroform extraction. The samples were supplemented with 45µl of water and 10µl of 4M ammonium acetate before the addition of 100µl of phenol. The tubes were vortexed and centrifuged for 5min at 15800 x *g* at room temperature. The top layer of the supernatant was then moved to a new tube and mixed with 250µl of 100% EtOH. The samples were vortexed and centrifuged twice at room temperature at 15800 x *g*, for 5min and 1min. After removal of ethanol, the pellets were dissolved in 20µl of water.

The digestion of the repair template was then prepared using 10µl of purified DNA. For each reaction, 6µl of water, 2µl of buffer, and 2µl of enzyme were added. The first homology arm of the repair template was digested using *NheI* (New England Biolabs; #R3131) and rCutSmart™ Buffer (New England Biolabs; # B6004S), while the second

homology arm required *NotI* (New England Biolabs; #R0189) and buffer H (50mM Tris-HCl, pH 7.5; 10mM MgCl₂; 1mM dithiothreitol (DTT); 100mM NaCl). The digestion reactions were left at 37°C overnight, and were then run on a 2% agarose gel with 0.001% ethidium bromide for validation. The separated bands were excised with a razor blade and eluted using the QIAquick PCR Purification Kit (Qiagen, #28106). Subsequently, a 3-way ligation was prepared using 1µl of the T4 DNA Ligase (Roche; #DNALIG-RO) and 2µl of LX10 buffer. The reaction was made up to 20µl, with 2µl of homology arms DNA each, and 0.5µl of a cut pcDNA6 vector. After 2h at room temperature, 10µl of the ligation mix was transformed into 100µl of competent *E. coli* cells (NM541 strain).

The bacteria were grown overnight on LB agar plates with 100µg/ml ampicillin, and after the formation of individual colonies, the clones were screened for the incorporation of the plasmid with the repair template. PCR reactions of 20µl were set up using the GoTaq® Hot Start Polymerase (Promega; #M5001) according to the enclosed protocol. The DNA was added to the reaction by swirling bacteria-coated toothpicks in the individual tubes. The amplified PCR products were run on a 2% agarose gel with 0.001% ethidium bromide to identify colonies harbouring the correct construct. The candidate clones were then picked again and set up for overnight inoculation in 200µl of 2xYT broth. The remainder of the protocol was performed correspondingly to the gRNAs preparation, leading to the production of 1µg/µl repair template construct ready for cell transfections.

2.1.2.3. Cell transfections

Following the preparation of the constructs, cells were transfected with the edited DNA. Typically, for 2ml of cells in culture a mix of 200µl jetPRIME® buffer, 4µl jetPRIME® transfection reagent (VWR; #101000046), and 2µg of DNA was prepared. The 2µg of DNA included 0.8µg of the repair template and 0.6µg of forward and reverse gRNAs

each. The components were mixed gently and left for 10min at room temperature, before being added to the cells. The transfected cells were then kept in an incubator, and after 24h the medium with the transfection mix was removed and changed to a fresh one.

2.1.2.4. Antibiotic selection

Transfected colonies were treated with antibiotics to select for cells which had accepted the plasmids with constructs. The cells were incubated at the same time with 10µg/ml of Blasticidin to select for the pcDNA6 plasmids carrying the repair template, and with 2µg/ml of Puromycin to select for the px462 plasmids with gRNAs. The antibiotics were kept on the cells for 48h before changing the medium.

2.1.2.5. Positive selection method of CDK1-F80G mutants

Owing to the CDK1 being a non-redundant cell-cycle regulator, mutants for the F80G substitution could be further screened using a novel positive selection method established during this study. Following the antibiotic treatment, the cells were given 24h to recover, and were then incubated simultaneously with 10µM 1-NM-PP1 and 0.1µM Taxol (gift from the Gruneberg lab). The incubation lasted for 24h, after which the media was changed and the cells were given a further 24h for recovery.

2.1.2.6. Screening of clones

Following the selection of transfected clones, individual cells were placed for inoculation into 96-well plates containing 200µl medium (50% fresh, 50% preconditioned) in each well. The sorting was performed using BD FACSAria III in the Sir William Dunn School of Pathology Flow Cytometry Facility. After the appearance of colonies in individual wells, the cells were pelleted and resuspended in 40µl of lysis buffer used for ChIP (see **sections 2.1.8. and 2.2.4.2.**). The samples were then sonicated for 3min at 100% amplitude and 30s on/off cycle using Q800R2 Sonicator (Qsonica).

2.1.2.6.1. Polymerase chain reaction (PCR)

The DNA obtained from the clones was taken to set up PCR reactions with HyperPFU™ high-fidelity DNA polymerase according to the manufacturer's instructions. For a final volume of 50µl, 2µl of DNA was added. The primers used for screening of clones are listed in **section 2.2.5.1**. Following the completion of PCR cycles, 5µl of the mix was run on a 2% agarose gel with 0.001% ethidium bromide to verify the success of the reaction.

2.1.2.6.2. Restriction enzyme digestion

To identify potential mutants, PCR products were digested with restriction enzymes to screen for the artificially-introduced restriction sites. Reactions of 10µl amplified DNA, 2µl buffer, 0.5µl enzyme and 7.5µl water were prepared. The samples were then incubated at 37°C for 2h. The F80G mutation was screened for using the EcoRI restriction enzyme (New England Biolabs; #R0101) and NEBuffer™ r2.1 (New England Biolabs; #B6002S), while EaeI (New England Biolabs; #R0508) and rCutSmart™ Buffer were used for the M32V screens. The digested DNA was then run on a 2% agarose gel with 0.001% ethidium bromide and samples with visible separation of bands were marked as possible mutants.

2.1.2.6.3. DNA sequencing

Candidates identified during the restriction enzyme digestion had their amplified DNA extracted using the QIAquick PCR Purification Kit. The purified PCR products were then sent for sequencing with a custom sequencing primer (found in **section 2.2.5.1**) to the SourceBioscience Sanger Sequencing Service.

2.1.3. Whole-cell protein extraction

For obtaining whole-cell extracts, cells were collected by scraping and centrifuged twice at 15800 x *g* to get rid of media. The cellular pellets were then resuspended in 1x

NuPAGE™ LDS Sample Buffer (Invitrogen, #NP0007) with 50mM DTT (Sigma-Aldrich; #D9779), supplemented with 1.5µl Benzonase® Nuclease (Millipore, #70664), and lysed for 30min at room temperature.

2.1.4. Western blotting

Before running on a gel, samples were denatured by boiling at 70°C for 10min, with additional 50mM DTT. During Western blotting, 3µl of each sample was run on a NuPAGE™ 4–12% Bis-Tris Gel (Invitrogen, #NP0323BOX) at 100V in 1x NuPAGE™ MOPS SDS Running Buffer (Invitrogen, #NP0001). The proteins were subsequently transferred onto a 0.45µm nitrocellulose membrane (Thermo Scientific, #88018) using a Bio-Rad wet transfer tank. The transfer was performed at 100V in Tris-Glycine transfer buffer with 20% ethanol, at 4°C for 1h. Membranes were blocked in 5% Bovine Serum Albumin (BSA; Roche, #10735086001) in 1x Tris-Buffered Saline (TBS), for 1h at room temperature or overnight at 4°C. They were then moved to a 2.5% BSA in 1x TBS with 0.1% Tween (TBS-T) solution for primary antibody incubation, which lasted for 1h at room temperature, or overnight at 4°C. Membranes were washed 3 times for 5min in TBS-T, and were incubated in secondary antibodies in 2.5% BSA in 1x TBS-T for 1h at room temperature. List of antibodies used in this study can be found in **section 2.2.2**. Three TBS-T washes were then repeated. The proteins of interest were visualised on iBright FL1000 Imaging System (Thermo Scientific, #A32752) using SuperSignal™ West Pico PLUS Chemiluminescent Substrate (Thermo Scientific, #34580).

2.1.5.5-Ethynyl Uridine (EU) labelling

Cells of interest were plated on 1.5mm thick, 13mm round coverslips (VWR International, #631-0150) in 6-well plates, before administering treatments the following day. The cells were further incubated with 1mM 5-ethynyl uridine (EU), which was

added 30min before the end of treatments to label newly-synthesised RNA. Cell fixation was performed by transferring the coverslips into 3.7% formaldehyde in phosphate-buffered saline (PBS). After 15min fixation, the coverslips were washed once with PBS. They were subsequently permeabilised with 0.5% Triton X-100 diluted in PBS for 15min at room temperature, followed by another PBS wash. Nascent RNA with incorporated EU was visualised using Click-iT™ RNA Alexa Fluor™ 488 Imaging Kit (Thermo Scientific, #C10329). In accordance with the protocol, the coverslips were incubated with 500µl of Click-iT® reaction cocktail for 30min, shielded from light. They were then washed once with Click-iT® reaction rinse buffer and once with PBS. Hoechst 33342 was diluted to 0.01mg/ml in PBS, and 1ml was added to each coverslip to stain cellular DNA. The cells were incubated for 15min at room temperature, protected from light. Following two further PBS washes, the coverslips were mounted on Fisherbrand™ Microscope Slides with Ground Edges (Fisherbrand, #12373118) using Fluoromount-G (SouthernBiotech, #0100-01) mounting medium.

The slides were examined using inverted multi-channel fluorescence microscope EVOS™ M7000 Imaging System (Thermo Scientific, #AMF7000). For analysis, 200-280 non-overlapping images were acquired randomly per condition, using a 20x objective and z-stack autofocus. The images were taken of the GFP and DAPI channels under non-saturating conditions, which displayed Alexa Fluor 488-stained RNA and Hoechst 33342-stained DNA respectively. The same settings were used for all conditions in the same type of cell line. Subsequent evaluation of fluorescence intensity was established using Cell Profiler software, with DAPI channel used for masking nuclei. The masks were then transposed onto the GFP channel to calculate the mean fluorescence intensity of all pixels within a nucleus, and thus determine the level of new RNA production in a cell.

2.1.6. Cell synchronisation

Cell synchronisation at the G1/S transition was achieved by performing a double thymidine block on 30-40% confluent cells. Firstly, cells were treated with 2mM thymidine (Sigma-Aldrich, #T1895) for 18h. They were subsequently washed with PBS twice and left in fresh medium in normal growing conditions. The medium was changed again after 9h, and the cells were treated with 2mM thymidine for a second time. After 17h of incubation with the drug, the cells were released from the block by washing twice with PBS, and they were harvested for further experiments.

2.1.7. Propidium Iodide (PI) staining

The cells were collected by centrifugation at 400 x *g* for 5min, and washed once with PBS. Cell pellets were resuspended in 330µl of cold PBS and fixed by adding 660µl of cold 100% ethanol while gently vortexing. The samples were then left at 4°C for at least 30min. Subsequently, the cells were washed twice with PBS (with centrifugation at 800 x *g* for 5min), and resuspended in PBS with 40µg/ml propidium iodide (PI) and 100µg/ml RNase A (Thermo Scientific, #EN0531). The samples were then kept for 15min in the dark at room temperature. Flow cytometry was performed on BD LSRFortessa X-20, using 561nm excitation laser and 610/20 filter. Finally, cell cycle profile data were examined in FlowJo software.

2.1.8. Chromatin immunoprecipitation (ChIP)

For chromatin immunoprecipitation (ChIP), cells were first cross-linked by incubation with 1% (w/v) formaldehyde for 10min at room temperature, while shaking. The reaction was quenched by addition of 125mM of glycine and a further 5min incubation shaking at room temperature. The cells were then washed twice with ice-cold PBS and scraped into

1.5ml Eppendorf tubes. The cells were pelleted by 5min centrifugation at 200 x *g* at 4°C, and the pellets were resuspended in 1ml of ChIP lysis buffer supplemented with protease and phosphatase inhibitors. The samples were left on ice for 10min, before centrifuging at 1500 x *g* for 5min at 4°C. After the removal of ChIP lysis buffer, they were then resuspended in 1ml ChIP wash buffer with protease and phosphatase inhibitors, and centrifuged again for 5min at 1500 x *g* at 4°C. The pellets were resuspended in 600µl of ChIP sonication buffer with protease and phosphatase inhibitors, separated into two 300µl samples, and sonicated for 30min on high setting 30s on/30s off using a Bioruptor® Plus sonication device (Diagenode, #B01020001) at 4°C. The sonicated samples were centrifuged for 15min at 20000 x *g* at 4°C and the supernatant from both tubes was then combined. DNA quantities were determined using NanoDrop™ One spectrophotometer. For preclearing, 20µl per sample of Dynabeads™ Protein G for Immunoprecipitation (Invitrogen, #10003D) was washed twice with 1ml of radioimmunoprecipitation assay (RIPA) buffer and 20µl of beads was then added to each condition. The tubes were subsequently placed on a rotating wheel for 30min at 16rpm at 4°C. Following preclearing, samples of 50-100µg of chromatin diluted in 150µl of RIPA buffer were incubated with at least 1µg of antibody overnight, rotating on a wheel at 16rpm at 4°C. The list of antibodies used for ChIP can be found in **Section 2.2.2**. Additionally, 20µl of Dynabeads per IP was washed once with 200µl of RIPA buffer and saturated in RIPA buffer with 4mg/ml of BSA equating their original volume. They were also incubated overnight, on a wheel rotating at 16rpm at 4°C.

On the following day, pre-blocked beads were washed twice in 5 times their volume of RIPA buffer, and after resuspending in their original volume, 20µl of Dynabeads was added to each IP sample. The extracts were incubated for 1h rotating on a wheel at 16rpm at 4°C. Subsequently, 100µl of IgG IP samples were taken as inputs, and the beads were

washed three times with 300µl RIPA, three times with 300µl High Salt Wash Buffer, twice with 300µl LiCl Wash Buffer, and twice with 600µl TE buffer (all the buffers were kept ice-cold). To elute the chromatin, the beads were incubated twice with 50µl of Elution buffer with 10mM DTT in Eppendorf ThermoMixer® C set at 23°C 1400rpm for 15min. In parallel, 15µl of the input samples were combined with 85µl of Elution buffer with DTT. The eluted samples were incubated with 1µl RNase A for 30min at 37°C, and then decrosslinked by addition of 200mM NaCl and 5h incubation at 65°C. Further, 2.5x volume of 100% ethanol was added and the DNA precipitated at -20°C overnight.

The samples were centrifuged for 20min at 18800 x *g* at 4°C and additional 2min centrifugation was used to remove residual ethanol. The pellets were resuspended in a mix of 100µl TE buffer, 25µl 5x Proteinase K buffer, and 1.5µl of Proteinase K (Roche, #RPROTKSOL-RO), and were incubated for 1.5h at 45°C. Finally, DNA was purified using the QIAquick PCR Purification Kit.

2.1.9. Chromatin RNA extraction

Cells plated in 15cm dishes were first washed with 1x PBS before scraping into 1.5ml Eppendorf tubes. After spinning for 5min at 200 x *g* at 4°C, the pellets were resuspended in 15ml Falcon tubes in 4ml of chilled HLBN with protease and phosphatase inhibitors. The samples were then underlaid with 1ml of ice-cold HLBN with sucrose, also supplemented with protease and phosphatase inhibitors. The tubes were centrifuged at 1000 x *g* for 5min at 4°C, and the supernatant was removed. The nuclear pellets were resuspended in 120µl of chilled NUN1 with protease and phosphatase inhibitors and 0.1M DTT. Further, 1200µl of ice-cold NUN2 with protease and phosphatase inhibitors and 0.1M DTT was added, the samples were vortexed gently and incubated on ice for 15min, with additional vortexing every 5min. To obtain the chromatin pellet, the samples were

centrifuged for 10min at 21100 x *g* at 4°C and the nucleoplasmic supernatant was discarded. The pellets were subsequently resuspended in 200µl of HSB and 5µl of TURBO™ DNase (Invitrogen, #AM2238) and left for 30min in Eppendorf ThermoMixer® C set at 37°C 1400rpm. Afterwards, a 200µl cocktail containing 8µl of 10% SDS and 8µl of Proteinase K (Roche, # RPROTKSOL-RO) dissolved in water was added, and the samples were incubated for further 10min in the Eppendorf ThermoMixer® C at 37°C 1400rpm. To perform phenol/chloroform extraction of RNA, 800µl of acidic phenol (VWR; #0966-100ML) was added to the samples and they were mixed and incubated at room temperature for 3min. The tubes were centrifuged at 12000 x *g* for 10min at 4°C, and the upper phase was then moved to a new tube and combined with 400µl of ice-cold isopropanol. The RNA was left to precipitate overnight at -20°C, or occasionally at room temperature for 10min.

The samples were pelleted by centrifugation at 12000 x *g* for 10min at 4°C and the supernatant was discarded. The pellets were washed with 75% ethanol and centrifuged twice for 5min 12000 x *g* at 4°C to remove liquid. They were then air-dried briefly (~5min) before dissolving in 40µl water, 5µl DNase I Reaction Buffer, 2.5µl DNase I (New England Biolabs; #M0303S), and 2.5µl RNaseOUT™ Recombinant Ribonuclease Inhibitor (Invitrogen; #10777019). The RNA was incubated in the Eppendorf ThermoMixer® C for 10min at 37°C 1400rpm before proceeding to Trizol extraction. To obtain purified RNA from the mix, the samples were treated with 1ml of TRIzol™ Reagent (Invitrogen; #15596026) and left for 3min at room temperature. Subsequently, 220µl of chloroform was added to each tube, and they were then shaken vigorously and left at room temperature for further 3min. The samples were centrifuged for 15min at 12000 x *g* at 4°C, and the upper aqueous phase was transferred into fresh tubes and mixed with 550µl chilled isopropanol. In a similar manner to the phenol/chloroform extraction,

the precipitation of RNA was performed at -20°C overnight, or sporadically at room temperature for 10min.

The RNA samples were centrifuged for 10min at 12000 x *g* at 4°C and the pellets were washed with 75% ethanol. The supernatant was then removed by centrifuging twice for 5min at 12000 x *g* at 4°C and the pellets were air-dried for around 5min. Elution of RNA was performed by dissolving the pellets in 100µl of Nuclease-Free Water and leaving them to incubate for 10min in the Eppendorf ThermoMixer® C at 37°C 1400rpm. The samples were moved to Eppendorf™ DNA LoBind™ Tubes (Fisher Scientific; #10051232) and RNA quantity of each condition was determined using NanoDrop™ One Spectrophotometer. An additional DNase treatment was performed by supplementing 1µg of RNA with 1µl DNase I Reaction Buffer, 0.9µl DNase I, 0.1µl RNaseOUT™ Recombinant Ribonuclease Inhibitor and water to make up 10µl of reaction mix. The samples were then incubated for 1h at 37°C, followed by 10min at 75°C to deactivate the DNase.

2.1.9.1. First strand cDNA synthesis

Following the DNase treatment, RNA samples were split into two 5µl samples and reverse transcription was performed using SuperScript™ III Reverse Transcriptase kit (Invitrogen; #18080085) according to the manufacturer's instructions. In short, samples were diluted with additional 9.75µl of water and then supplemented with 1µl of Random Primers (Invitrogen; #48190011) and 1µl of 10mM dNTPs (Bioline; #BIO-39025). They were incubated for 15min at 65°C. The reverse transcriptase mixes were prepared and 8.25µl of either a positive (containing SuperScript) or a negative (without SuperScript) mix was added to the samples, which were left for 1h at 55°C and 15min at 70°C. The prepared cDNA was diluted with 25µl of water and kept at -20°C.

2.1.10. Quantitative reverse-transcription polymerase chain reaction (qRT-PCR)

DNA from ChIP samples and cDNA from chromatin RNA extraction was amplified by real-time qPCR using QuantiTect SYBR® Green PCR Kit (Qiagen; # 204145) in a Rotor-Gene 3000 thermocycler (Corbett Research). For ChIP experiments, values were normalised and presented as percentage of total DNA input, while changes in RNA levels were normalised to GAPDH RNA. The primers used for qRT-PCR can be found in **sections 2.2.5.2. to 2.2.5.3.**

2.1.11. RNA-seq

To prepare samples for chromatin RNA-seq, the cells were cultured and treated as described in **section 2.1.9.** After scraping the cells into tubes and getting rid of media, a suspension of *Drosophila* S2 cells was added to each sample to create a 10% *Drosophila* spike-in and the samples were pelleted again. The spiked cells were then resuspended in HLBN buffer and chromatin RNA was acquired as detailed in the aforementioned section. Following the final elution in 100µl of Nuclease-Free Water, the samples were quantified and 2µg of RNA was used for the DNase treatment, which was performed in 30µl of reaction mix volume. Subsequently, 20µl of water was added to the samples. To enrich for nascent transcripts, depletion of Poly(A)⁺ mRNA was conducted with the use of NEBNext® Poly(A) mRNA Magnetic Isolation Module (New England Biolabs; #E7490). The kit instructions were followed to separate Poly(A)⁺ and Poly(A)⁻ mRNA, and after binding of Poly(A)⁺ mRNA to the magnetic beads, the supernatant with Poly(A)⁻ mRNA transcripts was recovered. Trizol extraction of RNA was then performed on the supernatant, as described in **section 2.1.9.** After addition of isopropanol, the samples were incubated for 10min at room temperature, centrifuged, and the pellets were washed with 75% ethanol. The RNA was then resuspended in 12µl of Nuclease-Free

Water and incubated for 10min in the Eppendorf ThermoMixer® C at 37°C 1400rpm. The samples were then moved to Eppendorf™ DNA LoBind™ Tubes and RNA quantity was determined. For subsequent steps, samples of 1µg of RNA in 11µl of water were prepared.

To remove rRNA transcripts from the samples, NEBNext® rRNA Depletion Kit v2 (New England Biolabs; #E7405) was used according to manufacturer's instructions. RNA purification was performed using NEBNext® RNA Sample Purification Beads (New England Biolabs; #E6351). RNA libraries were prepared using NEBNext® Ultra™ II Directional RNA Library Prep Kit for Illumina® (New England Biolabs; #E7760). Throughout the protocol, all purification steps were completed using SPRIselect beads (Beckman Coulter; #B23318), and the final purification was repeated twice to increase sample quality. During the PCR Enrichment of Adaptor Ligated DNA, NEBNext® Multiplex Oligos for Illumina® Index Primers Sets 1 and 2 (New England Biolabs; #E7335, #E7500) were used to introduce barcodes for library multiplexing, and the libraries were amplified with 7 cycles of PCR. The concentration of the final 20µl of premade libraries was determined with Qubit™ 4 Fluorometer (Invitrogen; #Q33240) and the quality control of DNA was performed using 4150 TapeStation System (Agilent; #G2992AA). Finally, the libraries were pooled and sequenced on the Illumina NovaSeq X Plus PE150 platform in the Novogene UK facility in Cambridge.

2.1.11.1. RNA-seq bioinformatics analysis

The bioinformatics analysis of the RNA-seq data was performed by Dr Callum Henfrey. The ensuing evaluation of the results and graphs was a product of my own work.

2.1.11.1.1. Pre-processing

Relevant RNA-seq datasets were downloaded in fastq.gz format directly from Novogene website. Read quality metrics were assessed using FASTQC version 0.12.1 (Andrews, 2010). Adapter sequences were trimmed from .fastq.gz files using CUTADAPT version 1.18 (Martin, 2011), set with the following parameters in paired-end mode; --minimum-length 10 -q 15,10 -j 16 -A AGATCGGAAGAGCGTCGTGTAGGGAAAGAGTGTAGATCTCGGTGGTCGCCGTATCATT -a AGATCGGAAGAGCACACGTCTGAACTCCAGTCAC.

Reads were aligned to a hybrid genome comprising the human genome hg38 and the *Drosophila melanogaster* dm6 genome using STAR (Spliced Transcripts Alignment to a Reference) version 2.7.8 (Dobin et al., 2013), with parameters --runThreadN 16 --readFilesCommand gunzip -c -k --outReadsUnmapped Fastx --limitBAMsortRAM 20000000000 --quantMode GeneCounts --outSAMtype BAM SortedByCoordinate to produce .bam files. Reads mapping to rRNA repeat regions were initially removed to account for unequal rRNA depletion between samples.

The number of exon-mapping reads per gene were determined using the quantMode GeneCounts parameter during alignment of reads to the genome.

2.1.11.1.2. Differential Gene Expression analysis

Raw read count files were inputted into R version 4.0.5. and differential expression analysis was performed using the DESeq2 and apegglm packages. Counts mapping to the *Drosophila* genes were used as control genes for DESeq2 to determine size factors for spike-in normalisation. From the resulting outputs, genes were selected as either up-regulated or down-regulated with a log2FoldChange of $> |1|$ and adjusted p-value < 0.05

(adjusted p-value < 0.1 and no log2FoldChange cutoff for the less stringent test). The MA plot and metagene profiles were generated using the ggplot2 package.

2.1.11.1.3. Motif analysis

Genes of interest were input into Multiple Em for Motif Elicitation (MEME) software for the identification of new motifs (Bailey & Elkan, 1994). Simple Enrichment Analysis (SEA) was performed to recognise enriched any known motifs in a dataset (Bailey & Grant, 2021).

2.1.12. Data analysis and visualisation

All numerical data gathered in this study were analysed using GraphPad Prism software. Firstly, the data points were checked for normal distribution, after which the appropriate statistical tests were applied and the fitting graphs were created. Remaining figures and diagrams were compiled using Adobe Photoshop and Adobe Illustrator.

2.2. Materials

2.2.1. Cell lines

Cell line	Source	Identifier
HeLa	ATCC	CCL-2
HeLa AS-CDK1	Gift from Prof Hochegger	Rata et al., 2018
HEK293	ATCC	CRL-157
HEK293 AS-CDK1	This work	N/A
HEK293 AS-CDK7	Murphy lab	Velychko et al., 2024
HEK293 AS-CDK8	Murphy lab	N/A
HEK293 AS-CDK9	Murphy lab	Tellier et al., 2022
HEK293 AS-CDK10	Murphy lab	N/A
HEK293 AS-CDK12	Murphy lab	Tellier et al., 2020
HEK293 AS-CDK13	Murphy lab	N/A
HEK293 AS-CDK12/13	Murphy lab	N/A
HCT116	Horizon Discovery	HD PAR-073
HCT116 CDK1 ^{WT/AS}	This work	N/A
Drosophila S2	Gift from Proudfoot lab	N/A

2.2.2. Antibodies

Antibody	Source	Identifier	Dilution (WB) / amount (ChIP)
<i>Western blotting</i>			
Anti-beta Tubulin antibody	Abcam	ab6046	1:500
Anti-Histone H3 antibody	Abcam	ab1791	1:20000
Anti-Nucleolin antibody	Abcam	ab22758	1:10000
BMAL1 (D2L7G) Rabbit mAb	Cell Signalling Technology	14020S	1:1000
CDK1 Polyclonal Antibody	Bethyl Laboratories	A303-663A	1:1000
Per1 Antibody (E-8)	Santa Cruz Biotechnology	sc-398890	1:1000
Phospho-Histone H3 (Ser10) Polyclonal Antibody	Invitrogen	PA5-17869	1:1000
TXNIP Polyclonal antibody	Proteintech	18243-1-AP	1:1000
<i>Chromatin immunoprecipitation (ChIP)</i>			
CDK1 Polyclonal Antibody	Bethyl Laboratories	A303-663A	5µl (5µg)

Normal Rabbit IgG	Cell Signalling Technology	2729	1µl (1µg)
RNA pol II CTD phospho Ser5 antibody (mAb)	Active Motif	61085	2µl (2µg)
Rpb1 NTD (D8L4Y) Rabbit mAb	Cell Signalling Technology	14958	5µl

2.2.3. Chemicals and reagents

Chemicals and reagents	Source	Identifier
<i>Drugs and inhibitors</i>		
1-NM-PP1	Cayman Chemical	13330
3-MB-PP1	Sigma-Aldrich	567809
RO-3306	Santa Cruz Biotechnology	sc-358700
Taxol	Gift from Gruneberg lab	N/A
Thymidine	Sigma-Aldrich	T1895
<i>General</i>		
Benzonase® Nuclease	Millipore	70664
Bovine Serum Albumin	Roche	10735086001
cComplete™, EDTA-free Protease Inhibit	Sigma-Aldrich	5056489001
Dimethyl sulfoxide (DMSO)	Sigma-Aldrich	D8418

DL-Dithiothreitol (DTT)	Sigma-Aldrich	D9779
dNTP Set	Bioline	BIO-39025
Nuclease-Free Water (not DEPC-Treated)	Invitrogen	AM9937
PhosSTOP™	Roche	PHOSS-RO
Proteinase K	Roche	RPROTKSOL-RO
QIAquick PCR Purification Kit	Qiagen	28106
RNase A	Thermo Scientific	EN0531
<i>CRISPR methods</i>		
QIAprep Spin Miniprep Kit	Qiagen	27106
QIAGEN Plasmid Maxi Kit	Qiagen	12163
T4 Polynucleotide Kinase	Thermo Scientific	EK0031
NheI	New England Biolabs	R3131
rCutSmart™ Buffer	New England Biolabs	B6004S
NotI	New England Biolabs	R0189
GoTaq® Hot Start Polymerase	Promega	M5001
jetPRIME® DNA and siRNA transfection reagent	VWR	101000046

HyperPFU™ high-fidelity DNA polymerase	APExBIO	K1031
EcoRI	New England Biolabs	R0101
NEBuffer™ r2.1	New England Biolabs	B6002S
EaeI	New England Biolabs	R0508
<i>Western blotting</i>		
NuPAGE™ LDS Sample Buffer (4X)	Invitrogen	NP0007
NuPAGE™ MOPS SDS Running Buffer (20X)	Invitrogen	NP0001
SuperSignal™ West Pico PLUS Chemiluminescent Substrate	Thermo Scientific	34580
<i>5-Ethynyl Uridine (EU) labelling</i>		
Click-iT™ RNA Alexa Fluor™ 488 Imaging Kit	Thermo Scientific	C10329
<i>Chromatin immunoprecipitation (ChIP)</i>		
Dynabeads™ Protein G for Immunoprecipitation	Invitrogen	10003D
<i>Chromatin RNA extraction</i>		
TURBO™ DNase	Invitrogen	AM2238
Phenol : Chloroform, low pH	VWR	0966-100ML

DNase I (RNase-free)	New England Biolabs	M0303S
RNaseOUT™ Recombinant Ribonuclease Inhibitor	Invitrogen	10777019
TRIzol™ Reagent	Invitrogen	15596026
<i>First strand cDNA synthesis</i>		
SuperScript™ III Reverse Transcriptase	Invitrogen	18080085
Random Primers	Invitrogen	48190011
<i>qRT-PCR</i>		
QuantiTect SYBR® Green PCR Kit	Qiagen	204145
<i>RNA-seq</i>		
NEBNext® Poly(A) mRNA Magnetic Isolation Module	New England Biolabs	E7490
NEBNext® rRNA Depletion Kit v2 (Human/Mouse/Rat) with RNA Sample Purification Beads	New England Biolabs	E7405
NEBNext® RNA Sample Purification Beads (included in #E7405)	New England Biolabs	E6351

NEBNext® Ultra™ II Directional RNA Library Prep Kit for Illumina®	New England Biolabs	E7760
SPRIselect	Beckman Coulter	B23318
NEBNext® Multiplex Oligos for Illumina® (Index Primers Set 1)	New England Biolabs	E7335
NEBNext® Multiplex Oligos for Illumina® (Index Primers Set 2)	New England Biolabs	E7500

2.2.4. Buffers and solutions

2.2.4.1. Buffers for Western blotting

Tris-Glycine (20X): 0.2M Tris, 2M glycine

TBS (10X): 1500mM NaCl, 200mM Tris, pH 7.6

TBS-T: 150mM NaCl, 20mM Tris, 0.1% Tween

2.2.4.2. Buffers for ChIP

Lysis buffer: 0.25% Triton X-100, 10mM EDTA, 1% SDS, 10mM Tris-HCl pH 8.0

Wash buffer: 0.2M NaCl, 10mM Tris-HCl pH 8.0, 1mM EDTA

Sonication buffer: 100mM NaCl, 10mM Tris-HCl pH 8.0, 1mM EDTA, 0.1% SDS

RIPA buffer: 10mM Tris-HCl pH 8.0, 150mM NaCl, 1mM EDTA, 0.1% SDS, 1% Triton X-100, 0.1% Sodium deoxycholate

High Salt Wash buffer: 10mM Tris-HCl pH 8.0, 500mM NaCl, 1mM EDTA, 0.1% SDS, 1% Triton X-100, 0.1% Sodium deoxycholate

LiCl buffer: 10mM Tris-HCl pH 8.0, 250mM LiCl, 1mM EDTA, 1% NP-40, 1% Sodium deoxycholate

TE buffer: 10mM Tris-HCl pH 7.5, 1mM EDTA

Elution buffer: 100mM NaHCO₃, 1% SDS, 10mM DTT* (add fresh before use)

Proteinase K buffer (5X): 50mM Tris-HCl pH 7.5, 25mM EDTA, 1.25% SDS

2.2.4.3. Buffers for chromatin RNA extraction

HLBN: 10mM NaCl, 2.5mM MgCl₂, 10mM Tris-HCl pH 7.5, 0.1% NP-40

HLBN + sucrose: 10mM NaCl, 2.5mM MgCl₂, 10mM Tris-HCl pH 7.5, 0.1% NP-40, 24% sucrose

NUN1: 20mM Tris-HCl pH 8.0, 75mM NaCl, 0.5mM EDTA, 50% glycerol, 1 complete tablet of protease inhibitor (Sigma-Aldrich; #5056489001)

NUN2: 20mM HEPES pH 7.6, 7.5mM MgCl₂, 0.2mM EDTA, 0.3M NaCl, 1M urea, 1% NP-40

HSB: 10mM Tris-HCl pH 7.5, 500mM NaCl, 10mM MgCl₂

2.2.5. Primers

All primers used in the investigation were synthesised by Thermo Scientific as desalted 25nmol powder with no modifications. The sequences of all oligonucleotides in the 5'-3' direction are listed in **sections 2.2.5.1. to 2.2.5.3.**

2.2.5.1. Primers for CRISPR

CRISPR primers			
F80G	gRNAs	1F	CACCGCATCTTTGAGTTTCTTTCCA
		1R	AAACTGGAAAGAACTCAAAGATGC

		2F	CACCGCTCAAAGATGAGATATAACC
		2R	AAACGGTTATATCTCATCTTTGAGC
		3F	CACCGTACTTGGATTCTATCCCTCC
		3R	AAACGGAGGGATAGAATCCAAGTAC
		4F	CACCGCAAGTATTTCTTCAGATCCA
		4R	AAACTGGATCTGAAGAAATACTTGC
	Repair template homology arms	Homology arm 1 F	GTCAGCTAGCTTGGGGAGCTCTAGTT CCTG
		Homology arm 1 R	GATGAGATATAACCTGGAATCCTGC
		Homology arm 2 F	GCGAGTTTCTTTCCATGGATCTGAA G
		Homology arm 2 R	GTCAGCGGCCGCGGTTTGGTAGAACT GGTGCAAG
		Screening F	GAGAACAGGCTGTTAAAGCAGG
		Screening R	GCATATTCCCACAAAATGCAGGG
	Screening and sequencing	Sequencing	GTTTTGAGTCTCTTCCATTAGGG
M32V	gRNAs	1F	CACCGAGACTAGAAAGTGAAGAGGA
		1R	AAACTCCTCTTCACTTTCTAGTCTC
		2F	CACCGGACTAGAAAGTGAAGAGGAA
		2R	AAACTTCCTCTTCACTTTCTAGTCC
		3F	CACCGGGGTTCTTAGTACTGCAATT
		3R	AAACAATTGCAGTACTAGGAACCCC
		4F	CACCGTTCTAGTCTGATTTTTTTCA

		4R	AAACTGAAAAAAAAATCAGACTAGAAC
	Repair template homology arms	Homology arm 1 F	GTCAGCTAGCGAAGCAATGAGGGTA TGAGTAAG
		Homology arm 1 R	GGCCACCACTTGACCTGTAG
		Homology arm 2 F	GTGAAAAAAAAATCAGACTAGAAAGTG
		Homology arm 2 R	GTCAGCGGCCGCACCCAATGCAAATA CTTTAAGG
	Screening and sequencing	Screening F	GGGTAGGATATTGACAGAAGG
		Screening R	GGCTAGACCAAGAAATTGAGC
		Sequencing	GAGCCTTGATTGACCCACTGC

2.2.5.2. Primers for ChIP

ChIP-qPCR primers		
Negative	Negative F	TGGTACAACCACAGCTCAGTG
	Negative R	AAGCTGGACATGGTTGTGTG
KPNB1	KPNB1 TSS+0.2 F	TTACTTCCTCCCTCCAAATGGG
	KPNB1 TSS+0.2 R	ACAGCCTCCCTTCCTTCTTTC
	KPNB1 TSS+3.2 F	GCCCAGAGAACAAGAAATCG
	KPNB1 TSS+3.2 R	GGAATGGACAAGCTGTGTTG
	KPNB1 TSS+20.2 F	TGCAAGAGCCAGTGGGAACACTT
	KPNB1 TSS+20.2 R	CCTCTACTCAGCAATGATACTTC
	KPNB1 pA-4.8 F	CTGAGGAAACTGAAGAACCAAG
	KPNB1 pA-4.8 R	GAAGGCAGTGCTTGCCAGAAT
	KPNB1 pA-2.9 F	GAGGAGTGTGCACGGATGCTGAA
	KPNB1 pA-2.9 R	CCAAGATGGCCGATGTTATGG
	KPNB1 pA-0.4 F	TAGTTACCGTCTGCTTGGAAGATG
	KPNB1 pA-0.4 R	CCTCTGACAGCAAGTCCAACATT

	KPNB1 pA+1.4 F	GACTCATCACACCAAGGTCAC
	KPNB1 pA+1.4 R	GATAGTGCTGGGAAGGAAATGG
	KPNB1 pA+2.6 F	GTACATCTCAGCTTTGGCATATG
	KPNB1 pA+2.6 R	GCCCAGAACATAGCAGGCATTGC
CD44	Beginning F	AAAAGTGCAGCCAACTTCCG
	Beginning R	GCGAAAGGAGCTGGAGGATC
	Middle F	GAAGGTTGCTTCAGCTACACTG
	Middle R	TCTCAAGCGGTCTGATAACAGG
	End F	GCCCATTGTTCAATTCTTGTGC
	End R	CAAAAACCAGAGGAAGGGTGTG
KLF4	Beginning F	GAAAAGGCTGTAGCGAAGGAAG
	Beginning R	ACACGTTTCGTTCTCTCTGGTC
	Middle F	TGCGGCAAAACCTACACAAAG
	Middle R	TTCTGCAGTTTGTCCCCCTAC
	End F	AGTTTTCTGTGGAGGCCTGTC
	End R	ACGTTCTTAGGGTTGTCTGCTG
PER1	Promoter F	CAGTGCCCGGCATTATGCAAC
	Promoter R	GCAGAGATGCCTACCTGGTCG
	Beginning F	CGTGGTGGCCTCTCTGCCTTC
	Beginning R	GGACGCCCCCAGGACAAAATG
	Middle F	CAGTGCTCCTGTTCTCTGCATC
	Middle R	GGACCAAGAGACACAACCAC
	Middle 2 F	GGGGGTGGGTCTTAAGGGAGG
	Middle 2 R	CAGCCCGGAACCCCATCCTAG
	End F	CAGATGGGAGGAGAGCCTGAG
	End R	CACAAATGCCATCGGCAGAGG
	Readthrough F	GAATCCTGCTCACAGCGTCAC
	Readthrough R	GACGGCCGAGTCTCTGAGATTC
TXNIP	Promoter F	GACGTTTCCGCCTCCTGCTTG
	Promoter R	CGCCGCTGGTTACACTAAGC
	Beginning F	GATCCTGAGGGGCGTTGCCTC
	Beginning R	GCAGCAAGTTGCCAAGCCCTG
	Middle F	GGCGGGTGTCTGTCTCTGCTC
	Middle R	CCTCTACCCAGTCCCATGCC
	Middle 2 F	GCAATGGAGTGTGGGTCCACC
	Middle 2 R	CCCTGTATCACAAACATGGGCGC

	End F	CAGCTGCCAGCACTTGGTCAG
	End R	CTTCAGCCCACACTTTCTGGCTG
	Readthrough F	CTCTCCGCCCCTTTCTCACCTG
	Readthrough R	CTTCACCCCTCTAGGCCCTGG

2.2.5.3. *Primers for chrRNA*

<i>chrRNA-qPCR primers</i>		
CD44	CD44 F	TGCCGCTTTGCAGGTGTATT
	CD44 R	CCGATGCTCAGAGCTTTCTCC
KLF4	KLF4 F	ATCTTTCTCCACGTTGCGTCTG
	KLF4 R	AAGCACTGGGGGAAGTCGCTTC
GAPDH	GAPDH F	CCCCTTCATTGACCTTCAACTA
	GAPDH R	GAGTCCTTCCACGATACCAAAG
PER1	PER1 F	CTGCTACAGGCACGTTCAAG
	PER1 R	CTCAGGGACCAAGGCTAGTG
TXNIP	TXNIP F	ACTCGTGTCAAAGCCGTTAGG
	TXNIP R	TCCCTGCATCCAAAGCACTT
PDK4	PDK4 F	GGAGCATTTCTCGCGCTACA
	PDK4 R	ACAGGCAATTCTTGTCGCAAA
CASP9	CASP9 F	ATGACCACCACAAAGCAGTCC
	CASP9 R	CGTGACCATTTTCTTGGCAG
ETS2	ETS2 F	CTGGGCATTCCAAAGAACCC
	ETS2 F	CCAGACTGAACTCATTGGTGG

3.GENERATION AND CHARACTERISATION OF AS-CDK1 CELL LINES

3.1. Background

Despite the critical importance of cyclin-dependent kinases (CDKs) in mammalian cell function, the process of studying this family of enzymes remains challenging. In yeast, different compensatory mechanisms are seen mainly between cyclins but not CDKs, in mammals however, there is a level of redundancy between CDKs which often exhibit overlapping roles (Satyanarayana & Kaldis, 2009b). Thus, inhibition or deletion of individual kinases does not always result in a visible phenotype, such as in the case of CDK3-knockout mice, which are indistinguishable from wild-type mice (Satyanarayana & Kaldis, 2009b; Ye et al., 2001). Further, the effects of ablation of CDK activity are heavily context-dependent, with the results varying between cells and organisms, and the duration of CDK depletion. Mouse models with CDK2 knockouts are viable but sterile, but mice lacking both CDK2 and CDK4 die during or shortly after embryonic development (Barrière et al., 2007; Berthet et al., 2006). In contrast, deletion of CDK2 in adult mice lacking CDK4 does not have any severe consequences for the animals (Barrière et al., 2007). Interestingly, the studies by Barrière et al. and Berthet et al. also reported contrasting *in vitro* results on the phenotypes of CDK2- and CDK4-deficient mouse embryonic fibroblasts, with one study showing that the cells become immortal and proliferate well, while the other observed that they have decreased proliferation rates and premature senescence (Barrière et al., 2007; Berthet et al., 2006). It is therefore not always straightforward to unpick the functions of individual CDKs.

While mouse knockout models are a popular method of investigating CDKs, many research groups also turn to small molecule inhibitors to modulate CDK activity. As mentioned previously (**Section 1.8.**), the development of selective CDK inhibitors has been a formidable task, with the first wave of attempts resulting in the creation of “pan-CDK inhibitors”. Many such compounds, although showing promise in slowing down the

cell cycle and reducing tumour cell proliferation, did not ultimately pass clinical trials due to their lack of selectivity (Asghar et al., 2015; Łukasik et al., 2021; Mughal et al., 2023; Y. T. Wang et al., 2024; M. Zhang et al., 2021). Currently, over 40 pan-CDK inhibitors are being studied and optimised further, both in therapeutic and academic settings (M. Zhang et al., 2021). It was only after the focus shifted to creating CDK inhibitors targeted at individual cell-cycle CDKs that more promising outcomes were seen, with palbociclib and its subsequent relatives reaching the clinic (Asghar et al., 2015; Kalra et al., 2017; Mughal et al., 2023; Y. T. Wang et al., 2024; Whittaker et al., 2017).

Outside of drug development, research into inhibition of CDKs has also greatly benefited from the chemical-genetic technique called analogue-sensitive (AS) kinase technology (Bishop et al., 1999, 2000, 2001; Elphick et al., 2007; Lopez et al., 2014). The challenges in elucidating the functions of individual kinases stem largely from the highly-conserved fold of their catalytic domains, and the resulting difficulty in targeting specific members of the family (Bishop et al., 1999; Elphick et al., 2007; Gray et al., 1998). Importantly, almost all of the members of the kinome harbour a relatively large amino acid (e.g. phenylalanine or methionine) at a conserved position in their active site, which is right next to the N⁶-amino group of a bound ATP molecule (Umana et al., 2018). This bulky residue was found to guard the entrance to the ATP-binding pocket, earning it the name of “gatekeeper” (Y. Liu et al., 1999). Mutation of this amino acid from a bulky residue to alanine or glycine, both of which have smaller side chains, leads to the enlargement of the active site; this, in turn, allows for binding of bigger ATP-analogues with modifications at the N⁶ position (Elphick et al., 2007). As a result, the engineered kinases, while still able to bind and utilise normal ATP, are also able to bulky ATP analogues, lending them the name of “analogue-sensitive” kinases.

The AS mutation of kinases is functionally useful in two distinct ways. Firstly, the AS technology allows for identification of downstream substrates of the kinase of interest. When an AS kinase binds an ATP analogue which carries an alternative γ -phosphate tag, it can transfer the altered phosphate group to its substrates and directly label them for analysis (Elphick et al., 2007; Hertz et al., 2010; Umana et al., 2018). The tag can be used to purify the labelled substrates, allowing for identification of any direct binding partners using mass spectrometry. However, despite the continuous development of this method, the efficacy of different tagging techniques and how they relate to other substrate-identifying protocols are still under debate (Blethrow et al., 2008; Elphick et al., 2007). The second way in which AS kinases can be studied is through direct and specific inhibition using ATP analogues (Figure 3.1.) (Bishop et al., 2001). Engineering of small molecule inhibitors has led to the creation of a broad toolkit of custom drugs that bind the AS kinases, with the most widely-used ones coming from a class of compounds derived from 4-Amino-5-(4-methylphenyl)-7-(*t*-butyl)pyrazolo[3,4-*d*]- pyrimidine (PP1) (Bishop et al., 1999, 2001; Y. Liu et al., 1999). These “bulky”, cell-permeable molecules enter the enlarged ATP-binding pocket of the AS kinases, interfering with their ability to accept ATP from the environment, and thereby inhibiting their activity.

As naturally-occurring wild-type kinases do not possess an enlarged ATP-binding pocket, the AS technique ensures that the ATP analogues, whether for labelling or inhibiting, should specifically bind to the mutated protein of interest. The added attractiveness of the AS approach lies in the unchanged substrate specificity of the studied kinase, as the functionally “silent” gatekeeper mutation lies deep in the ATP-binding pocket, effectively minimising any unwanted effects on substrate and regulatory interactions (Elphick et al., 2007). Further, not deleting the kinase of interest through gene knockdown also ensures that any functions it may perform that are not connected to its catalytic activity, such as

acting as a protein scaffold, can be mechanistically distinguished from its enzymatic roles. Additionally, the AS methodology stops the cells from developing any compensatory mechanisms for the missing kinase over the course of time (Bishop et al., 2000). The AS kinase technology offers therefore the specific approach of a genetic modification, with the speed and reversibility of small-molecule inhibition. This leads to greater resolution and precision of experimental design, as the effects of inhibition start to appear within minutes of drug administration (Lopez et al., 2014).

Shokat and colleagues, who pioneered the AS technology using the oncogenic tyrosine kinase v-Src (Bishop et al., 1998; Y. Liu et al., 1998; Shah et al., 1997), also used budding yeast CDK1 (Cdc28) as a testing ground for some of their earliest studies of the system (Bishop et al., 2000, 2001; Blethrow et al., 2008). Due to its function as the only non-redundant cell-cycle kinase in yeast, the efficacy of the as-Cdc28 inhibition could be easily determined by checking for cell cycle arrest in treated cells. As expected, the as-Cdc28 cells incubated with a PP1-derivative at high concentrations stopped progressing through the cell cycle, instead becoming trapped at the G2/M transition (Bishop et al., 2000). Notably, before the AS kinase technology, study of yeast Cdc28 was dependent mostly on the analysis of temperature-sensitive mutant alleles, majority of which display a G1 arrest at 37°C (Reed, 1980). Results derived from the as-Cdc28 studies showed therefore greater cohesion with the remaining research documenting the primary role of CDK1 in eukaryotes at mitotic entry (Bishop et al., 2000). Further, as-Cdc28 later served in multiple projects to identify Cdc28 substrates in *S. cerevisiae* (Blethrow et al., 2008; Holt et al., 2010; Ubersax et al., 2003). Among others, the use of as-Cdc28 in yeast cells has also helped elucidate the kinetics of mitotic transcription (Asfaha et al., 2022), principles of the mitotic exit (Keaton et al., 2023), and even the involvement of Cdc28 in basal transcription (Chymkowitch et al., 2012).

The expansion of the AS kinase technology to CDK1 research has had a number of substantial advantages. Because of its status as the only non-redundant cell-cycle kinase, CDK1 is an essential gene, knockouts of which lead to embryonic lethality and cell death (Hochegger et al., 2008; Malumbres, 2005; Satyanarayana et al., 2008; Satyanarayana & Kaldis, 2009b). Further, similarly to the remaining CDKs, there is a considerable lack of highly-specific small molecule inhibitors that would target CDK1 with no side effects, leaving tools such as siRNAs when higher levels of experimental precision are needed (Q. Wang et al., 2023; Wijnen et al., 2021). Therefore, after the appearance of as-Cdc28 in *S. cerevisiae*, the AS-CDK1 system has been predictably extended to a range of various other model organisms, including *S. pombe* (Aoi et al., 2014), chicken cells (Hochegger et al., 2007), mouse embryonic stem cells (Michowski et al., 2020) and *A. thaliana* (Harashima et al., 2016). The cited plant models were heterozygous as-CDKA;1 mutants (CDKA;1 being the plant CDK1 homologue), and the introduced CDKA;1^{F80G} cDNA construct was controlled by an endogenous CDKA;1 promoter (Harashima et al., 2016). In the case of chicken DT40 cells, the endogenous CDK1 was firstly abolished using gene targeting, with a *X. laevis* AS-CDK1 cDNA expressed exogenously (Hochegger et al., 2007). One of the first examples of the use of AS-CDK1 in human cells followed the same principle, with HT2-19 human fibrosarcoma cells transfected with *X. laevis* AS-CDK1 (Gravells et al., 2013). A more robust human AS-CDK1 system was introduced in the study on the mitotic switch conducted by Rata et al., who generated AS-CDK1 HeLa cells through delivering Cdk1-F80G-myc into cells in a pcDNA3.1 plasmid and knocking out endogenous CDK1 (Rata et al., 2018).

Upon closer examination of the AS-CDK1 literature, two things are worth noting. Firstly, the overwhelming majority of the research on this particular AS kinase, similarly to studies using non-AS models, tends to focus on its role in orchestrating mitosis. Secondly,

there exists a very limited number of genetically-modified organisms carrying the AS-CDK1 variant that was mutated from the endogenous CDK1, with the authors often employing transgenic plasmids, or *in vitro* AS kinase assays in eukaryotic cell extracts to examine CDK1 functions instead (Blethrow et al., 2008; Hocheegger et al., 2007; Ubersax et al., 2003). One notable exception in both of these cases is the aforementioned study on the role of CDK1 in transcription in mouse embryonic stem cells, in which CDK1 was found to promote pluripotency during early embryonic development (Michowski et al., 2020). Here, the authors, using CRISPR-modified mESCs, utilised both labelling and inhibition approaches to elucidate the transcriptional role of CDK1 in these cells. They concluded that the high CDK1 activity typical for mESCs was necessary to stop them from differentiating, and that this was achieved by CDK1 phosphorylating multiple downstream chromatin modifiers (Michowski et al., 2020).

Apart from embryonic stem cells, heightened CDK1 activity has also been often observed in multiple cancers (M. Li et al., 2020; Q. Li et al., 2019; Piao et al., 2019; Yamamura et al., 2020). Additionally, dedifferentiated tumour cells and undifferentiated stem cells share some of their properties, such as self-renewal (Rossi et al., 2020; Shackleton, 2010). Taken together, it becomes conceivable that, to an extent, the transcriptional landscape in cancer cells could possibly be controlled by CDK1, which would shape their pluripotent stem cell-like characteristics. The most elegant method of examining this additional role of CDK1 was deemed to be through the use of one of the AS-CDK1 cell lines, as it allows for the precise and rapid inhibition of the kinase without any of the prolonged effects seen during knockout studies.

The work presented in this chapter describes the characterization of the HeLa AS-CDK1 cell line obtained from Prof Helfrid Hocheegger (Rata et al., 2018), and the generation of

novel AS-CDK1 cell lines HEK293 AS-CDK1 and HCT116 CDK1^{WT/AS}. The validation of the HeLa AS-CDK1 line, as well as the creation of the HEK293 AS-CDK1 and HCT116 CDK1^{WT/AS} cell lines, laid the necessary groundwork for examining transcriptional roles of CDK1, detailed in the subsequent chapters.

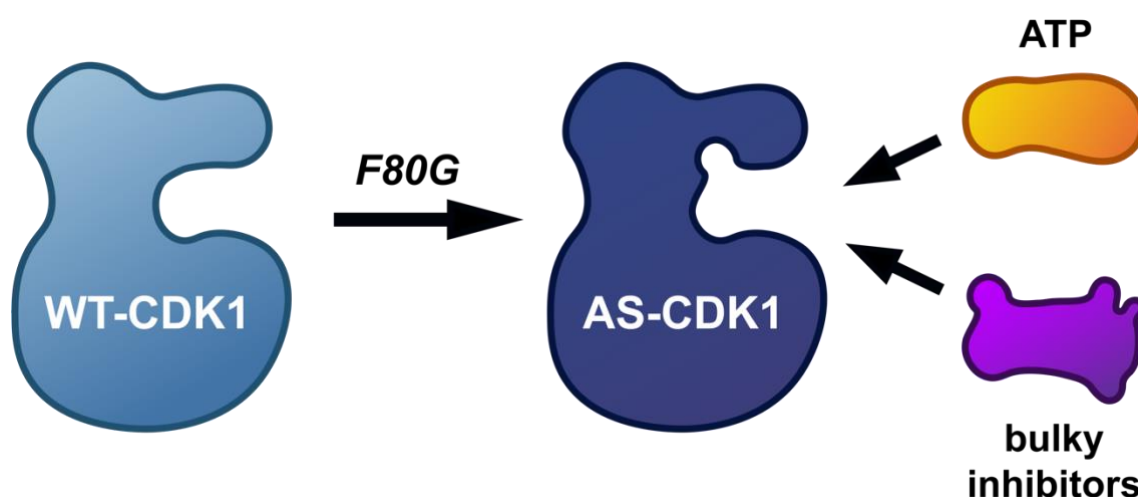


Figure 3.1. Diagram of analogue-sensitive CDK1 (AS-CDK1). Wild-type CDK1 (WT-CDK1) can be transformed into analogue-sensitive CDK1 (AS-CDK1) by introduction of the F80G substitution, which enlarges its ATP-binding pocket. AS-CDK1, while still able to utilise normal ATP, can now accept bulky ATP analogues that inhibit its catalytic activity. The bulky inhibitors are unable to bind wild-type kinases.

3.2. Characterisation of the HeLa AS-CDK1 cell line

Having chosen the AS-CDK1 system as the preferred model for this project, two concomitant avenues of investigation were undertaken. HeLa AS-CDK1 cell line, originally described in a study by Rata et al., was obtained from Prof Hochegger for characterisation and further experiments (Rata et al., 2018). However, it should be noted that AS-CDK1 expression in these cells is driven by a CMV promoter. To obtain cells with a mutated endogenous CDK1, the development of a new AS-CDK1 cell line also commenced.

3.2.1. AS-CDK1 can be selectively inhibited by 1-NM-PP1 in HeLa AS-CDK1

In order to determine whether HeLa AS-CDK1 cells could be used for investigating the transcriptional roles of CDK1, it was necessary to confirm that AS-CDK1 could be inhibited in these cells using a bulky ATP analogue. As mentioned before, inhibition of CDK1 blocks mitosis, as CDK1 is the only CDK necessary for cell cycle progression (Santamaría et al., 2007; Satyanarayana et al., 2008). Therefore, to judge successful inhibition of CDK1, cells incubated with the drug were checked for the disappearance of a well-established mitotic mark – the phosphorylation of histone 3 on serine 10 (H3S10p) (Johansen & Johansen, 2006; Komar & Juszczynski, 2020; Prigent & Dimitrov, 2003).

The HeLa AS-CDK1 cells were incubated for 2h with increasing concentrations of 1-NM-PP1, which is one of the PP1 derivatives most commonly used for AS kinase inhibition experiments. Its resuspension vehicle, DMSO, was used as control. Whole cell extracts were then taken of the samples, and Western blots were performed to establish

the levels of proteins of interest. The focus was placed on the H3S10p mitotic mark, and H3 and nucleolin served as controls.

The addition of 1-NM-PP1 to HeLa AS-CDK1 cells blocked mitotic entry, as judged by the almost complete loss of phosphorylation of H3 at S10 (**Figure 3.2.**). Although the effect was seen even at the lowest concentrations of the inhibitor, a marginally more thorough block was observed for the two highest concentrations of the drug. Therefore, the disappearance of H3S10p clearly indicated the successful inhibition of AS-CDK1 using 1-NM-PP1. The remaining faded signal could perhaps be attributed to baseline H3S10p marks that function throughout interphase to activate transcription (Komar & Juszczynski, 2020). Importantly, in unmodified HeLa cells the addition of the drug did not change the level of H3S10p at any of the used concentrations, confirming that 1-NM-PP1 does not target wild-type CDK1.

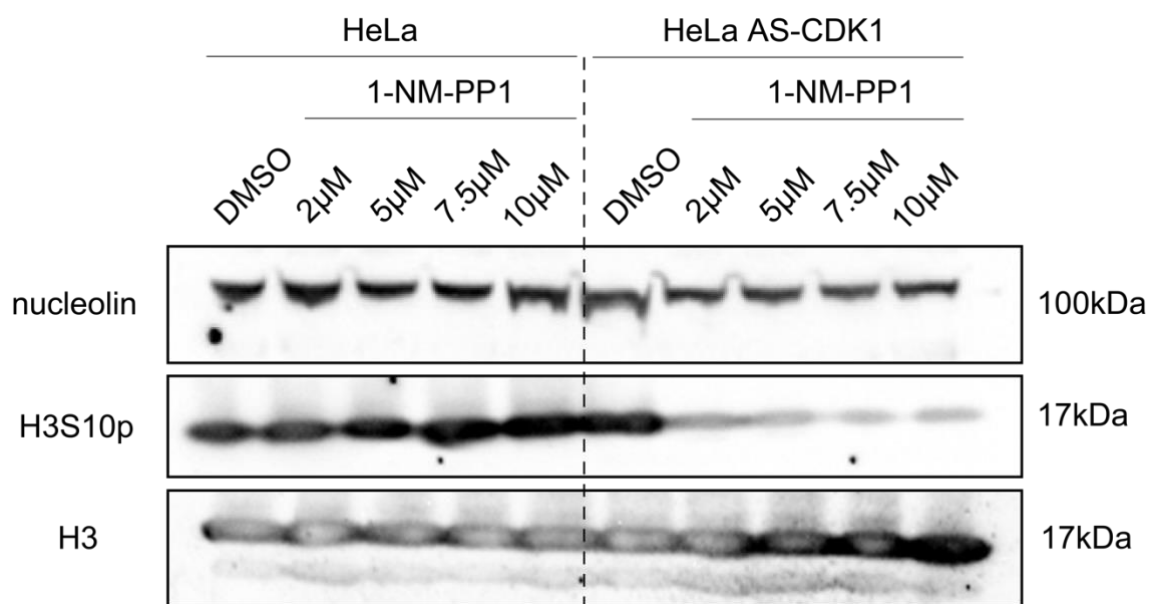


Figure 3.2. AS-CDK1 can be selectively inhibited by 1-NM-PP1 in HeLa AS-CDK1.

Western blots were prepared on whole cell extracts of HeLa and HeLa AS-CDK1 cells after 2h inhibition with 1-NM-PP1. The blots were probed with antibodies against nucleolin as a loading control, H3, and H3 phosphorylated on serine 10 residue (H3S10p), which acted as the mitotic mark.

3.2.2. AS-CDK1 levels in HeLa AS-CDK1 correspond to CDK1 levels in HeLa

Next, it was crucial to ascertain whether the levels of AS-CDK1 in HeLa AS-CDK1 cells were comparable to the levels of CDK1 in unmodified HeLa cells. As the AS-CDK1 gene in HeLa AS-CDK1 is expressed from a powerful CMV promoter (Rata et al., 2018), there was some concern that its level of expression might differ from CDK1 in unmodified HeLa cells. Western blotting of whole cell extracts was used to compare the AS-CDK1 levels to CDK1 levels found in HeLa, with tubulin used as loading control (**Figure 3.3.**). Although the HeLa line used in the experiments is not the direct parental line to the AS-CDK1 HeLa line, the levels of CDK1 protein in both of the cell lines were found to be comparable.

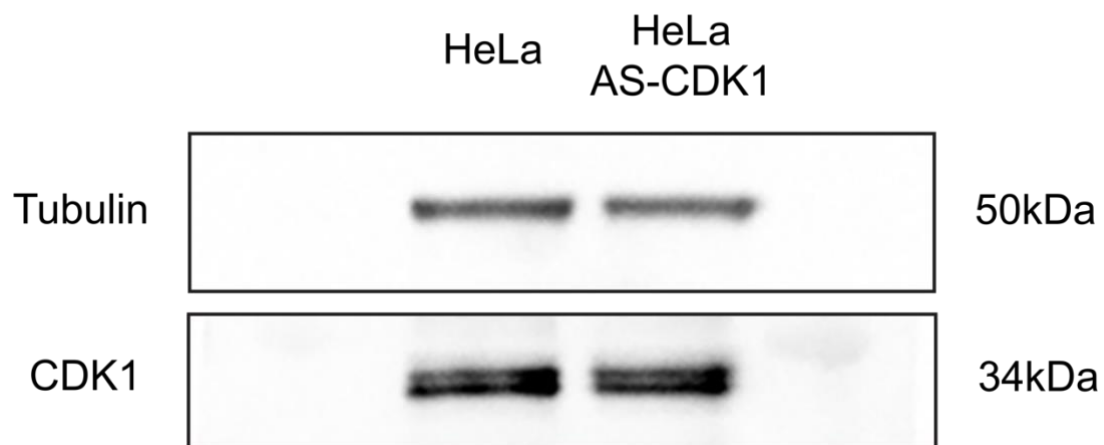


Figure 3.3. The levels of AS-CDK1 HeLa in HeLa AS-CDK1 correspond to the levels of CDK1 in HeLa. Western blots were prepared on whole cell extracts of HeLa and HeLa AS-CDK1 cells. The blots were probed with antibodies against tubulin as a loading control, and against CDK1.

3.2.3. HeLa AS-CDK1 can be synchronised using a double thymidine block

With the aim of this work to investigate the roles of CDK1 outside of mitosis, cell synchronisation was used as a way of eliminating cells undergoing cell division, which have high CDK1 activity. In the future, this would allow for selectively investigating interphase-specific roles of CDK1 in transcription. Despite a wide variety of cell synchronisation protocols available, double thymidine block (DTB) was chosen as the most appropriate, as its effects are well-established in both HeLa and HCT116 cells (Griesbach et al., 2021; Ligasová & Koberna, 2021; R. C. Wang & Wang, 2022).

The DTB was used on both HeLa and HeLa AS-CDK1, which were subsequently harvested and their DNA contents were stained with propidium iodide (PI). PI-stained cells were analysed in the Sir William Dunn School of Pathology Flow Cytometry Facility and their cell cycle distribution profiles were obtained (**Figure 3.4.**). For both cell lines a peak was observed at the beginning of S phase, suggesting an arrest of the cell cycle at the G1/S transition following thymidine treatment. DTB was therefore confirmed as a successful tool in synchronising both HeLa and HeLa AS-CDK1.

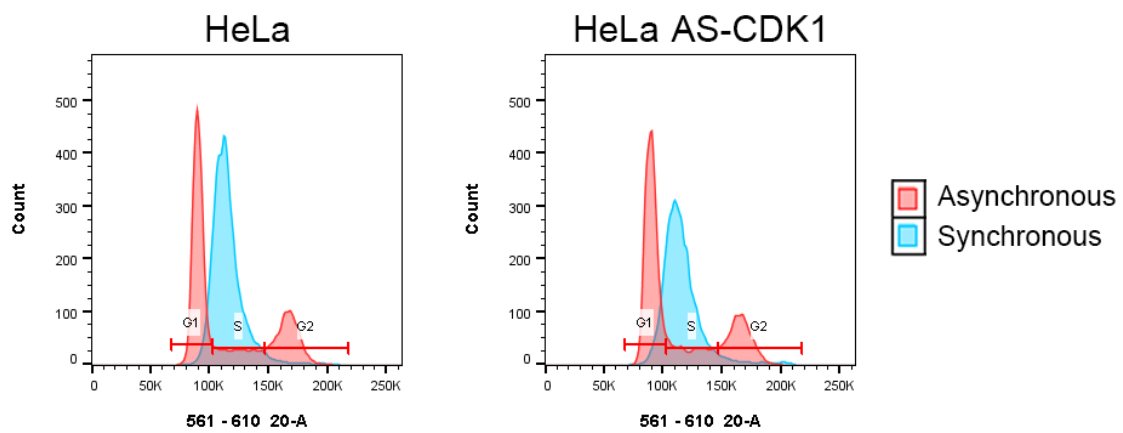


Figure 3.4. HeLa AS-CDK1 can be synchronised using a double thymidine block.

HeLa and HeLa AS-CDK1 cell lines were synchronised using a double thymidine block. Cells were stained with propidium iodide and DNA content per cell was measured using flow cytometry.

3.2.4. Short 1-NM-PP1 treatment does not disturb the cell cycle profile of AS-CDK1

HeLa cells

Prolonged inhibition of AS-CDK1 with 1-NM-PP1 halts cell cycle progression and can be used for cell synchronisation at the G2/M transition (Michowski et al., 2020; Rata et al., 2018; Santamaría et al., 2007). For this project, it was therefore important to confirm to what extent short 1-NM-PP1 treatment could alter the cell-cycle distribution of HeLa AS-CDK1 cells, as any deregulation of the cell cycle could influence transcription as a secondary effect.

HeLa AS-CDK1 cells were synchronised using a DTB, or left as an unsynchronised culture, and were then treated with 7.5 μ M 1-NM-PP1 for 0.5h. After harvesting and PI staining, their cell-cycle profiles were analysed using flow cytometry (**Figure 3.5.**). Brief inhibitor treatment did not noticeably distort the cell-cycle distribution of the asynchronous and synchronised HeLa AS-CDK1 in comparison to DMSO. However, it is important to remember that the disappearance of H3S10p following 1-NM-PP1 treatment translates to a block of mitotic entry (see **section 3.2.1.**). This means that during the flow cytometry analysis of an asynchronous, 1-NM-PP1-treated population, no cells are in the M phase of the cell cycle, although the unchanged DNA content between G2 and M cannot reflect that after PI staining. Based on the two figures (**Figure 3.2., Figure 3.5.**), it can be therefore said that the overall cell cycle distribution of the asynchronous HeLa AS-CDK1 inhibited by the drug is not markedly disrupted, but the modest population of mitotic cells has been eliminated. Importantly, the 1-NM-PP1 treatment did not change the cell-cycle distribution in a synchronised population of HeLa AS-CDK1 cells. This allows for the generation of a matched population of cells which are both in the same cell cycle stage, but where one of the populations has abolished CDK1 activity.

Such a set-up creates the ideal situation to assess the possible roles of CDK1 in transcription in interphase.

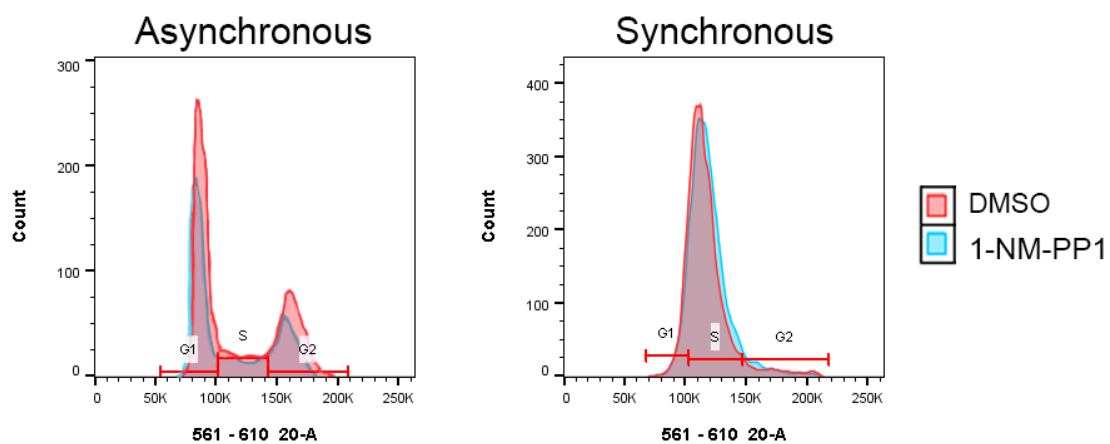


Figure 3.5. Short 1-NM-PP1 treatment does not disturb the cell cycle profile of HeLa AS-CDK1 cells. Two populations of HeLa AS-CDK1 cells, one unsynchronised and one synchronised using a double thymidine block, had their DNA contents stained with propidium iodide. Their cell cycle profiles were then visualised using flow cytometry.

3.3. Generation of AS-CDK1 cell lines using CRISPR-Cas9

Although the HeLa AS-CDK1 cell line was validated to be a valuable tool for studying CDK1 function, the endeavour of creating a true knock-in AS-CDK1 system was also pursued throughout the duration of this project. There exists a lack of available cell lines, or model organisms, where the F80G mutation required for the creation of AS-CDK1 is introduced in the native gene. Such a substitution would ensure that the same mechanisms which oversee the transcription and translation of wild-type CDK1 would therefore govern the mutant AS-CDK1, further solidifying its status as the ideal CDK1 model.

3.3.1. Positive selection of F80G cells with Taxol and 1-NM-PP1

During the generation of the new AS-CDK1 cell lines, the transfected populations were screened using antibiotics to select for cells that had taken up the plasmids with desired constructs (see **Figure 3.6.** for CRISPR diagram). It is important to note, however, that this project introduced a second novel method of positive selection of AS-CDK1 mutants. Owing to CDK1 being the only non-redundant cell-cycle kinase, inhibition of CDK1 stops the cells from progressing through the mitosis (Santamaría et al., 2007; Satyanarayana et al., 2008). On the other hand, taxanes target and kill cells that are progressing through mitosis. It was therefore speculated, that incubation of a population of cells transfected with F80G constructs with both paclitaxel (Taxol), and an ATP analogue that inhibits AS-CDK1, would stop any mutated cells from progressing through the cell cycle, therefore sparing them from the antimitotic drug. The unmutated cells, where CDK1 cannot be inhibited, would proceed into mitosis and be susceptible to the cytotoxic effects of Taxol, creating a positive selection process to enrich for AS-CDK1 clones (**Figure 3.7.**).

Before trying this method on transfected HCT116 and HEK293 cell lines, the system was tested with the existing HeLa and HeLa AS-CDK1 cells (**Figure 3.6**). In the pilot experiment, cells were incubated with 10 μ M 1-NM-PP1 and either 0.1 μ M or 50nM of Taxol. The cells were left in normal culture conditions and the drugs were allowed to act on them for 24h. When checked under a microscope, it could be observed that addition of 1-NM-PP1 to HeLa AS-CDK1 line prevented the cells from dying when incubated with Taxol (**Figure 3.8**). In contrast, HeLa cells were not protected by 1-NM-PP1, which cannot be utilised by unmodified CDK1, and Taxol was able to kill majority of these cells. This confirmed that the long exposure to 1-NM-PP1 inhibited AS-CDK1 in the modified cells, preventing mitosis, and as a consequence guarding them from the antimitotic actions of Taxol. Following this validation, the same treatment was then used on transfected HCT116 and HEK293 cells to facilitate the screening process by selecting against unedited cells, improving the overall screening efficiency.

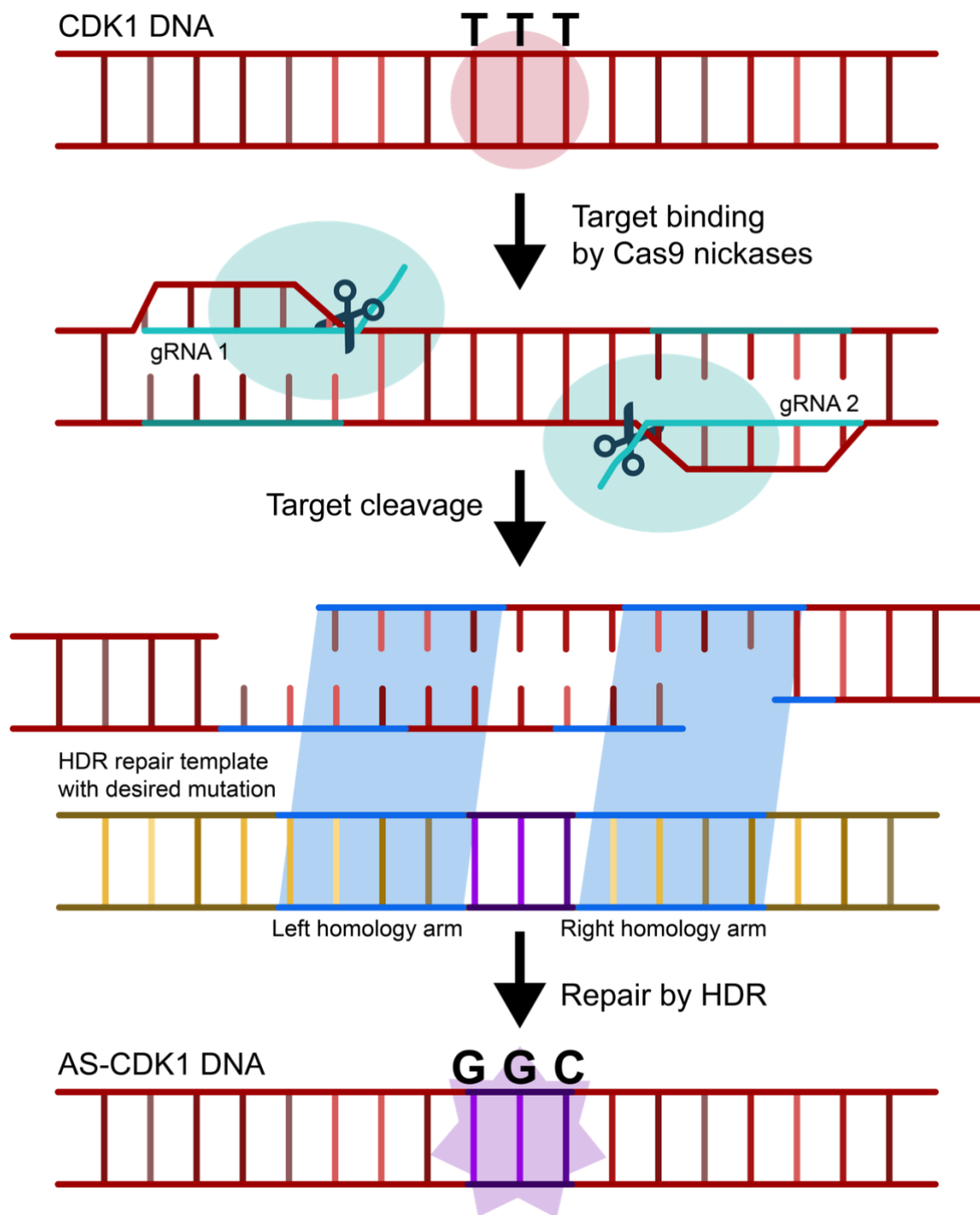


Figure 3.6. Generation of AS-CDK1 cells using the CRISPR-Cas9 system. Wild-type cells are transfected with constructs carrying a repair template with the desired F80G mutation and Cas9 nickase mutants with appropriate guide RNAs (gRNAs). Following cleavage, the breaks are repaired using homology-directed repair (HDR) and the DNA is restored with the new TTT to GGC substitution.

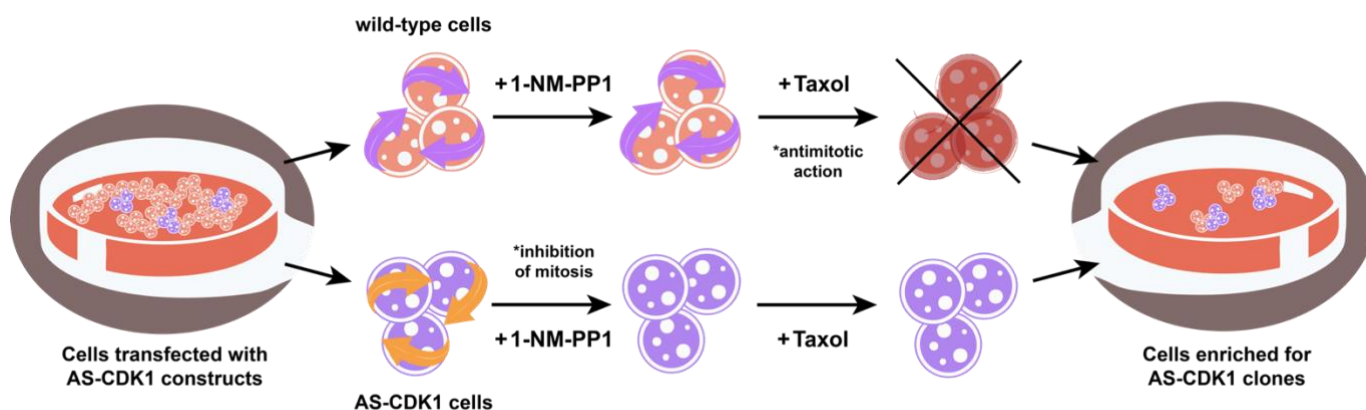


Figure 3.7. The principle of positive selection of AS-CDK1 mutants using 1-NM-PP1 and Taxol. To enrich for AS-CDK1 clones harbouring the F80G mutation, transfected cells are incubated simultaneously with 1-NM-PP1, which blocks mitosis in AS-CDK1 cells, and with Taxol which has a cytotoxic effect on proliferating cells. As a result, the vast majority of wild-type cells are removed from the population and the screening for AS-CDK1 cells is facilitated.

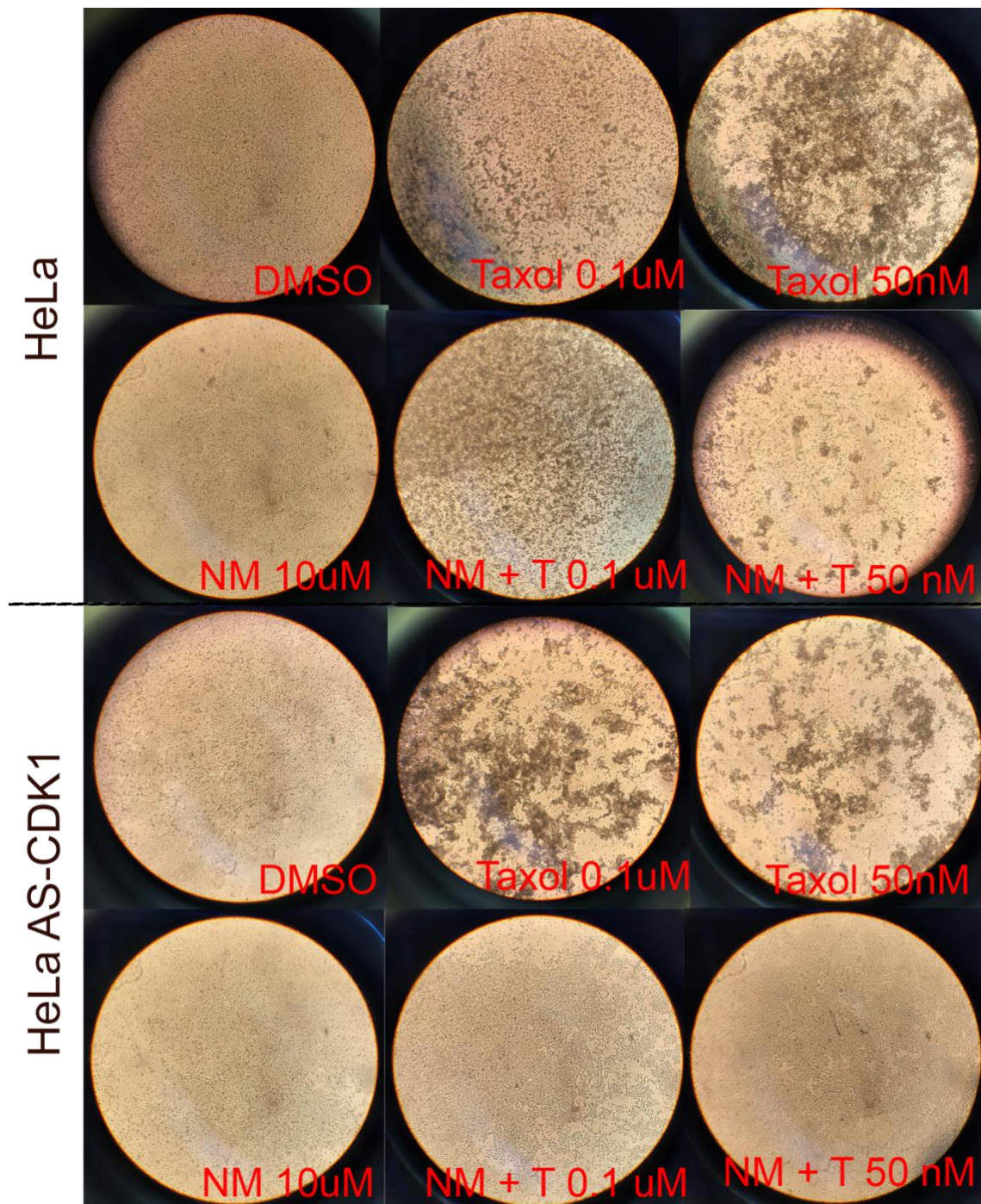


Figure 3.8. Low-power micrographs showing the principle of positive selection of AS-CDK1 cells using Taxol and 1-NM-PP1 treatment, on the example of HeLa and HeLa AS-CDK1 cell lines. During generation of the AS-CDK1 cell lines, AS-CDK1 mutants were selected using a 24h incubation with 10 μ M 1-NM-PP1 (NM) and 0.1 μ M Taxol (T) to enrich for cells where CDK1 was successfully inhibited.

3.3.2. Characterisation of the HCT116 CDK1^{WT/AS} cell line

The cell line originally chosen for the generation of the new AS-CDK1 system was the HCT116 human colon cancer cell line. This decision was supported by the growing evidence of increased CDK1 activity driving human colorectal tumours (Bury et al., 2019; Ghafouri-Fard et al., 2022; C. Li et al., 2023; J. Li et al., 2020; Q. Wang et al., 2023). Furthermore, HCT116 cells are near-diploid and have two copies of the CDK1-containing chromosome 10, which again made it the preferred model compared to the aneuploid HeLa AS-CDK1.

The generation of a HCT116 AS-CDK1 cell line was initially started by the Genome Engineering Oxford facility at the Sir William Dunn School of Pathology. Firstly, the Cpf1 (Cas12) system was used with a ssDNA repair template. Subsequently, in order to increase the homology-directed repair efficacy, different electroporator machine settings were trialled and cells were pre-treated with 200 ng/ml nocodazole for 17h or 15µM RS-1 for 5h. Nocodazole is used to synchronise the cells at the point of nuclear envelope breakdown to enhance the delivery of plasmid DNA, while RS-1 stimulates homologous recombination activity of hRAD51 (Jayatilaka et al., 2008; Lin et al., 2014). Performing a cold-shock by putting the nucleofected cells in a 32°C incubator for 48h after transfections was also tried. These attempts yielded one heterozygous HCT116 CDK1^{WT/AS} mutant, which was then used in this study. The final tests involved a Prime Editing system, with a SpRY form of Cas9 enzyme delivered in the form of a Nanoblade vector. Unfortunately, this method also ultimately failed to produce a homozygous mutant clone.

Following these unsuccessful attempts, plasmids expressing CRISPR-Cas9 nickase and guides, and an F80G homology template were created to introduce the AS mutation into

HCT116 cells (see **Section 2.1.2.** and **Figure 3.6.**). As the Cas9 plasmid carries puromycin resistance and the homology template carries blasticidin resistance, successfully transfected cells could be selected with both antibiotics after transfection. In addition to carrying out transfections of these plasmids into wild-type HCT116, the heterozygous CDK1^{WT/AS} clones were also transfected in an attempt to generate homozygotes. Both of these approaches eventually proved unsuccessful, and the heterozygous HCT116 CDK1^{WT/AS} mutants were used for the remainder of the project. The gDNA and cDNA of the heterozygous HCT116 CDK1^{WT/AS} cells were confirmed to carry the same mutation during sequencing (**Figure 3.9.**).

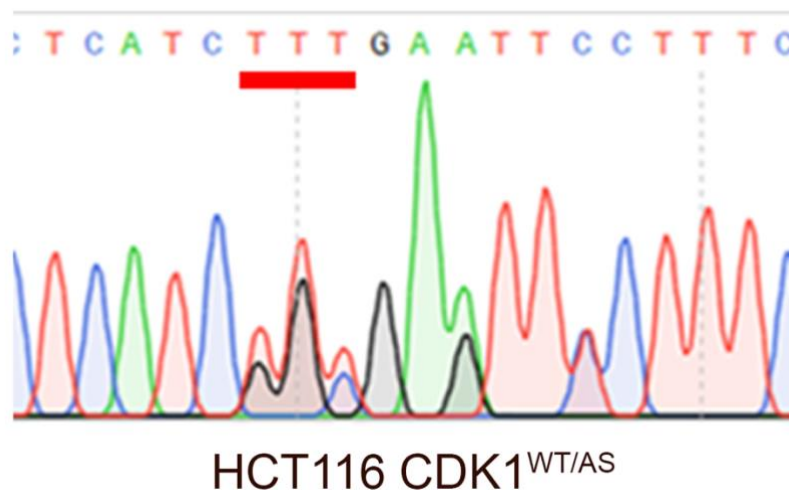


Figure 3.9. Sequencing trace of HCT116 CDK1^{WT/AS} cell line genomic DNA. The **TTTGAGTTT > GGCGAATTC** sequence change carries the F80G (TTT > GGC) base substitution to mutate CDK1 into an AS-CDK1 by enlarging its ATP-binding pocket. The changes at the additional positions insert an additional restriction site for *EcoRI* (G^vAATTC), which facilitates screening for AS-CDK1 clones. The mutations were introduced into one allele of wild-type CDK1 in HCT116 cells using CRISPR-Cpf1.

3.3.2.1. *CDK1^{WT/AS} in HCT116 can be selectively inhibited by 1-NM-PP1*

Correspondingly to the tests performed on HeLa AS-CDK1 cells, the HCT116 CDK1^{WT/AS} mutants were checked to see if the heterozygous CDK1 could still be inhibited with an ATP analogue. To determine this, wild-type HCT116 and HCT116 CDK1^{WT/AS} cells were cultured for 2h in increasing concentrations of 1-NM-PP1, or in DMSO. Whole cell extracts were then prepared and used for Western blots, and the behaviour of the H3S10p mitotic mark was observed.

Following incubation with the drug, the mitotic mark started vanishing from the HCT116 CDK1^{WT/AS} cells, however, it did not disappear entirely (**Figure 3.10.**). This suggests incomplete inhibition of CDK1 in this cell line, presumably because the unmodified CDK1 was unaffected by the presence of 1-NM-PP1. Similarly to HeLa cells, addition of 1-NM-PP1 to the wild-type HCT116 line did not affect the mitotic mark at all.

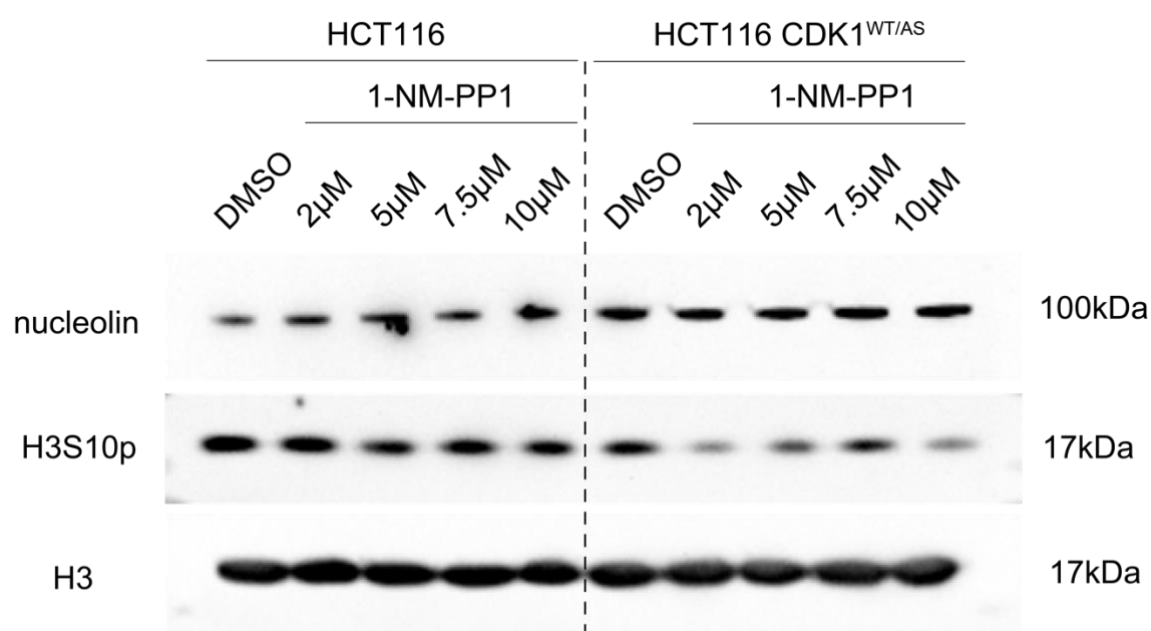


Figure 3.10. AS-CDK1 can be partly and selectively inhibited by 1-NM-PP1 in HCT116 CDK1^{WT/AS}. Western blots were prepared on whole cell extracts of HCT116 and HCT116 CDK1^{WT/AS} cells after 2h inhibition with 1-NM-PP1. The blots were probed with antibodies against nucleolin as a loading control, H3, and H3 phosphorylated on serine 10 residue (H3S10p), which acted as the mitotic mark.

3.3.3. Characterisation of the HEK293 AS-CDK1 cell line

The creation of the HEK293 AS-CDK1 cell line was performed entirely using the CRISPR-Cas9 constructs and F80G homology template described above (Section 2.1.2.). The double selection technique was also performed during the screening for the HEK293 AS-CDK1 cells. The editing efficiency in HEK293 cells was intrinsically higher than for the HCT116 cell line, which led to more cell surviving after the antibiotic selection. After trying out different gRNA combinations, the successful HEK293 AS-CDK1 clone was generated using gRNAs 2 and 3 (see **section 2.2.5.1.**). It is worth noting that, unlike the heterozygous HCT116 CDK1^{WT/AS} cell line, the homozygous HEK293 CDK1 cells had poorer recovery following sub-culturing compared to their wild-type HEK293 counterpart. They were therefore plated at higher density during passaging, allowing for otherwise normal culture. The cDNA and gDNA of the HEK293 AS-CDK1 cells were checked for the presence of the mutation, and the prepared cell line could be used in subsequent studies (**Figure 3.11.**).

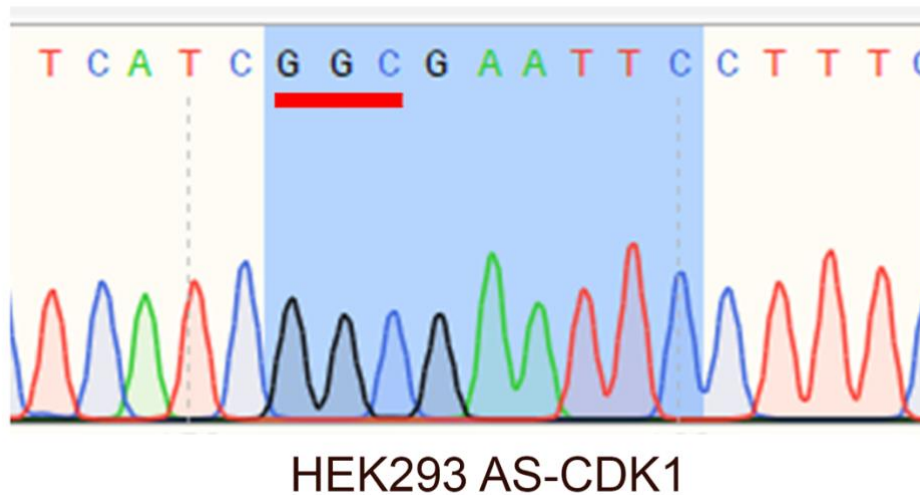


Figure 3.11. Sequencing trace of HEK293 AS-CDK1 cell line genomic DNA. The **TTTGAGTTT > GGCGAATTC** sequence change carries the F80G (TTT > GGC) base substitution to mutate CDK1 into an AS-CDK1 by enlarging its ATP-binding pocket. The changes at the additional positions insert an additional restriction site for *EcoRI* (G^vAATTC), which facilitates screening for AS-CDK1 clones. The mutations were introduced into wild-type CDK1 in HEK293 cells using CRISPR-Cas9.

3.3.3.1. AS-CDK1 in HEK293 can be selectively inhibited by 1-NM-PP1

The H3S10p mitotic mark was monitored to determine the response of HEK293 AS-CDK1 cells to AS-CDK1 inhibition. HEK293 and HEK293 AS-CDK1 cell lines were incubated for 2h with DMSO and increasing concentrations of 1-NM-PP1, after which whole cell extracts were prepared. Western blots were then performed using antibodies against H3S10p, H3, and nucleolin as the loading control.

The addition of 1-NM-PP1 to HEK293 AS-CDK1 cells caused a nearly complete loss of H3S10 phosphorylation, even at the lowest concentrations (**Figure 3.12.**). In contrast, H3S10p remained at the same level in DMSO- and 1-NM-PP1-treated HEK293 cells. Similarly to the other cell lines used, this signifies that the ATP analogue specifically inhibits AS-CDK1 in the mutated cells, but does not affect CDK1 in wild-type HEK293.

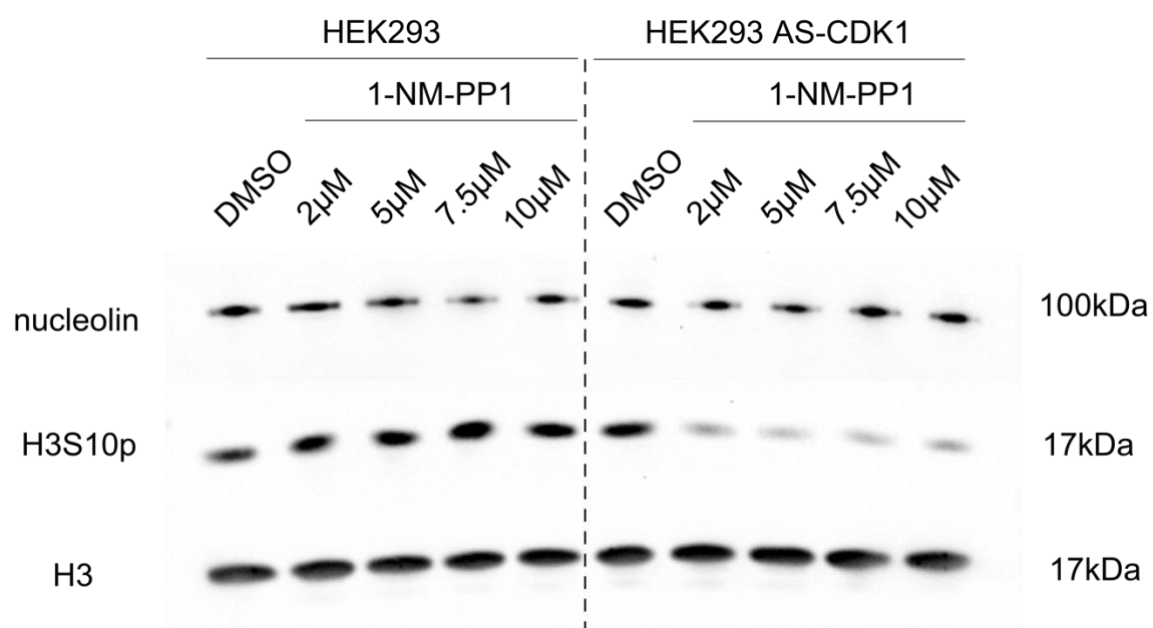


Figure 3.12. AS-CDK1 can be selectively inhibited by 1-NM-PP1 in HEK293 AS-CDK1. Western blots were prepared on whole cell extracts of HEK293 and HEK293 AS-CDK1 cells after 2h inhibition with 1-NM-PP1. The blots were probed with antibodies against nucleolin as a loading control, H3, and H3 phosphorylated on serine 10 residue (H3S10p), which acts as a mitotic mark.

3.4. Discussion

Despite nearly half a century of investigating CDK1, good experimental models to study its function are lacking. This rings especially true for studies where the cell cycle effects of CDK1 are not the primary focus of the project, as any prolonged inhibition or knockdown of the protein will skew the results into the field of cell cycle deregulation. Therefore, the primary objective of this work was to create a new, precisely-mutated AS-CDK1 cell line, while concomitantly validating the existing HeLa AS-CDK1 model. The HeLa AS-CDK1 cells have proven to be specifically inhibited by the 1-NM-PP1 drug, and the subsequent generation of HCT116 CDK1^{WT/AS} and HEK293 AS-CDK1 cell lines has confirmed the validity of the AS-CDK1 system across multiple models.

As discussed above, the AS kinase approach has several crucial advantages, with some being especially relevant for this study. Importantly, inhibition of AS kinases occurs very rapidly. It is also anticipated that the use of ATP analogues would allow for testing dose-dependent kinase inhibition, with the view of decreasing CDK1 activity without abolishing it completely. With this work centred around the possible transcriptional roles of CDK1 during interphase, swift inhibition of the kinase was particularly advantageous as it allowed to minimise the impact of its deregulation on mitosis and avoid any dominant cell-cycle effects seen with longer times of ablation of CDK1 activity. As the vast majority of cells in an asynchronous population are in interphase, short inhibition times of CDK1 help to ensure that most of the recorded changes in transcription can be attributed to the cells outside of the M phase.

In literature, a common CDK1 inhibition method is a 20h drug treatment, which creates a mitotic block of the cell cycle, suggesting the complete inhibition of the protein (Rata et al., 2018; Vassilev et al., 2006). The specificity and speed of the AS technology allows

for minimising the duration of treatment, as shown in mESCs where the initial characterisation of CDK1 substrates was conducted after only 30min of CDK1 inhibition with 3-MB-PP1 (Michowski et al., 2020). This permits to keep greater focus on any subtle changes in transcription, which occur at much finer time scales (as seen in AS-tCDK studies, such as Tellier et al., 2020, 2022; Velychko et al., 2024). The rapid inhibition of the kinase also means that any observed shifts in the transcriptional landscape are more likely to be direct effects of CDK1 inhibition, instead of, for instance, being a secondary consequence of cell cycle disruption. The lack of previous studies that would use shorter CDK1 inhibition times possibly stems from the primary focus of CDK1 literature lying in its regulation of mitosis, making the G2/M block the desired outcome of its inhibition.

Notably, the reversibility of the AS kinase inhibition provided a novel way to select and enrich for AS-CDK1 mutants during the creation of AS-CDK1 cell lines, when the transfected cells were incubated with an inhibitor and an antimetabolic drug. With so few AS-CDK1 models currently available, this screening technique could help other groups establish new AS-CDK1 cell lines in the future. During this work, the technique was tested and validated for two different cell lines: HEK293 and HCT116. It is worth noting that the treatment might be too harsh for less robust cells, and in this project additional recovery days were given before and after the double incubation to increase the yield of any possible mutants. Unfortunately, this method is only viable when editing CDK1, due to its non-redundant function in governing mitosis (Santamaría et al., 2007; Satyanarayana et al., 2008).

However, the work done during this project highlights the difficulty of introducing the AS-CDK1 mutation into mammalian cells by editing the endogenous gene using CRISPR-Cas9/Cas12. The HCT116 line has proven challenging to modify efficiently,

suggesting perhaps that its homologous recombination levels are not high enough to allow for efficient editing. Nevertheless, there have been no previous reports of HCT116 cells having impaired homologous recombination repair (HRR) pathways, and several studies in these cells have succeeded in introducing gene modifications using CRISPR-Cas9 (Hu et al., 2023). The strong tumorigenicity of HCT116 cells, which is driven by an oncogenic KRAS mutation, could even suggest that the levels of HRR should be higher due to their increased proliferative capabilities (Kalimutho et al., 2017). The generation of AS-CDK1 HCT116 cells could be therefore impeded at the level of transfection and DNA delivery inside of the cells, although the exact mechanism behind this low mutation efficiency remains elusive.

The HCT116 cell line also seems to be sensitive to transfecting and passaging in short periods of time, as well as to the harsh sorting process, as it was difficult to recover live cells afterwards. To counteract the difficulties with creating the HCT116 AS-CDK1 cell lines, introduction of the suppressor of the gatekeeper M32V mutation was attempted before the harsher F80G substitution. However, the editing efficiency seemed unchanged, which again underlined that HCT116 line might prove difficult to mutate, regardless of the desired genetic alteration.

The HEK293 cells were then introduced to the study, firstly as their high transfection efficiency allowed for corroborating the efficacy of the CRISPR-Cas9 plasmids, and secondly to establish a further AS-CDK1 cell line that the HeLa AS-CDK1 could be compared to. The HEK293 cell line is of human embryonic kidney origin, and was immortalised by the transfection with sheared adenovirus 5 (Ad5) DNA. Despite being known for its high transfection efficiency, the exact process driving this phenomenon in HEK293 cells has yet to be uncovered (Abaandou et al., 2021). HEK293 are hypotriploid

and have 3 copies of chromosome 10, which harbours the gene for CDK1. Importantly, the successful substitution of F80G in the CDK1 gene of the HEK293 cells confirmed the effectiveness of the prepared plasmids. Additionally, the production of a HEK293 AS-CDK1 cell line, as well as evidence from many other studies, supported the view that cells can tolerate the introduction of the F80G mutation without the previous M32V change (Bishop et al., 2000; Lopez et al., 2014; Rata et al., 2018).

With that in mind, it is important to again underline that HeLa AS-CDK1 cell line was not created through a mutation of the endogenous kinase, but rather through delivering the AS-CDK1 gene on a pcDNA3.1 plasmid and deleting the remaining wild-type CDK1 (**Section 3.1.**) (Rata et al., 2018). Furthermore, when analysing other AS-CDK1 studies, it could be speculated that the initial creation of as-Cdc28 in *S. cerevisiae* was enabled by the high homologous recombination rates naturally occurring in yeast cells (Bishop et al., 2000). Similarly, Dr Michowski credited the successful creation of the AS-CDK1 mESCs to their characteristically high recombination frequency and emphasised that even then the generation of homozygous AS-CDK1 mESCs proceeded in two steps after first creating heterozygous CDK1^{WT/AS} mutants (personal communication; Michowski et al., 2020). He further explained that during their study, the generation of fully-homozygous AS-CDK1 mice has proven practically impossible, strongly suggesting that although the AS mutation is described as functionally-silent, it might be poorly tolerated when introduced into an essential gene such as CDK1. Correspondingly, outside of the AS-CDK1 HeLa and the HT2-19 study (Gravells et al., 2013), a very limited amount of literature on human AS-CDK1 cells exists to date. It is therefore possible that the HEK293 AS-CDK1 cell line created during the course of this work is the first human cell line in which the F80G mutation has been introduced into the endogenous CDK1 gene.

Nonetheless, although the creation of the HEK293 AS-CDK1 line provided the project with an invaluable model to study the transcriptional effects of CDK1, the origin of HEK293 cells somehow deviates from the original aim of looking at CDK1 function in cancer cells. To this end, a homozygous HCT116 AS-CDK1 cell line would have been better suited for the purpose, as HCT116 cells are better characterised in terms of their cancer-relevant phenotypes, making them a preferred model for epithelial cancer biology. Additionally, in contrast to the highly aneuploid HeLa, HCT116 cells are near-diploid and their CDK1 is expressed from its own promoter (Knutsen et al., 2010; Rata et al., 2018; Thompson & Compton, 2008). Still, the creation of two additional AS-CDK1 models provided the study with important additional points of reference, as the universality of any CDK1 findings in HeLa could be checked against HEK293 or HCT116 backgrounds.

The main model of this study therefore became the HeLa AS-CDK1 cell line, whose mutation, despite not being endogenous, is well-understood and described in detail by its creators (Rata et al., 2018). Since their study focused on the role of CDK1 in the mitotic switch, the inhibition of the AS-CDK1 was primarily used for cell synchronisation at the G2/M transition, with 2 μ M 1-NM-PP1 acting on the cells for 20h, and afterwards increasing concentrations of 1-NM-PP1 were tested to determine the thresholds for mitotic entry and exit (Rata et al., 2018). In contrast, the transcriptional study of AS-CDK1 mESCs used 1 μ M of 3-MB-PP1 instead of 1-NM-PP1, and their inhibition times varied from 30min to 4 days depending on the experiment (Michowski et al., 2020).

Unfortunately, the direct comparison of the aforementioned experimental systems to that used in this study is complicated by the varied range of drug concentrations and timescales used for CDK1 inhibition during these experiments. Throughout this work, the

7.5 μ M concentration of 1-NM-PP1 was chosen to ensure that enough of the inhibitor was targeting CDK1 during the short inhibition times. To focus on the transcriptional changes, the inhibition timescales were kept between 0.5-2 hours, as within this timeframe a relatively small fraction of cells are undergoing mitosis, and any observed changes could therefore be assigned to the possible interphase functions of CDK1. This decision was further supported by the minimal cell cycle disturbance seen after PI staining of inhibitor-treated cells, both before and particularly after synchronisation, although as stated above, the unsynchronised cells underwent the loss of some mitotic cells. The same result was observed by Michowski et al. when they inhibited unsynchronised mESCs with 1 μ M of 3-MB-PP1 for 30min (Michowski et al., 2020).

Different 1-NM-PP1 concentrations were also used to inhibit CDK1 in both HCT116 CDK1^{WT/AS} and HEK293 AS-CDK1 cells, to check for any direct resemblance to the HeLa AS-CDK1. While the HEK293 AS-CDK1 cell line behaved predictably and its AS-CDK1 was completely inhibited after addition of the drug, the heterozygous mutant underwent only partial inhibition of the kinase. In literature, in diploid *S. cerevisiae* cells with both wild-type Cdc28 and a mutant as-Cdc28 allele the addition of a PP1 analogue produced no visible effect (Bishop et al., 2000). This comparison could suggest that, unlike in yeast cells where the heterozygous mutation was deemed recessive, a single AS-CDK1 allele in the heterozygous HCT116 CDK1^{WT/AS} cells can still partially enforce the AS phenotype. This in turn allows for possibility of testing the effect of approximately 50% reduction in CDK1 activity on transcription. Similarly, heterozygous CDK1^{WT/AS} mice displayed no abnormalities during growth, but their harvested organs could still be used for thiolabelling with ATP analogues, implying that one AS-CDK1 allele still allows for exploiting the AS system (Michowski et al., 2020).

Taken together, the production and characterisation of the AS-CDK1 cell lines described in this chapter are a necessary first step to investigating the role of CDK1 in transcription. The HeLa AS-CDK1 experiments helped establish the value of this research tool for studying CDK1 function, while the new HCT116 CDK1^{WT/AS} and HEK293 AS-CDK1 cell lines allow for the identification of cell-type-specific effects of CDK1 inhibition.

4.THE EFFECT OF CDK1 INHIBITION ON TRANSCRIPTION IN HELA CELLS

4.1. Background

CDK1 is the archetypal cell cycle regulator and master of mitosis, however, there is growing evidence of CDK1 functions in processes unrelated to the cell cycle as outlined in **sections 1.4.** and **1.7.3.** In *S. cerevisiae*, Cdc28 has been shown to phosphorylate pol II on the S5 residue of its CTD, and to further regulate the basal transcription of highly-expressed housekeeping genes such as *PMA1* (Chymkowitch et al., 2012; Embo et al., 1982; Enserink & Chymkowitch, 2022; Kõivomägi et al., 2021). In mouse embryonic stem cells, CDK1 controls the epigenetic landscape of the cells through phosphorylation of chromatin modifiers (Michowski et al., 2020). In human prostate cancer cells, CDK1 phosphorylates and stimulates the activity of the AR transcription factor, after its association with ABCC5 (Ji et al., 2021). Taken together, these findings indicate that CDK1 is not a purely cell-cycle kinase, and that the scope of its transcriptional functions could be more extensive. Additionally, the ways in which CDK1 can control transcription seem to be very context-dependent, with different mechanisms employed in different types of cells.

As stated before, overexpression of CDK1 is linked to various types of cancer (M. Li et al., 2020; Q. Li et al., 2019; Piao et al., 2019; Yamamura et al., 2020). If part of the cancer phenotype is indeed driven by CDK1 affecting transcription, it is possible that uncoupling the cell-cycle and transcriptional roles of this kinase by, for example, dose-dependent inhibition of its action, could improve the cancerous transcriptional landscape of affected cells, without compromising the proliferative roles of CDK1 in healthy tissues. It is also conceivable that part of the anti-tumour actions of FDA-approved CDK4/6 inhibitors such as palbociclib, in fact reflects inhibition of CDK1 (Finn et al., 2009). However, before therapeutic avenues can be properly explored, there is still a lack of understanding of the potential transcriptional roles of CDK1 in tumours. Using HeLa AS-CDK1 as the

experimental tool, CDK1 was inhibited with 1-NM-PP1 and the effects on transcription in de-differentiated cancer cells were investigated.

4.2. Global nascent RNA levels increase following CDK1 inhibition

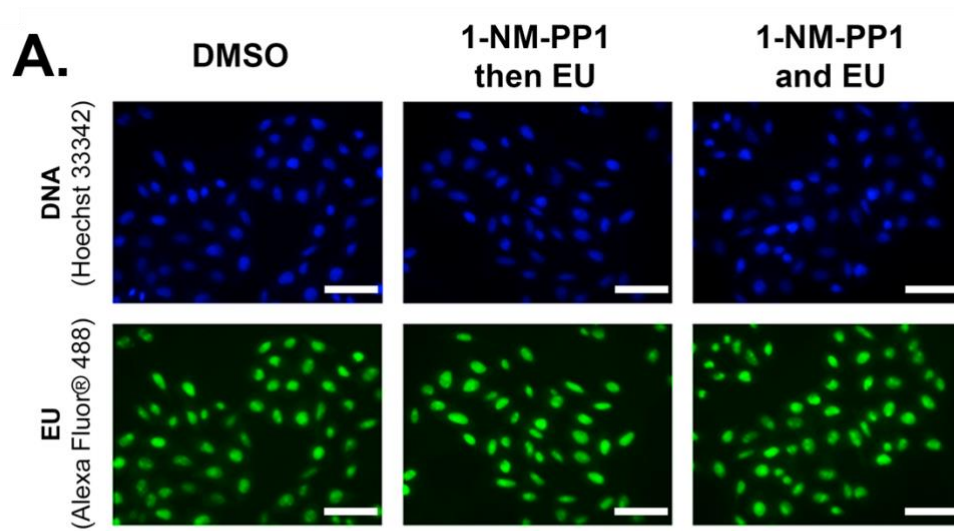
Global trends in transcription in a population of cells can be visualised *in vivo* using the 5-ethynyl uridine-incorporation assay. 5-ethynyl uridine (EU) is a nucleotide analogue of uracil, which is incorporated into newly-transcribed RNA (Jao & Salic, 2008). Alkyne-containing EU can then be detected using an azide-modified fluorophore as a result of a click-chemistry reaction (Breinbauer & Köhn, 2003).

In this study, transcription of nascent RNA in HeLa AS-CDK1 was measured following inhibition of CDK1 to determine whether there were any effects on global transcription. HeLa AS-CDK1 cells were first seeded on coverslips, before administering 0.5h treatment of 7.5 μ M 1-NM-PP1 the following day. Incubation with 1mM EU was performed both before, or at the same time as 1-NM-PP1 inhibition. The cells were then fixed and stained with Alexa Fluor 488, which recognised the EU incorporated into RNA, and Hoechst 33342, which intercalates into DNA. Images of the coverslips were taken on a fluorescence microscope, for both GFP and DAPI channels. The DAPI channel, which displays the Hoechst 33342-stained DNA, was used to mask cell nuclei. The mean fluorescence intensity of each nucleus was calculated using the GFP channel with Alexa Fluor 488-stained RNA, allowing for the evaluation of nascent RNA production in the cell.

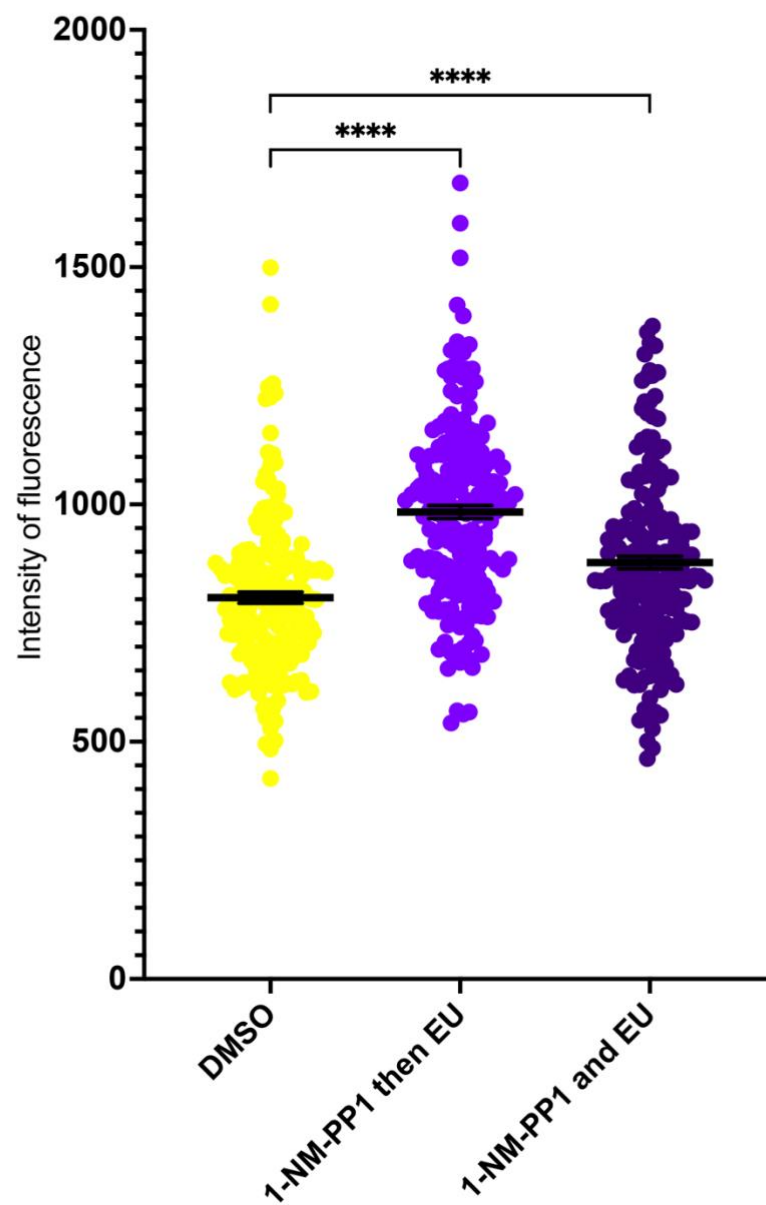
The analysis of the microscopy images showed that, following inhibition of CDK1 with 1-NM-PP1, the intensity of the Alexa Fluor 488 signal significantly increased compared to untreated cells (**Figure 4.1.**). Although the intensity was higher for both conditions, it was the strongest for cells which were inhibited with 1-NM-PP1 before the addition of EU. As the intensity of fluorescence was higher when CDK1 was inhibited, RNA production increases in these conditions, indicating a role for CDK1 as a transcriptional

repressor. CDK1 is well-recognised to inhibit transcription during mitotic transcriptional silencing, and the results was further in line with the transcriptional repression seen in mouse embryonic stem cells (Michowski et al., 2020).

Figure 4.1. The level of global RNA transcription increases following CDK1 inhibition. CDK1 in HeLa AS-CDK1 cells was inhibited with 7.5 μ M 1-NM-PP1 for 0.5h. EU was administered after (1-NM-PP1 then EU) or during (1-NM-PP1 and EU) the treatment to assess the level of nascent RNA production. **A.** Representative images of the analysed samples, taken using a fluorescence microscope. Images from the DAPI channel show DNA stained with Hoechst 33342, while GFP channel shows EU incorporated into newly-synthesised RNA stained with Alexa Fluor 488. Scale bars: 100 μ m. **B.** Quantification of the fluorescence intensity of the nuclei in each condition (****p<0.0001; Kruskal-Wallis test; n=198). Error bars show SEM.



B. EU incorporation after CDK1 inhibition



4.3. Effects of CDK1 inhibition on *KPNB1* transcription

As CDK1 appears to act as a global transcriptional repressor, it was essential to determine how its inhibition affects individual genes. Karyopherin β 1 (encoded by *KPNB1*) is a highly-expressed nuclear importin, which serves as a model housekeeping gene when investigating transcription of protein-coding genes (Marfori et al., 2011; Tellier et al., 2020, 2022). With well-established tools to study the transcription of *KPNB1* available in the lab, the changes observed on this gene suggest general modes of action of any potential transcriptional regulators, such as CDK1. The behaviour of RNA polymerase II (pol II) on *KPNB1* after the inhibition of CDK1 was determined using chromatin immunoprecipitation (ChIP) followed by real-time quantitative polymerase chain reaction (qRT-PCR). Additionally, as Cdc28 in *S. cerevisiae* phosphorylates pol II on serine 5 residue (S5) of its carboxy-terminal domain (CTD) (Enserink & Chymkowitch, 2022; Kõivomägi et al., 2021), the levels of S5 phosphorylation before and after CDK1 inhibition were also investigated. HeLa AS-CDK1 cells were incubated with 7.5 μ M 1-NM-PP1 for 0.5h, after which anti-pol II and anti-S5p antibodies were used to precipitate chromatin crosslinked to the proteins of interest.

Pol II ChIP of *KPNB1* gives the typical occupancy pattern of the polymerase, with a high peak close to the transcriptional start site (TSS), and a lower peak around the polyadenylation site (pA) (**Figure 4.2.**). No significant differences in pol II occupancy were observed between untreated cells and cells where CDK1 was inhibited at any point along the gene. Similarly, the signal for the phosphorylated S5 (S5p) did not change between untreated and 1-NM-PP1-treated cells. Taken together, the ChIP data indicate that CDK1 does not affect *KPNB1* transcription, and therefore, that it probably does not act as a transcriptional repressor for all protein-coding genes.

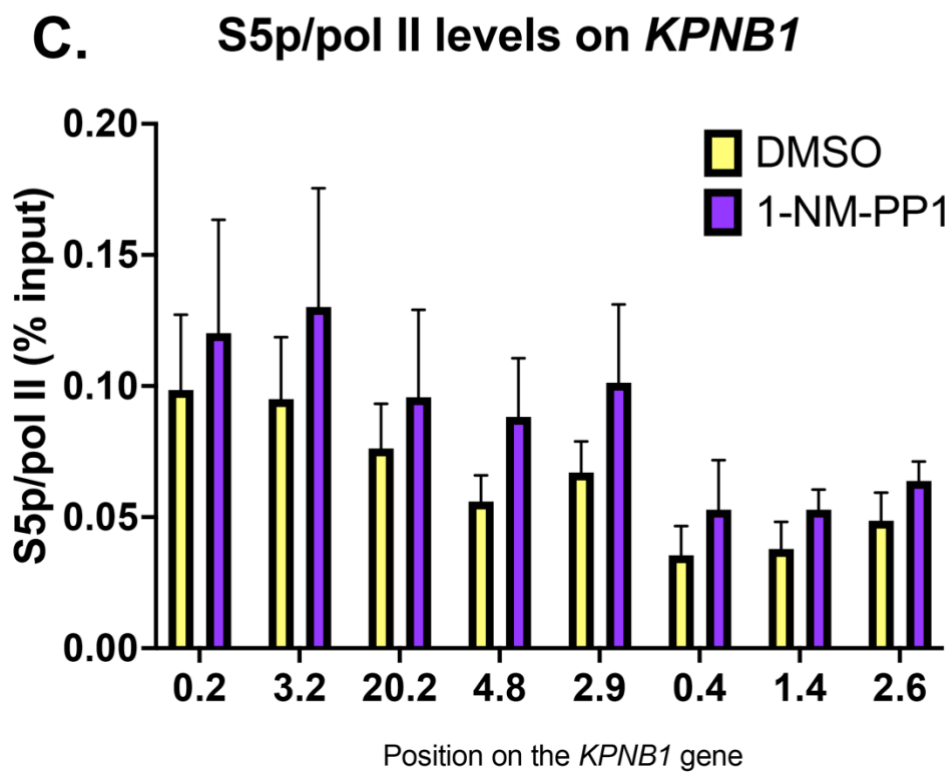
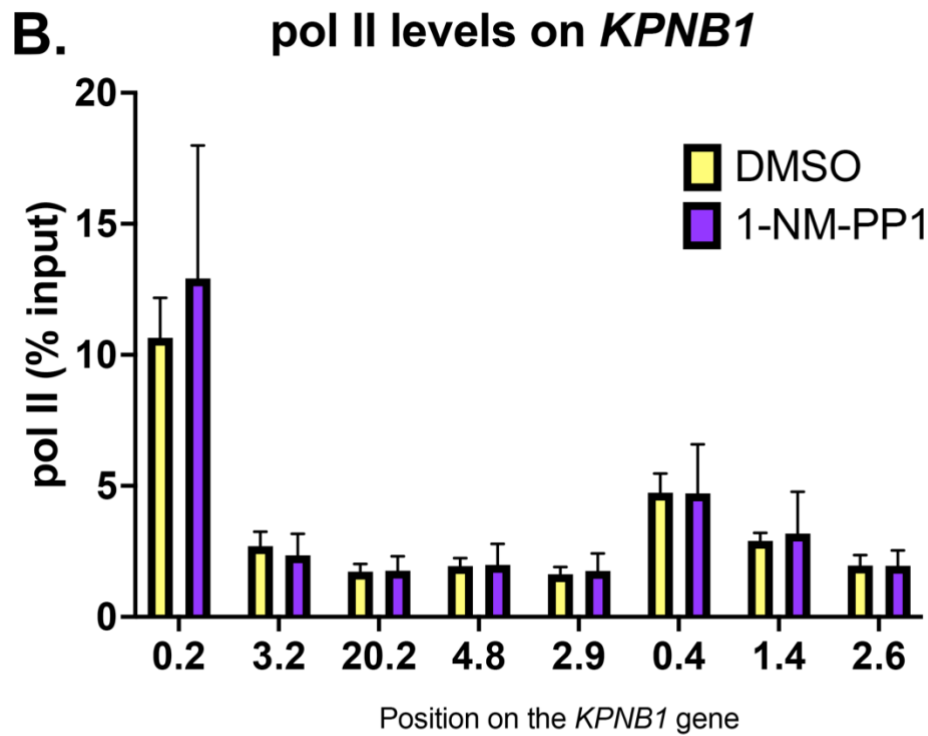
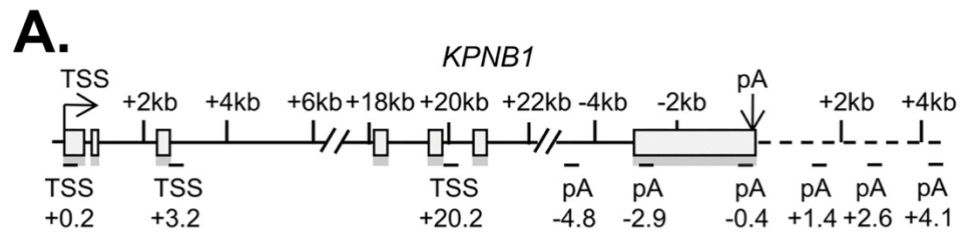


Figure 4.2. Effects of CDK1 inhibition on *KPNB1* gene. HeLa AS-CDK1 cells were treated with 7.5 μ M 1-NM-PP1 for 0.5h, after which chromatin immunoprecipitation (ChIP) was performed with anti-pol II and anti-S5p antibodies. **A.** Schematic of the *KPNB1* gene with marked primer locations used for ChIP-qPCR. **B.** RNA polymerase II (pol II) levels on the *KPNB1* gene following CDK1 inhibition. **C.** Levels of phosphorylated serine 5 residue (S5p) of pol II on the *KPNB1* gene following CDK1 inhibition. Values were normalised and presented as percentage of total DNA input, with the S5p data additionally normalised to pol II ($p>0.05$; ANOVA test; $n=3$). Error bars show SEM.

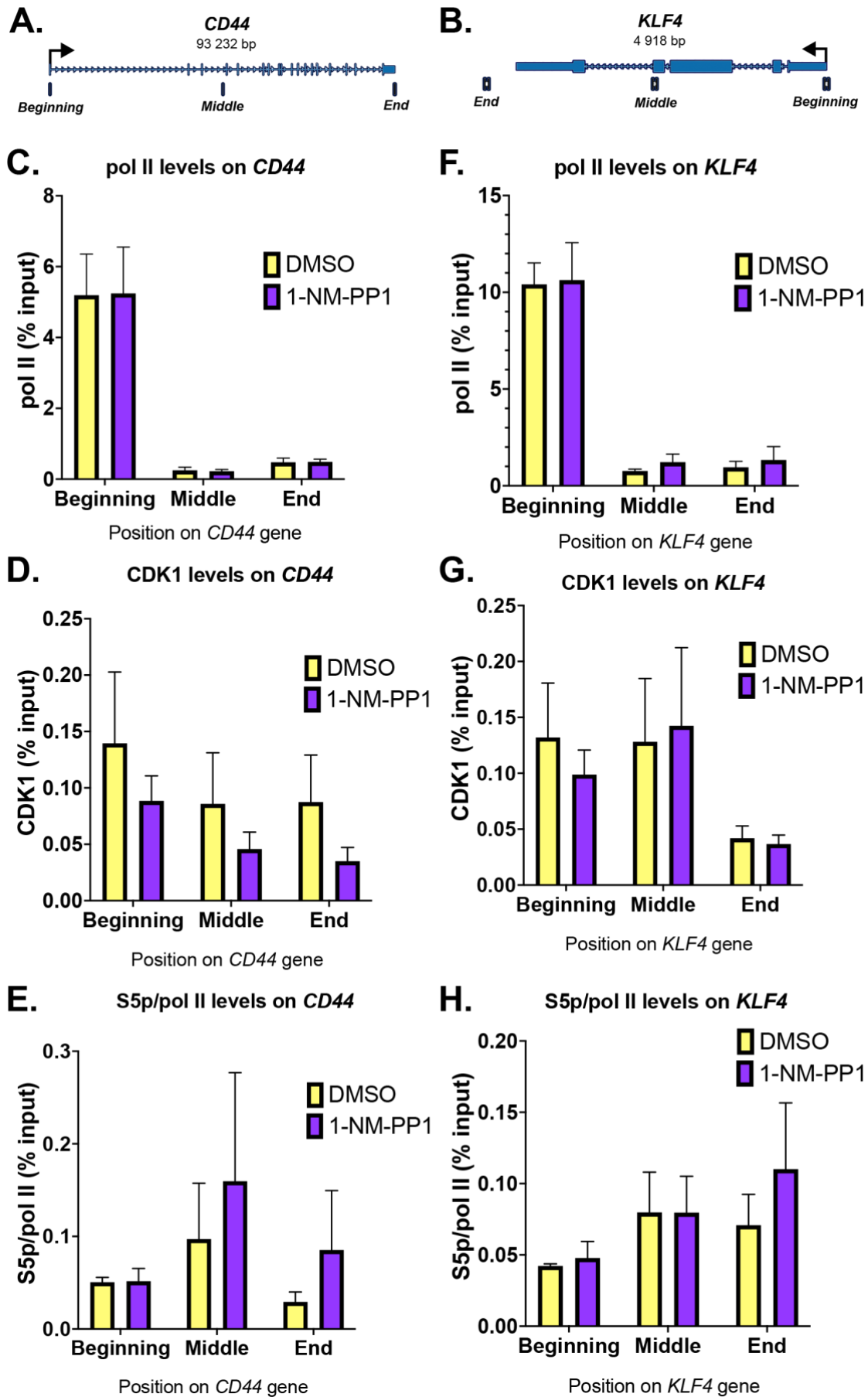
4.4. Effects of CDK1 inhibition on *CD44* and *KLF4* transcription

Given the evidence against a role of CDK1 as a general transcriptional repressor, the effects of its inhibition were then determined on genes with roles in differentiation and stemness. The two genes selected for this purpose were *CD44*, which is a cell-surface adhesion receptor and a marker of cell stemness (C. Chen et al., 2018; Morath et al., 2016; Senbanjo & Chellaiah, 2017; Thapa & Wilson, 2016; Underhill, 1992), and *KLF4*, which is a transcription factor controlling stem cell maintenance (Bourillot & Savatier, 2010; Ghaleb & Yang, 2017; Z. He et al., 2023; K. Takahashi & Yamanaka, 2006; P. Zhang et al., 2010). Both genes are present and actively transcribed in HeLa cells. Additionally, they were found to be dysregulated following the knockout of DOT1L, a substrate of CDK1 in mouse embryonic stem cells (mESCs) (Michowski et al., 2020), making these suitable candidates to test the transcriptional role of CDK1 in human cells. If CDK1 would affect one or both these genes, it would strongly suggest it possesses specialised transcriptional functions that could possibly tie it to the regulation of stem cell-like phenotype of cancer cells. Two sets of three primer pairs were designed to check the levels of pol II, S5p, and CDK1 along the chosen genes using ChIP-qPCR (**Figure 4.3.A.-B.**). CDK1 was added to this part of the study to further determine whether the kinase itself is associated with chromatin. In accordance with previous experiments, HeLa AS-CDK1 cells were inhibited with 7.5 μ M 1-NM-PP1 for 0.5h, after which the crosslinked chromatin was precipitated with the relevant antibodies.

Both *CD44* and *KLF4* have a peak of pol II activity at the beginning of the gene, which then drastically decreases for the middle and end primer sets (**Figure 4.3.C.-H.**), a profile common for protein-coding genes (Archuleta et al., 2024; Petrenko & Struhl, 2021; Quinodoz et al., 2014). However, no differences were seen in pol II levels between 1-

NM-PP1-treated and untreated samples at any point throughout the genes. Similarly, ChIP using anti-CDK1 antibody detected no shifts in CDK1 level throughout the genes. For both *CD44* and *KLF4*, there is no clear pattern of CDK1 occupancy across the gene, although it seems lower at the end of *KLF4*. Lastly, there are no significant differences in the phosphorylation of serine 5 before or after CDK1 inhibition. It was therefore concluded that CDK1 does not exert any direct transcriptional control over *CD44* or *KLF4* in human cancer cells, in contrast to what has been shown for mESCs.

Figure 4.3. Effects of CDK1 inhibition on *CD44* and *KLF4* genes. HeLa AS-CDK1 cells were treated with 7.5 μ M 1-NM-PP1 for 0.5h, after which chromatin immunoprecipitation (ChIP) was performed. Three primer pairs spaced along the *CD44* and *KLF4* genes were used for qPCR to determine the levels of different proteins on the chromatin. For *CD44*, levels of **A.-B.** Schematics of the *CD44* and *KLF4* genes with marked primer locations used for ChIP-qPCR. **C.-E.** RNA polymerase II (pol II), phosphorylated serine 5 residue (S5p) of pol II, and CDK1 levels on the *CD44* gene following CDK1 inhibition **F.-H.** RNA polymerase II (pol II), phosphorylated serine 5 residue (S5p) of pol II, and CDK1 levels on the *KLF4* gene following CDK1 inhibition. Values were normalised and presented as percentage of total DNA input, with the S5p data additionally normalised to pol II ($p>0.05$; ANOVA test; $n=3$). Error bars show SEM.



4.5. CDK1 inhibition upregulates the transcription of a subset of genes

As the ChIP of individual genes did not support the previous EU-staining data showing a general de-repression of transcription after CDK1 inhibition, it was decided to use a next-generation sequencing approach to obtain a more global picture of the transcriptional changes following CDK1 inhibition, and to identify any individual dysregulated genes. Since thus far there was no strong evidence suggesting that CDK1 acted directly through pol II, RNA-seq was elected as the method of choice instead, to determine whether CDK1 affects nascent transcript production. The inhibition time was raised to 2h instead of the former 0.5h to make sure that any changes caused by the absence of CDK1 activity would be detectable. Nascent transcripts were investigated by performing RNA-seq on chromatin-associated RNA (chrRNA-seq), which was further depleted of poly(A)⁺ and rRNA transcripts. It is unlikely that levels of mature mRNA would detectably change within the short (0.5h-2h) inhibition times used in this study. Additionally, 10% *Drosophila* cell spike-in was introduced, to control for any global changes in starting material. The combined procedures presented a relatively straightforward approach of examining global changes in RNA expression, while the exclusion of more mature mRNA allowed for maintaining greater focus on more subtle shifts in nascent transcription.

The HeLa AS-CDK1 cells were therefore treated with DMSO or 7.5μM 1-NM-PP1 for 2h, after which they were collected and *Drosophila* S2 cells were added as spike-in. Cell fractionation was performed, and the chromatin-associated RNA was purified and checked for quality quality using 4150 TapeStation System. After preparation of libraries

and sequencing, the data on the transcription of pol II-regulated protein-coding genes was analysed bioinformatically by Dr Callum Henfrey.

4.5.1. Chromatin RNA-seq analysis: Differential expression of genes after 1-NM-PP1 treatment of HeLa AS-CDK1 cells

The differential expression of genes following 2h inhibition of CDK1 is represented on an MA plot (**Figure 4.4.**). The horizontal axis of the graph shows the levels of expression of the individual genes, with genes on the right-hand side being more highly expressed in cells compared with the genes on the left. The vertical axis shows the log ratio of untreated and treated samples, which depicts the difference in expression between conditions.

The first important trend observed on the MA plot is the upwards shift of a subset of genes following the inhibition of CDK1 with 1-NM-PP1. This suggests that normally CDK1 acts to downregulate these genes, which would be in accordance with a possible role as a transcriptional repressor. Nevertheless, only a couple of hundred genes were found to be upregulated following CDK1 inhibition, and only a handful of those were found to be significantly dysregulated.

Using a low-stringency cut-off point of $p < 0.1$, 23 genes were classified as significantly dysregulated, with only one of them being significantly downregulated. The list of these genes can be found in **Table 4.1.** As can be seen from the list, there was no clear functional link between the dysregulated genes. In order to establish whether CDK1 inhibition affects any particular pathways, gene set enrichment analysis (GSEA) was performed. GSEA takes into account all genes regardless of whether they were significantly dysregulated or not during the differential expression analysis. 4010/4301 gene sets were upregulated following CDK1 inhibition, although the deregulation did not reach a necessary threshold of significance. Similarly, gene ontology (GO) analysis, which examines any sets of genes from the significantly dysregulated “hits”, did not produce a statistically-significant outcome.

As can be seen on the heatmap on **Figure 4.5.**, the differences in gene expression between the control samples and samples where CDK1 was inhibited were minimal. Nevertheless, two genes from the differential expression analysis were isolated as significantly-dysregulated ($p < 0.05$) following 1-NM-PP1 treatment. The significantly upregulated gene was *PER1*, which is one of the core circadian rhythm genes, and the significantly downregulated one was *TXNIP*, which functions in the oxidative stress response and several other pathways. Importantly, both were determined to be significant hits in all three repeats of the experiment. Despite their seemingly unrelated functions, these genes were therefore selected for further subsequent analysis to try to uncover the mechanism of their regulation by CDK1.

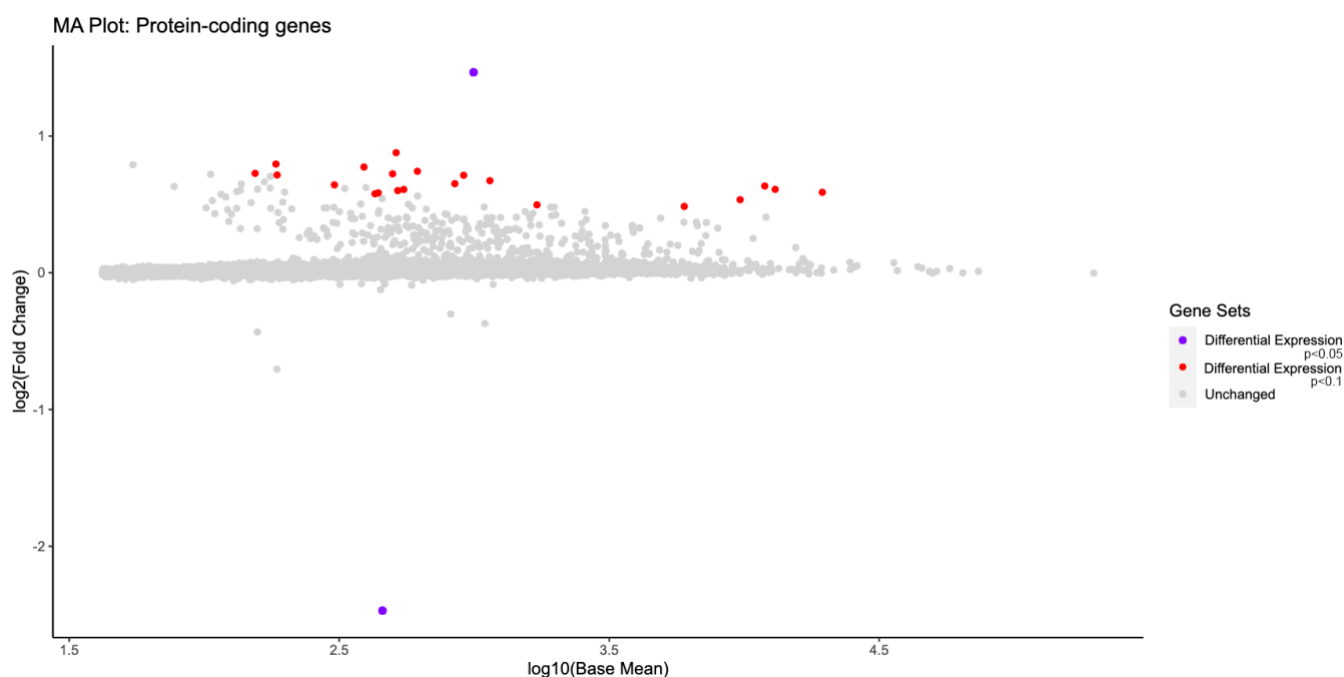


Figure 4.4. Differential expression of genes after 1-NM-PP1 treatment of HeLa AS-CDK1 cells. RNA-seq of chromatin-associated RNA was performed on HeLa AS-CDK1 cells treated with 7.5 μ M 1-NM-PP1 for 2h. The samples were supplemented with a 10% *Drosophila* cell spike-in and were further depleted of poly(A)⁺ and rRNA transcripts before analysis. The MA plot shows the differences in expression of protein-coding genes between treated and untreated conditions, with genes above the 0-mark correlating to upregulation following CDK1 inhibition. The red dots signify hits with a cut-off point of $p < 0.1$, while the purple dots represent statistically-significant hits with $p < 0.05$ (Benjamini-Hochberg adjusted P value; $n=3$).

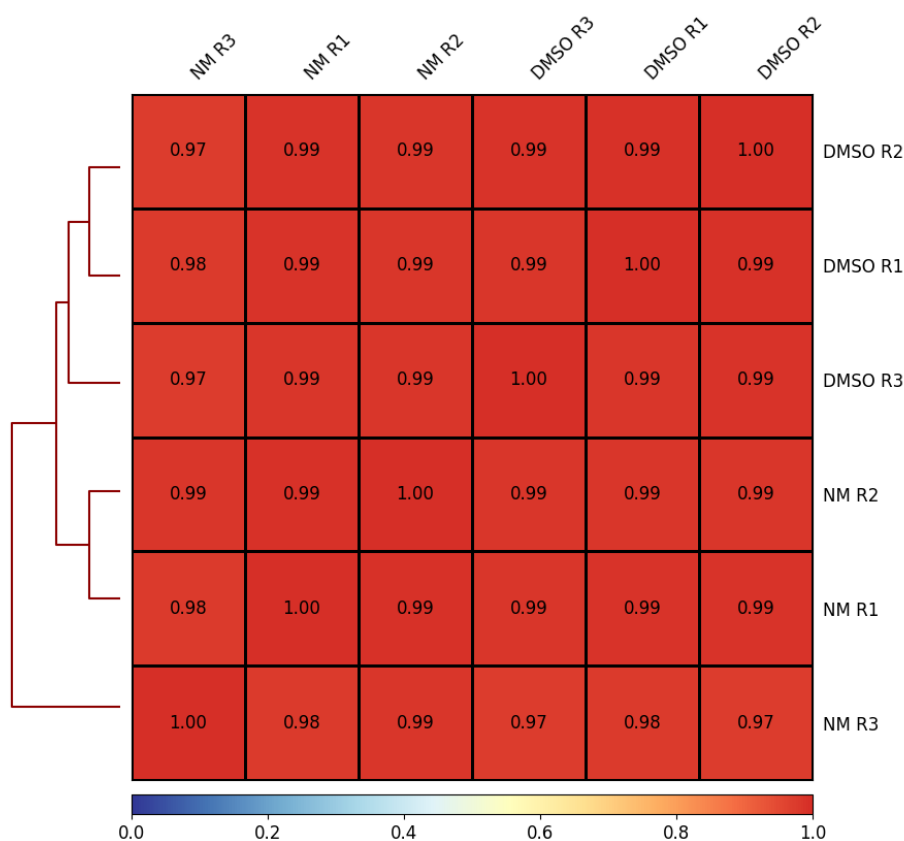


Figure 4.5. Differences in gene expression between DMSO- and 1-NM-PP1-treated HeLa AS-CDK1 cells. RNA-seq of chromatin-associated RNA was performed on HeLa AS-CDK1 cells treated with 7.5 μ M 1-NM-PP1 for 2h. The samples were supplemented with a 10% *Drosophila* cell spike-in and were further depleted of poly(A)⁺ and rRNA transcripts before analysis. The heatmap shows the degree of similarity of expression of protein-coding genes between individual samples (n=3; 3 repeats of DMSO, 3 repeats of 1-NM-PP1 (NM)).

Table 4.1. Genes dysregulated following the inhibition of CDK1 in HeLa AS-CDK1 cells (p<0.1).

Gene	Description
<i>ACTB</i>	Actin, cytoplasmic 1; part of the cytoskeleton
<i>ACTG1</i>	Actin, cytoplasmic 2; part of the cytoskeleton
<i>ADGRG1</i>	Adhesion G protein-coupled receptor G1; member of the adhesion G protein-coupled receptor family
<i>CASP9</i>	Caspase 9; initiator caspase of the apoptotic pathway
<i>ETS2</i>	Protein C-ets-2; transcription factor, usually upregulates genes and is implicated in development and apoptosis
<i>FAM124A</i>	Family with sequence similarity 124 Member A; function unknown
<i>GRINA</i>	Glutamate ionotropic receptor NMDA type subunit associated protein 1; member of NMDA glutamate receptor family
<i>ITPRIP</i>	Inositol 1,4,5-trisphosphate receptor interacting protein; enhances inhibition of inositol 1,4,5-triphosphate receptor
<i>KDELRL1</i>	KDEL endoplasmic reticulum protein retention receptor 1; receptor that recycles proteins back to the endoplasmic reticulum
<i>MAP2K6</i>	Dual specificity mitogen-activated protein kinase kinase 6; acts in the MAP kinase signal transduction pathway
<i>MCF2L</i>	MCF.2 cell line derived transforming sequence like; guanine nucleotide exchange factor involved in RHOA and CDC42 signaling pathways
<i>PDK4</i>	Pyruvate dehydrogenase kinase 4; regulates glucose metabolism
<i>PER1*</i>	Period circadian regulator 1; one of core circadian rhythm genes
<i>PPIAP22</i>	Peptidylprolyl isomerase A pseudogene 22; function unknown

<i>SIK1B</i>	Salt inducible kinase 1B; serine/threonine protein kinase from the adenosine monophosphate-activated kinase (AMPK) subfamily
<i>SLC52A2</i>	Solute carrier family 52 member 2; transporter protein, carries vitamin riboflavin (B ₂) across the cell membrane
<i>SLC7A5</i>	Large neutral amino acids transporter small subunit 1; transporter for large amino acids
<i>TKT</i>	Transketolase; participates in the pentose phosphate pathway
<i>TNS4</i>	Tensin 4; promotes cell migration
<i>TOB1</i>	Protein Tob1; regulates cell growth and has anti-proliferative activity
<i>TSC22D3</i>	TSC22 domain family member 3; glucocorticoid-induced anti-inflammatory protein
<i>TUBB</i>	Tubulin beta chain; structural component of microtubules
<i>TXNIP</i> ^{*†}	Thioredoxin-interacting protein; multifunctional protein, increases intracellular oxidative stress

*significantly dysregulated genes (p<0.05); †downregulated genes

4.5.2. RNA-seq analysis: metagene analysis, differential transcript usage and motif analysis

The RNA-seq data were subjected to further bioinformatical analysis to try to uncover how CDK1 influences transcription in HeLa AS-CDK1 cells. A metagene profile of transcribed genes was created to examine the average read density across genes, and establish any patterns in gene expression before and after CDK1 inhibition (**Figure 4.6.**). The metagene profile confirmed the trend of upregulated gene transcription following the inhibition of CDK1 activity, and it further showed that the biggest differences can be seen towards the 3' end of genes, closer to the transcription termination site (transcription end site, TES), compared to the relatively small changes at the transcription start site (TSS). This could imply that the consequences of CDK1 inhibition appear only after the early stages of gene transcription or that they accumulate with time as the gene is transcribed. Another step of the bioinformatics evaluation involved the analysis of differential transcript usage following CDK1 inhibition, in order to look for any changes in ratios of different isoforms of genes that could suggest dysregulation of splicing. As can be seen in **Figure 4.7.**, there was a small shift in the isoform usage across a subset of genes, however, it was not correlated with the differences in gene expression. Importantly, the genes with the strongest changes in transcript usage (*CDC40*, *TSPAN3*) were ultimately deemed to be bioinformatical artefacts, as the programme used for the analysis classified one base substitutions as different transcripts, leading to recording significant changes that were most likely false positives. The lack of correlation between transcript usage and differential gene expression suggests that any transcriptional effects of CDK1 inhibition are not caused by CDK1 affecting splicing pathways.

In order to uncover any further potential links between the genes found to be dysregulated after CDK1 inhibition, motif analysis was conducted on the region 1kb upstream of the

transcription start site of all differentially-expressed genes. Multiple Em for Motif Elicitation (MEME) software was used to uncover any novel motifs common between the dysregulated genes, while Simple Enrichment Analysis (SEA) was employed to check for any known enriched motifs. During MEME, the programme failed to find any meaningful similarities between the sets of differentially-expressed genes, identifying repetitive sections as possible novel motifs (**Figure 4.8.**). SEA, in turn, showed a couple of enriched motifs of the KLF family of transcription factors, as well as a binding motif for MYC. Although the latter candidate seemed interesting, it remains to be seen why changes in gene expression following the abolition of the activity of CDK1, a known oncogene, would be mediated by another oncogene (H. Chen et al., 2018).

The two significantly dysregulated genes, *PER1* and *TXNIP*, were further examined by looking at their individual read density on genome browser coverage tracks (**Figure 4.9.**).

In accord with *PER1* being the most upregulated gene following CDK1 inhibition, the density of reads along the gene uniformly increases following 1-NM-PP1 treatment. Correspondingly, the reads along *TXNIP* are all lower after 1-NM-PP1 treatment compared to DMSO. As the read increase and decrease are quite consistent along the length of the respective genes, this would suggest that CDK1 affects their transcription evenly across the gene, differing from the average metagene profile seen in **Figure 4.6.**

Finally, despite not being the primary focus of this study, the transcription of non-protein coding genes transcriptionally-regulated by pol II was also investigated. The MA plot of differential gene expression for different kinds of non-protein coding genes can be found in **Figure 4.10.** No significant differences were found for any class, indicating that CDK1 dysregulation primarily affects protein-coding genes.

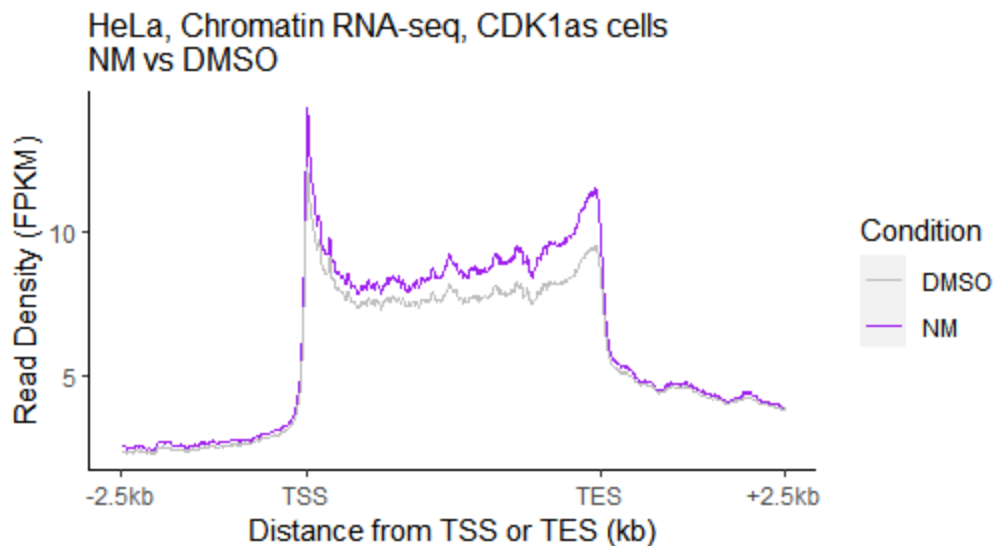


Figure 4.6. Metagene profile of protein-coding genes in HeLa AS-CDK1 cells after 1-NM-PP1 treatment. RNA-seq of chromatin-associated RNA was performed on HeLa AS-CDK1 cells treated with 7.5 μ M 1-NM-PP1 for 2h. The samples were supplemented with a 10% *Drosophila* cell spike-in and were further depleted of poly(A)⁺ and rRNA transcripts before analysis. A metagene profile was created to represent read density across transcribed genes in DMSO- or 1-NM-PP1-treated conditions (n=3). NM = 1-NM-PP1; TSS = transcriptional start site; TES = transcriptional end site.

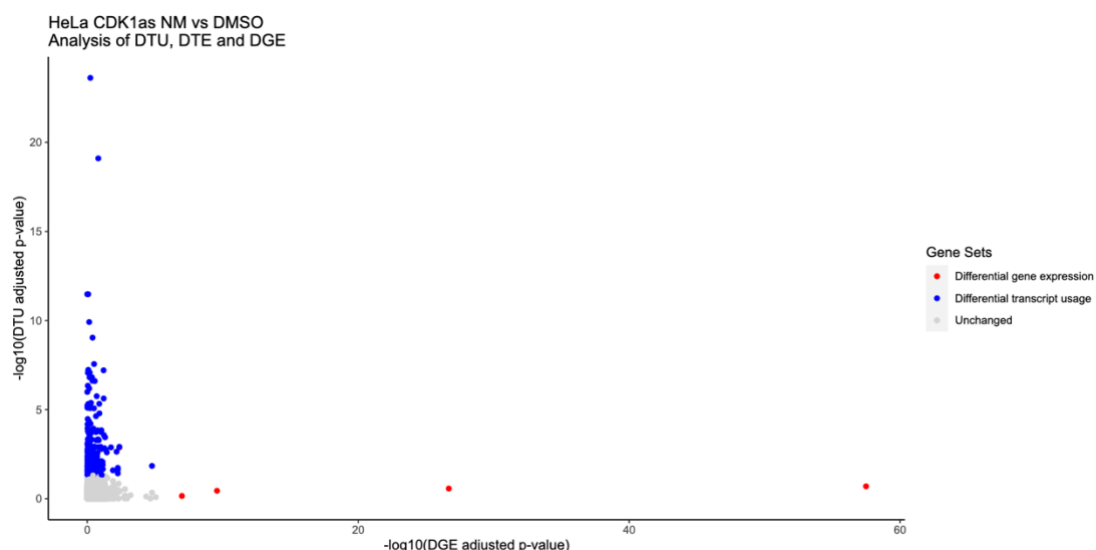
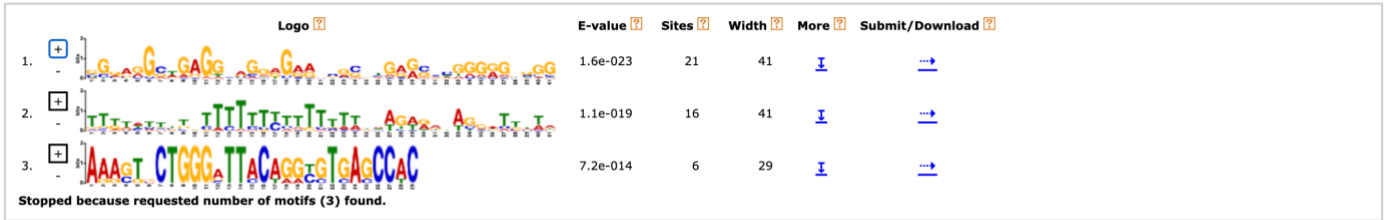


Figure 4.7. Differential transcript usage in HeLa AS-CDK1 cells after 1-NM-PP1 treatment. RNA-seq of chromatin-associated RNA was performed on HeLa AS-CDK1 cells treated with 7.5 μ M 1-NM-PP1 for 2h. The samples were supplemented with a 10% *Drosophila* cell spike-in and were further depleted of poly(A)⁺ and rRNA transcripts transcripts before analysis. The graph shows the relationship between differential gene expression (x-axis) and differential transcript usage (y-axis) following the inhibition of CDK1 (n=3). DTU – differential transcript usage; DTE – differential transcript expression; DGE – differential gene expression.

A. DISCOVERED MOTIFS



B. ENRICHED MOTIFS

Found 43 motifs with E-values ≤ 10.

Logo	Database	ID	Alt ID	P-value	E-value	Q-value	TP	FP	Enrichment Ratio	Score Threshold
	HOCOMOCov11 core HUMAN mono meme format	KLF15_HUMAN.H11MO.0.A		2.66e-5	1.07e-2	5.69e-3	12 / 21 (57.1%)	0 / 21 (0.0%)	13.00	13.87
	HOCOMOCov11 core HUMAN mono meme format	MYCN_HUMAN.H11MO.0.A		2.40e-4	9.61e-2	2.35e-2	10 / 21 (47.6%)	0 / 21 (0.0%)	11.00	10.97
	HOCOMOCov11 core HUMAN mono meme format	KLF6_HUMAN.H11MO.0.A		4.61e-4	1.85e-1	2.35e-2	14 / 21 (66.7%)	2 / 21 (9.5%)	5.00	11.14

Figure 4.8. Motif analysis for differentially-expressed genes in HeLa AS-CDK1 cells after 1-NM-PP1 treatment. RNA-seq of chromatin-associated RNA was performed on HeLa AS-CDK1 cells treated with 7.5μM 1-NM-PP1 for 2h. The samples were supplemented with a 10% *Drosophila* cell spike-in and were further depleted of poly(A)+ and rRNA transcripts before analysis. For differentially-expressed genes, the region 1kb upstream of the transcription start site was checked for any shared **A.** novel motifs using Multiple Em for Motif Elicitation (MEME) software; or for **B.** known enriched motifs using Simple Enrichment Analysis (SEA) (n=3).

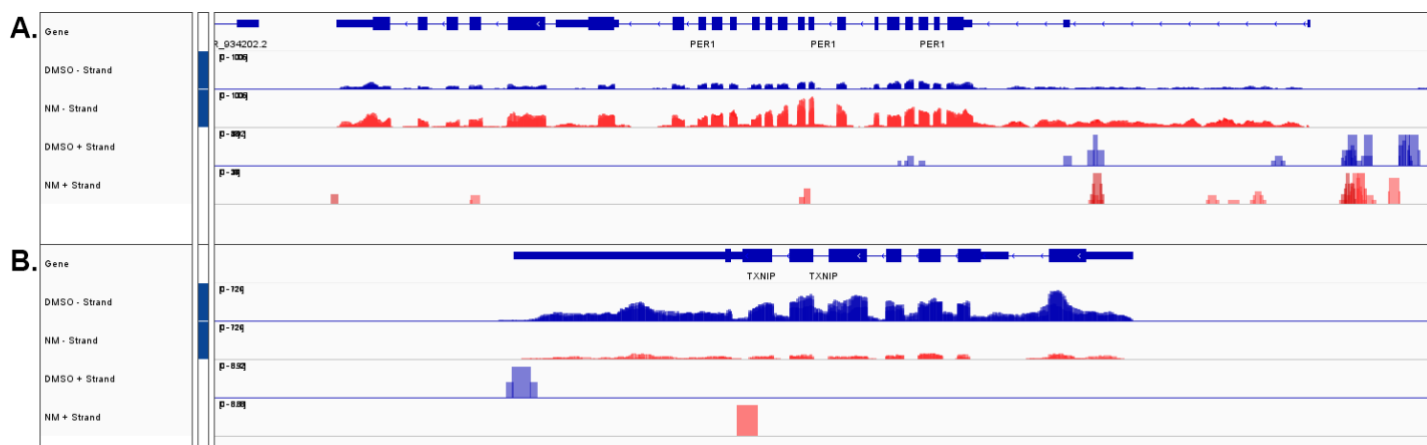


Figure 4.9. Genome browser coverage tracks of *PER1* and *TXNIP* genes in HeLa AS-CDK1 cells after 1-NM-PP1 treatment. RNA-seq of chromatin-associated RNA was performed on HeLa AS-CDK1 cells treated with 7.5 μ M 1-NM-PP1 for 2h. The samples were supplemented with a 10% *Drosophila* cell spike-in and were further depleted of poly(A)⁺ and rRNA transcripts before analysis. Only two genes were found to be significantly dysregulated following the inhibition of CDK1: *PER1* was found to be significantly upregulated, while *TXNIP* was significantly downregulated. Read density for both genes was checked using genome browser coverage tracks: **A.** *PER1* coverage track; **B.** *TXNIP* coverage track. Blue tracks show DMSO repeats and red tracks show 1-NM-PP1-treated samples (n=3).

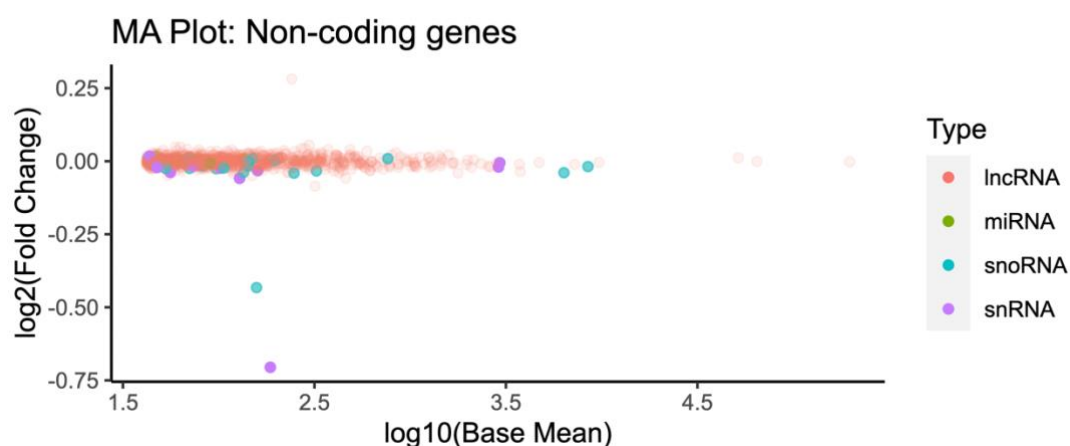


Figure 4.10. Differential expression of non-protein coding genes after 1-NM-PP1 treatment of HeLa AS-CDK1 cells. RNA-seq of chromatin-associated RNA was performed on HeLa AS-CDK1 cells treated with 7.5 μ M 1-NM-PP1 for 2h. The samples were supplemented with a 10% *Drosophila* cell spike-in and were further depleted of poly(A)⁺ and rRNA transcripts before analysis. The MA plot shows the differences in expression of non-protein coding genes between treated and untreated conditions, with genes above the 0-mark correlating to upregulation following CDK1 inhibition. No genes were found to be significantly dysregulated after 1-NM-PP1 treatment ($p < 0.05$; $n = 3$).

4.6. The effects of different durations of inhibition of CDK1 on chromatin RNA levels of *PER1* and *TXNIP*

In order to further validate the results seen in the RNA-seq, chromatin-associated RNA was purified from further HeLa AS-CDK1 samples treated with 7.5 μ M 1-NM-PP1. Supplementary time points were added to the analysis to obtain a more complete view of the consequences of CDK1 inhibition. Cells were therefore treated for 0.5h, the original shorter time point for more direct effects, for 2h as previously, and finally for 10h to examine long-term outcomes of CDK1 inhibition. The extracted chromatin RNA was reverse-transcribed into cDNA and amplified with qRT-PCR using primers corresponding to different genes of interest. *PER1* and *TXNIP* were investigated as the only significantly-dysregulated genes from the RNA-seq, and additional primers were prepared for *ETS2* and *PDK4*, two of the genes identified using a less stringent p-value. As seen in **Figure 4.11.**, the inhibition of HeLa AS-CDK1 with 1-NM-PP1 for 2h did not cause the same increase in the levels of *PER1* chrRNA as seen previously in the RNA-seq. The trend changes at the 10h timepoint, with the difference becoming significant compared to the DMSO control. Intriguingly, the increase in *PER1* chrRNA was not straightforward, as the 0.5h inhibition of CDK1 first caused a significant decrease in the *PER1* chrRNA, before its level started rising. The case was more linear for *TXNIP*, as its chrRNA dropped quickly after 0.5h inhibition to then become significantly decreased at the 2h timepoint, echoing the results seen in the RNA-seq.

The two borderline genes, *ETS2* and *PDK4*, did not show any clear changes after inhibition of CDK1, which corresponds to the RNA-seq data. The levels of *ETS2* chrRNA remained relatively stable at the different times of inhibition. For *PDK4*, there was a stronger increase of chrRNA levels after 2h of inhibition, with a significant increase after 10h mark. Taken together, the data seemed to confirm that CDK1 inhibition can affect

the transcription of both *PER1* and *TXNIP*, and that the more borderline genes such as *ETS2* and *PDK4* do not seem to be directly affected by its dysregulation.

Because of the changes in the regulation of *PER1* by CDK1 over time, a finer time course was conducted to try to elucidate the kinetics of CDK1 control over this gene. HeLa AS-CDK1 cells were treated with 7.5 μ M 1-NM-PP1 for 0.25h (15min), 0.5h, 0.75h (45min), 1h and 2h to generate a more detailed picture of the transcriptional changes seen for both *PER1* and *TXNIP*. Similar to before, the purified chrRNA from the cells was reverse-transcribed and analysed by qRT-PCR. The data confirm that immediately after the inhibition of CDK1 with 1-NM-PP1 the levels of *PER1* first decrease, before starting to rise again around the 0.75h mark of inhibition, and going back to the original level after 1h of inhibition (**Figure 4.12.**). The analysis of the *TXNIP* gene did not provide the same definite results, with the levels remaining relatively stable throughout the finer time course. The noticeable decrease in *TXNIP* chrRNA expression during the initial CDK1 inhibition time course (**Figure 4.11.**) could not be detected again, highlighting the need for further repeats. While the difference in *TXNIP* gene expression between treated and control samples during RNA-seq was statistically significant (**Figure 4.4.**), it was rather modest, suggesting that slight changes in conditions or other cellular factors could affect its significance. Nevertheless, the collected data highlighted the value of shorter inhibition times in studying the immediate transcriptional effects of inhibiting CDK1, and indicated that these effects might be overcompensated with time by potential feedback mechanisms, as seen for *PER1* regulation.

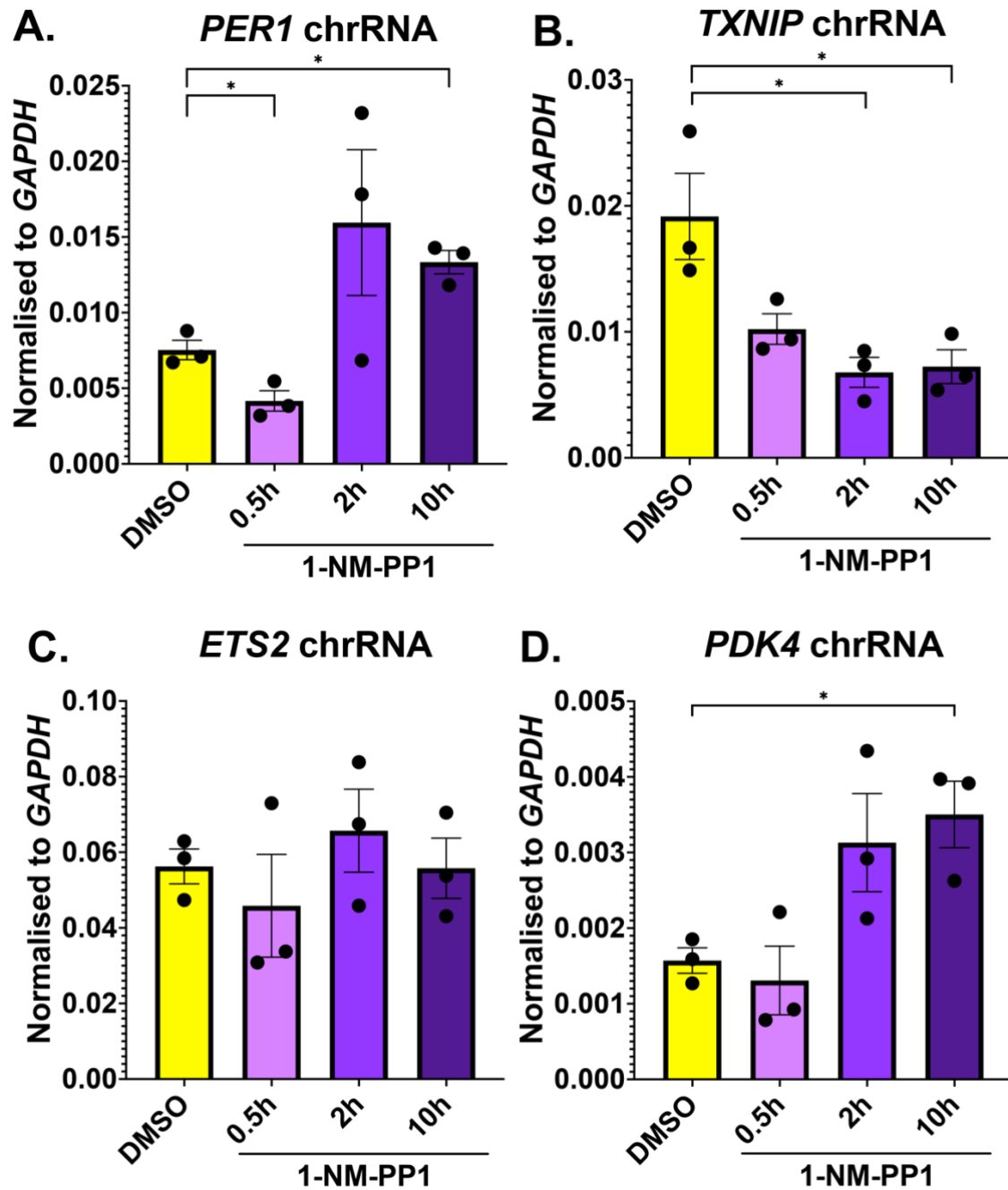


Figure 4.11. Effects of different durations of CDK1 inhibition on chromatin-associated RNA levels of *PER1*, *TXNIP*, *ETS2* and *PDK4* genes. HeLa AS-CDK1 cells were treated with 7.5 μ M 1-NM-PP1, after which their chromatin-associated RNA (chrRNA) was purified, reverse-transcribed, and quantified using qRT-PCR. The levels of chrRNA after extending inhibition times were determined for **A. *PER1***, **B. *TXNIP***, **C. *ETS2***, and **D. *PDK4***. Values were normalised to the levels of *GAPDH* chrRNA (* $p < 0.05$; Kruskal-Wallis test; $n = 3$). Error bars show SEM.

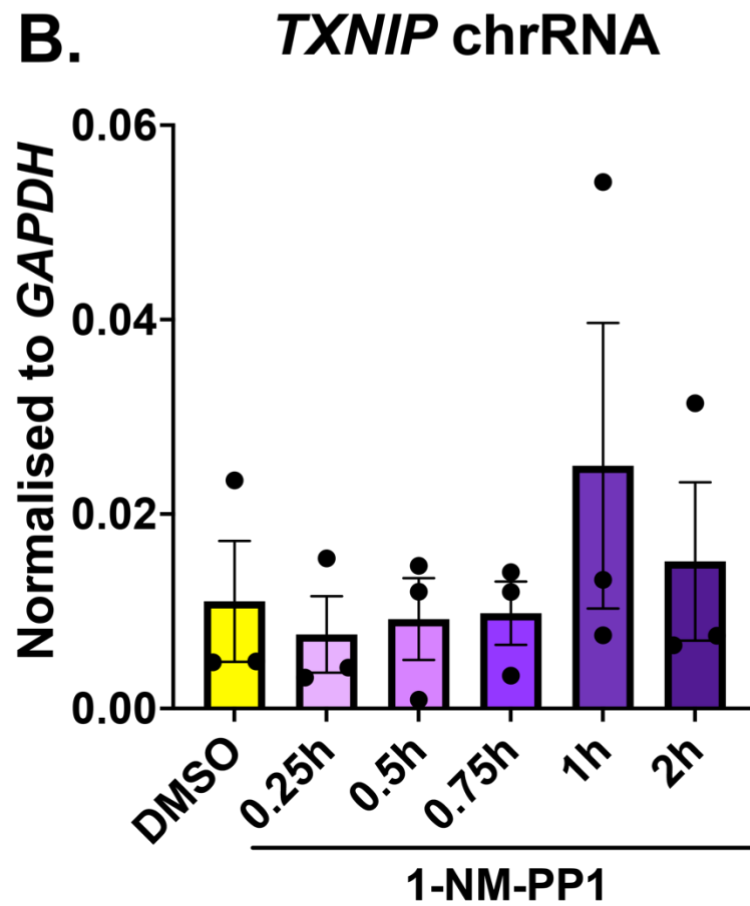
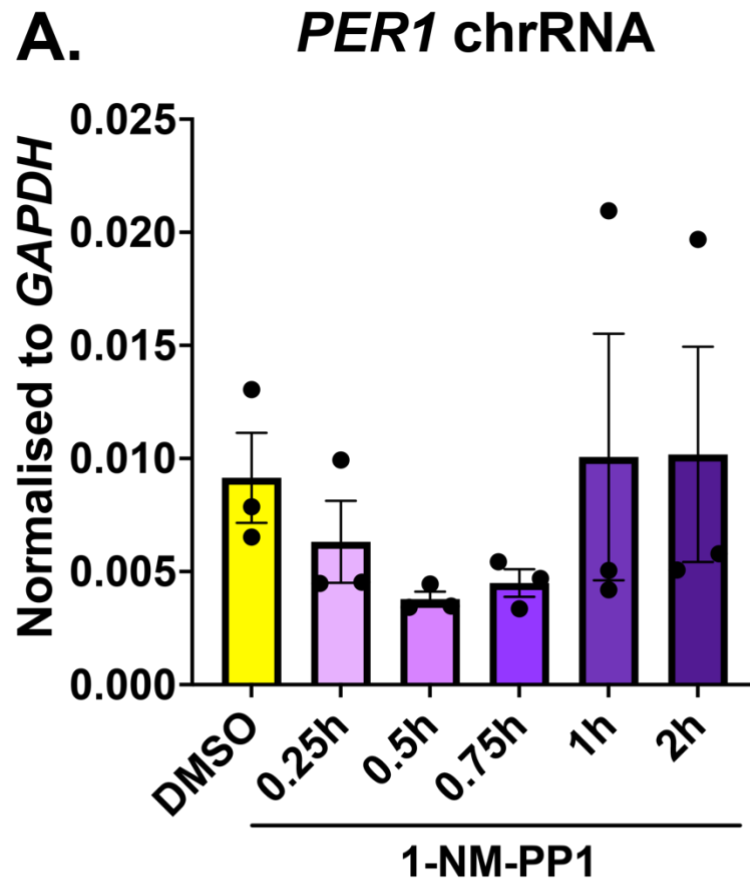


Figure 4.12. Effects of short CDK1 inhibition on chromatin-associated RNA levels of *PER1* and *TXNIP* genes. HeLa AS-CDK1 cells were treated with 7.5 μ M 1-NM-PP1, after which their chromatin-associated RNA (chrRNA) was purified, reverse-transcribed, and quantified using qRT-PCR. The levels of chrRNA after short inhibition times were determined for **A.** *PER1* and **B.** *TXNIP*. Values were normalised to the levels of *GAPDH* chrRNA ($p>0.05$; Kruskal-Wallis test; $n=3$). Error bars show SEM.

4.7. Protein levels of PER1 and TXNIP following CDK1 inhibition

Having the indication that CDK1 exerts transcriptional control over *PER1* and *TXNIP* genes, the next step was to determine whether inhibition of CDK1 activity could also affect their protein levels. HeLa AS-CDK1 cells, as well as normal HeLa cells, were treated with 7.5 μ M 1-NM-PP1 for 0.5, 2, or 10 hours, before whole cell protein extracts were prepared and analysed by Western blotting. The blots were probed with antibodies against PER1 and TXNIP, and against H3S10p to validate the correct inhibition of CDK1. Additionally, BMAL1 antibody was added to this part of the study to provide possible further clues on what happens to the circadian rhythm following the abolition of CDK1 activity. BMAL1, one of the core circadian rhythm regulators, acts as a transcriptional activator of *PER1*, while PER1 downregulates the expression of *BMAL1* (Buhr & Takahashi, 2013; Gery et al., 2006; Reppert & Weaver, 2002; Shearman et al., 2000; J. S. Takahashi, 2016). It is interesting to note that the only clear link of CDK1 regulation of the circadian rhythm is through its indirect upregulation of *BMAL1*, by targeting its repressor REV-ERB α for degradation (X. Zhao et al., 2016).

As can be seen on **Figure 4.13.**, the inhibition of CDK1 in the HeLa AS-CDK1 cells was successful based on the disappearance of the mitotic mark, H3S10p. The protein levels of both PER1 and BMAL1 remained constant throughout the duration of the experiment, indicating that although CDK1 regulates the transcription of *PER1*, the consequences of this control do not affect PER1 protein levels within 10 hours. Perhaps the circadian rhythm proteins require longer experimental timepoints for any shifts in expression to become detectable at the protein level; or they are controlled by more stringent feedback mechanisms that prevent their amount from fluctuating after the disappearance of only one regulator.

The protein levels of TXNIP instead decrease during the inhibition of CDK1 in HeLa AS-CDK1 cells. Surprisingly, the same trend was observed for wild type HeLa cells, where the 10h treatment with 1-NM-PP1 gives the same effect seen as the analogue-sensitive cells. This result implies that although 1-NM-PP1 inhibits the actions of CDK1 in AS-CDK1 cells, the regulation of TXNIP might not be controlled by this kinase, but rather through an off-target effect of the drug.

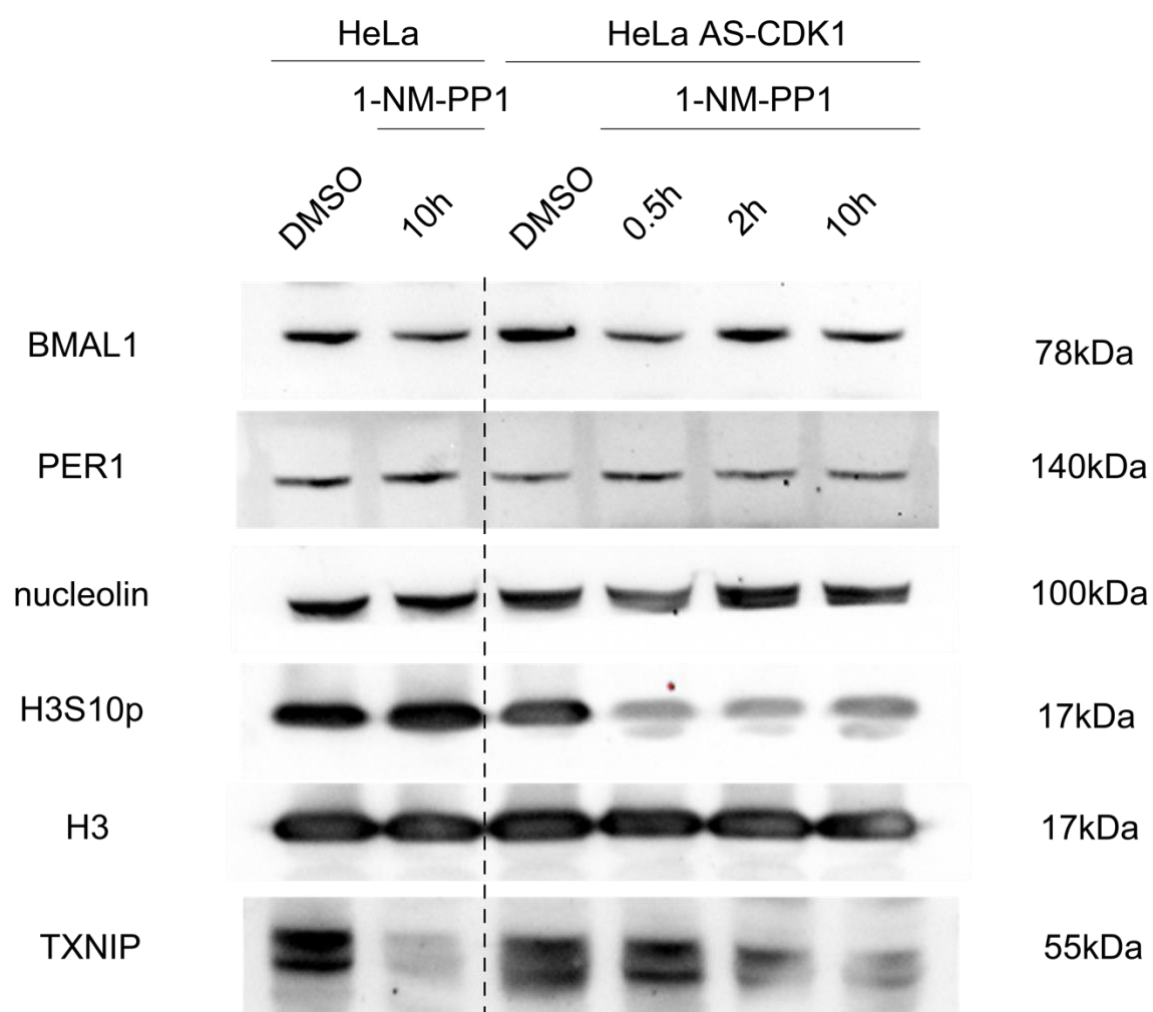


Figure 4.13. Protein levels of PER1 and TXNIP following CDK1 inhibition. Western blots were prepared on whole cell extracts of HeLa and HeLa AS-CDK1 cells after inhibition with 7.5 μ M 1-NM-PP1. The blots were probed with antibodies against nucleolin as a loading control, H3, H3 phosphorylated on serine 10 residue (H3S10p), which acted as the mitotic mark, as well as TXNIP, PER1 and BMAL1.

4.8. 1-NM-PP1 treatment affects transcription in HeLa cells

Following the western blot experiment, where the levels of TXNIP protein change in HeLa cells treated with 1-NM-PP1, it was crucial to establish whether the transcriptional changes seen in RNA-seq and chrRNA purifications (**sections 4.5.–4.6.**) could also be caused by an off-target effect of the drug instead of inhibition of AS-CDK1. HeLa AS-CDK1 cells and HeLa cells were treated for 2h with 7.5 μ M 1-NM-PP1. The samples were then fractionated and their chromatin-associated RNA was purified. The RNA was reverse-transcribed and amplified with qRT-PCR, using primers for the genes *PER1*, *TXNIP*, *ETS2*, and *PDK4*.

For HeLa AS-CDK1 cells, the results remained relatively the same as in previous experiments (see **section 4.3.**), with the 1-NM-PP1-treated samples exhibiting an increase in the chrRNA levels of *PER1* and *PDK4*, and to a lesser extent *ETS2*, and a marked decrease in *TXNIP* chrRNA. However, it was now clear that the same changes also occur in unmutated HeLa cells. In the cases of *PER1*, *ETS2* and *PDK4*, 1-NM-PP1 treatment causes an upregulation of chrRNA production, with relative ratios similar to their HeLa AS-CDK1 counterparts. The biggest difference could be observed for *TXNIP* in HeLa cells, where the clear decrease in chrRNA levels is also statistically significant. Since normal HeLa cells do not possess a mutated kinase with an enlarged ATP-binding pocket that should be the primary target of molecules such as 1-NM-PP1, the results obtained in this experiment strongly suggest that the drug has a secondary, off-target effect in both HeLa and HeLa AS-CDK1 cell lines, and that some the transcriptional changes observed after inhibition of AS-CDK1 are due to the effect on that unknown target.

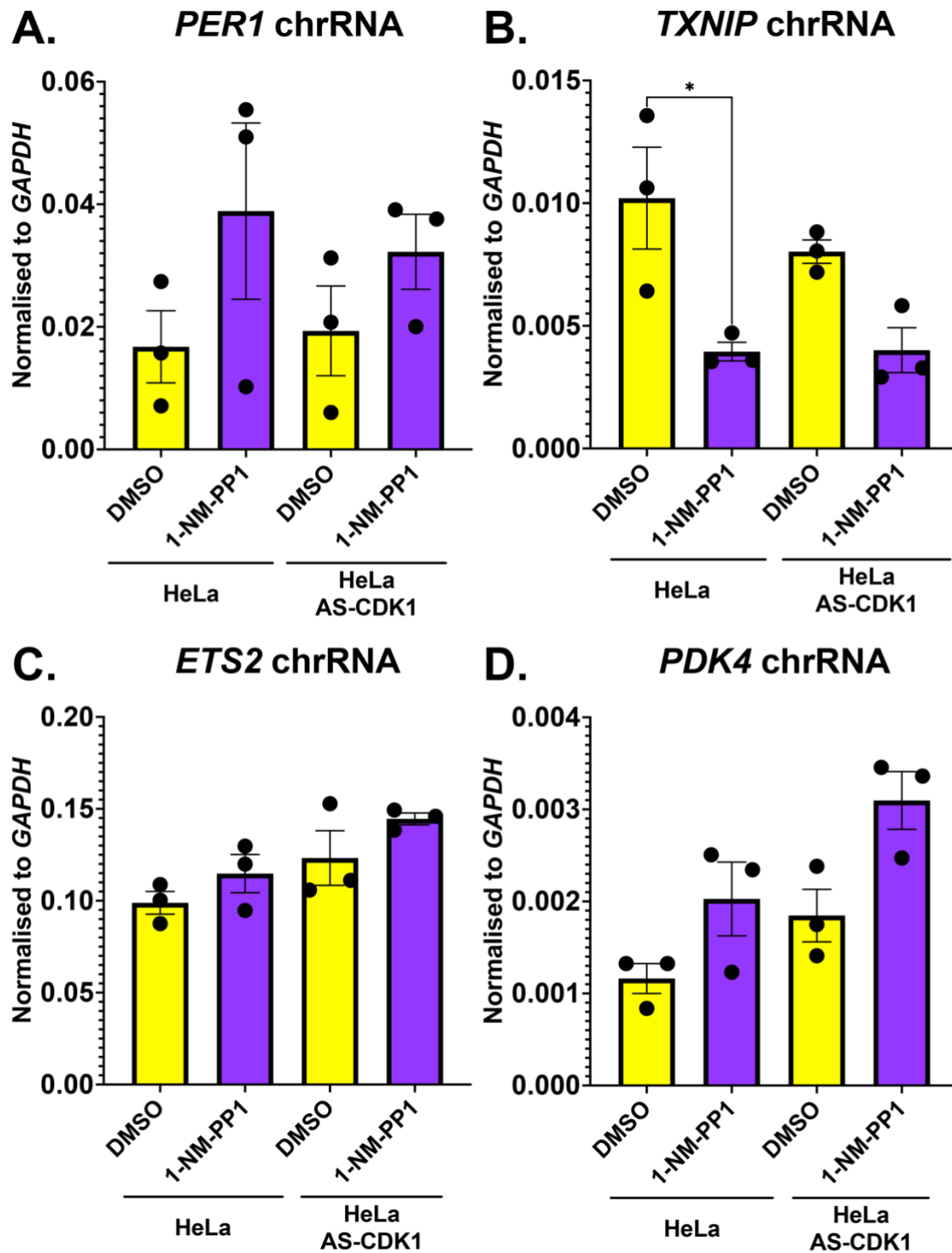


Figure 4.14. 1-NM-PP1 treatment affects HeLa. HeLa and HeLa AS-CDK1 cells were treated with 7.5 μ M 1-NM-PP1 for 2h, after which their chromatin-associated RNA (chrRNA) was purified, reverse-transcribed, and quantified using qRT-PCR. The levels of chrRNA with or without the inhibitor were determined for **A. *PER1***, **B. *TXNIP***, **C.**

ETS2, and **D.** *PDK4*. Values were normalised to the levels of *GAPDH* chrRNA (* $p < 0.05$; ANOVA test; $n=3$). Error bars show SEM.

4.9. Discussion

Following the validation of the experimental model, the transcriptional effects of CDK1 in the HeLa AS-CDK1 cell line could be investigated. Throughout the course of this study, the results indicate that in HeLa AS-CDK1 cells CDK1 acts as a transcriptional repressor, but that it controls the expression of only a limited subset of genes. Despite no evidence of it being a general transcriptional repressor, the underlying elements linking the genes dysregulated after its inhibition remain elusive. The two genes significantly altered following the abolition of CDK1 activity were the upregulated *PER1*, and the downregulated *TXNIP*. However, the first attempts at trying to find the molecular mechanism governing their regulation proved challenging. It was also shown that the two might not be affected by CDK1 at all, but rather by an off-target effect of 1-NM-PP1.

The initial EU-staining analysis of nascent RNA transcription yielded clear results, pointing to a possible role of CDK1 as a transcriptional repressor. This was also in agreement with previous literature, as in mouse embryonic stem cells inhibition of CDK1 activity led to upregulation of gene transcription (Michowski et al., 2020). CDK1 was also linked to the mitotic silencing of transcription during cell division in human cell extracts (Long et al., 1998). However, it is worth noting that the quickly-dividing mESCs have a shortened interphase, and the latter investigation also focused on *in vitro* experiments on mitotic extracts, which could explain why the role of CDK1 in those cells was mostly to repress transcription. In an asynchronous population of cells, like in the EU-staining test, the majority will be in interphase, so the defined increase in transcription during the short inhibition times in this experiment could suggest that CDK1 acts as a transcriptional repressor also outside of mitosis. Repeating the same experiment on synchronised cell populations would further solidify this claim.

Additionally, it is worth remembering that EU-staining analysis looks at global changes in transcription. Firstly, this means that the results give little indication as to what kind of genes are being up- or downregulated, as was illustrated in the subsequent search for dysregulated genes using ChIP-qPCR experiments. Secondly, the transcriptional changes indicated by the increased EU-incorporation and fluorescence intensity could indicate shifts in RNA transcribed by polymerase I and polymerase III, which transcribe rRNA and tRNA (Paule & White, 2000). Since this project focused on protein-coding genes transcribed by polymerase II, the EU-staining results had to be interpreted with a degree of caution, especially that in *S. cerevisiae* Cdc28 was shown to increase the transcription of tRNA by pol III in a cell cycle-dependent manner (Herrera et al., 2018). It is speculated however, that in mammalian cells CDK1 could actually decrease the production of tRNAs as part of mitotic transcriptional silencing (Enserink & Chymkowitch, 2022; White et al., 1995).

Unfortunately, the ChIP experiments did not result in any substantial clues as to how CDK1 exerts the transcriptional repression seen by EU incorporation. The *KPNB1* data showed a clear lack of change in pol II occupancy along the gene following 1-NM-PP1 treatment, which was then repeated in the studies on individual genes connected to differentiation, *CD44* and *KLF4*. The two genes were chosen due to their dysregulation in DOT1L-KO mESCs, which were left in normal or differentiating media (Michowski et al., 2020), and due to the fact that both of them could be found in HeLa cells. In retrospect, since both *CD44* and *KLF4* were not classified as primary CDK1 targets in the inhibition approach used in the mESCs, it could have been useful to conduct the ChIP on some additional genes that were direct targets of CDK1 in those cells. That being said, the subsequent RNA-seq experiment showed that many of the genes found upregulated following CDK1 inhibition in mESCs did not correspond to the genes upregulated in

HeLa AS-CDK1 after 1-NM-PP1 treatment. Having obtained the data from the RNA-seq, it seems clear that finding any genes with significant changes in pol II occupancy by checking individual candidates would have been a highly unlikely achievement.

The additional ChIPs of S5p and CDK1 were conducted to scan for further possible clues of CDK1 regulation of transcription, however, with no changes in pol II levels it was fairly improbable changes would be seen for any of these two proteins. Despite this, little additional work was needed during the experiment to add these additional controls, allowing for the supplementing the pol II data with these further results. As stated before, in *S. cerevisiae* CDK1 homologue Cdc28 can actually phosphorylate the CTD on S5 (Enserink & Chymkowitch, 2022; Kõivomägi et al., 2021), and there is speculation about CDK1 being able to phosphorylate the CTD in mammals, given that the murine CDK1 was the first CDK shown to do so in *in vitro* (Chymkowitch & Enserink, 2013; Cisek & Corden, 1989; Pines, 1995). Unfortunately, with no dysregulated genes identified during the ChIPs, it was difficult to ascertain whether the transcriptional control exerted by CDK1 could be achieved through phosphorylating the CTD, or if it can act directly on the chromatin.

In order to generate a more detailed picture of what kind of genes were regulated by CDK1 in HeLa AS-CDK1 cells, it was decided to try the next generation sequencing approach. RNA-seq was selected as the method of choice, as it would allow to see practical changes in transcript production, with the molecular mechanism behind it able to be delineated once dysregulated genes were identified. Additionally, the RNA purification protocol was well established for HeLa cells. As mentioned before, focus was placed on more nascent transcripts by performing the RNA-seq on chromatin-associated RNA purified from fractionated cells (chrRNA-seq). This allowed to look for more subtle

shifts in transcription, as it was speculated that the short inhibition times of CDK1 would not result in any pronounced changes in the mature RNA composition of the cells. In the library preparation step, outside of rRNA depletion, poly(A)+ RNA was also removed to further highlight changes in the nascent transcripts. Although there exist alternative methods of examining nascent transcription, for instance the NET-seq (Native Elongating Transcript sequencing), the chrRNA-seq approach was deemed the most feasible, owing to its simplicity and efficient RNA preparation (Nojima et al., 2015; Wissink et al., 2019).

Importantly, inhibition time of CDK1 was extended for the RNA-seq, from 0.5 to 2h. Although this risked more cell cycle-related genes being hit, it was decided that the slightly longer inhibition would give more time for any changes caused by the lack of CDK1 activity to come into view. With the inconclusive results from the ChIP experiments, it was clear that the transcriptional changes caused by CDK1 could be rather subtle, and that perhaps more time is needed for them to transpire. Additionally, cell-cycle targets would be fairly easily distinguishable during the subsequent analysis of the RNA-seq data, and any identified genes of interest could be then checked again with shorter inhibition times to confirm how direct their regulation by CDK1 is.

However, despite the extended 2h inhibition, the RNA-seq showed very limited changes between samples treated with DMSO or 1-NM-PP1. As could be seen in the heatmap (**Figure 4.5.**), the differences between samples were minimal. Although this was a welcome result for repeats of the same condition, as it increased confidence in the experimental technique, the repeats between conditions also remained incredibly similar. The discovery of only 2 significantly dysregulated genes was also rather disappointing, leading to the additional search for the “borderline” CDK1-controlled genes, identified by decreasing the significance threshold to $p < 0.1$. The data were also compared with and

without the *Drosophila* spike-in to see clearer relative changes in gene expression. Although the spike-in is needed to account for any global changes in total RNA level, the data from the experiment showed very little fluctuations in the starting material. When analysed without the spike-in, the RNA-seq still showed only two significantly-dysregulated genes (*PER1* and *TXNIP* – as before), however, this time more genes were classified as borderline ($p < 0.1$). The utility of the spike-in still meant that the subsequent analysis was conducted on the data with spike-in rather than without.

Interestingly, in studies of the transcriptional roles of Cdc28 in *S. cerevisiae*, the genes controlled by the kinase were only uncovered after multiple rounds of different next generation sequencing methods analysed without spike-in (personal communication with Prof Chymkowitch; Chymkowitch et al., 2012; Chymkowitch & Enserink, 2013). Although this could highlight the similarities between mammalian CDK1 and yeast Cdc28 even in the context of transcriptional regulation, it also suggests that any transcriptional roles of these kinases might be secondary to their mitotic functions. While CDK1 is non-redundant when it comes to cell cycle control, it is conceivable that its interphase transcriptional roles might overlap with those of other transcriptional kinases, which is why its short inhibition does not produce an obvious phenotype. On the other hand, it has been suggested that the lack of noticeable changes following the inhibition of CDK1 could be assigned to the adaptability and resilience of HeLa cells, which are more robust than many other available cell lines. While studying the consequences of inhibiting CDK1 activity in disturbed HeLa cells, for instance following UV irradiation, could result in more transcriptional differences before and after 1-NM-PP1 treatment, it is possible that the changes seen in such a set-up would be largely stress induced.

The issue with the low number of hits following CDK1 inhibition in the RNA-seq was the subsequent difficulty with uncovering any linking factors between the dysregulated genes. The clear upregulation of transcription for a subset of genes once again hinted at the potential role of CDK1 as a transcriptional repressor, however, the mechanism by which it achieves this repression still remained ambiguous. The borderline genes classified as dysregulated using a less-stringent cut-off point had no common motifs upstream of their transcription start site, leading to no clues on what kind of transcription factors might be involved in their regulation. Moreover, GSEA also did not show any common affected pathways between the whole subset of upregulated genes, regardless of the magnitude of their dysregulation. No non-protein coding genes were altered following the 1-NM-PP1 treatment, and the differential transcript usage analysis also suggested minimal effects of CDK1 inhibition on splicing machinery, ultimately providing very little hints as to how the lack of CDK1 activity resulted in upregulation of hundreds of genes. Although longer inhibition of CDK1 could likely produce more pronounced changes in the transcriptional landscape of HeLa AS-CDK1 cells, its inevitable effect on the cell cycle would diverge from the original aims of this study. Additionally, in mESCs, a 0.5h inhibition was used to detect CDK1 substrates, which led to changes in phosphorylation of 2228 peptides (Michowski et al., 2020), suggesting that depending on the system used, even a shorter inhibition can generate substantial changes in phenotype.

Despite these challenges, two genes stood out in the RNA-seq analysis as strongly affected by CDK1 inhibition. *PER1* and *TXNIP*, which were significantly dysregulated in all three repeats of the experiment, became therefore the primary focus of subsequent examinations. Unfortunately, transcription dysregulation of these two genes also occurs in unmutated HeLa cells treated with NM-PP1, indicating that the changes seen are due to an off-target effect of the drug.

It is therefore unsurprising with hindsight that the analysis trying to uncover linking factors of the genes dysregulated during the RNA-seq experiment did not result in any significant hints regarding what was supposed to be a CDK1-intermediate, but could now be the unknown 1-NM-PP1 target or its downstream effector. No common motifs nor modes of regulation were found, making it difficult to theorise on the possible identity of the substrate. Notably, the human kinome comprises of over 500 members, and current literature focuses on a relatively small fraction of them, with 50% of kinases still largely uncharacterised and over 100 with completely unknown function (Burkard & Jallepalli, 2010; Fedorov et al., 2010). Further, the off-target effects of PP1-derived inhibitors are also starting to be uncovered, with different studies listing several possible wild-type kinases that can be affected by treatment with 1-NM-PP1 or its close relatives (Bain et al., 2007; C. Zhang et al., 2013). 1-NM-PP1 was found to have similar affinity to the same several wild-type kinases as its parental molecule, PP1, and it inhibited targets such as RIP2, GAK, CK1, p38 α / β MAPK, Src, Lck, Csk, or PKA (Bain et al., 2007). All 3 PP1-derived inhibitors targeted protein kinase D (PKD) as well, which is believed to have a lysine side chain (K667) allowing for greater flexibility in the inhibitor binding orientation (C. Zhang et al., 2013). The study by Zhang et al. suggested that the lipophilicity of small molecule inhibitors is what makes them more likely to bind to hydrophobic ligand-accepting sites of unmutated kinases, and that their poor solubility might lead to the formation of aggregates that could also target some of the kinases (McGovern et al., 2003; C. Zhang et al., 2013). However, they argue that the high IC₅₀ values of the AS inhibitors against AS kinases (<100nM) make it very unlikely for them to bind to wild-type targets at the concentrations usually used in experimental set-ups, and they suggest using inhibitor concentrations of less than 5 μ M to minimise such effects (C. Zhang et al., 2013). Importantly, it was found that PP1-derived inhibitors are able to

bind to CK1 δ and CK1 ϵ , molecules involved in the circadian rhythm, which phosphorylate PER1 and allow it for nuclear entry (Bain et al., 2007; H. M. Lee et al., 2011; Richards et al., 2012; C. Zhang et al., 2013). There are fewer hints, however, on how 1-NM-PP1 could affect the expression of *TXNIP*.

It is worth noting that it is not uncommon not to include wild-type controls in analogue-sensitive kinase studies, and many of the investigations cited in this text did not check the effect of treating wild-type cells with ATP analogues following the initial results that validated the AS-CDK1 cells. The principle behind the AS system should make such checks unnecessary, however, the data presented here highlight that this is not always the case. Unfortunately, in this study the results were tested against the wild-type controls only after the RNA-seq, as the previous negative data did not necessitate a negative control. It is also possible that the short inhibition of CDK1, with any differences in transcription potentially compensated by other factors, have caused the off-target effects of 1-NM-PP1 to take centre stage, which was not the case during investigations with high CDK1 dependence or with longer inhibition times.

With the possibility of the off-target effects of 1-NM-PP1, it is crucial to remember that the initial validation of the AS methodology (described in **Chapter 3.**) showed a clear abolition of CDK1 activity following the administration of 1-NM-PP1 in HeLa AS-CDK1 cells, but not in HeLa cells. It could be therefore possible that apart from the unidentified substrate acting on, for instance *PER1* and *TXNIP*, some of the transcriptional dysregulation seen in the EU staining or the RNA-seq could still be due to CDK1 inhibition. Additionally, it is unclear whether the 1-NM-PP1 off-target actions are particularly potent in the HeLa cells specifically, or whether they would affect other cell lines as well, since the yeast and mESCs studies also noted *TXNIP* and its homologues to

be dysregulated following treatment with an ATP-analogue. Notably, mESCs were inhibited with an alternative PP1 derivative, 3-MB-PP1, raising the question of whether the off-target effects seen in this study were 1-NM-PP1-specific, or whether it could be a problem with the family of PP1-derived drugs in general.

When viewed together, the data collected in this chapter has shown the potential of CDK1 to act as a transcriptional repressor in HeLa AS-CDK1 cells. However, more evidence is needed to determine specifically to what extent the transcriptional changes visible following CDK1 inhibition are caused by the lack of the activity of the kinase, and how much of what was observed was influenced by the unidentified target, or targets, of 1-NM-PP1. The next chapter details experiments designed to address these questions, which investigate the actions of additional CDK1 inhibitors and the effect of 1-NM-PP1 on the new AS-CDK1 cell lines created during this study.

5. TRANSCRIPTIONAL EFFECTS OF COMMON CDK1 INHIBITORS IN WILD-TYPE AND AS-CDK1 CELL LINES

5.1. Background

No CDK1 inhibitors have yet reached the clinic, in part due to their lack of specificity, and basic research into CDK1 functions is also complicated as small-molecule inhibitors usually target other kinases. One approach to countering this lack of precision is the analogue-sensitive approach (Bishop et al., 2000, 2001; Blethrow et al., 2008; Holt et al., 2010; Ubersax et al., 2003). The use of bulky ATP-analogues has helped elucidate the functions of multiple kinases, but very few studies have explored how these molecules affect unmutated cells. This project has revealed unanticipated off-target effects of 1-NM-PP1 (**Chapter 5**). Despite this, 1-NM-PP1 was shown to inhibit CDK1, leaving open the possibility that some of the dysregulation seen in 1-NM-PP1-treated HeLa AS-CDK1 cells was caused by reduced CDK1 activity.

While the mitotic role of CDK1 is considered to be universal across eukaryotes, additional functions of this kinase appear context-dependent. Inhibiting such supplementary CDK1 roles, while the universal mitotic functions of CDK1 remain intact, could help target specific types of cells, for instance during cancer therapy. However, the lack of reliable systems for investigating non-mitotic functions of CDK1 has made it challenging to use the same experimental methods across multiple cell lines or organisms. In this part of the project, different AS-CDK1 cell lines and inhibitors were used to elucidate how the method and model of CDK1 inhibition could influence differences seen in the transcriptional landscape. Importantly, it was essential to establish to what degree the off-target effects of 1-NM-PP1 complicate the interpretation of CDK1 inhibition. Using comparisons between different cell types and inhibitors, it was crucial to determine whether the influence of CDK1 on transcription in cancer cells could be isolated and characterised.

5.2. Effects of 1-NM-PP1 treatment on chromatin RNA levels in HCT116 CDK1^{WT/AS} and HEK293 AS-CDK1

With 1-NM-PP1 treatment affecting the transcription of at least some genes in both HeLa and HeLa AS-CDK1 cell lines, it was important to establish whether the drug could affect other unmodified cell lines. During the course of this investigation, two new cell lines have been developed: HCT116 CDK1^{WT/AS} and HEK293 AS-CDK1. The use of both may help indicate to what extent the off-target effects of 1-NM-PP1 affect other cell types.

Four cell lines: HCT116 and HCT116 CDK1^{WT/AS}, as well as HEK293 and HEK293 AS-CDK1, were all treated for 2h with 7.5μM 1-NM-PP1. Their chromatin-associated RNA was purified, reverse-transcribed into cDNA and qRT-PCR was performed using primer pairs for *PER1* and *TXNIP* genes, the only genes in HeLa AS-CDK1 cells significantly dysregulated following 1-NM-PP1 treatment (Section *).

Following treatment of HCT116 CDK1^{WT/AS} with 1-NM-PP1, no significant changes occurred in the levels of chromatin-associated *PER1* or *TXNIP* RNA (**Figure 5.1.**). Similarly, no significant differences were noted for the parental HCT116, for HEK293 or HEK293 AS-CDK1 cells treated with DMSO or 1-NM-PP1.

Taken together, these results further indicated that the changes in *PER1* and *TXNIP* chrRNA levels seen in HeLa and HeLa AS-CDK1 cells were most probably caused by an off-target effect of 1-NM-PP1 rather than the inhibition of CDK1. The lack of any significant differences in *PER1* and *TXNIP* expression in all four of the other cell lines following treatment with 1-NM-PP1 suggested that the off-target effect was confined to the HeLa and HeLa AS-CDK1 cell lines.

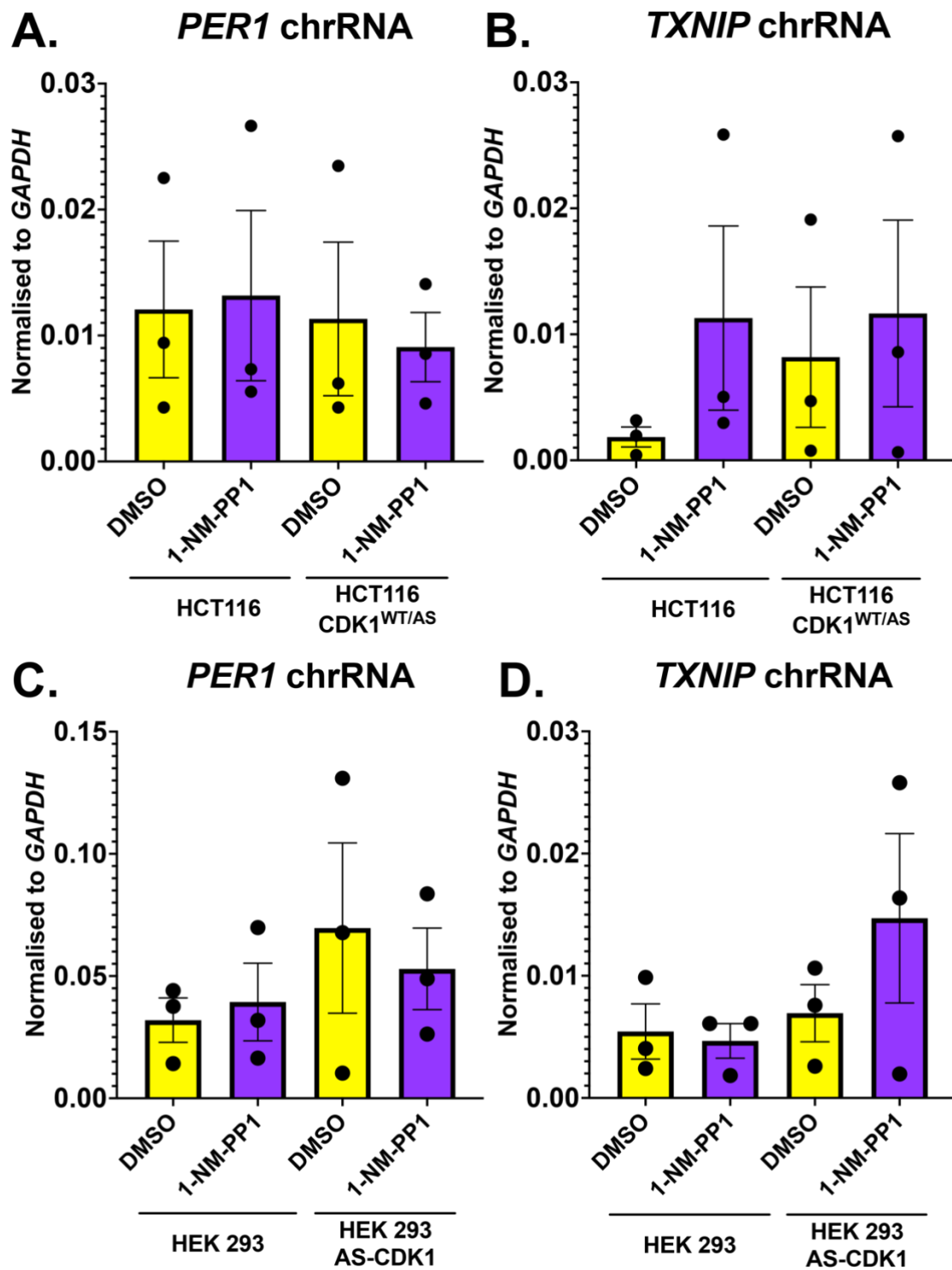


Figure 5.1. Effects of 1-NM-PP1 treatment on chromatin RNA levels of *PER1* and *TXNIP* in HCT116 CDK1^{WT/AS} and HEK293 AS-CDK1. HCT116, HCT116 CDK1^{WT/AS}, HEK293 and HEK293 AS-CDK1 cells were treated with 7.5μM 1-NM-PP1 for 2h, after which their chromatin-associated RNA (chrRNA) was purified, reverse-transcribed, and quantified using qRT-PCR. The levels of chrRNA after the treatment were determined for **A. *PER1*** and **B. *TXNIP*** in HCT116/ HCT116 CDK1^{WT/AS} cells, as well as **C. *PER1***, and **D. *TXNIP*** in HEK293/HEK293 AS-CDK1 cells. Values were normalised to the levels of *GAPDH* chrRNA in the respective cell lines (p>0.05; ANOVA test; n=3). Error bars show SEM.

5.3. The effects of other CDK1 inhibitors in wild-type and AS-CDK1 cell lines

Having established that 1-NM-PP1 had off-target effects in HeLa cells, it was important to investigate whether these could be avoided using alternative means to inhibit CDK1. 1-NM-PP1 belongs to a range of analogue-sensitive kinase inhibitors, and is one of three widely-used PP1-derived compounds alongside 1-NA-PP1 and 3-MB-PP1 (Bain et al., 2007; Lopez et al., 2014; C. Zhang et al., 2013). Additionally, various small-molecule CDK inhibitors have been developed against individual members of the family, though there are considerable doubts about their specificity (Łukasik et al., 2021; Mughal et al., 2023; Q. Wang et al., 2023; M. Zhang et al., 2021). The comparison between the effects of different CDK1-targeting drugs on HeLa and HeLa AS-CDK1 and the dysregulation of *PER1* and *TXNIP* could further clarify the presumptive off-target effects of 1-NM-PP1 on these genes in previous experiments.

5.3.1. 3-MB-PP1 treatment does not affect the levels of *PER1* and *TXNIP* chrRNA in HeLa cells

1-NM-PP1 is a widely-used PP1-derived inhibitor of analogue-sensitive kinases, and nearly all of the aforementioned AS-CDK1 studies in human or yeast cells were conducted using this drug (Blethrow et al., 2008; Hochegger et al., 2007; Ubersax et al., 2003). One exception was the investigation showing that AS-CDK1 in mESCs could be inhibited by both 1-NM-PP1 and 3-MB-PP1, before proceeding to use 3-MB-PP1 for the remainder of the experiments (Michowski et al., 2020). The impact of 3-MB-PP1 on the HeLa AS-CDK1 cell line was therefore examined.

Firstly, to check for specific inhibition of CDK1, both HeLa and HeLa AS-CDK1 cells were incubated in DMSO or in increasing concentrations of 3-MB-PP1 for 2h. Whole-cell protein extracts were run on Western blots; membranes were probed with antibodies against nucleolin and H3 as controls, and against phosphorylated H3S10 to monitor inhibition of mitosis.

Following the addition of 3-MB-PP1 even at the lowest concentration, the level of H3S10p markedly decreased in HeLa AS-CDK1 cells (**Figure 5.2.A.**), suggesting inhibition of mitotic entry. The levels of H3S10p in unmodified HeLa cells were not affected at any concentration of 3-MB-PP1. These results reflect what was seen in HeLa and HeLa AS-CDK1 cells treated with 1-NM-PP1, and imply that 3-MB-PP1 can also selectively inhibit AS-CDK1 in HeLa AS-CDK1 cells, while not affecting CDK1 in unmodified HeLa cells.

Having this confirmation, the level of *PER1* and *TXNIP* chrRNA following 3-MB-PP1 treatment were checked. HeLa and HeLa AS-CDK1 cells were incubated for 2h with DMSO or 1 μ M 3-MB-PP1, after which chrRNA was extracted. The RNA was reverse-transcribed and amplified using qRT-PCR with primer pairs corresponding to the genes of interest.

In contrast to the incubation with 1-NM-PP1, the treatment of HeLa AS-CDK1 cells with 3-MB-PP1 for 2h did not cause any significant change in the chrRNA level of either *PER1* or *TXNIP* (**Figure 5.2.B.-C.**). Correspondingly, the expression of the two genes did not change for unmodified HeLa cells. These data suggest that 3-MB-PP1 is more specific than 1-NM-PP1 in its inhibition of AS-CDK1 and support the conclusion that the altered expression of *PER1* and *TXNIP* following 1-NM-PP1 treatment was due to off-target effects.

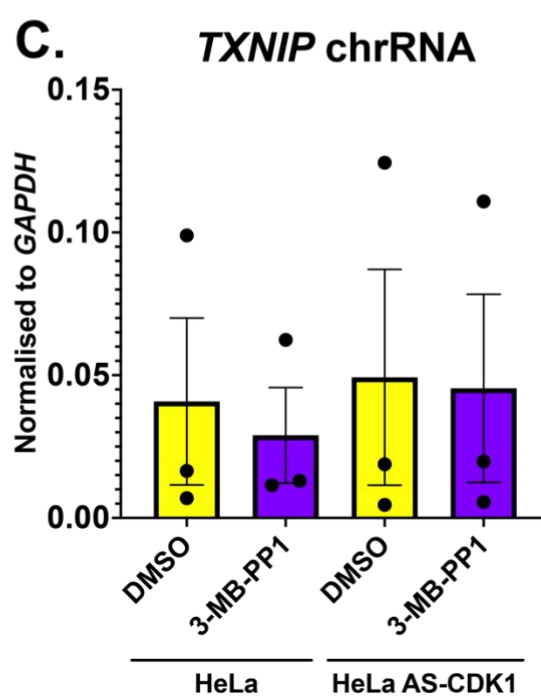
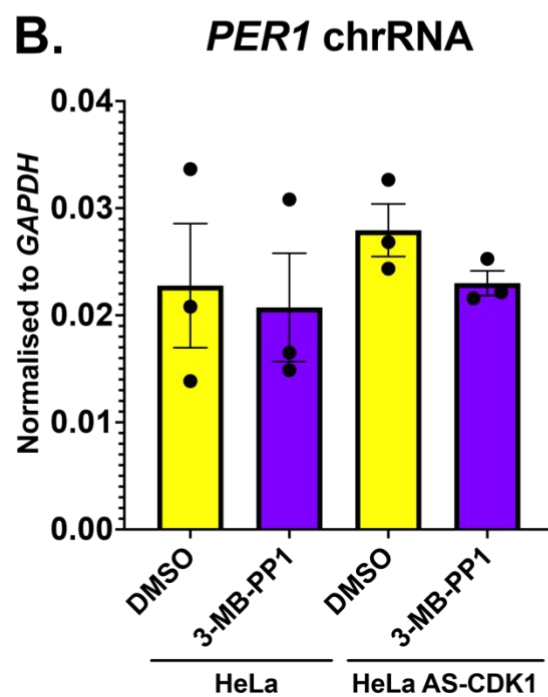
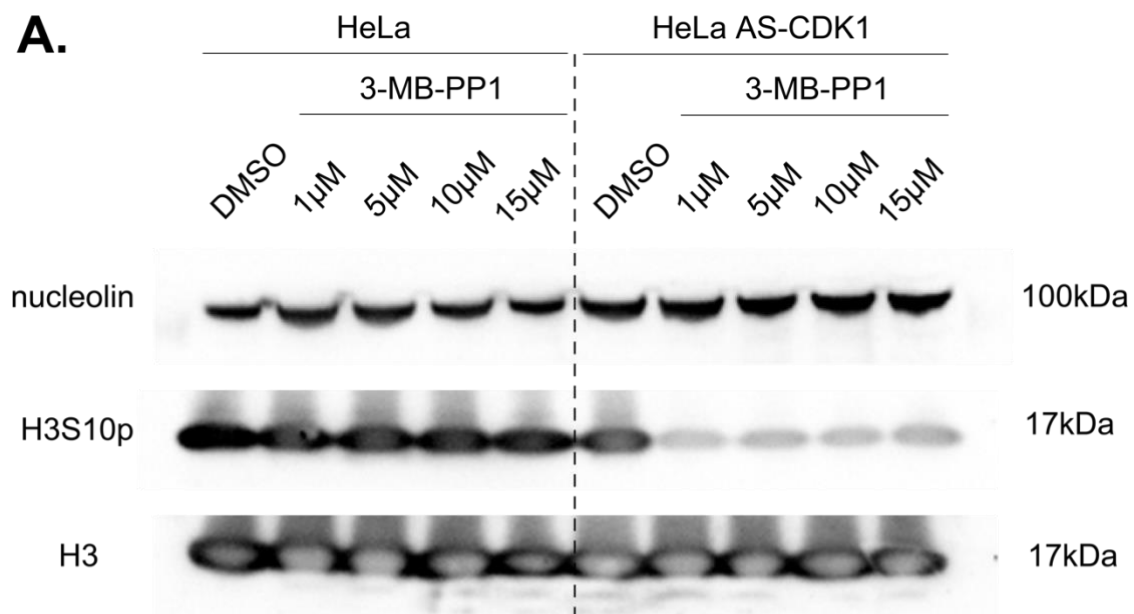


Figure 5.2. 3-MB-PP1 treatment does not affect the levels of *PER1* and *TXNIP* chrRNA. **A.** Western blots were prepared on whole cell extracts of HeLa and HeLa AS-CDK1 cells after 2h inhibition with 3-MB-PP1. The blots were probed against nucleolin as a loading control, H3, and H3 phosphorylated on serine 10 residue (H3S10p). **B-C.** HeLa and HeLa AS-CDK1 cells were treated with 1 μ M 3-MB-PP1 for 2h, after which chromatin-associated RNA (chrRNA) was purified, reverse-transcribed, and quantified using qRT-PCR. The levels of chrRNA after the treatment were determined for **B. *PER1*** and **C. *TXNIP***. Values were normalised to the levels of *GAPDH* chrRNA ($p>0.05$; ANOVA test; $n=3$). Error bars show SEM.

5.3.2. Inhibition of CDK1 with RO-3306 has opposite effects of AS-CDK1 inhibition with 1-NM-PP1

Truly specific small-molecule CDK1 inhibitors are currently lacking. Although many pan-CDK inhibitors, such as Flavopiridol, act also to inhibit CDK1, the wide range of their CDK targets makes them unsuitable for studying CDK1 function individually – for example, Flavopiridol inhibits CDK1, CDK2, CDK4, CDK6, CDK7, and CDK9. To date, the most widely-used comparatively CDK1-specific inhibitor is RO-3306, which competes with ATP for binding to CDK1 (Schwermer et al., 2015; Vassilev et al., 2006; C. X. Wu et al., 2018). Despite this, RO-3306 is also fairly potent against the closely-related CDK2. Overall, the activity of RO-3306 against CDK1/cyclin B1, CDK1/cyclin A, CDK2/cyclin E, and CDK4/cyclin D showed inhibition at K_i of 35nM, 110nM, 340nM, and over 2000nM, respectively (Vassilev et al., 2006). There exist other CDK1 inhibitors such as CGP-74514A, which like RO-3306 is currently undergoing pre-clinical trials (Garcia-Sampedro et al., 2021; Larsson et al., 2009; Sojung et al., 2014; Q. Wang et al., 2023).

Because of the lack of specificity of small molecule CDK inhibitors, the analogue-sensitive approach is regarded as the more precise alternative when examining this family of kinases. However, with the RNA-seq data in this project pointing at the off-target effects of the ATP-analogue drug in HeLa cells, its accuracy was also brought in question. To establish a more complete overview of the actions of 1-NM-PP1 as a CDK1 inhibitor, additional experiments were undertaken to compare the effects of 1-NM-PP1 with those of RO-3306.

Initially, western blots were performed to check for the inhibition of CDK1 using RO-3306. Different concentrations of the drug were used to treat HeLa cells, and afterwards whole-cell protein extracts were taken of the samples. 1-NM-PP1-treated HeLa and HeLa

AS-CDK1 samples were included as additional controls. Western blots were run and probed with antibodies against nucleolin and H3, as well as the mitotic mark H3S10p.

The addition of 1-NM-PP1 to HeLa and HeLa AS-CDK1 cells caused the decrease of H3S10 phosphorylation in the latter but not the former, as described previously (**Figures 3.2.** and **5.3.**). The incubation with RO-3306 caused a block of mitotic entry in the HeLa cells, as judged by the marked decrease in H3S10p levels (**Figure 5.3.**). The use of 10 μ M concentration appeared to partly alleviate the block. The Western blots supported the targeting of CDK1 by RO-3306, and although the block seemed less complete for 10 μ M, this concentration was used for subsequent experiments in this project. The original RO-3306 study used 9 μ M of the drug to inhibit CDK1, while the mESCs investigation used 10 μ M for their checks of non-AS cells, so it was decided that the use of 10 μ M of RO-3306 in this part of the investigation would allow to keep a better reference point to previous literature (Michowski et al., 2020; Vassilev et al., 2006).

For comparison, HeLa and HeLa AS-CDK1 cells were incubated for 2h with 7.5 μ M 1-NM-PP1, and additional HeLa samples were treated with 10 μ M of RO-3306. Following the incubation, the chrRNA from the cells was purified, reverse-transcribed, and amplified with qRT-PCR. For analysis, primer pairs for *PER1* and *TXNIP* were used, as well as *ETS2* and *PDK4*, the borderline genes that were slightly upregulated in previous experiments (**Figures 4.4.**, **4.11.** and **4.14.**; Table **4.1.**).

Inhibition of CDK1 with RO-3306 in HeLa cells gave very different results to the treatment of HeLa AS-CDK1 cells with 1-NM-PP1 (**Figure 5.4.**). Whereas 2h treatment of cells with 1-NM-PP1 led to increased transcription of *PER1*, inhibition with RO-3306 caused no changes in the levels of *PER1* chrRNA. For *PDK4*, a significant upregulation of expression was observed after inhibition with RO-3306, and no change was recorded for *ETS2*. The biggest differences were seen for *TXNIP* chrRNA, which while decreased

in both HeLa and HeLa AS-CDK1 cells following the 1-NM-PP1 inhibition, showed a large and significant upregulation when HeLa cells were treated with RO-3306. This could imply that CDK1 does in fact execute some level of control over *TXNIP*, but in contrast to what the previous 1-NM-PP1 data suggested, CDK1 seems to repress *TXNIP* expression, rather than activate it. Collectively, this part of the investigation managed to highlight the contrasting results caused by the use of distinct CDK1 inhibitors. The difference between 1-NM-PP1 and RO-3306 underlines that the *PER1* and *TXNIP* dysregulation observed during the RNA-seq was most probably caused by off-target effects of 1-NM-PP1, if it is assumed that RO-3306 is mono-specific for CDK1. However, given its ability to inhibit CDK2 as well, the conflicting results could still be possibly attributed to the latter.

The effects of RO-3306 on the new AS-CDK1 cell lines, HCT116 CDK1^{WT/AS} and HEK293 AS-CDK1, were then investigated. As before, the comparison of 1-NM-PP1 effects with those of RO-3306 could help indicate to what extent the choice of the drug affects the results seen after CDK1 inhibition. Additionally, if CDK1 acts similarly in all three cell lines, the RO-3306 results from HCT116 and HEK293 should reflect those seen in HeLa. Otherwise, the comparison could give greater indication of differences in CDK1 roles between cancerous and non-cancerous cells.

The final part of RO-3306 tests involved the examination of how it affects the new AS-CDK1 cell lines, HCT116 CDK1^{WT/AS} and HEK293 AS-CDK1. As before, the comparison of 1-NM-PP1 results with those from RO-3306 could help indicate to what extent the choice of the drug affects the results seen after CDK1 inhibition. Additionally, assuming CDK1 acts similarly in all 3 types of cell lines, the RO-3306 results from HCT116 and HEK293 should reflect those seen in HeLa. Otherwise, the comparison

could give greater indication of differences in CDK1 roles between cancerous and non-cancerous cells.

To obtain chrRNA, HCT116 and HCT116 CDK1^{WT/AS}, HEK293 and HEK293 AS-CDK1 cells were treated for 2h with 7.5μM 1-NM-PP1 or 10μM RO-3306, before being fractionated. Their chromatin-associated RNA was purified and reverse-transcribed into cDNA. Finally, qRT-PCR was performed with primer pairs for *PER1* and *TXNIP*.

In contrast to what was seen in the HeLa cell lines, RO-3306 treatment of none of the additional cell lines, HCT116, HCT116 CDK1^{WT/AS}, HEK293 and HEK293 AS-CDK1, resulted in any significant changes in chrRNA levels of either *PER1* or *TXNIP* (**Figure 5.5.**). Although not entirely surprising for *PER1*, as this gene was also not significantly dysregulated in HeLa cells treated with the inhibitor, the lack of *TXNIP* dysregulation in RO-3306-treated HCT116 and HEK293 cells compared to HeLa could suggest that in the latter CDK1 has acquired some level of repressive control over this gene. Additionally, the tests confirmed that the results of CDK1 inhibition can vary considerably depending on both the inhibitor and the cell line being used in the investigation.

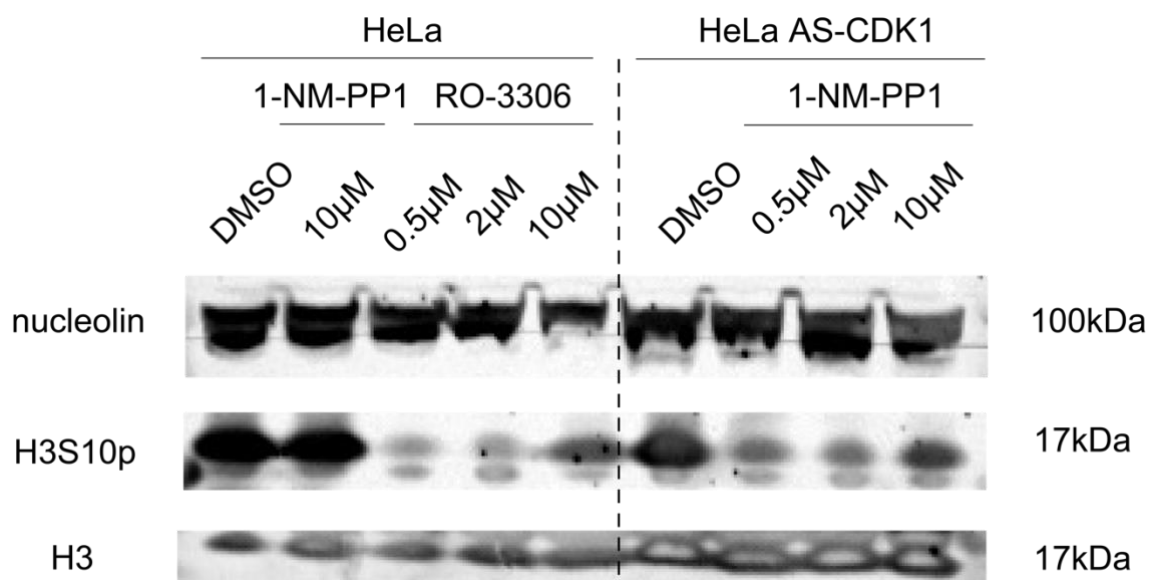


Figure 5.3. CDK1 can be selectively inhibited by RO-3306 in HeLa cells. Western blots were prepared on whole cell extracts of HeLa and HeLa AS-CDK1 cells after 2h inhibition with RO-3306 or 1-NM-PP1. The blots were probed with antibodies against nucleolin as a loading control, H3, and H3 phosphorylated on serine 10 residue (H3S10p), which acted as a mitotic mark.

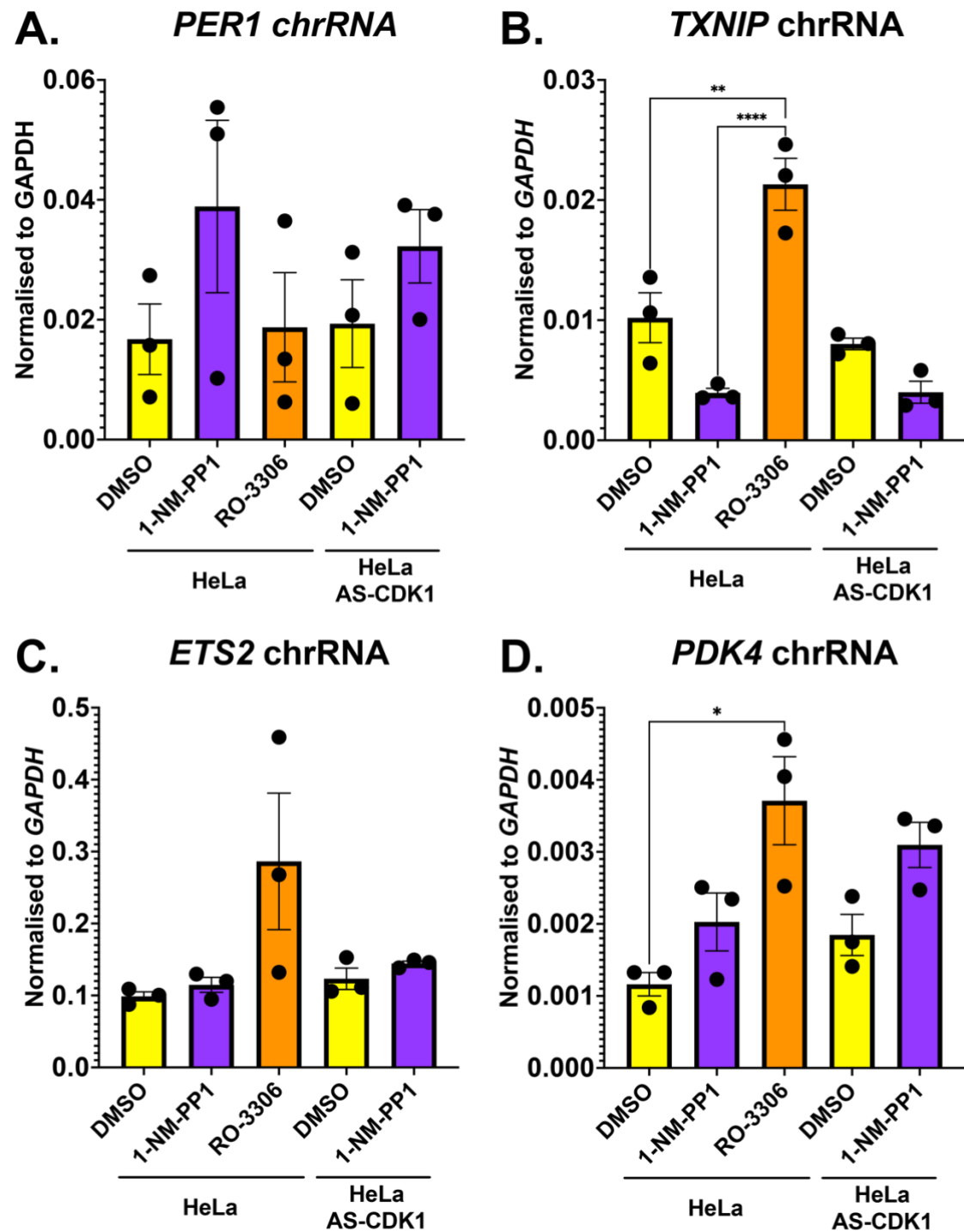
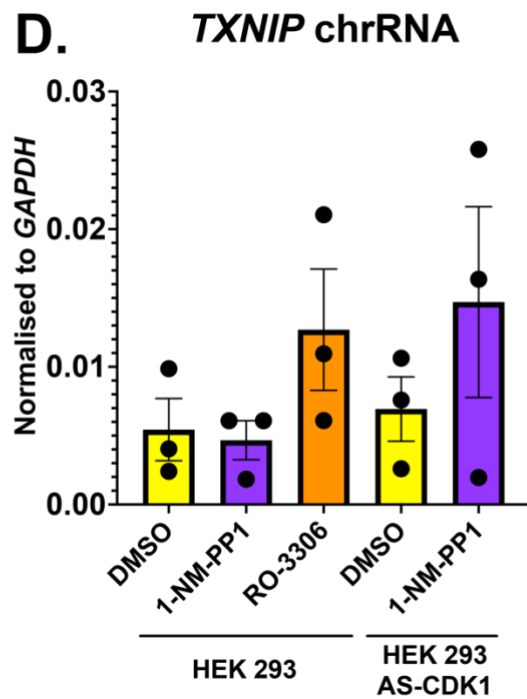
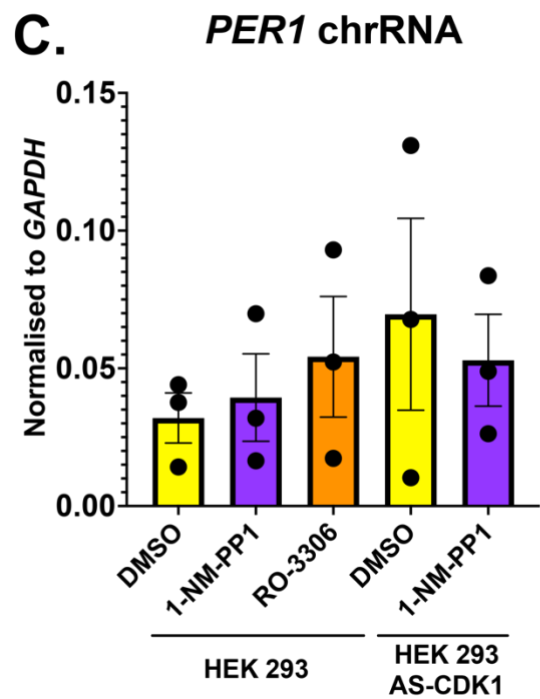
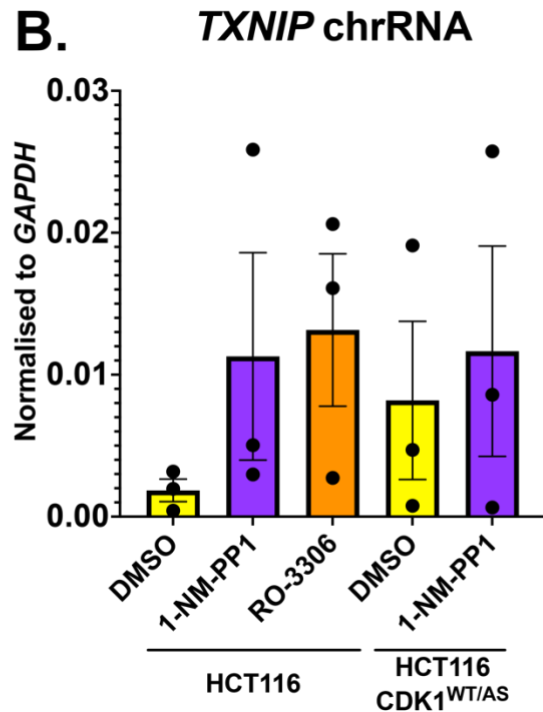
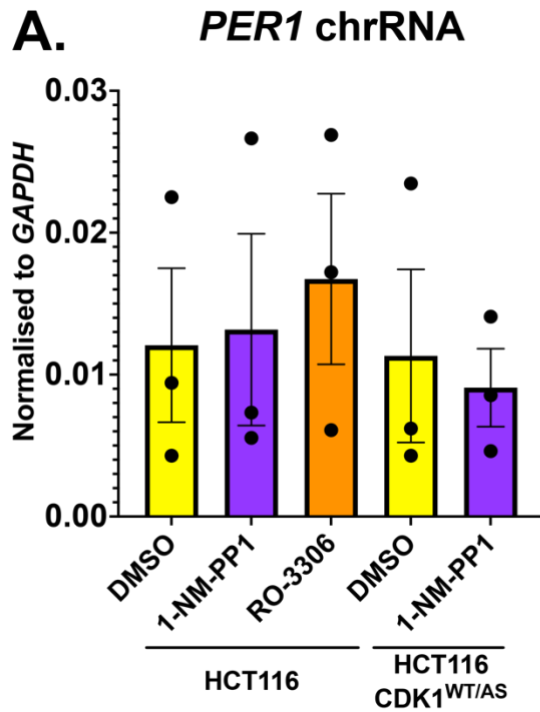


Figure 5.4. Inhibition of CDK1 in HeLa cells with RO-3306 has opposite effects to AS-CDK1 inhibition with 1-NM-PP1. HeLa and HeLa AS-CDK1 cells were treated with 7.5 μ M 1-NM-PP1 or 10 μ M RO-3306 for 2h, after which their chromatin-associated RNA (chrRNA) was purified, reverse-transcribed, and quantified using qRT-PCR. The

levels of chrRNA after the treatment were determined for **A. *PER1***, **B. *TXNIP***, **C. *ETS2***, and **D. *PDK4*** genes. Values were normalised to the levels of *GAPDH* chrRNA (**** $p < 0.0001$, ** $p < 0.01$, * $p < 0.05$; ANOVA test; $n=3$). Error bars show SEM.

Figure 5.5. Effects of RO-3306 treatment on chromatin RNA levels of *PER1* and *TXNIP* in HCT116 and HEK293 cell lines. HCT116, HCT116 CDK1^{WT/AS}, HEK293 and HEK293 AS-CDK1 cells were treated with 7.5 μ M 1-NM-PP1 or 10 μ M RO-3306 for 2h, after which their chromatin-associated RNA (chrRNA) was purified, reverse-transcribed, and quantified using qRT-PCR. The levels of chrRNA after the treatment were determined for **A. *PER1*** and **B. *TXNIP*** in HCT116/ HCT116 CDK1^{WT/AS} cells, as well as **C. *PER1***, and **D. *TXNIP*** in HEK293/HEK293 AS-CDK1 cells. Values were normalised to the levels of *GAPDH* chrRNA in the respective cell lines ($p > 0.05$; ANOVA test; $n=3$). Error bars show SEM.



5.4. The level of global RNA transcription decreases following CDK1 inhibition in HEK293 AS-CDK1

Previously, 1-NM-PP1 treatment of HeLa AS-CDK1 cells was shown to cause a marked upregulation of a subset of genes, with two of them, *PER1* and *TXNIP*, being significantly dysregulated (sections 4.5.1., 4.6.). Although these were ultimately found to be off-target effects of the drug, it remained unclear whether the widescale upregulation of transcription seen in HeLa AS-CDK1 was similarly attributable to off-target effects of 1-NM-PP1, or whether the shift was a genuine effect of CDK1 inhibition. This hypothesis was not without foundation, as it had already been shown that CDK1 can act as a transcriptional repressor (Michowski et al., 2020).

To clarify whether CDK1 was responsible for transcriptional repression in HeLa AS-CDK1 cells, the effects of 1-NM-PP1 treatment on global transcription in unmodified HeLa cells were examined using EU staining. In HEK293 AS-CDK1 cells 1-NM-PP1 also inhibited CDK1, but did not change *PER1* and *TXNIP* expression as it did in HeLa cells (Figure 3.10., Figure 5.1.C.-D.). The HEK293 AS-CDK1 cell line was created during this study using CRISPR-Cas9, with AS-CDK1 being expressed from its endogenous promoter. As HEK293 AS-CDK1 may prove to be a reliable testing ground for the transcriptional roles of CDK1, HEK293 samples were also added to the EU-staining experiment to see if inhibition of CDK1 in these cells would result in the same upregulation of transcription seen in HeLa AS-CDK1. Parental HEK293 cells were also included in the EU staining as a reference point for possible changes observed in HEK293 AS-CDK1 cells. Finally, 3-MB-PP1-treated samples were added to the investigation to see how the two AS-kinase inhibitors compare to each other.

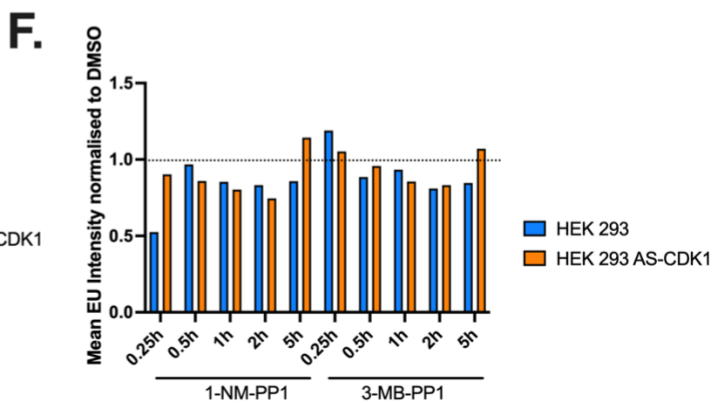
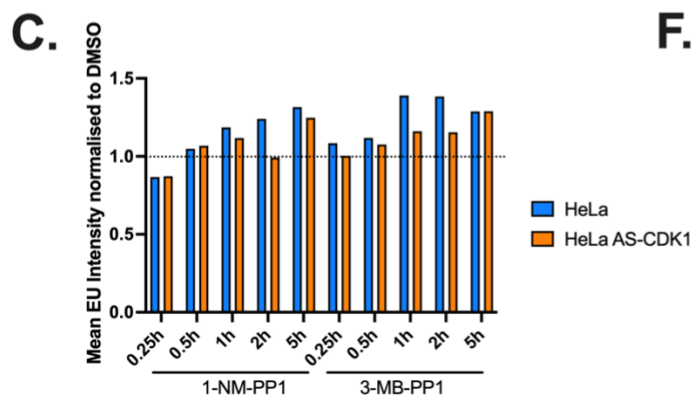
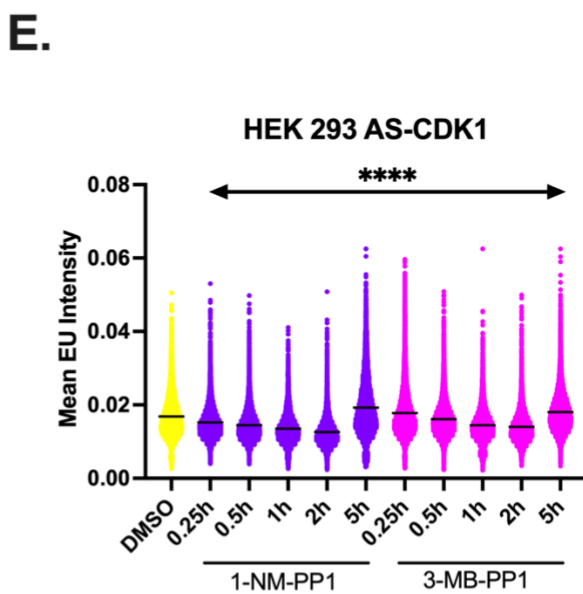
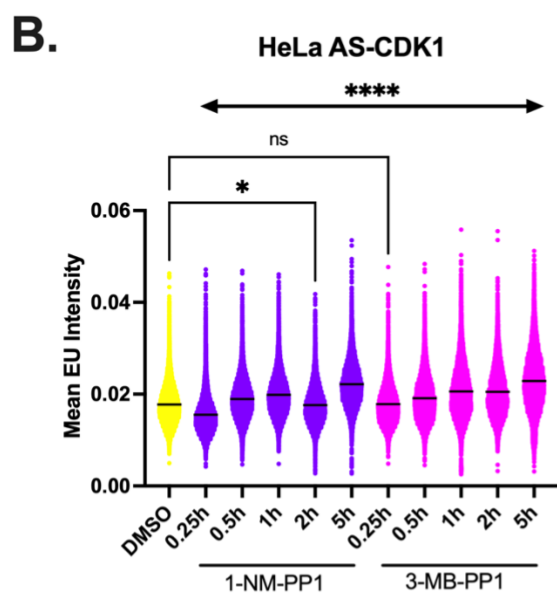
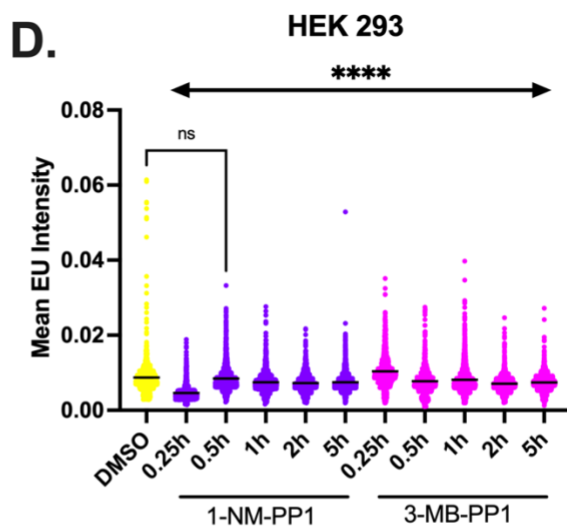
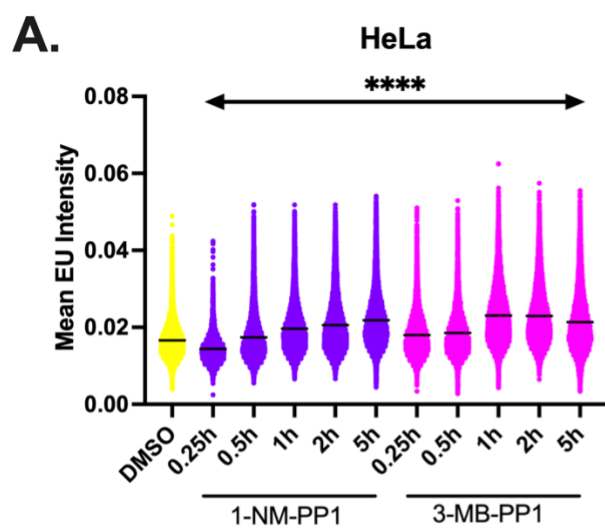
The cell lines of interest, HeLa, HeLa AS-CDK1, HEK293 and HEK293 AS-CDK1, were seeded on coverslips and incubated with 7.5 μ M 1-NM-PP1 or 1 μ M 3-MB-PP1 the next

day. 1mM EU 1mM was added to the cells 30 min before the end of the incubation. The cells were fixed and stained with Hoechst 33342 and Alexa Fluor 488, which recognise DNA and EU incorporated into RNA, respectively. The samples were imaged on a fluorescence microscope, with the DAPI channel used to create outlines of cell nuclei, and the GFP channel used to measure the mean fluorescence intensity of Alexa Fluor 488 in each nucleus. Lastly, the data were pooled to establish the levels of nascent RNA production in each sample. It is important to note that, so far, the experiment has been performed only once and additional repetitions will be completed in the future.

The EU-staining data indicated a couple of important trends in the global levels of transcription of inhibited cells, as seen on **Figure 5.6**. Firstly, the HeLa AS-CDK1 samples treated with 1-NM-PP1 confirmed the previously-observed trend of transcriptional upregulation, as the nascent RNA production in these cells increased. Incubation with 3-MB-PP1 also resulted in increased gene expression, with the strength of the upregulation corresponding to the length of the treatment. Surprisingly, the same behaviour was observed in HeLa cells. For these samples, inhibition with 1-NM-PP1 or 3-MB-PP1 also caused an upregulation of RNA production, which increased the longer the incubation was. The changes in RNA production in HEK293 cells did not follow the same pattern. On the contrary, incubation of HEK293 AS-CDK1 cells with 1-NM-PP1 or 3-MB-PP1 resulted in a decrease of global transcription, with an increasing effect with extending treatment times. The fluctuations in the RNA production of wild-type HEK293 cells treated either with 1-NM-PP1 or 3-MB-PP1 were also significant. It is worth noting that, when compared to DMSO, both pairs of cell lines actually followed mirrored patterns of transcriptional upregulation for HeLa and HeLa AS-CDK1, and a downregulation of global transcription for HEK293 and HEK293 AS-CDK1.

Altogether, the EU-staining results suggested that the global transcriptional changes seen previously in HeLa AS-CDK1 cells, and now also in HEK293 AS-CDK1 cells, could have been caused by the off-target actions of the ATP-analogues rather than precise CDK1 inhibition. The same changes were observed in their wild-type counterparts, which do not have a mutated CDK1 able to accept the analogue inhibitor in its ATP-binding pocket. Interestingly the two inhibitors caused opposite effects in HeLa and HEK293 cell lines, which perhaps may be due to differences in cancerous (HeLa) versus non-cancerous (HEK293) transcriptional phenotypes.

Figure 5.6. The level of global transcription in HeLa and HEK293 cells lines following 1-NM-PP1 or 3-MB-PP1 inhibition. HeLa, HeLa AS-CDK1, HEK293, and HEK293 AS-CDK1 cells were incubated with 7.5 μ M 1-NM-PP1 or 1 μ M of 3-MB-PP1. EU was administered 0.5h before the end of treatment to assess the level of nascent RNA production. The graphs show the quantification of the fluorescence intensity of the nuclei for A. HeLa, B. HeLa AS-CDK1, D. HEK293, E. HEK293 AS-CDK1; or the mean fluorescence intensity normalised to DMSO for C. HeLa cell lines and F. HEK293 cell lines. In graphs A., B., D., and E., DMSO served as the control for statistical tests (****p<0.0001, * p<0.05; Kruskal-Wallis test; n=1).



5.5. CDK1 inhibition upregulates the transcription of a subset of genes in HEK293 AS-CDK1 cells

The EU-staining data strongly suggested that the changes in bulk transcription in AS-CDK1 cells are caused by the off-target effects of the inhibitors rather than the inhibition of CDK1 itself. However, it is difficult to gauge whether a part of the observed dysregulation could still be attributed to CDK1 inhibition, particularly as EU staining measures RNA produced by pol II as well as pol I and III in the nucleoplasm. An additional RNA-seq experiment was therefore designed to perform a more thorough analysis of the effects of 1-NM-PP1 treatment on transcription of pol II-dependent genes in wild-type and AS-CDK1 cells. The aim of this experiment was to compare directly differential expression of protein-coding genes between unmodified and AS-CDK1 cells. Any differences could provide clues to which genes are targeted by CDK1 and which are deregulated due to the lack of specificity of 1-NM-PP1.

Due to its endogenously-mutated CDK1, HEK293 AS-CDK1 was chosen as the model of choice for this part of the project. Additionally, based on the lack of deregulation of *PER1* and *TXNIP* in wild-type HEK293 (**Figure 5.1.C.-D.**), it was possible that 1-NM-PP1 would not cause as many off-target effects in this cell line as in the HeLa and HeLa AS-CDK1 cells. The RNA-seq comparison between HeLa AS-CDK1 cells and HEK293 cells could also shed light on the contrasting downregulation of gene expression seen following EU staining of HEK293 cells.

Both HEK293 AS-CDK1 cells and unmodified HEK293 cells were used for the experiment, to control for off-target effects of 1-NM-PP1. Moreover, 0.5h inhibition time points were added to the treatment of both cell lines to capture the more direct changes

caused by CDK1 inhibition, and because previous data pointed to changes in effect between 0.5h and after 2h of inhibition (**section 4.6.**).

As before, the RNA-seq was performed on chromatin-associated RNA. The HEK293 and HEK293 AS-CDK1 cells were incubated with 7.5 μ M 1-NM-PP1 for 0.5h or 2h, after which they were fractionated and the chromatin-associated RNA was purified. During library preparation, greater focus was placed on nascent transcripts by discarding polyadenylated mRNA, and rRNA was also removed from the samples. The libraries were checked for quality using 4150 TapeStation System and sent for sequencing. The received data were handled by Dr Callum Henfrey, who performed the bioinformatical analysis.

5.5.1. Chromatin RNA-seq analysis: Differential expression of genes after 1-NM-PP1 treatment of HEK293 and HEK293 AS-CDK1 cells

Four MA plots were created to show the differential expression of genes in HEK293 and HEK293 AS-CDK1 cells following 0.5h and 2h inhibition with 1-NM-PP1 (**Figure 5.7.**). The horizontal axis shows the level of expression of indicated genes, with genes closer to the right-hand side being more highly-transcribed. The vertical axis shows the expression difference between DMSO and 1-NM-PP1-treated conditions, represented as a log ratio of control and experimental samples. The genes above the horizontal line were found to be upregulated following 1-NM-PP1 treatment, with two-fold change in either direction considered significant.

Minimal changes in gene expression were observed for both HEK293 and HEK293 AS-CDK1 after 0.5h treatment with 1-NM-PP1 (**Figure 5.7.A. and C.**). In HEK293 AS-CDK1 cells, the vast majority of genes showed little deregulation following treatment, and none was significantly down- or upregulated. The HEK293 cells incubated with 1-

NM-PP1 for 0.5h showed a very limited downregulation of gene expression, reflecting the previous data obtained during EU staining (**Figure 5.6.C.**). Additionally, in these cells one gene was found significantly downregulated: *H1-3*, encoding histone H1.3.

More pronounced changes were observed after 2h treatment of HEK293 and HEK293 AS-CDK1 cells with 1-NM-PP1 (**Figure 5.7.B. and D.**). In the case of HEK293, incubation with the drug caused a marked downregulation of a subset of genes, also confirming the results from EU staining (**Figure 5.6.C.**). After 2h of inhibition, a set of 15 genes was found to be significantly-downregulated compared to cells treated with DMSO. These genes included the *H1-3* gene mentioned before, as well as *TXNIP* found downregulated in the original HeLa AS-CDK1 RNA-seq, but not in the chrRNA purifications of HEK293 cell lines (**Figures 4.11., 5.1.**). Surprisingly, out of 15 downregulated genes, 12 were histone protein-coding genes. The two-hour treatment of HEK293 also caused the significant upregulation of a single gene – *CHAC1*, which encodes the glutathione-specific gamma-glutamylcyclotransferase 1 and acts in the unfolded protein response.

Finally, in HEK293 AS-CDK1 cells treatment with 1-NM-PP1 induced a significant upregulation of a small subset of 61 genes, despite the expression of most of genes remaining fairly stable between control and treated samples. Most of the upregulated genes were not usually highly-transcribed in HEK293 AS-CDK1 cells. Accordingly, the magnitude of the deregulation of these genes was much larger than what was seen for the unmodified HEK293 or HeLa AS-CDK1 cells. The most upregulated gene was *CPNE7*, encoding Copine-7, a calcium-dependent phospholipid-binding protein. On the other hand, incubation of HEK293 AS-CDK1 cells with 1-NM-PP1 also caused a significant downregulation of four genes including *TXNIP*, but the most downregulated gene in this

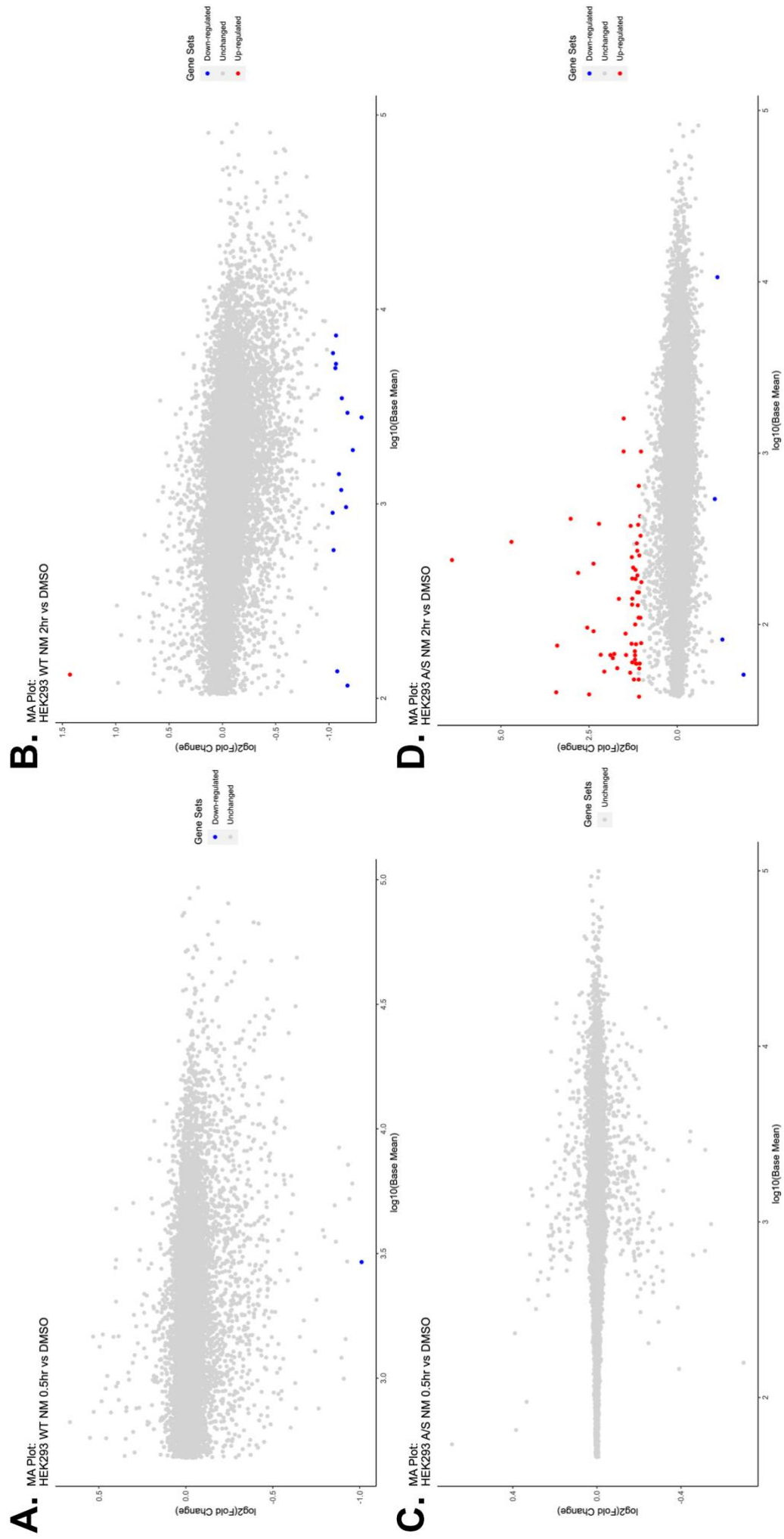
set was *HSPA1A*, encoding heat shock protein 70. This could suggest that the cells were under stress after the 1-NM-PP1 inhibition.

Having identified genes dysregulated in HEK293 and HEK293 AS-CDK1 cells following treatment with 1-NM-PP1, links between these genes were sought. The 61 significantly-upregulated genes from HEK293 AS-CDK1 cells were referenced against the 23 upregulated genes from HeLa AS-CDK1, as well as the top 74 upregulated genes with a $p < 0.1$ cut-off point in HEK293 cells. None of the named genes were repeated between the cell lines, perhaps underlining their different baseline phenotypes. Gene set enrichment analysis (GSEA) of the data also did not reveal any significant results for any dysregulated gene sets. For instance, for HEK293 AS-CDK1 cells treated for 2h with 1-NM-PP1, roughly half of the gene sets were upregulated and half were downregulated, further accentuating the relatively small dysregulation of general gene expression in these cells. Gene Ontology enrichment analysis indicated that, for unmodified HEK293 cells, the dysregulated genes were mostly implicated in nucleosome assembly (**Figure 5.8**). For genes dysregulated in the HEK293 AS-CDK1 cells, the enriched class of genes was involved in glycosaminoglycan biosynthetic process. However, of the several dozen dysregulated genes, only five were assigned to this category. Additionally, the very little evidence of CDK1 functions in polysaccharide metabolism calls into question the validity of this result. The ambiguous output of the GO analysis, especially for the HEK293 AS-CDK1 dataset, could stem from the limited number of significant hits that could be examined using the software. The results should therefore be interpreted with a degree of caution.

Altogether, the differential gene expression results from the second round of chRNA-seq highlights that the 1-NM-PP1 inhibition can affect distinct cell lines in different ways,

possibly due to the discrete phenotypes of cancerous and non-cancerous cells. 1-NM-PP1 repeatedly affected unmodified cells not harbouring an AS kinase, and the overall dysregulation of gene expression in HEK293 cells after 2h treatment was marginally greater than in HEK293 AS-CDK1 cells. It was difficult to elucidate the connecting links between the dysregulated genes in these cell lines, although more of them were found in HEK293 and HEK293 AS-CDK1 cells than in HeLa AS-CDK1. The significant link between genes involved in nucleosome assembly in HEK293 cells presumably represents an off-target effect of 1-NM-PP1. So far, the analysis did not clarify the extent to which transcriptional dysregulation in HEK293 AS-CDK1 cells may be caused by the inhibition of CDK1.

Figure 5.7. Differential expression of genes after 1-NM-PP1 treatment of HEK293 and HEK293 AS-CDK1 cells. RNA-seq of chromatin-associated RNA was performed on HEK293 and HEK293 AS-CDK1 cells treated with 7.5 μ M 1-NM-PP1 for 0.5h or 2h. The samples were depleted of poly(A)⁺ mRNA and rRNA transcripts before analysis. The MA plot shows the differences in expression of protein-coding genes between treated and untreated conditions, with genes above the 0-mark correlating to upregulation following 1-NM-PP1 treatment. The coloured dots signify statistically significant hits (Benjamini-Hochberg adjusted P value<0.05; n=3).



A. HEK293, 2h 1-NM-PP1 treatment

	Homo sapiens (REF)	upload_1 (▼ Hierarchy_ NEW! ?)					
GO biological process complete	#	#	expected	Fold Enrichment	+/-	raw P value	FDR
nucleosome assembly	90	4	.05	83.15	+	1.10E-07	3.33E-04
↳ protein-DNA complex assembly	201	4	.11	37.23	+	2.76E-06	6.96E-03
↳ protein-containing complex organization	2044	7	1.09	6.41	+	2.17E-05	4.69E-02
↳ protein-DNA complex organization	878	7	.47	14.92	+	7.13E-08	2.70E-04
↳ nucleosome organization	110	5	.06	85.04	+	1.79E-09	2.71E-05
↳ chromatin remodeling	637	7	.34	20.56	+	7.80E-09	5.90E-05
↳ chromatin organization	786	7	.42	16.66	+	3.33E-08	1.68E-04
heterochromatin formation	112	3	.06	50.11	+	2.51E-05	4.74E-02

B. HEK293 AS-CDK1, 2h 1-NM-PP1 treatment

	Homo sapiens (REF)	upload_1 (▼ Hierarchy_ NEW! ?)					
GO biological process complete	#	#	expected	Fold Enrichment	+/-	raw P value	FDR
glycosaminoglycan biosynthetic process	65	5	.19	25.95	+	1.39E-06	2.11E-02
↳ aminooglycan biosynthetic process	69	5	.20	24.45	+	1.88E-06	1.42E-02

Figure 5.8. Gene Ontology enrichment analysis of genes dysregulated following 2h 1-NM-PP1 treatment of HEK293 and HEK293 AS-CDK1 cells. RNA-seq of chromatin-associated RNA was performed on HEK293 and HEK293 AS-CDK1 cells treated with 7.5µM 1-NM-PP1 for 2h. The samples were depleted of poly(A)+ mRNA and rRNA transcripts before analysis. The screenshots show the significant shared GO terms between genes found to be dysregulated in **A. HEK293** and **B. HEK293 AS-CDK1** cells (False Discovery Rate-adjusted q-value<0.05).

5.5.2. RNA-seq analysis: continued

To investigate how 1-NM-PP1 treatment affects the HEK293 cell lines, and especially CDK1, further bioinformatics analysis was performed (**Figure 5.9.**). Following 0.5h inhibition with 1-NM-PP1, no non-protein coding genes were found dysregulated in either HEK293 or HEK293 AS-CDK1 cells. HEK293 cells treated with 1-NM-PP1 for 2h showed a significant upregulation of two poorly characterised long non-coding RNA genes (*ENSG00000230454*, *ENSG00000232611*). The biggest differences were observed for HEK293 AS-CDK1 cells after 2h of 1-NM-PP1 inhibition, as around 50 genes were found to be mostly upregulated following the treatment. Although the majority did not exceed a 2-fold change in expression, one unknown long-coding RNA, *ENSG00000286385*, was seen to be upregulated almost 8-fold after the addition of 1-NM-PP1. Although the data implied that 1-NM-PP1 can affect the transcription of non-protein coding genes even in wild-type HEK293, the stronger dysregulation seen for HEK293 AS-CDK1 cells suggests that CDK1 could influence the transcription of non-protein coding genes to some degree as well.

Genome browser tracks were used to analyse the read density for the two most dysregulated genes when HEK293 AS-CDK1 cells were treated with 1-NM-PP1 for 2h (**Figure 5.10.**). *CPNE7*, the most upregulated gene, was expressed at a very low level in the untreated condition, and even after 0.5h of 1-NM-PP1 incubation. Only after 2h was the effect of the drug seen, with the reads appearing uniformly across the track. This underlined that the abolition of CDK1 activity does not have an immediate effect on *CPNE7*, possibly pointing to the involvement of a transcriptional mediator downstream of CDK1 that acts evenly throughout the gene. The most downregulated gene, *HSPA1A*, behaved similarly, as its downregulation was consistent across the gene, and only appeared after 2h of 1-NM-PP1 incubation. The behaviour of the two genes seemed to

confirm that any transcriptional changes caused by 1-NM-PP1 treatment need longer incubation times to materialise, perhaps suggesting that the effects of the inhibition are indirect.

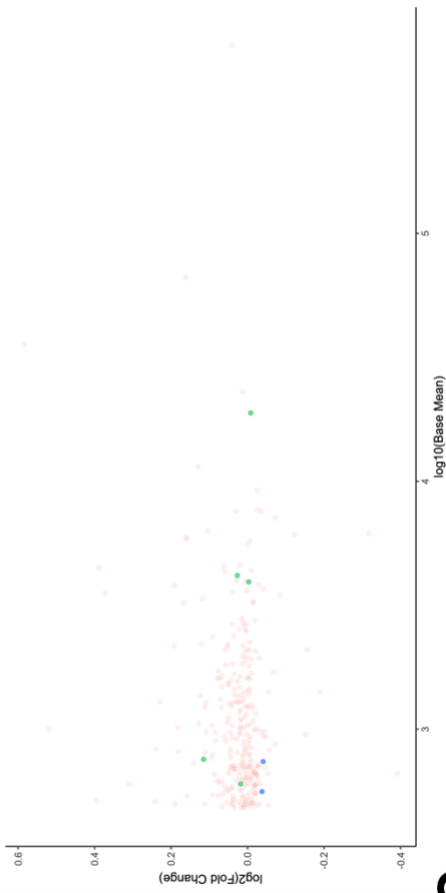
Finally, motif analysis of the regions 1kb upstream of transcription start sites was conducted to find any potential links between the genes dysregulated after 1-NM-PP1 treatment. Multiple Em for Motif Elicitation (MEME) was performed to uncover any novel motifs, and Simple Enrichment Analysis (SEA) was used to find for any known enriched motifs that would be common between the dysregulated genes. Similarly to before (**Figure 4.8.**), MEME analysis of the upregulated genes in 1-NM-PP1-treated HEK293 AS-CDK1 cells showed sets of repetitive sequences as the most significantly enriched motifs, providing few clues to the potential mechanism of action (**Figure 5.11.**). The 74 upregulated genes ($p < 0.1$) from HEK293 cells were also analysed using MEME, resulting in the discovery of more diverse motifs, which could potentially shed light on the off-target effects of 1-NM-PP1.

SEA was conducted on both the 61 significantly-upregulated genes from HEK293 AS-CDK1 cells after 2h 1-NM-PP1 treatment, as well as the 74 upregulated genes in HEK293 after 2h 1-NM-PP1 treatment (**Figure 5.11.**). Although by themselves the sequences looked quite repetitive, the discovered motifs were compared between the two sets of cells, as well as with the previous motifs discovered from 23 genes upregulated in HeLa AS-CDK1 cells (**Table 5.1.**). By finding the motifs common between dysregulated genes in all three of the cell lines, a better understanding of 1-NM-PP1 actions could be gained. The sequences suggested common ways in which the drug can affect all cells, regardless of whether they are analogue-sensitive or wild-type. More importantly, motifs shared between the two AS-CDK1 cell lines were collected, and the motifs that appeared in the HEK293 analysis were subtracted from that group. The remaining seven motifs: *OSR2*,

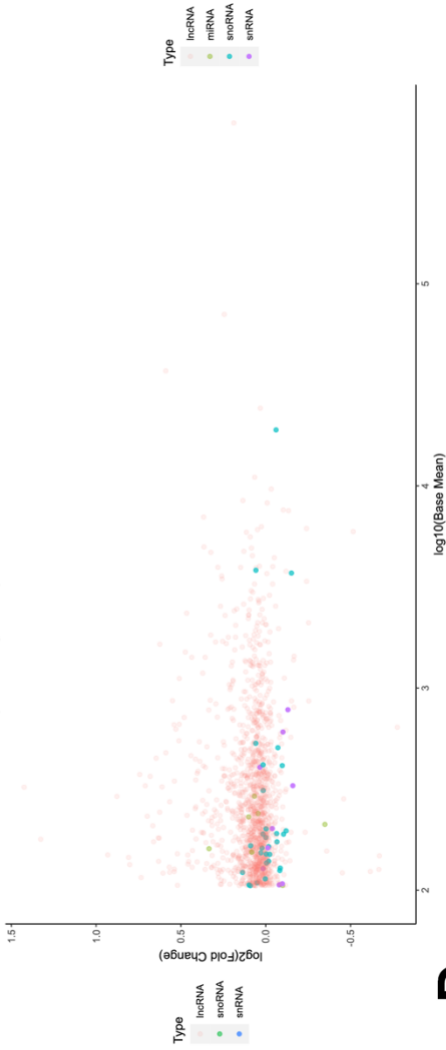
ZN467, *MAZ*, *VEZF1*, *PATZ1*, *ZN418*, and *ERR3* are common between HeLa AS-CDK1 and HEK293 AS-CDK1 cells, but not the wild-type HEK293, finally giving clear evidence of pathways that could be affected by the inhibition of CDK1 that occurs in the mutated cell lines. Although no immediate connections can be drawn between any of these transcriptional factors and CDK1, this could suggest either a substantial degree of novelty of these results, or possibly that additional mediators are involved in their regulation by CDK1. Basing on the order of significance of the identified motifs in HeLa AS-CDK1 and HEK293 AS-CDK1 cells, the two sequences that seem the most common between the dysregulated genes in the two cell types are the *OSR2* and *MAZ*, then followed by *ZN467* and *VEZF1*, suggesting that these four could constitute as the most encouraging leads for any future investigations.

The RNA-seq data analysis gave further clues on how 1-NM-PP1 treatment affects HEK293 and HEK293 AS-CDK1 cells, but relatively few differences were found that could help establish the background effects caused by off-target actions of 1-NM-PP1. The non-protein coding genes expression showed that the treatment had very limited effect on this class of genes. The track coverage showed that the biggest changes in gene expression occurred after 2h of inhibition rather than 0.5h, and because the shift was not gradual, it can be assumed that the dysregulation of those genes was most probably an indirect effect. The most promising results came from the motif analysis section of the investigation, which helped discriminate individual pathways that were affected in AS-CDK1, but not in wild-type cells. The seven found motifs serve as an indication of what kind of methods CDK1 might employ to regulate the transcriptional phenotype of both cancerous, and non-cancerous cells, and investigation into the exact links between them and CDK1 could provide exciting new insights into the universal transcriptional roles of this kinase.

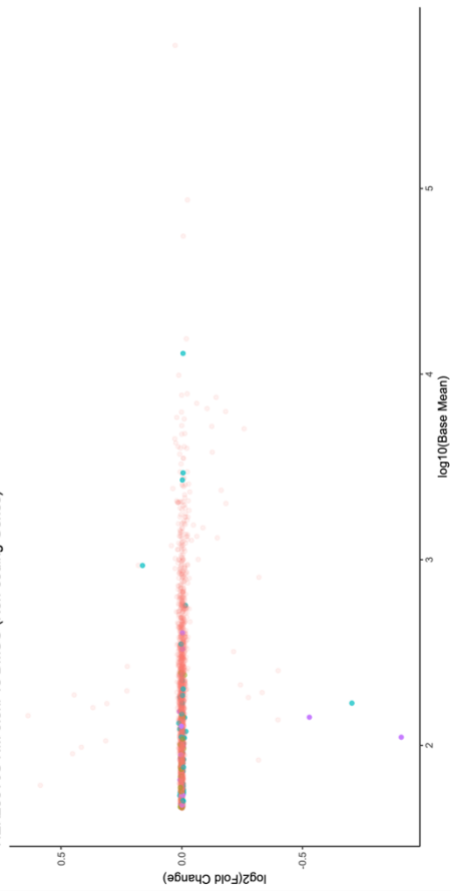
A. MA Plot:
HEK293 WT NM 0.5hr vs DMSO (Non-coding Genes)



B. MA Plot:
HEK293 WT NM 2hr vs DMSO (Non-coding Genes)



C. MA Plot:
HEK293 A/S NM 0.5hr vs DMSO (Non-coding Genes)



D. MA Plot:
HEK293 A/S NM 2hr vs DMSO (Non-coding Genes)

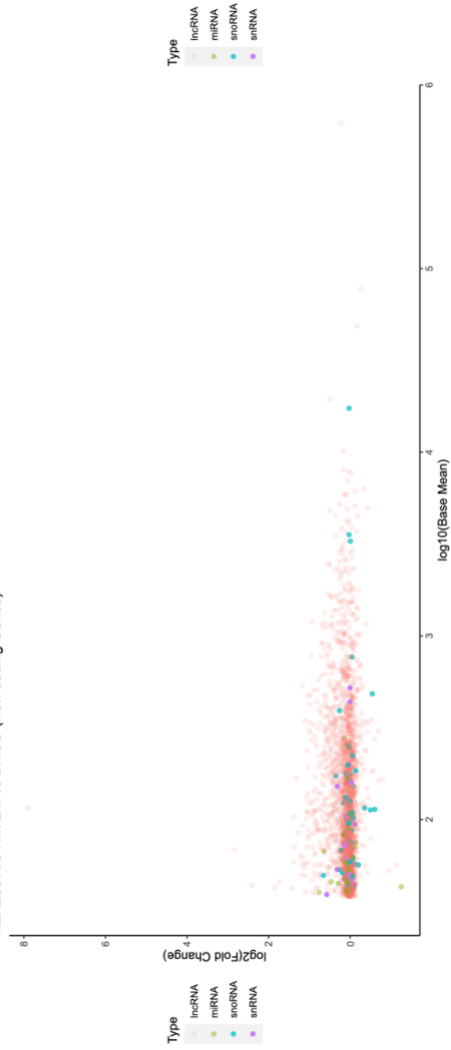


Figure 5.9. Differential expression of non-protein coding genes after 1-NM-PP1 treatment of HEK293 and HEK293 AS-CDK1 cells. RNA-seq of chromatin-associated RNA was performed on HEK293 and HEK293 AS-CDK1 cells treated with 7.5 μ M 1-NM-PP1 for 0.5h or 2h. The samples were depleted of poly(A)+ mRNA and rRNA transcripts before analysis. The MA plot shows the differences in expression of protein-coding genes between treated and untreated conditions, with genes above the 0-mark correlating to upregulation following 1-NM-PP1 treatment.

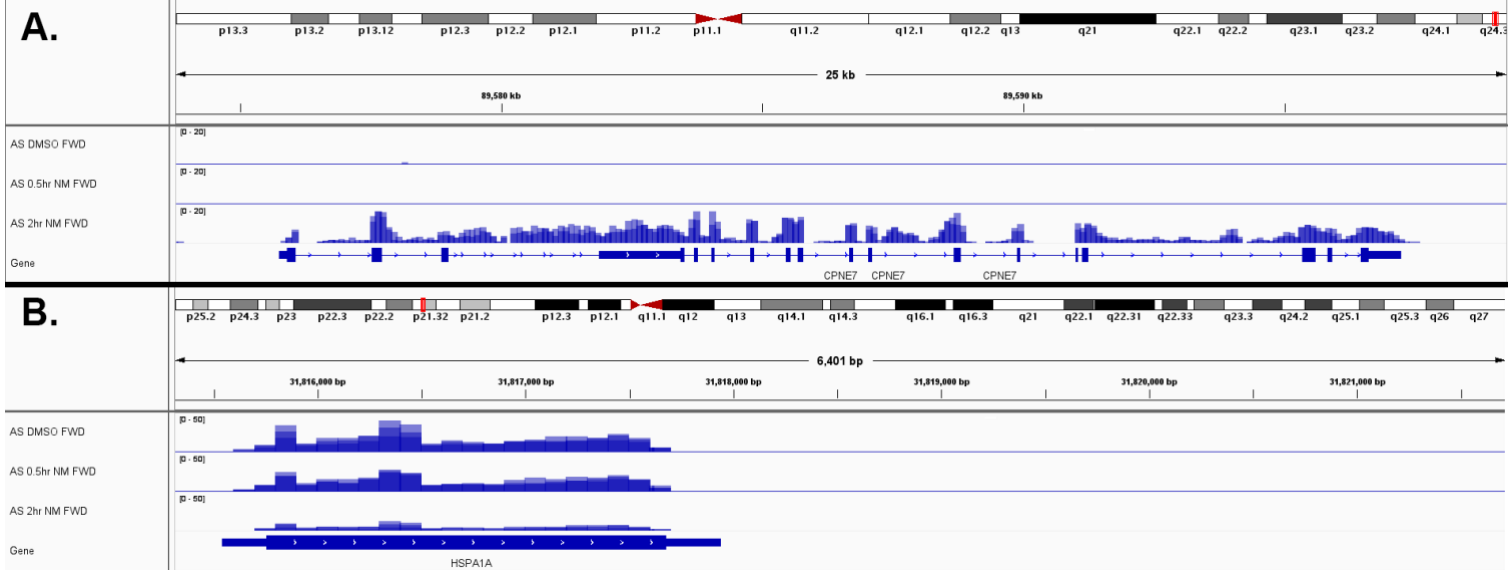


Figure 5.10. Genome browser coverage tracks of *CPNE7* and *HSPA1A* in HEK293 AS-CDK1 cells after 1-NM-PP1 treatment. RNA-seq of chromatin-associated RNA was performed on HEK293 AS-CDK1 cells treated with 7.5μM 1-NM-PP1 for 0.5h or 2h. The samples were depleted of poly(A)+ mRNA and rRNA transcripts before analysis. Read density for both genes was checked using genome browser coverage tracks for the most upregulated gene after 2h of treatment: **A.** *CPNE7*; and the most downregulated gene: **B.** *HSPA1A*. The tracks for each gene show reads for DMSO-treated cells (top), followed by 0.5h 1-NM-PP1 treatment, and 2h 1-NM-PP1 treatment at the bottom (n=3).

Table 5.1. Known motifs found in Simple Enrichment Analysis (SEA) of upregulated genes from cells treated for 2h with 1-NM-PP1.

HeLa AS-CDK1 (in order of significance)	KLF15, MYCN, KLF6, OSR2, SP2, ZN467, IRF3, KLF3, BHE40, NKX61, SP3, ZFP82, MAZ, SP1, ZN770, KLF5, FOXJ3, SP4, KLF9, MAX, VEZF1, ERG, ETV5, TFEB, EGR2, PATZ1, ZN140, ZN263, PO2F2, ZN418, HXA10, HXB7, MEIS1, NANOG, KLF4, SOX4, THAP1, ZN281, KLF12, TFE3, ERR3, CDX2, PDX1
HEK293 AS-CDK1 (in order of significance)	ZBT17, MAZ, VEZF1, KLF15, SP2, KLF6, SP1, IRF3, ZN341, WT1, OSR2, PATZ1, SP3, EGR1, HNF6, FLI1, KLF12, ZN467, RXRB, KLF9, KLF1, BC11A, ZN770, ZSC22, SALL4, ELF5, ETS2, E2F7, Z354A, SPI1, RORG, E2F4, SPIB, KLF3, GFI1, ZBTB6, ERR3, MEF2C, ZN418, MAF, CREB1, ETV2
HEK293 (in order of significance)	KLF1, RARA, FOXQ1, ZSC31, KLF3, ZN263, MYCN, ZN770, KLF5, SP3, KLF12, IRF1, ANDR, ZN121, FOXJ3, IRF3, ZSC22, MEF2B, SP1, SOX2, MEF2D, ZN281, SP2, PAX5, NRF1, SMAD2, KAISO, KLF4, MYF6, KLF6, FOXA1, NFAC1, HNF1A, KLF9, COT2, ZNF85, NR6A1, RFX3, SRBP2, RORA, SP4, ZN140, KLF15, ZN341, PRD14, CEBPD, TFEB, IRF2, SOX10, BMAL1, OVOL1
Common between all three cell lines	KLF15, SP2, KLF6, SP1, IRF3, SP3, KLF12, KLF9, ZN770, KLF3
Common between AS-CDK1 cell lines, but not HEK293	OSR2, ZN467, MAZ, VEZF1, PATZ1, ZN418, ERR3

5.6. Discussion

In the final part of the project, the role of CDK1 in transcription was studied across different cell lines and with the use of different inhibitors, to better understand the universality of its actions, and to delineate the best methods to elucidate any possible functions of this protein in transcription of de-differentiated cells. It was found that the off-target effects of 1-NM-PP1 differ between the HeLa, HCT116, and HEK293 cell lines. Another PP1-derived inhibitor, 3-MB-PP1, was shown to specifically inhibit CDK1 in the HeLa AS-CDK1 cell line, without affecting the *PER1* and *TXNIP* genes dysregulated by 1-NM-PP1. Additionally, a separate small molecule inhibitor of CDK1, RO-3306, had opposing transcriptional effects on the genes included in this study when compared to 1-NM-PP1. Interestingly, the incubation of the two HEK293 cell lines with 1-NM-PP1 showed opposing trends in transcription to the HeLa and HeLa AS-CDK1 cells. Finally, chromatin RNA-seq of the HEK293 and HEK293 AS-CDK1 cell lines helped to clarify how CDK1 might influence transcription across multiple cell types.

The examination of HCT116 CDK1^{WT/AS} and HEK293 AS-CDK1 cell lines began by looking at the two genes, *PER1* and *TXNIP*, dysregulated by 1-NM-PP1 in HeLa AS-CDK1. The lack of changes in the expression of these genes following the treatment of the two new AS-CDK1 cell lines suggested that the actions of 1-NM-PP1 are cell-type dependent, and it helped to clarify that the two genes were affected by 1-NM-PP1 alone.

3-MB-PP1 inhibition of the HeLa and HeLa AS-CDK1 cell lines indicated that the off-target factor affecting *PER1* and *TXNIP* was not common between the PP1-derived AS kinase inhibitors. Despite their similar structures, the three PP1-derived molecules – 1-NM-PP1, 3-MB-PP1, and 1-NA-PP1 – have different off-target effects (C. Zhang et al., 2013). A 2013 study by Zhang and colleagues outlined the structural basis of the three

inhibitors, and how their differences affect their specificity and off-target interactions (C. Zhang et al., 2013). In short, the enlarged C3 substituents of PP1-analogues (for instance, naphthylmethyl and 3'-methylbenzyl substituents of 1NM-PP1 and 3MB-PP1, respectively) are able to occupy the enlarged ATP-binding pocket of analogue-sensitive proteins, and the inhibitors form hydrogen bonds with the hinge region of the kinase. In contrast, the bulky groups prevent binding of the inhibitors to wild-type kinases due to a steric clash with the native gatekeeper residue. However, the degree of the clash differs between compounds, with 1-NM-PP1 experiencing the greatest overlap with the gatekeeper side chain, and 1-NA-PP1 the weakest. Additionally, both 1NM-PP1 and 3MB-PP1 possess a methylene linker that buries the substituents deeper into the binding pocket maximizing the steric clash with the gatekeeper. Importantly, 1-NM-PP1 differs from 3-MB-PP1 in that its additional atoms of the naphthyl ring (C6', C7', and C8') further overlap with the gatekeeper and several other residues of the ATP-binding pocket. Zhang and colleagues hypothesise therefore that the intrinsic variety in the structure of ATP-binding pockets between distinct kinases could be responsible for the increased, or decreased, interactions between the atoms and residues, and hence the altered affinity of 1-NM-PP1 binding (C. Zhang et al., 2013).

3-MB-PP1 was developed because 1-NA-PP1 and 1-NM-PP1 were not capable of targeting all analogue-sensitive kinases (C. Zhang et al., 2013). As the different members of this class of inhibitors have varying degrees of affinity to ATP-binding pockets of AS kinases, their affinity to other unmutated members of the kinome also changes. The previously mentioned (**section 4.9.**) PER1-phosphorylating CK1 ϵ has a slightly higher binding affinity to 1-NM-PP1 when compared to 3-MB-PP1, but it remains unclear whether the small difference could account for the discrepancy in *PER1* upregulation seen during this investigation (C. Zhang et al., 2013). A more significant difference in

inhibition efficacy was seen for its relative CK1 δ , but this one in turn was more efficiently inhibited by 3-MB-PP1, in contrast to the results seen here. In mouse ES cells, 3-MB-PP1 was used as the inhibitor of choice because of its higher potency (personal communication; Michowski et al., 2020). It is conceivable that since 3-MB-PP1 treatment requires only 1 μ M of the drug compared to 7.5 μ M of 1-NM-PP1, the latter might be more prone to off-target effects.

The inhibition of CDK1 in HeLa cells with RO-3306 showed a completely different behaviour of the *TXNIP*, *ETS2* and *PDK4* genes, but not so much of *PER1*, compared to the treatment of HeLa AS-CDK1 cells with 1-NM-PP1. These results underline the differences that come from using different cell lines and inhibitors to study CDK1 function. When looking at an AS-CDK1 cell line by itself, some of the genes dysregulated by inhibition of CDK1 with RO-3306 might be missed. Additionally, since RO-3306 also inhibits CDK2 and CDK4, the differences between inhibition with RO-3306- and a CDK1-specific ATP-analogue could potentially help discriminate the different CDK functions – assuming the ATP-analogue lacks the off-target effects seen with 1-NM-PP1 (Vassilev et al., 2006). The comparison between the two could also help to identify potential off-targets of 1-NM-PP1 if the regulation of the gene by CDK1 seems completely opposing, as was seen for *TXNIP* in this study. Interestingly, incubation of HCT116 and HEK293 with RO-3306 did not cause any change in the transcription of *TXNIP*, while in HeLa cells the difference was stark. This could potentially serve as further evidence that certain cell lines evolve to acquire dependencies on processes driven by CDKs, and that such relationships are context-dependent.

Inhibition of CDK1 with RO-3306 in HeLa cells was achieved even at lower drug concentrations. Inhibition of CDK1 has been achieved using 10 μ M of RO-3306 in many

different models, although the concentration range can vary between 5 μ M to 2mM (Michowski et al., 2020; Sunada et al., 2021; Vassilev et al., 2006; Voets et al., 2015; C. X. Wu et al., 2018). In some investigations, 10 μ M RO-3306 allowed for complete inhibition of CDK1 activity, although most of the cited studies use inhibition times that well exceed the 2h used here (Voets et al., 2015). Lower concentrations of the drug actually increased the mitotic index of RPE1 and U2OS cells, and only 10 μ M treatment created a full arrest of cells at G2 (Voets et al., 2015). Ultimately, the 10 μ M RO-3306 concentration was used in this study to create a clearer point of reference to how this CDK1 inhibitor was used in other studies. Direct monitoring of mitotic index by microscopy would help gauge the exact level of CDK1 inhibition in these cells.

Despite gaining a clearer picture of the actions of 1-NM-PP1 and other inhibitors across the different cell lines, there were still relatively few clues as to how CDK1 might act as a transcriptional regulator. Since the previous data (**sections 4.2., 4.5.1.**) implied that 1-NM-PP1 treatment of HeLa AS-CDK1 cells resulted in upregulation of expression of a subset of genes, but that the most dysregulated genes were off-targets, it was crucial to determine whether the whole, or just a part, of the overall gene upregulation was caused by NM-PP1 targets other than CDK1. The EU-staining results (**section 4.2.**) corroborated the chrRNA-seq data in HeLa AS-CDK1 cells (**section 4.5.1.**), so the dysregulation of genes in HeLa cells treated with 1-NM-PP1 was also checked with fluorescence microscopy first. Additional samples of the two HEK293 cell lines, as well as 3-MB-PP1-treated cells were added to this part of the study to create a broader overview of the actions of the ATP-analogues. Due to time constraints only one iteration of this experiment was conducted and repeats will be performed in the future.

The EU-staining analysis showed that the upregulation of gene expression following inhibition with 1-NM-PP1 was not confined to HeLa AS-CDK1 cells, but was also seen in unmodified HeLa cells. The changes began after 15min of treatment, and increased with longer drug exposure. The trend was similar with both 1-NM-PP1 and 3-MB-PP1, implying that despite the latter not targeting genes like *PER1* or *TXNIP*, it also has a range of off-target substrates in unmodified cell lines. Moreover, the levels of gene upregulation in treated HeLa and HeLa AS-CDK1 cells were similar, suggesting that the transcriptional effect of CDK1 inhibition in these cell lines is very limited, and most of the observed changes were actually caused by the ATP analogues targeting other cellular components.

The EU staining of HEK293 and HEK293 AS-CDK1 cells showed a completely opposite trend, with both cell lines experiencing a downregulation of gene transcription following incubation with either ATP analogue. As in HeLa and HeLa AS-CDK1 cells, the dysregulation was progressive, similar between 1-NM-PP1 and 3-MB-PP1, and experienced by both unmodified and analogue-sensitive cells. Although this reinforced some of the conclusions drawn from HeLa cell data, the important question remained of why these cells experienced primarily global gene downregulation in response to the drugs. For instance, it can be speculated that the two cell lines evolved to have distinct sets of kinases that can be targeted by 1-NM-PP1 or 3-MB-PP1. Alternatively, the differences could stem from the deregulation of non-protein coding genes that are governed by pol I or III, which could be examined in the future by excluding nucleoli from the analysis.

That being said, the changes occurring in inhibitor-treated HEK293 cells during EU staining (**Figure 5.6.**) seemed marginally smaller than in their analogue-sensitive

counterparts. It was therefore decided, that an additional round of chromatin-associated RNA-seq could shed more light on the level of CDK1 involvement in the dysregulation of the transcriptional phenotype following 1-NM-PP1 treatment. Based on the previous experiences, this time the importance of having an unmodified control was recognised. Additionally, because of the results seen during a short inhibition time course of *PER1*, 0.5 h treatment with 1-NM-PP1 was also included. The comparisons between the different conditions served therefore to identify individual genes dysregulated in HEK293 AS-CDK1, but not HEK293, that could lead to evidence of the involvement of CDK1 in governing their transcriptional landscape.

For both HEK293 and HEK293 AS-CDK1, very few changes in gene expression appeared after 0.5h inhibition with 1-NM-PP1. This suggested that longer inhibition times might be needed, though changes seen after 2h of inhibition were considered more likely to be indirect, potentially contributing to difficulties in finding common motifs or links between the dysregulated genes. For both HEK293 and HEK293 AS-CDK1 cells more significantly-dysregulated genes were found in comparison to the previous HeLa AS-CDK1 RNA-seq investigation. However, HEK293 AS-CDK1 cells despite having more individual significantly-dysregulated genes of $p < 0.05$, did not seem to undergo the same shifts of gene expression as HeLa AS-CDK1 or HEK293. Rather, the vast majority of the genes remained closer to the middle of the y-axis, signifying no major differences in gene expression in 1-NM-PP1-treated condition compared to DMSO.

This result did not correlate entirely with the previous EU-staining data, which showed a stronger downregulation of genes in HEK293 AS-CDK1 cells incubated with 1-NM-PP1. This was in contrast to the HEK293 cell line, whose results from the EU-incorporation assay and RNA-seq matched, both showing a downregulation of gene expression. One

possible explanation for this result could lay in the aforementioned limitation of the EU-staining approach, which only looks at global changes in transcription. This means that some of the transcriptional downregulation occurring in inhibited HEK293 AS-CDK1 cells during EU staining could be caused by the decreased transcription of tRNA and rRNA governed by pol I and III. However, since no such result was observed in HEK293 cells, this could suggest that this action is CDK1-dependent, and that the kinase can repress tRNA and/or rRNA transcription in HEK293 cells. This would be in line with previous suggestions that in mammals CDK1 might decrease tRNA production during mitotic silencing (Enserink & Chymkowitch, 2022; White et al., 1995).

A more speculative hypothesis to explain the relatively small overall changes in the gene expression of 1-NM-PP1-treated HEK293 AS-CDK1 during the RNA-seq lies in the two actions of the inhibitor cancelling each other out. Since unmutated HEK293 cells showed stronger downregulation of overall gene expression following treatment (**Figure 5.7.B.**), it can be assumed that 1-NM-PP1 acts as a transcriptional repressor in these cells. If, based on the HeLa AS-CDK1 data (**sections 4.2., 4.5.1.**) and previous literature, one were to consider CDK1 a transcriptional repressor, its inhibition by 1-NM-PP1 in HEK293 AS-CDK1 would cause the upregulation of gene expression. If the action pathways of both 1-NM-PP1 and CDK1 (inhibited by 1-NM-PP1) overlay to some degree, there could exist a possibility of the two mechanisms counteracting each other and creating the dampened transcriptional effect seen in the HEK293 AS-CDK1 cells.

The GSEA of HEK293 AS-CDK1 cells confirmed that the split of upregulated and downregulated gene sets was divided almost equally, although several dozen individual genes were more upregulated. The GO enrichment analysis highlighted the aforementioned ‘glycosaminoglycan biosynthetic process’ term, which was connected to

just a handful of the dysregulated genes. Although very limited literature exists that could help explain the connection of CDK1 to this process, some studies described a link between CDKs and how they regulate TGF- β -mediated mRNA expression of glycosaminoglycan-synthesizing enzyme in vascular cells through phosphorylation of Smad2 linker region (Kamoto et al., 2014; Rostam et al., 2016). The motif for Smad2 was actually one of known motifs identified as common between dysregulated genes in the subsequent part of RNA-seq analysis – however, it was found to be involved in genes upregulated in HEK293 cells rather than HEK293 AS-CDK1. The aforementioned investigations inhibited CDKs with small molecule inhibitors such as flavopiridol, while the Smad2 motif identified in this study would have been a 1-NM-PP1 target motif instead.

The most dysregulated genes in 1-NM-PP1-treated HEK293 AS-CDK1 cells were the upregulated *CPNE7*, and the downregulated *HSPA1A*. *CPNE7* was not among the 74 most upregulated genes in HEK293 ($p < 0.1$), and *HSPA1A* was not significantly downregulated in the unmodified cells either ($p < 0.5$), suggesting that they might be genuine targets of CDK1. Little is known about the relationship between CDK1 and *CPNE7*, suggesting a novel unidentified link between these proteins. In mESCs, *CPNE1* and 3 were identified as potential CDK1 substrates during the inhibition approach, but *CPNE7* was not identified in this context (Michowski et al., 2020). Connections between CDK1 and *HSPA1A* are, however, much clearer. Importantly, the *S. cerevisiae* homologue of Hsp70 is known to be phosphorylated by Cdc28, which causes it to bind and promote the degradation of Cln3 cyclin, impeding the entry into S phase (Truman et al., 2012; Velasco et al., 2019). The same study has also recorded the phosphorylation of the mammalian Hsp70 pulled down from HEK293 cells, which leads it to interact with cyclin D1 (Truman et al., 2012). Although it was shown that the phosphorylation is

“CDK-dependent”, the exact kinase responsible was not identified, leading to the possible conclusion that it might be, in fact, CDK1. Whether CDK1 could also control the transcription of this protein remains to be examined. In a study on the effects of baicalein in hepatocellular carcinoma (HCC), the construction of a protein-protein interaction (PPI) module network of baicalein linked CDK1 as a target inhibited by baicalein, and Hsp70 as a downregulated differentially-expressed gene (DEG), mirroring the results of this investigation (Ma et al., 2019). Additionally, it was reported that overexpression of both *CDK1* and *HSPA1A* is linked to significantly worse overall survival in HCC patients (Ma et al., 2019).

The most dysregulated genes in HEK293 cells treated with 2h for 1-NM-PP1 were used to look for hints to the nature of off-target effects of the inhibitor in unmutated and AS cells. The only significantly upregulated gene identified in this study was the *CHAC1*, which acts as a pro-apoptotic factor in the unfolded protein response (UPR) pathway. The mRNA of *CHAC1* was found to be upregulated following drug treatments that induce the UPR, particularly through the actions ATF3 and CHOP transcription factors (Mungrue et al., 2009). It could be therefore speculated that 1-NM-PP1 either acts to upregulate the UPR itself, or that it modifies the transcription factors involved in this process.

The most downregulated genes in treated HEK293 cells belonged, in turn, to the histone family. Some of the kinases found to be deregulated by off-target actions of 1-NM-PP1, such as PKA or ACK, are known to phosphorylate some of the histones influencing the transcriptional landscape – although it remains unclear how they could affect the transcription of histone genes (Bain et al., 2007; Ha et al., 2010; Mahajan et al., 2017; C. Zhang et al., 2013). If 1-NM-PP1 treatment genuinely affected the epigenetic remodelling pathways, not only could it explain the overall dysregulation of transcription seen during

inhibition of HEK293 and HEK293 AS-CDK1 cell lines, but it would perhaps cast some doubt on the findings in mESCs, where inhibition of CDK1 with an ATP-analogue resulted in dysregulation of epigenetic regulators (Michowski et al., 2020). It has to be said, however, that the epigenetic regulators identified as CDK1 substrates in that study were also verified by the labelling approach and contained the S/T-P consensus CDK1 phosphorylation motif.

The dysregulation of transcription of pol II-transcribed non-protein coding genes in HEK293 and HEK293 AS-CDK1 treated with 1-NM-PP1 was also minimal, further suggesting that the downregulation of transcription seen during EU staining of HEK293 AS-CDK1 cells was most probably caused by the actions of pol I, pol III, or both. The single long non-coding RNA gene (*ENSG00000286385*) that was very strongly upregulated in the treated AS-CDK1 cells remains uncharacterised, and at the moment provides no information on how CDK1 might be linked to its regulation. Similarly, the genome browser track coverage snapshots of the two genes dysregulated in HEK293 AS-CDK1 only allowed for speculations whether they could be indirect, due to their sudden change after 2h of inhibition, but not after 0.5h. The novel motifs uncovered in MEME for both HEK293 and HEK293 AS-CDK1 cells could potentially be worth investigating; especially with the less-repetitive sequences seen for HEK293, which could give hints on the common links between off-target effectors of 1-NM-PP1.

Finally, the analysis of known motifs identified using SEA software provided significant new insights into the 1-NM-PP1 inhibition of wild-type and AS-CDK1 cells. Although by themselves the motifs were already showing some interesting links between dysregulated genes, such as in the case of the aforementioned Smad2, the comparison between sequences found in HEK293, HEK293 AS-CDK1, as well as HeLa AS-CDK1

cell lines allowed to generate leads on how the transcriptional dysregulation of gene expression in these cells might be controlled between DMSO- and 1-NM-PP1-treated conditions.

The 74 most upregulated genes from HEK293 were found to be linked by several dozen motifs. Interestingly, one of them was the motif for *BMAL1* transcriptional factor, which solidified the previous data showing dysregulation of *PER1*, and provided an additional link to how 1-NM-PP1 might inadvertently affect the circadian rhythm in cells. Additionally, genes in treated HEK293 cells were enriched for the *MYCN* motif, which showed up as the second most significant sequence in the inhibited HeLa AS-CDK1 cells. Although at the time promising, now it could be concluded that those genes were probably dysregulated by the off-target effects of the ATP-analogue rather than inhibition of CDK1 itself.

When looking at motifs common for the dysregulated genes in all three 1-NM-PP1-treated cell lines, HEK293, HEK293 AS-CDK1, and HeLa AS-CDK1, the large prevalence of sequences for Krüppel-like factor (KLF) family members can be immediately seen. Out of 17 members of the family, motifs for KLF3, 6, 9, 12, and 15 were highlighted as significant in all three sets of dysregulated genes. These transcriptional factors possess a crucial role in maintaining the pluripotency of the transcriptional landscape in mammalian cells, and because of this they are also involved in reprogramming cells into induced pluripotent stem cells (Bourillot & Savatier, 2010; McConnell & Yang, 2010; K. Takahashi & Yamanaka, 2006). Having this insight, it could be hypothesised whether the decreased pluripotency of mESCs treated with an ATP-analogue could, in part, have been caused by 3-MB-PP1 also affecting members of the KLF family as an additional effect to its inhibition of CDK1 (Michowski et al., 2020).

The ways in which the 1-NM-PP1 drug could affect the KLF transcriptional factors is not entirely clear. The transcriptional activity of several KLF proteins can be controlled through phosphorylation, for instance KLF1 gets activated by casein kinase II, while KLF11 activity is hindered by mitogen-activated protein kinase (MAPK) (McConnell & Yang, 2010). Perhaps 1-NM-PP1 targets a kinase that controls the phosphorylation of the KLF members listed above, although no obvious candidates for this role are currently identifiable. Additionally, KLF15 was also characterised as a protective factor in UPR and autophagy signalling (Jung et al., 2013). Since the most upregulated gene in inhibited HEK293 cells, *CHAC1*, functions in the same pathways, together this could potentially imply that 1-NM-PP1 treatment starts to affect the UPR across different cell types.

Importantly, the motif analysis comparisons between the three different cell lines used for RNA-seq allowed for identification of seven known sequences shared between the HeLa AS-CDK1 and HEK293 AS-CDK1 cells, but not present in the HEK293 dataset. These motifs provided evidence on how CDK1 inhibition with 1-NM-PP1 might affect gene transcription, while subtracting the off-target effects of the drug. The most significantly enriched motifs were those bound by OSR2 and MAZ, with ZN467 and VEZF1 almost as highly enriched.

OSR2 (Odd-Skipped Related Transcription Factor 2) contains a DNA-binding zinc-finger domain and functions in development of several organs (Kawai et al., 2010; Lan et al., 2001). Further, it has an important role in osteoblast proliferation stimulated by vitamin D (Kawai et al., 2007; Verlinden et al., 2013). It was also found that, in mice, induction of *OSR2* expression positively regulates cell proliferation (Kawai et al., 2010). Because of its previously documented upregulation in cells experiencing serum starvation or confluence, which usually arrests the cell cycle at G1, the authors of the study speculated

whether *OSR2* could be also involved in cell differentiation. This hypothesis matches the data gathered during this work, as CDK1 was found to repress differentiation in mouse ES cells (Michowski et al., 2020), and in this investigation *OSR2*-regulated genes were upregulated after its inhibition. The same group has also described the upregulation of *OSR2* mRNA during the state of cellular quiescence (Kawai & Amano, 2010). However, in proliferating bone tumours *OSR2* emerged as one of the most active transcriptional factors, while CDK1 was shown to be also overexpressed in osteosarcomas, contradicting the relationship shown during motif analysis of the RNA-seq data (Mao et al., 2024; Mo et al., 2021).

Little clear-cut evidence is currently available on the possible links between CDK1 and *OSR2*, despite both having an established role in proliferation. The closest connection between the transcriptional factor and the CDK family was found when *OSR2* overexpression increased the levels of CDK6 and cyclin D2 (Kawai et al., 2010). Intriguingly, despite not being identified as a CDK1 target during the inhibition approach in mESCs, *OSR2* was shown to have a CDK1 substrate associated with it during thiopeak labelling of the DNA (Michowski et al., 2020). Of the seven factors found during the motif analysis (**Table 5.1.**), *OSR2*, *MAZ*, *VEZF1*, and *PATZ1* had thiopeaks around their TSS regions, and *PATZ1* was also identified as a potential substrate during the inhibition approach (Michowski et al., 2020). One could therefore speculate that there exists an additional upstream substrate that CDK1 employs to regulate *OSR2* indirectly, leading to subsequent dysregulation of genes identified in this study.

Similar conclusions might be drawn for the regulation of *MAZ* by CDK1. *MAZ* (Myc-associated zinc finger protein) is also a transcription factor implicated in development and other cellular processes (Haller et al., 2018; Medina-Martinez et al., 2020).

Knockdowns of MAZ in both HEK293 and mice during developmental studies showed decreased cell proliferation and Wnt signalling (Haller et al., 2018; Medina-Martinez et al., 2020). Further, abolition of MAZ activity in HEK293 caused an upregulation of cyclin A1, but downregulation of cyclins D1 and A2, with cyclin D1 also depleted in MAZ-lacking mouse models *in vivo* (Haller et al., 2018). The HEK293 cells with knocked-out MAZ also showed a defect in S phase entry (Haller et al., 2018). MAZ was shown to act at both the initiation and termination stages of transcription in *CMYC*, the gene that it was first associated with (Bossone et al., 1992; Marcu et al., 1997; Pyre et al., 1992). It was also found to bind and modulate the promoters of several genes, for instance activating the insulin promoter in HeLa cells (Her et al., 2003; Kennedy & Rutter, 1992; Ray & Ray, 1998). Interestingly, MAZ was also able to alter the pol II elongation complex to affect splicing, and complemented CTCF to modify genome organisation and cohesion sequestering (Robson-Dixon & Garcia-Blanco, 2004; Xiao et al., 2021).

Because of MAZ's paired functions as a transcriptional activator and repressor, it is difficult to speculate how CDK1 could exert control over gene expression through the actions of this transcription factor. MAZ positive regulation of the cell cycle also seems counterintuitive, as downregulation of its activity by CDK1, as implied by the RNA-seq, would act to decrease cell proliferation, perhaps creating an indirect negative feedback mechanism with the mitotic kinase. Moreover, MAZ overexpression promotes progression of a number of tumours, so its repression by CDK1 is also in contrast to the oncogenic actions of CDK1 itself (Maity et al., 2018; M. Wang et al., 2023; C. Zheng et al., 2022). Perhaps, therefore, the regulation of *MAZ* and *OSR2* by CDK1 is not necessarily connected to tumorigenesis, especially that their motifs were found in genes dysregulated both in HeLa AS-CDK1, and in non-cancerous HEK293 AS-CDK1 cells. The two transcription factors identified during this study were not found in any previous

lists of mammalian CDK1 substrates (Massacci et al., 2023; Michowski et al., 2020; Petrone et al., 2016; Q. Wang et al., 2023). This, together with the dysregulation of gene expression occurring only after 2h inhibition of CDK1, could indicate that they are not directly phosphorylated by CDK1, but rather, that they act downstream of its direct substrates.

It is important to remember that the Gene Set Enrichment Analysis did not show any significantly dysregulated gene groups in any of the chrRNA-seq data sets. This was further true for the examination of gene sets associated with individual transcription factors. Although the small groups of upregulated genes in 1-NM-PP1-treated HeLa AS-CDK1 or HEK293 AS-CDK1 cells were enriched for specific TF motifs, the overall target gene sets of these TFs showed no significant dysregulation. Possibly, the remaining targets of such transcription factors require longer experimental timepoints to become deregulated, or their expression is controlled by additional balancing agents.

The GSEA database was also investigated to examine whether any known targets of the highlighted TFs correspond to dysregulated genes in the chrRNA-seq experiments. Curiously, only one specific gene – *FGF17*, a known target of PATZ1 – was also significantly upregulated in the inhibited HEK293 AS-CDK1 cells. This suggests that many upregulated genes in 1-NM-PP1-treated AS-CDK1 cells may not yet be classified as official targets of the seven identified transcription factors. *FGF17* encodes for fibroblast growth factor 17, which is expressed primarily during embryonic development and plays an important role in brain formation (Hoshikawa et al., 1998; Zanni et al., 2011; X. Zhang et al., 2006). Additionally, its expression is also activated during limb development, potentially tying it to the actions of OSR2 in limb patterning (X. Zhang et al., 2006). Although expressed primarily during embryonic stages, its recorded role in

morphogenesis and upregulation following CDK1 inhibition could correspond to the possible role of CDK1 as a differentiation repressor. However, *FGF17* is also overexpressed in different types of cancer, such as prostate, where it is said to stimulate tumour growth (Heer et al., 2004; Ling & Du, 2022; Polnaszek et al., 2004). It remains to be elucidated therefore how the downregulation of *FGF17* by CDK1 influences the balance between cellular proliferation and differentiation.

The identification of CDK1 substrates is performed in two major ways in literature. Firstly, there exists the inhibition approach, where CDK1 (or AS-CDK1) is inhibited and the dysregulated proteins are then identified. On the other hand, there are phosphoproteomics methods, that directly label the downstream substrates of CDK1. However, no method is without its limitations – as was seen here, the inhibition approach can be susceptible to the off-target effects of the drugs of choice, but the different labelling approaches also deal with a level of background signal that needs to be eliminated. Currently, studies often try to mix the different methods to establish lists of de facto substrates of CDK1. Frequently, the substrate lists are restricted to contain only proteins that possess the CDK1 consensus sequence of S/T-P (a consensus sequence common for a wide variety of proline-directed kinases), as was in the case of the mESCs investigation (Michowski et al., 2020). However, such methods might overlook some of the lesser-known CDK1 substrates, as it is starting to be more widely-recognised that not all CDK1 targets contain the S/T-P consensus motif (Suzuki et al., 2015).

It seems that many CDK1 targets, especially those not connected to the cell cycle, or those which are cell type-dependent, still remain to be described. The trend is only exacerbated for non-mitotic proteins which could be connected to CDK1, but are not their direct substrates. The work presented here underscores the value of employing controls across

multiple models, and mechanisms of inhibition, to create results that help gain better understanding of actions of CDK1. The limited effects seen in HeLa and HEK293 cells when CDK1 was inhibited suggest that, in contrast to some of the other cell types like mESCs, the transcriptional role of CDK1 in these cells might be more ambiguous and needs more time to exhibit its effects. Although more work needs to be conducted to elucidate the exact molecular mechanisms by which CDK1 can affect gene transcription through the actions of *OSR2*, *MAZ*, or other identified factors, the experiments presented in this part of the thesis contribute to the characterisation of transcriptional functions of CDK1 outside of the cell cycle, as well as advance our understanding of the limitations connected to using ATP-analogues such as 1-NM-PP1 in studying protein kinases.

6.CONCLUDING REMARKS

6.1. Conclusions

The work presented in this thesis highlights the value of using the AS-CDK1 system for studying the transcriptional functions of CDK1, while providing crucial insights into the possible limitations of this approach. Most of the existing literature on CDK1 focuses on its role in mitosis, but its non-mitotic functions are now gradually gaining more interest. Because of this, there is a need for robust and reliable systems for examining additional functions of this kinase. Previous studies of CDK1 have described some transcriptional roles of this kinase. However, they are mostly focused on transcriptional silencing during mitosis. Despite some hints pointing at CDK1 exerting transcriptional control also in interphase, it is not as well-understood. Whereas the biological rationale for mitotic transcriptional silencing is fairly clear, it remains to be seen how CDK1 could influence transcription during the remaining parts of the cell cycle and for what purpose.

Due to its role as a transcriptional repressor in mouse embryonic stem cells, as well as its overexpression in several types of cancer, it was speculated that in tumours CDK1 could also downregulate interphase gene expression to maintain the proliferative, undifferentiated landscape characteristic of those cells. This hypothesis was of particular interest, as if proven true, dose-dependent inhibition of CDK1 could decrease its transcriptional functions in cancer, while maintaining enough activity to orchestrate mitosis in healthy tissues.

The task of creating new models for studying CDK1 function was undertaken using CRISPR, and led to the generation of two new AS-CDK1 cell lines: HCT116 CDK1^{WT/AS} and HEK293 AS-CDK1. The essential status of CDK1 meant that modifications to the endogenous kinase were especially challenging. Trialling different gene editing methods ultimately ended in the characterisation of a successful CRISPR-Cas9 pipeline, which

could potentially be used for the creation of additional AS-CDK1 cell lines in the future. Importantly, this work established a new unique screening method for AS-CDK1 cells, which involves their inhibition with an ATP-analogue and additional treatment with an antimitotic drug to positively select for viable mutants. Based on the available information, the two new cell lines described in this study are also the first human AS-CDK1 cell lines in which CDK1 was mutated endogenously. As the genome editing of new AS-CDK1 cell lines was time-consuming, HeLa AS-CDK1 cells were employed at first to study the potential transcriptional roles of CDK1.

The data collected during this study managed to expand on the current restricted understanding of CDK1 functions in transcription, by utilising a range of CDK1 inhibition models and drugs. Although within the given timeframe limited evidence was gathered on the exact molecular mechanisms which connect CDK1 to transcriptional control in cancerous and non-cancerous cells, the investigation contributed to creating a stronger framework for any future CDK1 and AS-CDK1 studies. It also hinted at novel CDK1 effectors that could function universally across different cell lines.

Performing chrRNA-seq on unmodified HeLa cells would allow comparison between genes dysregulated in HeLa and HeLa AS-CDK1, potentially identifying cancer-specific pathways transcriptionally affected by CDK1. Correspondingly, an RNA-seq analysis of 1-NM-PP1-treated HCT116 CDK1^{WT/AS} cells, or a future HCT116 AS-CDK1 cell line, would help test the generality of such findings across different cancer types, with potential implications for future therapies targeting CDK1.

Despite ultimately finding several factors that could mediate the effects of CDK1 in transcription, the very limited dysregulation of gene expression in CDK1-inhibited cells leads to the conclusion that the transcriptional roles of CDK1 in interphase are quite

specialised. This could be due to a possible degree of redundancy exhibited by CDK1 and other transcriptional kinases. Alternatively, CDK1 could primarily perform interphase functions by phosphorylating proteins in signalling cascades, that do not necessarily involve changes at a transcriptional level. That being said, the studies characterising substrates of CDK1 have classified many of them as proteins involved in transcription and chromatin maintenance. It is also clear, as was hinted at in previous literature, that the roles of CDK1 depend heavily on the type of cell being investigated. It is also hard to exclude the possibility of publication bias against negative findings.

Lastly, despite the obstacles encountered during the use of the AS-CDK1 approach, the value of this method for studying CDK function was upheld. In this project, utilising comparisons between different cell lines and ATP-analogues helped to discriminate a lot of the background caused by the off-target effects of 1-NM-PP1. Above all, the gathered data underscored the importance of having a wild-type control when performing AS kinase studies, even if the principle behind this method should make such a comparison obsolete. Taken together, this data helped shed more light on the limitations that come with using the AS kinase methodology, and will hopefully contribute to enhancing the efficacy of future kinase research.

The redundancy between individual family members and low selectivity of inhibitors have made investigation of CDK roles challenging, but their molecular functions are now coming more sharply into focus. The transcriptional functions of CDK1, although certainly elusive, will without a doubt remain an enticing avenue of research, that will greatly benefit from the constant improvement of the AS kinase technology.

6.2. Future directions

Due to the novelty of the project, substantial time was spent on optimising the experimental set-up and models before any transcriptional roles of CDK1 began to be uncovered. Because of this, several important lines of questioning remain, which when answered, could help create a more complete picture of the cellular relationships found over the course of this investigation.

Primarily, it would be crucial to try and elucidate the molecular mechanisms linking CDK1 to some of the identified transcription factors, such as OSR2 or MAZ. Based on the collected data, it is probable that these are regulated indirectly by an intermediate substrate of CDK1, and it would be valuable to establish a possible chain of signalling that leads from the abolition of CDK1 activity to the upregulation of genes controlled by OSR2 or MAZ. It would be also interesting to determine the exact downstream genes affected by the identified transcription factors, and what kind of phenotypic changes they cause in the cells by, for instance, upregulating and downregulating the activities of the TFs themselves. Production of HCT116 CDK1^{AS/AS} cells would help to confirm whether these relationships exist in another experimental model.

Importantly, the results found during this study should be translated to synchronised cells, to truly focus on the non-mitotic, interphase actions of CDK1. The initial steps to establish the necessary workflow were undertaken during this project, however, the subsequent difficulties with 1-NM-PP1 specificity and lack of gene expression changes following CDK1 inhibition meant that this part of the investigation was not possible in the given timeframe. Having clearer links on how CDK1 influences transcription, it could be now determined if the changes are temporally-localised to one of the interphase stages: G1, S, or G2.

Over the duration of this project, the focus has also shifted from CDK1 transcriptional regulation of cancer cells to gene expression regulation in HEK293 cells, due to the problems with finding and creating a reliable AS-CDK1 model in cancer cells. That being said, it would still be extremely valuable to return to this original goal, by for example conducting RNA-seq analyses of HeLa, HCT116, and HCT116 AS-CDK1 cell lines. These would hopefully provide more possible transcription factor motifs that when compared with the previous HeLa AS-CDK1 data could help discriminate: 1) sequences that occur both in HeLa AS-CDK1 and HeLa cells, making them likely 1-NM-PP1 targets, and segregating the remaining more viable CDK1 targets in HeLa AS-CDK1; 2) sequences common between HCT116 and HCT116 AS-CDK1 cells for the same reasons; 3) sequences that are affected in both HeLa AS-CDK1 and HCT116 AS-CDK1 cells, but not unmutated cells, to find transcriptional mechanisms common between different types of cancers. The examination of molecular mechanisms regulating such targets would surely provide further insight into how CDK1 overexpression could affect the transcriptional landscape in cancer cells, and help confirm the hypothesis whether this is achieved through regulation of differentiation pathways. In the long term, the dose-dependent inhibition of CDK1 could be investigated to try and pharmacologically uncouple the cell-cycle and transcriptional functions of this kinase. Even further, the actions of the FDA-approved CDK inhibitors such as palbociclib on CDK1 could be examined in a cancer context.

To enhance the scope of this project and fully utilise the possibilities provided by the AS technology, the AS-CDK1 inhibition approach could be supplemented by phosphoproteomics to gain a better understanding of CDK1 function in cancer cells and beyond. Employing techniques such as stable isotope labelling by amino acid in cell culture (SILAC), could potentially provide clues on how the proteome changes following

the inhibition of CDK1. On the other hand, thiophosphate labelling approach could help with the characterisation of direct targets phosphorylated by CDK1, which when identified, could perhaps provide the missing link to the regulation of transcriptional factors such as OSR2 and MAZ.

The proposed studies would certainly contribute to advancing our understanding of the transcriptional functions of CDK1 in interphase in cancer cells, with the view of contributing the acquired knowledge to the development of new therapeutic opportunities.

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