

# Role of Chk1 and Chk2 in mitotic checkpoint control in vertebrate cells

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## Abstract

Two conserved protein kinases, Chk1 and Chk2, are activated in response to genotoxic stress and mediate multiple cell cycle checkpoint mechanisms that ensure genomic integrity. The establishment of mitotic checkpoint delay in response to DNA damage or incompletely replicated DNA is conventionally thought to be accomplished through inhibition of the cyclin-dependent kinase, Cdc2. Both Chk1 and Chk2 have the potential to operate in this pathway. Therefore, the initial aim of this study was to investigate the relative requirement of Chk1 and Chk2 for mitotic delay mechanisms triggered by DNA damage and DNA replication arrest in avian and human cells.

These studies demonstrated that Chk1 is the principal regulator of the G2/M checkpoint with a direct role in the establishment and maintenance of the mitotic delay in response to DNA damage. Chk1 was also found to be required for the S/M checkpoint in response to DNA replication arrest; however, detailed analysis indicated that its role is to maintain rather than initiate this checkpoint, as cells lacking functional Chk1 can initially delay mitosis for many hours before they enter a premature mitosis with unreplicated DNA.

In avian cells, mitotic phosphorylation of cyclinB2 was found to be mediated by cyclin dependent kinases and suppressed by checkpoint signalling. However, accumulation of potentially active phospho-cyclinB2/Cdc2 complexes was observed during the initial mitotic delay in the absence of functional Chk1, suggesting that other factors apart from the conventionally known mechanisms can restrain mitotic Cdc2 activity. In addition, avian cells were able to delay mitosis effectively during replication arrest in the presence of the ATM/ATR inhibitor caffeine, further emphasizing the possibility of mitotic delay mechanisms that operate independently of ATM/ATR and Chk1.

Furthermore, this study revealed that endogenous Cdc6 accumulates in a Chk1-dependent manner during replication arrest. To test whether Cdc6 might function upstream or downstream of Chk1 in the replication checkpoint pathway, Cdc6 was ectopically expressed in both checkpoint-proficient and checkpoint-deficient Chk1-depleted cells. The results from these intervention experiments give preliminary evidence that places Cdc6 downstream of Chk1 in the S/M checkpoint response.

The ability of cells to delay the onset of mitosis while DNA replication is stalled independently of ATM/ATR/Chk1 is consistent with the general idea of an inherent relationship between the process of DNA replication and mitosis. The replication

machinery might be able to signal either normal DNA replication in progress or the presence of stalled replication structures and thereby intrinsically link the successful completion of DNA synthesis to progression into mitosis.

# Table of Contents

ABSTRACT .....	2
TABLE OF CONTENTS .....	4
LIST OF TABLES .....	7
LIST OF FIGURES .....	8
ACKNOWLEDGEMENTS.....	10
AUTHOR'S DECLARATION.....	11
ABBREVIATIONS .....	12
<b>1 INTRODUCTION .....</b>	<b>14</b>
1.1 DNA Damage and replication checkpoints .....	14
1.2 Checkpoint pathways in fission yeast .....	15
1.2.1 Outline of the checkpoint signalling cascade in fission yeast .....	17
1.2.2 Chk1 and Cds1 kinases in fission yeast.....	18
1.2.3 Targets of the mitotic checkpoint mechanisms .....	20
1.2.4 DNA replication and checkpoint signalling .....	21
1.3 Checkpoint pathways in budding yeast.....	23
1.4 Checkpoint pathways in vertebrate cells .....	24
1.4.1 Sensing checkpoint-activating DNA structures.....	24
1.4.2 Checkpoint kinases Chk1 and Chk2.....	30
1.4.3 Targets of cell cycle checkpoint signalling .....	33
1.5 Mitotic checkpoint signalling pathways.....	34
1.5.1 The G2/M checkpoint in response to DNA damage .....	34
1.5.2 The S/M checkpoint in response to DNA replication arrest.....	41
1.5.3 Replication Recovery functions .....	43
1.5.4 DNA replication and checkpoint control.....	43
1.6 Checkpoint signalling and cancer therapy.....	47
1.7 Project aims .....	47
<b>2 MATERIALS AND METHODS.....</b>	<b>49</b>
2.1 Materials .....	49
2.1.1 Chemicals and reagents .....	49
2.1.2 Enzymes and kits.....	49
2.1.3 Bacteriology .....	49
2.1.4 Cell culture .....	50
2.1.5 Antibodies .....	51
2.1.6 Immunofluorescence .....	52
2.1.7 Plasmids, Primers, Probes .....	52
2.1.8 siRNA sequences.....	54
2.1.9 Miscellaneous.....	55
2.2 Methods .....	56
2.2.1 Bacteriology .....	56
2.2.2 DNA protocols .....	57
2.2.3 Cell Culture .....	59
2.2.4 Elutriation of living cells.....	61
2.2.5 Protein Extraction and Analysis .....	61
2.2.6 Flow cytometry protocols.....	64
2.2.7 Immunofluorescence .....	65

<b>3 RESULTS: REQUIREMENT FOR CHK1 AND CHK2 FUNCTION FOR S/M AND G2/M CHECKPOINTS.....</b>	<b>67</b>
3.1 Mitotic checkpoint responses in avian DT40 cells.....	67
3.1.1 Analysis of mitotic delay proficiency in DT40 WT, Chk1 <sup>-/-</sup> , and Chk2 <sup>-/-</sup> knock-out cell lines in response to replication block and DNA damage .....	67
3.1.2 Analysis of mitotic delay proficiency in Chk1 mutant cell lines in response to replication block and DNA damage .....	72
3.1.3 Analysis of Chk1 activation in response to replication stress and DNA damage .....	78
3.2 Mitotic checkpoint responses in human BE cells.....	80
3.2.1 Requirement of Chk1 for S/M checkpoint proficiency in BE cells.....	80
3.2.2 Analysis of Chk1 and Chk2 requirement for mitotic checkpoint proficiency in BE cells.....	85
3.3 Discussion .....	90
<b>4 RESULTS: THE G2/M AND S/M CHECKPOINTS ARE MECHANISTICALLY DISTINCT.....</b>	<b>94</b>
4.1 Kinetic analysis of mitotic checkpoint proficiency .....	94
4.1.1 Comparison of mitotic checkpoint responses in asynchronous DT40 WT and Chk1 <sup>-/-</sup> cell cultures .....	94
4.1.2 Comparison of mitotic checkpoint responses in BE cells .....	95
4.1.3 Comparison of mitotic checkpoint responses in synchronised DT40 WT and Chk1 <sup>-/-</sup> cells .....	102
4.2 Timing of S/M checkpoint failure in absence of Chk1 and Chk2 activity.....	108
4.2.1 Attempts to generate a Chk1/Chk2 double knock-out DT40 cell line .....	108
4.3 Effect of ATM/ATR inhibition with caffeine on mitotic checkpoint responses ...	112
4.3.1 Effect of caffeine on S/M and G2/M checkpoint responses in BE cells .....	114
4.3.2 Effect of caffeine on mitotic checkpoint proficiency in DT40 WT and Chk1 <sup>-/-</sup> cells.....	116
4.3.3 Effect of caffeine on the timing of S/M checkpoint failure in Chk1 <sup>-/-</sup> cells.	122
4.3.4 Timing of S/M checkpoint failure in Chk1 mutant cells is not affected by expression of catalytically inactive or S345A Chk1 .....	124
4.4 Discussion .....	126
<b>5 RESULTS: BIOCHEMICAL ANALYSIS OF POTENTIAL S/M AND G2/M CHECKPOINT CONTROL MECHANISMS .....</b>	<b>131</b>
5.1 Analysis of Cdc2 kinase activity during mitotic checkpoint responses in DT40 WT and Chk1 <sup>-/-</sup> cells .....	131
5.2 Biochemical analysis of Cdc2/CyclinB2 regulation during an unperturbed cell cycle in DT40 WT and Chk1 <sup>-/-</sup> cells.....	134
5.3 Biochemical analysis of Cdc2/CyclinB2 regulation during mitotic checkpoint responses .....	138
5.4 CyclinB2 phosphorylation is a target of mitotic checkpoints in DT40 cells.....	143
5.5 CyclinB2 phosphorylation is a target of Cdks in the DT40 cells.....	145
5.6 Discussion .....	147
<b>6 RESULTS: DNA REPLICATION AND CHECKPOINT CONTROL .....</b>	<b>150</b>
6.1 Documentation of the relationship between DNA replication and entry to mitosis ...	150
6.1.1 Analysis of the timing of PCNA loss and the onset of mitosis .....	150
6.1.2 PCNA distribution during an unperturbed cell cycle .....	151
6.1.3 PCNA distribution during normal and premature mitosis.....	156

6.2	Checkpoint regulation of Cdc6.....	160
6.2.1	Role for Cdc6 in the S/M checkpoint response in BE cells .....	160
6.2.2	Regulation of Cdc6 during an unperturbed cell cycle in BE cells .....	162
6.2.3	Regulation of Cdc6 stability by Chk1 in BE cells.....	164
6.2.4	Role of Cdc6 during S/M checkpoint - Intervention experiments .....	169
6.3	Discussion .....	171
<b>7</b>	<b>CONCLUSIONS AND FUTURE PERSPECTIVES .....</b>	<b>175</b>
	<b>LIST OF REFERENCES .....</b>	<b>185</b>

## List of Tables

Table 1: Primary and secondary antibodies .....	51
Table 2: siRNA duplex sequences.....	54
Table 3: SDS PAGE resolving and stacking gels.....	62

## List of Figures

Figure 1.1: DNA damage and replication checkpoints .....	16
Figure 1.2: Outline of the checkpoint signalling cascade in fission yeast.....	19
Figure 1.3: Targets of the mitotic checkpoint mechanisms in fission yeast.....	22
Figure 1.4: Sensing and transducing DNA damage in vertebrate cells.....	29
Figure 1.5: Sensing and transducing the checkpoint signal from DNA lesions induced by DNA replication arrest .....	31
Figure 1.6: Model for Cdc2 regulation in vertebrate cells .....	37
Figure 1.7: Model for the establishment of mitotic delay in response to DNA damage.....	40
Figure 1.8: Model for the establishment of mitotic delay in response to replication arrest	42
Figure 1.9: Initiation of DNA replication in vertebrates .....	45
Figure 3.1: Measurement of S/M checkpoint proficiency by flow cytometry .....	68
Figure 3.2: S/M and G2/M checkpoint proficiency in DT40 WT, Chk1 <sup>-/-</sup> , Chk2 <sup>-/-</sup> cell lines .....	70
Figure 3.3: Expression levels of Chk1 and mutant derivatives in DT40 cell lines .....	73
Figure 3.4: S/M and G2/M checkpoint proficiency in Chk1 Revertant and Kinase Dead cell lines .....	74
Figure 3.5: S/M and G2/M checkpoint proficiency in Chk1 phosphorylation mutant cell lines .....	75
Figure 3.6: Comparison of S/M and G2/M checkpoint proficiency in DT40 cell lines.....	77
Figure 3.7: Chk1 phosphorylation in response to replication stress and DNA damage.....	79
Figure 3.8: Analysis of effect of Chk1 inhibition with UCN-01 on S/M checkpoint proficiency.....	81
Figure 3.9: Analysis of Chk1 down-regulation with siRNA.....	83
Figure 3.10: Comparison of effect of Chk1 siRNA and Chk1 inhibitor UCN-01 on S/M checkpoint proficiency .....	84
Figure 3.11: Analysis of S/M and G2/M checkpoint proficiency in BE cells depleted of Chk1 or Chk2 function.....	87
Figure 3.12: Analysis of S/M and G2/M checkpoint proficiency in BE cells depleted of Chk1 and Chk2 function.....	88
Figure 3.13: Comparison of S/M and G2/M checkpoint proficiency in BE cells depleted of Chk1 and/or Chk2 expression .....	89
Figure 4.1: Kinetic analysis of mitotic checkpoint responses in DT40 WT cells .....	96
Figure 4.2: Kinetic analysis of mitotic checkpoint responses in Chk1 <sup>-/-</sup> cells .....	97
Figure 4.3: Comparison of kinetic analysis of mitotic checkpoint responses in DT40 WT and Chk1 <sup>-/-</sup> cells .....	98
Figure 4.4: Kinetic analysis of mitotic checkpoint responses in BE cells .....	100
Figure 4.5: Quantification of kinetic analysis of mitotic checkpoint responses in BE cells .....	101
Figure 4.6: Mitotic checkpoint responses in G1/S synchronised DT40 WT cells .....	103
Figure 4.7: Mitotic checkpoint responses in G1/S synchronised Chk1 <sup>-/-</sup> cells .....	105
Figure 4.8: Comparison of mitotic checkpoint responses in DT40 WT and Chk1 <sup>-/-</sup> cells	107
Figure 4.9: Schematic overview of Chk2 gene targeting vector and knock-out strategy..	109
Figure 4.10: Analysis of Chk2 targeting event by PCR and Southern blotting .....	111
Figure 4.11: Effect of caffeine treatment on Chk1 phosphorylation.....	113
Figure 4.12: Effect of ATM/ATR inhibition with caffeine on mitotic checkpoint responses in BE cells .....	115

Figure 4.13: Effect of ATM/ATR inhibition with caffeine on mitotic checkpoint responses in DT40 WT cells .....	117
Figure 4.14: Comparison of the effect of ATM/ATR inhibition with caffeine on mitotic checkpoint responses in DT40 WT cells .....	118
Figure 4.15: Effect of ATM/ATR inhibition with caffeine on mitotic checkpoint responses in Chk1 <sup>-/-</sup> cells .....	120
Figure 4.16: Comparison of the effect of ATM/ATR inhibition with caffeine on mitotic checkpoint responses in Chk1 <sup>-/-</sup> cells .....	121
Figure 4.17: Effect of ATM/ATR inhibition with caffeine on the timing of S/M checkpoint failure in Chk1 <sup>-/-</sup> cells.....	123
Figure 4.18: Timing of S/M checkpoint failure in Chk1 mutants .....	125
Figure 5.1: Cdc2 kinase activity in checkpoint assays in synchronised DT40 cells .....	132
Figure 5.2: Centrifugal Elutriation of DT40 WT cells .....	135
Figure 5.3: Cdc2/CyclinB2 regulation during unperturbed cell cycle in DT40 WT and Chk1 <sup>-/-</sup> .....	137
Figure 5.4: Analysis of CyclinB2 regulation and during mitotic checkpoint responses in DT40 WT and Chk1 <sup>-/-</sup> cells.....	140
Figure 5.5: Analysis of Cdc2 regulation and inhibitory phosphorylation on T14/Y15 during mitotic checkpoint responses in DT40 WT and Chk1 <sup>-/-</sup> cells .....	142
Figure 5.6: CyclinB2 phosphorylation is a checkpoint target in cells with functional Chk1 .....	144
Figure 5.7: Analysis of cyclinB2 phosphorylation in DT40 WT and Chk1 <sup>-/-</sup> cells treated with various protein kinase inhibitors .....	146
Figure 6.1: Flow cytometry analysis of PCNA expression in DT40 WT and Chk1 <sup>-/-</sup> cells .....	152
Figure 6.2: Expression and localisation of PCNA during an unperturbed cell cycle.....	154
Figure 6.3: Time-lapse analysis of PCNA distribution during an unperturbed cell cycle	155
Figure 6.4: Analysis of PCNA distribution in DT40 WT and Chk1 <sup>-/-</sup> cells.....	157
Figure 6.5: Analysis of PCNA distribution during S/M checkpoint failure in Chk1 <sup>-/-</sup> cells .....	158
Figure 6.6: Accumulation of endogenous Cdc6 during replication arrest in BE cells .....	161
Figure 6.7: Analysis of Cdc6 protein levels throughout an unperturbed cell cycle in BE cells.....	163
Figure 6.8: Inhibition of the proteasome with MG 132 stabilises Cdc6 protein levels.....	165
Figure 6.9: Analysis of Cdc6 half life in BE cells.....	167
Figure 6.10: Effect of Cdc6 over-expression on S/M checkpoint response in BE cells ...	170
Figure 7.1: Model for mitotic delay mechanisms in response to DNA damage .....	177
Figure 7.2: Model for mitotic delay mechanisms in response to DNA replication block.	183

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## Author's Declaration

I am the sole author of this thesis. All the work presented in this thesis was performed by myself, unless otherwise stated.

## Abbreviations

A	Alanine
APC	Anaphase promoting complex
ATM	Ataxia telangiectasia mutated
ATP	Adenosine triphosphate
ATR	Ataxia telangiectasia and Rad3-related
ATRIP	ATR-interacting protein
BRCA1	Breast cancer gene 1
BrdU	5-Bromo-2-deoxyuridine
CAK	Cdc2-activating kinase
Cdc2	also known as Cdk1, cyclin-dependent kinase 1
Cdk	Cyclin-dependent kinase
Dapi	4',6-diamidino-2-phenylindole
DMEM	Dulbecco's Modified Eagle Medium
dNTP	deoxyribonucleotides
FHA	Forkhead-associated domain
FITC	Fluorescein isothiocyanate
H	Histone
IR	Ionising radiation
K	Lysine
MAPK	Mitogen-activated protein kinase
MDC1	Mediator of DNA damage checkpoint 1
MRE11	Meiotic recombination 11
MRN	MRE11-Rad50-NBS1
NBS1	Nijmegen breakage syndrome 1
NHEJ	Non-homologous end joining
ORC	Origin-recognition complex
P	Phospho-/Phosphorylation
PAGE	Polyacrylamide gel electrophoresis
PCNA	Proliferating Cell Nuclear Antigen
PCR	Polymerase Chain Reaction
PE	Phycoerythrin
PI	Propidium Iodide
PIKK	Phosphoinositide 3-kinase related kinase
P-S10-H3	Phosphorylation of Serine 10 on Histone H3

R	Arginine
RFC	Replication factor C
RPA	Replication protein A
RT	Room temperature
S	Serine
SDS	Sodium dodecyl sulphate
T	Threonine
TopBP1	Topoisomerase binding protein 1
TRITC	Tetramethyl Rhodamine Isothiocyanate
UCN-01	7-hydroxy-staurosporine
UV	Ultra-violet
WT	Wild-type
Y	Tyrosine
53BP1	p53 binding protein 1
9-1-1	Rad9-Hus1-Rad1

# 1 Introduction

The progression of normal cells into cancer cells is accompanied by a series of genetic changes. The deregulation of cell cycle components has been implicated in tumorigenesis. One of the major aims during development is to ensure the fidelity of cell division without any damage to the integrity of the genome (Nyberg et al., 2002). To preserve genomic stability, cells have developed a variety of mechanisms that enable them to deal with any endogenous and exogenous assaults. These surveillance mechanisms ensure the correct sequence of cell cycle events and also coordinate repair mechanism; they are commonly known as cell cycle checkpoints (Hartwell and Weinert, 1989).

## 1.1 DNA Damage and replication checkpoints

In response to genotoxic stress, cells can activate various checkpoint responses which will delay the progression of the cell cycle until any lesions have been resolved. Depending on the type of stress as well as the position in the cell cycle, several checkpoints can be triggered, that can halt cell cycle progression, induce repair processes, or activate cell death mechanisms. The eukaryotic cell cycle can be divided into interphase, in which cells grow, accumulate nutrients and replicate their DNA, and mitosis, the process of cell division which results in the production of two daughter cells. Interphase consists of three stages: the first gap or growth phase G<sub>1</sub>, which is followed by S-phase in which DNA synthesis takes place and another gap or growth phase G<sub>2</sub>. Multiple cell cycle checkpoints have been identified throughout the cell cycle, during each of these phases and also at transition stages in between (Elledge, 1996; Hartwell and Weinert, 1989).

Both exogenous and endogenous factors can lead to DNA damage, for example ionising radiation (IR) can result in DNA single- and double-strand breaks whereas metabolic processes can lead to oxidation of the DNA. In general, checkpoint proficient cells will attempt to repair the damage before cells progress any further through the cell cycle. If the damage is un-repairable, other potential responses include the initiation of programmed cell death or even adaptation and re-entry into the cell cycle. DNA damage checkpoints are thought to constantly monitor the DNA for lesions and are presumably active at all times. The simple model in Figure 1.1 illustrates three of the best characterized DNA damage checkpoint responses that act at the G<sub>1</sub>/S transition, but also throughout S phase, and at the G<sub>2</sub>/M transition. Relevant in particular for this study of mitotic checkpoint mechanisms is

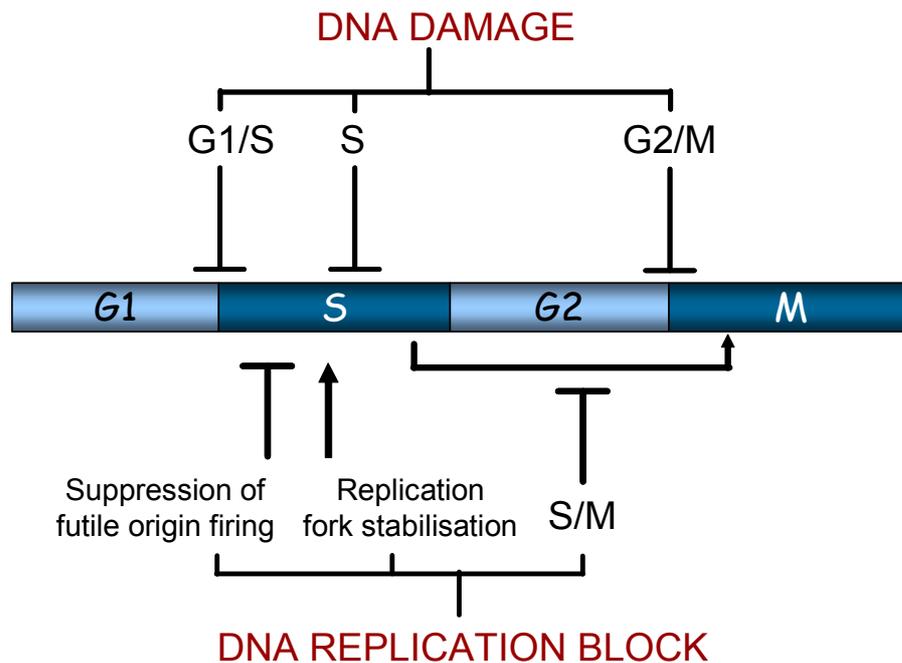
the G2/M checkpoint which can arrest cells in G2 phase and thereby prevent cell division in the presence of damaged DNA.

Replication forks will slow down or halt their progress if they encounter any obstacles, such as DNA adducts or when DNA precursor pools are limiting. Drugs such as hydroxyurea or aphidicolin are commonly used to block DNA replication in experimental systems. In general, blocks to the replication machinery can activate at least three checkpoint responses: the stabilisation of stalled replication forks and the suppression of futile origin firing which can be grouped into the replication recovery functions (Figure 1.1). The third response, which is very much relevant for this study, the S/M checkpoint, delays the onset of mitosis in the presence of unreplicated DNA.

The known checkpoint proteins that are involved in the execution of cell cycle checkpoint mechanisms can be broadly divided into sensors, transducers, mediators and effectors, although some proteins fit in more than one category. Initially, sensor proteins detect damaged DNA or stalled replication structures and set a signalling cascade in motion that includes transducer proteins which transmit and amplify the checkpoint signal to downstream effector proteins which are directly involved in cell cycle progression, repair mechanisms or apoptosis (Nyberg et al., 2002; Sancar et al., 2004). The majority of checkpoint proteins and signalling pathways have initially been identified and characterised in budding and fission yeast. As many checkpoint proteins are conserved from yeast to higher organisms, it was assumed that most of these observations could be extrapolated, however, although many general checkpoint properties are indeed similar, significant differences were also identified. Therefore, general checkpoint mechanisms will be introduced first with a focus on the model organisms *Schizosaccharomyces pombe* (fission yeast) and *Saccharomyces cerevisiae* (budding yeast). After that, the current knowledge of these mechanisms in higher eukaryotes will be discussed.

## 1.2 Checkpoint pathways in fission yeast

Fission yeast has been a powerful model organism for cell cycle research in general and for the investigation of cell cycle checkpoint mechanisms in particular as they are easy to grow and manipulate. There are basically two major checkpoint responses in fission yeast: during S phase, replication stress can create structures such as stalled replication forks which result in the activation of the replication recovery functions and lead to mitotic delay (S/M checkpoint), whereas the DNA damage checkpoint response delays cells in G2 phase from entering mitosis with damaged DNA (G2/M checkpoint).



**Figure 1.1: DNA damage and replication checkpoints**

Schematic overview of the phases of the cell cycle and the most relevant cell cycle checkpoints activated in response to either DNA damage (i.e. through Irradiation) or DNA replication block (i.e. with the DNA polymerase inhibitor aphidicolin) throughout the cell cycle. In response to DNA damage, cell cycle checkpoints can be induced at the G1/S transition, throughout S phase, and at the G2/M transition. In response to DNA replication block, cells can activate the replication recovery functions, such as the suppression of futile origin firing and the stabilisation of stalled replication forks. In addition, the S/M checkpoint ensures that cells do not enter mitosis in the presence of unreplicated DNA.

### **1.2.1 Outline of the checkpoint signalling cascade in fission yeast**

Several proteins responsible for monitoring aberrant DNA structures have been identified in fission yeast, most notably the Rad9, Hus1, Rad1, and Rad17 proteins. These four proteins are structurally related to replication proteins. The 9-1-1 complex consists of the three proteins: Rad9-Hus1-Rad1 which were found to share sequence similarity with the proliferating cell nuclear antigen (PCNA), a DNA polymerase processivity factor, and form a trimeric complex that structurally resembles a PCNA trimer (Thelen et al., 1999). The Rad17 protein contains five domains with sequence similarity to the subunits of replication factor C (RFC) and was shown to interact with four RFC subunits and form a pentameric complex Rad17-RFC (Griffiths et al., 1995). In analogy to the replication proteins, it is possible that the 9-1-1 complex might slide along the DNA scanning for sites of DNA damage or stalled replication forks. A model has been proposed where the Rad17-RFC complex can load the 9-1-1 complex onto the DNA as a checkpoint sliding clamp. Upon ATP-binding the clamp loading complex can undergo conformational changes that result in the opening of the complex allowing DNA binding (Venclovas et al., 2002).

In addition to these two sensory complexes there are two more Rad-proteins, Rad3 and Rad26, which are homologues of vertebrate ATR and ATRIP and are also involved in sensing DNA lesions. Importantly, Rad3 is the phosphoinositide 3-kinase related kinase (PIKK) that becomes activated in response to genotoxic stress and transduces the checkpoint signal to downstream effector kinases Chk1 and Cds1 (the fission yeast homologue of vertebrate Chk2). Thus, Rad3 has both sensor and transducer properties. Most parts of the signalling pathway, including Chk1 and Cds1 activation, are dependent on the Rad17-RFC and 9-1-1 complexes, but the phosphorylation of Rad26 by Rad3 in response to ionising radiation does not require the two sensor complexes, suggesting that Rad3 and Rad26 may bind damage independently (Edwards et al., 1999).

In general, the checkpoint signal leads to the activation of either of the two downstream checkpoint kinases: Whereas Chk1 is selectively activated in response to DNA damage, Cds1 becomes mainly activated in response to replication stress (Murakami and Okayama, 1995; Walworth et al., 1993). It is known that Rad3 relays the checkpoint signal to its downstream kinases via mediator or adaptor proteins. The adaptor protein Crb2 plays a crucial role in the establishment of the DNA damage checkpoint. Crb2 is a BRCT-domain protein similar to vertebrate 53BP1 and mediates the checkpoint response through interactions with both Rad3 and Chk1 (Mochida et al., 2004). In contrast, in response to replication stress, Rad3 transduces the checkpoint signal via Mrc1, the mediator of

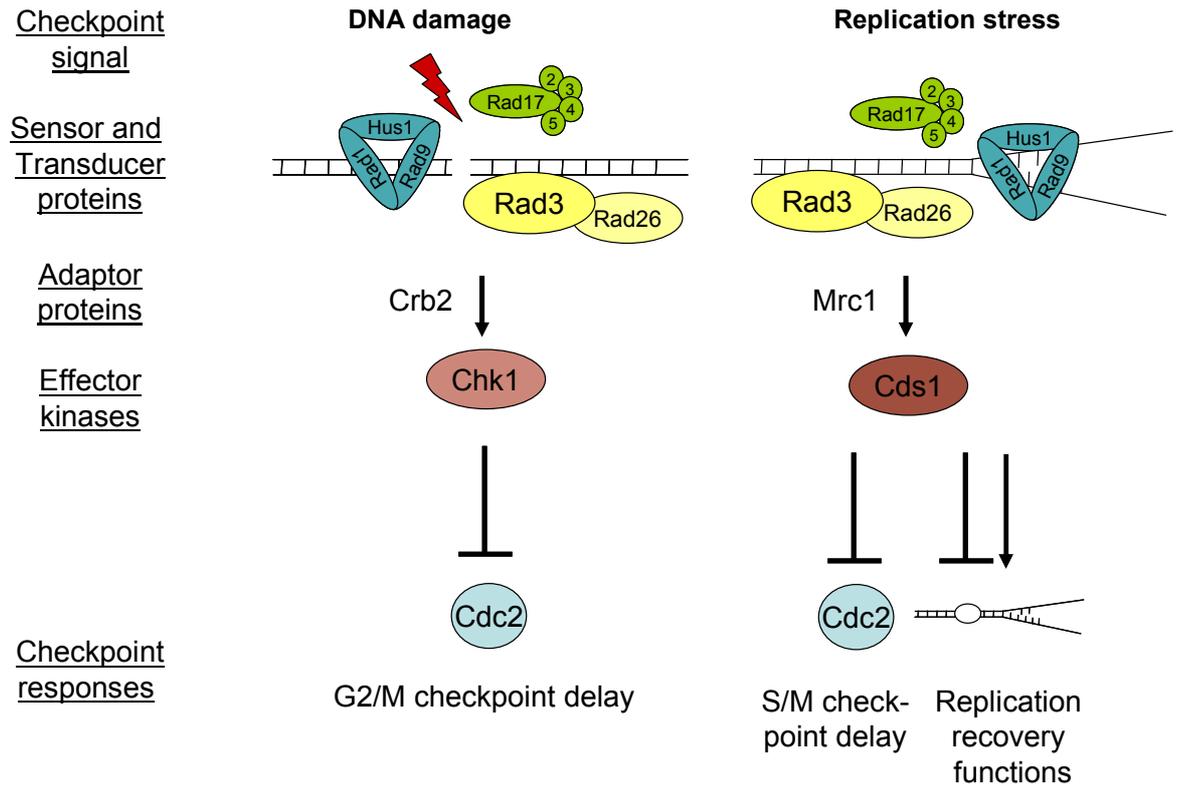
replication checkpoint comparable to vertebrate Claspin, which is required for the initial phosphorylation of Cds1 by Rad3 (Tanaka and Russell, 2001; Tanaka and Russell, 2004). In addition, the Rad3-related kinase Tel1, which is involved in telomere maintenance, has been shown to regulate Mrc1 in combination with Rad3 by differential phosphorylation (Zhao et al., 2003). Aside from Tel1, recently the closely related Tel2 has also been implicated in the activation of the Mrc1-mediated replication checkpoint (Shikata et al., 2007).

Once the checkpoint signal reaches the effector kinases Chk1 and Cds1, they can subsequently phosphorylate a wide variety of downstream targets that will lead to the implementation of appropriate checkpoint responses. The cyclin dependent kinase Cdc2, which executes the transition into mitosis, is the major downstream target of mitotic checkpoints in response to DNA damage or replication block. A simplified diagram for the checkpoint signalling cascades in fission yeast is shown in Figure 1.2.

### **1.2.2 *Chk1 and Cds1 kinases in fission yeast***

In general, the two checkpoint kinases are thought to operate in two different pathways according to the type of genotoxic stress that leads to their activation. However, there is evidence for some kind of overlap or interplay between the two kinases as demonstrated with the checkpoint pathway in response to replication stress: early studies demonstrated that the S/M checkpoint in fission yeast prevents the onset of mitosis if DNA replication is blocked by hydroxyurea (Enoch and Nurse, 1990). The checkpoint response is thought to be accomplished by Cds1, which is primarily activated by hydroxyurea in these cells. However, in the absence of Cds1, fission yeast mutants still retain the capacity to delay mitosis, probably due to Chk1 which becomes strongly activated in these cells. Moreover, mutants deficient for both Chk1 and Cds1 completely lack mitotic delay, further confirming this model of compensatory activation of the two kinases in S/M checkpoint control (Boddy et al., 1998). It has been suggested that failure to stabilize protein complexes at stalled replication forks in Cds1-deficient yeast cells might cause DNA damage and therefore activation of Chk1 (Lindsay et al., 1998).

Interestingly however, this study by Lindsay et al. proposed two models for Cds1 function in response to S phase perturbations. The first model implied that Cds1 has two separate targets: on one side it can stabilise components of the replication machinery and on the other side it can additionally target proteins that regulate Cdc2 activity, as described in the diagram in Figure 1.2 (Lindsay et al., 1998). This model is the generally accepted one but



**Figure 1.2: Outline of the checkpoint signalling cascade in fission yeast**

DNA damage induced lesions can be detected by the 9-1-1 and Rad17-RFC complex and also by Rad3-Rad26. The signal is transduced by Rad3 to the effector kinase Chk1 via the mediator protein Crb2. Chk1 activation leads to downstream responses that result in the execution of mitotic delay via inhibition of Cdc2.

In response to replication stress, stalled replication structures can be detected by 9-1-1 and Rad17-RFC and the signal is transduced by Rad3. Mediator proteins such as Mrc1 are also directly involved in sensing and transmitting the checkpoint signal to the effector kinase Cds1 which in turn will activate downstream responses that result in the execution of mitotic delay via inhibition of Cdc2 and the replication recovery functions: suppression of origin firing and stabilisation of stalled replication forks.

interestingly, another alternative model was also proposed. This second model suggested that Cds1 has only one single target, the replication machinery. Accordingly, Cds1 would stabilise the replication structures during S phase and they could themselves generate a signal leading to the inhibition of Cdc2 and mitotic delay in a manner that would be independent of the action of the checkpoint Rad proteins (Lindsay et al., 1998).

### **1.2.3 Targets of the mitotic checkpoint mechanisms**

The ultimate target of the mitotic checkpoint mechanisms is to block the onset of mitosis in the presence of damaged or incompletely replicated DNA. During a normal cell cycle the transition to mitosis requires the activation of the cyclin dependent kinase Cdc2 which is stimulated through its association with a regulatory mitotic B-type cyclin subunit and an activatory phosphorylation on Threonine 167 (T167). Cdc2 activity is also regulated through inhibitory phosphorylation on a Tyrosine 15 (Y15) residue. The phosphorylation status of the critical Cdc2-residue Y15 is controlled by the Wee1 and Mik1 kinases, which are responsible for the phosphorylation, and the Cdc25 phosphatase which is able to remove it (Lundgren et al., 1991; Millar et al., 1991).

The inhibition of mitosis during checkpoint responses is generally thought to be achieved by preventing the activation of Cdc2. The checkpoint kinases Chk1 and Cds1 were both implicated in the regulation of the Wee1 and Mik1 kinases and the Cdc25 phosphatase. In response to DNA damage, Chk1 was shown to be able to activate Wee1 which can in turn contribute to the maintenance of inhibitory Y15 phosphorylation on Cdc2 (O'Connell et al., 1997). In a similar manner, Cds1 appears to be able to bind and phosphorylate Wee1 in response to hydroxyurea (Boddy et al., 1998). In addition to Wee1, the other Y15-kinase Mik1 was found to be important for both the establishment and the maintenance of the DNA damage and replication checkpoints (Rhind and Russell, 2001). Thus, the activation of Wee1 and Mik1 by Chk1 and Cds1 during checkpoint activation in fission yeast is associated with the maintenance of the inhibitory Cdc2-Y15 phosphorylation.

Another study identified the phosphatase Cdc25 as target of the DNA damage checkpoint in fission yeast by demonstrating that Chk1 can associate with Cdc25 *in vivo* and furthermore phosphorylate Cdc25 *in vitro* (Furnari et al., 1997). In addition to that, it was established that Cds1 can phosphorylate Cdc25 in response to replication stress. In the absence of Cds1 in fission yeast, the other checkpoint kinase Chk1 can be activated and phosphorylate Cdc25 in a similar manner (Zeng et al., 1998). In either case, Cdc25 phosphatase activity is inhibited, either directly or by association with another protein of

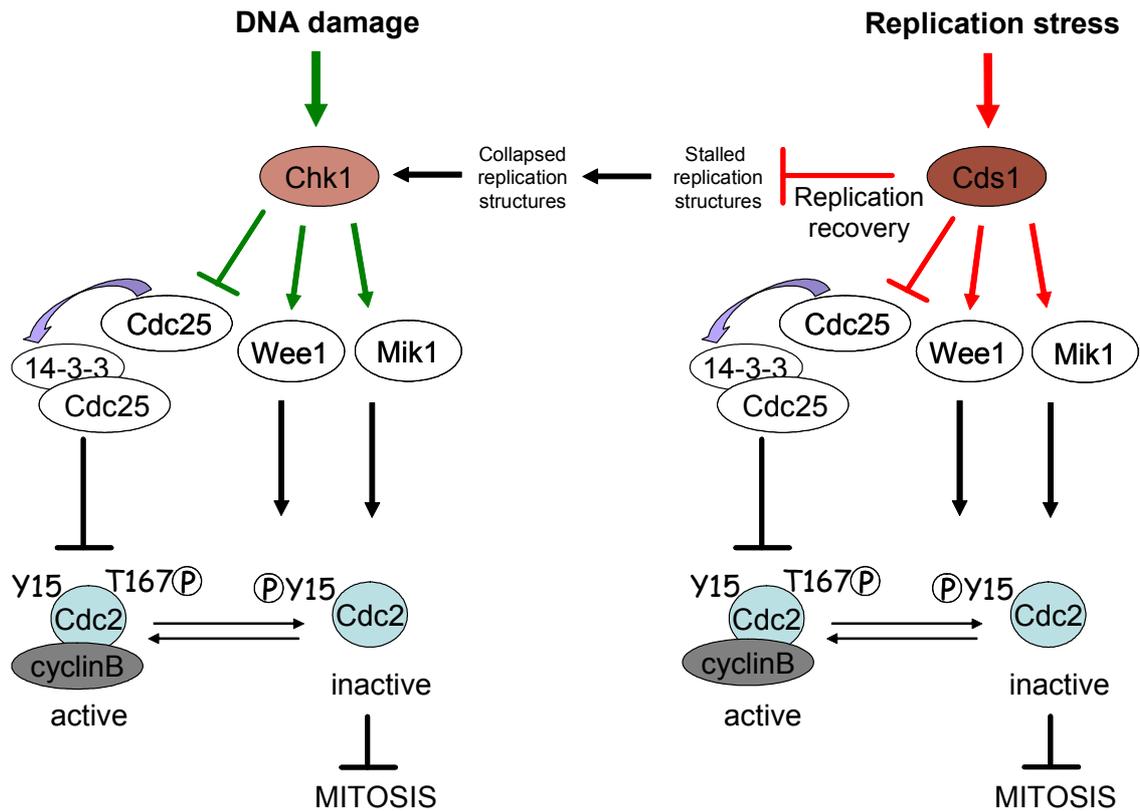
the 14-3-3 family. The phosphorylation of Cdc25 on several specific sites (Serines 99, 192, 359) leads to its association with Rad24 and Rad25, two fission yeast homologues of 14-3-3 proteins (Ford et al., 1994; Zeng et al., 1998). In this complex Cdc25 is unable to remove the inhibitory Y15 phosphorylation on Cdc2 and thus maintains the inactive kinase form.

In summary, Chk1 and Cds1 can execute mitotic checkpoint responses by targeting the downstream kinases Wee1 and Mik1 as well as the Cdc25 phosphatase. As shown in the model in Figure 1.3, Chk1 mainly becomes activated in response to DNA damage and leads to Cdc2 inhibition through Wee1 and Mik1 induced inhibitory Y15 phosphorylation and inhibition of the Cdc25 phosphatase. In response to replication stress, Cds1 is mainly activated and can inhibit Cdc2 in the same manner as Chk1 by targeting Wee1, Mik1, and Cdc25. In addition to that, the replication recovery functions of Cds1 ensure the stabilisation of stalled replication structures by suppressing any futile firing of origins and furthermore by stabilising stalled replication forks. However, in the absence of Cds1, Chk1 activation is responsible for the checkpoint response, presumably because the stalled replication structures collapse and can then be detected by the DNA damage sensors.

#### **1.2.4 DNA replication and checkpoint signalling**

As mentioned above, one study has previously suggested a model where the stalled replication machinery itself could potentially contribute to the checkpoint signal leading to the inhibition of mitosis. There has been some speculation about whether proteins involved in DNA replication might be able to sequester Cdc2 on stalled replication structures and thereby inhibit the onset of mitosis (Lindsay et al., 1998; Russell, 1998).

For example, it has been proposed that the presence of replication proteins Cdc18 (also known as Cdc6) and Orp1 (an ORC subunit homologue) in combination with stalled replication structures is important for Cds1 activation during replication checkpoint responses (Murakami et al., 2002). Cdc18 is an essential factor for the initiation of DNA synthesis in fission yeast. It has been shown to interact with Cdc2, and it is thought that the Cdc2-dependent phosphorylation of Cdc18 is involved in preventing the re-activation of replication origins once DNA synthesis has finished (Lopez-Girona et al., 1998). These findings make Cdc18 a prime candidate to be involved in replication checkpoint signalling. Only recently Rad26 has been shown to physically interact with Cdc18 and it was also demonstrated that this interaction allows a more stable recruitment of Rad3 to the chromatin when DNA replication is stalled thereby enforcing long-term maintenance of the checkpoint (Hermand and Nurse, 2007).



**Figure 1.3: Targets of the mitotic checkpoint mechanisms in fission yeast**

In response to DNA damage, Chk1 becomes activated and phosphorylates downstream targets Cdc25, Wee1, and Mik1. Phosphorylation of Cdc25 creates a binding site for 14-3-3 proteins and inhibits the phosphatase activity of Cdc25. Phosphorylation of Wee1 and Mik1 further induces their kinase activity towards Cdc2 and ensures the maintenance of the inhibitory Y15 phosphorylation which keeps Cdc2 in an inactive state and blocks the onset of mitosis.

In response to replication stress, Cds1 becomes activated and can phosphorylate downstream targets Cdc25, Wee1, and Mik1, which results in mitotic delay in a similar fashion as Chk1 activation described above. Additionally, the replication recovery functions of Cds1 are responsible for stabilising stalled replication structures. In the absence of Cds1, Chk1 becomes activated presumably because stalled replication structures would collapse and then be detected by the DNA damage sensors. In this scenario, in the absence of Cds1, Chk1 would be responsible for the block to mitosis in response to replication block.

### 1.3 Checkpoint pathways in budding yeast

Checkpoint proteins and signalling pathways have also been identified and extensively studied in the budding yeast model organism. Similar to fission yeast, the budding yeast proteins involved in checkpoint signalling can be roughly grouped into sensors, mediators, transducers, and effectors, although very often one protein can be assigned to more than one of these categories.

To briefly summarize budding yeast checkpoint control, the monitoring and detection of aberrant DNA structures is thought to be mainly accomplished by Rad-family protein complexes, whereby the Rad24 protein complex can function as a clamp-loader similar to fission yeast Rad17-RFC. Accordingly, the budding yeast Rad17 complex, consisting of Rad17, Mec3 and Ddc1, can be viewed as the sliding clamp that is loaded onto the DNA by the clamp loader Rad24 complex (Green et al., 2000; Thelen et al., 1999). Several other proteins have been implicated in detecting checkpoint-activating structures and mediating checkpoint signalling, including the Rad9 protein, the Mrc1 protein, as well as various repair proteins, such as for example the double strand recognition and repair proteins Mre11, Rad50, Xrs2 (D'Amours and Jackson, 2002).

The major transducer kinase in budding yeast is called Mec1, the homologue of fission yeast Rad3, and the main effector kinases responsible for budding yeast checkpoint signalling are Chk1 and Rad53, the latter being the homologue of fission yeast Cds1. In response to both DNA damage and replication arrest, Rad53 has been identified as the major effector kinase that is phosphorylated in a Mec1-dependent manner (Pelliccioli et al., 1999; Santocanale and Diffley, 1998). The other effector kinase Chk1 has been shown to be mainly activated in response to DNA damage, and as for Rad53, this is accomplished in a Mec1-dependent fashion. In response to DNA damage, it is thought that Chk1 and Rad53 function in parallel pathways, preventing progression through mitosis by maintaining the anaphase inhibitor Pds1 and by regulating the Polo kinase Cdc5, respectively (Sanchez et al., 1999). Another important component of checkpoint signalling in budding yeast is Dun1, a downstream kinase of Mec1/Rad53, which leads to the transcriptional activation of repair genes and has been shown to regulate the abundance of the ribonucleotide reductase inhibitor Sml1 in response to DNA damage (Zhao and Rothstein, 2002). As for the checkpoint response to replication arrest or DNA damage in S phase, Rad53 alone has emerged as main effector kinase responsible for preventing the firing of late origins (Santocanale and Diffley, 1998).

## 1.4 Checkpoint pathways in vertebrate cells

Many of the basic principles of cell cycle checkpoint mechanisms were first described in the yeast model organisms. As many checkpoint proteins have homologues in higher eukaryotes, the question of whether the findings in budding and fission yeast could be extrapolated to vertebrate cells was raised. The cell cycle checkpoint research has rapidly progressed using various cell lines and several animal model systems, most notably work on *Xenopus* oocytes, knock-out mice models, and avian DT40 knock-out cell lines.

Similar to fission yeast, the components of the checkpoint pathways in vertebrate cells can be broadly divided into sensor proteins, adaptor/mediator proteins, transducers and effectors, although some proteins fit into more than one category. The next sections will first examine how DNA damage and replication stress are thought to be sensed in vertebrate cells, and then the actual details of the checkpoint signalling cascades will be discussed with a focus on mitotic checkpoint responses.

### 1.4.1 Sensing checkpoint-activating DNA structures

In general, cells are constantly exposed to endogenous and exogenous forms of DNA damage. In order to study individual cell cycle checkpoint responses, cells are often exposed to DNA damage by ionising radiation (IR) or ultra-violet (UV) radiation. These two types of radiation induce quite different types of DNA damage; IR treatment induces DNA single- and double-strand breaks whereas UV light has cross-linking properties and frequently creates pyrimidine dimers. Apart from radiation, DNA damage can also be induced by various genotoxic chemotherapeutic agents, for example cisplatin or etoposide.

During S phase, when cells replicate their DNA, they are susceptible to lesions that compromise replication fork progression and ultimately endanger genomic integrity. In general, drugs that interfere with the process of DNA replication directly are used to study replication checkpoint responses in experimental systems. Hydroxyurea (HU) is a ribonucleotide reductase inhibitor that causes the depletion of dNTP pools and thus inhibits DNA synthesis. Another drug that is frequently used for checkpoint analysis is aphidicolin, which inhibits the DNA polymerases  $\alpha$  (also  $\delta$  and  $\epsilon$ ) and therefore blocks DNA replication (Liu et al., 2003).

As in fission yeast, the vertebrate Rad17-RFC complex and the 9-1-1 complex have been identified as 'sensors' of DNA lesions in vertebrate cells. Correspondingly, the hetero-

pentameric Rad17-RFC complex can bind to the hetero-trimeric 9-1-1 complex in an ATP-dependent manner but binding to DNA is independent of ATP. Also in vertebrates, it is thought that the Rad17-RFC complex can act as a clamp loader that loads the 9-1-1 checkpoint clamp onto DNA in a manner reminiscent to RFC and PCNA during DNA replication (Bermudez et al., 2003; Lindsey-Boltz et al., 2001).

Apart from the Rad17-RFC and 9-1-1 complex, several other proteins have been identified that are in some way involved in sensing DNA damage or stalled replication structures. Protein kinases such as ATM and ATR provide not only sensory functions; they also transduce the checkpoint signal and are generally thought to be essential components of the cell cycle checkpoint machinery.

#### **1.4.1.1 PI3-kinase related protein kinases: ATM, ATR, DNA-PK**

Two members of the PIKK family are primarily required for the cell cycle checkpoint mechanisms in vertebrate cells: ATM (Ataxia-Telangiectasia-mutated) and ATR (ATM and Rad3-related). Whereas ATM appears to be mainly activated by DNA damage, such as double-strand breaks, ATR responds mainly to stalled replication structures and single-stranded DNA, however this distinction is not completely clean-cut as their activatory patterns sometimes overlap (Yang et al., 2003).

Another member of the PIKK family is DNA-PK (DNA-dependent protein kinase), a serine/threonine protein kinase involved in the repair of DNA double-strand breaks by the process of non-homologous end joining (NHEJ). This repair mechanism accomplishes the ligation of the ends of DNA strand breaks without the need for a homologous template. DNA-PK functions in this response as a hetero-trimeric complex in association with two Ku subunits, Ku70 and Ku80, which form a DNA end binding complex and recruit and activate the DNA-PK catalytic subunit (Doherty and Jackson, 2001).

The ATM protein kinase received its name Ataxia-telangiectasia-mutated because mutations in the gene cause the condition Ataxia-telangiectasia in humans. This disease is characterised by neurological defects, radiation sensitivity, genome instability, and cancer predisposition, amongst other symptoms (Savitsky et al., 1995). ATM knock-out mice are viable and recreate much of the phenotype of Ataxia-telangiectasia (Xu et al., 1996). ATM is mainly involved in the recognition of DNA double-strand breaks and even a very small number of DNA double-strand breaks are already sufficient to induce ATM activation in cells (Bakkenist and Kastan, 2003). The intra-molecular auto-phosphorylation of ATM on

a specific serine residue (S1981) converts the dimeric ATM protein into monomers. Activated ATM rapidly phosphorylates histone H2AX on S319 ( $\gamma$ -H2AX), thereby marking sites of DNA damage, particularly DNA double-strand breaks, and initiating the recruitment of various checkpoint proteins, including mediator proteins which further contribute to the activation of several ATM downstream targets to transduce and amplify the checkpoint signal (Burma et al., 2001; Paull et al., 2000; Rogakou et al., 1998). The major downstream target of ATM in vertebrate cells is thought to be the checkpoint kinase Chk2 which will be discussed in more detail at a later point.

ATR and ATRIP (ATR-interacting protein) are the vertebrate homologues of Rad3 and Rad26, and as with their fission yeast counterparts they are involved in sensing DNA lesions. ATR is another member of the PIKK family and has essential functions during early embryonic development as ATR knock-out mice are embryonically lethal and furthermore display high levels of chromosome fragmentation (Brown and Baltimore, 2000). A mutation that alters splicing of the ATR gene and affects the expression of ATR has been associated with Seckel syndrome, an autosomal recessive disorder. The disease is characterised by growth retardation and mental retardation and also presents symptoms indicating impaired DNA damage responses (O'Driscoll et al., 2003).

ATR and ATRIP are thought to be mainly involved in sensing single-stranded DNA stretches, which are generated for example when a replication fork stalls. The replication protein A (RPA) can bind to the single-stranded DNA and it is thought that RPA-coated DNA preferentially recruits the ATR-ATRIP complex, although it has been shown that ATR can also bind directly to DNA. The ATR-ATRIP complex is able to induce the phosphorylation of the Rad17 sensor protein in the presence of RPA-coated DNA. However, the loading of Rad17, the 9-1-1 complex and other checkpoint mediator factors appears to be independent of ATR (Unsal-Kacmaz et al., 2002; Zou and Elledge, 2003).

Similar to ATM, ATR can trigger the phosphorylation of histone H2AX, for example in response to UV light when DNA repair intermediates trigger the response (Hanasoge and Ljungman, 2007). In addition, when UV light-induced bulky DNA adducts collide with replication forks, resulting DNA double-strand breaks can also lead to histone H2AX phosphorylation by ATM (Rogakou et al., 1998). Furthermore activated DNA-PK has been shown to induce H2AX phosphorylation (Park et al., 2003). Overall, ATM, ATR and DNA-PK can be involved in the phosphorylation of histone H2AX, which can be used as a marker for DNA lesions and sites to which several checkpoint proteins are recruited. These

sites are detectable within one minute of IR treatment and can be visualised as distinct nuclear foci using antibodies specific for  $\gamma$ -H2AX (Sedelnikova et al., 2002).

Interestingly, ATR seems to play an important role during normal S phase progression, as it is recruited to the chromatin in the absence of DNA damage (Dart et al., 2004).

Moreover, both ATM and ATR have been shown to contribute to the regulation of the timing of origin firing during an unperturbed S phase (Shechter et al., 2004). Thus, ATM and ATR are thought to contribute to the regulation of a normal unperturbed cell cycle as well as to checkpoint signalling in response to DNA damage and replication stress.

In any case, all three PIKK family members possibly will associate with a variety of adaptor/mediator proteins in order to transduce and amplify the checkpoint signal to their respective downstream targets.

#### **1.4.1.2 Adaptor and mediator proteins involved in checkpoint signalling**

Checkpoint mediator proteins can transiently associate with sensor proteins and transducer proteins and thereby help to specifically transmit checkpoint signals. In response to DNA damage or replication stress, at least five mediator proteins are involved in checkpoint signalling in vertebrate cells: the mediator of DNA damage checkpoint 1 (MDC1), the topoisomerase binding protein 1 (TopBP1), the p53 binding protein 1 (53BP1), breast cancer gene 1 (BRCA1), and Claspin. Some of these proteins do not only act as mediators but also as sensor proteins, and many of these mediator proteins contain one or more BRCT domains, of which the first was identified in the C-terminal part of BRCA1. The BRCT domain is an evolutionary conserved interaction motif that is found in many proteins involved in cell cycle checkpoint function (Bork et al., 1997).

The BRCT domain containing protein MDC1 is one of the first proteins to localise to sites of DNA damage. Following H2AX-phosphorylation by ATM, MDC1 can directly associate with  $\gamma$ -H2AX via its BRCT domain and subsequently recruit further checkpoint proteins, such as the MRN repair complex, 53BP1, and BRCA1 (Stucki et al., 2005). The MRN complex consists of three proteins which are called meiotic recombination 11 (MRE11), Rad50, and Nijmegen breakage syndrome 1 (NBS1). The mediator protein MDC1 can furthermore interact with the ATM downstream target Chk2 and is also phosphorylated in an ATM/Chk2-dependent manner in response to DNA damage (Canman, 2003; Lou et al., 2003).

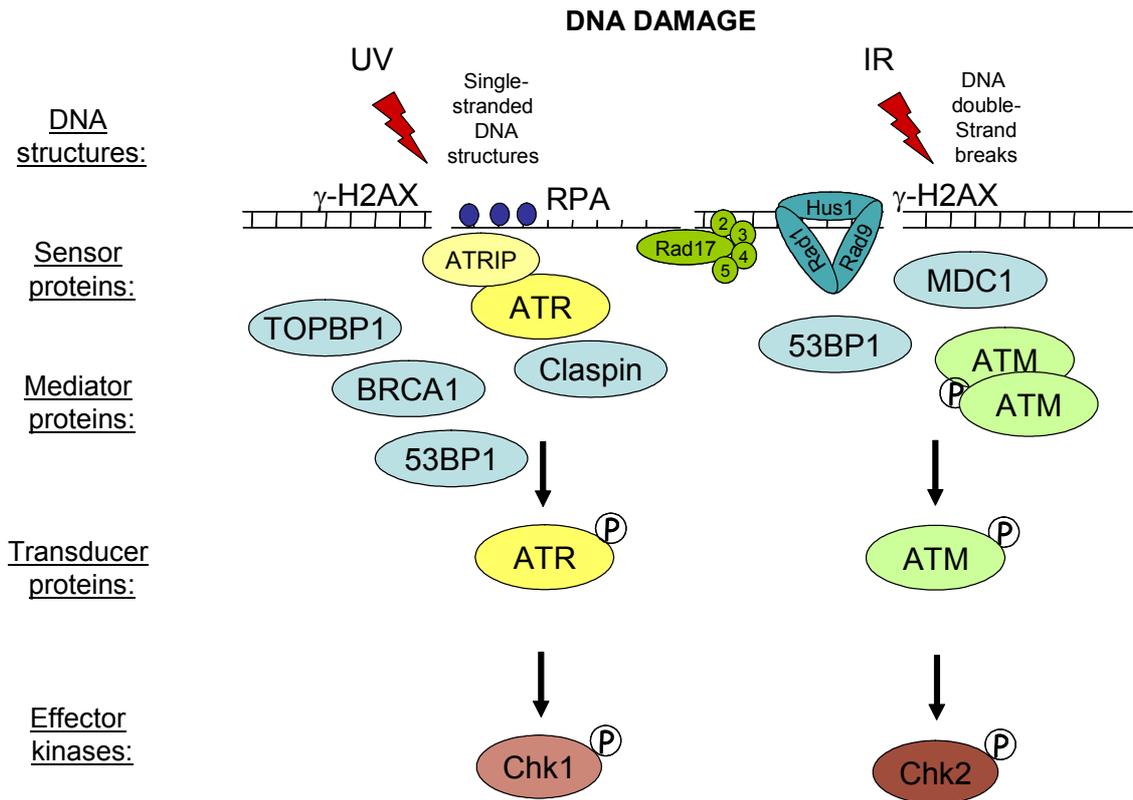
The multiple BRCT domain-containing protein TopBP1 is phosphorylated in response to DNA damage and replication blocks. TopBP1 has a crucial role in ATR-dependent checkpoint signalling and has been shown to activate the ATR-ATRIP complex in *Xenopus* (Kumagai et al., 2006). This interaction as well as ATR activation appears to be dependent on the checkpoint clamp 9-1-1 complex (Lee et al., 2007). Furthermore, ATM can also regulate TopBP1 by phosphorylation and thereby enhance the TopBP1-ATR association (Yoo et al., 2007).

The p53 binding protein 53BP1 is another BRCT domain containing protein involved in checkpoint signalling. 53BP1 is phosphorylated in response to DNA damage and forms nuclear foci that co-localize with  $\gamma$ -H2AX, mainly to DNA double-strand breaks (Rappold et al., 2001). However, depending on the type of damage, both ATM and ATR have been associated with 53BP1 phosphorylation (Jowsey et al., 2007). Apart from stimulating p53-dependent transcription, 53BP1 has been shown to function in the process of non-homologous end joining of DNA double-strand breaks (Nakamura et al., 2006).

BRCA1 is a breast cancer susceptibility gene that is involved in DNA damage repair. It contains a C-terminal BRCT domain and as previously mentioned, BRCA1 is recruited to sites of damage, such as DNA double-strand breaks. It has also been proposed that a direct interaction between ATRIP and BRCA1 is required for the checkpoint function of ATR (Bork et al., 1997; Venere et al., 2007).

Another adaptor protein, Claspin, has been identified in *Xenopus* as a protein that is required for Chk1 activation during the replication checkpoint response (Kumagai and Dunphy, 2000). Claspin also acts as a sensor protein and can bind to chromatin in the absence or presence of DNA damage. It is involved in the ATR-dependent activation of Chk1 and the stabilisation of Claspin is thought to be part of the checkpoint in response to replication arrest (Bennett and Clarke, 2006).

The mechanistical details of the functions and interactions of these mediator proteins are rather complex. Depending on the type of DNA damage, different lesions such as DNA double-strand breaks or sections of single-stranded DNA, result in the recruitment of the appropriate mediator proteins. Figure 1.4 shows a simplified model for the checkpoint signalling components that participate in the recognition of DNA lesions in response to DNA damage. For example, UV-light-induced DNA lesions may result in the generation of single-stranded DNA and thus activate mainly ATR with the possible participation of mediator proteins like TopBP1, BRCA1, 53BP1, and Claspin.



**Figure 1.4: Sensing and transducing DNA damage in vertebrate cells**

UV light induced DNA damage DNA lesions, including single-stranded DNA. ATR can directly bind to these lesions or ATR-ATRIP is recruited to stretches of RPA-coated single-stranded DNA. Rad17-RFC and 9-1-1 are also involved in sensing DNA damage. ATR can phosphorylate histone H2AX at the sites of damage. The complete activation of ATR is achieved through the involvement of several mediator proteins, such as TopBP1, BRCA1, 53BP1, and Claspin. These mediator proteins may also bind directly to the DNA and they communicate the checkpoint signal from ATR to the effector kinase Chk1.

DNA damage, such as DNA double-strand breaks induced by IR, lead to the recruitment of ATM. The intra-molecular phosphorylation of ATM converts the dimer into active monomers which can phosphorylate histone H2AX. Damage can also be sensed by the Rad17-RFC and 9-1-1 complexes. Mediator proteins such as MDC1 and 53BP1 communicate the checkpoint signal to the effector kinase Chk2.

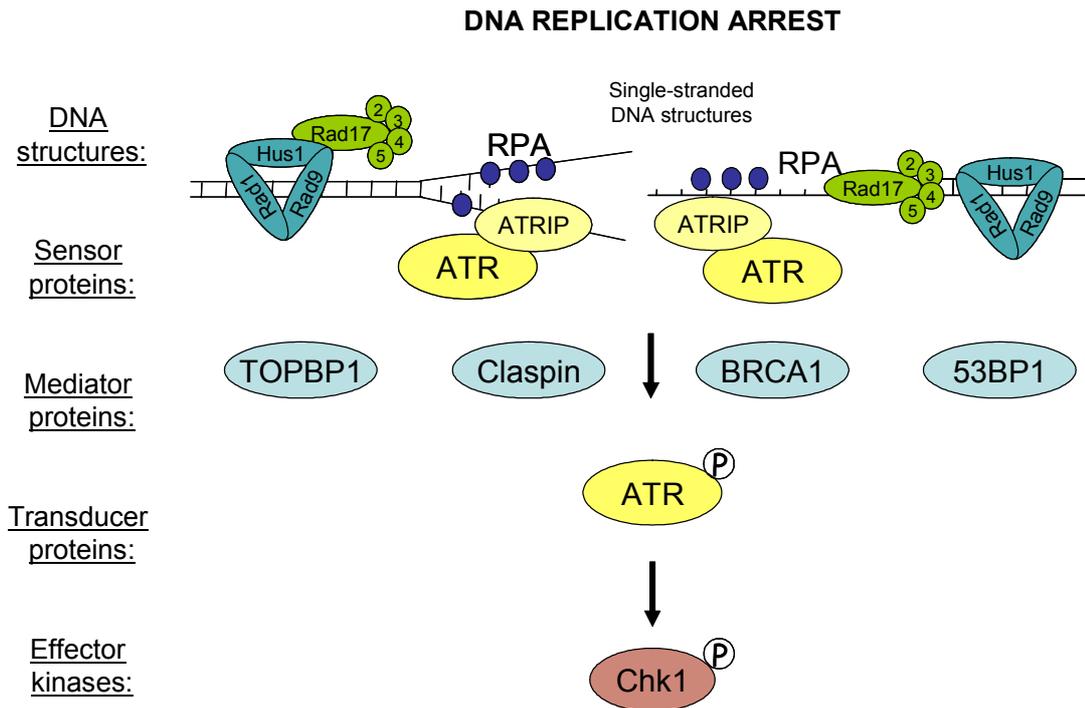
Other types of DNA damage, such as DNA double-strand breaks induced by IR, may lead mainly to the activation of ATM in cooperation with mediator proteins such as MDC1 and 53BP1. Besides, the DNA double-strand breaks may additionally lead to the generation of other structures, such as DNA repair intermediates and single stranded DNA which could subsequently lead to the activation of ATR (Figure 1.4).

Figure 1.5 illustrates the structures and proteins involved in sensing and transducing the checkpoint signal in response to DNA replication block. Inhibitors of DNA replication, such as aphidicolin or hydroxyurea, stall the replication machinery and can generate stretches of single-stranded DNA which will mainly result in the activation of ATR in association with appropriate mediator proteins, such as TopBP1, Claspin, BRCA1, and 53BP1.

Ultimately, in response to either DNA damage or replication arrest, the activation of ATM/ATR ensures that the specific checkpoint signal is transduced to the two major checkpoint effector kinases: Chk1 and/or Chk2.

### **1.4.2 Checkpoint kinases Chk1 and Chk2**

The two checkpoint kinases Chk1 and Chk2 are serine/threonine protein kinases and were initially identified in yeast as has been described above. Whereas fission yeast Chk1 is mainly activated in response to DNA damage, Cds1 becomes mainly activated in response to DNA replication arrest (Murakami and Okayama, 1995; Walworth et al., 1993). In contrast to fission yeast however, the vertebrate homologue of Cds1 is called Chk2 and becomes mainly activated in response to DNA damage (Chaturvedi et al., 1999), whereas Chk1 can be active even during an unperturbed cell cycle and is further activated in response to both DNA damage and replication arrest in vertebrate cells (Jiang et al., 2003; Sanchez et al., 1997). The main function of Chk1 and Chk2 is to transduce the checkpoint signal from their upstream kinases ATM and ATR to downstream targets of the checkpoint signalling cascade. Originally, it was thought that Chk1 does exclusively depend on ATR, and Chk2 on ATM, but there appears to be some cross talk between the two pathways as will be addressed where appropriate in the following sections.



**Figure 1.5: Sensing and transducing the checkpoint signal from DNA lesions induced by DNA replication arrest**

DNA replication inhibitors such as aphidicolin or hydroxyurea which block DNA polymerase or deplete nucleotide pools generate single-stranded DNA structures. RPA-coated single-stranded DNA may result in the recruitment of the ATR-ATRIP complex. Other sensor proteins are Rad17-RFC and 9-1-1. Mediator proteins such as TopBP1, Claspin, BRCA1, and 53BP1 communicate the checkpoint signal to the effector kinase Chk1 and may also have roles in the sensing and effector steps. 53BP1 and BRCA1 also have roles in double-strand break repair.

### 1.4.2.1 Chk1 and Chk2 knock-out models

In mammals, Chk1 function is essential for embryonic development as Chk1 knock-out mice die before embryonic day 7. Chk1-deficient blastocysts and embryonic stem cells show severe proliferation defects and impaired cell cycle checkpoint responses. These cells fail to arrest in response to DNA damage and replication block, demonstrating the essential function of Chk1 in cell cycle checkpoint control (Liu et al., 2000; Takai et al., 2000).

Interestingly however, Chk1-deficient tumour cells can be viable, as was shown with the generation of a somatic avian B-lymphoma DT40 cell line deficient for Chk1 (Zachos et al., 2003). These knock-out cells demonstrated that Chk1 has essential functions during many cell cycle checkpoint responses as the Chk1<sup>-/-</sup> cells exhibit an impaired G2/M arrest and are hypersensitive to killing by IR. Furthermore, Chk1 function is required for replication checkpoint responses, such as the replication recovery functions including the stabilisation of stalled replication forks and the suppression of futile origin firing (Zachos et al., 2005). Only recently it has been shown that Chk1 is also involved in the spindle checkpoint functions (Zachos et al., 2007).

In humans, very few Chk1 mutations have been associated with cancer so far, examples are frame-shift mutations in sporadic and hereditary colorectal cancers and sporadic stomach tumours (Bertoni et al., 1999; Menoyo et al., 2001). Furthermore, a study using conditional Chk1-heterozygous mice has demonstrated that Chk1 is haploinsufficient for many functions that are critical for tumour suppression (Lam et al., 2004).

In contrast to Chk1, Chk2 function is not essential for embryonic development as Chk2 knock-out mice are viable, fertile and only display minor defects in cell cycle checkpoint control (Hirao et al., 2000; Takai et al., 2002). There is evidence from these studies that Chk2 plays a role in p53-mediated apoptosis. Furthermore, Chk2 has been identified as a putative tumour suppressor; a specific deletion mutation (CHEK2 1100delC) has been associated with an increased risk for breast cancer (Meijers-Heijboer et al., 2003). In addition to that, Chk2 mutations have been found in a particular subset of the multi-cancer phenotype Li Fraumeni-syndrome (Lee et al., 2001b).

As for Chk1, the avian B-lymphoma DT40 cell line has also been used to generate a Chk2-deficient cell line (Rainey et al., 2007). The Chk2<sup>-/-</sup> cells are perhaps not surprisingly viable and display only subtle cell cycle checkpoint impairments. Importantly, it was demonstrated that Chk2 is required for the optimal G2/M delay of G2 phase cells after IR

treatment. Furthermore, it was shown that in the DT40 cells, Chk2 is activated in response to DNA damage but not replication arrest (Rainey, 2003; Rainey et al., 2007).

Viable knock-out models for the simultaneous deletion of both Chk1 and Chk2 do not yet exist. However, the generation of Chk1/Chk2 double knock-out thymocytes has been reported recently (Zaugg et al., 2007). In this specific study it was shown that Chk1 is essential for the T-lineage development and that the cross-talk between Chk1 and Chk2 is involved in the regulation of normal thymocyte development. Also, under some circumstances, Chk2 appeared to be at least partially able to compensate for the loss of Chk1 in these cells (Zaugg et al., 2007). These Chk1/Chk2 double knock-out thymocytes have not yet been analysed with respect to their response to genotoxic stress, such as DNA damage or replication arrest.

### **1.4.3 Targets of cell cycle checkpoint signalling**

The effector kinases Chk1 and Chk2 can phosphorylate several known downstream targets, yet many more potential targets still remain to be identified. Overall, a number of cell cycle checkpoints are responsible for the establishment of cell cycle delay and thus target regulators of the cell cycle transitions, the cyclin dependent kinases. Some of the key targets for the negative regulation of Cdks are members of the Cdc25 phosphatase family, which consists of Cdc25A, Cdc25B, and Cdc25C in vertebrate cells. All of these phosphatases are involved in the regulation of Cdks at various stages of the cell cycle. Cdc25A can de-phosphorylate and activate Cdk2 in complex with cyclinE or cyclinA and the Cdk1 (Cdc2)-cyclinB complex, whereas Cdc25B and Cdc25C mainly act on Cdc2-cyclinB and thus control mitotic entry. Both Chk1 and Chk2 are able to phosphorylate Cdc25, and thereby inhibit the Cdc25 phosphatase activity through various mechanisms, which ultimately lead to the inhibition of the relevant Cdk and thus cell cycle arrest (Chaturvedi et al., 1999; Peng et al., 1997; Sanchez et al., 1997).

Another major downstream target of cell cycle checkpoint activation is the tumour suppressor p53, which has been shown to accumulate in response to DNA damage and can subsequently induce cell cycle arrest, for example at the G1/S transition by activation of the Cdk inhibitor p21 (Agarwal et al., 1995). Furthermore, p53 can induce apoptosis by up-regulating the expression of apoptosis-promoting genes, such as Bax, Puma or Fas (Haupt et al., 2003).

The tumour suppressor p53 is mutated or lost in a high percentage of human cancers. The avian B-lymphoma DT40 cell lines and the human colon carcinoma BE cells that have been used in the experimental section of this project are both cancer cell lines. As many transformed cell lines, the DT40 cells do not express p53 (Takao et al., 1999) and the BE cells appear to express a non-functional mutated version of p53. Therefore, these cells are not susceptible to any p53-mediated checkpoint responses but they are suitable for the investigation of mitotic checkpoint responses in a p53-deficient background.

## **1.5 Mitotic checkpoint signalling pathways**

This study focuses on mitotic checkpoint pathways in response to either DNA damage or DNA replication block in vertebrate cells. The following section will discuss how these checkpoint signalling cascades are thought to function according to current knowledge.

### **1.5.1 The G2/M checkpoint in response to DNA damage**

The G2/M arrest ensures that cells with damaged DNA do not enter mitosis and undergo cell division. For example, IR-induced DNA double-strand breaks or UV-induced bulky DNA adducts and resulting single-stranded stretches of DNA can be detected by various sensors and adaptor proteins, such as Rad17-RFC, 9-1-1, ATR and ATRIP, MDC1, BRCA1, TopBP1, Claspin and 53BP1 as described in Figure 1.4. and 1.5. The cooperative action of these proteins consequently leads to the activation of the transducer kinases ATM and ATR.

In response to DNA double-strand breaks, ATM becomes activated as it undergoes an intra-molecular auto-phosphorylation on S1981 which converts the dimeric ATM protein into active monomers (Bakkenist and Kastan, 2003). In addition, ATR can be activated in response to DNA damage and it is thought that the resection of the double-strand break is required. In this way generated single-stranded stretches of DNA may then be coated by RPA and signal to recruit the ATR-ATRIP complex (Zou and Elledge, 2003).

More recently, several studies have discussed the relatively new role for ATR in the DNA damage response to double-strand breaks as previously mainly ATM has been implicated in this type of response. It has been shown that ATR activation in response to DNA double-strand breaks requires both ATM and the nuclease activity of the MRE11 protein (Jazayeri et al., 2006; Myers and Cortez, 2006).

The major downstream target of ATM is thought to be Chk2, whereas ATR mainly targets Chk1 for phosphorylation (Matsuoka et al., 2000; Zhao and Piwnica-Worms, 2001). However, as with ATM and ATR activation in response to DNA damage, also at the level of their downstream targets appears to be some cross-talk between the ATM-Chk2 and ATR-Chk1 signalling pathways. For example, the phosphorylation of Chk1 in response to IR-induced DNA damage appears to be dependent on ATM and NBS1 (Gatei et al., 2003). Furthermore, a recent study has suggested that ATR can phosphorylate Chk2 in response to DNA damage, at least in the absence of ATM (Wang et al., 2006).

In either case, activated ATM/ATR can subsequently phosphorylate the effector kinases Chk1 and Chk2. Two C-terminal serine residues, S317 and S345 of Chk1, were identified as the major ATR phosphorylation sites, although other putative sites have also been suggested (Zhao and Piwnica-Worms, 2001). The identification of an auto-inhibitory domain has led to the proposal that Chk1 activation is associated with conformational changes promoted at least partly by ATR-phosphorylation that disrupt an interaction between the kinase domain and the auto-inhibitory domain of Chk1 (Katsuragi and Sagata, 2004). In general, the phosphorylation of Chk1 on the two serine residues (S317 and S345) can be used as an indicator of Chk1 activation (Zhao and Piwnica-Worms, 2001).

The major phosphorylation site on Chk2 is a threonine residue, T68, which is phosphorylated by ATM in response to IR and required for the activation of Chk2 (Ahn et al., 2000). The multi-step activation process of Chk2 involves the initial phosphorylation on T68, which can then bind in *trans* to the FHA (fork-head associated) domain of another Chk2 molecule (Ahn et al., 2002). This step is thought to promote the oligomerisation of Chk2 which in turn enhances kinases activity (Xu et al., 2002b). Recent analyses of the crystal structure of Chk2 led to the proposal that once two Chk2 kinases are dimerized, an activation segment exchange can occur between the two adjacent kinase domains and promote the *trans*-autophosphorylation of two threonine residues (T383 and T387) in the catalytic domain in the T-loop region (Oliver et al., 2006).

Activated Chk1 and/or Chk2 can then relay the checkpoint signal to their downstream targets. For the G2/M checkpoint the ultimate target is the cyclin dependent kinase Cdc2.

#### **1.5.1.1 Regulation of Cdc2 throughout the cell cycle**

The kinase activity of Cdc2 is regulated through various changes in the phosphorylation status of specific residues and the association with cyclin subunits throughout the cell

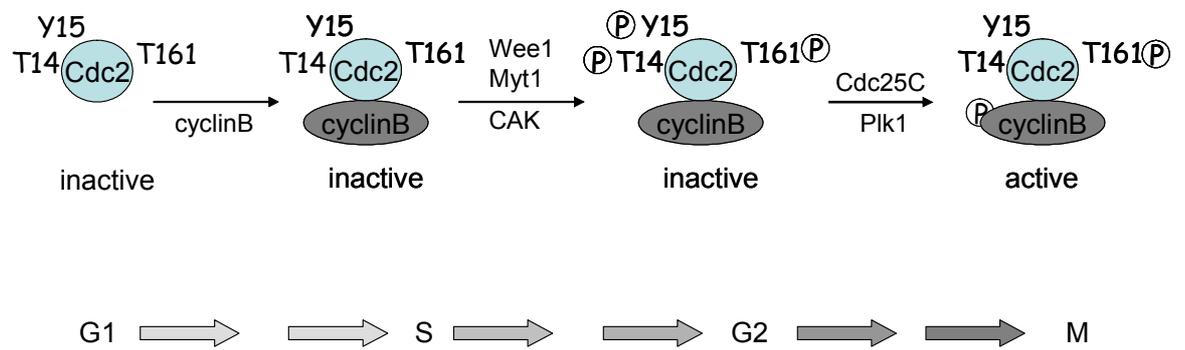
cycle. Cdc2 is able to bind to cyclins A and B and in particular the gradual accumulation of the latter and its association with Cdc2 are important for the mitotic kinase activity (Solomon et al., 1990). It has been proposed that cyclin binding is required for stabilising the catalytic subunit and also induces changes in the conformation which then allows threonine 161 (T161) to be phosphorylated (Draetta, 1993). This activatory T161 phosphorylation is absolutely required for Cdc2 kinase activity and is thought to be catalysed by the CAK (Cdc2-activating kinase) complex. There is some speculation about the actual nature of this CAK kinase(s), but in general it is believed that CAK is a trimer containing cdk7, cyclinH, and Mat1 which are all subunits of the transcription factor IIIH (Kaldis and Solomon, 2000).

During interphase, the Cdc2 kinase is kept inactive through the inhibitory phosphorylation on two specific residues, T14 and Y15. There are two kinases that catalyse this inhibitory phosphorylation in vertebrate cells: Wee1, which specifically phosphorylates Y15, and Myt1, which is a dual-specificity kinase and has been shown to phosphorylate both T14 and Y15 (McGowan and Russell, 1993; Mueller et al., 1995). Active Cdc2 is generated by the reversal of the inhibitory phosphorylation by the phosphatase activity of Cdc25(C) (Gautier et al., 1991).

The polo-like kinase Plk1 appears to be involved in the regulation of several components of the mitosis promoting complex. It has been implicated in both the activation of Cdc25C and the targeting of cyclinB to the nucleus by phosphorylation. Moreover, Myt1 has been identified as a substrate for Plk1 during mitosis (Nakajima et al., 2003; Toyoshima-Morimoto et al., 2002; Toyoshima-Morimoto et al., 2001).

For illustration purpose, Figure 1.6 shows the generally accepted model for Cdc2/cyclinB regulation throughout the cell cycle. Cdc2 can complex with cyclinB, subsequently undergo phosphorylation on T14, Y15, and T161. The phosphatase activity of Cdc25C leads to the removal of the inhibitory phosphorylation on Cdc2 and in concert with the mitosis-promoting functions of Plk1 the Cdc2/cyclinB becomes abruptly activated at the G2/M transition (Figure 1.6).

The major B-type cyclin in the vast majority of vertebrate cells is cyclinB1; however in chicken cells the major B-type cyclin is cyclinB2 which in general fulfils the same functions as cyclinB1 (Gallant and Nigg, 1992). Although both vertebrate cyclins are regulated at the level of sub-cellular localisation, there are some differences in respect to their phosphorylation status which will be addressed in the results section of this thesis.



**Figure 1.6: Model for Cdc2 regulation in vertebrate cells**

In G1 phase, Cdc2 exists as an inactive monomer. Upon cyclinB binding during S-phase, vertebrate Cdc2 gets phosphorylated on T14, Y15, and T161 by Wee1, Myt1, and CAK. The Cdc2/cyclinB complex is kept inactive through the inhibitory phosphorylation on residues T14 and Y15 until the end of G2 phase. De-phosphorylation of T14 and Y15 by the Cdc25C phosphatase activates the complex and mitosis can be initiated. Complete Cdc2/cyclinB activation possibly involves Plk1-mediated phosphorylation of cyclinB.

In general, it is thought that the activation of Cdc2 occurs very abruptly with a peak in the Cdc2 kinase activity at the G2/M transition resulting in the onset of mitosis. Originally, it was believed that the individual vertebrate Cdks are indispensable, for example Cdc2 has always been assigned the role of the mitotic Cdk, whereas for example Cdk2 was thought to be the major Cdk responsible for the G1/S transition and S-phase. Recently however, the strictness of these assignments has been questioned.

The generation of Cdk2 knock-out mice revealed that Cdk2 is not an essential gene as these animals, although infertile, were found to be viable (Berthet et al., 2003; Ortega et al., 2003). Interestingly, it was proposed that in the absence of Cdk2, Cdc2 might be able to substitute for loss of Cdk2 (Aleem et al., 2005). This study suggested that Cdc2 could complex with cyclinE to perform the G1/S transition and S phase functions and further on in the cell cycle complex with cyclinB to perform the usual mitotic Cdc2 functions (Aleem et al., 2005). Importantly, the recent generation of Cdk2-deficient avian DT40 cells supported these ideas and furthermore demonstrated that Cdc2 is indeed essential for the initiation of replication as well as mitotic events in these Cdk2 knock-out cells (Hochegger et al., 2007). This study made use of Cdk2 knock-out cells and an analog-sensitive mutant of Cdc2 and the results led to the proposal that the functions of these two Cdks can completely overlap during S phase control. Interestingly, the results from this particular study also indicate that the presence of active Cdc2 is possible early on in the cell cycle, independently of mitotic events.

### **1.5.1.2 Mitosis and cytokinesis**

During a normal unperturbed cell cycle and in the absence of any lesions that may result in checkpoint activation, cells are able to undergo the process of cell division which starts with the segregation of sister chromatids (mitosis) and ends with the division of the cytoplasm and organelles onto the two daughter cells (cytokinesis).

It has been well established that the process of cell division can be grouped into several distinct phases, beginning with prophase, in which the chromatin visibly condenses and the two centrosomes start to nucleate microtubules. In prometaphase, the nuclear envelope breaks down and microtubules can attach to kinetochores at the centrosome. The next stage, metaphase, is the alignment of the chromosomes at the metaphase plate. During the subsequent stages of anaphase, the two sister chromatids are pulled apart towards opposite poles of the cell and the poles themselves further separate. During the next phase, telophase, the two new nuclear envelopes form and chromatin de-condensation begins.

Around the same time, cytokinesis starts which eventually completes the process of cell division (Nigg, 2001).

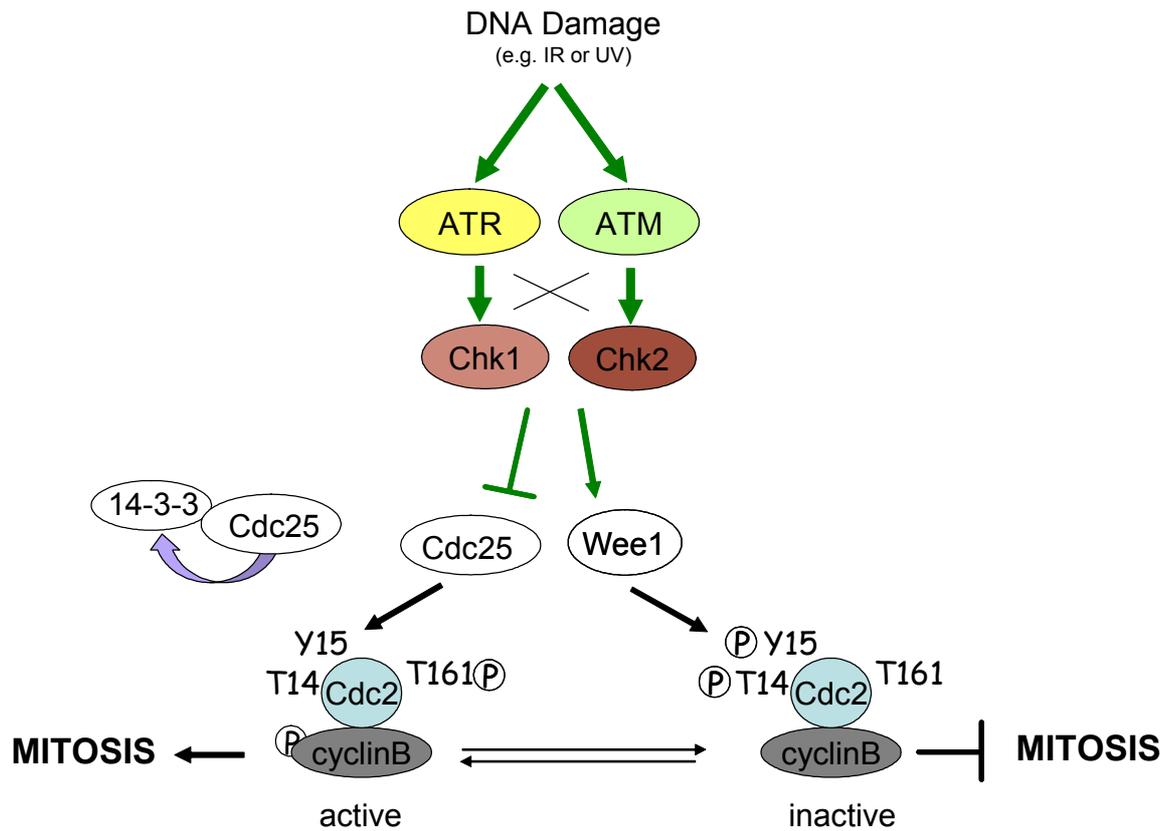
Cdc2 is thought to be involved in many aspects of the cell division process, including centrosome assembly, mitotic spindle assembly, and nuclear envelope breakdown. Apart from Cdc2, other important mitotic kinases include Plk1 and members of the Aurora kinase family, for example Aurora A, B and C (Nigg, 2001).

### **1.5.1.3 Mitotic delay mechanisms induced by Chk1/Chk2**

In general, the onset of mitosis is blocked or delayed in the presence of any DNA lesions that lead to the activation of checkpoint responses, such as the G2/M checkpoint in response to DNA damage. The effector kinases Chk1 and Chk2 can both phosphorylate Cdc25 and in this way negatively regulate the kinase activity of Cdc2. The N-terminal phosphorylation of serine 216 (S216) on Cdc25C by Chk1 and Chk2 creates a binding site for 14-3-3 proteins (Chaturvedi et al., 1999; Peng et al., 1997; Sanchez et al., 1997). This interaction results in the nuclear export and subsequent degradation of Cdc25C. In addition, the N-terminal phosphorylation of Cdc25C can also directly inhibit the phosphatase activity (Blasina et al., 1999). Chk1 has also been shown to phosphorylate several Cdc25 family members on specific C-terminal sites, which as a result inhibits the interaction of the relevant Cdc25 phosphatase with its respective Cdk/cyclin substrates (Uto et al., 2004). As a consequence of these inhibitory mechanisms in response to DNA damage, the Cdc25C phosphatase cannot de-phosphorylate Cdc2 on residues T14 and Y15 and the Cdk is kept in an inactive state and blocks the G2/M transition.

Apart from preventing Cdc25 phosphatase activity, Chk1 can phosphorylate and thereby further activate Wee1 which in turn promotes the inhibitory phosphorylation of Cdc2 (O'Connell et al., 1997). In addition to that, Chk1-phosphorylation of Wee1 can also promote 14-3-3 protein binding and further enhance the inhibitory Wee1 kinase activity (Lee et al., 2001a).

An illustration of the DNA damage-induced mitotic delay mechanisms is shown in Figure 1.7. In general, it is believed that mitotic delay is always imposed through these two conventional delay mechanisms: the inhibition of Cdc25 phosphatase and the promotion of Wee1 kinase activity which ensure the maintenance of inhibitory Cdc2 phosphorylation, keeping the Cdc2/cyclinB complex inactive and hence preventing the onset of mitosis.



**Figure 1.7: Model for the establishment of mitotic delay in response to DNA damage**

In response to DNA damage, Chk1 and Chk2 become activated via ATM/ATR. Chk1 can subsequently phosphorylate downstream targets Cdc25 and Wee1; Chk2 is able to phosphorylate Cdc25. The phosphatase activity of Cdc25 can be inhibited either directly or through association with 14-3-3 proteins and nuclear export or by Chk1-mediated C-terminal phosphorylation which inhibits Cdc25 binding to substrates. Furthermore, Chk1-mediated Wee1 phosphorylation promotes the inhibitory phosphorylation on Cdc2-Y15. Through maintenance of the inhibitory phosphorylation, Cdc2 is kept inactive and consequently mitosis is blocked.

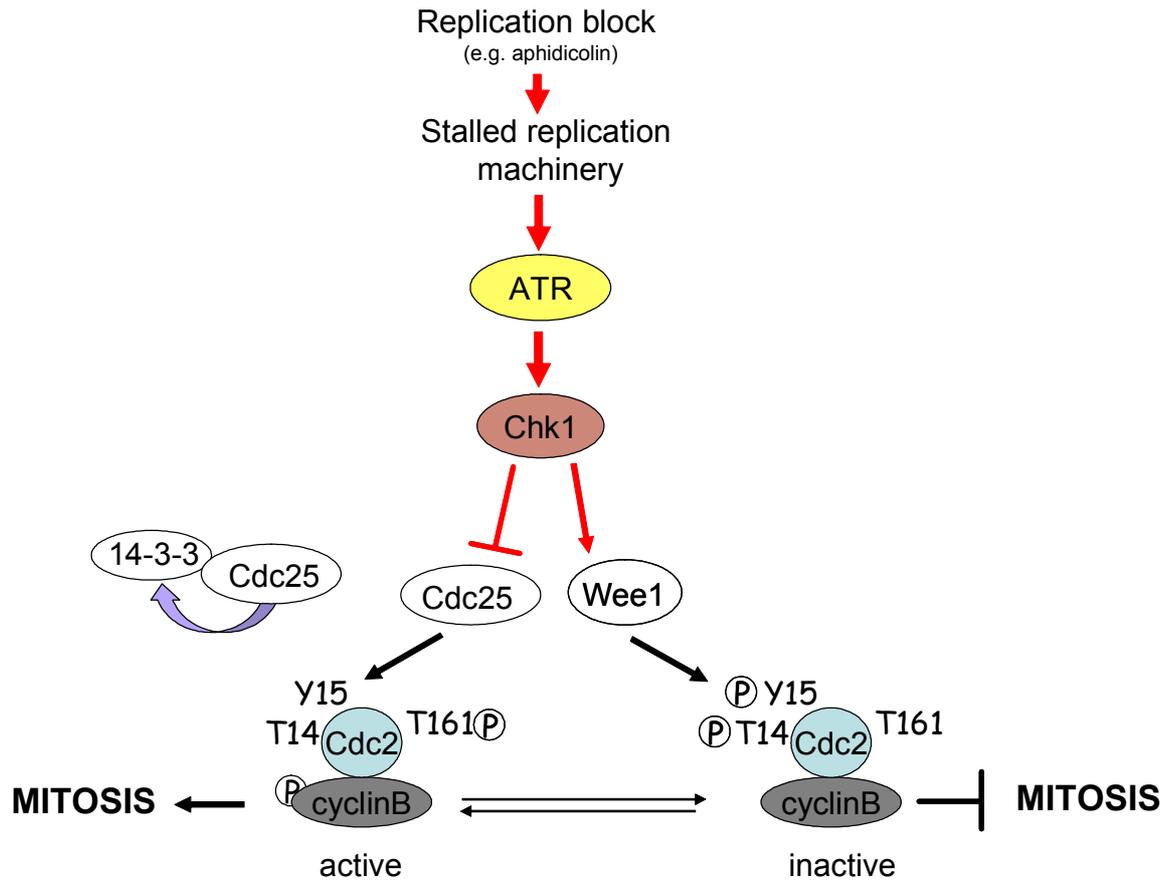
### **1.5.2 The S/M checkpoint in response to DNA replication arrest**

The S/M checkpoint response ensures that cells do not enter mitosis and divide in the presence of incompletely replicated DNA. In marked contrast to fission yeast, immunodepletion experiments in *Xenopus* oocytes demonstrated that Chk1, rather than Cds1/Chk2, becomes activated in response to replication blocks (Kumagai et al., 1998). The same is true for mouse embryonic cells where Chk1-deficiency leads to premature mitotic entry of cells in the presence of replication inhibitors (Takai et al., 2000).

In an experimental system DNA replication can be blocked with various drugs, for example aphidicolin, which inhibits DNA polymerase and stalls the replication process. There are several sensor proteins that are involved in the detection of stalled replication forks. ATR-ATRIP can bind to single-stranded RPA-coated DNA and ATR can also bind to DNA independently of ATRIP. The Rad17-RFC complex can interact with regions of DNA that have been primed by the DNA polymerase and subsequently act as a clamp loader for the 9-1-1 complex. Furthermore, the adaptor protein Claspin can bind to branched structures in the stalled replication fork independently of ATR and Rad17-RFC (Sar et al., 2004; Zou and Elledge, 2003).

The activated transducer kinase ATR consequently phosphorylates and activates the effector kinase Chk1, which in turn phosphorylates downstream targets, such as Cdc25C and Wee1 in the same manner as described above. Hence, as for the mitotic G2/M delay in response to DNA damage, it is believed that the mitotic delay in response to DNA replication block is also always imposed through the two above described conventional delay mechanisms: the inhibition of Cdc25 phosphatase and the promotion of Wee1 kinase activity which ensure the maintenance of inhibitory Cdc2 phosphorylation, thereby keeping the Cdc2/cyclinB complex inactive and preventing the onset of mitosis as illustrated in the simple model in Figure 1.8.

Thus, the S/M checkpoint is thought to operate via ATR and Chk1, ultimately targeting Cdc2 via maintenance of inhibitory T14/Y15-Cdc2 phosphorylation. However, in the last few years there have been a few reports challenging that pathway. For example, a mutant version of Cdc2 that cannot be phosphorylated on T14 and Y15 can still respond to the aphidicolin-induced replication checkpoint in *Xenopus* oocytes (Kumagai and Dunphy, 1995). Furthermore, primary mouse cells with conditionally deleted ATR, and thus minimised Chk1 activation, still display a functional S-M checkpoint response when DNA replication is blocked (Brown and Baltimore, 2003). This interesting observation led to the



**Figure 1.8: Model for the establishment of mitotic delay in response to replication arrest**

In response to DNA replication block, Chk1 become activated via ATR and can subsequently phosphorylate downstream targets Cdc25 and Wee1. The phosphatase activity of Cdc25 can be inhibited either directly or through association with 14-3-3 proteins and nuclear export or by phosphorylation of Cdc25 on the C-terminus which inhibits binding to substrates. Furthermore, Chk1-mediated Wee1 phosphorylation promotes the inhibitory phosphorylation on Cdc2-Y15. Through maintenance of the inhibitory phosphorylation, Cdc2 is kept inactive and consequently mitosis is blocked.

suggestion of the existence of an ATR-independent replication checkpoint. Another recent study has reported that Chk1 activation in response to hydroxyurea treatment in HeLa cells is mediated by Claspin but independently of ATM/ATR activity as judged by caffeine sensitivity. This study also suggested that the block to mitosis was associated with the down-regulation of cyclinB (Rodriguez-Bravo et al., 2006).

### **1.5.3 Replication Recovery functions**

Apart from the S/M checkpoint, blocks to DNA replication can also lead to the activation of another set of checkpoint functions which are often referred to as the replication recovery functions. These checkpoint mechanisms ensure that the replication machinery is kept in a stable condition so that DNA replication can resume once the respective lesions have been resolved. As previously described, Cds1 is the main effector kinase for these responses in fission yeast; however in vertebrate cells, Chk1 appears to be responsible for most of these recovery functions.

Initially, studies using a selective Chk1 inhibitor were able to show that Chk1 is required for the suppression of late origin firing in response to replication stress (Feijoo et al., 2001). Furthermore, the generation of the Chk1-deficient DT40 cell line allowed the investigation of replication recovery functions on a genetic basis (Zachos et al., 2003). Using a dual labelling technique to monitor replication fork progression and origin firing, it was shown that in response to replication arrest, a high percentage of the Chk1-deficient cells lost the ability to recover from replication arrest, which was furthermore associated with the appearance of new sites of replication. Thus, this study demonstrated that Chk1 is essential for the replication recovery functions, such as the stabilisation of stalled replication forks and the suppression of futile origin firing (Zachos et al., 2003).

### **1.5.4 DNA replication and checkpoint control**

To maintain genomic integrity it is important for cells to successfully complete DNA synthesis before they undergo mitosis and divide and it seems only logical that at least some of the checkpoint-activating proteins might actually be proteins that participate in the process of DNA replication. As previously mentioned, several replication proteins, such as fission yeast Cdc18 (Cdc6) and Orp1 have been implicated to some extent in Cds1 activation during replication checkpoint responses (Murakami et al., 2002). Especially Cdc6, which is an essential factor for the initiation of DNA synthesis, has emerged as a prime candidate to be involved in replication checkpoint signalling.

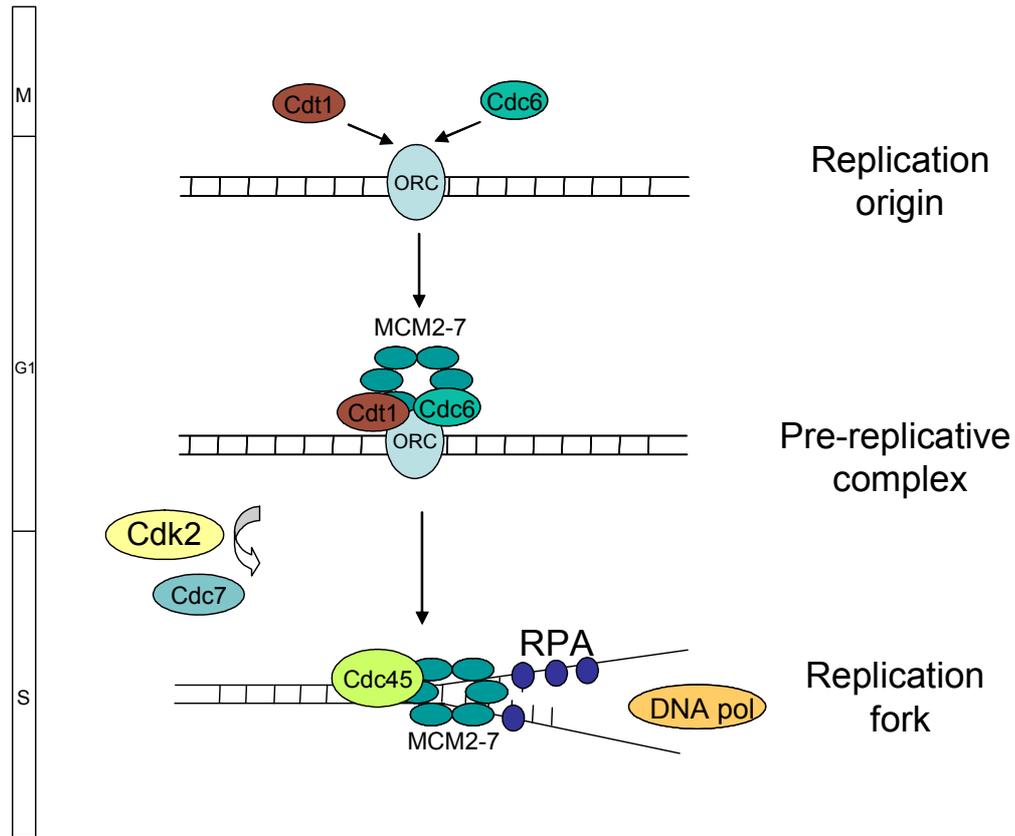
### 1.5.4.1 DNA replication

The major function of Cdc6 is in the establishment of the pre-replicative complex. As illustrated in the simple model in Figure 1.9, the process of DNA replication is initiated at origins of replication which are bound by the origin recognition complex (ORC). The ORC subsequently recruits two replication proteins, namely Cdt1 and Cdc6. Together, these three proteins (ORC, Cdt1 and Cdc6) enable the loading of the Mcm2-7 (minichromosome maintenance) hexamer onto the origin and as a consequence the pre-replicative complex is formed (Bell and Dutta, 2002). Once the DNA has been prepared for DNA synthesis in this manner, termed 'licensing', S phase is thought to be initiated through the action of Cdks, mainly Cdk2, as well as the Dbf4-dependent kinase Cdc7, which promote the loading of replication components, such as Cdc45, RPA, and DNA polymerase  $\alpha$  onto the chromatin (Walter and Newport, 2000). The pre-replicative complexes are disassembled after the initiation of DNA replication until the passage through the following mitosis. The coordinated assembly and disassembly of these complexes is thought to control that the process of DNA replication only occurs once per cell cycle (Stillman, 1996).

The exact fate of Cdc6 after S phase has not been completely clear and there has been some degree of discrepancy between some studies. Several studies using epitope-tagged versions of Cdc6 observed the translocation of the protein from the nucleus to the cytoplasm at the G1/S transition (Petersen et al., 2000; Saha et al., 1998). Overall however, it appears that in vertebrate cells, endogenous Cdc6 remains nuclear and chromatin bound throughout S phase, whereas exogenous, i.e. ectopically expressed, Cdc6 is able to translocate from the nucleus to the cytoplasm in a Cdk2-dependent manner (Alexandrow and Hamlin, 2004). The differences in the localisation patterns of endogenous and ectopically expressed Cdc6 might be attributed to the use of an epitope tag and also to differences in expression levels of the protein. Another study has shown that the Cdc6 protein appears to be degraded by the anaphase promoting complex (APC) in G1 phase (Petersen et al., 2000).

### 1.5.4.2 Cdc6 and checkpoint control

Besides studies in both budding and fission yeast, only a very small number of studies have addressed any potential functions for Cdc6 in checkpoint control in vertebrate cells. One study showed that the over-expression of human Cdc6 in G2 phase cells resulted in a mitotic delay that was associated with Chk1 phosphorylation and could also be abrogated by chemical inhibition of Chk1 (Clay-Farrace et al., 2003). Another study showed that the



**Figure 1.9: Initiation of DNA replication in vertebrates**

DNA replication is initiated at origins of replication which are bound by the origin recognition complex (ORC), which subsequently recruits Cdt1 and Cdc6. Together, ORC, Cdt1 and Cdc6 enable the loading of the Mcm2-7 hexamer onto the origin and as a consequence the pre-replicative complex is formed. Once the DNA is licensed, S phase can be initiated through the action of Cdk2 and the Dbf4-dependent kinase Cdc7, which promote the loading of replication components, such as Cdc45, RPA, and DNA polymerase  $\alpha$  onto the chromatin.

presence of Cdc6 is required for Chk1 phosphorylation in response to replication block in the *Xenopus* system (Oehlmann et al., 2004). These observations predict a model in which Cdc6 would function upstream of Chk1 in the replication checkpoint response.

However, as explained in an earlier section, there is also some indication that Cdc6 might be able to directly interact with Cdc2. At least in yeast, several studies have shown that Cdks can bind to Cdc6/Cdc18 in a cyclin-dependent manner, for example the mitotic Cdk in both fission and budding yeast can interact with an N-terminal domain in Cdc6/Cdc18 and this process is mediated by the mitotic B-type cyclin subunit (Lopez-Girona et al., 1998; Mimura et al., 2004). As Chk1 is generally required for the stabilisation of stalled replication structures, it could be predicted that in this kind of scenario Cdc6 might function downstream of Chk1 in the checkpoint response. Thus, there appears to be potential for the Cdc6 protein to function either upstream or downstream of Chk1 in replication checkpoint control.

#### **1.5.4.3 Regulation of DNA synthesis and mitosis**

Overall, the regulation of DNA replication and mitosis has been most extensively studied in yeast. However, some of the earliest studies addressing the regulation of these processes were classic cell fusion experiments performed with HeLa cells (Rao and Johnson, 1970). As an example, the fusion of the nuclei from a G1 and an S phase cell showed that S phase nuclei appear to contain certain inducers of DNA synthesis, termed S phase promoting factors (SPF), that can transmit and rapidly initiate S phase in the G1 nucleus. Interestingly however, these experiments also revealed that when the nucleus from an S phase cell was fused with the nucleus of a G2 phase cell, the progression of the resulting heterokaryon into mitosis was dependent on the completion of DNA synthesis in the S phase nucleus (Rao and Johnson, 1970). Thus, the presence of certain S phase components was able to delay the onset of mitosis in the G2 nucleus until DNA synthesis in the S phase nucleus was complete. Under these conditions, any checkpoint kinases should have hardly been activated and thus, it seems likely that these observations indicate the functioning of an intrinsic link between DNA replication and mitosis. Alternatively, a low level of activation of checkpoint kinases during normal unperturbed DNA replication might be sufficient to delay mitosis.

## 1.6 Checkpoint signalling and cancer therapy

In general, cell cycle checkpoint mechanisms aim to ensure the integrity of the genome and promote the survival of the whole organism (Nyberg et al., 2002). Tumour cells very often display mutations in components of the checkpoint signalling machinery. One of the major approaches of cancer therapy is chemotherapy which involves the treatment with cytotoxic drugs in order to kill tumour cells. Another approach is radiotherapy which aims to sensitize cells to killing by radiation. As these types of therapy might often target both normal and tumour cells, it is of interest to improve these treatments by ensuring greater selectivity towards the tumour cells. By studying the molecular details of cell cycle checkpoint control in both normal and tumour cells, exploration of the basic aspects of these mechanisms should enable the development of more effective and targeted cancer therapies (Hartwell and Kastan, 1994; Zhou and Bartek, 2004).

## 1.7 Project aims

Chk1 and Chk2 participate in various cell cycle checkpoints, their roles have been shown to vary between different organisms. The initial aim of this project was to determine the specific requirement of Chk1 and Chk2 for mitotic checkpoint responses in vertebrate cells. The main focus was directed towards two checkpoints in response to two distinct types of genotoxic stress that both delay the onset of mitosis: the G2/M checkpoint in response to DNA damage and the S/M checkpoint in response to DNA replication block.

Two different vertebrate cell lines were deployed for this purpose: avian B-lymphoma DT40 cells, where wild-type and Chk1 and Chk2 knock-out cell lines are available, and human colon carcinoma BE cell line in which Chk1 and Chk2 function can be abrogated by siRNA or using a selective Chk1 inhibitor. The generation of the Chk1- and Chk2-deficient DT40 cells has been described previously (Rainey et al., 2007; Zachos et al., 2003). The avian DT40 and human BE cell lines were used for detailed kinetic analysis of the two mitotic checkpoint mechanisms. In addition, it was of interest to investigate the mechanisms of mitotic delay by biochemical analyses with particular interest in the regulation of the mitotic cyclin dependent kinase Cdc2 and the mitotic B-type cyclin.

A recent study by Zachos et al. suggested that Chk1 functions indirectly in the S/M checkpoint by maintaining any stalled but viable replication structures (Zachos et al., 2005). Chk1-deficient DT40 cells were able to initially delay mitosis in response to

replication block but eventually entered mitosis with un-replicated DNA, suggesting a defect in the maintenance of the delay. As these cells still expressed functional Chk2, the question was raised of whether the initial mitotic delay in response to replication block could be executed by Chk2. Interestingly, when Chk2-deficient DT40 cells were treated with a selective Chk1-inhibitor, they still retained an initial mitotic delay after DNA replication block, however whether the kinetics of checkpoint failure were altered was not resolved. Building on these observations, it was of interest to attempt to generate a DT40 Chk1/Chk2 double-knock-out cell line in order to study checkpoint function in the absence of both kinases. These initial observations also raised the possibility of the existence of intrinsic control mechanisms that might operate independently of Chk1 and Chk2 function, a notion that could benefit from further investigation.

Furthermore, it was of interest to determine how cells deficient for Chk1 can execute normal cell cycle progression as well as checkpoint responses, such as the initial S/M delay after DNA replication arrest. The previous study by Zachos et al. indicated that the premature mitotic entry of Chk1-deficient cells appeared to be associated with the loss of all viable replication structures. This hypothesis was derived from the observation that the loss of the surrogate marker PCNA (proliferating cell nuclear antigen) appeared to precede the onset of premature mitosis (Zachos et al., 2005). However, this study did not document the exact timing of the loss of viable replication structures and the onset of premature mitosis in individual cells, an issue which was aimed to be addressed here in order to gain insight into the mitotic delay mechanism.

In addition to that, it was of interest to further test the hypothesis of whether the replication protein Cdc6 functions in replication checkpoint control in vertebrate cells. Moreover, if this was the case, the two vertebrate systems could potentially be employed to identify the exact pathway in which Cdc6 functions. For example, the issue of whether Cdc6 might operate upstream or downstream of Chk1 could be resolved using the Chk1-proficient in combination with the Chk1-deficient cell systems available.

## 2 Materials and Methods

### 2.1 Materials

#### 2.1.1 Chemicals and reagents

Unless specified otherwise, chemicals and reagents were purchased in the highest available quality from Fisher Scientific UK, GE Healthcare, Invitrogen Ltd, Severn Biotech Ltd, and Sigma-Aldrich.

#### 2.1.2 Enzymes and kits

**Amaxa:** Cell line nucleofector kit T

**Applied Biosystems:** GeneAmp RNA PCR Core Kit, GeneAmp PCR Core Reagents

**GE Healthcare:** ECL Western Blotting Detection Reagents, Rediprime II DNA labelling system

**Invitrogen Ltd:** Restriction enzymes and reaction buffers

**New England Biolabs Ltd:** Restriction enzymes and reaction buffers

**Promega:** Shrimp Alkaline Phosphatase

**QIAGEN:** QIAprep Spin Mini-Prep Kit, QIAGEN Plasmid Maxi Kit, QIAquick PCR purification Kit, QIAquick Gel Extraction Kit

**Roche:** Rapid DNA Ligation Kit

**Stratagene:** Herculase Enhanced DNA polymerase and 10x reaction buffer

#### 2.1.3 Bacteriology

**Beatson Institute Central Services:** LB broth culture medium, LB plates with 100µg/ml Ampicillin or 30µg/ml Kanamycin, sterile glycerol

**Invitrogen:** *E.coli* DH5 $\alpha$  competent cells, SOC medium

**Sigma-Aldrich:** Ampicillin, Kanamycin

### **2.1.4 Cell culture**

**Beatson Institute Central Services:** sterile distilled water, sterile PBS (Phosphate Buffered Saline), sterile PE (Phosphate Buffered Saline/EDTA)

**Beckman Coulter:** JE-5.0 Elutriation System and Avanti J-20XP centrifuge with Masterflex L/S easy-load II pump system

**Autogen Bioclear:** Blasticidin S, Foetal Bovine Serum, Zeocin

**Invitrogen:** DMEM (Dulbecco's Modified Eagle's Medium), 200mM L-Glutamine (100x), Lipofectamine 2000 Reagent, Oligofectamine Reagent, OptiMEM 1 (Reduced Serum Medium), 2.5% Trypsin (10x)

**Fisher Scientific UK:** DMSO (Dimethyl sulfoxide), HCl (Hydrochloric acid)

**Sigma-Aldrich:** Amphotericin B (Fungizone), Aphidicolin, 5-Bromo-2-deoxyuridine (BrdU), Caffeine, Chicken Serum, Cycloheximide, 10x DMEM, MG132, Nocodazole, Penicillin G, Poly-L-Lysine, Puromycin, Roscovitine, Sodium bicarbonate solution 7.5%, Sodium pyruvate 100mM, Streptomycin Sulphate

**Merck:** Allsterpaullone, Purvalanol A, Wortmannin

**NCI (Bethesda, MD, USA):** UCN-01 (7-hydroxy-staurosporine)

## 2.1.5 Antibodies

**Table 1: Primary and secondary antibodies**

Antibody	Host species	Company	Dilution Duration
Monoclonal Anti-Actin, clone AC-40 A4700	mouse monoclonal	Sigma-Aldrich	1:1000 1h
Bromodeoxyuridine, Bu20a	mouse monoclonal	Dako Ltd.	1:40 1h
Cdc2 p34 (17): sc-54	mouse monoclonal	Santa Cruz Biotechnology, Inc.	1:1000 1h
p-Cdc2 p34 (Thr14/Tyr15): sc-12340	rabbit polyclonal	Santa Cruz Biotechnology, Inc.	1:1000 overnight
p-Cdc2 (Tyr 15): sc-7989	rabbit polyclonal	Santa Cruz Biotechnology, Inc.	1:1000 overnight
Phospho-cdc2 (Thr161) antibody #9114	rabbit polyclonal	Cell Signaling Technology	1:1000 overnight
CDC47/MCM7 Ab-2 (clone 47DC141)	mouse monoclonal	NeoMarkers	1:1000 1h
Cdc6 (H-304): sc-8341	rabbit polyclonal	Santa Cruz Biotechnology, Inc.	1:1000 overnight
Chk1 (G-4): sc-8404	mouse monoclonal	Santa Cruz Biotechnology, Inc.	1:1000 1h
Phospho-Chk1 (S317) antibody #2344	rabbit polyclonal	Cell Signaling Technology	1:1000 overnight
Phospho-Chk1 (Ser345) antibody #2341	rabbit polyclonal	Cell Signaling Technology	1:1000 overnight
Anti-CHK2, clone 7, Catalog #05-649	mouse monoclonal	Chemicon	1:1000 overnight
Cyclin B2, raised against C-terminal peptide from chicken (CSQLNSKTIKDLAASLL)	rabbit polyclonal	In house: Cancer Research UK	1:1000 1h
Anti-phospho-Histone H3 (Ser10), Mitosis Marker, Catalog # 06-570	rabbit polyclonal	Chemicon	1:33 3h
PCNA (PC10): sc-56	mouse monoclonal	Santa Cruz Biotechnology, Inc.	1:1000 1h
HRP-Linked anti Mouse antibody IgG		New England Biolabs	1:5000 1h
HRP-Linked anti Rabbit antibody IgG		New England Biolabs	1:5000 1h
Goat Anti-mouse FITC	goat	Stratech Scientific LTD	1:100 1h
Goat Anti-rabbit FITC	goat	Stratech Scientific LTD	1:100 1h
Goat Anti-mouse TRITC	goat	Stratech Scientific LTD	1:100 1h
Goat Anti-rabbit Phycoerythrin	goat	Stratech Scientific LTD	1:100 1h
Goat Anti-rabbit TRITC	goat	Stratech Scientific LTD	1:100 1h

## **2.1.6 Immunofluorescence**

**Fisher Scientific UK:** Acetone, Methanol

**Science Services Ltd:** Paraformaldehyde solution 16%

**Sigma-Aldrich:** Poly-L-Lysine, Propidium Iodide (PI)

**Vector Laboratories, Inc.:** Vectashield mounting medium, Vectashield with DAPI (4',6-diamidino-2-phenylindole)

## **2.1.7 Plasmids, Primers, Probes**

### **Plasmids**

pcDNA 3.1Zeo RFP-PCNA based on pcDNA 3.1Zeo from Invitrogen

pEGFP-C1 RFP-PCNA obtained from CG Morrison, based on pEGFP-C1 from BD Biosciences Clontech

Chk2 gene targeting construct adapted from pBS-LA/Puro/RA2 designed by M. Rainey (Rainey, 2003): LA-left arm was based on PCR product amplified from chicken genomic DNA with primers LAa and LAb, the RA-right arm was based on the product obtained from a restriction digest with BamH1 and KpnI of a PCR product amplified from chicken genomic DNA with primers RAc and RAd. The selectable marker cassette Puro was replaced by BSR from a ploxBSR vector obtained from JM Buerstedde (Arakawa et al., 2001). Targeting construct backbone pBluescript SK(II) was obtained from Stratagene. More detailed description of the generation of the original targeting vectors can be found in the thesis of M. Rainey (Rainey, 2003) and general chicken genome sequence information can be obtained from [http://www.ensembl.org/Gallus\\_gallus](http://www.ensembl.org/Gallus_gallus).

**Primers**

A	Forward	5'-ATG TCT CGA GAG ACG AGG AGC-3'
C	Reverse	5'-ACC AGT ACT CAT CAT TCA CAC-3'
Bsr1	Reverse	5'-GAT CTG GGC TAG CCC TGA TC-3'
Bsr2	Forward	5'-CTC ATC AAT GTA TCT TAT CAT GTC-3'
F	Forward	5'-GTG GAC TGC TGG AGT TTA GGA-3'
H	Forward	5'-CAC TCG TGG AGA ATA CAC ATT-3'
I	Reverse	5'-AAT GTG TAT TCT CCA CGAGTG-3'
LA1	Forward	5'-GTG TGC GCG CTG CTC CT-3'
R	Reverse	5'-GCC AGG GAT GCT CTA AGG CTT CCT C-3'
RA1	Reverse	5'-GGC TGT ACT TAA TAG ACT CAA T-3'
RA2	Reverse	5'-CAT GTT GGA TAC GTG TTT CCA-3'
V	Forward	5'-TCG GCG CTC GCC ATG TCT-3'
LAA	Forward	5'- <u>TTGCGGCCGCAA</u> AGACGAGGAGCGACGCGC-3'
LAb	Reverse	5'- <u>CGGGATCCCCGA</u> ACCAGTACTCATCATTACAC-3'
RAc	Forward	5'- <u>GAAGATCTTCAGC</u> ACTTGTAATAAAGTTGCGG-3'
RAd	Reverse	5'- <u>GGGGTACCCA</u> ATGTGTATTCTCCACGAGTGA-3'

**Probes**

Probe A used for Southern blotting was generated by M. Rainey as previously described (Rainey, 2003).

### 2.1.8 siRNA sequences

All siRNA SMARTpool reagents were obtained from DHARMACON RNA Technologies.

**Chk1:** siGENOME SMART pool reagent (of four duplexes) and individual siRNA, raised against Human CHEK1, NM\_001274

**Chk2:** siGENOME SMART pool reagent (of four duplexes) and individual siRNA, raised against Human CHEK2, NM\_007194

**Table 2: siRNA duplex sequences**

siRNA	Sequence
Chk1 siGENOME duplex (6)	Sense sequence: G.C.A.A.C.A.G.U.A.U.U.U.C.G.G.U.A.U.A.U.U Antisense sequence: 5'- P.U.A.U.A.C.C.G.A.A.A.U.A.C.U.G.U.U.G.C.U.U
Chk1 siGENOME duplex (7)	Sense sequence: G.G.A.C.U.U.C.U.C.U.C.C.A.G.U.A.A.A.C.U.U Antisense sequence: 5'- P.G.U.U.U.A.C.U.G.G.A.G.A.G.A.A.G.U.C.C.U.U
Chk1 siGENOME duplex (8)	Sense sequence: A.A.A.G.A.U.A.G.A.U.G.G.U.A.C.A.A.C.A.U.U Antisense sequence: 5'- P.U.G.U.U.G.U.A.C.C.A.U.C.U.A.U.C.U.U.U.U.U
Chk1 siGENOME duplex (9)	Sense sequence: C.C.A.C.A.U.G.U.C.C.U.G.A.U.C.A.U.A.U.U.U Antisense sequence: 5'- P.A.U.A.U.G.A.U.C.A.G.G.A.C.A.U.G.U.G.G.U.U
Chk2 siGENOME duplex (5)	Sense sequence: G.A.A.A.U.U.G.C.A.C.U.G.U.C.A.C.U.A.A.U.U Antisense sequence: 5'- P.U.U.A.G.U.G.A.C.A.G.U.G.C.A.A.U.U.U.C.U.U
Chk2 siGENOME duplex (13)	Sense sequence: C.U.C.A.G.G.A.A.C.U.C.U.A.U.U.C.U.A.U.U.U Antisense sequence: 5'- P.A.U.A.G.A.A.U.A.G.A.G.U.U.C.C.U.G.A.G.U.U
Chk2 siGENOME duplex (14)	Sense sequence: A.A.A.C.G.C.C.G.U.C.C.U.U.U.G.A.A.U.A.U.U Antisense sequence: 5'- P.U.A.U.U.C.A.A.A.G.G.A.C.G.G.C.G.U.U.U.U.U
Chk2 siGENOME duplex (15)	Sense sequence: G.C.U.A.A.A.U.C.A.U.C.C.U.U.G.C.A.U.C.U.U Antisense sequence: 5'- P.G.A.U.G.C.A.A.G.G.A.U.G.A.U.U.U.A.G.C.U.U

### **2.1.9 Miscellaneous**

**BioRad:** Bio-Spin 30 Chromatography Columns

**Fuji Photo Film:** Super RX Medical X-ray film

**GE Healthcare:** Redivue ( $\gamma$ - $^{32}\text{P}$ )-ATP, Redivue ( $\alpha$ - $^{32}\text{P}$ )-dCTP, Full-Range Rainbow Molecular Weight Marker, Hybond N<sup>+</sup> Nylon membrane

**Invitrogen:** 100bp DNA Ladder, 1kb DNA Ladder,  $\lambda$  DNA Hind III fragments

**React Scientific:** GE Nitrocellulose Supported Transfer Membrane (0.45 micron)

**Sigma-Aldrich:** Protein A-Sepharose 4B

**Whatman International Ltd.:** Whatman 3mm filter paper

## **2.2 Methods**

### **2.2.1 Bacteriology**

#### **Transformation of DNA into bacterial hosts**

*E. coli* DH5 $\alpha$  competent cells were thawed on ice and a 50 $\mu$ l aliquot was mixed with 2 $\mu$ l of DNA solution (of varying concentrations) in a pre-chilled polypropylene tube. After incubation on ice for 45min, cells were subjected to heat-shock at 42°C for 45s and then chilled on ice for 2 min before 180 $\mu$ l of SOC medium was added and the tube incubated for a further hour at 37°C while shaking. The transformation mixture was spread onto LB-agar plates containing the appropriate antibiotic and incubated overnight at 37°C.

#### **Bacterial culture**

Single bacterial colonies were picked from LB-agar plates and inoculated into 5ml of LB-medium containing the appropriate antibiotic. The culture was grown overnight at 37°C while shaking. For long-term storage, 500 $\mu$ l of bacterial culture were mixed with 500 $\mu$ l of sterile glycerol in a cyrotube and stored at -70°C.

#### **Small-scale plasmid DNA preparation**

1.5ml of bacterial overnight culture was pelleted by centrifugation at 5000rpm for 5min in a micro-centrifuge. The pellet was resuspended and processed using the QIAprep Spin Mini-Prep Kit according to the manufacturer's instructions. The eluted DNA was stored at -20°C.

#### **Large-scale plasmid DNA preparation**

100ml of bacterial overnight culture was pelleted by centrifugation at 6000rpm for 15min at 4°C in a Sorvall RC3C DuPont centrifuge. The pellet was resuspended and processed using the QIAGEN Plasmid Maxi Kit according to the manufacturer's instructions. The concentration and purity of the eluted DNA was determined using an Eppendorf biophotometer. The DNA was stored at -20°C.

## **2.2.2 DNA protocols**

### **Isolation of genomic DNA from cell cultures**

Phenol extraction was used to isolate genomic DNA for PCR and Southern blotting from up to  $10^7$  cells. The cells were harvested, washed, and resuspended in 100 $\mu$ l PBS and incubated overnight at 55°C with the addition of 200 $\mu$ l 1.5x Lysis buffer (0.3M Tris pH 8.0, 0.15M EDTA pH 8.0, 1.5% SDS) plus proteinase K (0.15mg/ml). After addition of another 200 $\mu$ l of 1.5x Lysis buffer, 1 volume (500 $\mu$ l) of phenol/chloroform/isoamyl alcohol was added. Samples were centrifuged at 14,000rpm in a micro-centrifuge and the top aqueous layer transferred to a new tube. Another extraction step was performed using 1 volume of chloroform/isoamyl alcohol and the resulting top aqueous layer was transferred to a new tube again. DNA was precipitated by addition of  $\frac{1}{2}$  volume of 7.5M ammonium acetate and 2 volumes of absolute ethanol. After washing the DNA in 70% cold ethanol, the DNA pellet was resuspended in TE buffer (10mM Tris pH 8.0, 1mM EDTA pH 8.0) and stored at 4°C.

### **Polymerase chain reaction (PCR)**

PCR was performed using the GeneAmp PCR Core Reagents or the Herculase Enhanced DNA polymerase PCR reagents. The PCR protocol was designed according to the manufacturer's instructions. In general 1 $\mu$ l of genomic DNA was used per reaction. Primers were obtained from VHBIO (for sequences see 2.1.7). The reaction was performed in a PTC-200 thermal cycler with a heated lid. In general, the protocol consisted of an initial denaturation step at 95°C for 2min, followed by 35 amplification cycles (94°C denaturation for 1min, 59°C annealing for 1min and 72°C elongation for 1min/1kb of product) and an additional elongation step at the end. PCR products were stored at 4°C.

### **Agarose gel electrophoresis**

1% (w/v) agarose gels were poured by dissolving ultra pure electrophoresis grade agarose in 1xTAE buffer (40mM Tris, 0.114% (v/v) glacial acetic acid, 1mM EDTA pH 8.0) and placed in a horizontal gel apparatus from Stratagene containing 1xTAE running buffer. DNA samples were diluted in 1x DNA loading buffer (for 10x: 50% (v/v) 20x TAE, 50% (v/v) glycerol, 1% (w/v) bromophenol blue) and loaded alongside suitable DNA ladder markers in the wells of the gel. Electrophoresis was performed at 80-100V and the DNA visualised using the BIO-RAD GelDoc 1000 system.

## Cloning protocols

DNA restriction digests were performed using the appropriate restriction enzyme and the corresponding reaction buffer from Invitrogen or New England Biolabs Inc. Reactions were carried out according to the manufacturer's instructions and samples generally incubated in a 37°C water bath for the duration of the digest. DNA from restriction digests or PCR reactions was purified using the QIAquick PCR purification Kit according to the manufacturer's instructions. For purification of DNA fragments from agarose gels the respective band was excised using a scalpel and the DNA recovered using the QIAquick Gel Extraction Kit according to the manufacturer's instructions. The Rapid DNA Ligation Kit from Roche was used for the ligation of DNA fragments into plasmids with T4 DNA ligase. The plasmid DNA used in ligation reactions was pre-treated with Shrimp Alkaline Phosphatase according to the manufacturer's instructions to prevent vector re-ligation. In general, DNA was purified by ethanol precipitation: Potassium acetate was added to a final concentration of 0.3M. After addition of 2 volumes of ethanol, the mixed samples were cooled on ice and then centrifuged at high speed. The DNA pellet was washed with 70% ethanol and then resuspended in an appropriate amount of TE buffer.

## Southern blotting

Genomic DNA isolated from Chk1<sup>-/-</sup> DT40 clones transfected with the Chk2 targeting construct was analysed for targeted integration by Southern blotting. The DNA was digested in an excess of EcoRI restriction enzyme overnight and separated on a 0.8% agarose gel alongside a  $\alpha$ -<sup>32</sup>P-labelled DNA ladder. In preparation for transfer, the gel was subjected to an acid depurination wash in 0.25M HCl for 30min, an alkali denaturation wash in 1.5M NaCl/0.5M NaOH for 1h, and a neutralisation wash in 1M Tris-HCl pH8.0/1.5M NaCl for 1h. The transfer was set up in a large dish filled with 20xSSC (3M NaCl, 300mM trisodium citrate, pH7.0). Two sheets of 33mm paper with 5cm overhangs into the buffer solution were placed on a glass plate on top of four rubber stoppers. The inverted gel was positioned on the paper, removing any air bubbles, and the nylon filter membrane adjusted on top of the gel. A further two sheets of 33mm paper and a stack of paper towels and a weight were sat on top and saran wrap placed around the sides of the gel. This setup allowed the flow of 20xSSC buffer from the dish up through the gel and the nylon membrane, thereby transferring DNA fragments onto the membrane. In general, the transfer was left to proceed for 16-24h, and then the DNA was cross-linked to the membrane using 2400J/m<sup>2</sup> in a UV Stratalinker 1800.

## **Probe hybridisation**

The nylon filter membrane was placed on mesh into an Appligene hybridisation bottle containing 30ml of pre-warmed hybridisation buffer (0.25M Na<sub>2</sub>HPO<sub>4</sub> pH7.2, 7% (w/v) SDS, 1mM EDTA pH8.0) and incubated in an Appligene rotating mini-hybridisation oven at 65°C for 3-4h. After pre-hybridisation, the solution was replaced with a 30ml aliquot of hybridisation buffer containing the labelled probe and incubation continued at 65°C overnight. The probe was generated using the Rediprime II DNA labelling system according to the manufacturer's instructions. In general, 50ng diluted and denatured DNA was combined with the Rediprime reaction mix and 5µl (50µCi) of α-<sup>32</sup>P-dCTP and incubated at 37°C for 10 min. The reaction was stopped by addition of 5ml 0.2M EDTA and the labelled DNA was passed through a Bio-Spin 30 Columns to remove any unincorporated nucleotides. Prior to hybridisation, the DNA was denatured for 5min. After probe-hybridisation, the nylon filter was washed 2x in 5% wash buffer (20mM Na<sub>2</sub>HPO<sub>4</sub> pH 7.2, 1mM EDTA pH8.0, 5% (w/v) SDS) and 2x in 1% wash buffer (20mM Na<sub>2</sub>HPO<sub>4</sub> pH 7.2, 1mM EDTA pH8.0, 1% (w/v) SDS) for 10min each at 65°C in the rotating oven. The nylon filter was briefly dried, wrapped in saran wrap and exposed to X-ray film at -70°C.

### **2.2.3 Cell Culture**

All cell culture work was performed using aseptic techniques inside a laminar flow hood.

#### **Growth conditions for avian DT40 cell lines**

Chicken B-lymphoma DT40 suspension cells were grown at 39°C and 5% CO<sub>2</sub> in a humid incubator. The DT40 culture medium consists of DMEM supplemented with 10% foetal bovine serum (FBS), 1% chicken serum (heat-inactivated at 56° for 2 hours), 2mM L-glutamine, 10µM β-mercaptoethanol, 30µg/ml penicillin, 50µg/ml streptomycin. DT40 cells grow in suspension and were routinely passaged every 3-4 days by dilution (1:10) into fresh culture medium. For long-term storage cells were frozen in 10% DMSO in FBS and stored in liquid nitrogen vapour phase tanks.

#### **Growth conditions for human BE cell line**

Human colon carcinoma BE adherent cells were grown at 37°C and 5% CO<sub>2</sub> in a humid incubator. The BE culture medium consists of DMEM supplemented with 10% FBS, 2mM

L-glutamine, 30µg/ml penicillin, 50µg/ml streptomycin. BE cells were routinely passaged every 3-4 days. Cells were briefly exposed to 0.25% trypsin in PE buffer, loosened off the culture dish by agitation and then resuspended and diluted (1:10) into fresh medium. For long-term storage cells were frozen in 10% DMSO in FBS and stored in liquid nitrogen vapour phase tanks.

### **Transfection of BE cells with siRNA**

For transfection of siRNA with Oligofectamine reagent, BE cells were seeded at  $2.5 \times 10^5$ /ml 16-24h prior to transfection. Oligofectamine was used according to the manufacturer's instructions. In general, both siRNA (e.g. 50nM of siRNA pool, individual duplex or negative control) and Oligofectamine were diluted in OptiMEM, incubated at RT for 5min, then combined and incubated for a further 20min. The mixture was added directly to the cells. Down regulation of protein levels was generally achieved after 48h-72h in culture and confirmed by Western blotting.

### **Transient transfection of cells with plasmid DNA**

For transient transfection with plasmid DNA, cells were set up at an appropriate density in a culture dish (e.g. BE at  $1.2 \times 10^5$ /ml). After 16-24h transfection of the DNA of choice was performed using Lipofectamine 2000 according to the manufacturer's instructions. In general, both DNA (e.g. 5-10µg) and Lipofectamine 2000 were diluted in OptiMEM, incubated at RT for 5min, then combined and incubated for a further 20min, and then added directly to the cells. Transgene expression was in general tested after 24-48 hours.

### **Stable transfection of DT40 cells with plasmid DNA**

The Amaxa nucleofactor kit T was routinely used for the stable transfection of DT40 cells. The DNA of choice was linearised with the appropriate restriction enzyme, ethanol precipitated and resuspended in TE (Tris-EDTA) buffer.  $1-5 \times 10^6$  cells were pelleted, resuspended in 100µl of solution T /DNA, transferred to a cuvette and placed in the Nucleofactor. Program A23 was routinely used. Cells were then returned to culture, allowed to recover overnight and plated into 96 well plates in the presence of the appropriate antibiotic. After approximately 2 weeks, when cell colonies appear visibly in the wells, clones were picked and expanded for subsequent analysis.

### **2.2.4 Elutriation of living cells**

This method was used to separate an asynchronous cell population into fractions enriched in a specific phase of the cell cycle: G1-S-G2/M. The method was also used to synchronise an asynchronous cell population by elutriating only cells that were in G1/S phase of the cell cycle and then returning them to culture to perform time course experiments. The principle of the elutriation method is that a cell population inside a rotating flow chamber is exposed to two opposing forces, the centrifugal force and the flow force. Cells can be eluted according to their size by changing either the flow rate or in this case by changing the rotation speed. Decrease of the rotation speed in small intervals will release small cells (such as those in G1) first, and then with further decreases in rpm larger cells at later stages of the cell cycle are progressively eluted.

Elutriation of living cells was performed using the Beckman Coulter JE-5.0 Elutriation System in combination with an Avanti J-20XP centrifuge and Masterflex L/S easy-load II pump system. DT40 cells were set up at  $0.3 \times 10^6$ /ml in large culture flasks and after 16-24h cells were pelleted, and resuspended in 7ml of medium. Best results were achieved with approximately  $2-3 \times 10^8$  cells, maximum capacity of the flow cell is  $10^9$  cells. BE cells were set up to be 80% confluent, washed, trypsinised and then pelleted and resuspended in 7ml of medium. The cell solution was inserted into the loading chamber of the elutriator and then allowed to enter the flow chamber at a constant flow of 40ml/min of elutriation medium at 4000rpm. Once all cells were loaded, cell fractions were elutriated by gradual reduction of rotor speed by 200rpm increments while the medium flow rate was kept at a constant speed of 40ml/min. Cells were collected in 100-150ml aliquots and then either further analysed by FACS and Western blotting or returned into culture and treated as required.

### **2.2.5 Protein Extraction and Analysis**

#### **Whole cell extracts**

Cells were treated as required and then harvested by pelleting them directly (suspension cells) or scraping them into PBS (adherent cells) and pelleting them into an eppendorf tube. Cell pellets were washed in ice cold PBS and resuspended in whole cell extract lysis buffer (20mM HEPES, 5mM EDTA, 10mM EGTA, 5mM NaF, 50ng/ml Okadaic acid, 1mM DTT, 0.4M KCl, 0.4% Triton X-100, 10% Glycerol, 5µg/ml Leupeptin, 50µg/ml PMSF, 1mM Benzamidin, 5µg/ml Aprotinin, 1mM Sodium Vanadate). Samples were

then incubated on ice for 20-30 min and subsequently centrifuged at 14,000rpm in a cooled micro-centrifuge. Supernatants were removed, analysed for protein concentration and stored at -80oC.

### Determination of protein concentration: Bradford Assay

Bovine serum albumin (BSA) was used to prepare a dilution series of standard protein concentrations in lysis buffer ranging from 0mg/ml to 2mg/ml. Each BSA standard sample was diluted with a 50% Coomassie protein assay reagent solution and the absorbance determined at a wavelength of 595 nm using an Eppendorf biophotometer. The absorbance values were used to generate a standard curve. 10µl of each protein sample was measured in the same way and the protein concentration determined from the standard curve.

### Protein analysis:

Protein samples were resolved on SDS-polyacrylamide gels composed of a 10% resolving gel solution and a stacking gel solution.

**Table 3: SDS PAGE resolving and stacking gels**

<b>10% resolving gel</b>	<b>40ml</b>
H <sub>2</sub> O	15.8ml
30% Acrylamide mix 37.5:1 Bis Acrylamide ratio	13.3ml
1.5M Tris (pH8.8)	10.0ml
10% SDS	0.4ml
10% Ammonium persulfate (APS)	0.4ml
N,N,N',N'-Tetramethylethylenediamine (TEMED)	0.016ml
<b>10% phosphorylation shift gel</b>	<b>40ml</b>
H <sub>2</sub> O	12.9ml
40% Acrylamide mix 2% Bis-Acrylamide	10ml 1.7ml
1M Tris (pH8.8)	15ml
10% SDS	0.4ml
10% APS	0.4ml
TEMED	0.016ml
<b>Stacking gel</b>	<b>10ml</b>
H <sub>2</sub> O	6.8ml
30% Acrylamide mix 37.5:1 Bis-Acrylamide ratio	1.7ml
1M Tris (pH6.8)	1.25ml
10% SDS	0.1ml
10% APS	0.1ml
TEMED	0.01ml

In general, 50µg of protein in SDS sample buffer (4 volumes of 5% SDS, 25% glycerol, 125mM Tris pH6.8, bromophenolblue and 1 volume of 1M DTT) were boiled for 5 min and then loaded into individual wells alongside a full-range molecular weight marker. Gels were run in a gel tank in SDS Running buffer (25mM Tris, 0.1% SDS, 0.19M glycine) at 20 to 250 V until dye front migrated to the bottom of the gel tank. Gels were transferred onto nitrocellulose by Western blotting.

### **Protein analysis: Western blotting**

Polyacrylamide gels were transferred onto a nitrocellulose filter using a semi-dry electroblotter. The nitrocellulose and 10 pieces of 3mm paper were soaked in transfer buffer (48mM Tris pH9.2, 39mM glycine, 1.3mM SDS, 20% (v/v) methanol) and assembled on the blotter, from anode to cathode, 5 pieces of 3mm paper, nitrocellulose, resolving polyacrylamide gel, and 5 pieces of 3mm paper. The transfer was performed at 20V, 200mA, 10W for 1 hour.

The nitrocellulose filter blot was washed briefly in TBST (10mM Tris pH7.6, 100mM NaCl, 0.1% (v/v) Tween 20) and then blocked in 5% (w/v) Marvel in TBST for an hour. Appropriate dilutions of primary antibody were prepared in 5% Marvel/TBST or for phospho-specific antibodies in 5% (w/v) BSA in TBST. The blot was incubated for at least one hour at room temperature or over night at 4°C. Next, blots were washed 3 x 5min in TBST before being incubated in 5% Marvel/TBST containing a 1/500 dilution of appropriate horseradish-peroxidase-conjugated secondary antibody for an hour. After washing once for 15min in 5% Marvel/TBST and a further 3 x5 min in TBST, the blots were analysed by enhanced chemiluminescence with ECL detection reagent, exposed to film and developed with a Kodak processor.

### **Measurement of Cdc2 kinase activity: IP-Kinase Assay**

Cdc2 kinase activity was measured by an immunoprecipitation (IP) kinase assay. DT40 cells were elutriated and the G1/S population was returned to culture and treated as required. Cells were harvested after 14h, washed in ice-cold PBS and resuspended in the appropriate amount of IP lysis buffer (50 mM Hepes pH 7.5, 150mM NaCl, 1mM EDTA, 2.5mM EGTA, 10% Glycerol, 1mM DTT, 0.1% Tween20, 10mM β-glycerophosphate, 1mM NaF, 0.1mM NaVO<sub>3</sub>, 2µg/ml Aprotinin, 5µg/ml Leupeptin, 0.1M PMSF) and frozen

on dry-ice for at least 5min. Samples were thawed on ice and centrifuged at 14,000rpm in a cooled micro-centrifuge. The protein concentration of the supernatants was analysed by Bradford assay. Per IP reaction, 0.2-1mg of protein was diluted in IP lysis buffer and pre-cleared with 40 $\mu$ l of a 50% Protein A Sepharose slurry by rotating for 1h at 4°C. The sample was centrifuged at high speed for 1min and the supernatant transferred into a new tube. The appropriate amount (e.g. 2 $\mu$ g) of immune or pre-immune serum was added and the sample incubated for at least 3h or overnight at 4°C on a rotating wheel. After addition of 40 $\mu$ l of 50% Protein A Sepharose slurry, the sample was incubated for another hour at 4°C while rotating and then centrifuged at high speed for 1min. The supernatant was discarded and the pellet washed 3x in IP lysis buffer and 3x in kinase buffer (50mM HEPES pH 7.5, 50mM NaCl, 10mM MgCl<sub>2</sub>, 10mM MnCl<sub>2</sub>, 1mM DTT). The kinase reaction was performed in a 20 $\mu$ l reaction mix containing 1 $\mu$ g Histone H1 substrate, 50 $\mu$ M ATP, 2 $\mu$ Ci  $\gamma$ -<sup>32</sup>P ATP in kinase buffer. The pellet was incubated in the reaction mixture for 30min at 30°C. The sample was boiled in SDS sample buffer, run on a 10% SDS PAGE gel. The gel was transferred to a nitrocellulose filter, which was briefly dried, wrapped in saran wrap and exposed to an X-ray film at -70°C.

## **2.2.6 Flow cytometry protocols**

### **Preparation and fixation of cells**

DT40 or BE cells were set up and treated as required. An appropriate amount (5-10x10<sup>5</sup>/ml) were harvested (trypsinised if adherent), washed in PBS, and fixed in 70% Ethanol in PBS at 4°C for at least 30min or overnight.

### **Cell Cycle Analysis: PI staining (DNA content)**

Propidium iodide is a fluorescent dye that intercalates with nucleic acids and can be used to analyse the DNA content of a cell population. Fixed cells were pelleted and resuspended in 1ml of PBS containing 25 $\mu$ g/ml propidium iodide and 250 $\mu$ g/ml RNaseA and incubated for 30min at RT in the dark before being analysed by flow cytometry.

### **Cell Cycle Analysis: BrdU/PI staining (proliferating cells)**

Bromodeoxyuridine is a synthetic thymidine analogue which can be incorporated into newly synthesised DNA in replicating cells. In general, 25 $\mu$ M BrdU was added to the cells prior to harvesting for 30min. Once fixed, cells were resuspended in 1ml of PBS and the

DNA denatured at RT for 15min with addition of 1ml of 4N HCl. Cells were then pelleted, washed in PBT (PBS-0.5%BSA, 0.1%Tween20), pelleted again, and resuspended in PBT containing a 1/40 dilution of Anti-BrdU mouse monoclonal antibody and incubated at RT for 30min. Samples were washed again, pelleted, and resuspended in PBT containing a 1/40 dilution of anti-mouse FITC conjugate and incubated for 30 min at RT in the dark. After an additional wash, cells were pelleted and resuspended in 1ml of PBS containing 25µg/ml propidium iodide and 250µg/ml RNaseA and incubated for 30min at RT in the dark before being analysed by flow cytometry.

### **Cell Cycle Analysis: P-S10-H3/PI staining (mitotic cells)**

The phosphorylation of Serine 10 on Histone H3 is an early event during mitosis and can be used as a tool for quantification of the percentage of mitotic cells in a population. In general, fixed cells were pelleted and resuspended in 1ml PBS containing 0.1% Triton X-100 and incubated on ice for 1h. Cells were pelleted and resuspended in 1% BSA/PBS containing a 1/33 dilution of Anti-P-S10-H3 rabbit polyclonal antibody for at least 1h at RT. Samples were washed and pelleted again and resuspended in 1% BSA/PBS containing a 1/33 dilution of anti-rabbit FITC conjugate and incubated for 30min at RT in the dark. After an additional wash, cells were pelleted and resuspended in 1ml of PBS containing 25µg/ml propidium iodide and 250µg/ml RNaseA and incubated for 30min at RT in the dark before being analysed by flow cytometry.

### **Flow cytometry**

Samples were analysed using a Becton Dickinson FACScan with a laser at 488nm and Macintosh CellQuest software. Data was then further analysed and quantified using PC WinMDI software.

## **2.2.7 Immunofluorescence**

### **Fixing and staining cells for confocal microscopy**

Cells were set up at appropriate concentration (e.g. DT40 at  $2.5 \times 10^5$ /ml). Suspension cells were grown on poly-L-lysine coated cover slips and adherent cells were grown on uncoated cover slips generally in 6 well plates. After respective treatment, cells were fixed either in 4% paraformaldehyde for 15 min, or in 50:50 Methanol/Acetone mix for 10 min. Cover slips were washed and permeabilised 3x 5min in 0.1% Triton X-100 in PBS and

blocked for 30 min in 10% FCS, 0.5% BSA in PBS. The cover slips were transferred to a humid black box and the blocking solution was replaced with a 1/100 dilution of primary antibody/antibodies in blocking buffer. After 1-3h incubation at room temperature, the cells were washed 3x 5 min in blocking buffer. Then the secondary antibody/antibodies/PI was added in a 1-2/100 dilution in blocking buffer for 40 min in the dark at room temperature. Finally cover slips were washed twice in blocking buffer and once in permeabilising buffer and then mounted on a glass slide with vectashield (clear or with Dapi) and sealed with clear nail varnish. The cover slides were analysed using Leica confocal microscopes and windows based LCS software.

### **Time-lapse microscopy**

DT40 H2B-GFP PCNA-RFP suspension cells were grown in Iwaki 35mm glass bottom dishes. Time-lapse microscopy was performed using an inverted Zeiss microscope system with temperature humidity 37°C and CO<sub>2</sub> control. Pictures were taken using a 40x objective and three channels (Bright field, GFP, RFP) at 2 min intervals over up to 20 hours. Analysis of experimental data was performed using AndorIQ software.

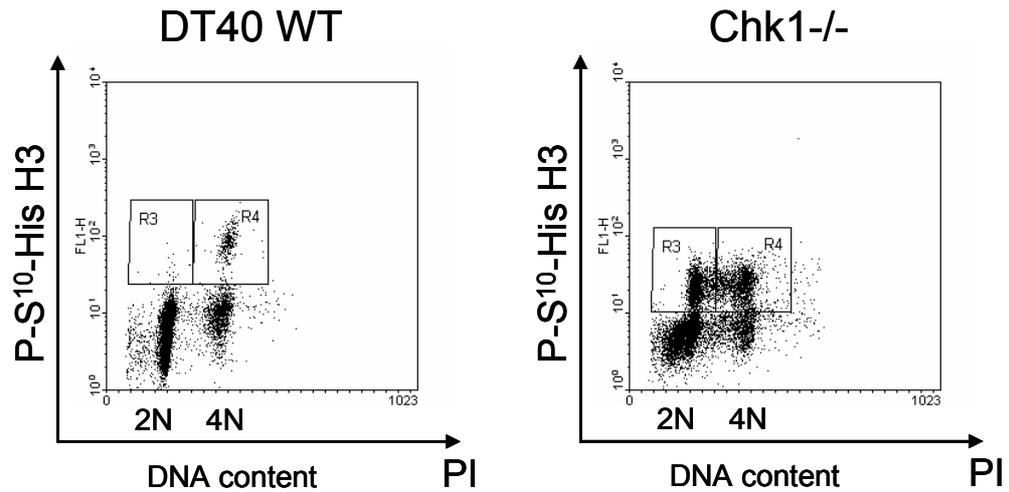
## **3 Results: Requirement for Chk1 and Chk2 function for S/M and G2/M checkpoints**

### **3.1 Mitotic checkpoint responses in avian DT40 cells**

#### ***3.1.1 Analysis of mitotic delay proficiency in DT40 WT, Chk1<sup>-/-</sup>, and Chk2<sup>-/-</sup> knock-out cell lines in response to replication block and DNA damage***

The role of Chk1 and Chk2 in different checkpoint responses has been shown to vary between different organisms. Furthermore it has been difficult to study Chk1 function genetically in mammalian cells due to the lack of a viable knock-out model. In order to investigate and compare Chk1 and Chk2 function in vertebrate cells, this study used avian DT40 knock-out cell lines which are viable in the absence of either Chk1 or Chk2. The generation of the Chk1-deficient and Chk2-deficient DT40 cell lines has been described previously (Rainey et al., 2007; Zachos et al., 2003).

This study focused on checkpoint responses to two distinct types of genotoxic stress that both delay the onset of mitosis: replication block and DNA damage. When DNA replication is blocked, for example with the DNA polymerase inhibitor aphidicolin, checkpoint proficient cells will arrest in S-phase and delay the onset of mitosis. This S/M checkpoint response ensures that cells do not enter mitosis in the presence of un-replicated DNA. Failure of the S/M checkpoint is characterised by an increase in the total number of cells entering mitosis when DNA replication is blocked and more specifically by the appearance of mitotic cells with a DNA content of less than 4N. Throughout this study mitotic checkpoint proficiency has been measured by staining for both the DNA content and a mitotic marker, the phosphorylation of Histone H3 on Serine 10 (P-S10-H3), and subsequent analysis of the samples by flow cytometry. A typical example of a functional mitotic delay in response to replication block as observed in DT40 WT cells can be seen in Figure 3.1. Cells were treated with the DNA polymerase inhibitor aphidicolin and grown in the presence of the spindle poison nocodazole which traps cells in mitosis and therefore allows an accurate measurement of both the proportion and DNA content of cells that enter mitosis during the course of the experiment. As shown in Figure 3.1, after prolonged treatment with aphidicolin and nocodazole, relatively few mitotic cells are observed and all of these exhibit 4N DNA content, i.e. they have completed DNA replication.



**Figure 3.1: Measurement of S/M checkpoint proficiency by flow cytometry**

DT40 WT and Chk1<sup>-/-</sup> cells were treated with the DNA polymerase inhibitor aphidicolin for 16h in the presence of nocodazole. Samples were analysed by flow cytometry for DNA content and mitotic indices (P-S10-H3/PI). Gates R3 and R4 depict mitotic cells with 2N and 4N DNA content respectively.

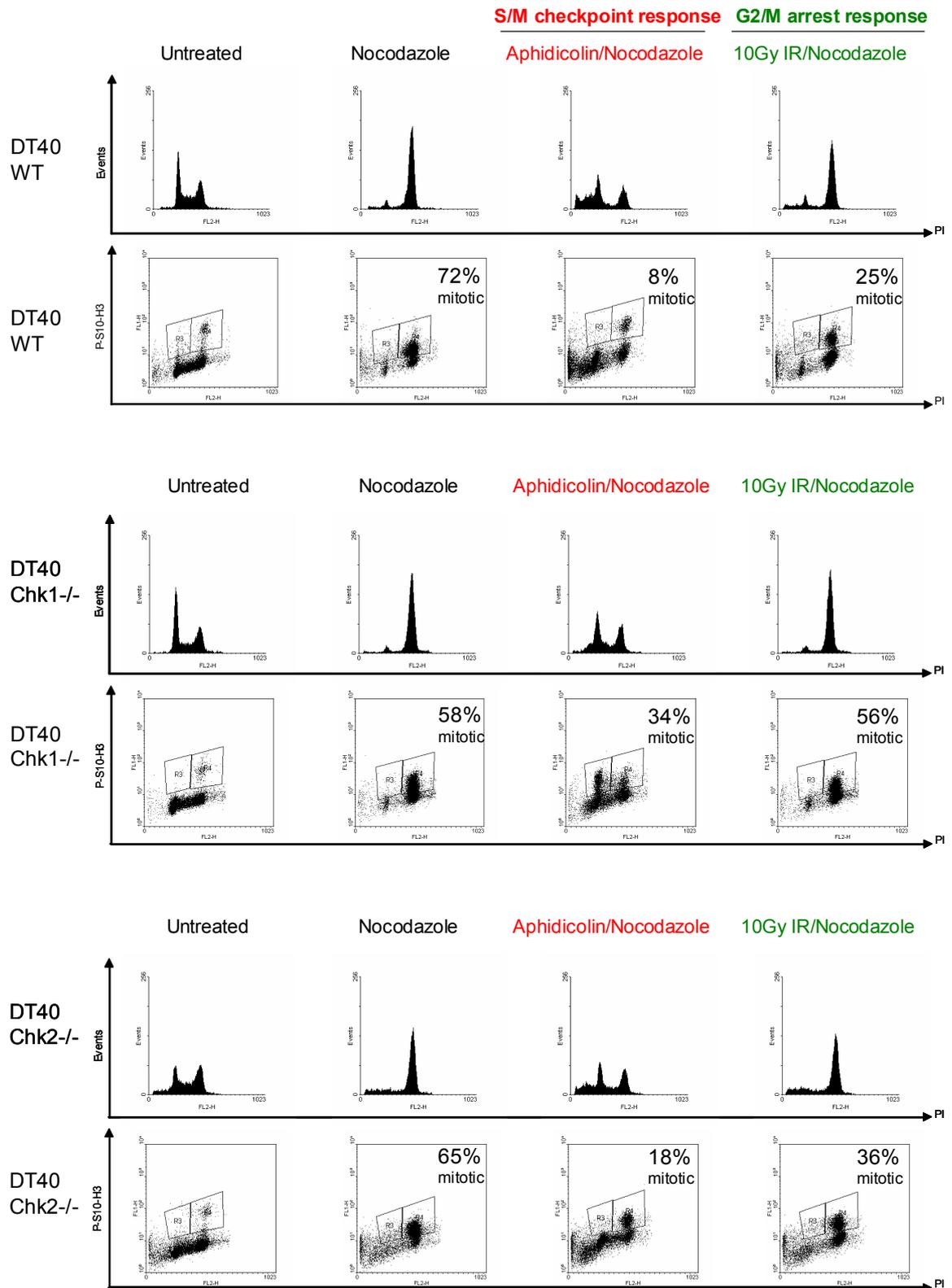
As described in more detail below, DT40 cells which are deficient for Chk1 (Chk1<sup>-/-</sup> cells) exhibit S/M checkpoint failure under these conditions, resulting in an increase in the total number of mitotic cells and the appearance of a large number of 2N mitotic cells (Figure 3.1).

The other mitotic checkpoint response of interest is activated when cells are subjected to DNA damage, such as DNA double-strand breaks induced by irradiation (IR). This G2/M arrest ensures that cells with damaged DNA are unable to divide and arrest in G2 until the DNA damage has been resolved. Failure of the G2/M checkpoint is characterised by cells entering mitosis without any measurable arrest, which can be detected as an increase in the number of 4N mitotic cells if nocodazole is present in the experiment.

The response of DT40 WT, Chk1<sup>-/-</sup>, and Chk2<sup>-/-</sup> cells to these two distinct genotoxic stresses that delay mitosis was analysed by flow cytometry. Cells were subjected to either 10Gy of IR to induce DNA damage or treated with the DNA polymerase inhibitor aphidicolin to block DNA replication. The cells were grown in the presence of the spindle poison nocodazole and an untreated control was also included in the analysis.

As shown in Figure 3.2, after 16h in the presence of nocodazole alone, a majority (72%) of DT40 WT cells progressed into mitosis. In response to DNA polymerase inhibition with aphidicolin, a high proportion of DT40 WT cells were blocked from entering mitosis and no cells entering mitosis with 2N DNA content were observed, demonstrating activation of the S/M checkpoint. When DT40 WT cells were challenged with 10Gy of IR and then returned to culture, approximately two thirds of the cells arrested in G2 phase with a DNA content of 4N, displaying a functional G2/M checkpoint after DNA damage. It should also be mentioned that some cells died after both treatments, probably through apoptosis (i.e. less than 2N DNA content but P-S10-H3-negative).

The cell cycle profile of control Chk1<sup>-/-</sup> cells and their progress into mitosis in the absence of perturbation was similar to control DT40 WT cells (Figure 3.2). In response to DNA replication block with aphidicolin almost half of the Chk1<sup>-/-</sup> cell population stained positive for P-S10- H3. Interestingly, the majority of these mitotic cells displayed a DNA content of only 2N. This premature mitosis of cells that have not yet completed DNA replication is characteristic of S/M checkpoint failure. In comparison, after exposure to IR, Chk1<sup>-/-</sup> cells progressed through the cell cycle without any measurable arrest and accumulated in mitosis with 4N DNA content comparable to un-stressed control cells (56% and 58% respectively, Figure 3.2). Moreover, in the absence of Chk1, hardly any non-



**Figure 3.2: S/M and G2/M checkpoint proficiency in DT40 WT, Chk1<sup>-/-</sup>, Chk2<sup>-/-</sup> cell lines**

Flow cytometry data for DNA content (PI profiles) and mitotic indices (P-S10-H3/PI staining) of DT40 WT, Chk1<sup>-/-</sup> and Chk2<sup>-/-</sup> cell lines after 16h treatments with the DNA polymerase inhibitor aphidicolin or 10Gy of IR in the presence of nocodazole. The percentage of total mitotic cells after each treatment is annotated in the mitotic indices profile.

mitotic cells with sub 2N DNA content were detected after either treatment. Taken together these results demonstrated that both S/M and G2/M checkpoint responses were severely compromised in the absence of Chk1.

Unperturbed Chk2-deficient DT40 cells showed slight differences in their cell cycle profile as compared to control DT40 WT cells (compare DNA content histograms in Figure 3.2). The relative decrease in G1 and increase in G2 cells in asynchronous Chk2<sup>-/-</sup> cultures has been described previously and may suggest a slower rate of progression through G2/M in these cells (Rainey, 2003). Nevertheless, after 16h the vast majority (65%) of Chk2<sup>-/-</sup> cells had also progressed through the cell cycle and accumulated in mitosis in the presence of nocodazole. Block of DNA replication with aphidicolin resulted in mitotic delay of a high proportion of Chk2<sup>-/-</sup> cells and no mitotic cells with less than 4N DNA content were observed, demonstrating that the S/M checkpoint was still functional in the absence of Chk2. As for the G2/M arrest in response to DNA damage, only approximately 50% of Chk2<sup>-/-</sup> cells arrested in G2 in comparison to IR treated DT40 WT cells where the majority of cells arrested in G2 (Figure 3.2). This result indicates that the G2/M arrest is slightly compromised in the absence of Chk2 in these cells, consistent with the findings of Rainey et al. (Rainey et al., 2007).

A graphical summary of the flow cytometry data for all DT40 cell lines is shown in Figure 3.6, including data for the DT40 WT, Chk1<sup>-/-</sup>, and Chk2<sup>-/-</sup> cells as shown in Figure 3.2, as well as data from checkpoint proficiency assays shown subsequently in Figures 3.4 and 3.5. The total percentage of P-S10-H3-positive cells in each sample with respect to the nocodazole treated control (designated 100%) is shown in Figure 3.6a. The percentage of 2N mitotic cells (Figure 3.6b) provides a specific measure of S/M checkpoint failure, whereas the amount of 4N mitotic cells with respect to the nocodazole treated control allows an assessment of G2/M checkpoint failure in irradiated cells (Figure 3.6c).

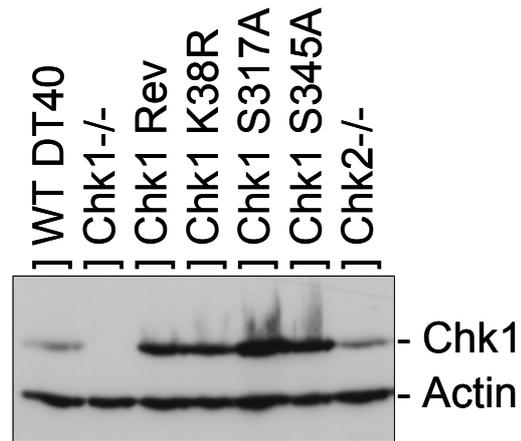
### ***3.1.2 Analysis of mitotic delay proficiency in Chk1 mutant cell lines in response to replication block and DNA damage***

These checkpoint proficiency assays performed in the DT40 WT and knock-out cell lines demonstrated that Chk1 is essential for normal S/M and G2/M checkpoint delays. To further characterize the function of Chk1, various mutant versions of Chk1 were re-introduced into the Chk1-deficient cell line (by Mark Walker). These cell lines were then analysed by flow cytometry for proficiency in the S/M and G2/M checkpoint assays as described above.

The Chk1 Revertant cell line was established by transfecting Chk1<sup>-/-</sup> cells with an expression vector encoding an avian Chk1 cDNA. To enable an optimal comparison, a clone was chosen that expressed similar Chk1 protein levels as the WT DT40 cells. This approach was used for all mutant cell lines generated as far as it was possible. The protein expression levels of Chk1 in all the seven cell lines that have been analysed are shown in Figure 3.3. The Chk1 protein levels were somewhat higher in the Chk1 mutant cell lines, but importantly, re-introduction of functional Chk1 was able to completely restore normal S/M and G2/M checkpoint function, confirming that Chk1 is essential for both of these responses (Figure 3.4; Chk1 Revertant).

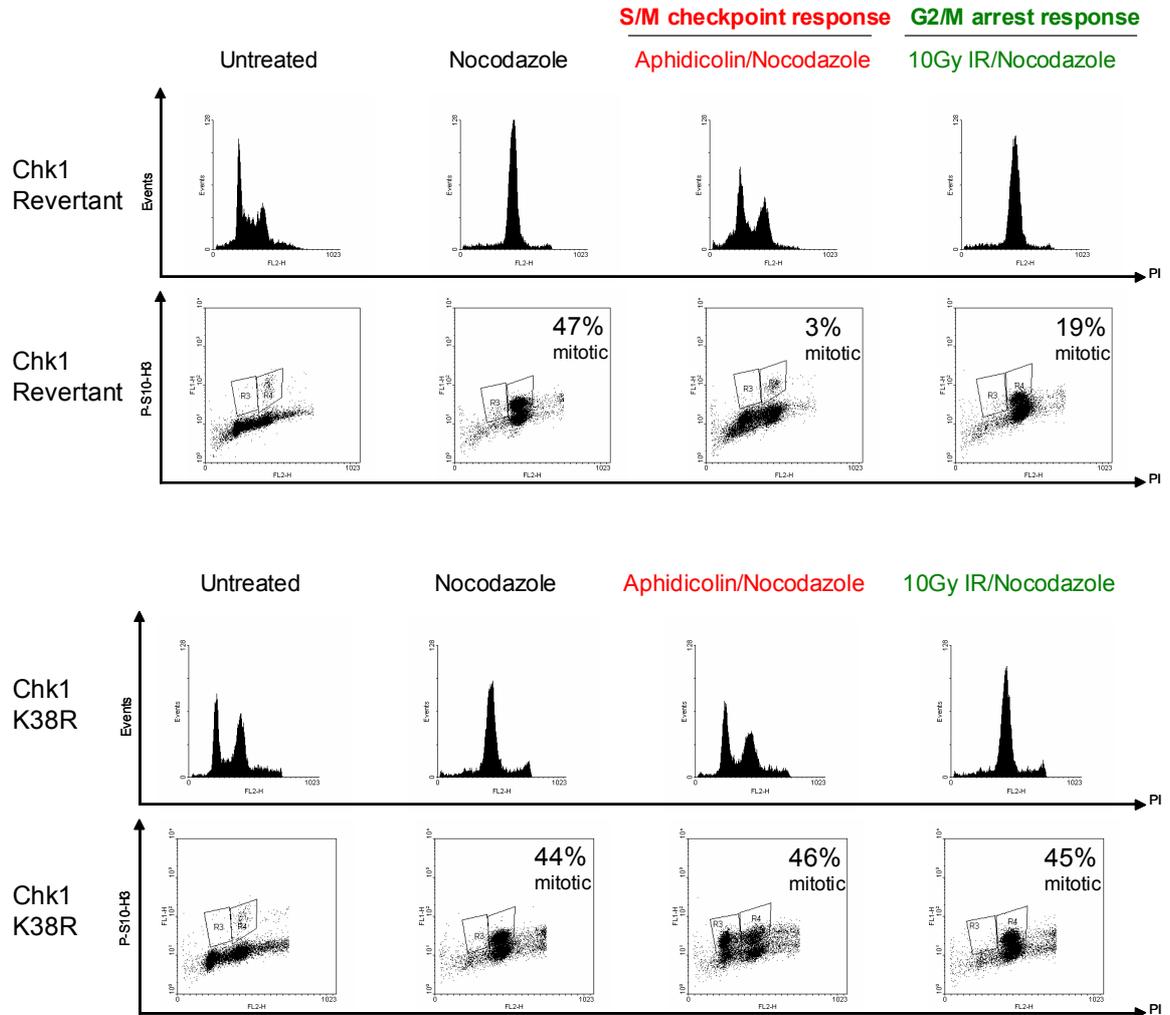
To establish whether Chk1 kinase activity per se is essential for checkpoint proficiency, a kinase dead version of the protein was generated. An expression vector encoding a mutant version of Chk1 where lysine 38 was replaced by an arginine (Chk1 K38R) was stably introduced in the Chk1<sup>-/-</sup> cells. This mutation interferes with ATP binding in the catalytic domain of Chk1. As shown in Figure 3.4, the Chk1 K38R cells showed high levels of premature mitosis (26%) after block to DNA replication and progressed into mitosis without any detectable arrest 16h after exposure to DNA damage. Thus, both S/M and G2/M checkpoints were impaired or abolished in the Chk1 K38R cell line, demonstrating that Chk1 kinase activity is essential for these checkpoint responses.

The upstream kinase ATR has been shown to phosphorylate Chk1 on serines 317 and 345 (Zhao and Piwnica-Worms, 2001). In order to investigate the requirement for phosphorylation of these sites during checkpoint responses, two mutant versions of Chk1 were created. In each case the respective serine residue was mutated to an alanine residue that cannot be phosphorylated. As shown in Figure 3.5, in the presence of the DNA polymerase inhibitor aphidicolin a majority of the Chk1 S317A cells were able to delay mitosis, indicating that the S/M checkpoint is active in these cells.



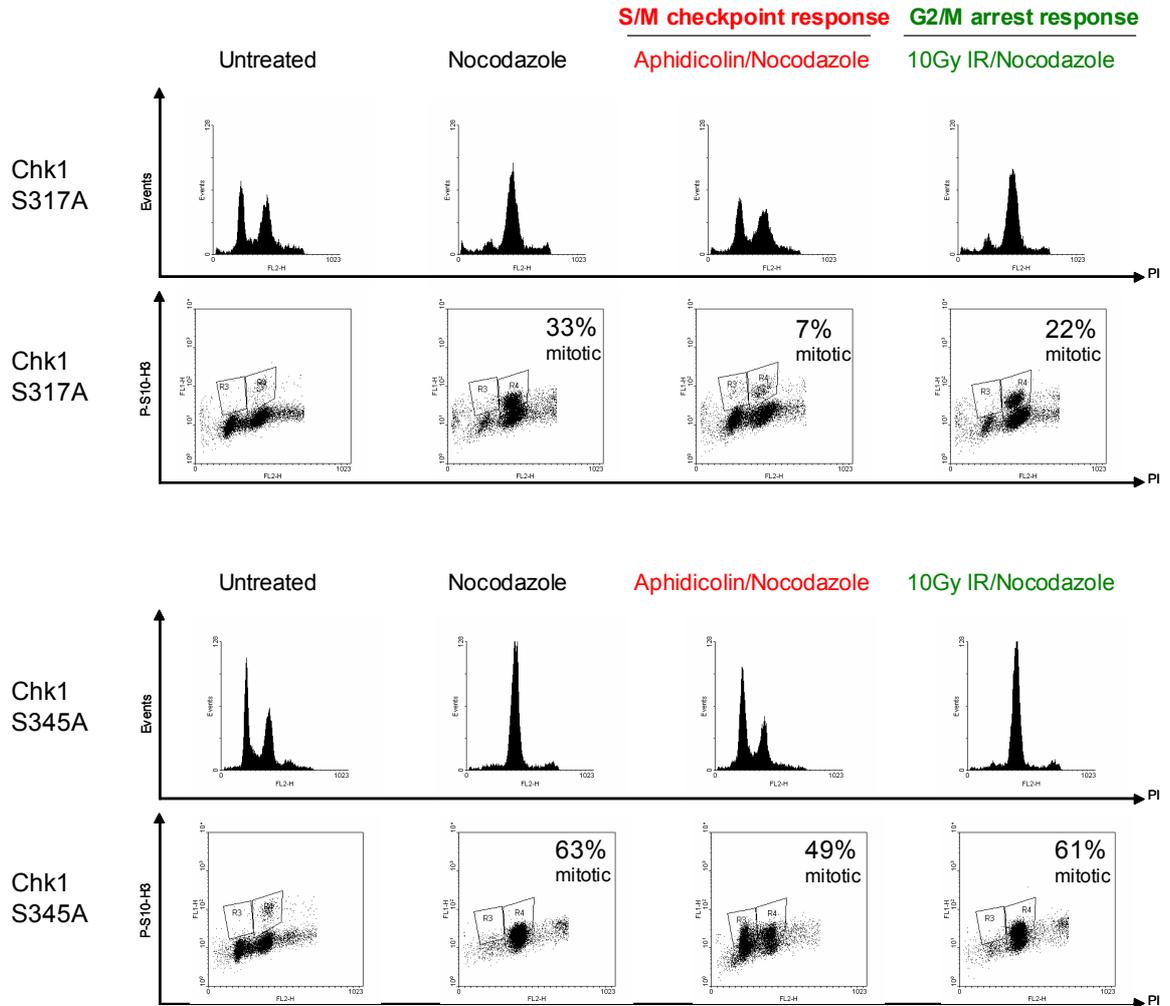
**Figure 3.3: Expression levels of Chk1 and mutant derivatives in DT40 cell lines**

To determine the expression levels of Chk1 in all seven cell lines tested in the checkpoint assays whole cell extracts were separated on a 10% SDS PAGE gel. Chk1 protein levels were analysed by Western blotting for Chk1 and Actin was used as a loading control.



**Figure 3.4: S/M and G2/M checkpoint proficiency in Chk1 Revertant and Kinase Dead cell lines**

Flow cytometry data for DNA content (PI profiles) and mitotic indices (P-S10-H3/PI staining) of Chk1 Revertant and Chk1 K38R cell lines after 16h treatments with the DNA polymerase inhibitor aphidicolin or 10Gy of IR in the presence of nocodazole. The percentage of total mitotic cells after each treatment is annotated in the mitotic indices profile.



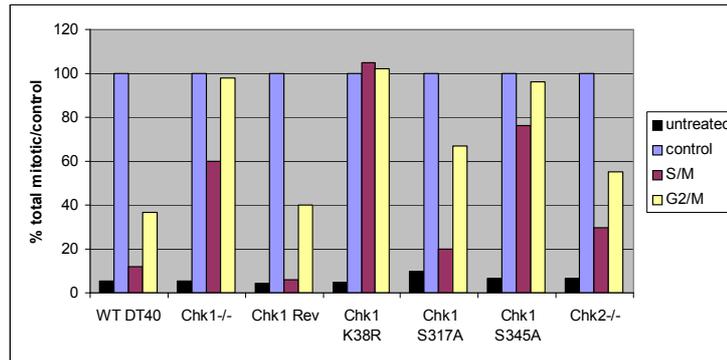
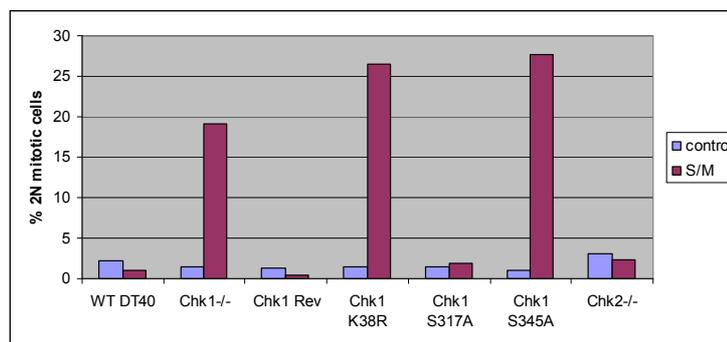
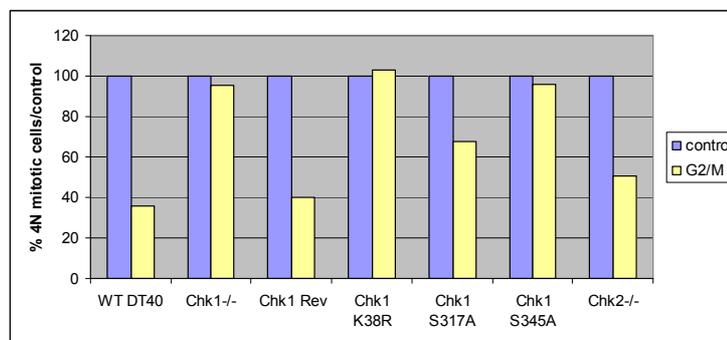
**Figure 3.5: S/M and G2/M checkpoint proficiency in Chk1 phosphorylation mutant cell lines**

Flow cytometry data for DNA content (PI profiles) and mitotic indices (P-S10-H3/PI staining) of Chk1 S317A and Chk1 S345A phosphorylation mutant cell lines after 16h treatments with the DNA polymerase inhibitor aphidicolin or 10Gy of IR in the presence of nocodazole. The percentage of total mitotic cells after each treatment is annotated in the mitotic indices profile.

After exposure to DNA damage (IR), a high percentage of Chk1 S317A cells entered mitosis, however, in comparison to complete G2/M checkpoint failure observed in Chk1<sup>-/-</sup> cells, the Chk1 S317A mutant displayed an intermediate phenotype (Figure 3.5). Taken together, these results suggest that phosphorylation of Chk1 on S317 is not essential for the S/M checkpoint response; however it appears to be to some extent important for the proper establishment of the G2/M arrest in response to DNA damage.

The second phosphorylation mutant, Chk1 S345A, exhibited a more severe phenotype (Figure 3.5). In response to block to DNA replication with aphidicolin a high percentage of Chk1 S345A cells (28%) entered mitosis prematurely with 2N DNA content. Also, there was no measurable G2 arrest in response to DNA damage after IR exposure in the Chk1 S345A cells. This observation suggests that phosphorylation of Chk1 on S345 is an essential event for the proper functioning of both the S/M and the G2/M checkpoint responses.

To summarise this section, failure of the S/M checkpoint as judged by detection of premature 2N mitotic cells was observed in Chk1<sup>-/-</sup>, Chk1 K38R Kinase Dead, and Chk1 S345A phosphorylation mutant cell lines (Figure 3.6b). In comparison, G2/M arrest was abolished in Chk1<sup>-/-</sup>, Chk1 K38R, Chk1 S345A, and significantly compromised in the Chk1 S317A mutant. The Chk2<sup>-/-</sup> cell lines showed an intermediate G2/M checkpoint response (Figure 3.6c).

**(a) Total mitotic cells (2N and 4N)****(b) 2N mitotic cells (S/M checkpoint)****(c) 4N mitotic cells (G2/M checkpoint)****Figure 3.6: Comparison of S/M and G2/M checkpoint proficiency in DT40 cell lines**

Graphical illustration of representative examples for the flow cytometry data of checkpoint proficiency experiments which were each performed several times with reproducible results:

(a) Percentage of total mitotic cells (i.e. both 2N and 4N) calculated as a percentage of the nocodazole treated mitotic control (blue: 100%). Untreated control cells are depicted in black, cells treated with aphidicolin are shown in red (S/M checkpoint response), and cells treated with 10Gy IR are represented in yellow (G2/M checkpoint response).

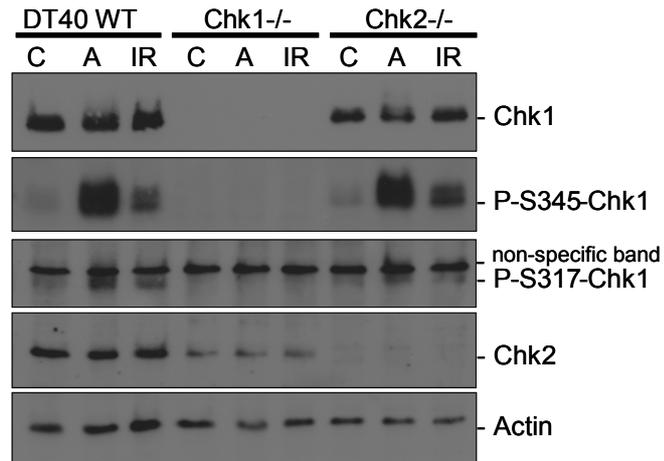
(b) S/M checkpoint response (red) after 16h replication block represented as percentage of 2N premature mitosis in each cell line.

(c) G2/M checkpoint response (yellow) after 16h IR treatment represented as percentage of 4N mitotic cells compared to mitotic control (blue: 100%).

### **3.1.3 Analysis of Chk1 activation in response to replication stress and DNA damage**

Checkpoint assays with DT40 cells expressing a kinase dead Chk1 mutant demonstrated that Chk1 kinase activity is essential for S/M and G2/M checkpoint responses in DT40 cells. Furthermore, analysis of Chk1 phosphorylation mutants has shown that phosphorylation on S317 and S345 of Chk1 is also important for proper functioning of these mitotic checkpoints. To complement this analysis it was of interest to document Chk1 phosphorylation at these two regulatory sites. Previous studies have shown that the phosphorylation of Chk1 by upstream kinase ATR can serve as a read-out of kinase activity (Zhao and Piwnica-Worms, 2001). Therefore, the phosphorylation status of Chk1 on critical residues S317 and S345 was analysed as a surrogate marker to determine Chk1 activation in response to block to DNA replication or DNA damage.

DT40 WT, Chk1<sup>-/-</sup> and Chk2<sup>-/-</sup> cells were treated either with the DNA polymerase inhibitor aphidicolin or 10Gy of IR and returned to culture. Cells were harvested after 1h and whole cell extracts analysed on a phosphorylation shift SDS PAGE gel (see materials and methods). As shown in Figure 3.7, Western blotting with Chk1 phospho-specific antibodies revealed that the level of basal Chk1 phosphorylation was very low under untreated control conditions. In response to aphidicolin treatment, Chk1 became very strongly phosphorylated on S345 and also modified on S317 in DT40 WT and Chk2<sup>-/-</sup> cells. After exposure to DNA damage induced by IR, both S317 and S345 of Chk1 were phosphorylated within one hour of treatment; however the overall extent of S345 phosphorylation was much lower than after DNA replication block. This result implied a difference in Chk1 activation depending on the kind of stress the cells were exposed to. Chk1 became more strongly phosphorylated, and thus presumably activated, in response to DNA replication block than after DNA damage with 10Gy IR. The levels of S317 and S345 Chk1 phosphorylation were comparable in DT40 WT and Chk2<sup>-/-</sup> cells showing that Chk1 activation was not impaired in the absence of Chk2. Interestingly, the total Chk2 protein level in Chk1<sup>-/-</sup> cells was much lower than in DT40 WT cells. However, the reason and significance of this difference has not yet been determined.



**Figure 3.7: Chk1 phosphorylation in response to replication stress and DNA damage**

DT40 WT, Chk1<sup>-/-</sup>, Chk2<sup>-/-</sup> cells were either untreated (C), aphidicolin treated (A), or irradiated with 10Gy (IR), returned to culture for 1h and harvested. Whole cell extracts were separated on a phosphorylation shift SDS PAGE gel. Chk1 protein levels and phosphorylation status on S317 and S345 as well as Chk2 protein levels and Actin protein levels were analysed by Western blotting.

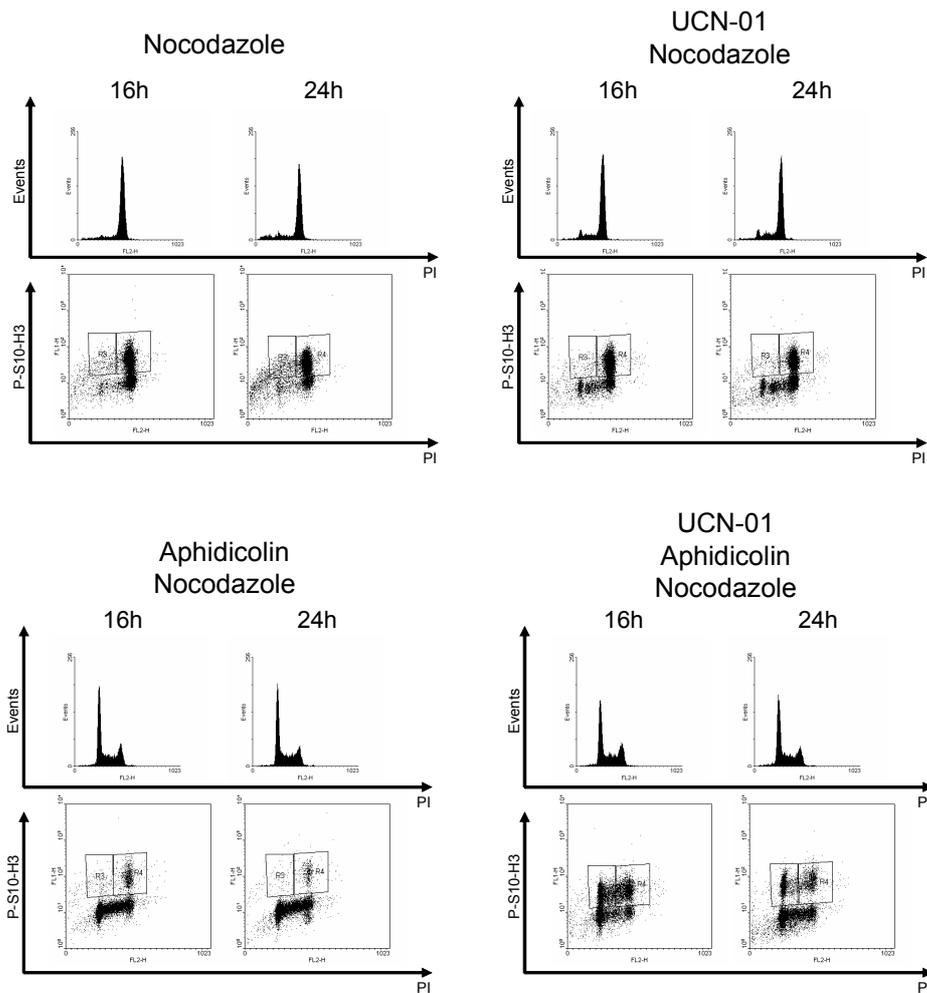
## 3.2 Mitotic checkpoint responses in human BE cells

The avian Chk1<sup>-/-</sup> cell line has been established as a very useful experimental system. However, to determine whether the requirement for Chk1 in mitotic checkpoint responses is general, it was necessary to study Chk1 function in a different background, such as a human cell line. Therefore, the colon carcinoma BE cell line was chosen as a second vertebrate system to study Chk1 function in addition to the DT40 knock-out cell lines. The human BE cells were selected as, like the DT40 cells, they do not express functional p53 and furthermore they have been established as a cell line that is very suitable for the use of small interfering RNA (siRNA). Thus, two approaches were employed to abrogate Chk1 function in the BE cells: Firstly, using a small molecule inhibitor with a high selectivity for Chk1, and secondly, using siRNA in order to down-regulate Chk1 expression levels. Initially, the effect of both Chk1 inhibition and Chk1 siRNA were tested in the S/M checkpoint assay.

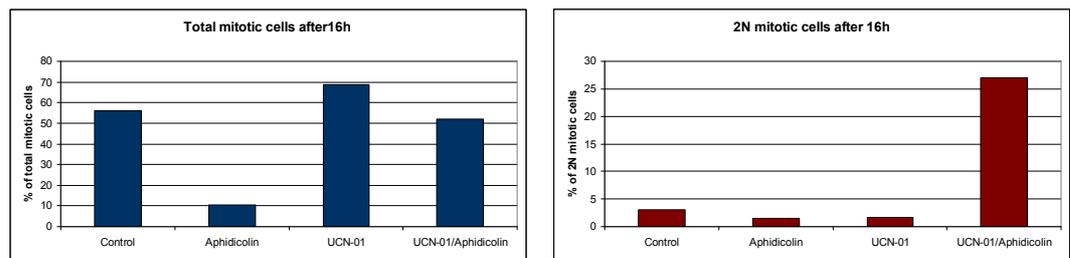
### 3.2.1 Requirement of Chk1 for S/M checkpoint proficiency in BE cells

The small molecule inhibitor 7-hydroxy-staurosporine (UCN-01) has been identified as a potent anticancer agent and was found to target Chk1 and the Cdc25C pathway (Graves et al., 2000). To investigate the effect of pharmacological inhibition of Chk1 on the S/M checkpoint response, BE cells were treated with aphidicolin and nocodazole in the presence or absence of 300nM UCN-01. Cells were harvested after 16 or 24h and analysed by flow cytometry. Control cells treated with nocodazole alone and cells treated with UCN-01 and nocodazole progressed through the cell cycle and the majority of cells had accumulated in mitosis by 24h (Figure 3.8a). In response to replication block with aphidicolin the BE cells were able to delay entry into mitosis. In contrast, in the presence of UCN-01 and aphidicolin, there was an increase in the total number of mitotic cells after 16h and 24h. Furthermore, more than a quarter of UCN-01 treated BE cells had entered mitosis prematurely with 2N DNA content after 16h of replication block with aphidicolin. The graph in Figure 3.8b illustrates the quantification of the flow cytometry data for the 16h time point in more detail. These results demonstrated that inhibition of Chk1 with UCN-01 in BE cells recapitulated the phenotype of the avian Chk1<sup>-/-</sup> cells in response to replication block.

(a)



(b)



**Figure 3.8: Analysis of effect of Chk1 inhibition with UCN-01 on S/M checkpoint proficiency**

BE cells were treated as required with UCN-01 and/or aphidicolin and grown in the presence of nocodazole for 16 or 24h respectively. Data for untreated control cells is not shown. Cells were harvested and analysed for DNA content (PI profiles) and mitotic indices (P-S10-H3/PI) and analysed by flow cytometry (a). Quantification of flow cytometry data for the 16h time point (b).

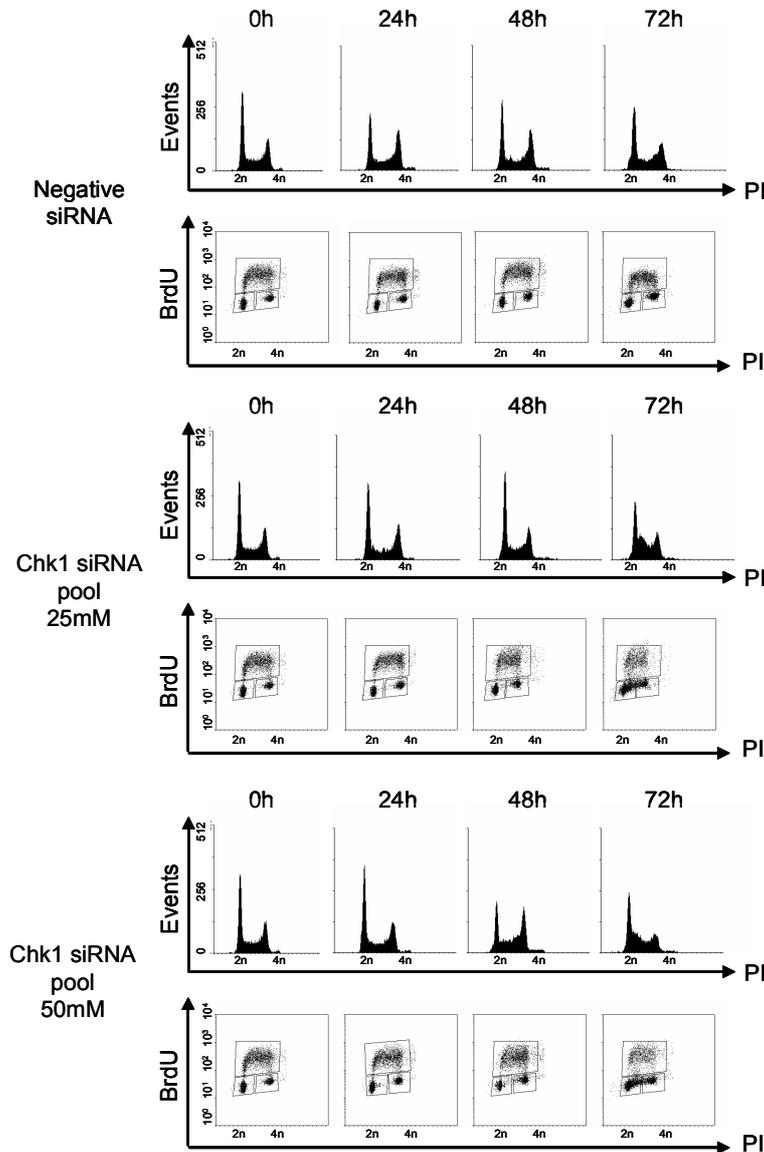
Down-regulation of Chk1 protein expression was achieved using siRNA directed against human Chk1. As shown in the Western blot analysis in Figure 3.9a, four individual siRNA duplexes as well as a pool of all four in combination successfully ablated Chk1 protein expression after 72h of transfection at both 50 and 100nM. A time course experiment was performed to establish the best dose and time for siRNA administration and to determine any negative effects on cell cycle progression. A concentration of 50nM of Chk1 siRNA pool was sufficient to effectively down-regulate Chk1 protein expression after 48h (Figure 3.9a, right panel). Flow cytometry analysis of BrdU incorporation suggested that after 72h of transfection with Chk1 siRNA some cells exhibited perturbation of S phase progression as indicated by loss of BrdU incorporation; however this was not seen at 48h (Figure 3.9b). From these results it was concluded that a concentration of 50nM of Chk1 siRNA pool for 48h of transfection was suitable for further experiments.

To evaluate the effectiveness of Chk1 inhibition versus down-regulation of Chk1 protein expression, BE cells were analysed for S/M checkpoint proficiency after exposure to either UCN-01 or Chk1 siRNA. As shown in Figure 3.10, after block to DNA replication with aphidicolin, cells treated with UCN-01 or Chk1 siRNA entered mitosis prematurely with 2N DNA content (9% and 15% respectively). The observed impairment of the S/M checkpoint response was comparable to previous observations of S/M checkpoint failure made in Chk1<sup>-/-</sup> DT40 cells. In conclusion, both approaches of abrogating Chk1 function, either by inhibition with UCN-01 or down-regulation of Chk1 protein levels with siRNA, showed that Chk1 is required for the S/M checkpoint in BE cells.

(a)

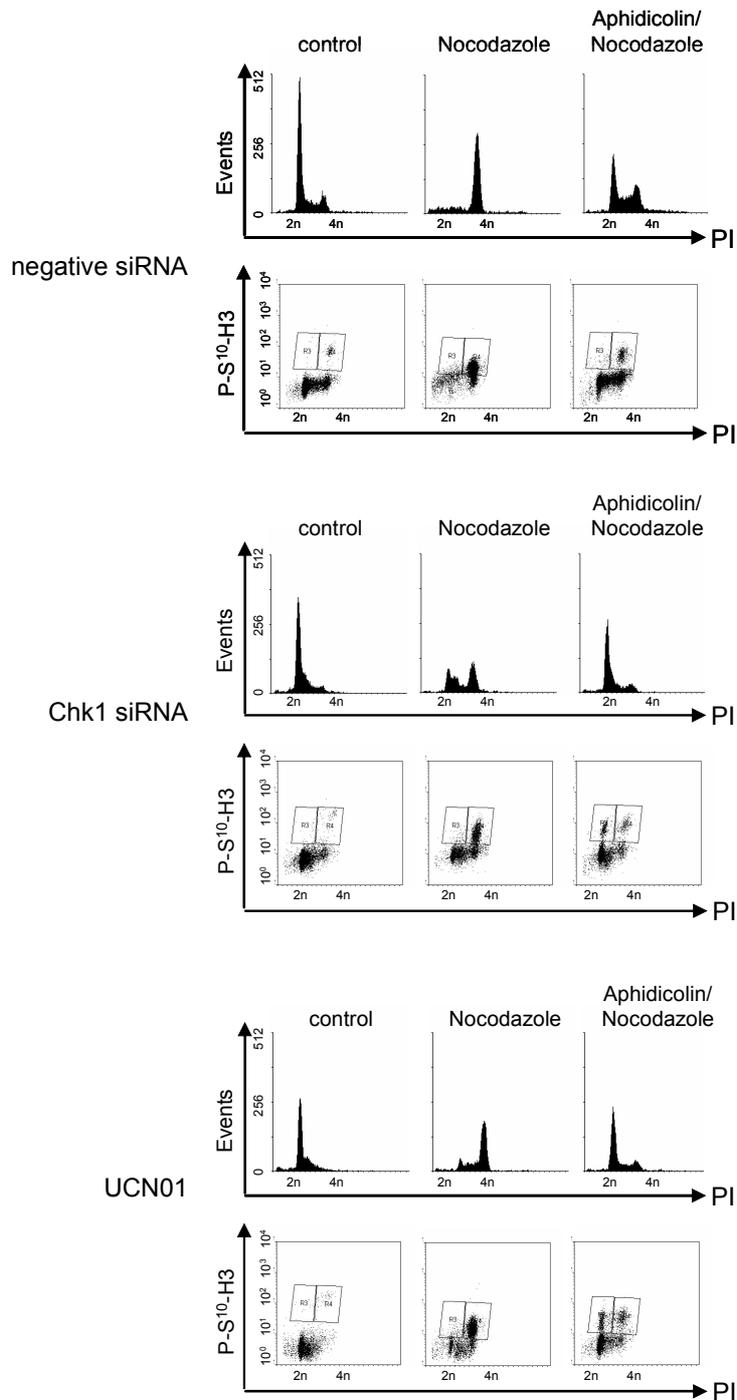


(b)



**Figure 3.9: Analysis of Chk1 down-regulation with siRNA**

(a) Left panel: Western blot analysis of Chk1 protein levels, Actin served as loading control. Cells were un-transfected (U) or transfected with either mock (M), negative control siRNA (Neg), positive control siRNA (Lam), 50 or 100nM of either individual Chk1 siRNA duplex or Chk1 pool siRNA. Right panel: Western blot analysis of Chk1 levels for Chk1 siRNA time and dose response experiment, Actin was used as loading control. Cells were un-transfected cells (U), transfected with either mock (M), 25 or 50mM of Chk1 siRNA pool. (b) FACS profiles of DNA content (PI) and analysis of proliferating cells (BrdU/PI) for Chk1 siRNA time and dose response experiment.



**Figure 3.10: Comparison of effect of Chk1 siRNA and Chk1 inhibitor UCN-01 on S/M checkpoint proficiency**

Chk1 function was abrogated in BE cells by either transfection with Chk1 siRNA (for 48h) or Chk1 inhibition with UCN-01. Respective drug treatments of nocodazole, aphidicolin or UCN-01 were administered 24h before harvesting. Samples were analysed by flow cytometry for DNA content (PI profiles) and mitotic indices (P-S10-H3/PI).

### **3.2.2 Analysis of Chk1 and Chk2 requirement for mitotic checkpoint proficiency in BE cells**

To compare the relative requirement of Chk1 and Chk2 for S/M and G2/M checkpoint proficiency in BE cells the expression of one or both proteins was silenced using small interfering RNA. The effectiveness of Chk1 siRNA in BE cells has already been described (3.2.1). In the case of Chk2, protein expression was also successfully ablated within 48h with either of four individual siRNA duplexes as well as a Chk2 siRNA pool. An example of Chk2 protein depletion after siRNA treatment can be seen in Figure 3.13b.

In order to determine the consequences for S/M and G2/M checkpoint proficiency, BE cells were transfected with siRNA(s) for 48h and then exposed to either the DNA polymerase inhibitor aphidicolin or 10Gy of IR and grown in the presence of nocodazole for a further 24h. As shown in Figure 3.12, mock transfected control cells progressed through the cell cycle and accumulated efficiently in mitosis in the presence of nocodazole. In contrast, mitosis was effectively delayed in response to both DNA replication block with aphidicolin and DNA damage induced by IR, as assessed by the appearance of 2N and 4N mitotic cells, respectively.

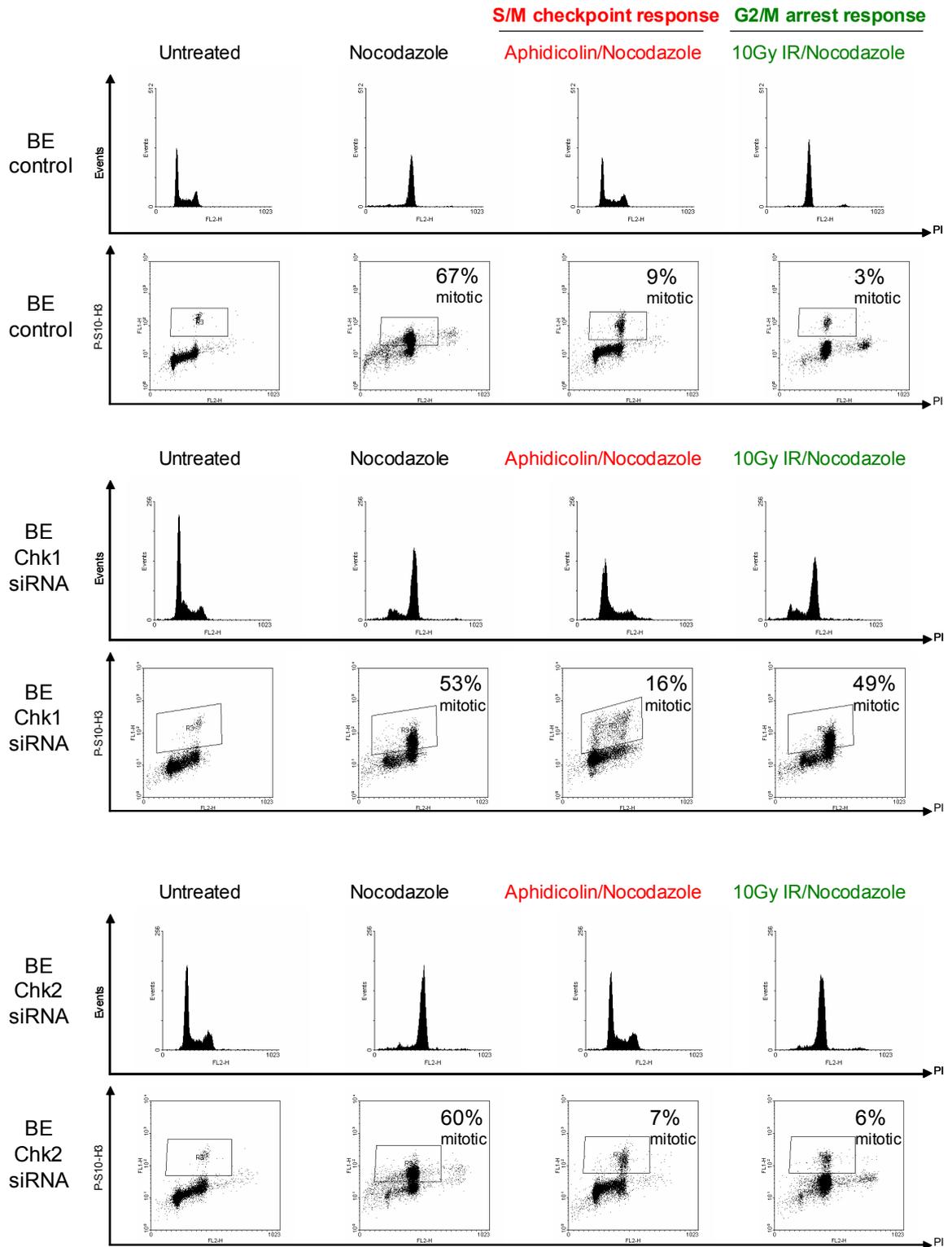
When Chk1 expression was down-regulated with siRNA, the majority of cells accumulated in mitosis in the presence of nocodazole (Figure 3.12). After aphidicolin treatment an increase in the total number of mitotic cells was observed and there was also a small but significant amount of premature mitotic cells with 2N DNA content, indicating impaired S/M checkpoint function. Between individual Chk1 siRNA experiments a certain degree of variation in the amount of premature mitosis was seen (compare for example Figures 3.10 and 3.12), however overall the effect was very reproducible. When Chk1siRNA treated cells were exposed to DNA damage induced by IR, they were not able to delay mitosis efficiently as most cells progressed through the cell cycle to mitosis at a rate similar to cells treated with nocodazole alone (49% and 53% respectively in Figure 3.12). The absence of any measurable arrest in response to DNA damage demonstrated that Chk1 function was essential for the G2/M checkpoint in BE cells.

In contrast, when BE cells were treated with Chk2 siRNA little effect on S/M or G2/M checkpoint proficiency was observed (Figure 3.12). In the presence of Chk2 siRNA, most cells arrested in S phase and delayed the onset of mitosis after block to DNA replication. Entry to mitosis was also blocked after exposure to DNA damage with 10Gy of IR and

almost all cells arrested in G2 (Figure 3.12). Thus, both S/M and G2/M checkpoint responses were functioning properly even in the absence of Chk2.

The siRNA approach has also been applied to investigate checkpoint responses in BE cells in the absence of both Chk1 and Chk2 function (Figure 3.13a). Western blot analysis was performed to confirm efficient down regulation of the protein levels of both kinases (Figure 3.13b). In the checkpoint proficiency assays, the S/M checkpoint response was impaired as judged by the appearance of a small population of premature mitotic cells with 2N DNA content after replication block with aphidicolin. In comparison, the G2/M arrest in response to DNA damage was essentially abrogated in these cells as the majority of cells entered mitosis without any measurable arrest in G2. This phenotype was very similar to that of Chk1 down regulation alone as shown in the graphical representation of all siRNA experiments in this section as calculated from the flow cytometry data and presented in graph form to allow ready comparison (Figure 3.11) This result further emphasises the significant function of Chk1 in these two mitotic checkpoint responses in BE cells.

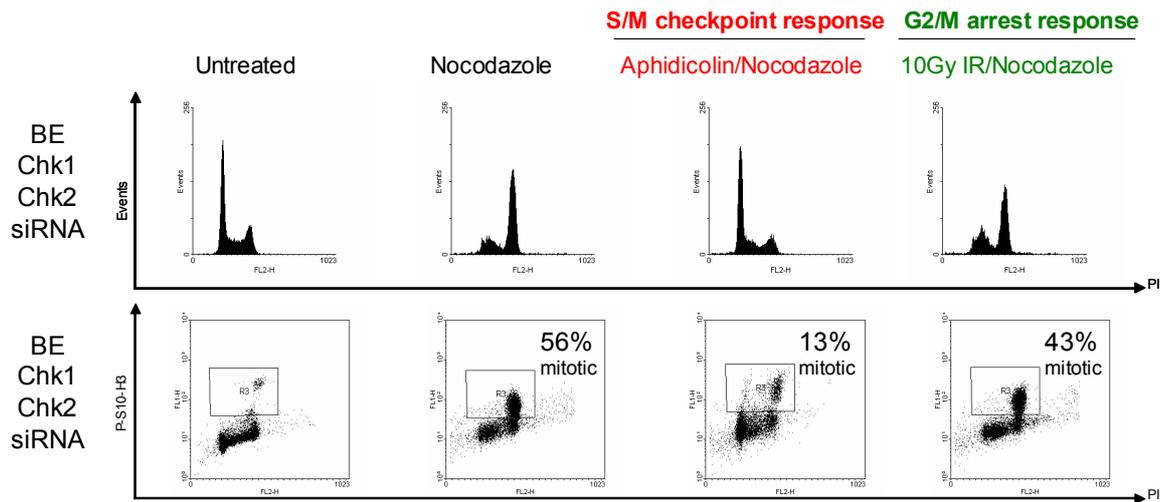
Taken together, the results from the siRNA experiments in BE cells confirmed several observations made in the avian DT40 knock out system: Chk1 function is essential for the G2/M arrest in response to DNA damage. However, while Chk2 plays a minor role in the proper execution of the G2/M checkpoint in DT40 cells, this does not seem to be the case in the BE cells. Furthermore, the proper functioning of the S/M checkpoint is also dependent on Chk1 function in DT40 and BE cells, whereas Chk2 is not required for mitotic delay after replication block. Taken together, Chk1 emerged as the major effector kinase of the S/M and G2/M checkpoint responses in both vertebrate systems that were analysed in this study.



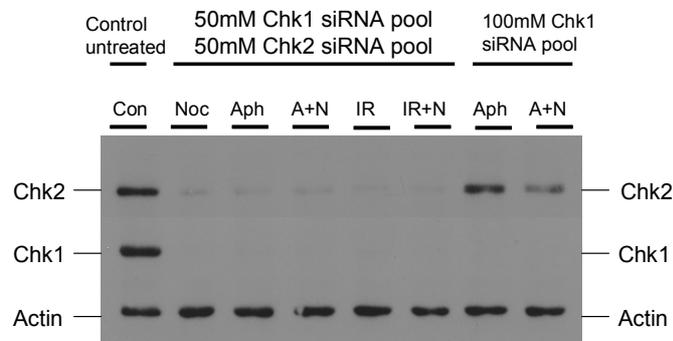
**Figure 3.11: Analysis of S/M and G2/M checkpoint proficiency in BE cells depleted of Chk1 or Chk2 function**

BE cells were transfected with siRNA against Chk1 or Chk2 or with a negative control. After 48h of transfection, cells were exposed to either block to DNA replication with aphidicolin or DNA damage with 10Gy of IR and cultured in the presence of nocodazole. At the experimental end point, samples were fixed and analysed by flow cytometry for DNA content (PI profiles) and mitotic indices (P-S10-H3/PI). The percentage of total mitotic cells after each treatment is annotated in the mitotic indices profile.

(a)



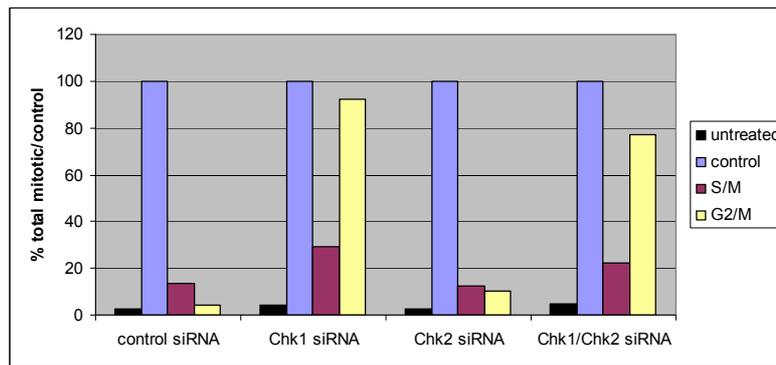
(b)



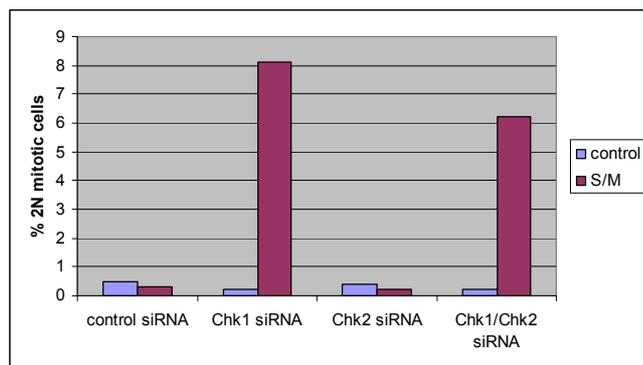
**Figure 3.12: Analysis of S/M and G2/M checkpoint proficiency in BE cells depleted of Chk1 and Chk2 function**

BE cells were transfected with siRNA against Chk1 and Chk2 or with a negative control. After 48h of transfection, cells were exposed to either block to DNA replication with aphidicolin or DNA damage with 10Gy of IR and cultured in the presence of nocodazole. At the experimental end point, samples were fixed and analysed by flow cytometry for DNA content (PI profiles) and mitotic indices (P-S10-H3/PI) as shown in (a). Figure (b) shows the Western blot analysis of Chk1 and Chk2 protein levels after siRNA treatment with either Chk1 and Chk2 siRNA together (lanes 2-6) or Chk1 siRNA alone (lanes 7-8), Actin was used as loading control.

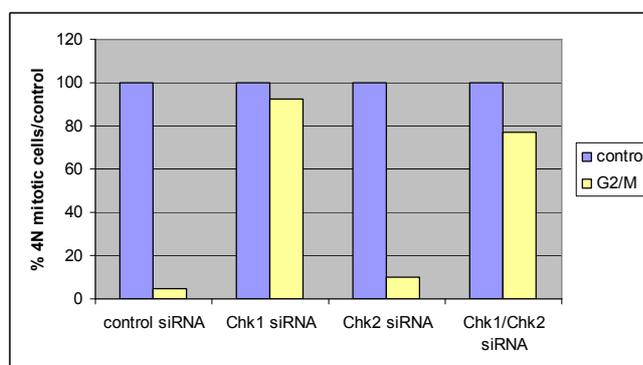
(a)



(b)



(c)



**Figure 3.13: Comparison of S/M and G2/M checkpoint proficiency in BE cells depleted of Chk1 and/or Chk2 expression**

Graphical illustration of a representative example of the flow cytometry data of experiments that have been performed several times with reproducible results:

(a) Percentage of total mitotic cells (i.e. both 2N and 4N) calculated as a percentage of the nocodazole treated mitotic control (blue: 100%). Untreated control cells are depicted in black, cells treated with aphidicolin are shown in red (S/M checkpoint response), and cells treated with 10Gy IR are represented in yellow (G2/M checkpoint response).

(b) S/M checkpoint response (red) after replication block represented as percentage of 2N premature mitosis in each cell line.

(c) G2/M checkpoint response (yellow) after IR treatment represented as percentage of 4N mitotic cells compared to mitotic control (blue: 100%).

### 3.3 Discussion

The requirement of Chk1 and Chk2 for mitotic checkpoint proficiency in response to DNA replication block and DNA damage was analysed in two vertebrate systems: avian B-lymphoma DT40 cells, where WT and knock-out cell lines for each kinase could be compared, and in human colon carcinoma BE cells in which Chk1 and Chk2 function was abrogated by siRNA or using a selective Chk1 inhibitor.

Chk1 emerged as the major effector kinase for the S/M delay in both systems. Flow cytometry analysis revealed that avian Chk1<sup>-/-</sup> cells were not able to delay mitosis when DNA replication was blocked. Instead, a significant number of cells entered mitosis prematurely with un-replicated DNA. Re-expression of Chk1 in the Chk1<sup>-/-</sup> cells completely rescued the phenotype, demonstrating that the effect on checkpoint proficiency was directly dependent on Chk1. Further experiments established that the S/M delay was dependent on Chk1 kinase activity as cells expressing a kinase dead version of Chk1 were not able to delay the onset of mitosis after DNA replication block and also displayed high levels of premature mitosis. These observations were consistent with previous studies demonstrating a role for Chk1 in response to replication stress in vertebrate cells (Feijoo et al., 2001; Zachos et al., 2003).

The analysis of Chk1 phosphorylation mutants revealed that phosphorylation of Serine 345 by upstream kinases ATM/ATR was essential for the S/M checkpoint response whereas phosphorylation of Serine 317 was dispensable. This result was in agreement with the findings of a recent study using conditional Chk1 knock-out ES cells (Niida et al., 2007). This also correlated with the finding that Chk1 became very strongly phosphorylated on Serine 345 within one hour of aphidicolin treatment. Serine 317 was found to be phosphorylated also, however to a much weaker extent. These observations demonstrated that Serine 345 is the major phosphorylation site of Chk1 by upstream kinases after DNA replication block and it is essential for Chk1 biological function. Serine 317 is a second site that is phosphorylated after replication stress; however this does not appear to be essential for the initiation or maintenance of the S/M checkpoint delay.

Chk1 was also shown to play a significant role for S/M checkpoint proficiency in the human BE cells. When DNA replication was blocked in the presence of either the Chk1 inhibitor UCN-01 or Chk1 siRNA, BE cells entered mitosis prematurely. The degree of checkpoint failure in BE cells did not seem as severe as in the Chk1<sup>-/-</sup> cells. Differences between the two experimental systems could be due to the fact that the avian system used a

knock-out approach to study Chk1 function and therefore was void of any Chk1 gene or protein expression as shown by Western blot analysis for Chk1 protein levels. In contrast, the human system was first investigated using the Chk1 inhibitor UCN-01, which might not achieve complete inhibition of Chk1 function. In this situation a residual amount of active Chk1 and also the actual presence of the Chk1 protein itself, whether active or not, could have been responsible for the residual checkpoint response in the BE cells as the actual amount of premature mitosis was lower than during checkpoint failure in the avian system. The same could apply to the siRNA approach, as although Chk1 protein levels were greatly diminished, some residual protein might have remained and could have had an effect on the checkpoint response. Alternatively, it is also possible that the degree of the S/M checkpoint response simply varies between the two different vertebrate systems. In either case, Chk1 plays a crucial role for the proper execution of this mitotic checkpoint in both DT40 and BE cells.

Chk1 also emerged as the major effector kinase for the G2/M arrest in response to DNA damage. This mitotic delay was completely abrogated in avian Chk1<sup>-/-</sup> cells and human BE cells treated with Chk1 siRNA. Cells entered mitosis to same levels as control cells without any measurable cell cycle arrest, clearly demonstrating that Chk1 is absolutely essential for the G2/M arrest. Furthermore, the checkpoint could also be restored by re-introducing Chk1 in the Chk1<sup>-/-</sup> cells. The essential requirement of Chk1 for DNA damage checkpoint pathways in vertebrate cells has previously been described in studies of Chk1<sup>-/-</sup> blastocysts and conditional Chk1<sup>-/-</sup> ES cells (Liu et al., 2000; Takai et al., 2000).

Furthermore, the analysis of Chk1 phosphorylation mutants revealed that phosphorylation of Serine 345 is also a crucial event for the G2/M checkpoint function of Chk1 as was previously observed for the S/M checkpoint response. Interestingly however, the Chk1 Serine 317 phosphorylation mutant displayed an intermediate phenotype, suggesting that phosphorylation on this residue is necessary for a full G2/M checkpoint response, albeit secondary to Serine 345 phosphorylation. This observation suggests that both Serines 317 and 345 need to be phosphorylated for maximal G2/M checkpoint response. Analysis of Chk1 phosphorylation after exposure to 10Gy of IR by Western blotting revealed that both Serine residues become phosphorylated within one hour after DNA damage. Interestingly, the amount of phosphorylation on S345 and possibly on S317 of Chk1 was much less after the doses of IR used as compared to the levels of phosphorylation after DNA replication was blocked with aphidicolin. A possible explanation for this could be that the two types of genotoxic stress result in different amounts of checkpoint activating structures. For example, when replication is blocked with aphidicolin, this presumably affects all active

replication forks and results in quite a high number of single-stranded stretches of DNA that can be bound by sensory proteins such as RPA. Subsequent binding of the ATR-ATRIP complexes will result in a strong activation of the signalling cascade including high levels of Chk1 phosphorylation. In comparison, exposure to IR will create DNA damage such as DNA double-strand breaks which may result in a smaller number of DNA repair intermediates and single stranded DNA. In addition, treatment with aphidicolin was continuous compared to a brief IR treatment. Thus, it is difficult to compare these two different treatment regimens.

A very different picture emerged for the requirement of Chk2 for mitotic checkpoint proficiency. Chk2 does not play a significant role in the S/M checkpoint response at least in the presence of functional Chk1. Instead it was found to be dispensable as both avian Chk2<sup>-/-</sup> cells and Chk2 siRNA treated human BE cells were still able to delay mitosis after DNA replication was blocked. The S/M checkpoint response was almost certainly executed by Chk1 which is still functioning in these cells. In the absence of both Chk1 and Chk2 function, achieved by down-regulation of their protein expression with siRNA in the BE cells, the S/M checkpoint response was noticeably impaired. There was no obvious quantitative difference between the S/M checkpoint failures in cells depleted of Chk1 alone or Chk1 and Chk2 together, suggesting that Chk2 does not contribute to S/M checkpoint proficiency independently of Chk1. The significant function of Chk1 in the S/M checkpoint response is also in line with previous studies that have demonstrated a role for Chk1 in suppressing futile origin firing and stabilizing stalled replication forks in response to replication arrest (Feijoo et al., 2001; Zachos et al., 2003). Taken together these observations further confirm that Chk1 is the major effector kinase of the S/M checkpoint in vertebrate cells whereas Chk2 is dispensable.

In contrast, Chk2 does contribute to the G2/M checkpoint response in DT40 cells as it plays a role in establishing optimal mitotic delay in G2 cells after DNA damage. Previously, a study by Xu et al. described and characterised the G2/M arrest after IR and demonstrated that two distinct checkpoint responses become activated depending on the cell cycle stage that the cells are in at the time of stress (Xu et al., 2002a). An important observation in the study presented here was that Chk2<sup>-/-</sup> cells displayed an intermediate phenotype after DNA damage as some cells arrested in G2, however almost 50% of cells did eventually enter mitosis. This result was in agreement with a recent study that established a role for Chk2 in the optimal execution of mitotic delay after DNA damage (Rainey et al., 2007). This study found that cells lacking Chk2 that were in G1 or S phase at the time of DNA damage with IR were able to arrest properly in G2; however a greater

proportion of cells that were in G2 at the time of damage did enter mitosis compared to wild-type cells. The contribution of Chk2 to the G2/M arrest was suggested to be epistatic to Chk1. The intermediate phenotype of the Chk2<sup>-/-</sup> cells in this study can therefore be explained by the mixture of cells in an asynchronous cell population, whereby approximately half of the cells were in G1 and S phase at the time of damage and therefore arrested in G2, while the other half of the cells was mainly in G2 and thus progressed into mitosis with DNA damage.

Analysis of Chk2 requirement in response to DNA damage in human BE cells did not reveal a similar role for Chk2 in this checkpoint response as cells treated with Chk2 siRNA displayed a functional G2/M arrest comparable to control cells. This could be a general difference between the two vertebrate systems or the fact that siRNA down-regulation of Chk2 gene expression might not have been as effective and left some residual amounts of protein that preserved a functional response. A more careful analysis using various approaches of interfering with Chk2 function could be applied to clarify the situation, however from the presented data in this study Chk2 appears dispensable for the G2/M arrest in BE cells. Taken together, the role of Chk2 appears to vary between the two vertebrate systems that were investigated in this study. Under the experimental conditions used, Chk2 plays a minor role for the optimal execution of the G2/M arrest in DT40 cells but it is basically dispensable for the G2/M arrest in BE cells. When both Chk1 and Chk2 expression levels were down-regulated with siRNA in BE cells, the G2/M arrest was completely abrogated, further emphasising the key role of Chk1.

In conclusion, Chk1 emerged as the major effector kinase for the G2/M arrest in response to DNA damage and for the S/M checkpoint arrest in response to blocks to DNA replication in both the avian and human cell systems that were investigated in this study. A more detailed analysis and comparison of the mechanisms of mitotic delay after the two types of genotoxic stress including respective downstream targets of Chk1 can reveal more aspects of the role of Chk1 in mitotic checkpoint responses in vertebrate cells.

## **4 Results: The G2/M and S/M checkpoints are mechanistically distinct**

### **4.1 Kinetic analysis of mitotic checkpoint proficiency**

The experiments in chapter 3 established Chk1 as the major effector kinase for both S/M and G2/M checkpoint proficiency in avian DT40 and human BE cells. As these checkpoints are activated by two distinct types of stress, i.e. block to DNA replication on one side and DNA damage on the other, their actual response mechanism could be different even though the eventual outcome is the same: to delay the onset of mitosis. Thus, to determine whether the timing of mitotic entry was the same in the absence of Chk1, the mitotic checkpoint responses were analysed kinetically.

#### ***4.1.1 Comparison of mitotic checkpoint responses in asynchronous DT40 WT and Chk1<sup>-/-</sup> cell cultures***

To document and compare the S/M and G2/M checkpoint responses over time in the presence or absence of functional Chk1, a kinetic analysis was performed with the DT40 WT and Chk1<sup>-/-</sup> cell lines. Cells were either exposed to DNA damage with IR or treated with the DNA polymerase inhibitor aphidicolin to block DNA replication. Subsequently cells were cultured in the presence of the spindle poison nocodazole and samples taken at 0, 2, 4, 6, 9, and 12h, and analysed for DNA content and mitotic indices by flow cytometry.

As shown in Figure 4.1, control DT40 WT cells progressed steadily through the cell cycle with a majority of cells accumulating in mitosis after 12h. In response to DNA damage induced by IR, entry to mitosis was completely blocked, although cells progressed through S phase and arrested in G2. An initial increase in sub 2N cells for up to 4h was also noted. In comparison, when DNA replication was blocked with aphidicolin, cells were also able to delay mitotic entry successfully. However in this case they did not progress through the cell cycle and appeared to be arrested predominantly in G1 and S phase. Furthermore, a significant number of sub 2N DNA content cells could be detected throughout the time course. The quantification of the flow cytometry data for this time course experiment is presented in Figure 4.3. By comparing the numbers of either the total mitotic or the 4N mitotic cells in the graphs on the left side it is apparent that DNA damage and replication

arrest were equally effective at delaying the onset of mitosis in DT40 WT cells. The percentages of total mitotic cells were very low after both DNA damage with IR (10%) and replication block (4%), whereas 62% of the control cells entered mitosis (Figure 4.3).

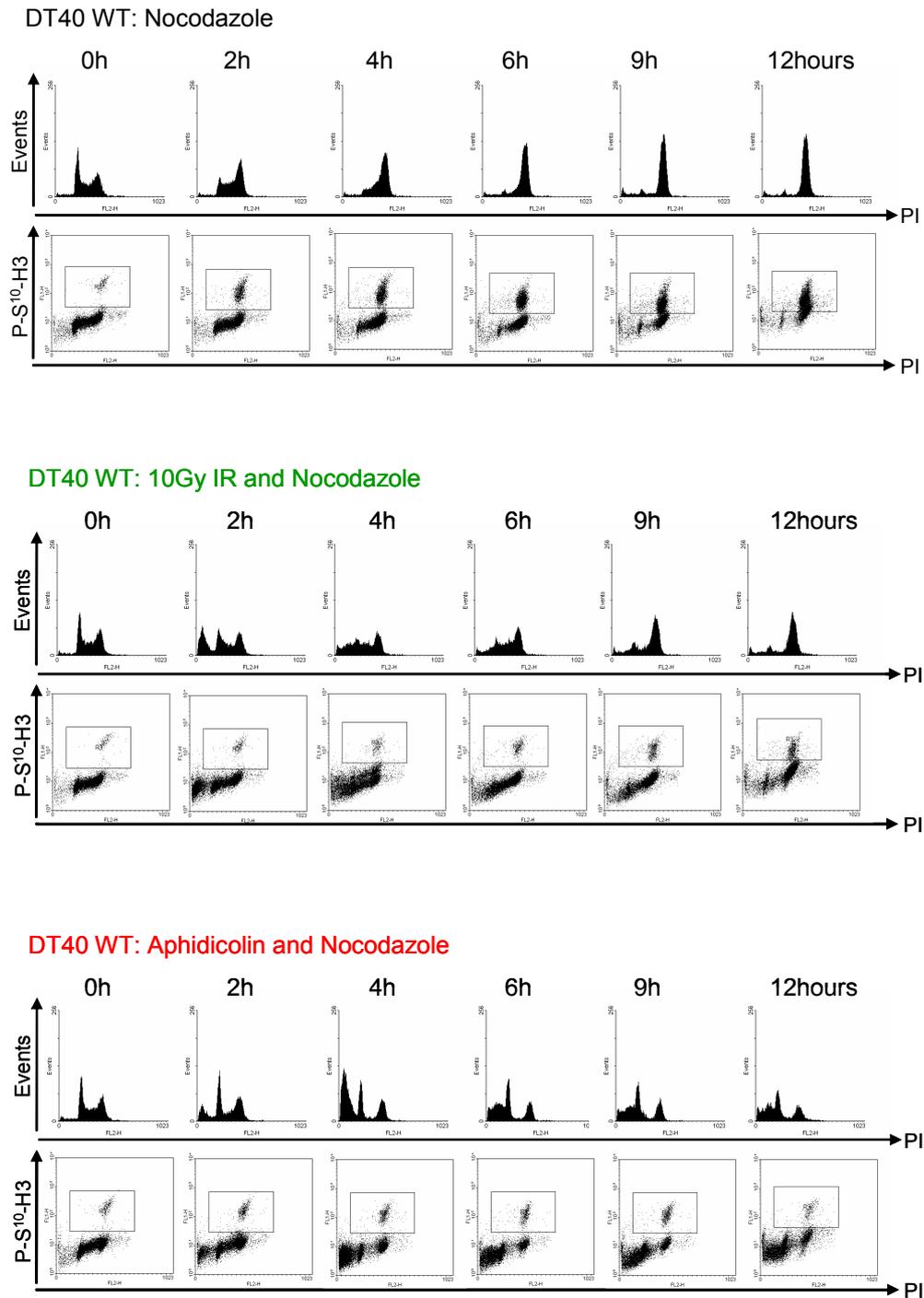
A similar analysis was also performed on the Chk1<sup>-/-</sup> cells. As shown in Figure 4.2, control Chk1<sup>-/-</sup> cells progressed through the cell cycle at a comparable rate to the DT40 WT cells, with approximately two thirds of the cells accumulating in mitosis after 12h. After irradiation with 10Gy, Chk1<sup>-/-</sup> cells advanced through the cell cycle and accumulated in mitosis with 4N DNA content at a rate similar to the control cells and without any measurable arrest in G2. Furthermore, the actual number of either total or 4N mitotic Chk1<sup>-/-</sup> cells was found to be basically identical after 12h in both control cells (56%) and in cells that had been exposed to DNA damage (58%).

The response of Chk1<sup>-/-</sup> cells to DNA replication block with aphidicolin was different. Initially, the total rate of cells entering mitosis was the same as in control or IR treated cells, however this was entirely due to mitotic cells with a DNA content of 4N. Over time however, there was a distinct difference in the actual DNA content of the mitotic cells as they were comprised of both 2N and 4N cells. As illustrated in the quantification of the flow cytometry data in Figure 4.3, only Chk1<sup>-/-</sup> cells with 4N DNA content initially entered mitosis and after approximately 6h this number reached a plateau level and did not increase any further. By contrast, Chk1<sup>-/-</sup> cells with 2N DNA content, and thus unreplicated DNA, started to enter mitosis only after 6h of treatment and this number increased progressively until the 12h time point.

Taken together, these experiments revealed a difference in the actual timing of checkpoint failure in the different cell populations after DNA damage or DNA replication arrest in the Chk1<sup>-/-</sup> cells. Whereas cells with damaged DNA entered mitosis at a similar rate to control cells in the absence of Chk1, there was a difference in the timing of cell populations entering normal 4N or premature 2N mitosis after DNA replication was blocked, as the onset of premature mitosis occurred only at later times.

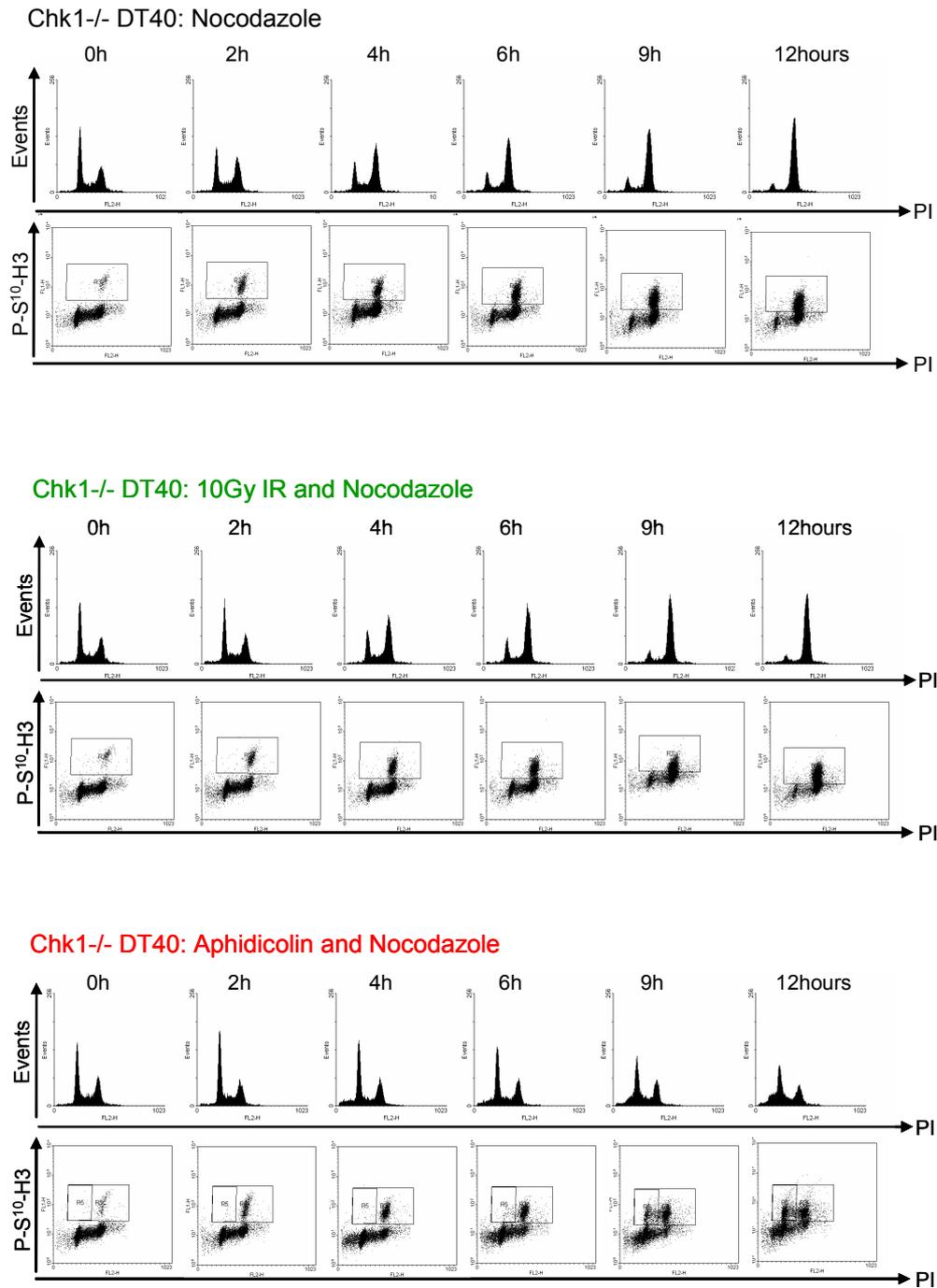
#### **4.1.2 Comparison of mitotic checkpoint responses in BE cells**

A similar kinetic analysis was performed in human BE cells. Time course experiments were performed with asynchronous BE cells and Chk1 function was abrogated using the inhibitor UCN-01. Cells were exposed to either DNA damage with IR or replication block with aphidicolin and cultured in the presence of nocodazole for up to 16h.



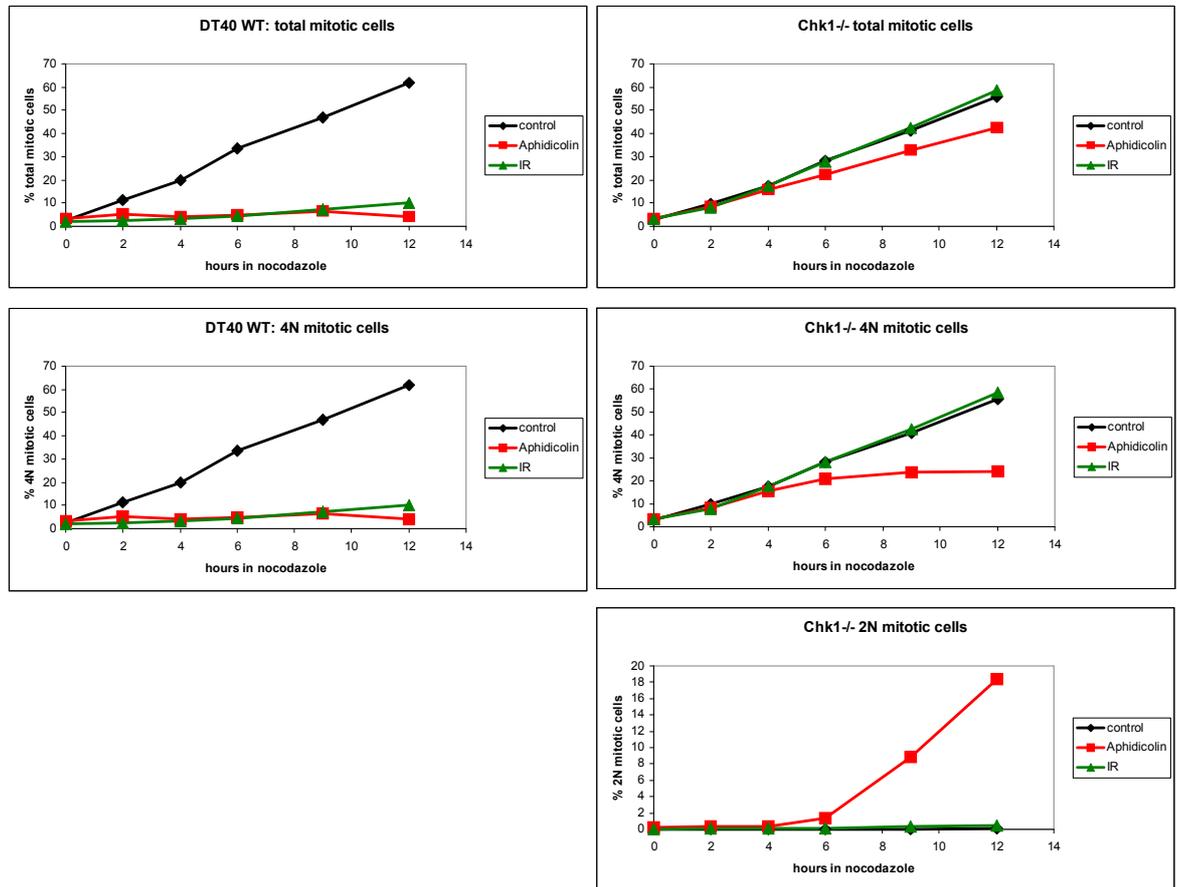
**Figure 4.1: Kinetic analysis of mitotic checkpoint responses in DT40 WT cells**

Asynchronous DT40 WT cells were treated with either 10Gy of IR or the DNA polymerase inhibitor aphidicolin and subsequently cultured in the presence of nocodazole. Cells were grown for 12h and harvested at the indicated time points. Samples were fixed and analysed for their DNA content (PI profiles) and mitotic indices (P-S10-H3/PI) by flow cytometry.



**Figure 4.2: Kinetic analysis of mitotic checkpoint responses in Chk1<sup>-/-</sup> cells**

Asynchronous Chk1<sup>-/-</sup> cells were treated with either 10Gy of IR or the DNA polymerase inhibitor aphidicolin and subsequently cultured in the presence of nocodazole. Cells were grown for 12h and harvested at the indicated time points. Samples were fixed and analysed for their DNA content (PI profiles) and mitotic indices (P-S10-H3/PI) by flow cytometry.



**Figure 4.3: Comparison of kinetic analysis of mitotic checkpoint responses in DT40 WT and Chk1-/- cells**

Graphical illustration of representative examples of the flow cytometry data for the kinetic analysis of mitotic checkpoint responses in DT40 WT (graphs on the left side) and Chk1-/- cells (graphs on the right side) which has been performed on several occasions with reproducible results (see also Figures 4.13 and 4.15 for statistical analysis).

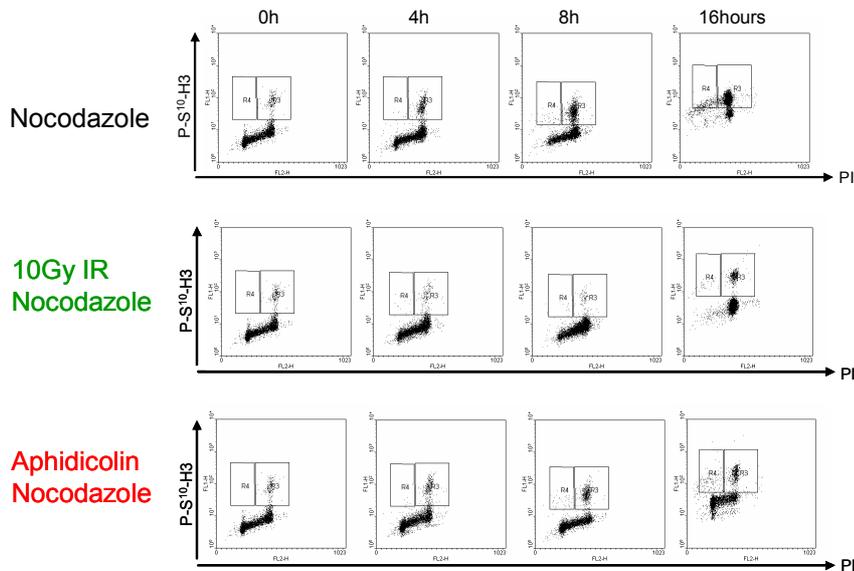
Cells were treated with either 10Gy of IR (green) or the DNA polymerase inhibitor aphidicolin (red) and cultured in the presence of nocodazole. Apart from the total number of mitotic cells (i.e. 2N and 4N), the graphs also depict the percentages of 4N mitotic cells and 2N mitotic cells (only for Chk1-/- cells).

As shown in Figure 4.4, the majority of control BE cells progressed through the cell cycle and accumulated in mitosis after 16h in the presence of nocodazole alone. After irradiation however, mitosis was blocked. Cells did advance through S phase but by 16h a very high percentage arrested in G2 phase with 4N DNA content. In comparison, after DNA replication was blocked with aphidicolin, BE cells also effectively delayed the onset of mitosis. The cells appeared to be mainly in G1 and S phase of the cell cycle. Quantification of the flow cytometry data revealed that whereas up to 73% of control cells entered mitosis, only 11% and 15% of cells entered mitosis after treatment with IR and aphidicolin respectively (Figure 4.5). The numbers of total and 4N mitotic cells were basically the same as essentially no mitotic cells with less than 4N DNA content were observed.

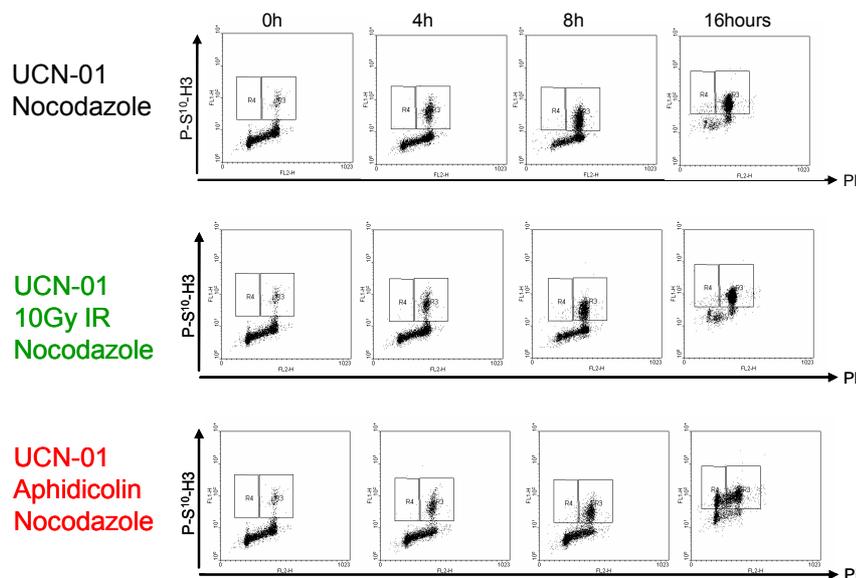
To inhibit Chk1 function, BE cells were treated with the inhibitor UCN-01 and subsequently analysed for mitotic checkpoint proficiency. As shown in Figure 4.4, UCN-01 alone did not interfere with cell cycle progression. After DNA damage, UCN-01-treated cells progressed through the cell cycle almost as rapidly as controls and accumulated in mitosis to a similar extent (84% and 78% respectively). In comparison, when DNA replication was blocked with aphidicolin, the rate of cells entering mitosis appeared to reach similar levels as control cells; however, when separating the total number of mitotic cells according to their DNA content, it became apparent that initially only cells with 4N DNA content entered mitosis and that from 8h to 16h their number did not increase much further and reached a plateau level. By contrast, mitotic cells with 2N DNA content were only observed at the 16h time point when a large percentage (32%) of cells entered mitosis with unreplicated DNA. Figure 4.5 shows the quantification of 2N and 4N mitotic cells from the flow cytometry data for this experiment.

In summary, quantification of the time course data indicated that there was a difference in the timing of checkpoint failure in different cell populations in response to the two types of genotoxic stress. The results from the UCN-01 treated BE cells were very reminiscent of observations previously made in the avian Chk1<sup>-/-</sup> DT40 cells. In both systems, cells without Chk1 function were unable to delay mitosis after DNA damage as cells progressed through the cell cycle and accumulated in mitosis at the same rate as unstressed control cells. In contrast, when DNA replication was blocked with aphidicolin, there was a difference in the timing of cell populations entering normal 4N or premature 2N mitosis in the absence of functional Chk1. Similar to Chk1<sup>-/-</sup> cells, the UCN-01 treated BE cells entered premature mitosis with un-replicated DNA only at later times.

(a)

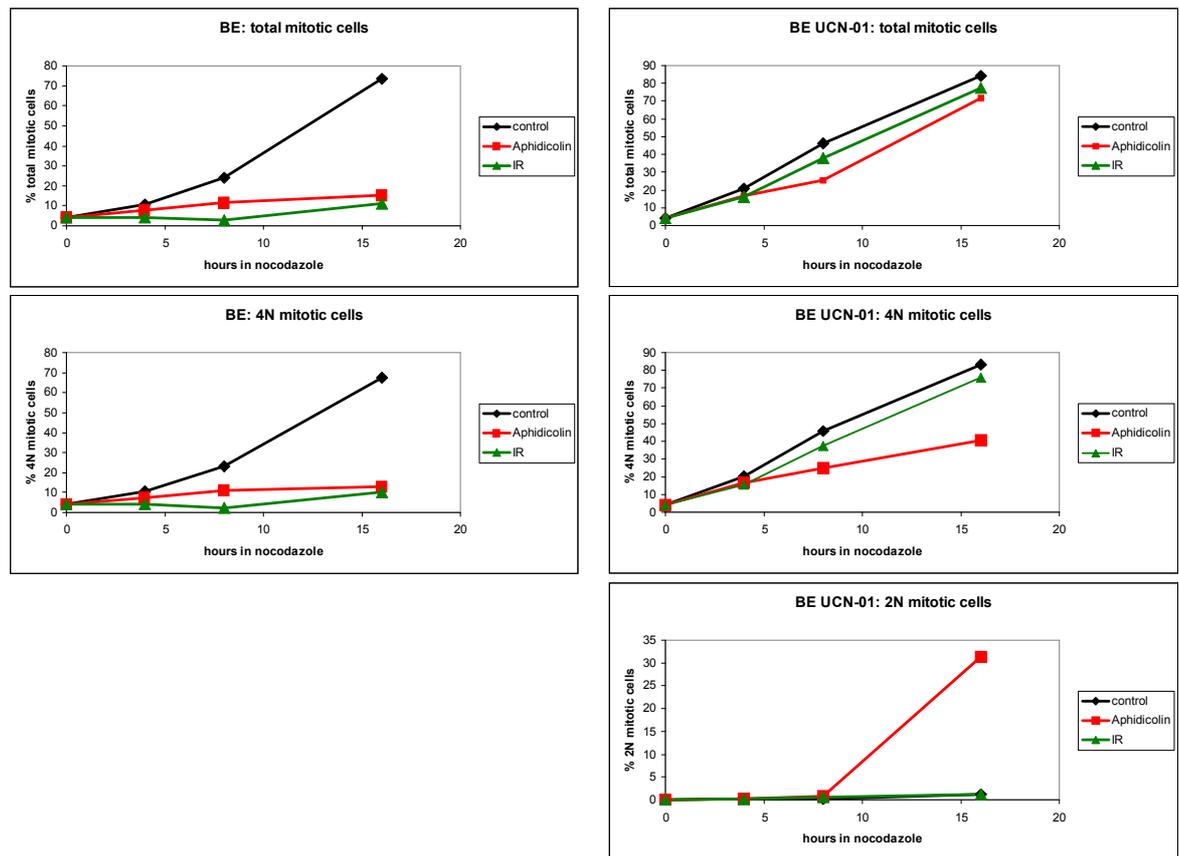


(b)



**Figure 4.4: Kinetic analysis of mitotic checkpoint responses in BE cells**

The timing of S/M and G2/M checkpoint responses of BE cells was compared in normal wild-type cells (a) or cells that were grown in the presence of the Chk1 inhibitor UCN-01 (b). Cells were treated with either the DNA polymerase inhibitor aphidicolin or 10Gy of IR and then cultured in the presence of nocodazole. Cells were grown for up to 16h and harvested at the indicated time points. Samples were fixed and analysed for their DNA content (PI profiles-not shown) and mitotic indices (P-S10-H3/PI) by flow cytometry.



**Figure 4.5: Quantification of kinetic analysis of mitotic checkpoint responses in BE cells**

Graphical illustration for a representative example of the flow cytometry data for the kinetic analysis of mitotic checkpoint responses for BE cells (graphs on the left side) and BE cells treated with the Chk1 inhibitor UCN-01 (graphs on the right side). Cells were treated with either 10Gy of IR (green) or the DNA polymerase inhibitor aphidicolin (red) and cultured in the presence of nocodazole. Apart from the total number of mitotic cells (i.e. 2N and 4N), the graphs also depict the percentages of 4N mitotic cells and 2N mitotic cells (only for BE UCN-01).

### **4.1.3 Comparison of mitotic checkpoint responses in synchronised DT40 WT and Chk1<sup>-/-</sup> cells**

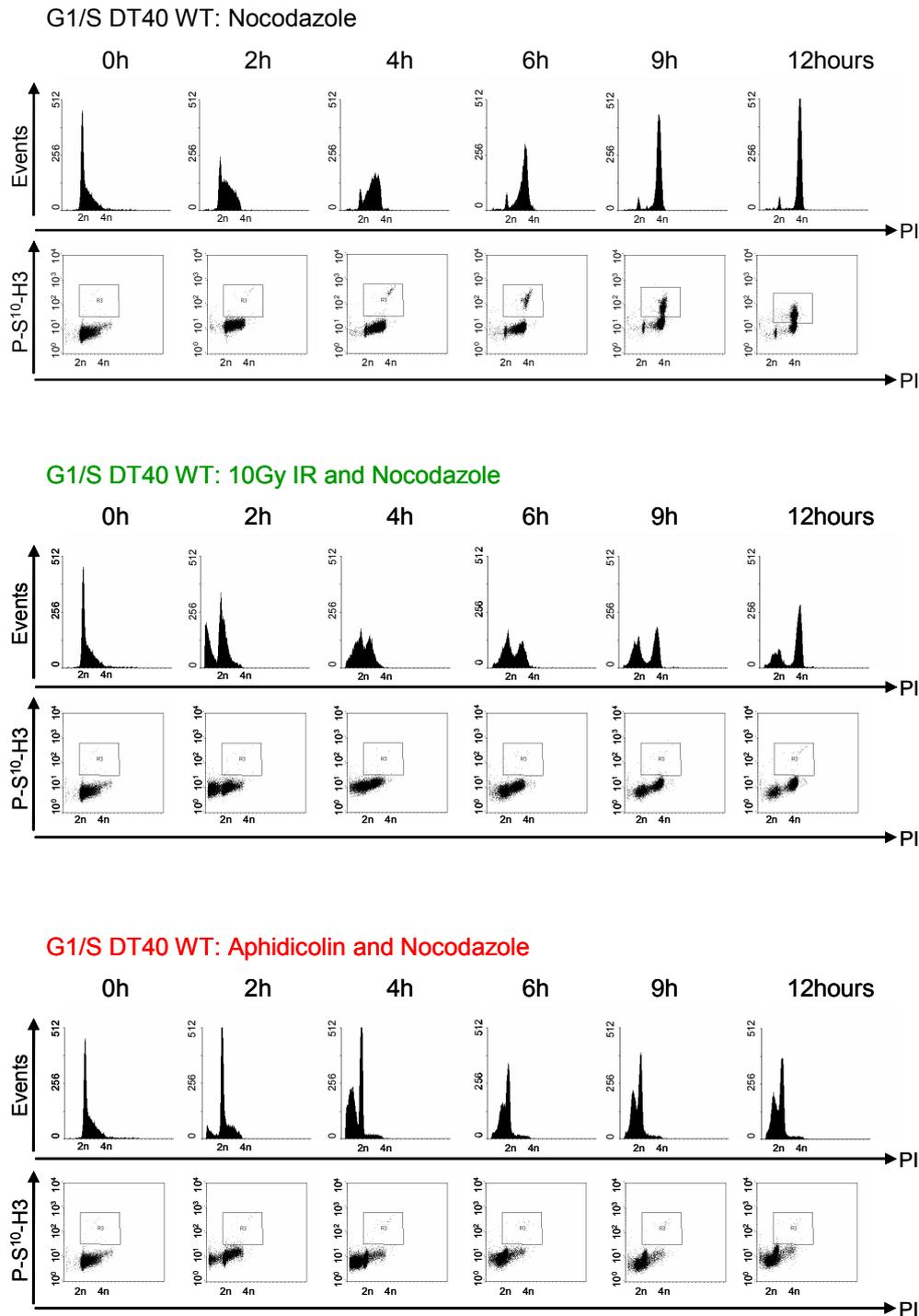
The time course analysis of mitotic checkpoint responses in asynchronous DT40 cells and BE cells revealed differences in the timing of the checkpoint failure in different cell populations in response to either DNA damage or DNA replication block. To document and compare these timing differences in more detail, the kinetic analysis of mitotic checkpoint responses was performed with synchronised cells. Also, asynchronous cell populations are not ideal for a detailed biochemical analysis as the presence of cells in various cell cycle phases could obscure biochemical changes occurring in a specific subpopulation.

To obtain a purified cell population, asynchronous DT40 WT or Chk1<sup>-/-</sup> cultures were synchronised by centrifugal elutriation, a method that allows separation of cells based on their size (see method section 2.2.4). Populations of cells in the G1/S phase of the cell cycle were elutriated and then either exposed to DNA damage with 10Gy of IR or treated with the DNA polymerase inhibitor aphidicolin to block DNA replication. Cells were then returned to culture and grown in the presence of the spindle poison nocodazole. This procedure made it possible to observe the progress of a synchronised cell population through the cell cycle to mitosis as well as their actual DNA content in much more detail.

Figure 4.6 shows synchronised G1/S DT40 WT control cells progressing unperturbed through the cell cycle in the presence of nocodazole. Most cells reached S phase 4h after release as can be seen clearly in the PI profiles in Figure 4.6. The cells then passed through G2 phase and eventually after 12h almost all cells entered mitosis.

In comparison, when cells were exposed to DNA damage with IR, they did not enter mitosis. A small but significant proportion of DT40 WT cells appeared to arrest in S phase after IR treatment, indicating the existence of an intra S phase checkpoint in response to DNA damage in these cells (Figure 4.6). Aside from this specific population, the vast majority of irradiated DT40 WT cells progressed through S phase by 4 to 6h and eventually accumulated in G2 phase with 4N DNA content. There was also some level of cell death after DNA damage as apparent in the sub 2N population, most notably at the 2h time point (Figure 4.6).

In contrast, when DNA replication was blocked with aphidicolin, accumulation of mitotic cells was also completely blocked. However, the purified cell population did not progress



**Figure 4.6: Mitotic checkpoint responses in G1/S synchronised DT40 WT cells**

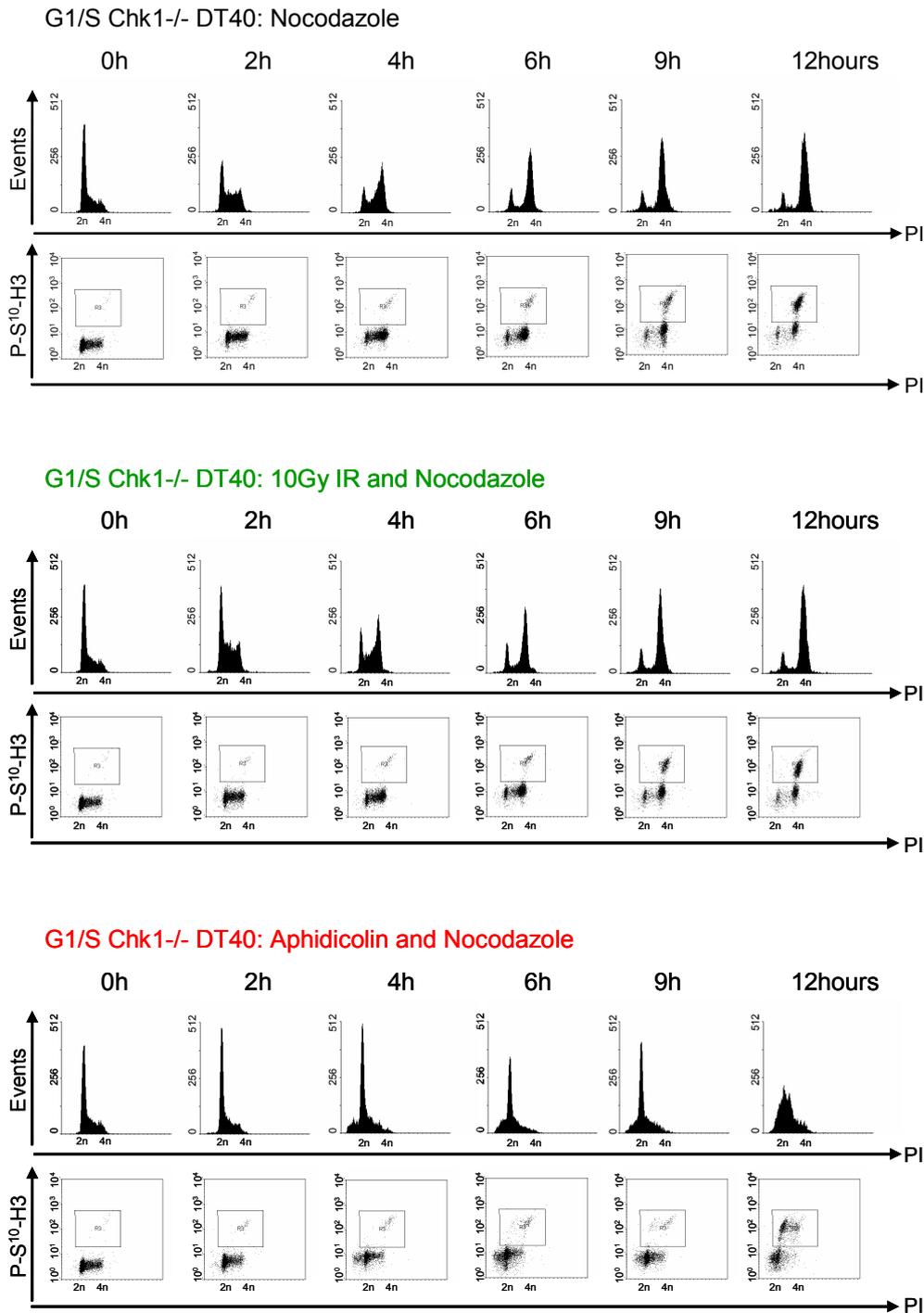
A purified G1/S cell population was isolated from DT40 WT cells by centrifugal elutriation and returned to culture. Cells were subsequently treated with either the DNA polymerase inhibitor aphidicolin or 10Gy of IR and released into culture in the presence of nocodazole. Cells were grown for 12h and harvested at the indicated time points. Samples were fixed and analysed for their DNA content (PI profiles) and mitotic indices (P-S10-H3/PI) by flow cytometry.

through the cell cycle and appeared to still be in G1/S phase, or, most likely early S, after 12h of aphidicolin treatment. There was some level of cell death, which was greatest after 4h but still apparent at 12h. Taken together, these results showed that DT40 WT cells were blocked from entering mitosis in the presence of either damaged or un-replicated DNA and accumulated in G2 phase or G1/S phase, respectively. The results obtained from this kind of time course experiment were very reproducible. The average of the flow cytometry data of four independent elutriation experiments is shown in Figure 4.8.

A similar kinetic analysis was also performed with the Chk1<sup>-/-</sup> cells. Elutriated purified G1/S Chk1<sup>-/-</sup> control cells progressed through the cell cycle in a similar manner to their DT40 WT counterparts in the absence of perturbation (Figure 4.7). In response to DNA damage with IR, the G1/S cell population of Chk1<sup>-/-</sup> cells advanced through S phase and G2 phase without any measurable arrest and eventually accumulated in natural 4N mitosis at levels comparable to control cells. In contrast to DT40 WT cells, the Chk1<sup>-/-</sup> cells displayed no measurable arrest or slowing in S phase or G2 phase in response to DNA damage, indicating that Chk1 function is required for an intra S phase checkpoint as well as the G2/M checkpoint response in the DT40 cells.

In marked contrast, when the purified Chk1<sup>-/-</sup> cells were treated with the DNA polymerase inhibitor aphidicolin and then released back into culture, accumulation of mitotic cells was almost completely blocked for up to 6h (Figure 4.7). The initial mitotic delay of Chk1<sup>-/-</sup> cells lasted for up to 9h, after which the majority of arrested cells entered mitosis prematurely. This was not due to cells progressing through S phase to reach G2 and then divide; instead these cells entered mitosis with unreplicated DNA. The quantification of the flow cytometry data from four independent experiments demonstrated how reproducible these observations were. As shown in the graph in Figure 4.8, there were significant differences in the timing of the appearance of mitotic cells after aphidicolin or IR treatment. Whereas the amount and rate of progression into mitosis was basically the same after DNA damage as in the unstressed control cells, there was absolutely no increase in the number of mitotic cells after aphidicolin treatment for up to 9h. However, after this prolonged mitotic delay Chk1<sup>-/-</sup> cells did eventually enter mitosis, although prematurely with unreplicated DNA.

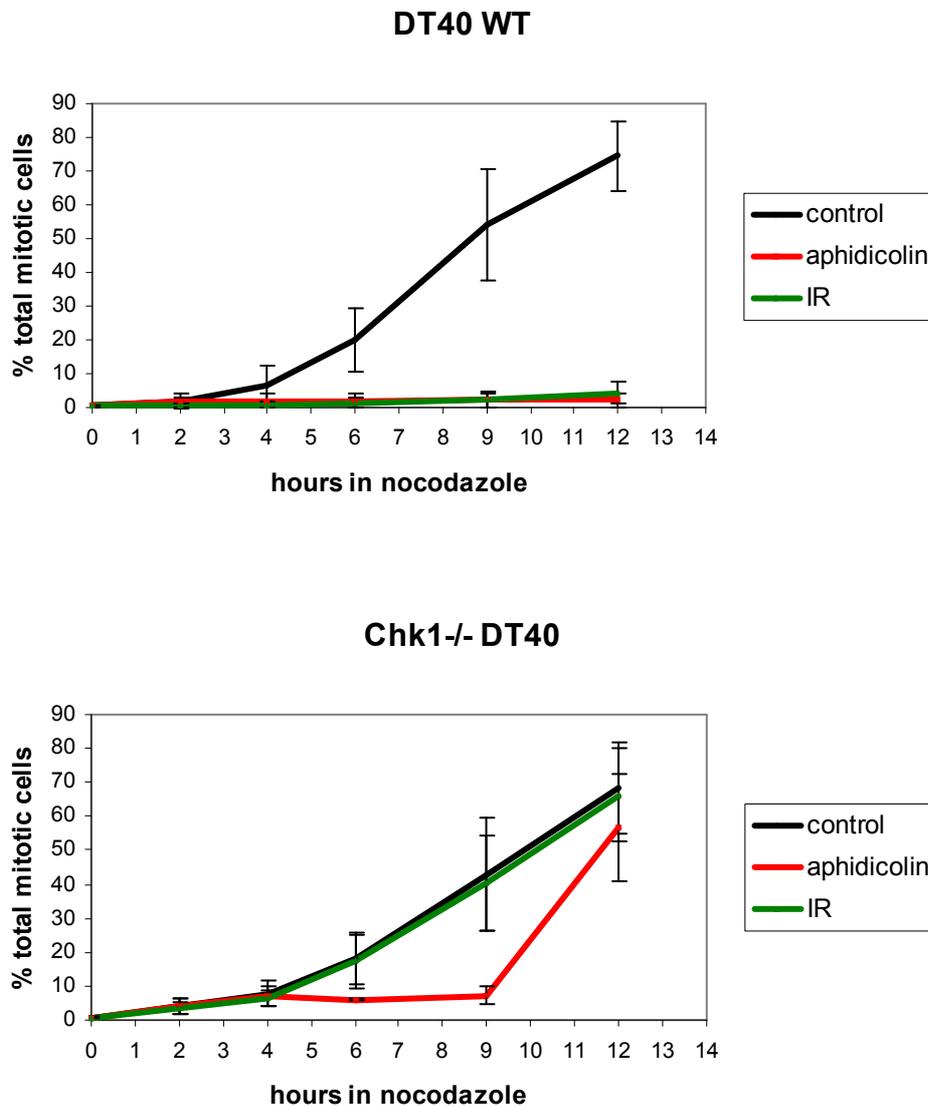
Furthermore, the levels of cell death in the Chk1<sup>-/-</sup> cells after both IR and aphidicolin treatment were practically negligible, as judged by the amount of cells with a DNA content of less than 2N (Figure 4.7). This observation could indicate a potential role for Chk1 in stress-induced cell death mechanisms, a notion that will require further investigation.



**Figure 4.7: Mitotic checkpoint responses in G1/S synchronised Chk1<sup>-/-</sup> cells**

A purified G1/S cell population was isolated from Chk1<sup>-/-</sup> cells by centrifugal elutriation and returned to culture. Cells were subsequently treated with either the DNA polymerase inhibitor aphidicolin or 10Gy of IR and released into culture in the presence of nocodazole. Cells were grown for 12h and harvested at the indicated time points. Samples were fixed and analysed for their DNA content (PI profiles) and mitotic indices (P-S<sup>10</sup>-H3/PI) by flow cytometry.

The use of synchronised cells allowed the exact documentation of the behaviour of a specific cell population in response to DNA damage or DNA replication arrest and revealed significant differences between the two mitotic checkpoint mechanisms. The G2/M arrest, as well as the intra S phase arrest, is completely absent in Chk1<sup>-/-</sup> cells and therefore absolutely dependent on Chk1 function. In marked contrast, only the maintenance of the S/M checkpoint is dependent on Chk1, whereas the initiation of the checkpoint is not. Chk1<sup>-/-</sup> cells in G1/S phase can initially delay the onset of mitosis for more than 9h and only after this extended time period do they enter mitosis with unreplicated DNA. This strongly suggests that some timing mechanism other than cell cycle position and progression per se is operating and is responsible for the initial S/M checkpoint delay in the absence of Chk1.



**Figure 4.8: Comparison of mitotic checkpoint responses in DT40 WT and Chk1<sup>-/-</sup> cells**

Quantification of the flow cytometry data from the kinetic analysis of mitotic checkpoint responses in synchronised DT40 WT (top) and Chk1<sup>-/-</sup> cells (bottom): The percentage of total mitotic cells was judged by P-S10-H3 staining and the average was calculated from four independent experiments, including the standard deviation. Untreated control cells are depicted in black, cells treated with 10Gy IR are represented in green (G2/M checkpoint response) and cells treated with aphidicolin are shown in red (S/M checkpoint response).

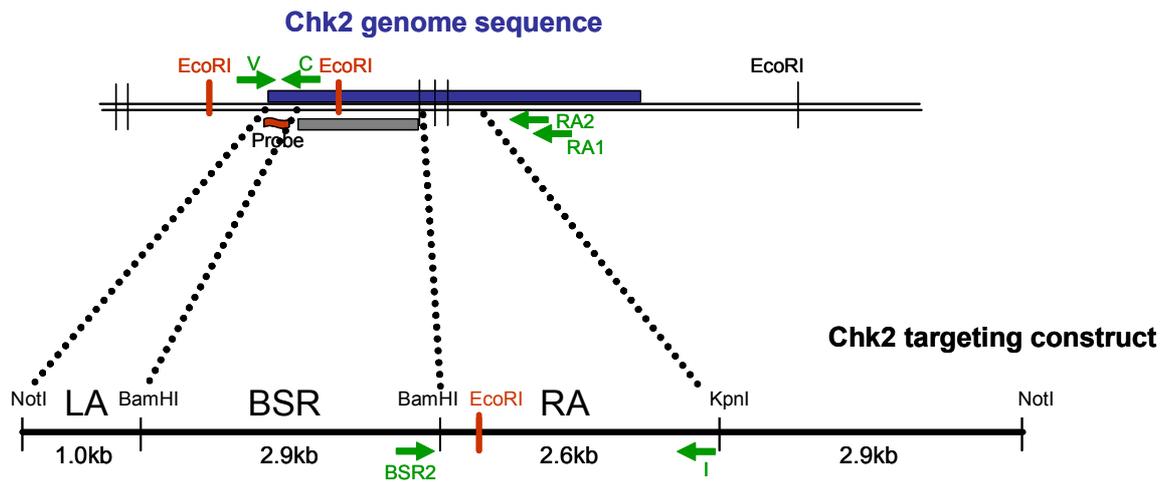
## 4.2 Timing of S/M checkpoint failure in the absence of Chk1 and Chk2 activity

Chk1<sup>-/-</sup> cells still express functional Chk2, therefore this raises the question of whether the initial mitotic delay during aphidicolin treatment could be executed by Chk2. When Chk2<sup>-/-</sup> cells were treated with the Chk1-inhibitor UCN-01 they still retained an initial mitotic delay after DNA replication block, however whether the kinetics of checkpoint failure were altered was not resolved (Zachos et al., 2005). Building on these observations, one aim of this study was to investigate checkpoint responses in DT40 cells devoid of both Chk1 and Chk2 function.

### 4.2.1 Attempts to generate a Chk1/Chk2 double knock-out DT40 cell line

To address this issue genetically, a gene knock-out approach was attempted. It was decided to try to knock out the Chk2 gene in the Chk1<sup>-/-</sup> background by homologous recombination and thereby obtain a Chk1/Chk2 double knock-out DT40 cell line. DT40 cells have a high ratio of targeted to random integration after transfection (Buerstedde and Takeda, 1991) and have previously been successfully used to establish a wide range of knock-out cell lines including the Chk1<sup>-/-</sup> and Chk2<sup>-/-</sup> cell lines.

The design of the Chk2 gene targeting vector was based on the Chk2 targeting vector that had been used previously to generate the Chk2<sup>-/-</sup> cells (Rainey et al., 2007), although a few important changes were introduced. In order to knock-out the two chicken Chk2 alleles it was necessary to generate a replacement construct containing two regions of homology and a selectable marker cassette which would then replace essential regions of the Chk2 gene. As the Chk1<sup>-/-</sup> and the Chk2<sup>-/-</sup> cell lines are resistant to puromycin and neomycin due to the design of their original respective gene targeting vectors, the selectable marker cassette of the new Chk2 targeting vector had to be changed. The regions of homology were created by amplifying stretches of genomic DNA using PCR with primers based on the chicken Chk2 cDNA sequence (Rainey, 2003). Briefly, the left arm of homology was of 1kb length whereas the right arm of homology was of 2.6kb length and with these regions in the targeting vector it would be possible to achieve disruption of the region of the Chk2 gene that encoded the FHA domain and most of the kinase domain. Figure 4.9 shows a schematic diagram of the design of the targeting vectors and the targeting strategy.



**Figure 4.9: Schematic overview of Chk2 gene targeting vector and knock-out strategy**

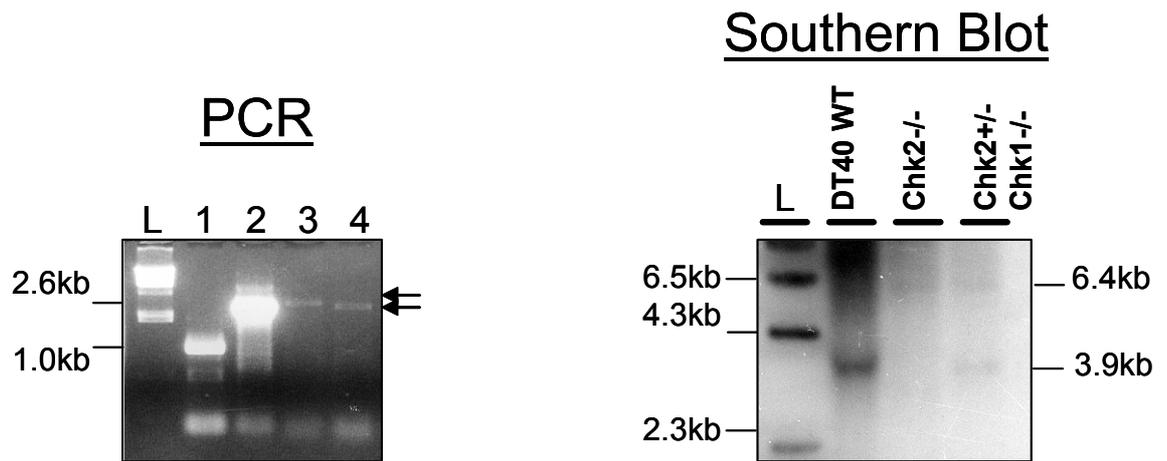
This simplified schematic overview shows the Chk2 genome sequence (blue box) that encodes the Chk2 protein. The Chk2 targeting construct has been aligned to show how the two regions of homology (LA and RA) relate to the genomic sequence of Chk2. The region of genomic Chk2 that would get disrupted by targeted integration is also highlighted (grey box). PCR primers are annotated in green (primers BSR2, C, I, RA1, RA2, V) and EcoRI restriction enzymes and the probe relevant for Southern blotting are highlighted in red.

The newly integrated BSR cassette was flanked by two loxP sites and would confer resistance to blasticidin. The presence of loxP sites would allow recycling of the marker cassette by excising it with Cre recombinase after the first round of targeting and therefore rendering the targeting vector re-usable for disruption of the second Chk2 allele.

The targeting vector was linearised and transfected into Chk1<sup>-/-</sup> cells in order to obtain clones that had stably integrated the targeting construct (see methods). Over the course of several rounds of targeting a large number of blasticidin resistant clones was analysed for targeted integration by PCR and Southern blotting. Altogether however, only two targeted clones could be identified. An example of the PCR/Southern blot analysis for the targeting event is shown in Figure 4.10. To summarise briefly, the PCR products in lanes 3 and 4 were of the predicted size for amplification of a stretch of DNA from a region within the selectable BSR marker cassette of the targeting construct to a region of genomic DNA that lay outside of the targeting construct. The presence of these PCR products therefore confirmed targeting of at least one of the two Chk2 alleles in this particular clone. As for the Southern blotting analysis (see methods), a probe was chosen that would recognize a fragment of 3.9kb if the gene was wild-type and a fragment of 6.4kb if the gene was targeted as integration of the vector would have destroyed one restriction site of the wild-type gene and created a new site that was located within the targeting vector (Figure 4.9). Figure 4.10 shows a Southern blot analysis with DT40 WT, Chk2<sup>-/-</sup>, and a targeted Chk1<sup>-/-</sup>; Chk2<sup>+/-</sup> clone. Due to differences in the amount of genomic DNA in each lane, the two bands at 6.4kb and 3.9kb are quite faint but still visible and confirmed targeting of one of the two Chk2 alleles in this clone.

Unfortunately, neither clone could be propagated for an extended period of time as they died within a few weeks of culturing. Several attempts to grow the cells for a longer time period failed and therefore made it impossible to characterise these clones thoroughly. This issue remains to be resolved; however there are several reasons why the approach to generate Chk1/Chk2 double knock-out cells did not have a positive outcome.

Over the course of several rounds of targeting, only two targeted clones were identified. Reasons for this could have been that the targeting strategy and the design of the targeting vector was not optimal and also that the transfection efficiency in general was quite low. It was not possible to grow Chk1<sup>-/-</sup>;Chk2<sup>+/-</sup> cells in culture for an extended amount of time, implying that Chk2 was important for the growth of these cells in the absence of Chk1 and that a double knock-out was likely to be lethal. There are several ways to address these problems: apart from trouble-shooting the design of the targeting vector, the transfection



**Figure 4.10: Analysis of Chk2 targeting event by PCR and Southern blotting**

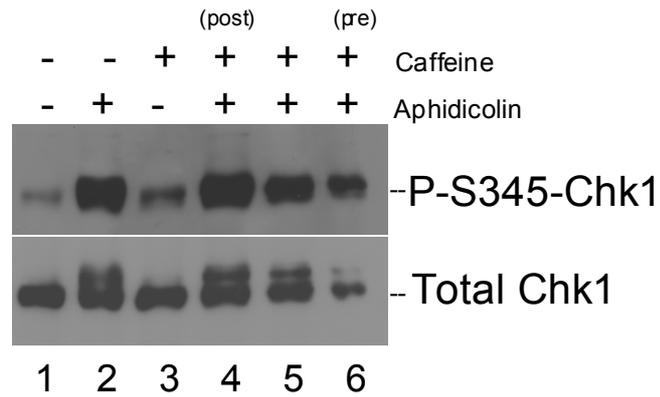
Left: Agarose gel photograph depicting results from a PCR analysis. L:  $\lambda$ /HindIII DNA ladder, PCR reactions with primers: for 1: V-C control genomic DNA, 2: BSR2-I control vector, 3: BSR2-RA1 and 4: BSR2-RA2 targeting event. Right: Southern blot analysis with probe A on genomic DNA after digest with EcoRI restriction enzyme. DT40 WT (3.9kb), Chk2<sup>-/-</sup> (6.4kb), and Chk2<sup>+/-</sup>;Chk1<sup>-/-</sup> clone (3.9kb and 6.4kb).

method and the screening process, it would be logical to try a conditional knock-out approach where Chk1 and Chk2 gene expression could be switched off for the duration of an experiment. This method could also clarify whether the issue of lethality of the cells in culture is true.

### **4.3 Effect of ATM/ATR inhibition with caffeine on mitotic checkpoint responses**

As part of the signalling cascade activated in response to genotoxic stress, the upstream kinases ATM/ATR, in particular ATR, can phosphorylate and activate Chk1 kinase (Zhao and Piwnica-Worms, 2001). In contrast, Chk2 is phosphorylated and activated mainly by ATM (Matsuoka et al., 2000). Interfering with components of this signalling pathway can result in checkpoint failure, as has been shown in the case of Chk1 previously in this study. When Chk1 function was abrogated in DT40 and BE cells both S/M and G2/M checkpoint response were severely compromised. If ATM/ATR and Chk1/Chk2 operate in the same signalling cascade, then inhibition of ATM and ATR could potentially mimic the inhibition of both Chk1 and Chk2.

The natural compound caffeine is a widely used reagent that has been shown to inhibit the catalytic activity of both ATM and ATR kinases (Sarkaria et al., 1999). Therefore, caffeine was used to investigate the role of ATM/ATR in the G2/M and S/M checkpoint responses in avian DT40 and human BE cells. The effectiveness of inhibition of ATM/ATR activity with caffeine was initially tested in the DT40 WT cells. To achieve this, cells were treated with aphidicolin which was known to induce a strong checkpoint response associated with Chk1 activation within minutes. The phosphorylation of Chk1 on S345 is not only a read-out of Chk1 kinase activity but can also serve as a read-out for ATM/ATR kinase activity as S345 of Chk1 is a known ATM/ATR phosphorylation site (Zhao and Piwnica-Worms, 2001). Thus, analysing the levels of Chk1 S345 phosphorylation by Western blotting allowed an estimation of ATM/ATR activity. As shown in Figure 4.11, when comparing the effect of caffeine in relation to the time of addition to the cells, the strongest inhibitory effect on aphidicolin-induced Chk1 phosphorylation at S345 was observed when cells had been pre-treated with caffeine. Significantly weaker inhibition was obtained if caffeine was added at the same time as aphidicolin, and none at all if it was added afterwards. Therefore, for all following experiments ATM/ATR inhibition was accomplished by pre-treating cells with caffeine for 30min.



**Figure 4.11: Effect of caffeine treatment on Chk1 phosphorylation**

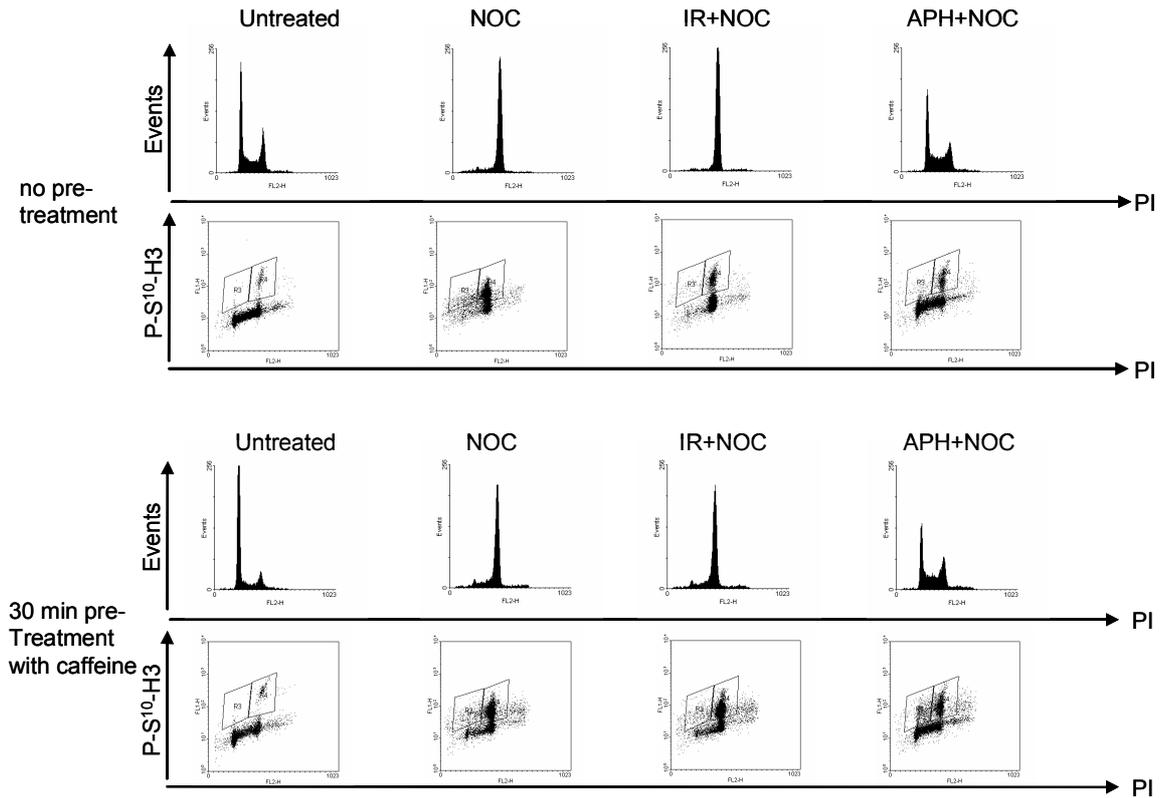
The effect of caffeine on Chk1 phosphorylation as a read-out of ATM/ATR kinase activity after DNA replication block with aphidicolin was analysed in DT40 WT cells. The cells were either untreated (1), treated with aphidicolin alone (2), with caffeine alone (3), caffeine was added 15min after aphidicolin treatment (4), caffeine and aphidicolin were added at the same time (5), or cells were pre-treated with caffeine for 15min before aphidicolin was added (6). Samples were harvested after 1.5h in the absence or presence of 5mM caffeine and analysed by SDS PAGE and Western blotting with antibodies specific for phosphorylation of Chk1 on S345 and total Chk1.

### **4.3.1 Effect of caffeine on S/M and G2/M checkpoint responses in BE cells**

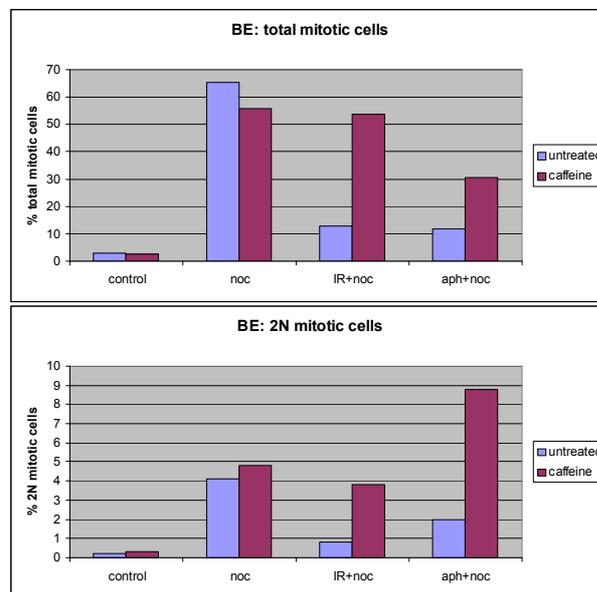
To evaluate the role of ATM/ATR in human cells, BE cells were pre-treated with caffeine for 30min and subsequently analysed for checkpoint proficiency by flow cytometry. As in previous experiments, cells were exposed to DNA damage with IR or DNA replication block with aphidicolin and cultured in the presence of nocodazole for 16h.

As shown in Figure 4.12a, caffeine did not inhibit normal cell cycle progression as seen in the nocodazole treated samples. However, in response to DNA damage with IR, caffeine severely impaired the G2/M checkpoint as cells entered mitosis without any measurable arrest. This is also illustrated in the graphical representation of the flow cytometry data in Figure 4.12b. Whereas only 10% of untreated BE cells entered mitosis after DNA damage, more than 50% of the cells with caffeine pre-treatment accumulated in mitosis after exposure to IR. In comparison, in response to DNA replication block with aphidicolin, caffeine also increased the overall number of mitotic cells and led to the appearance of cells that entered mitosis prematurely with 2N DNA content, which is a diagnostic feature of S/M checkpoint failure (Figure 4.12a). Thus, the effect of ATM/ATR inhibition on the S/M and G2/M checkpoint responses in the BE cells was comparable to the effect of Chk1 inhibition with UCN-01 or Chk1 down-regulation with siRNA.

(a)



(b)



**Figure 4.12: Effect of ATM/ATR inhibition with caffeine on mitotic checkpoint responses in BE cells**

ATM/ATR function was inhibited by pre-treating the cells for 30min with 5mM caffeine and then exposed to appropriate drug treatment. DNA replication was blocked by aphidicolin and DNA damage was induced with 10Gy of IR. Cells were grown in the presence of nocodazole and in the presence or absence of caffeine and harvested after 16h of treatment and subsequently analysed by flow cytometry for DNA content (PI profiles) and mitotic indices (P-S10-H3/PI) as shown in (a). Quantification of the percentage of mitotic cells is displayed in (b): total number of mitotic cells in the upper panel and number of 2N mitotic cells in the lower panel.

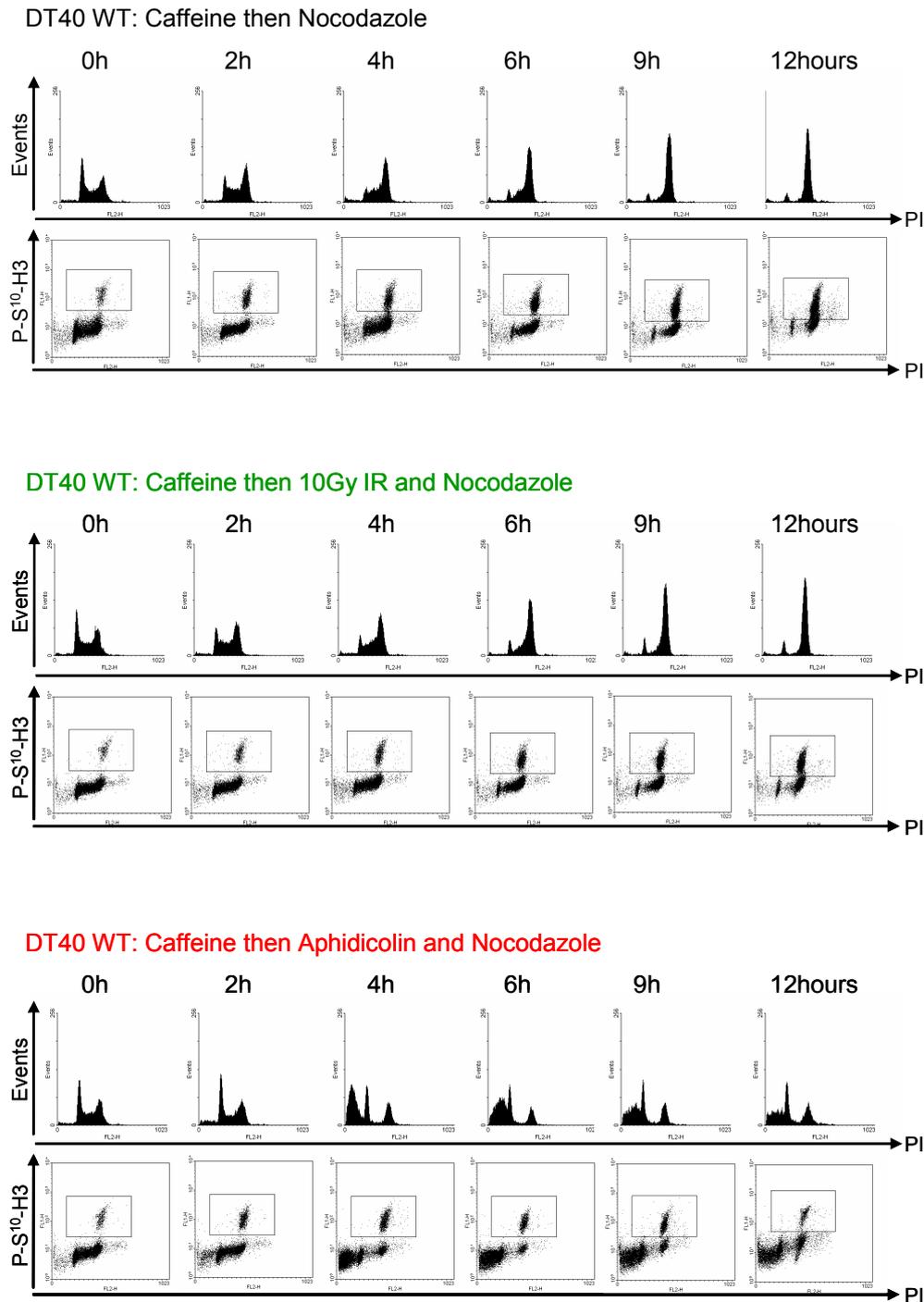
### **4.3.2 Effect of caffeine on mitotic checkpoint proficiency in DT40 WT and Chk1<sup>-/-</sup> cells**

To evaluate the role of ATM/ATR in the S/M and G2/M checkpoint responses in the avian DT40 cells, the effect of caffeine on checkpoint proficiency was first examined in genetically normal cultures of DT40 WT cells and subsequently in the Chk1<sup>-/-</sup> cells which were included for comparison.

Interesting differences in the behaviour of the DT40 WT cells in the mitotic checkpoint assays were observed when ATM/ATR activity was inhibited by a 30 min pre-treatment with caffeine prior to the start of 12h time course treatments. As shown in Figure 4.13, the majority of cells treated with caffeine alone accumulated in mitosis after 12h in nocodazole just as their untreated counterparts, indicating that caffeine did not interfere with normal cell cycle progression. In response to DNA damage, almost as many caffeine-treated cells entered mitosis as in the control cultures and only relatively few cells were observed to arrest in G2. Interestingly however, when DNA replication was blocked in the presence of caffeine, accumulation of mitotic cells was suppressed and no mitotic cells with 2N DNA content were observed. This indicated that a functional S/M checkpoint response persisted in caffeine-treated DT40 WT cells. Furthermore, these results also indicated the potential existence of a Chk1-dependent but caffeine-(ATM/ATR)-independent checkpoint in response to replication arrest in DT40 WT cells.

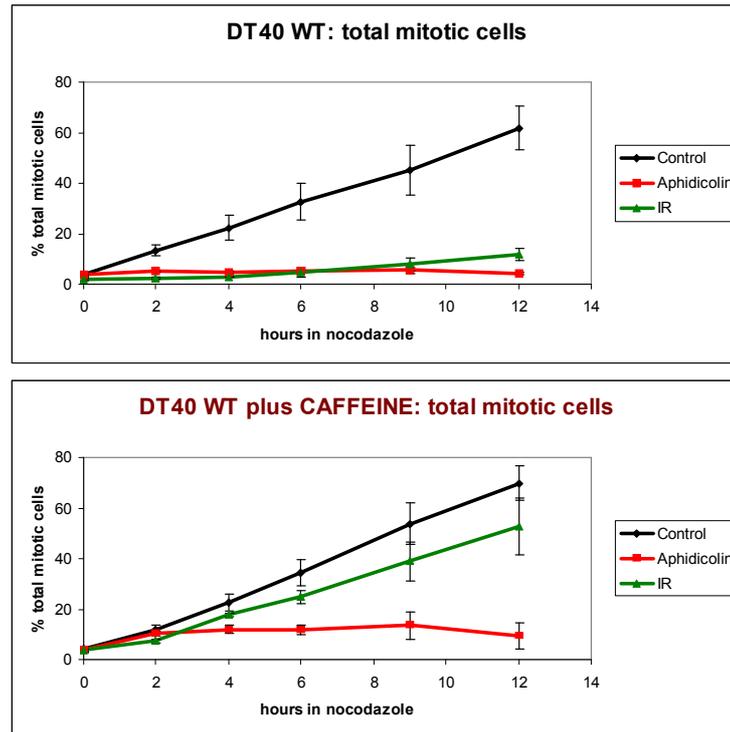
The quantification of three independent time course experiments with asynchronous DT40 WT cells is presented in Figure 4.14. For comparison, the upper panel shows the response of control DT40 WT cells to IR and aphidicolin as previously described in detail in chapter 4.1.1, and the lower panel presents the response of DT40 WT cells that had been pre-treated with caffeine (Figure 4.14). Taken together, these results show that ATM/ATR inhibition with caffeine in DT40 WT cells had little, if any, effect on the S/M checkpoint but strongly compromised the G2/M arrest.

The Chk1<sup>-/-</sup> cells were also included in the analysis to investigate any possible additive effects of dual ATM/ATR inhibition with caffeine and Chk1 loss. The flow cytometry data from a representative example for this analysis is shown in Figure 4.15. The quantification of the data from three independent experiments including the response of control Chk1<sup>-/-</sup> cells as previously described in detail in chapter 4.1.1 is illustrated in Figure 4.16.



**Figure 4.13: Effect of ATM/ATR inhibition with caffeine on mitotic checkpoint responses in DT40 WT cells**

Asynchronous cultures of DT40 WT cells were pre-treated with the ATM/ATR inhibitor caffeine for 30min and subsequently treated with either 10Gy of IR or the DNA polymerase inhibitor aphidicolin and cultured in the presence of nocodazole. Cells were grown for 12h and harvested at the indicated time points. Samples were fixed and analysed for their DNA content (PI profiles) and mitotic indices (P-S10-H3/PI) by flow cytometry.

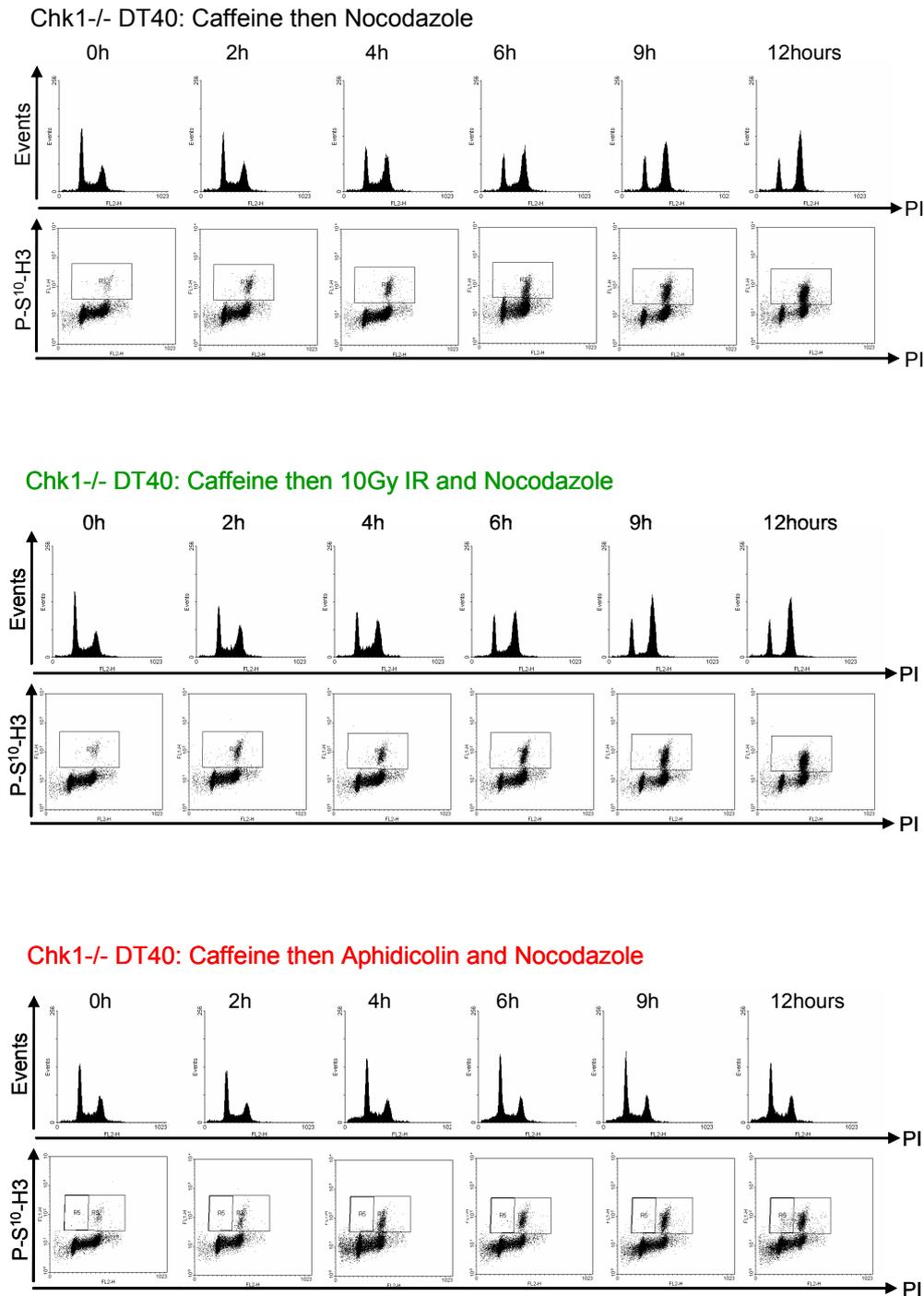


**Figure 4.14: Comparison of the effect of ATM/ATR inhibition with caffeine on mitotic checkpoint responses in DT40 WT cells**

Quantification of the flow cytometry data from the kinetic analysis of mitotic checkpoint responses in asynchronous cultures of DT40 WT cells: The graphs show control DT40 WT cells (top) versus caffeine pre-treated cells (bottom). The percentage of total mitotic cells was judged by P-S10-H3 staining and the average was calculated from three independent experiments, including the standard deviation. Untreated control cells are depicted in black, cells treated with 10Gy IR are represented in green (G2/M checkpoint response), and cells treated with aphidicolin are shown in red (S/M checkpoint response).

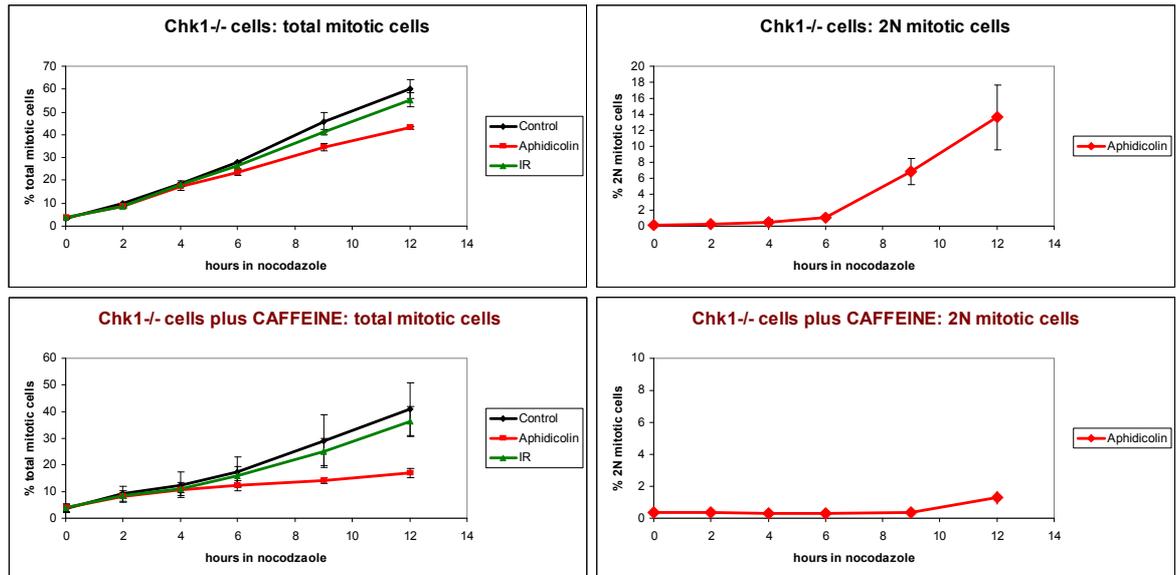
Several interesting observations were made when analysing the effect of caffeine on the Chk1<sup>-/-</sup> cells. Firstly, the overall number of cells progressing to mitosis was reduced by approximately one third compared to cells without caffeine pre-treatment (see graphs in Figure 4.16). Also, caffeine blocked some cells in G1/S as can be seen in the flow cytometry profiles in Figure 4.15. These observations indicated that ATM/ATR inhibition had a negative effect on cell cycle progression in the Chk1<sup>-/-</sup> cells. In response to DNA damage with IR, the majority of caffeine pre-treated Chk1<sup>-/-</sup> cells progressed into mitosis without measurable arrest (Figure 4.15). This result was very similar to the DNA damage response of Chk1<sup>-/-</sup> cells with functional ATM/ATR, albeit the total amount of mitotic cells was lower after caffeine pre-treatment. Another interesting observation was made when Chk1<sup>-/-</sup> cells were treated simultaneously with caffeine and the DNA polymerase inhibitor aphidicolin. In response to replication block, caffeine pre-treated Chk1<sup>-/-</sup> cells were able to delay mitosis for the duration of the experiment. The amount of mitotic cells with 2N DNA content at the 12h time point was extremely low (1%) in comparison to Chk1<sup>-/-</sup> cells with functional ATM/ATR (14%) as illustrated in the graphs in Figure 4.16 (right panels).

In summary, the effect of ATM/ATR inhibition with caffeine was found to be of a different nature for the mitotic checkpoint responses in the DT40 WT and Chk1<sup>-/-</sup> cells. The G2/M arrest was basically abrogated by caffeine pre-treatment in the DT40 WT cells. As Chk1<sup>-/-</sup> cells display G2/M arrest checkpoint failure in general, it was noted that ATM/ATR inhibition with caffeine did not have any additional effect on the extent of this checkpoint failure. However, while the S/M checkpoint response appeared to persist in caffeine-treated DT40 WT cells, caffeine-treated Chk1<sup>-/-</sup> cells surprisingly appeared to block the onset of premature mitosis in response to aphidicolin treatment, suggesting that caffeine somehow affected this checkpoint response, an observation that was further explored.



**Figure 4.15: Effect of ATM/ATR inhibition with caffeine on mitotic checkpoint responses in Chk1<sup>-/-</sup> cells**

Asynchronous cultures of Chk1<sup>-/-</sup> cells were pre-treated with the ATM/ATR inhibitor caffeine for 30min and subsequently treated with either 10Gy of IR or the DNA polymerase inhibitor aphidicolin and cultured in the presence of nocodazole. Cells were grown for 12h and harvested at the indicated time points. Samples were fixed and analysed for their DNA content (PI profiles) and mitotic indices (P-S10-H3/PI) by flow cytometry.



**Figure 4.16: Comparison of the effect of ATM/ATR inhibition with caffeine on mitotic checkpoint responses in Chk1<sup>-/-</sup> cells**

Quantification of the flow cytometry data from the kinetic analysis of mitotic checkpoint responses in asynchronous cultures of Chk1<sup>-/-</sup> cells: The graphs show control Chk1<sup>-/-</sup> cells (top) versus caffeine pre-treated cells (bottom). The percentage of total mitotic cells was judged by P-S10-H3 staining and the average was calculated from three independent experiments, including the standard deviation. Untreated control cells are depicted in black, cells treated with 10Gy IR are represented in green (G2/M checkpoint response), and cells treated with aphidicolin are shown in red (S/M checkpoint response).

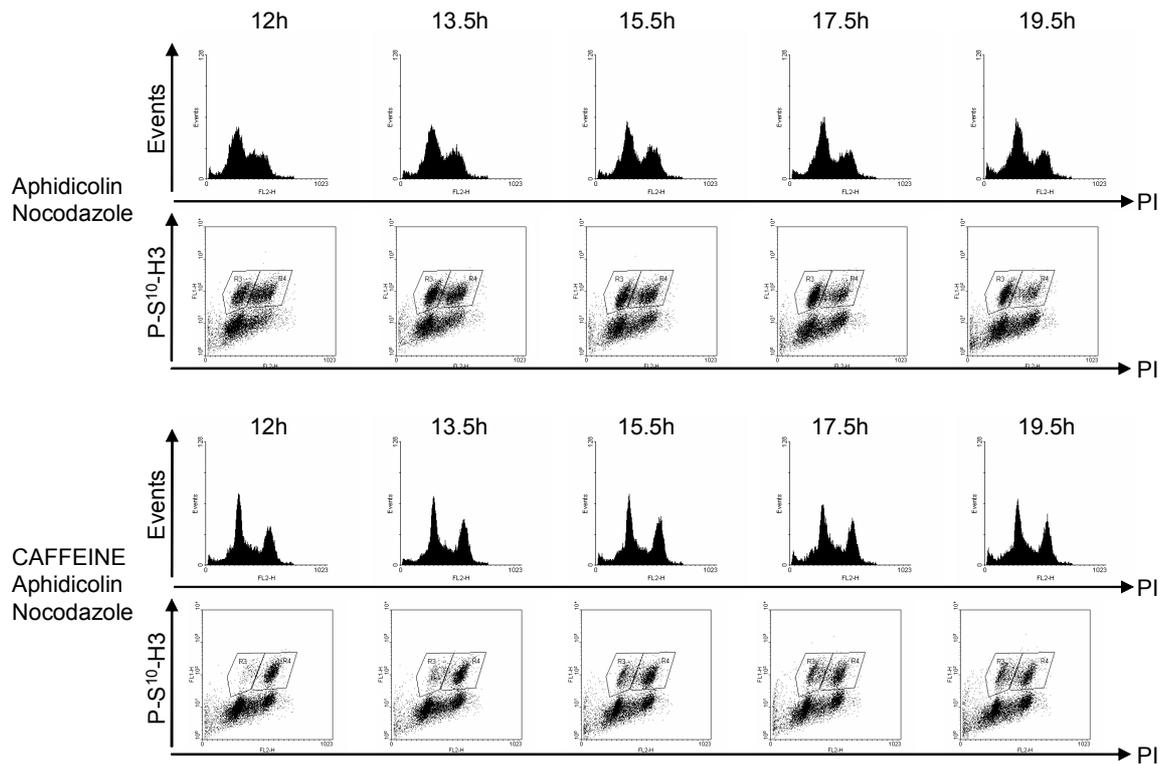
### **4.3.3 Effect of caffeine on the timing of S/M checkpoint failure in *Chk1*<sup>-/-</sup> cells**

Under the conditions used in this study, the inhibition of ATM/ATR with caffeine (including a 30min pre-treatment) did not override the S/M checkpoint in DT40 WT cells, which were still able to delay mitosis after replication block in the presence of caffeine. However, caffeine treatment changed the behaviour of *Chk1*<sup>-/-</sup> cells in response to DNA replication block. Caffeine had a negative effect on cell cycle progression specifically in the *Chk1*<sup>-/-</sup> cells. In addition, virtually no 2N mitotic *Chk1*<sup>-/-</sup> cells appeared after 12h of DNA replication block in combination with caffeine treatment. Since it seems unlikely that caffeine could restore a normal S/M checkpoint response in the absence of *Chk1*, these observations raised the question of whether it was altering the timing of the S/M checkpoint failure in the *Chk1*<sup>-/-</sup> cells.

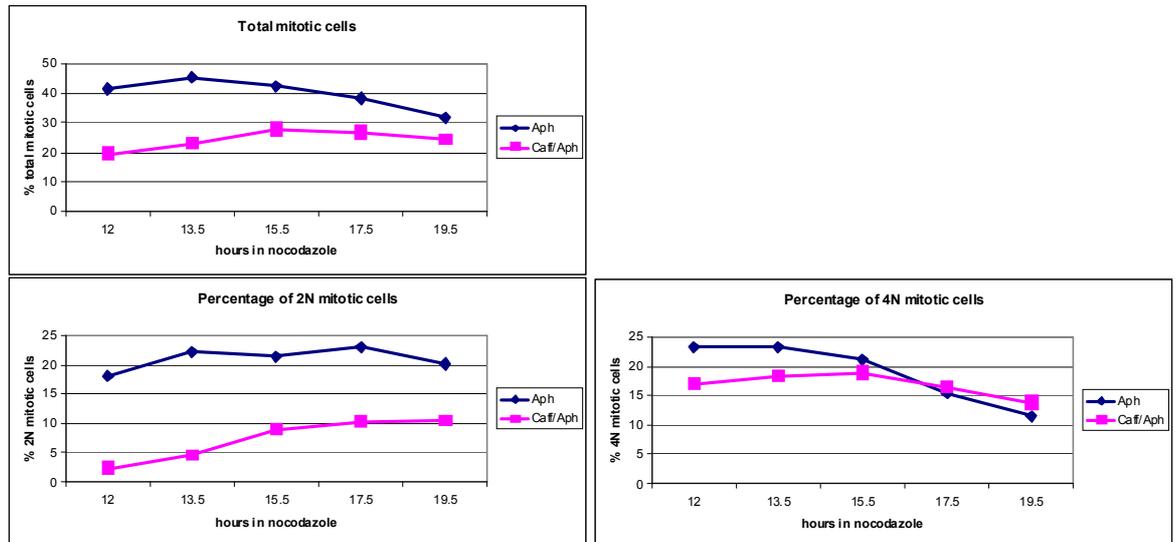
To test the possibility that ATM/ATR inhibition with caffeine could affect the timing of the S/M checkpoint failure, the time course analysis was extended for a longer time period. Figure 4.17 shows that when *Chk1*<sup>-/-</sup> cells were treated with aphidicolin alone in the presence of nocodazole, approximately 20% of the cells entered premature mitosis by 12h and this number did not increase much further until up to 19.5h. By contrast, caffeine pre-treated *Chk1*<sup>-/-</sup> cells displayed very low amounts of 2N mitotic cells after 12h of replication arrest. Interestingly however, after 14 and 16h more and more of these caffeine treated *Chk1*<sup>-/-</sup> cells entered mitosis prematurely, reaching more than 10% at the 19.5h time point as depicted in the graphical representation in Figure 4.17b.

These observations suggested that caffeine does not only have an effect on the timing of cell cycle progression in *Chk1*<sup>-/-</sup> cells in general, but also on the timing of the mitotic checkpoint in response to DNA replication arrest. Caffeine was able to delay the onset of S/M checkpoint failure in *Chk1*<sup>-/-</sup> cells for many hours. However, caffeine treatment did not have any effect on the timing of the G2/M checkpoint failure in response to DNA damage in *Chk1*<sup>-/-</sup> cells. Thus, these two mitotic checkpoint responses can be further distinguished by their sensitivity to caffeine.

(a)



(b)



**Figure 4.17: Effect of ATM/ATR inhibition with caffeine on the timing of S/M checkpoint failure in Chk1<sup>-/-</sup> cells**

Extended kinetic analysis of the effect of ATM/ATR inhibition on the onset of premature mitosis in Chk1<sup>-/-</sup> cells: Inhibition of ATM/ATR was achieved by a 30min pre-treatment with caffeine. Subsequently, cells were treated with aphidicolin and nocodazole for up to 19.5h before being analysed by flow cytometry for DNA content (PI profiles) and mitotic indices (P-S10-H3/PI) as shown in (a). Quantification of the amount of total, 4N, and 2N mitotic cells is presented in the graphs in (b). (Aph: Aphidicolin and Caff: Caffeine)

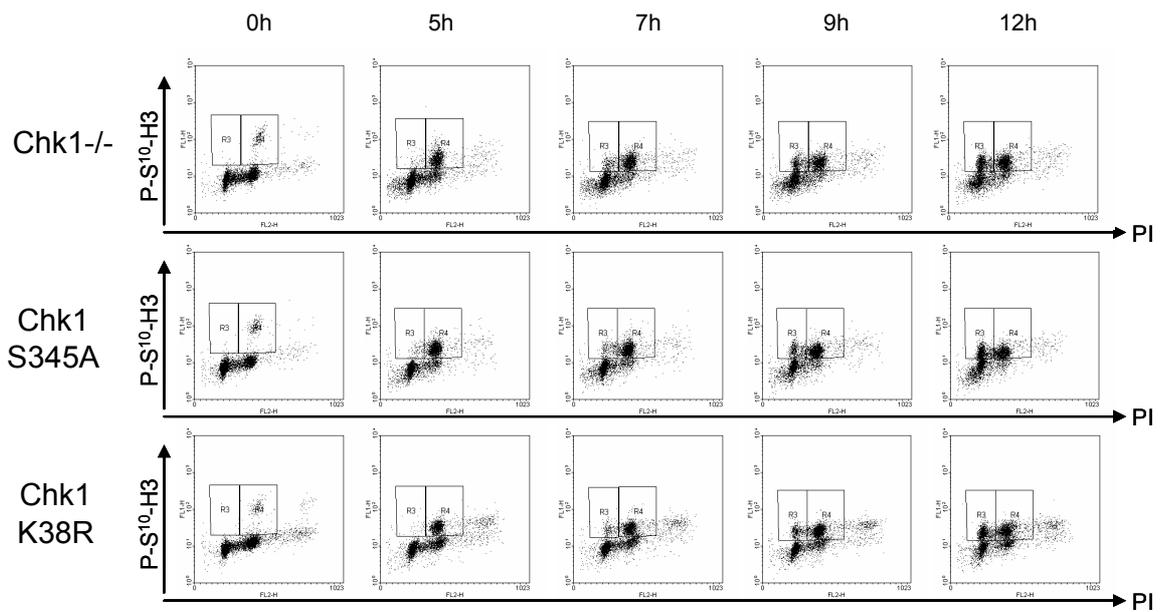
#### ***4.3.4 Timing of S/M checkpoint failure in Chk1 mutant cells is not affected by expression of catalytically inactive or S345A Chk1***

The analysis of the effect of ATM/ATR inhibition during replication block in Chk1<sup>-/-</sup> cells showed that the onset of S/M checkpoint failure was delayed in the presence of caffeine. This observation raised the question of whether mutants of Chk1 that for example can not be activated by ATM/ATR would exhibit similar changes in the timing of the onset of premature mitosis after DNA replication block with aphidicolin.

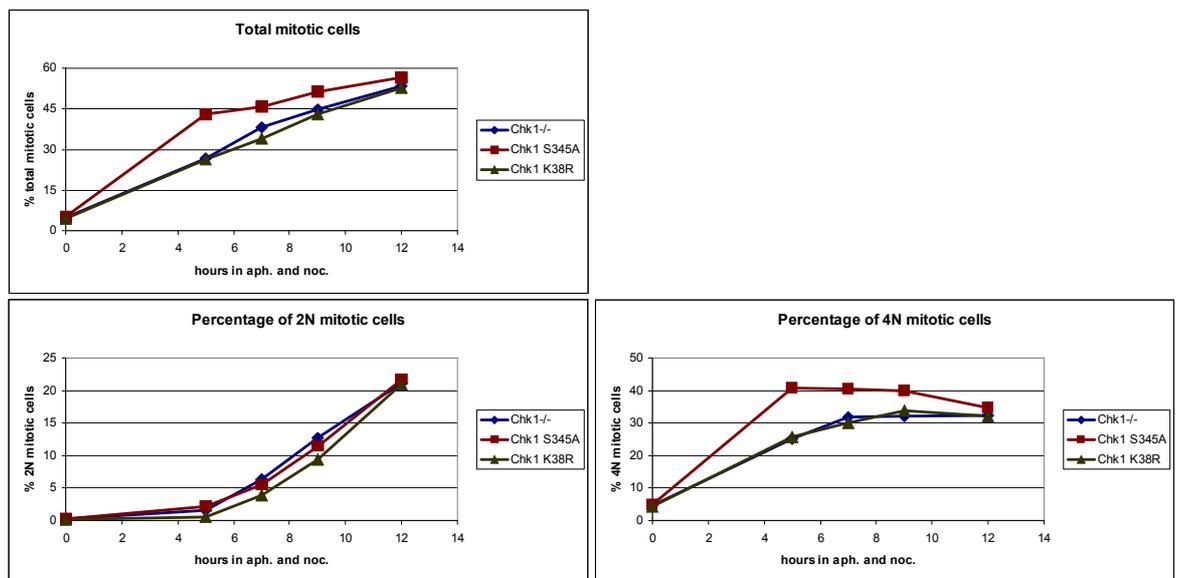
To further investigate this issue, time course experiments were performed with Chk1<sup>-/-</sup>, Chk1 S345A, and Chk1 K38R cells. The Chk1 S345A mutant can not be phosphorylated by upstream kinases ATM/ATR. The K38R mutant is a catalytically inactive version of Chk1 and was included to test any dominant-negative effects. The cells were treated with the DNA polymerase inhibitor aphidicolin and grown in the presence of nocodazole for up to 12h. As in previous experiments, the analysis of mitotic indices by flow cytometry revealed that the Chk1<sup>-/-</sup> cells initially delayed mitosis, but after 9h cells started to enter mitosis prematurely with 2N DNA content (Figure 4.18a). Quantification of the flow cytometry data showed that the rate of entry into mitosis of the 2N cells was basically the same in all three cell lines (Figure 4.18b). Although the Chk1 S345A mutant did accumulate more 4N mitotic cells by 5h compared to the Chk1<sup>-/-</sup> and Chk1 K38R cells, overall the timing of S/M checkpoint failure was very similar in all three cell lines.

Taken together, these observations indicated that cells expressing these mutants of Chk1, which are either catalytically inactive or can not be phosphorylated by upstream kinases ATM/ATR, display very similar phenotypes to Chk1<sup>-/-</sup> cells in response to replication block. The timing of S/M checkpoint failure was not affected in these cells, in contrast to the effect of ATM/ATR inhibition with caffeine which delayed the onset of premature mitosis.

(a)



(b)



**Figure 4.18: Timing of S/M checkpoint failure in Chk1 mutants**

To compare the timing of S/M checkpoint failure, Chk1<sup>-/-</sup>, Chk1 S345A, and Chk1 K38R cells were treated with aphidicolin and nocodazole for up to 12h. Samples were analysed for mitotic indices by flow cytometry. Figure (a) shows the P-S10-His H3/PI profiles and (b) illustrates the quantification of the flow cytometry data.

## 4.4 Discussion

One of the aims of this study was to characterise and compare the G2/M and S/M checkpoint mechanisms and their requirement for Chk1, which was established as the major effector kinase for mitotic checkpoint proficiency in both avian DT40 and human BE cells. Remarkably, kinetic analysis revealed that the G2/M and S/M checkpoint mechanisms can be distinguished in terms of the rate at which cells enter mitosis aberrantly in the absence of Chk1 function and they can furthermore be distinguished according to their sensitivity to the ATM/ATR inhibitor caffeine.

In response to DNA damage with IR, checkpoint proficient cells accumulate in G2 and can delay entry to mitosis in the presence of damaged DNA. Chk1 is absolutely essential for this G2/M arrest as Chk1<sup>-/-</sup> cells displayed immediate checkpoint failure with cells progressing through the cell cycle and entering mitosis without any measurable arrest in G2. The same was true for BE cells treated with the Chk1 inhibitor UCN-01 where the G2/M arrest in response to DNA damage was completely absent. These observations demonstrate a direct role for Chk1 in the G2/M arrest in vertebrate cells which is consistent with several previous studies analysing Chk1 requirement in response to DNA damage (Busby et al., 2000; Graves et al., 2000; Zachos et al., 2003).

In contrast, in response to DNA replication block with the DNA polymerase inhibitor aphidicolin, cells with a functional S/M checkpoint can delay mitosis in the presence of un-replicated DNA. This checkpoint response is also dependent on Chk1 function, but importantly, whereas Chk1 is essential for the maintenance of this mitotic delay, it is not essential for its initiation. This was initially documented in Chk1<sup>-/-</sup> cells where in response to replication block the cells behaved differently according to their position in the cell cycle at the time of arrest. Chk1<sup>-/-</sup> cells that were in G2 phase when aphidicolin was added initially entered mitosis at a rate similar to control cells until the respective G2-population had been depleted. This observation suggested that the DNA polymerase inhibitor aphidicolin can affect G2 phase cells and is able to induce a mitotic delay specifically in this cell population. This observation could suggest that the DNA polymerase might still be required after DNA synthesis has been completed, for example in post-replication DNA repair or recombination.

On the other hand, Chk1<sup>-/-</sup> cells that were in G1/S phase at the time of aphidicolin treatment behaved differently. Notably, cells with clearly un-replicated DNA (with only 2N DNA content) were only observed to enter mitosis after a significant delay. This delay

was not solely due to cell cycle position, as revealed by experiments with synchronised cells. Centrifugal elutriation was used to purify G1/S cells and then monitor the progress of these cells very carefully, obtaining information about the DNA content and progress into mitosis from a pure G1/S starting population without interference from cells in other cell cycle phases. The G1/S Chk1<sup>-/-</sup> cells were able to delay entry to mitosis for more than 9h after replication was blocked with aphidicolin. Only after this prolonged mitotic delay did cells enter mitosis abruptly with un-replicated DNA. This distinctive phenotype with prolonged mitotic delay in response to replication arrest was also observed in BE cells treated with the Chk1 inhibitor UCN-01, indicating that it is a fundamental property of vertebrate cells. Taken together, these results suggest that some other factors contribute to the timing of premature mitosis in the absence of Chk1.

The maintenance of the S/M checkpoint is dependent on Chk1, which is consistent with the fact that Chk1 is responsible for the replication recovery functions, such as suppression of origin firing and the stabilisation of stalled replication forks (Feijoo et al., 2001; Zachos et al., 2003). Thus, it has been suggested that Chk1<sup>-/-</sup> cells can only enter mitosis prematurely once all stalled replication structures have collapsed, and that the signal for mitotic delay might originate from these replication structures (Zachos et al., 2005).

ATM and ATR are known upstream kinases of Chk1 and Chk2 in the same signalling pathways that become activated in response to genotoxic stress. The ATR kinase has been shown to be able to phosphorylate and thereby activate Chk1 in response to replication blocks and certain types of genotoxic stress (Zhao and Piwnicka-Worms, 2001). Chk1 can then phosphorylate and inhibit the activity of downstream targets such as Cdc25, which in turn will lead to inhibition of Cdc2 and block of the onset of mitosis.

It is possible to make predictions from this scheme, as interfering with components of this signalling pathway can result in checkpoint failure as was shown when Chk1 function was abrogated. Accordingly, the inhibition of ATM/ATR should mimic the effects of dual Chk1 and Chk2 inhibition. Interestingly, experiments with the ATM/ATR inhibitor caffeine revealed that the mitotic checkpoint mechanisms were regulated differently in the two vertebrate systems. In the BE cells, ATM/ATR and Chk1 do indeed appear to operate in the same pathway, as inhibition of ATM/ATR resulted in the same phenotype as Chk1 inhibition with UCN-01 or Chk1 down-regulation with siRNA: in either case, cells without functional Chk1 or caffeine-inhibited ATM/ATR were unable to effectively delay mitosis in response to either DNA damage or replication block.

The situation turned out to be more complex in avian DT40 cells. Here, caffeine was able to largely override the G2/M arrest in DT40 WT cells, but it had no noticeable effect on the S/M checkpoint that appeared to be still intact in these cells. The differential requirement for ATM/ATR in the two checkpoint mechanisms in DT40 cells suggested that ATM/ATR is essential for the G2/M arrest in response to DNA damage but dispensable for the S/M checkpoint response when DNA replication is blocked in the DT40 WT cells. These differences between the avian and the human cells were not too surprising as it is known from the literature that the effects of caffeine can differ in various cell types: some checkpoint responses are dependent on ATM/ATR, others are independent. For example, while caffeine was able to override the S/M checkpoint in fission yeast and hamster cells, this was not the case in some of the human and mouse cells tested in earlier studies (Schlegel and Pardee, 1986; Steinmann et al., 1991; Wang et al., 1999). Furthermore, a study by Brown and Baltimore was able to show the existence of an ATR-independent S/M checkpoint in mouse embryo fibroblasts as ATM/ATR knock out cells were still able to block mitosis in response to replication block (Brown and Baltimore, 2003). This observation was further supported by the differential response of non-transformed and transformed mammalian cell lines indicating that most tumour cell lines might have lost the ATR-independent checkpoint response (Florensa et al., 2003).

The persistence of a functional S/M checkpoint in the caffeine pre-treated DT40 WT cells also indicated the possible existence of a Chk1-dependent but ATM/ATR-independent checkpoint response. A similar observation has previously been made in HeLa cells which are able to delay mitosis in response to DNA synthesis inhibition in a Chk1- and Claspin-dependent but Rad17- and ATM/ATR-independent checkpoint mechanism (Rodriguez-Bravo et al., 2006). The requirement for ATM/ATR for the checkpoint response in this particular study was assessed using caffeine, similar to the results presented here. It would be interesting to further confirm the existence of this Chk1-dependent but ATM/ATR-independent checkpoint mechanism by using additional approaches, such as for example studying the activation of Chk1 in ATM/ATR knock-out cells.

Interestingly, in the absence of Chk1, inhibition of ATM/ATR activity with caffeine affects the rate of cell cycle progression. After caffeine treatment the cell cycle progression of Chk1<sup>-/-</sup> cells was slowed down with only approximately two thirds of the cells entering mitosis compared to control cells at the same 12h time point. This observation suggests that ATM/ATR function is important for the normal rate of cell cycle progression in the Chk1<sup>-/-</sup> cells, although the exact details of this mechanism remain to be elucidated.

Surprisingly, under the conditions used, the inhibition of ATM/ATR with caffeine had an unexpected effect on the S/M checkpoint response in the Chk1<sup>-/-</sup> cells. The onset of premature mitosis in response to replication block with aphidicolin was delayed for many hours in the Chk1<sup>-/-</sup> cells. It is known that ATM and ATR function during the normal unperturbed cell cycle by regulating the timing of origin firing during DNA replication (Shechter et al., 2004). Also, it has been shown that Chk1<sup>-/-</sup> cells have a higher origin density in combination with reduced replication fork rates compared to their wild-type counterparts (Maya-Mendoza et al., 2007; Petermann et al., 2006). Perhaps, if ATM/ATR activity normally suppresses excessive origin firing in Chk1<sup>-/-</sup> cells, then caffeine treatment could interfere with this control mechanism and more origins would be able to fire. This could extend the time needed for complete collapse of all replication structures. However, it is unclear whether this would account for the general slowing of cell cycle progression. Another speculation could be that the reduced replication fork rate of Chk1<sup>-/-</sup> cells can somehow compensate for higher rates of origin firing in the presence of caffeine and actually slow down general cell cycle progression. This in turn could also affect the rate at which stalled replication structures eventually collapse.

Generally, caffeine is widely used to inhibit ATM/ATR activity and to study checkpoint signalling in many experimental systems. In this study, caffeine appeared to reduce the kinase activity of ATM/ATR in the DT40 cells as judged by the reduction of Chk1 phosphorylation on S345 alone (Figure 4.11). However, previously it has also been reported that under some circumstances caffeine can actually abrogate checkpoint responses while at the same time promoting increases in the phosphorylation of ATM/ATR substrates as well as increased auto-phosphorylation of ATM (Cortez, 2003). Furthermore, caffeine has been shown to inhibit many other protein kinases besides ATM/ATR, for example DNA-PK, Chk1, Akt, and mTOR (Bode and Dong, 2007). Based on these observations it is possible that some effects of caffeine on cell cycle progression might not be due to direct inhibition of the kinase activity of ATM/ATR. Therefore, additional approaches would be helpful to more satisfactorily address this issue in future experiments, such as for example the direct measurement of ATM/ATR kinase activity in the presence and absence of caffeine.

From this study it has emerged that the S/M and G2/M checkpoint responses can be distinguished by two factors, the timing of checkpoint failure in Chk1<sup>-/-</sup> cells and the sensitivity to caffeine. Also, it indicates that the initial mitotic delay in response to replication block must be due to other factors that contribute to the timing of premature mitosis. Nevertheless, both mitotic checkpoints presumably achieve inhibition of mitosis

by regulation of downstream effectors leading to the inhibition of Cdc2. Therefore, investigation of the regulation of Cdc2 in response to the two types of genotoxic stress could reveal the mechanism of mitotic delay. Moreover, the analysis of Cdc2 regulation in Chk1<sup>-/-</sup> cells could reveal how the initial Chk1-independent mitotic delay in response to DNA replication arrest is accomplished.

## **5 Results: Biochemical analysis of potential S/M and G2/M checkpoint control mechanisms**

The general idea of mitotic checkpoint control mechanisms is that they target Cdc2, the cyclin dependent kinase that initiates mitosis. Conventional models postulate that mitotic delay in higher eukaryotes is imposed through inhibitory phosphorylation of two residues on Cdc2, Threonine 14 and Tyrosine 15. The kinases Wee1 and Myt1 catalyze this phosphorylation whereas the phosphatase Cdc25 is able to reverse it (Kumagai and Dunphy, 1991; Mueller et al., 1995). Human Chk1 has been shown to be able to phosphorylate and inhibit Cdc25, by creating a binding site for 14-3-3 proteins and thereby inhibiting Cdc25 activity and de-phosphorylation of Cdc2 (Peng et al., 1997; Sanchez et al., 1997). Similarly, Chk2 was found to be able to inhibit Cdc25 and therefore Cdc2 activity and the onset of mitosis after DNA damage (Chaturvedi et al., 1999).

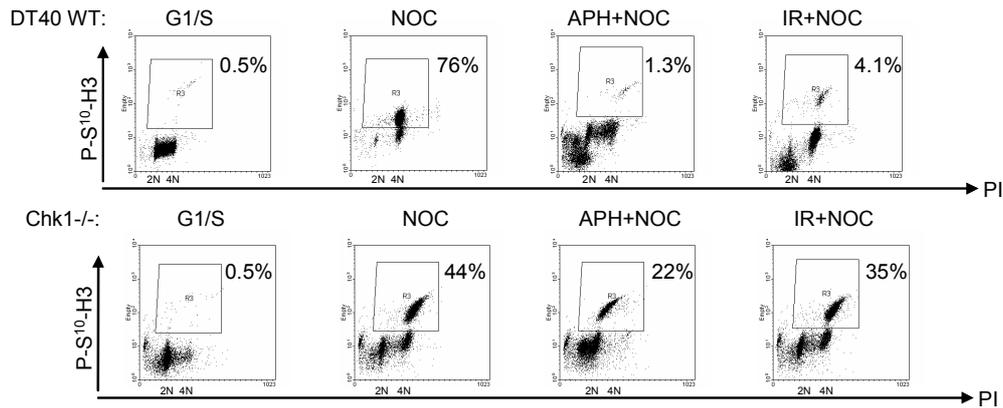
For this study, it was important to establish whether the S/M and G2/M checkpoint responses were accomplished via the conventional control mechanisms in the DT40 and BE cells. Furthermore, it was of interest to determine how cells deficient for Chk1 can execute mitotic checkpoint responses, such as the initial S/M delay after replication block.

### **5.1 Analysis of Cdc2 kinase activity during mitotic checkpoint responses in DT40 WT and Chk1<sup>-/-</sup> cells**

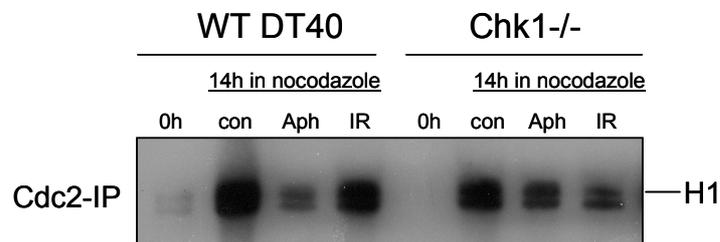
In order to investigate the effect of both DNA replication block and DNA damage on Cdc2 kinase activity during checkpoint responses, DT40 WT and Chk1<sup>-/-</sup> cells were subjected to Cdc2-Immunoprecipitation (IP) kinase assays using histone H1 as a substrate.

The assays were performed on synchronised cells which enabled optimal comparison of the changes in Cdc2 kinase activity. Purified G1/S cells were obtained from DT40 WT and Chk1<sup>-/-</sup> cells by centrifugal elutriation. Cells were subsequently returned to culture and treated either with the DNA polymerase inhibitor aphidicolin or they were exposed to DNA damage by IR and cultured in the presence of the spindle poison nocodazole for 14h. Samples were harvested and divided into two parts: one for the analysis of mitotic indices and DNA content by flow cytometry (Figure 5.1a), the other part for analysis of Cdc2 kinase activity (Figure 5.1b). Kinase assay reactions were performed with histone H1 as a substrate and Cdc2 that had been immunoprecipitated from cell lysates.

(a)



(b)



**Figure 5.1: Cdc2 kinase activity in checkpoint assays in synchronised DT40 cells**

Purified G1/S cell populations of DT40 WT and Chk1<sup>-/-</sup> cells were treated with aphidicolin (Aph) or irradiated with 10Gy (IR) and then cultured for 14h in the presence of nocodazole (Noc). (a) Samples were analysed for DNA content and mitotic indices (P-S10-H3/PI profiles) by flow cytometry as shown in. The percentage of P-S10-H3-positive cells for each sample is included in the profiles. (b) Cdc2 kinase activity was measured by immunoprecipitating Cdc2 from cells and performing kinase assays using Histone 1 (H1) as substrate. Samples were resolved by SDS PAGE and transferred to a membrane for autoradiography.

Figure 5.1b shows a representative example of the results obtained from the kinase assays, which were performed on three independent occasions with similar results.

The DT40 WT purified G1/S cells had very low Cdc2 kinase activity at the 0h time point directly after the elutriation. Control DT40 WT displayed a substantial increase in Cdc2 kinase activity after 14h in nocodazole when the majority of cells had entered mitosis (Figure 5.1b). In response to DNA replication block, cells did not progress through the cell cycle and the onset of mitosis was delayed. This was associated with a strong suppression of Cdc2 kinase activity compared to the mitotic control. However, when DT40 WT cells were exposed to DNA damage with IR, the majority of cells progressed into G2 but were still able to delay entry to mitosis. This was also associated with some degree of Cdc2 kinase activity suppression; however in this case the measured kinase activity was much higher than in the aphidicolin treated sample.

Thus, in DT40 WT cells, both DNA replication block and DNA damage lead to mitotic delay associated with suppression of Cdc2 kinase activity when compared to mitotic control cells. The actual amount of suppression of Cdc2 activity varied considerably between the two types of genotoxic stress. Whereas cells arrested in S phase displayed relatively low levels, cells arrested in G2 had relatively high levels of Cdc2 kinase activity, but mitosis was still effectively delayed in either case. The differences in kinase activity could be attributed to a number of facts. Firstly, Cdc2 kinase activity might increase throughout the cell cycle and only once a certain threshold level is reached will cells enter mitosis. Secondly, Cdc2 can associate with other cyclins such as cyclinA and this could enable a putative Cdc2/cyclinA complex to be catalytically active but not induce the onset of mitosis.

The Chk1<sup>-/-</sup> cells also showed barely detectable amounts of Cdc2 kinase activity in the G1/S starting population but exhibited very high levels after nocodazole treatment when they had mostly accumulated in mitosis (Figure 5.1b). In response to DNA replication block and DNA damage, Chk1<sup>-/-</sup> cells had entered either premature 2N mitosis or 4N mitosis after 14h, respectively. In each case, the S/M and G2/M checkpoint failure in Chk1<sup>-/-</sup> cells was associated with increased levels of Cdc2 kinase activity, although in neither case was the level as high as in nocodazole treated cells.

## **5.2 Biochemical analysis of Cdc2/CyclinB2 regulation during an unperturbed cell cycle in DT40 WT and Chk1<sup>-/-</sup> cells**

Prior to investigating any potential control mechanisms of mitotic checkpoint responses in DT40 WT and Chk1<sup>-/-</sup> cells, it was important to characterize the regulatory mechanisms affecting Cdc2 during an unperturbed cell cycle in the presence or absence of Chk1. As introduced earlier, Cdc2 activity is regulated by inhibitory and activatory phosphorylation on specific residues as well as its association with cyclins. In avian cells the major mitotic B-type cyclin that is associated with Cdc2 is cyclinB2. In order to study the regulation of Cdc2 and cyclinB2 throughout the phases of a normal cell cycle, the method of centrifugal elutriation was applied to separate an asynchronous cell population into fractions enriched in G1, S, G2, and mitotic cells. The analysis was performed in DT40 WT cells and importantly also in Chk1<sup>-/-</sup> cells to investigate whether the absence of Chk1 had any impact on the regulation of Cdc2 during a normal unperturbed cell cycle.

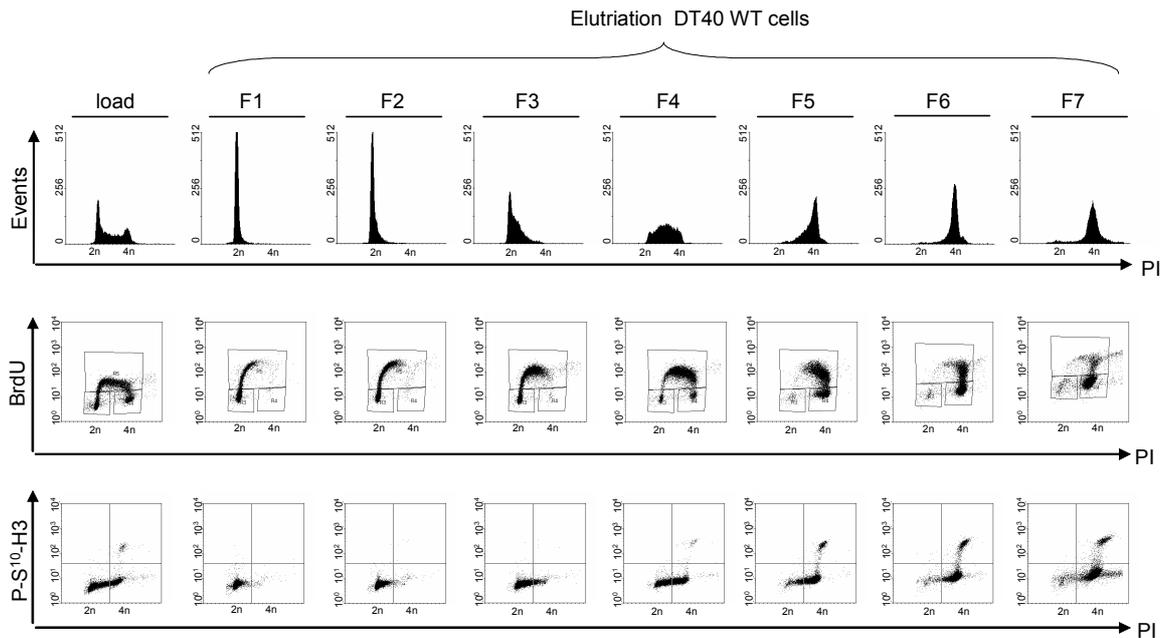
Asynchronous cultures of DT40 WT and Chk1<sup>-/-</sup> cells were separated into fractions by centrifugal elutriation (as described in methods). A typical elutriation experiment and the flow cytometry results are demonstrated using DT40 WT cells as an example. The actual speed at which each fraction was eluted is presented in Figure 5.2a. Each eluted fraction was divided into two parts and further processed for analysis by flow cytometry and Western blotting. The flow cytometry analysis was used to measure several parameters of each fraction: Samples were stained with PI for their DNA content, while staining for the mitotic marker P-S10-H3 revealed the amount of mitotic cells in each fraction. Prior to the elutriation, cells were pulse labelled for 30min with BrdU which is incorporated into newly synthesised DNA. Staining with a specific antibody for BrdU then allowed an assessment of the number of replicating cells in each sample.

As shown in Figure 5.2b (upper panel), the flow cytometry analysis demonstrated that the asynchronous culture starting population displayed a typical healthy cell cycle profile with two peaks in the PI profile. Fractions 1 to 7 that were obtained from the asynchronous load were of increasing DNA content as shown by their PI profiles, starting with only 2N cells in fraction 1 through to mainly intermediate 2-4N cells in fraction 4, and the last eluted fraction 7 contained mainly 4N cells. The number of replicating cells was analysed by studying BrdU incorporation (Figure 5.2b middle panel). The asynchronous starting population stained BrdU positive for approximately half of the cells, indicating that these

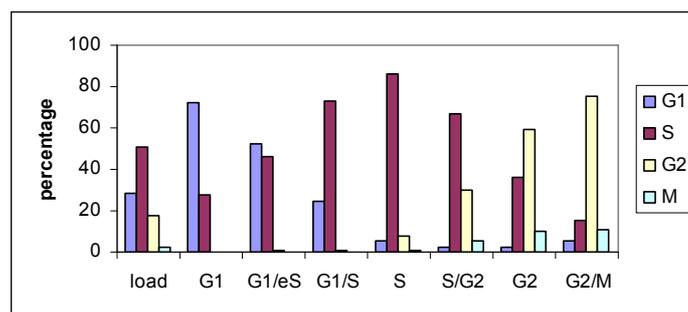
(a)

<b>Speed in rpm/rcf</b>	3800/ 2054	3600/ 1843	3400/ 1644	3200/ 1457	3000/ 1280	2800/ 1115	2600/ 962	0/0
<b>Eluted fraction</b>	Load	F1	F2	F3	F4	F5	F6	F7
<b>Cell cycle phase</b>	Asyn-chronous	G1	G1/earlyS	G1/S	S	S/G2	G2	G2/M

(b)



(c)



**Figure 5.2: Centrifugal Elutriation of DT40 WT cells**

Centrifugal elutriation was applied to separate an asynchronous cell population into fractions enriched in specific phases of the cell cycle. (a) Speed at which each fraction was eluted. (b) Data from DT40 WT elutriation showing the DNA content (PI profiles), analysis of replicating cells (BrdU/PI profiles) and mitotic cells (P-S10-H3/PI profiles) as analysed by flow cytometry. (c) Graph representing the percentage of cells in each cell cycle phase for the individual fractions.

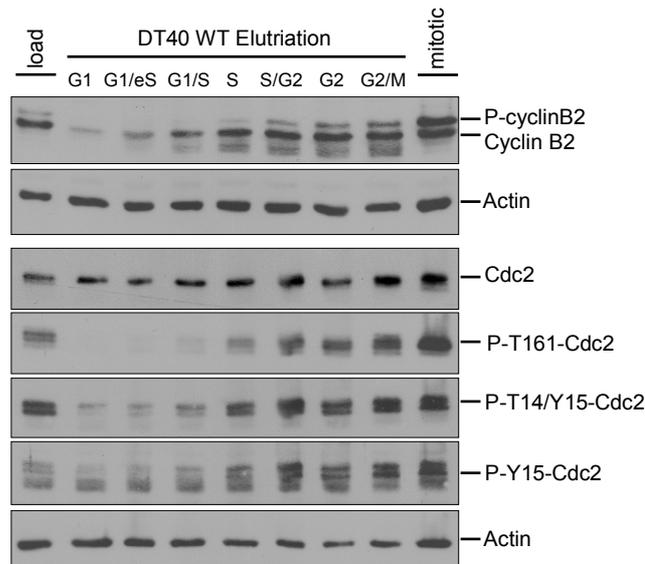
cells were in S phase. The degree of BrdU incorporation varied from fraction 1 to fraction 7, starting with low amounts of positive stained cells with 2N DNA content, reaching a maximum (86%) in fraction number 4, and in the last two eluted fractions again low levels of BrdU positive cells with 4N DNA content. Analysis of P-S10-H3 staining allowed the detection of mitotic cells in each fraction, which was found to be approximately 3-4% in the asynchronous starting population (Figure 5.2b lower panel). The first three eluted fractions 1 to 3 did not contain any mitotic cells and thereafter positive staining was detected from fraction 4 on and reached a maximum number of around 10% in fraction 7.

The percentage of cells in each cell cycle phase was calculated from the quantification of the flow cytometry data for each individual fraction (Figure 5.2c). Data obtained from the BrdU staining as well as the mitotic indices analysis was taken into account. For example, cells with 2N DNA content that were predominantly BrdU negative were judged as G1 cells, and cells with 4N DNA content that stained predominantly negative for BrdU were judged as mainly G2 cells.

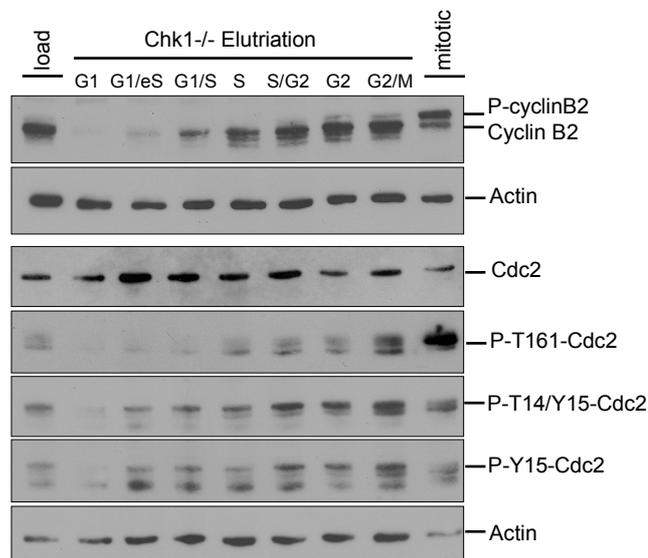
The biochemical analysis of Cdc2 and cyclinB2 in the DT40 WT and Chk1<sup>-/-</sup> cells was performed by Western blotting. For each cell type, whole cell extracts from the starting population and from the seven elutriated fractions were compared. An additional sample was also included in the analysis. As the maximum number of mitotic cells in the elutriation experiment did not exceed 10%, a sample enriched in mitotic cells was prepared by treating cells with nocodazole for 16h. These cells were at least 60-80% mitotic as judged by flow cytometry for mitotic indices (data not shown) and therefore suitable for the analysis of Cdc2/cyclinB2 regulation during mitosis.

As shown in Figure 5.3a, the Western blot analysis of the DT40 WT samples showed that the protein levels of cyclinB2 were very low in the G1 fraction, then increased progressively and reached a maximum in the G2/M fraction. In addition, in fractions 4 and 5 which contained mainly S and S/G2 cells, a slower migrating form of cyclinB2 appeared. This became more pronounced in the later G2 and G2/M fractions, with a maximum level being seen in the nocodazole treated mitotic sample. This slower migrating form has previously been identified as a phosphorylated isoform of cyclinB2 which has been shown to accumulate during mitosis and to be associated with the translocation of the cyclin to the nucleus in avian fibroblast cells (Gallant and Nigg, 1992). From both the flow cytometry data and the Western blot analysis of the elutriation experiment, it emerged that the amount of phosphorylated cyclinB2 isoform correlated closely with the percentage of mitotic cells in each sample.

(a)



(b)



**Figure 5.3: Cdc2/CyclinB2 regulation during unperturbed cell cycle in DT40 WT and Chk1<sup>-/-</sup>**

Comparison of cell cycle specific protein levels and phosphorylation status of Cdc2 and cyclinB2 in DT40 WT (a) and Chk1<sup>-/-</sup> (b) cells: Whole cell extract lysates were subjected to SDS PAGE and Western blotting analysis. Asynchronous cell cultures (Load) were separated into several fractions (G1-earlyS-S-G2-M) by centrifugal elutriation; to obtain a sample highly enriched in mitotic cells a lysate from 16h nocodazole treated DT40 WT or Chk1<sup>-/-</sup> cells respectively (mitotic) was included in the analysis. Actin served as loading control.

In contrast to cyclinB2, the total protein levels of Cdc2 were found to be the same in each sample (Figure 5.3a). However, various changes in the phosphorylation status of Cdc2 were observed. The activatory phosphorylation of Cdc2 on Threonine 161 was detectable from fraction 4 on and progressively increased in each following fraction. The highest levels of this activatory phosphorylation were observed in the nocodazole treated mitotic sample. Two different antibodies were used for the detection of inhibitory Cdc2 phosphorylation, one recognizing both Threonine 14 and Tyrosine 15 phosphorylation of Cdc2 and the other recognizing phospho-Y15-Cdc2 only. The results were almost identical with both antibodies. The levels of inhibitory phosphorylation of Cdc2 were very low in the G1 and G1/early S fractions and steadily increased until reaching maximum levels in the fraction with mainly G2 cells.

When the same analysis was performed in Chk1<sup>-/-</sup> cells, very similar results were obtained. As shown in Figure 5.3b, the total cyclinB2 protein levels were very low in G1 and then progressively increased in the subsequent fractions. The phosphorylated cyclinB2 isoform was detected from fraction 5 onwards and maximum levels were seen in the mitotic sample. The total protein levels of Cdc2 were constant throughout the cell cycle and the periodic changes in phosphorylation status were observed just as in the DT40 WT cells. The amount of activatory phosphorylation on T161 increased from fraction to fraction with highest levels in the mitotic sample, whereas the inhibitory phosphorylation on T14 and Y15 progressively increased until maximum levels were reached in G2. These observations demonstrated that the regulation of Cdc2 and cyclinB2 during an unperturbed cell cycle is not compromised by the absence of Chk1 in these avian cells.

### **5.3 Biochemical analysis of Cdc2/CyclinB2 regulation during mitotic checkpoint responses**

The regulation of Cdc2/cyclinB2 during an unperturbed cell cycle was essentially the same in DT40 WT and Chk1<sup>-/-</sup> cells. To investigate the checkpoint mechanisms responsible for mitotic delay during functional S/M and G2/M arrest, the regulation of Cdc2/cyclinB2 in the DT40 WT cells was analysed by Western blotting. In addition, the Chk1<sup>-/-</sup> cells were analysed in the same manner in order to assess the regulation of Cdc2/cyclinB2 during checkpoint failure and crucially also during the initial mitotic delay after replication block. To enable optimal analysis of the exact regulation of Cdc2 and cyclinB2, Western blotting was performed on samples obtained from the time course analyses with synchronised DT40 WT and Chk1<sup>-/-</sup> cells. By using samples from synchronised cells, the biochemical analysis

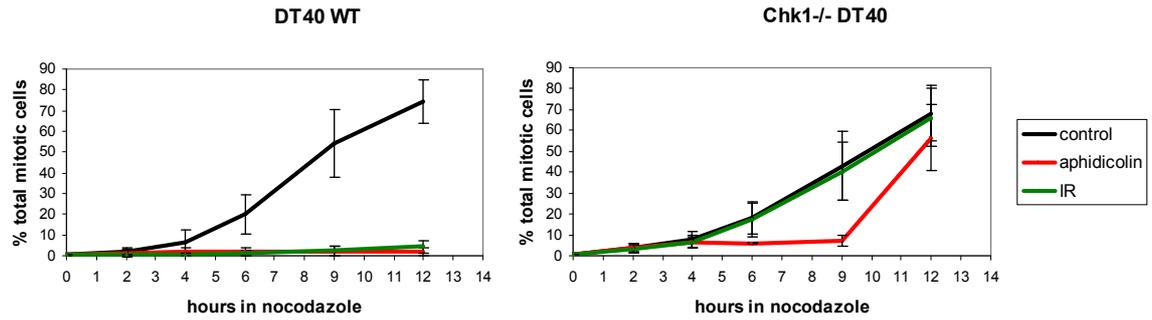
should not be compromised by the simultaneous presence of cell populations from multiple phases of the cell cycle.

Figure 5.4b shows two representative examples for the Western blot analysis of cyclinB2 regulation in the time course experiments. In DT40 WT cells, the G1/S starting population displayed very low levels of cyclinB2 which progressively increased with time. The phosphorylated cyclinB2 isoform was detectable from about 6h onwards and progressively increased and reached maximum levels at the 12h time point. As noted earlier, the amount of phosphorylated cyclinB2 isoform correlated with the percentage of mitotic cells in each sample. When the DT40 WT cells were exposed to DNA damage, they very effectively delayed entry into mitosis. For purpose of comparison, the graphs in Figure 5.4a illustrate again the results of the flow cytometry analyses of synchronised time course experiments (as previously shown in Figure 4.8). Interestingly, the mitotic delay in response to IR was associated with the suppression of cyclinB2 phosphorylation. The same observation was made when DT40 WT cells were treated with the DNA polymerase inhibitor aphidicolin. The cells were able to delay mitosis and this was associated with the suppression of cyclinB2 phosphorylation.

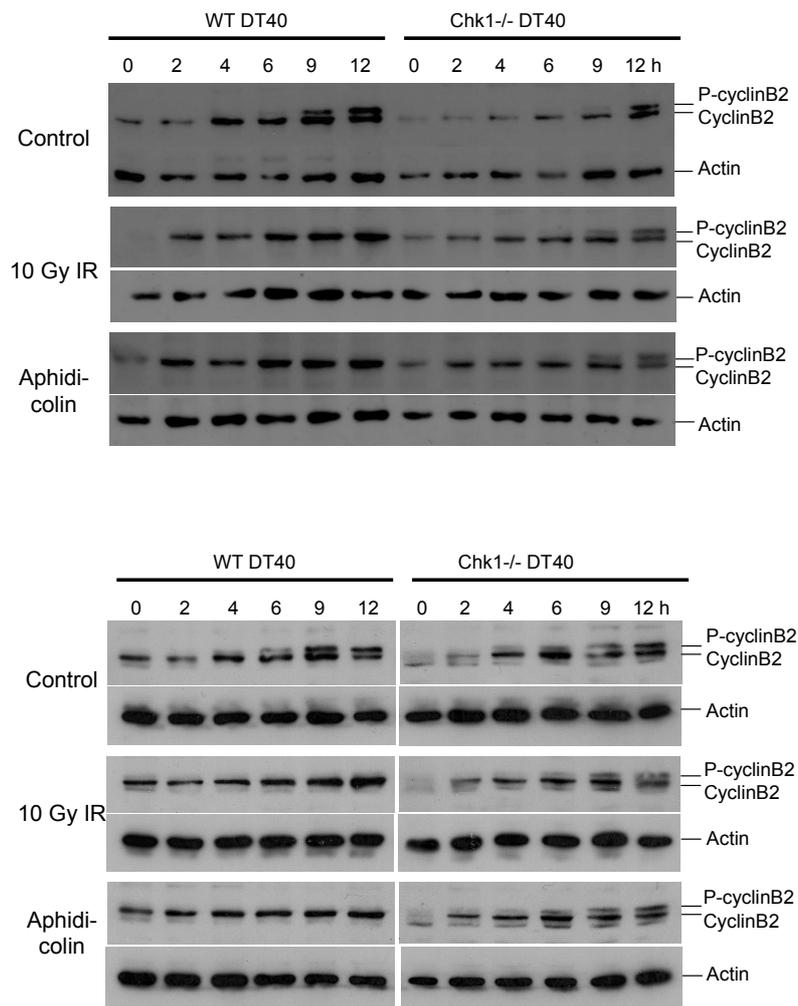
In comparison, control Chk1<sup>-/-</sup> cells also initially displayed very low levels of cyclinB2 at the 0h time point but the protein levels increased over time and the phosphorylated isoform of cyclinB2 was detectable from around 6h onwards with maximum amounts at the 12h time point (Figure 5.4b).

In marked contrast to DT40 WT cells, Chk1<sup>-/-</sup> cells failed to arrest after exposure to DNA damage and entered mitosis at a rate similar to control Chk1<sup>-/-</sup> cells as illustrated in the graph in Figure 5.4a. Strikingly, G2/M checkpoint failure was associated with a corresponding failure to suppress cyclinB2 phosphorylation. Thus, the onset and degree of cyclinB2 phosphorylation was basically identical in control cells and in cells that had been irradiated. In comparison, after replication block with aphidicolin, the Chk1<sup>-/-</sup> cells initially delayed mitosis for up to 9h prior to entering premature mitosis with unreplicated DNA. Western blot analysis established that cyclinB2 was regulated just as in the control cells, with increases in both total protein level and phosphorylated cyclinB2 isoform. Interestingly, in this case cyclinB2 phosphorylation did not strictly correlate with the percentage of mitotic cells, since the phosphorylated isoform was already detectable at 6h, when the onset of premature mitosis had not yet started.

(a)



(b)



**Figure 5.4: Analysis of CyclinB2 regulation and during mitotic checkpoint responses in DT40 WT and Chk1<sup>-/-</sup> cells**

(a) Graphs illustrating flow cytometry analysis results from the time course experiments with synchronised DT40 WT and Chk1<sup>-/-</sup> cells. (b) Whole cell extracts from the time course samples were separated on SDS PAGE gels and analysed by Western blotting with antibodies specific for CyclinB2. Actin served as loading control. Shown are two examples of the Western blot analysis.

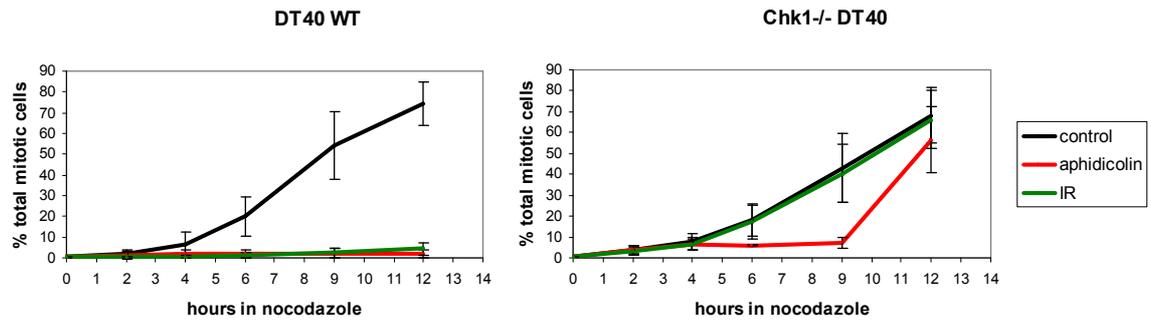
Total Cdc2 protein levels were found to be constant throughout the duration of the experiment, whereas the phosphorylation status of Cdc2 changed in response to genotoxic stress in the DT40 WT cells (Figure 5.5b). The nocodazole treated control cells displayed a progressive decrease in inhibitory T14/Y15-Cdc2 phosphorylation with lowest amounts at 12h when the majority of cells had accumulated in mitosis. In contrast, both DNA damage and DNA replication block affected the levels of inhibitory Cdc2 phosphorylation. In response to DNA damage, the phosphorylation of Cdc2 on T14/Y15 was maintained whereas the levels of inhibitory phosphorylation increased slightly over time in response to replication block. Very similar results were obtained with the anti-phospho-Y15-Cdc2 antibody (data not shown).

Total Cdc2 protein levels were also found to be constant throughout the time course experiments in Chk1<sup>-/-</sup> cells (Figure 5.5b). Furthermore, the Chk1<sup>-/-</sup> cells did not exhibit any significant changes in the amount of inhibitory phosphorylation on Cdc2 in response to either DNA replication block or DNA damage. In some experiments lower levels of P-T14/Y15-Cdc2 were observed at the 12h time point in IR or aphidicolin treated samples, however overall the inhibitory Cdc2 phosphorylation levels in the Chk1<sup>-/-</sup> cells were very similar. Taken together, the inhibitory phosphorylation of Cdc2 was not notably regulated in response to genotoxic stress in the absence of Chk1.

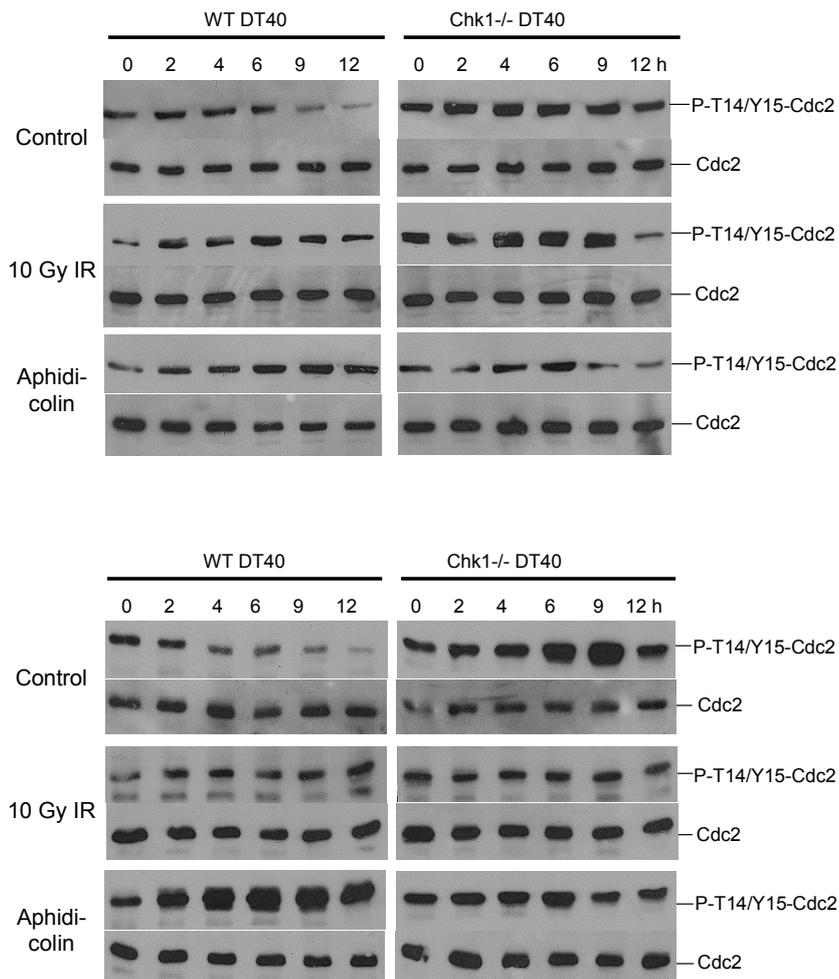
In summary, the mitotic delay in DT40 WT cells was presumably imposed through the conventional mechanism as mitotic delay in response to DNA damage and DNA replication block was associated with maintenance of inhibitory Cdc2 phosphorylation on T14 and Y15. In addition, the mitotic delay was also associated with the suppression of cyclinB2 phosphorylation. In contrast, the Chk1<sup>-/-</sup> cells did not show any significant regulatory changes to either cyclinB2 or Cdc2 phosphorylation in response to the two types of genotoxic stress, suggesting that proper functioning of these regulatory checkpoint mechanisms is dependent on Chk1. Interestingly, the accumulation of total cyclinB2 and especially the appearance of the phosphorylated isoform preceded the onset of premature mitosis when DNA replication was blocked with aphidicolin in the Chk1<sup>-/-</sup> cells.

The human BE cell line was also analysed for the regulation of Cdc2/cyclinB during checkpoint responses in the time course experiments with asynchronous cultures of BE cells that have been discussed earlier in chapter 4. The flow cytometry analysis showed that in response to either DNA damage by IR or replication block with aphidicolin, the BE cells efficiently delayed mitosis. Similar to DT40 WT cells, the mitotic delay in BE cells was associated with maintenance of inhibitory Cdc2 phosphorylation on T14 and Y15 (data not

(a)



(b)



**Figure 5.5: Analysis of Cdc2 regulation and inhibitory phosphorylation on T14/Y15 during mitotic checkpoint responses in DT40 WT and Chk1<sup>-/-</sup> cells**

(a) Graph illustrating flow cytometry analysis results from the time course experiments with synchronised DT40 WT and Chk1<sup>-/-</sup> cells. (b) Whole cell extracts from the time course samples were separated on SDS PAGE gels and analysed by Western blotting with antibodies specific for total Cdc2 levels and Cdc2 inhibitory phosphorylation on T14/Y15. Shown are two examples of the Western blot analysis.

shown). The regulation of human cyclinB1 was also analysed by Western blotting with an antibody recognizing the total protein, but the phospho-isoform of cyclinB1 was not detected and no significant regulatory changes associated with checkpoint activation could be observed (data not shown).

## **5.4 CyclinB2 phosphorylation is a target of mitotic checkpoints in DT40 cells**

The biochemical analysis of synchronised DT40 WT cells revealed that the phosphorylation of cyclinB2 was suppressed in response to both DNA replication block and DNA damage. This regulatory mechanism was completely absent in Chk1<sup>-/-</sup> cells, suggesting that the suppression of cyclinB2 phosphorylation is Chk1 dependent.

To explore this hypothesis further, a variety of DT40 cell lines were analysed in the S/M checkpoint assay. Apart from the DT40 WT and Chk1<sup>-/-</sup> cells, the Chk1 Revertant (Rev) as well as the Chk1 kinase dead (K38R) mutant cells were included in the analysis.

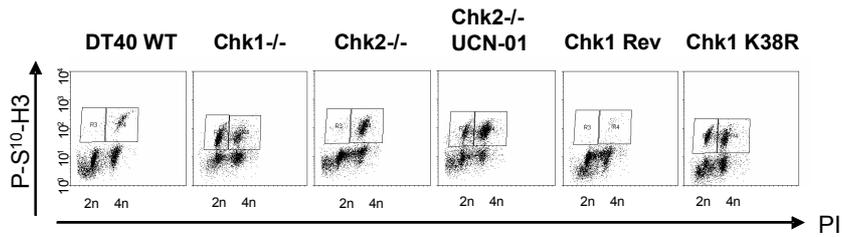
Furthermore, Chk2<sup>-/-</sup> cells in which Chk1 function was inhibited with UCN-01 were also investigated. All samples were analysed by flow cytometry and Western blotting.

As shown in the flow cytometry analysis in Figure 5.6a, the DT40 WT, Chk2<sup>-/-</sup>, and Chk1 Revertant cell lines were able to delay mitosis effectively when replication was blocked, whereas all cell lines without functional Chk1 (Chk1<sup>-/-</sup>, Chk1 K38R, Chk2<sup>-/-</sup> plus UCN-01) were unable to delay entry to mitosis. The results from the Western blot analysis showed that the mitotic delay observed in cells with functional Chk1, was in each case associated with the suppression of cyclinB2 phosphorylation (Figure 5.6b). In marked contrast, Western blotting analysis of samples from Chk1<sup>-/-</sup>, Chk1 K38R Kinase Dead mutant, and Chk2<sup>-/-</sup> cells treated with the Chk1 inhibitor UCN-01 revealed no differences in the total cyclinB2 levels or the phosphorylation status of cyclinB2 in these cells.

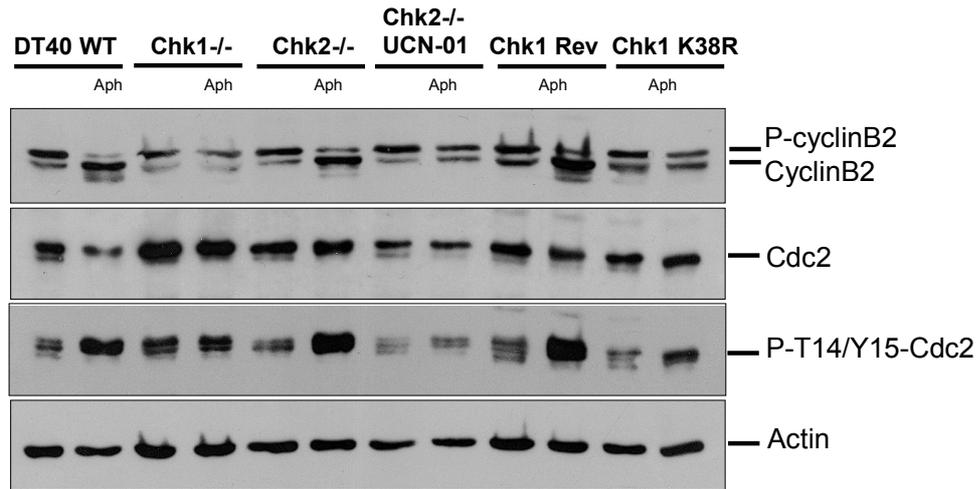
The total protein levels of Cdc2 were more or less the same in each sample, whereas a functional S/M checkpoint response was associated with high levels of inhibitory T14/Y15 phosphorylation of Cdc2. Actin served as a loading control.

In conclusion, mitotic delay in response to DNA replication block was associated with the suppression of cyclinB2 phosphorylation and this was dependent on functional Chk1. Thus, the data suggested that the phosphorylation of cyclinB2 is a target of mitotic checkpoints.

(a)



(b)



**Figure 5.6: CyclinB2 phosphorylation is a checkpoint target in cells with functional Chk1**

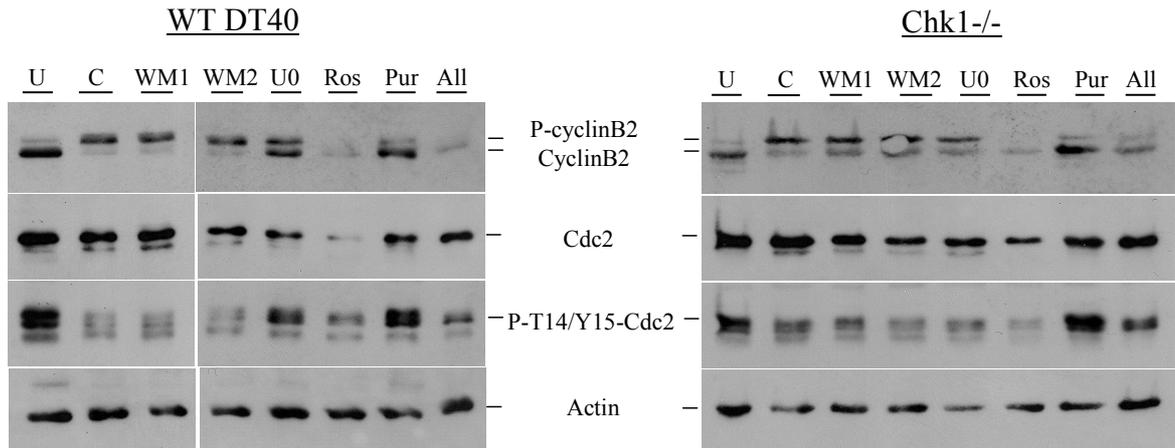
To investigate cyclinB2 modification in response to DNA replication block, cells were treated with the DNA polymerase inhibitor aphidicolin and cultured in the presence of nocodazole for 16h. Samples were analysed by flow cytometry for DNA content and mitotic indices (P-S10-H3/PI profiles) as shown in (a). Whole cell extracts were prepared and analysed by SDS PAGE and Western blotting with antibodies specific for cyclinB2, Cdc2, P-T14/Y15-Cdc2, and Actin served as loading control (b).

## 5.5 CyclinB2 phosphorylation is a target of Cdks in the DT40 cells

Avian cyclinB2 isoform accomplishes basically the same functions as human cyclinB1; however its regulation is not exactly the same. Both cyclins are able to bind to Cdc2 and the active state of this complex can bring about the onset of mitosis. CyclinB1 has been shown to be phosphorylated by Plk1 and this was associated with its nuclear translocation (Toyoshima-Morimoto et al., 2001). Studies on the starfish oocyte model system have shown that Cdc2 is the cyclinB-kinase and there has also been some speculation about a possible role for MAP kinase in the regulation of Cdc2/cyclinB1 (Borgne et al., 1999).

In order to clarify the issue of which kinase might be responsible for mitotic phosphorylation of cyclinB2 in DT40 cells, an inhibitor approach was taken. The phosphoinositide 3-kinase inhibitor wortmannin was used at concentrations that were shown to potently inhibit Plk1 activity (Liu et al., 2005). The MEK inhibitor U0126 was used to inhibit MAP kinase activity and in order to inhibit Cdc2 a panel of three different Cdk inhibitors was used: allsterpaullone, purvalanol A, and roscovitine. Although none is completely specific, all three inhibitors were used at concentrations which should be selective for Cdc2. DT40 WT and Chk1<sup>-/-</sup> cells were treated with the respective inhibitors and grown for 16h in the presence of the spindle poison nocodazole. Whole cell extracts were analysed by SDS PAGE and Western blotting with specific antibodies.

As shown in Figure 5.7, untreated DT40 WT and Chk1<sup>-/-</sup> cells displayed very low levels of the phosphorylated cyclinB2 isoform, whereas in cells that had been treated with nocodazole high levels of the phosphorylated isoform of cyclinB2 were detected. When the cells were treated with two different concentrations of wortmannin which should inhibit Plk1 activity, the phosphorylation of cyclinB2 was not affected compared to nocodazole treated control cells. However, whether these concentrations of wortmannin indeed potently inhibit Plk1 activity was not tested in this particular experiment. This issue could be clarified for example by performing a Plk1 kinase assay. The phosphorylation of cyclinB2 was slightly affected by MAP kinase inhibition with U0126 in the DT40 WT but not in the Chk1<sup>-/-</sup> cells. However, inhibition of Cdc2 with any of the three Cdk inhibitors abrogated cyclinB2 phosphorylation in the DT40 WT as well as the Chk1<sup>-/-</sup> cells. It was also noted that the cdk inhibitors, especially roscovitine, had a negative effect on total cyclinB2 protein levels in both cell types. The inhibition of cyclinB2 phosphorylation was also associated with inhibition of mitosis as was detected by flow cytometry (data not shown).



**Figure 5.7: Analysis of cyclinB2 phosphorylation in DT40 WT and Chk1<sup>-/-</sup> cells treated with various protein kinase inhibitors**

DT40 WT and Chk1<sup>-/-</sup> cells were treated with a panel of kinase inhibitors (C: DMSO control, WM1/WM2: 100mM/500mM of wortmannin, U0: 50mM of U0126, Ros: 100mM of roscovitine, Pur: 15mM of purvalanol A, All: 100mM of allsterpaullone) and grown in the presence of nocodazole for 16h (apart from U: untreated control). Whole cells extracts were analysed by SDS PAGE and Western blotting with antibodies specific for cyclinB2, Cdc2, and P-T14/Y15-Cdc2. Actin was used as a loading control.

The Western blot analysis was also used to study the regulation of Cdc2 in these samples. In general, total Cdc2 protein levels were similar in most samples, apart from the samples treated with roscovitine which displayed lower levels of Cdc2, indicating that roscovitine might have a negative effect on transcription. This property of roscovitine has also been described in a study in human cells (Ljungman and Paulsen, 2001). As for the phosphorylation status of Cdc2, suppression of cyclinB2 phosphorylation was associated with higher levels of inhibitory T14/Y15 phosphorylation in the samples treated with purvalanol A and allsterpaullone. The inhibitory phosphorylation was also to some extent increased in the U0126 treated DT40 WT cells, indicating that MAP kinase might be involved in the regulation of Cdc2/cyclinB2 in the DT40 WT cells, however this issue has so far not been addressed any further.

In summary, these observations suggest that the phosphorylation of cyclinB2 in avian cells is executed by Cdks. From the present data there is a strong implication for Cdc2 as the kinase responsible for cyclinB2 phosphorylation in these cells; however other cdks can not be excluded at this stage.

## 5.6 Discussion

Cell cycle arrest in response to checkpoint activation is generally believed to result from inhibition of cyclin dependent kinases. Cdc2 is the major Cdk responsible for triggering entry into mitosis and therefore the main target of the G2/M and S/M checkpoint responses. One aim of this study was to evaluate the mitotic checkpoint control mechanisms associated with mitotic delay in response to genotoxic stress in the DT40 WT and BE cells. Another focus was to determine how cells deficient for Chk1 can execute mitotic checkpoint responses, such as the initial S/M delay after replication block.

Avian DT40 cells are evidently able to regulate Cdc2 and cyclinB2 normally during an unperturbed cell cycle even in the absence of functional Chk1. Both in the presence and absence of Chk1, cyclinB2 protein levels accumulated progressively throughout the cell cycle and a phosphorylated isoform was observed which correlated with the proportion of mitotic cells as determined by flow cytometry. Furthermore, typical fluctuations in activatory and inhibitory Cdc2 phosphorylation status were observed in both cell types. These results demonstrated that normal Cdc2 regulation is not affected by loss of Chk1 in these cells, which was somewhat surprising as Chk1 has previously been implicated in the regulation of cell cycle progression in the absence of genotoxic stress, for example by

phosphorylation and negative regulation of Cdc25B on the centrosome (Kramer et al., 2004; Schmitt et al., 2006).

The mitotic delay in response to DNA damage or replication block in DT40 WT and BE cells was associated with Cdc2 inhibition via maintenance of inhibitory phosphorylation on T14 and Y15, which is broadly consistent with the proposed conventional checkpoint model. In contrast, the phosphorylation of Cdc2 did not appear to be significantly affected in response to DNA damage or replication block in Chk1<sup>-/-</sup> cells. These observations suggested that Chk1 is involved in the regulation of Cdc2 in response to genotoxic stress. This is in line with the fact that Chk1 is known to be able to phosphorylate Cdc25 in response to stress, thereby creating a binding site for 14-3-3 proteins, which will then export Cdc25 out of the nucleus and thus prevent de-phosphorylation of Cdc2 (Peng et al., 1997; Sanchez et al., 1997). In addition, it has been shown that Chk1 can phosphorylate and activate Wee1 kinase in response to DNA damage, thereby inducing inhibitory phosphorylation and inactivation of Cdc2 (O'Connell et al., 1997).

Apart from the conventional checkpoint regulation of Cdc2 by activatory and inhibitory phosphorylation, another potential checkpoint target was identified in the DT40 cells. The mitotic delay in response to either DNA damage or replication block was associated with the suppression of cyclinB2 phosphorylation. This regulatory mechanism was further shown to be dependent on functional Chk1 as cells with compromised Chk1 function (Chk1<sup>-/-</sup>, Chk1 K38R, and also Chk2<sup>-/-</sup> cells with Chk1 inhibitor UCN-01) were unable to suppress cyclinB2 phosphorylation in response to genotoxic stress.

Avian cyclinB2 has been shown to undergo rapid translocation to the nucleus at the onset of prophase and this process might be associated with the phosphorylated cyclinB2 isoform (Gallant and Nigg, 1992). This idea is also supported by the approximate correlation of the amount of phosphorylated isoform with the percentage of mitotic DT40 WT cells.

Interestingly, this does not account for the fact that cyclinB2 is able to undergo phosphorylation and nuclear translocation in the Chk1<sup>-/-</sup> cells even during the initial prolonged mitotic delay in response to replication block. The onset of premature mitosis in the Chk1<sup>-/-</sup> cells is dependent on Cdk activity as it could be blocked by inhibition of Cdc2 (Cdk) with either of three different Cdk inhibitors at concentrations selective for Cdc2 (data not shown). This indicates that the presence of the mitotic phosphorylated cyclinB2 isoform is necessary but not sufficient to induce mitosis and that some other factors are able to restrain Cdc2 activity. This notion could also explain why DT40 WT cells displayed relatively high levels of Cdc2 kinase activity while mitosis was effectively delayed in

response to DNA damage (Figure 5.1). Thus, apart from the conventional mitotic delay mechanism via maintenance of inhibitory Cdc2 phosphorylation, other factors can restrain Cdc2 activity and an obvious candidate involved in this regulation is the replication machinery itself.

It is known that Chk1 plays an important role for the suppression of origin firing and for the stabilisation of stalled replication forks and is therefore responsible for the maintenance of viable replication structures in response to DNA replication stress (Zachos et al., 2003; Zachos et al., 2005). However, the initial mitotic delay in response to DNA replication block in Chk1<sup>-/-</sup> cells was independent of Chk1 function, further emphasizing that some other intrinsic mechanism is responsible for mitotic delay in the presence of unreplicated DNA even in the absence of Chk1. This idea is further supported by the observation that DT40 WT cells displayed a functional S/M checkpoint response in the presence of the ATM/ATR inhibitor caffeine which severely compromised Chk1 activation, while their ability to delay mitosis in response to DNA damage was abrogated in the presence of caffeine (Figure 4.13).

Thus, in response to replication arrest, cells appear to be able to initially delay mitosis independently of ATM/ATR and Chk1 function. The replication machinery itself emerges as the most likely candidate for the origin of the checkpoint signal. Investigation of the properties of the stalled replication machinery could reveal the origin of the checkpoint signal for mitotic delay. Eventual checkpoint failure in Chk1<sup>-/-</sup> cells suggests that in the long term, Chk1 function does become important for maintaining the mitotic delay, probably due to its function in stabilising the stalled replication structures (Zachos et al., 2005). Maybe preventing the stalled replication structures from collapsing could hence extend the mitotic delay in the absence of Chk1, a hypothesis that remains to be tested.

To investigate the mechanism of mitotic delay in response to replication arrest in Chk1<sup>-/-</sup> cells, further characterisation of the relationship between the timing of DNA replication and the onset of normal as well as premature mitosis is required.

## 6 Results: DNA replication and checkpoint control

The experiments in the previous chapters have established that the initiation of the S/M checkpoint is independent of Chk1. When DNA replication is blocked with aphidicolin, Chk1<sup>-/-</sup> cells can delay the onset of mitosis for many hours even in the presence of potentially active Cdc2/cyclinB2. Furthermore, from observations both in this study as well as earlier studies it has been suggested that there is an intrinsic checkpoint mechanism that ensures that cells block entry to mitosis either while normal DNA replication is ongoing but also when the replication machinery is stalled (Rao and Johnson, 1970; Zachos et al., 2005). To further investigate this hypothesis it was important to document the relationship between the process of DNA replication and the onset of normal as well as premature mitosis.

### 6.1 Documentation of the relationship between DNA replication and entry to mitosis

Proliferating cell nuclear antigen (PCNA) is a clamp protein associated with DNA polymerase  $\delta$  during DNA synthesis, thus it is found at replication foci during S-phase and can therefore be used as a marker for DNA replication (Celis and Celis, 1985). Previously it has been suggested that loss of PCNA staining precedes the onset of premature mitosis in Chk1<sup>-/-</sup> cells (Zachos et al., 2005). Although this previous study described the staining pattern of PCNA and P-S10-H3 in cell populations, it did not document the exact timing of PCNA loss and onset of premature mitosis in individual cells.

#### 6.1.1 Analysis of the timing of PCNA loss and the onset of mitosis

One approach to document the timing of PCNA loss and the onset of normal or premature mitosis was by flow cytometry analysis. DT40 WT and Chk1<sup>-/-</sup> cells were treated with the DNA polymerase inhibitor aphidicolin and grown in the presence of nocodazole for 16h. Cells were stained for the DNA replication marker PCNA with a FITC-labelled secondary antibody and for the mitotic marker P-S10-H3 with a PE-labelled secondary antibody and subsequently analysed by flow cytometry. The anticipated objective was to follow the progress of PCNA positive cells as they presumably first become PCNA negative and then eventually P-S10-H3 positive when they enter mitosis prematurely.

As shown in Figure 6.1, untreated cultures of DT40 WT and Chk1<sup>-/-</sup> cells displayed predominantly positive PCNA staining and approximately 5% mitotic cells that stained for P-S10-H3. The appropriate negative and positive controls were included in the analysis and verified that the staining was indeed specific (data not shown). In the presence of nocodazole, the number of mitotic cells rose to about 50% in the DT40 WT and Chk1<sup>-/-</sup> cells as judged by staining for P-S10-H3, whereas the PCNA staining appeared over a somewhat broader intensity range compared to untreated controls (Figure 6.1).

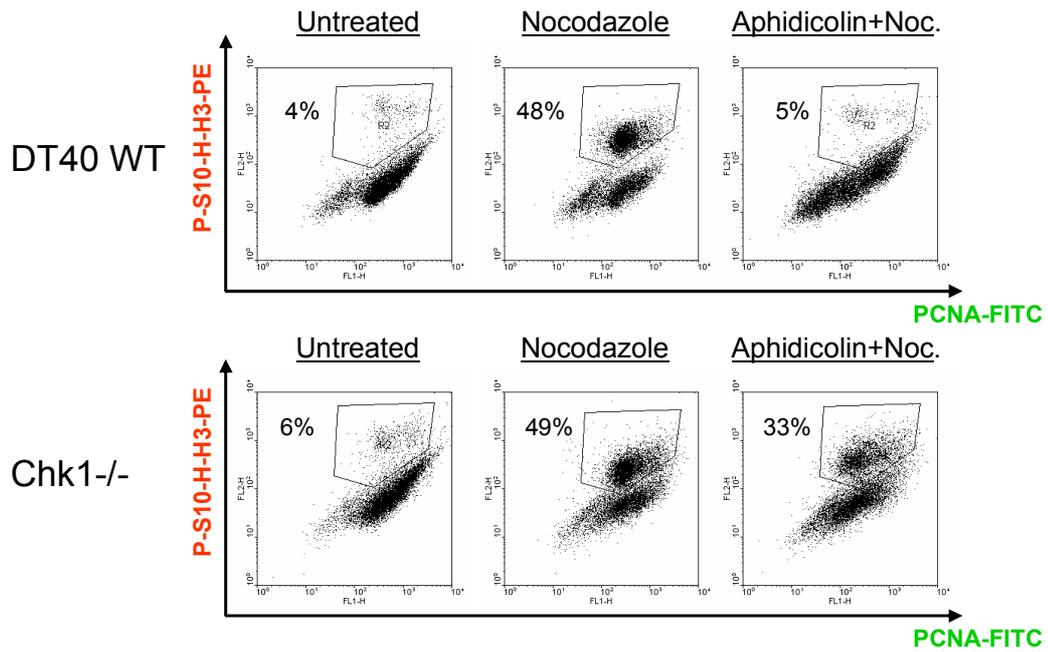
In comparison, while only about 5% of DT40 WT cells entered mitosis in the presence of aphidicolin, a high percentage (33%) of Chk1<sup>-/-</sup> cells stained positive for P-S10-H3 (Figure 6.1). The levels of intensity for PCNA seemed to vary slightly, however overall it was not possible to detect any significant changes in the PCNA staining in response to the different treatments. Moreover, the majority of nocodazole-treated DT40 WT and Chk1<sup>-/-</sup> cells stained positive for both PCNA and P-S10-H3. The same was true for Chk1<sup>-/-</sup> cells that had entered mitosis in the presence of aphidicolin and nocodazole.

This was somewhat unexpected as a previous study by Zachos et al. had shown that PCNA and P-S10-H3 staining was mutually exclusive and suggested that loss of PCNA staining preceded the onset of premature mitosis in the Chk1<sup>-/-</sup> cells (Zachos et al., 2005). However, the flow cytometry results presented here in this study indicated that DT40 WT and Chk1<sup>-/-</sup> cells in fact enter both normal and premature mitosis without loss of PCNA staining.

### **6.1.2 PCNA distribution during an unperturbed cell cycle**

The flow cytometry analysis of DT40 WT and Chk1<sup>-/-</sup> cells suggested that both natural and premature mitotic cells retained PCNA staining. To further investigate this observation, the expression patterns of PCNA were first analysed during an unperturbed cell cycle.

To achieve this, the protein levels of PCNA were analysed during the phases of the cell cycle and mitosis in whole cell extracts obtained by centrifugal elutriation of DT40 WT and Chk1<sup>-/-</sup> cells (as described in chapter 5.2). Interestingly, the total protein levels of PCNA were found to be constant in all fractions as shown in the Western blot analysis in Figure 6.2. This observation suggested that while PCNA protein levels remain the same throughout the cell cycle they might possibly undergo changes in sub-cellular localisation. To investigate changes in the sub-cellular localisation of PCNA, DT40 WT cells were



**Figure 6.1: Flow cytometry analysis of PCNA expression in DT40 WT and Chk1<sup>-/-</sup> cells**

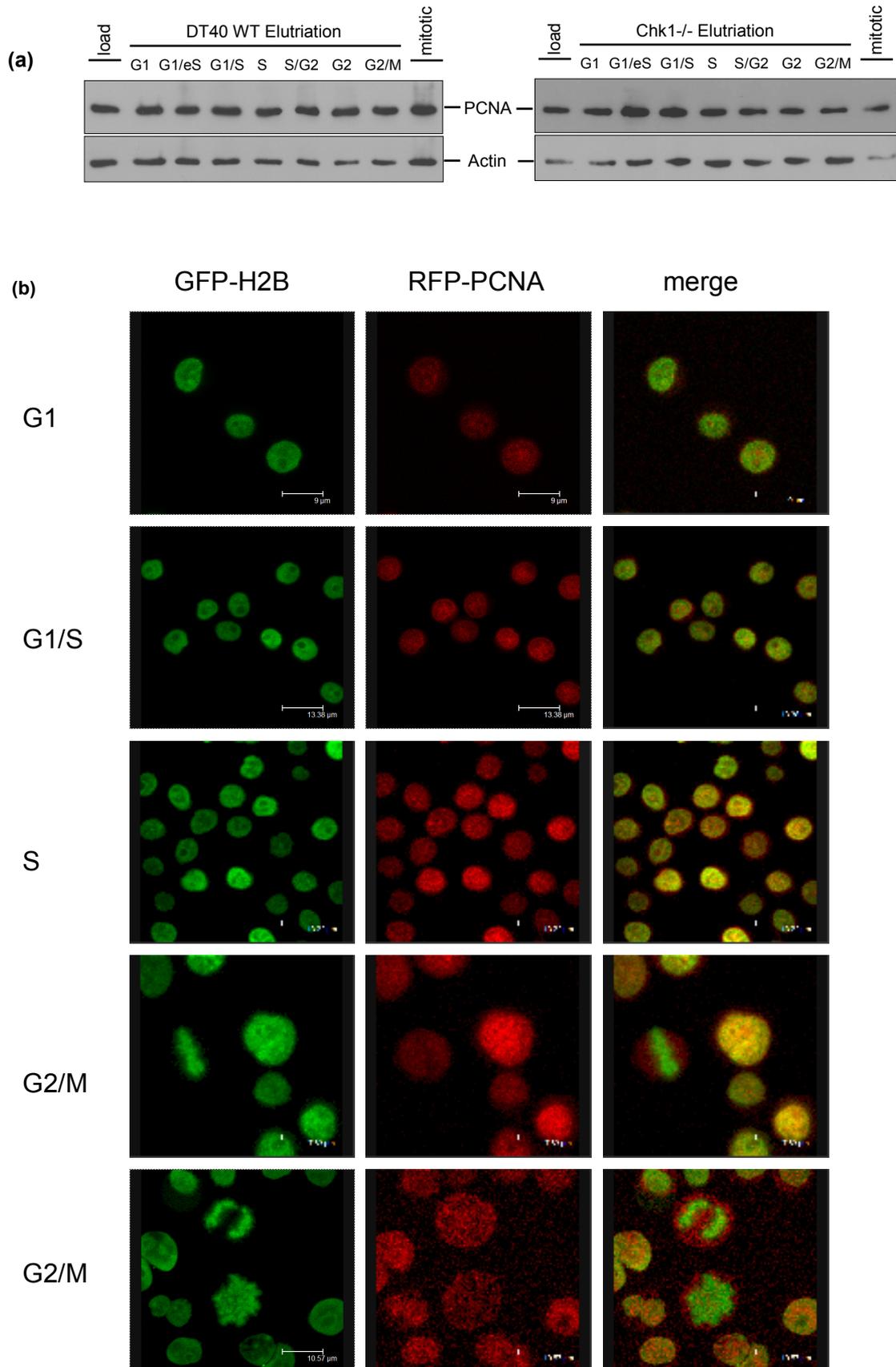
DT40 WT and Chk1<sup>-/-</sup> cells were treated with/without the DNA polymerase inhibitor aphidicolin and grown in the presence of nocodazole (Noc.) for 16h. Samples were stained with antibodies specific for PCNA and P-S10-H3 and analysed by flow cytometry (P-S10-H3/PCNA profiles).

established that stably expressed PCNA as a marker for DNA replication. RFP-tagged PCNA was introduced into a DT40 WT cell line expressing GFP-tagged Histone H2B which can be used as a marker to visualize chromatin condensation.

The RFP-PCNA/GFP-H2B/DT40 WT cells were separated into several fractions by centrifugal elutriation, a method that has been introduced previously in chapter 5. The cells were then analysed by confocal microscopy to study the expression pattern and localisation of RFP-PCNA and GFP-H2B throughout the phases of a normal unperturbed cell cycle. As shown in the first and second picture row in Figure 6.2b, G1 and G1/S phase cells displayed relatively low levels of PCNA expression in the nucleus. The third picture row in Figure 6.2b shows a typical example for cells in S phase with strong nuclear staining of PCNA with typical speckled structures in the nucleus, indicative of ongoing DNA synthesis. Whereas cells in G2 phase still contained mainly nuclear PCNA, cells undergoing mitosis displayed a dramatic change in PCNA localisation, as it appeared dispersed into the cytoplasm while the chromatin was condensed (Figure 6.2b). Overall, PCNA was found diffusely dispersed in the cytosol of all mitotic cells observed. These observations confirmed that while total PCNA protein levels remain constant, the sub-cellular localisation of the protein changes throughout the cell cycle.

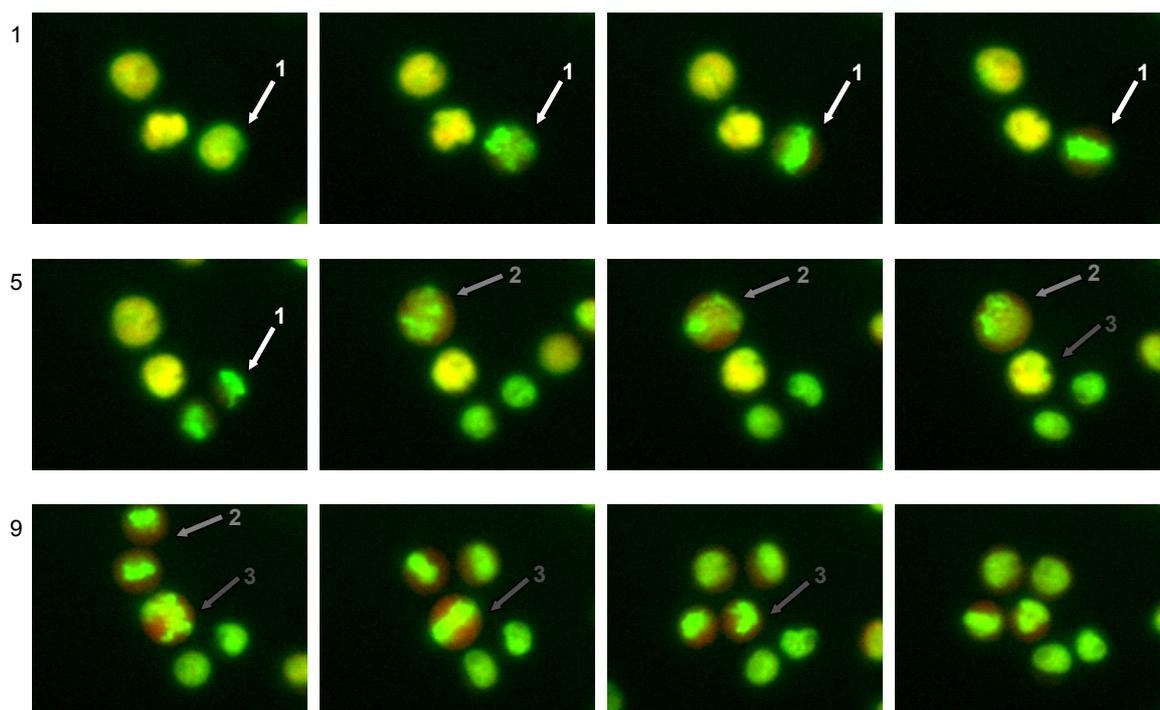
To study these regulatory patterns of PCNA in real time, GFP-H2B/RFP-PCNA DT40 WT cells were analysed by time-lapse microscopy. Overall the results confirmed the observations made in fixed cells with PCNA appearing diffusely distributed in the cytosol in G1 and accumulating in the nucleus during S phase forming bright speckles in places of DNA synthesis (data not shown). Importantly, the time-lapse movies made it possible to document the changes in sub-cellular localisation of PCNA in individual cells as they entered mitosis.

Although the resolution was not very good, the pictures in Figure 6.3 show three individual cells as they entered mitosis and each divided into two daughter cells. Firstly, the chromatin in cell no.1 started to condense as shown from picture 1 onwards. Then, PCNA started to appear in the cytosol from picture 2 on and is clearly visible in the cytoplasm of the metaphase cell in picture 4. The two daughter cells then presumably entered G1 and a new S phase. Cells no.2 and no.3 also underwent mitosis in this time frame. They displayed the same change in sub-cellular localisation of PCNA which dispersed into the cytosol at the same time as the chromatin condensed. This analysis showed that normal scheduled mitosis in the DT40 WT cells is associated with chromatin dissociation and cytosolic dispersion of PCNA.



**Figure 6.2: Expression and localisation of PCNA during an unperturbed cell cycle**

(a) Asynchronous DT40 WT and Chk1<sup>-/-</sup> cell cultures (Load) were separated into several fractions (G1-earlyS-S-G2-M) by centrifugal elutriation and samples analysed by SDS PAGE and Western blotting (including load and mitotic control) with antibodies specific for PCNA and Actin (upper panel). (b) GFP-H2B/RFP-PCNA DT40 WT cells were separated into several fractions (G1-S-G2-M) by centrifugal elutriation and analysed by confocal microscopy. Shown are five representative examples for the pattern of PCNA during G1, S, G2 phase, and in mitosis (lower panels).



**Figure 6.3: Time-lapse analysis of PCNA distribution during an unperturbed cell cycle**

Time-lapse microscopy of GFP-H2B RFP-PCNA DT40 WT cells: Shown is a selection of pictures (from 1 to 12) taken every 10 frames/20min corresponding to a total time frame of 3h 40min. Three cells (no.1, 2 and 3) are highlighted in selective pictures as they successively undergo mitosis.

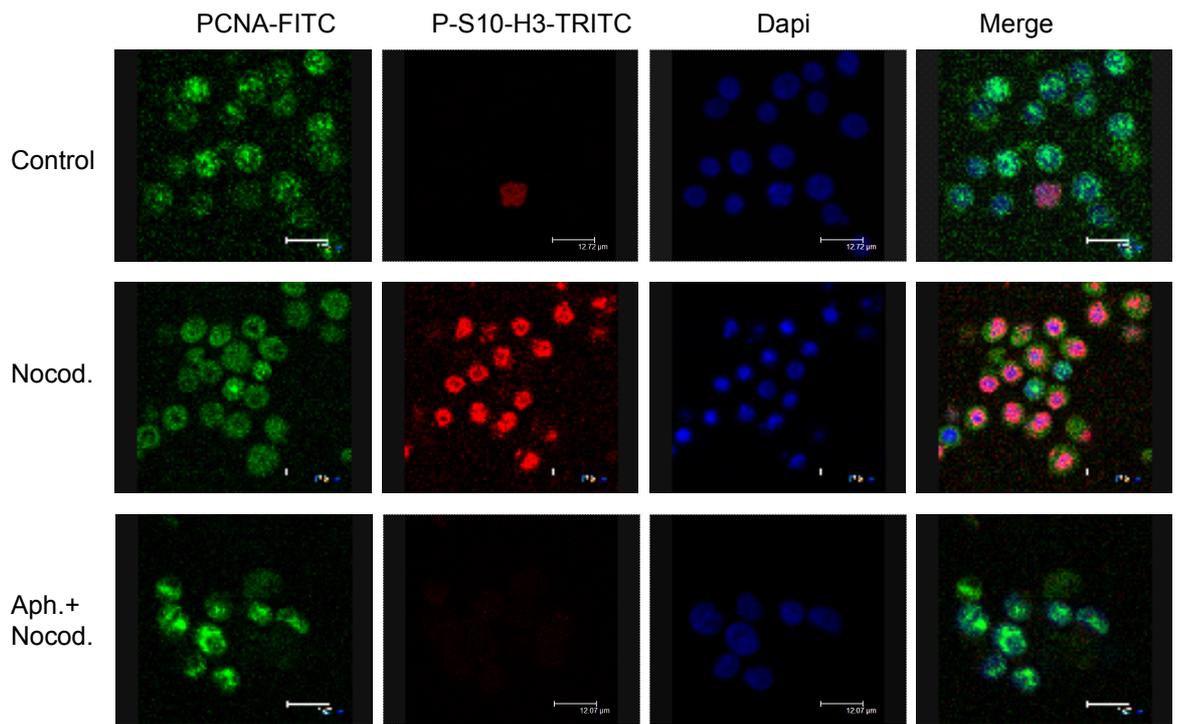
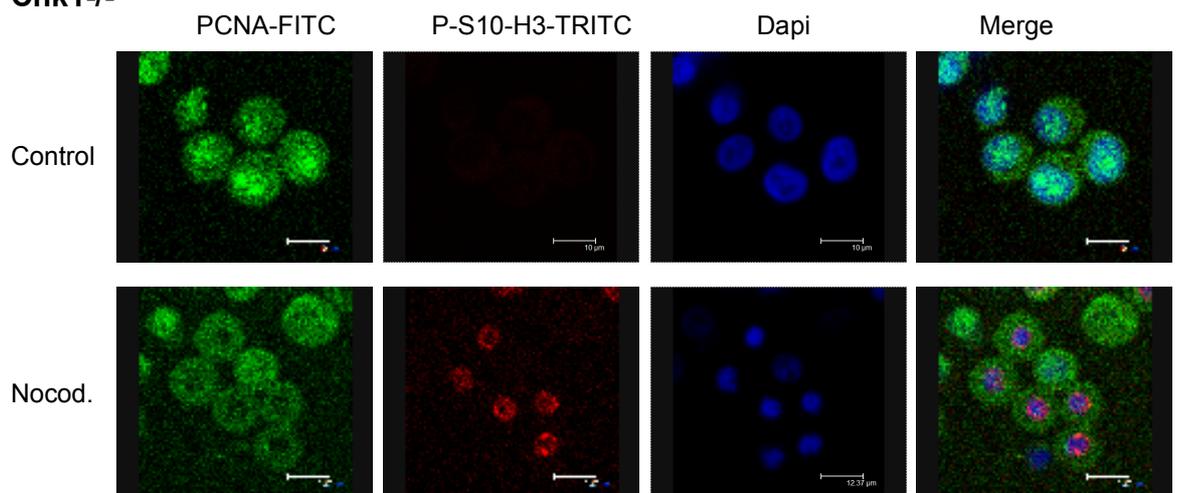
### **6.1.3 PCNA distribution during normal and premature mitosis**

To study the timing of changes in PCNA sub-cellular distribution and the onset of normal or premature mitosis, the response of cells to DNA replication block was analysed over a time course. DT40 WT and Chk1<sup>-/-</sup> cells were treated with aphidicolin and grown for up to 12h in nocodazole. Samples were taken at several time points and analysed by confocal microscopy. Cells were stained for PCNA with a FITC-labelled secondary antibody (green) and for P-S10-H3 with a TRITC-labelled secondary antibody (red). Subsequently, cover slips were mounted on Vectashield mounting medium containing DAPI to enable staining of the DNA of the cells (blue).

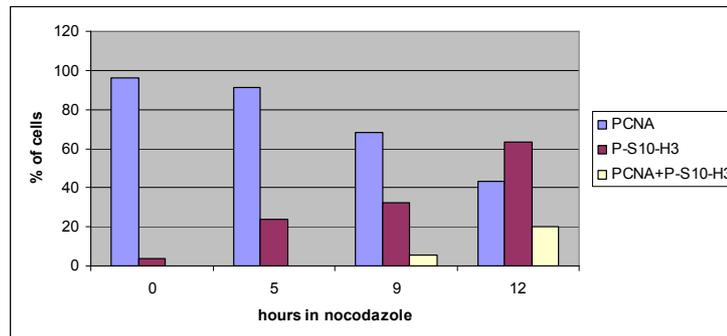
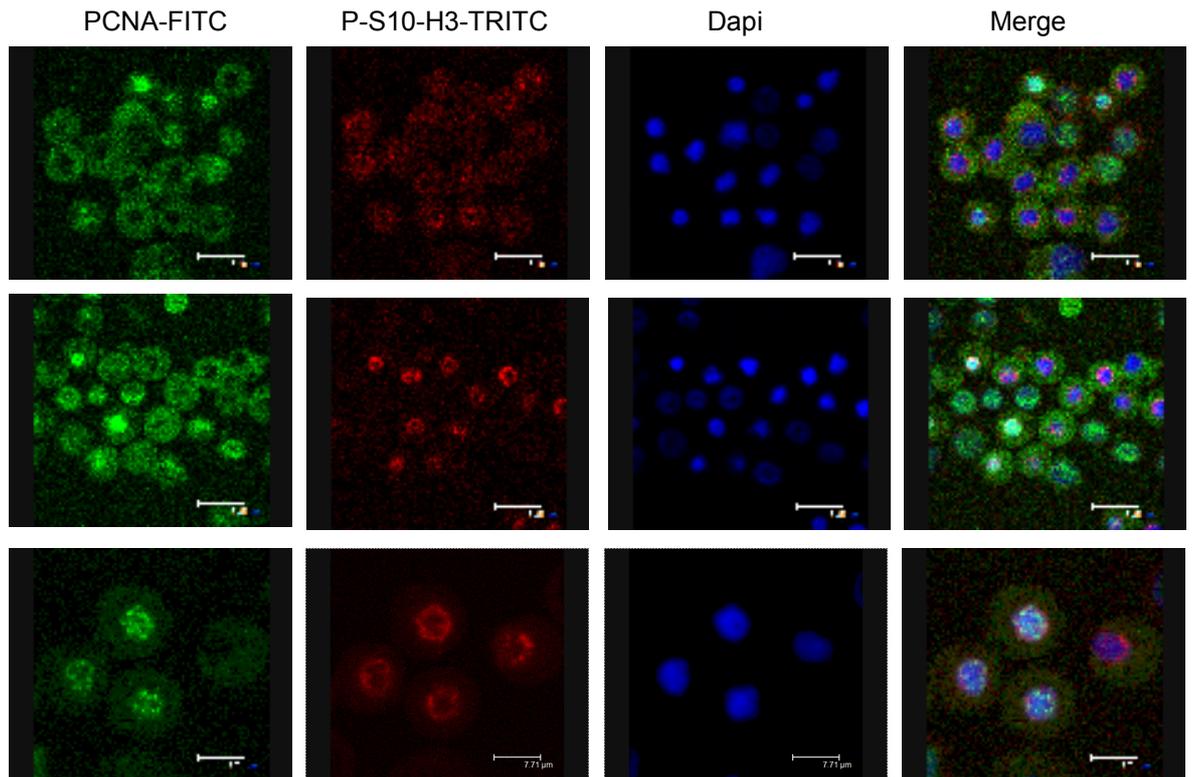
As shown in Figure 6.4, untreated DT40 WT cells stained mainly positive for PCNA in the nucleus, consistent with the fact that the majority of cells in an unperturbed cell population are either in S phase or just before or after DNA replication with PCNA located mainly in the nucleus. When DT40 WT cells were grown in the presence of nocodazole for 12h, they accumulated in mitosis as demonstrated by the high percentage of cells staining positive for P-S10-H3. These mitotic cells showed PCNA staining excluded from the nucleus. By contrast, when DNA replication was blocked with aphidicolin in the presence of nocodazole, DT40 WT cells retained nuclear PCNA staining and the vast majority of cells did not enter mitosis as judged by P-S10-H3 staining. Taken together, checkpoint proficient DT40 WT cells were able to delay entry to mitosis in response to replication block and this was associated with maintenance of nuclear PCNA staining in arrested cells, suggesting that these cells retained the capability to synthesise DNA.

In comparison, untreated Chk1<sup>-/-</sup> cells showed very similar staining as untreated DT40 WT cells with the majority of cells exhibiting strong mainly nuclear PCNA staining (Figure 6.4). When Chk1<sup>-/-</sup> cells were grown in the presence of nocodazole, they accumulated in mitosis and by 12h the vast majority of cells were P-S10-H3-positive and PCNA was excluded from the nucleus. Thus, in both DT40 WT and Chk1<sup>-/-</sup> cells the onset of normal 4N mitosis was associated with cytosolic dispersed PCNA and on no occasion was double staining of PCNA and P-S10-H3 on the chromatin observed.

In marked contrast, when Chk1<sup>-/-</sup> cells were treated with the DNA polymerase inhibitor aphidicolin and grown in the presence of nocodazole for 12h, most of the cells stained positive for P-S10-H3 as shown in Figure 6.5. Interestingly, approximately a third of these mitotic cells exhibited strong co-staining of both P-S10-H3 and PCNA on the condensed

**DT40 WT****Chk1<sup>-/-</sup>****Figure 6.4: Analysis of PCNA distribution in DT40 WT and Chk1<sup>-/-</sup> cells**

DT40 WT and Chk1<sup>-/-</sup> cells were treated with/without aphidicolin and nocodazole and grown for up to 12h on poly-L-lysine coated cover slips. The cover slips were stained with antibodies specific for PCNA (FITC) and P-S10-H3 (TRITC) and with Dapi and analysed by confocal microscopy. The displayed pictures represent the 12h time point for each respective treatment.

**Chk1<sup>-/-</sup> cells**

**Figure 6.5: Analysis of PCNA distribution during S/M checkpoint failure in Chk1<sup>-/-</sup> cells**

Chk1<sup>-/-</sup> cells were grown for up to 12h on poly-L-lysine coated cover slips. Cells were treated with aphidicolin and nocodazole. Cover slips were stained with antibodies specific for PCNA (FITC) and P-S10-H3 (TRITC) and with Dapi and analysed by confocal microscopy. The three displayed pictures represent examples for the typical staining pattern of aphidicolin and nocodazole treated Chk1<sup>-/-</sup> cells at the 12h time point.

The graph illustrates quantification of PCNA and P-S10-H3 staining throughout the time course experiment with Chk1<sup>-/-</sup> cells treated with aphidicolin and nocodazole.

chromatin, whereas the other two thirds were positive for P-S10-H3 but displayed diffuse PCNA staining in the cytosol as shown in several examples in Figure 6.5.

The failure of the S/M checkpoint is characterised by a general increase in the number of mitotic cells and more specifically by the appearance of premature mitotic cells with a 2N DNA content as has been documented previously in several experiments in this study. The percentage of mitotic Chk1<sup>-/-</sup> cells with strong PCNA staining was analysed over a time course after 0, 5, 9, and 12h and was found to correlate very closely with the percentage of 2N mitotic cells as estimated from quantification of previous flow cytometry analyses (Figure 6.5 graph). This observation suggested that those cells with dual P-S10-H3 and PCNA staining could actually be premature mitotic cells with unreplicated DNA. It also raised the question whether all premature mitotic cells stain simultaneously for nuclear PCNA or whether the dual staining can only be observed at certain times and PCNA will eventually disperse in the cytoplasm of the mitotic cell. However, unfortunately it was not possible to follow Chk1<sup>-/-</sup> cells in real-time which would be useful to distinguish between the two possibilities. Although GFP-H2B/RFP-PCNA Chk1<sup>-/-</sup> cells were generated during this study, the cells were unsuitable for analysis due to difficulties with stable RFP-PCNA expression and extreme variability in the fluorescence intensity. As both untreated as well as nocodazole-treated mitotic Chk1<sup>-/-</sup> cells never showed any dual PCNA/P-S10-H3 staining, it seems most likely that it really is a characteristic of premature mitosis associated with S/M checkpoint failure.

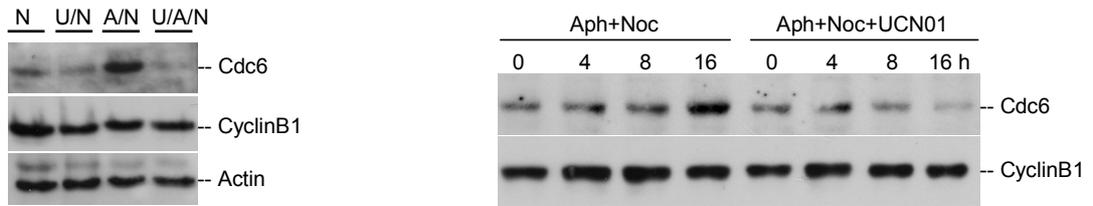
## 6.2 Checkpoint regulation of Cdc6

The observations in this and previous studies (Zachos et al., 2005) led to the hypothesis that the signal for mitotic delay in response to replication arrest might originate from the replication machinery itself. Potential candidates would presumably be involved in the process of DNA replication but also interact with mitotic controls and checkpoint signalling. One protein potentially fulfilling these criteria is the cell division cycle protein 6 (Cdc6), an important component of the pre-replication complex and essential for the initiation of DNA replication in yeast and vertebrate cells (Cocker et al., 1996; Coleman et al., 1996). Studies on the Cdc6 fission yeast homologue Cdc18 have shown that mitosis can be delayed or prevented through the production of large amounts of Cdc18 (Nishitani and Nurse, 1995). More recent studies have implicated a role for Cdc6 in checkpoint function as over-expression of human Cdc6 in G2 phase prevents HeLa cells from entering mitosis and it has been suggested that this control mechanism is Chk1-dependent (Clay-Farrace et al., 2003). It has also been suggested that the presence of Cdc6 during S phase is essential for Chk1 activation in response to replication inhibition in *Xenopus* (Oehlmann et al., 2004). Most of these observations however were made using ectopic expression of Cdc6. Thus, to evaluate any potential checkpoint function, it was of interest to determine whether the amount of endogenous Cdc6 protein might vary in a Chk1-dependent manner in response to DNA replication arrest.

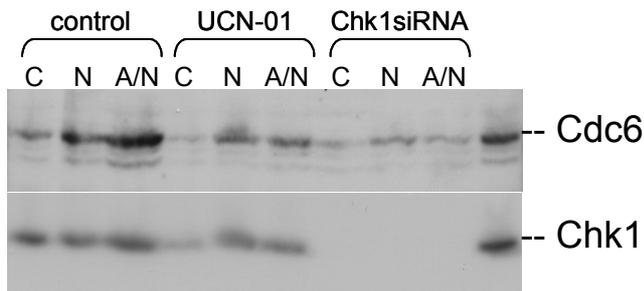
### 6.2.1 Role for Cdc6 in the S/M checkpoint response in BE cells

To investigate whether Cdc6 levels change during the S/M checkpoint in response to block to DNA replication, endogenous Cdc6 protein levels were analyzed in the mitotic checkpoint assay. Experiments were performed using the human BE colon carcinoma cell line and not the avian DT40 cells due to difficulties in obtaining an antibody specific for the avian Cdc6 protein. The cells were treated with the DNA polymerase inhibitor aphidicolin and grown in the presence of nocodazole for 16h. Samples were analyzed for mitotic indices by flow cytometry and also by Western blotting with an antibody specific for human Cdc6. Aphidicolin treated BE cells delayed the entry to mitosis and arrested mainly in G1 and S phase as previously described in detail in chapter 3. This was associated with a substantial increase of endogenous Cdc6 protein levels as shown in the Western blot analysis in Figure 6.6a (left panel). The protein levels of Cdc6 were increased in response to DNA replication block as compared to the levels in nocodazole treated cells that had mainly accumulated in mitosis. Interestingly, when cells were treated with

(a)



(b)



**Figure 6.6: Accumulation of endogenous Cdc6 during replication arrest in BE cells**

(a) BE cells were treated with the DNA polymerase inhibitor aphidicolin (A) and grown in the presence of nocodazole (N) for up to 16h. Chk1 inhibition was achieved by addition of UCN-01 (U).  
 (b) BE cells were transfected with Chk1 siRNA for 48h and then treated with aphidicolin and nocodazole for a further 24h. Whole cell extracts were analysed by SDS PAGE and Western blotting with antibodies specific for Cdc6, cyclinB1, Chk1, Actin.

aphidicolin while Chk1 was inhibited with UCN-01, Cdc6 levels did not increase. Also, under these conditions S/M checkpoint failure was detected by flow cytometry (data not shown).

Furthermore, analysis of a time course experiment of the response of BE cells to DNA replication arrest with aphidicolin revealed that Cdc6 protein levels appeared to accumulate throughout the experiment and reached highest levels at 16h (Figure 6.6a right panel). This was in stark contrast to UCN-01-treated BE cells which entered mitosis prematurely after 16h of aphidicolin treatment as detected by flow cytometry (data not shown) and displayed reduced Cdc6 levels at this time point.

A similar analysis was also performed in BE cells in which Chk1 protein expression was down-regulated with Chk1 siRNA. The analysis by both flow cytometry (not shown) and Western blotting (Figure 6.6b) revealed that a functional S/M checkpoint response in control BE cells was associated with a substantial accumulation of Cdc6 protein levels, whereas S/M checkpoint failure induced by either Chk1 siRNA or UCN-01 was associated with very low Cdc6 protein levels. These results suggested that the accumulation of endogenous Cdc6 during replication arrest was dependent on Chk1 function.

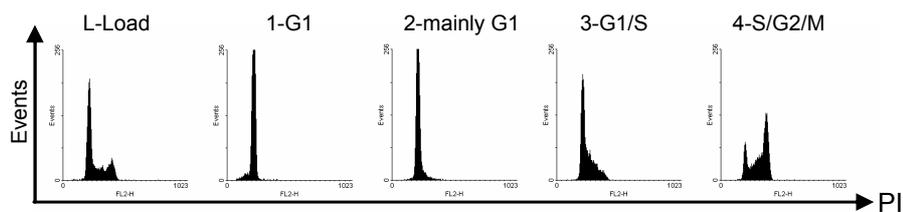
### ***6.2.2 Regulation of Cdc6 during an unperturbed cell cycle in BE cells***

The functional S/M checkpoint in response to replication arrest in BE cells was associated with an accumulation of Cdc6 protein levels. This could be due to either an increase in the levels of Cdc6 in cells specifically arrested in S phase, or because Cdc6 levels are in general higher in cells in S phase. To test this, BE cells were subjected to centrifugal elutriation and separated in several fractions according to cells size and then analysed by Western blotting with antibodies specific for Cdc6.

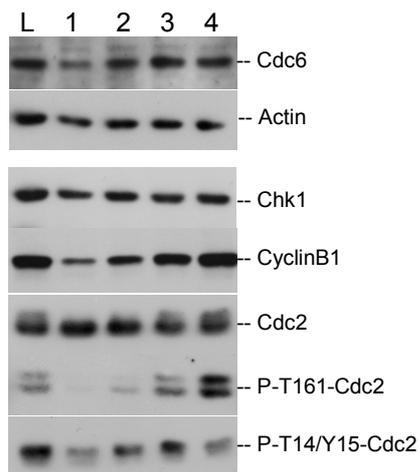
As shown in the DNA content histograms in Figure 6.7a, the asynchronous load was separated into four fractions with increasing DNA content. Separation of BE cells was not as efficient as compared to DT40 cells, which might be due to their greater variability in size and shape. However, each of the four fractions was enriched in G1, S, or G2/M cells and subsequently used for Western blot analysis.

As shown in Figure 6.7b, the protein levels of Cdc6 were lowest in G1 and increased slightly throughout the cell cycle in contrast to Actin levels that remained constant in each

(a)



(b)



**Figure 6.7: Analysis of Cdc6 protein levels throughout an unperturbed cell cycle in BE cells**

BE cells (L) were separated in several fractions (1 to 4) by centrifugal elutriation. Samples were analysed for DNA content (PI profiles) as shown in (a) and samples were also analysed by SDS PAGE and Western blotting with antibodies specific for Cdc6, cyclinB1, Chk1, Cdc2, P-Cdc2, and Actin served as loading control as shown in (b).

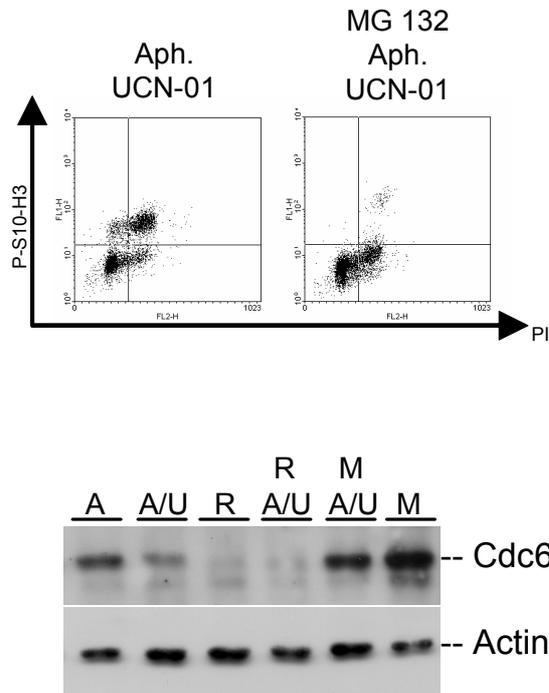
fraction. Samples were further analysed by Western blotting and cell cycle specific fluctuations were observed in cyclinB1 levels and in the phosphorylation status of Cdc2, with highest activatory T161-Cdc2 phosphorylation in fraction 4 with mainly G2/M cells. The inhibitory T14/Y15-Cdc2 phosphorylation levels reached a maximum in fraction 3 and then decreased again in fraction 4. These changes were observed while total Cdc2 and Chk1 protein levels were constant.

In summary, the data from the elutriation experiment showed that Cdc6 levels were low in G1 and increased throughout the cell cycle, very similar to cyclinB1 which is regulated by proteolysis by the APC. These observations were in accordance with previous studies in HeLa cells that demonstrated that Cdc6 is a target of the APC in G1 (Petersen et al., 2000). Thus, this suggests that the Cdc6 protein levels in the BE cells are also regulated by proteasomal degradation.

### **6.2.3 Regulation of Cdc6 stability by Chk1 in BE cells**

The observations made in the chapters above as well as previous studies in HeLa cells (Clay-Farrace et al., 2003) have suggested a role for Cdc6 in the S/M checkpoint response. In BE cells, endogenous protein levels of Cdc6 accumulated in a Chk1-dependent manner in response to replication arrest with aphidicolin. As Cdc6 protein levels have previously been shown to be regulated via the proteasome (Petersen et al., 2000), this raised the question of whether Cdc6 accumulation during replication arrest was due to inhibition of the proteasome.

To address this issue, BE cells were treated with aphidicolin and grown in the presence of nocodazole for 16h. Chk1 inhibition was achieved using UCN-01 and the proteasome inhibitor MG 132 was included to study the involvement of the proteasome. Samples were harvested and analysed by SDS PAGE and Western blotting with antibodies specific for Cdc6 and Actin was used as a loading control. As shown in Figure 6.8, the protein levels of Cdc6 were relatively high in response to replication arrest with aphidicolin and in contrast much lower when Chk1 was inhibited with UCN-01 and cells exhibited S/M checkpoint failure (upper panels). Interestingly, addition of the proteasome inhibitor MG 132 was able to rescue the S/M checkpoint response in the absence of functional Chk1 as shown in the flow cytometry profiles in Figure 6.8. The restored checkpoint response was associated with an increase in Cdc6 protein levels. However, it was also noticed that MG 132 alone was able to block entry to mitosis along with an increase in Cdc6 protein levels (flow



**Figure 6.8: Inhibition of the proteasome with MG 132 stabilises Cdc6 protein levels**

BE cells were grown in the presence of nocodazole for 16h. Cells were treated with the DNA polymerase inhibitor aphidicolin (A or Aph.), Chk1 inhibitor UCN-01 (U), cdk inhibitor roscovitine (R), or the proteasome inhibitor MG 132 (M) respectively. Samples were analysed for mitotic indices (P-S10-H3/PI) by flow cytometry and also by SDS PAGE and Western blotting with antibodies specific for Cdc6. Actin served as a loading control.

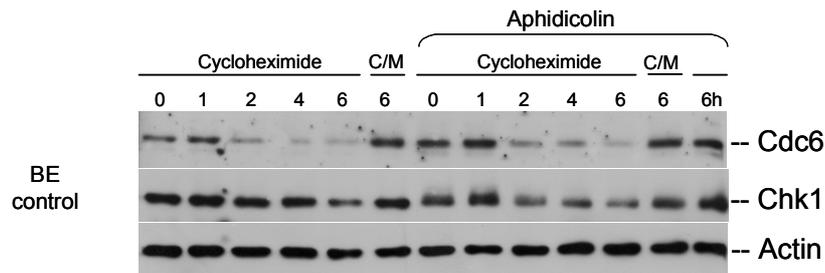
cytometry data not shown). Nevertheless, these results suggested that Chk1 might be able to stabilise Cdc6 during replication arrest via inhibition of the proteasome.

Another interesting observation was that while the Cdk inhibitor roscovitine was able to inhibit the onset of premature mitosis, it did not restore Cdc6 protein levels, as shown in lane 4 in the Western blot analysis in Figure 6.8. Moreover, roscovitine alone had a strong effect on Cdc6 levels. This could be due to the aforementioned negative effect of roscovitine on transcription (Ljungman and Paulsen, 2001). Aside from that, in budding yeast, the activation of S phase Cdks is associated with de-stabilization of Cdc6 and the fission yeast homologue of Cdc6 has been shown to be regulated by Cdk-dependent phosphorylation and proteolysis (Jallepalli et al., 1997; Piatti et al., 1996). However, the stability of Cdc6 in human Hela cells has been found to be independent of cdk phosphorylation (Petersen et al., 2000). Nevertheless, the observations presented here could suggest that Cdc6 stability or accumulation in response to replication arrest is not due to low levels of Cdk activity as inhibition of Cdks with roscovitine did not restore Cdc6 protein levels. On the other side, results from another study showed that within a limited time window prior to S phase Cdc6 can also be stabilized by Cdk2/cyclinE-dependent phosphorylation which prevents its association with the APC (Mailand and Diffley, 2005). It is therefore possible that Cdk inhibition with roscovitine could have affected this step of initial Cdc6 stabilisation; however a more detailed analysis would be required to clarify this issue.

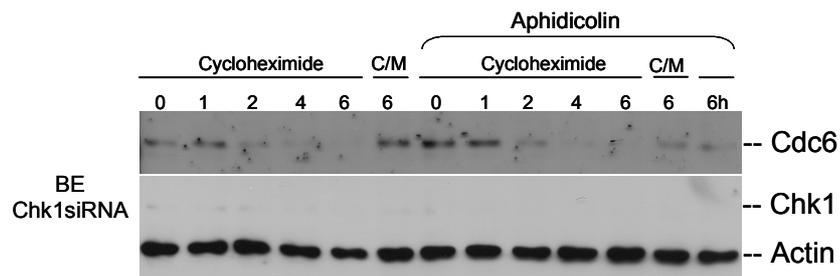
The replication protein Cdc6 is a target of proteasomal degradation in BE cells and Chk1 appears to play a role in the accumulation of Cdc6 during replication arrest. To test whether Chk1 has any effect on proteasomal degradation of Cdc6 in general and during replication arrest, the half life of Cdc6 was analysed in both checkpoint proficient BE cells and cells in which Chk1 protein levels were down-regulated with siRNA.

The half life of Cdc6 was analysed by performing a time course experiment with cells that were treated with the protein synthesis inhibitor cycloheximide which inhibits de novo synthesis of Cdc6 and therefore allows the rate at which the protein is degraded to be estimated. Samples were taken at 0, 1, 2, 4, and 6h time points and analysed by SDS PAGE and Western blotting. As shown in Figure 6.9a, control BE cells exhibited decreases in the total Cdc6 protein levels after cycloheximide treatment, which led to an estimation of an approximate Cdc6 half life of about 2h. In the presence of both cycloheximide and the proteasome inhibitor MG 132 the protein levels of Cdc6 did not decrease. Actin levels

(a)



(b)



**Figure 6.9: Analysis of Cdc6 half life in BE cells**

Cells were treated with cycloheximide (C or CHX) for up to 6h. Analysis was performed with control cells in (a) and in (b) Chk1 function was abrogated with Chk1 siRNA transfection for 48h. Where required, DNA replication was blocked with aphidicolin for 12h prior to start of the time course. MG 132 (M) was used for inhibition of the proteasome. Cells were analysed by SDS PAGE and Western blotting with antibodies specific for Cdc6, Chk1, and Actin which served as loading control.

were not affected by cycloheximide treatment, while total Chk1 protein levels appeared to be decreasing after 6h of treatment.

The cycloheximide time course analysis was also performed on BE cells that had been pre-treated with the DNA polymerase inhibitor aphidicolin for 12h. These cells displayed high levels of Cdc6 after 12h and still after an additional 6h of replication arrest (Figure 6.9a). At the beginning of the cycloheximide treatment Cdc6 levels were high but subsequently decreased over time at a rate similar to untreated control cells. The Cdc6 protein levels did not decrease when the proteasome inhibitor MG 132 was included. These observations suggested that the accumulation of Cdc6 protein in response to replication arrest was not primarily due to a longer half life of the protein.

A similar analysis was then performed on BE cells that had been transfected with Chk1 siRNA for 48h prior to starting the cycloheximide time course. As shown in Figure 6.9b, Chk1 protein levels were almost completely abrogated by siRNA treatment whereas Actin levels were constant throughout the duration of the experiment. When Chk1 siRNA transfected BE cells were treated with cycloheximide, total Cdc6 protein levels decreased over time with an estimated half life of about 2h as judged by density quantification of the Western blots. Overall, the half life of Cdc6 was very similar in both control cells and Chk1-depleted cells.

When Chk1 siRNA transfected BE cells were exposed to aphidicolin for 12h prior to the start of the cycloheximide time course, the Western blot analysis revealed that a certain amount of Cdc6 was detectable at the 0h time point (Figure 6.9b). In the presence of cycloheximide the Cdc6 protein levels in the Chk1-depleted cells decreased over time at a rate similar to control cells. When the proteasome inhibitor MG 132 was added, the levels of Cdc6 protein were restored to some extent.

These observations showed that the half life of Cdc6 is approximately 2h and does not appear to be affected by Chk1 status or aphidicolin treatment in BE cells. Taken together, although Chk1 appears to be important for promoting accumulation of Cdc6 in response to replication arrest, this is not obviously associated with an increased half life of the protein. Thus, other factors, such as for example transcriptional activation, might contribute to the accumulation of Cdc6 in response to replication block with aphidicolin.

### **6.2.4 Role of Cdc6 during S/M checkpoint - Intervention experiments**

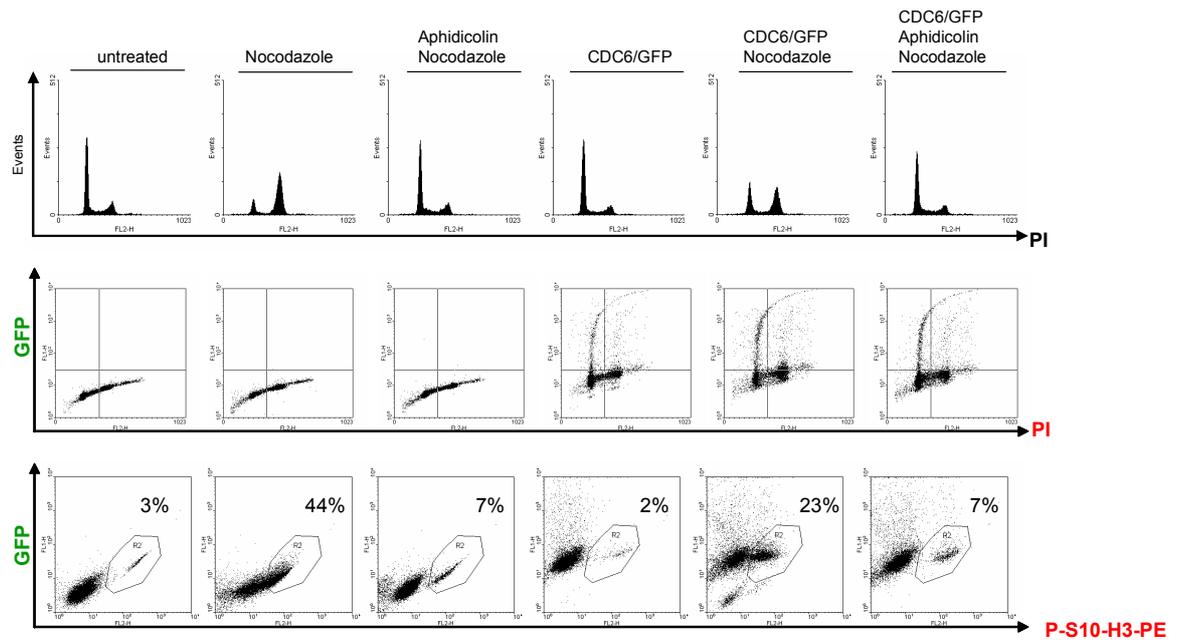
The functional S/M checkpoint response in BE cells was characterized by the ability of arrested cells to efficiently delay mitosis and this was also associated with an accumulation of endogenous Cdc6 protein levels. Previously it has been shown that ectopic expression of Cdc6 can block the entry to mitosis in checkpoint-proficient cells (Clay-Farrace et al., 2003). As S/M checkpoint failure in the absence of functional Chk1 was associated with very low levels of Cdc6, this raised the question of whether introduction of exogenous Cdc6 could restore the mitotic checkpoint response in checkpoint-deficient cells.

To test this, Cdc6 was ectopically expressed in checkpoint-proficient as well as Chk1-depleted checkpoint-deficient BE cells. The experimental procedure involved the co-transfection of cells with plasmids expressing Cdc6 and GFP for 24h. These cells were then treated with aphidicolin and nocodazole as required for a further 24h. Samples were analysed by flow cytometry for DNA content and mitotic indices in combination with GFP staining as shown in Figure 6.10.

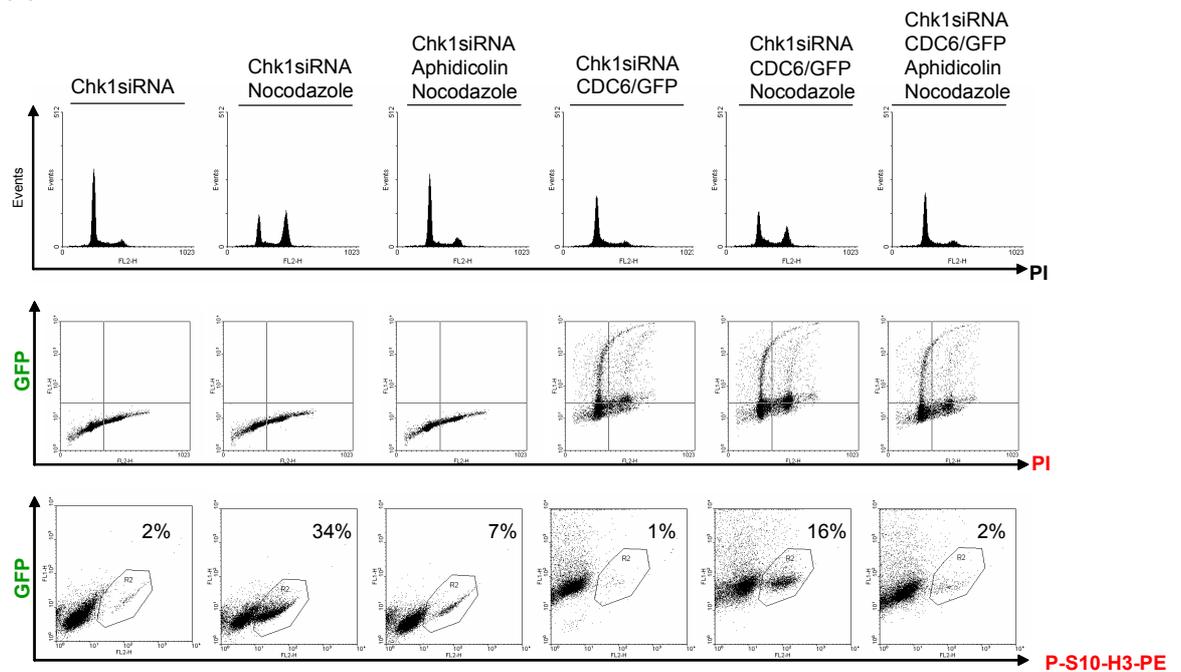
Un-transfected BE cells accumulated in mitosis in the presence of nocodazole (44%) but delayed mitosis effectively when replication was blocked with aphidicolin as only a small percentage of cells (7%) stained positive for the mitotic marker P-S10-H3 (Figure 6.10a). Transfection efficiency for Cdc6 was estimated by the amount of cells displaying positive GFP-staining. Although the levels of GFP staining varied in intensity, overall a high percentage of cells (~90%) were successfully transfected. Of the transfected cells only 23% were able to accumulate in mitosis in the presence of nocodazole. As the progression into mitosis was not compromised in GFP-only transfected control cells (data not shown), this suggested a negative effect of Cdc6 transfection on cell cycle progression. In response to aphidicolin treatment, the transfected cells were able to effectively delay mitosis (7% P-S10-H3-positive) similar to un-transfected cells under the same treatment.

To study the effect of Cdc6 over-expression on checkpoint-deficient cells, BE cells were first treated with Chk1 siRNA (48h), then transfected with Cdc6/GFP plasmids after 24h and finally treated with aphidicolin and nocodazole for the last 24h of the experiment. As shown in Figure 6.10b, un-transfected Chk1-depleted cells progressed into mitosis in the presence of nocodazole (34%), but not as effectively as Chk1-proficient cells. There was an increase in the amount of mitotic cells after aphidicolin treatment, indicative of S/M checkpoint failure; however, the overall increase in mitotic cells was rather small (7%),

(a)



(b)



**Figure 6.10: Effect of Cdc6 over-expression on S/M checkpoint response in BE cells**

The effect of Cdc6 over-expression was analysed in control cells (a) or cells transfected with Chk1 siRNA for 48h prior to plasmid transfection (b). Cells were co-transfected with Cdc6 and GFP expression vectors for 24h and treated for a further 24h with aphidicolin and/or nocodazole. Samples were analysed by flow cytometry for DNA content (PI profiles), GFP/PI staining, and mitotic indices by GFP/PS10-H3 staining by flow cytometry. Gate R2 served as approximate quantification of P-S10-H3 positive cells for each sample.

which might be due to variability in the extent of S/M checkpoint failure as has been previously mentioned (chapter 3.2.2). Interestingly, the number of mitotic cells in response to aphidicolin treatment was decreased in the Cdc6-transfected cells (2%). Furthermore, only 16% of the Chk1-depleted and Cdc6-transfected cells accumulated in mitosis after nocodazole treatment.

Taken together, the ectopic expression of Cdc6 resulted in a decrease in the number of cells entering mitosis in the presence of nocodazole in both checkpoint-proficient as well as checkpoint-deficient BE cells. Moreover, the amount of checkpoint-deficient cells entering mitosis in response to replication block was to some extent reduced. However, overall it was not yet possible to judge whether this effect of ectopic Cdc6 expression on the S/M checkpoint failure in Chk1-depleted BE cells was significant.

### 6.3 Discussion

The relationship between the process of DNA replication and the onset of mitosis was analyzed in order to gain insight into the S/M checkpoint mechanism as well as mitotic delay in response to replication block in the absence of functional Chk1.

The localization and distribution pattern of a marker for DNA replication, PCNA, was first analyzed during normal unperturbed cell cycle regulation in DT40 WT cells. Cell cycle specific distribution patterns of PCNA were identified. Whereas PCNA staining appears weak and cytosolic in G1, it accumulates in S phase cells in the nucleus forming foci with strong staining and thereafter the staining becomes weaker and diffuse again similar to descriptions in earlier studies (Celis and Celis, 1985). In DT40 WT and Chk1<sup>-/-</sup> cells the chromatin bound PCNA dispersed into the cytosol when cells started to enter natural mitosis associated with condensation of the chromatin. Interestingly, PCNA was found to be bound to chromatin in some of the Chk1<sup>-/-</sup> cells that entered mitosis prematurely after 12h of replication arrest with aphidicolin. This observation suggested that premature mitosis of cells with un-replicated DNA is associated with the continued presence of PCNA on the chromatin of these cells.

However, the experiments in this study did not address the question whether the dual PCNA and P-S10-H3 staining of Chk1<sup>-/-</sup> cells was a general feature of premature mitotic cells or a transient step until completion of mitosis. This issue could be further addressed using time-lapse microscopy on Chk1<sup>-/-</sup> cells stably expressing GFP-H2B and RFP-PCNA. Although respective DT40 WT counterparts have been generated successfully, it

was not yet possible to obtain Chk1<sup>-/-</sup> cells with the same properties. This was due to extreme variability in fluorescence intensity which made further characterization of these cells difficult.

The results from these experiments suggest that premature and normal mitosis can be distinguished by the difference in PCNA distribution, which appears still bound to the chromatin during the onset of premature mitosis whereas it is dispersed into the cytosol during chromatin condensation associated with normal scheduled mitosis.

While Chk1 function is dispensable for the initiation of mitotic delay, it appears to be important at later stages as a major factor for maintenance of the delay (Zachos et al., 2005). This notion is supported by the fact that Chk1 is responsible for the replication recovery functions, such as suppression of new origin firing and the stabilization of stalled replication forks (Feijoo et al., 2001; Zachos et al., 2003). Thus, the model is that the replication machinery itself is able to signal either the process of normal properly executed DNA replication or the presence of disrupted stalled replication machinery. This signal can initiate a mitotic delay independently of Chk1. However, in general the stabilization of stalled replication forks and the suppression of origin firing are dependent on Chk1 function, which could explain why Chk1-deficient cells eventually fail to maintain the mitotic delay. Intriguingly, this model also implies that as long as stalled replication structures are stabilized, the intrinsic mitotic delay mechanism will persist.

Accordingly, one prediction would be that Chk1<sup>-/-</sup> cells could block the onset of premature mitosis as long as the collapse of stalled replication structures is prevented. In this study, replication was routinely blocked with the DNA polymerase inhibitor aphidicolin. This drug inhibits the DNA polymerase activity and thereby stalls DNA replication. However, aphidicolin can also cause uncoupling of the MCM helicase from the replication fork and this can eventually result in the collapse of previously stalled replication structures (Byun et al., 2005; Walter and Newport, 2000). Thus, one could imagine that aphidicolin treatment will aid the collapse of stalled replication structures over time and this could be responsible for the premature mitotic entry of Chk1<sup>-/-</sup> cells. This scenario could suggest that the intrinsic checkpoint signal might persist for a longer time if the uncoupling of the polymerase and helicase activities during aphidicolin treatment could be prevented and the stalled replication structures would be kept stable. This is a concept that could be easily tested, for example with an inhibitor of MCM helicase, such as the Rb-400 fragment used in a recent study (Byun et al., 2005).

One component of the replication machinery, the replication protein Cdc6, has been identified as a potential candidate involved in checkpoint regulation. Interestingly, during replication arrest in human BE cells, the mitotic delay was associated with an accumulation of endogenous Cdc6 protein. Furthermore, the accumulation of Cdc6 stability is dependent on Chk1 function, as cells in which Chk1 was inhibited with UCN-01 or in which Chk1 levels were depleted with siRNA did not accumulate Cdc6 and also exhibited S/M checkpoint failure after several hours in aphidicolin. This observation is probably the first evidence for checkpoint regulation of endogenous Cdc6, as most previous studies have used ectopic expression of the protein and there has been some discrepancy in results obtained with endogenous and ectopically expressed Cdc6 protein which related to the degradation of the protein (Clay-Farrace et al., 2003; Coverley et al., 2000). An interesting experiment to confirm whether Cdc6 accumulation is only associated with arrested cells would be to separate mitotic and non-mitotic Chk1- depleted cells after replication block. The prediction would be that only the non-mitotic arrested cells would display high Cdc6 protein levels and only cells with low Cdc6 levels enter mitosis prematurely. Aside from that, the presented results further confirm that Cdc6 plays a role in replication checkpoint control in accordance with several recent studies in both human and yeast cells (Clay-Farrace et al., 2003; Fersht et al., 2007; Hermand and Nurse, 2007; Oehlmann et al., 2004).

The potential role of Chk1 in the accumulation of Cdc6 in response to replication arrest might be the stabilization of Cdc6 protein by inhibiting its proteasomal degradation, as the proteasome inhibitor MG 132 was able to maintain or even increase cdc6 levels. However, analysis of the half life of Cdc6 revealed no significant differences between cells with or without functional Chk1 in untreated or aphidicolin treated cells. Moreover, it appears that de novo Cdc6 protein synthesis contributes to some extent to the increase in Cdc6 protein levels after replication block. It would be interesting to analyze whether Cdc6 mRNA levels might be affected in response to replication block. Taken together, there are several factors determining the checkpoint regulation of Cdc6 and while Chk1 function appears to be important, the exact mechanism has not been elucidated yet.

One proposed model for mitotic delay in response to replication block is that the presence of replication proteins such as Cdc6 generates the signal for mitotic delay. This model would predict that intervention experiments using ectopic expression of the protein could presumably restore the checkpoint response in Chk1-depleted cells. Intervention experiments performed in this study gave some indication that this might be true as Chk1-depleted BE cells displayed lower levels of mitosis when replication was blocked while Cdc6 was ectopically expressed. However, further experiments are required to sufficiently

address this issue, for example, the use of an inducible Tet-On system for Cdc6 or other potential candidates in DT40 WT and Chk1<sup>-/-</sup> cells. Unfortunately, due to difficulties in obtaining an antibody specific for the avian protein, it was not yet possible to study Cdc6 in the DT40 cells. Apart from that, adherent BE cells could also be microinjected with plasmids of interest and then analyzed using UCN-01 or Chk1 siRNA to test the hypothesis of checkpoint restoration. Although this approach has been attempted during this study, it could not be pursued further due to low injection efficiency and low survival rates of the microinjected cells.

## 7 Conclusions and Future Perspectives

Vertebrate cells have evolved checkpoint control mechanisms that prevent the onset of mitosis in the presence of DNA damage or if DNA replication is blocked. Most of these control mechanisms have originally been identified in yeast but were later found to be similar in many aspects in vertebrate cells. The signaling pathway that was identified to be responsible for the establishment of mitotic delay can be summarized in the 'conventional' checkpoint model.

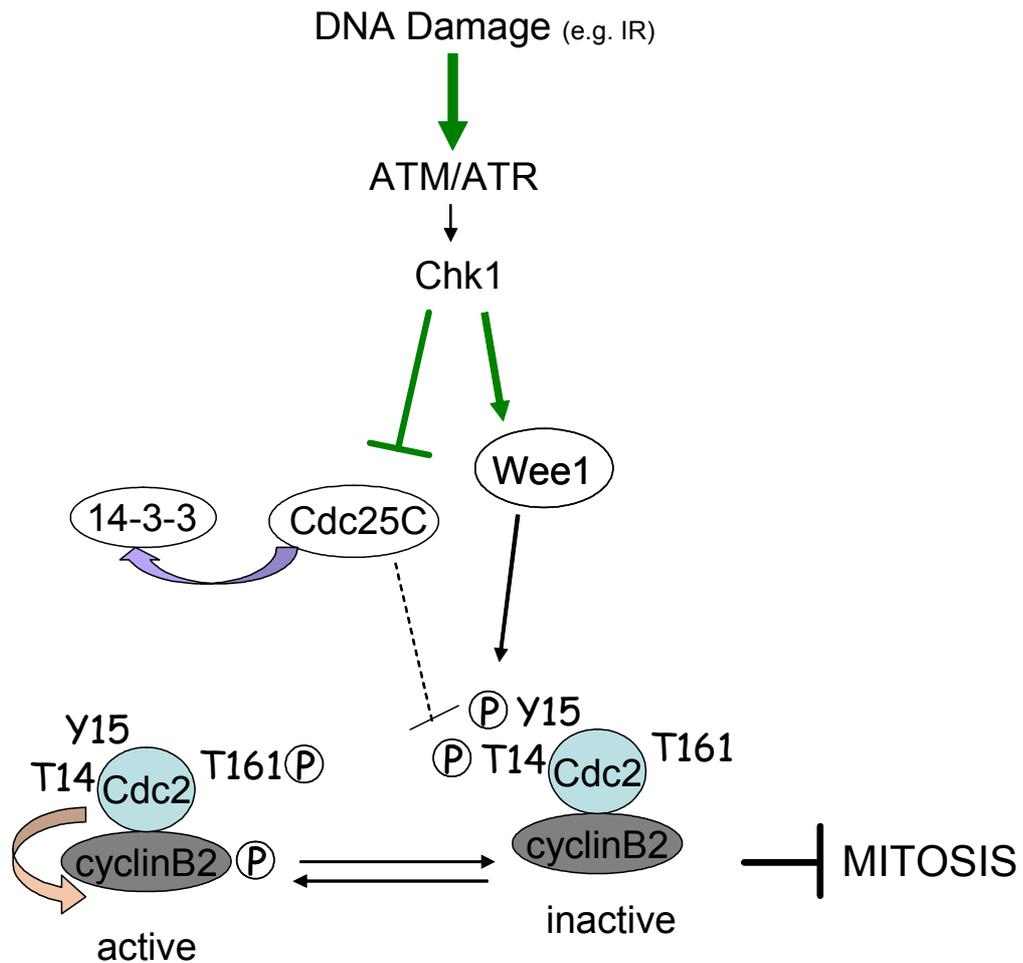
This conventional model states that the onset of mitosis is always blocked through inhibition of the cyclin dependent kinase Cdc2. Initially, sensor proteins can detect any DNA lesions or stalled replication structures and subsequently lead to the activation of a signalling cascade that involves Chk1/Chk2-activation via ATM/ATR and consequently results in the modulation of several downstream targets. Phosphorylation of the Cdc25C phosphatase by Chk1 and Chk2 on the N-terminus creates a binding site for 14-3-3 proteins and results in the nuclear export and degradation of Cdc25C (Chaturvedi et al., 1999; Peng et al., 1997; Sanchez et al., 1997). In addition, this N-terminal phosphorylation of Cdc25C can also directly inhibit Cdc25 phosphatase activity (Blasina et al., 1999). Apart from this, Chk1 has also been shown to phosphorylate several Cdc25 family members on C-terminal sites which as a result inhibits their interaction with their cdk/cyclin substrates (Uto et al., 2004). As a consequence of these inhibitory mechanisms, the Cdc25C phosphatase cannot de-phosphorylate Cdc2 on residues T14 and Y15 and the Cdk is kept in an inactive state. In addition, Chk1 can phosphorylate and activate Wee1 and thus promote inhibitory phosphorylation of Cdc2. In *Xenopus*, Chk1-mediated phosphorylation of Wee1 can promote 14-3-3 binding and further enhance the inhibitory Wee1 kinase activity (Lee et al., 2001a; O'Connell et al., 1997).

These two conventional checkpoint mechanisms, the inhibition of Cdc25 phosphatase and the promotion of Wee1 kinase activity, ensure the maintenance of inhibitory Cdc2 phosphorylation, keeping the Cdc2/cyclinB complex inactive and hence preventing the onset of mitosis. Both checkpoint kinases Chk1 and Chk2 have the potential to operate in the conventional checkpoint model. Therefore, the initial aim of this study was to investigate the relative requirement of Chk1 and Chk2 function for mitotic checkpoint responses in vertebrate cells. Using two different vertebrate cell systems, it was shown that Chk1 is the major effector kinase for mitotic delay in response to DNA damage. In the absence of Chk1, cells with damaged DNA entered mitosis at a similar rate to control cells.

The biochemical analysis of Cdc2 regulation showed that the inhibitory phosphorylation of Cdc2 was maintained in checkpoint proficient cells in response to DNA damage but not in untreated control cells or checkpoint-deficient cells. Furthermore, the suppression of cyclinB2 phosphorylation was identified as a checkpoint target in the avian DT40 cells. Overall, the results suggest that IR-induced DNA damage leads to the activation of a signalling cascade involving ATM/ATR and Chk1 and subsequent phosphorylation of downstream targets then results in the inhibition of mitosis via maintenance of inhibitory Cdc2 phosphorylation in a fashion that is in many aspects consistent with the conventional model as illustrated in Figure 7.1.

However, although the regulatory changes in Cdc2/cyclinB2 correlated with the conventional model of mitotic checkpoint delay, the actual Cdc2 kinase activity of IR-treated DT40 WT G2 cells was relatively high even though mitosis was effectively delayed. This observation seems initially counterintuitive, but could be attributed to a number of facts as discussed earlier. For example, Cdc2 kinase activity might increase progressively throughout the cell cycle and only once a certain threshold level is reached will cells enter mitosis. If this was the case, then the levels of Cdc2 kinase activity in the G2 arrested DT40 WT cells would have been greater than in the starting G1/S population but nevertheless below that threshold. Alternatively, Cdc2 could have been associated with another cyclin such as cyclinA, which could enable a putative Cdc2/cyclinA complex to be catalytically active but either not be in the appropriate location or below the threshold to induce the onset of mitosis.

As described in chapter 5, further studies of the Cdc2/cyclinB2 complex in the DT40 cells revealed that the phosphorylation of cyclinB2 is accomplished by Cdks and from experiments using a variety of kinase inhibitors it could be suggested that maybe Cdc2 itself can auto-phosphorylate the cyclinB2 subunit in these cells. Interestingly, this concept of Cdc2/cyclinB2 auto-phosphorylation has previously been proposed on the basis of earlier studies in starfish oocytes, which demonstrated that the phosphorylation of cyclinB requires Cdc2 activity both *in vivo* and *in vitro* (Borgne et al., 1999). In this particular system, it was shown that the Cdc2-mediated cyclinB phosphorylation is an intramolecular mechanism that occurs at the prophase/metaphase transition. It has also been suggested that Cdc2/cyclinB becomes activated in the cytoplasm prior to the transition to the nucleus in these starfish oocytes (Ookata et al., 1992). Interestingly, the Cdc2 kinase activity of purified de-phosphorylated Cdc2 complexed to the de-phosphorylated cyclinB subunit was almost identical to the kinase activity of the mitotic complex of de-phosphorylated Cdc2 complexed to the phosphorylated cyclinB (Borgne et al., 1999).



**Figure 7.1: Model for mitotic delay mechanisms in response to DNA damage**

DNA damage, such as DNA double strand breaks induced by IR, lead to the activation of a signalling cascade that involves ATM/ATR and Chk1. Subsequent phosphorylation of Chk1 downstream targets results in nuclear exclusion of the Cdc25C phosphatase by binding of 14-3-3 proteins and activation of the Wee1 kinase. Both events help to maintain inhibitory phosphorylation of Cdc2 on T14 and Y15 according to the conventional model of mitotic checkpoint delay mechanisms. In addition in DT40 cells, the phosphorylation of cyclinB2 is suppressed as part of the signalling cascade. Consequently, the Cdc2/cyclinB(2) complex is kept in an inactive state and the onset of mitosis is inhibited.

The fact that Cdc2 in combination with de-phosphorylated cyclinB appears to be catalytically active in the cytoplasm without inducing mitosis could therefore possibly account for the observation that IR-treated DT40 WT cells, which were mainly arrested in G2, displayed such high Cdc2 kinase activity levels although they blocked the entry to mitosis. This could also relate to the threshold model for Cdc2 activity which might increase progressively throughout the cell cycle but needs to exceed a certain level in order to induce mitosis. In the Cdc2-IP kinase assays, the relatively high Cdc2 kinase activity in response to IR was accompanied by the un-phosphorylated form of cyclinB2. As the phosphorylated isoform of cyclinB2 has been associated with Cdc2 nuclear translocation at the onset of prophase (Gallant and Nigg, 1992), this would further support the idea that mitosis is only initiated once Cdc2 has phosphorylated its cyclinB2 subunit and the complex has translocated to the nucleus.

In order to thoroughly investigate these issues, it would be interesting to repeat these experiments in the Cdk2 knock out cell line that has been generated recently in DT40 cells (Hochegger et al., 2007). In these cells it is thought that Cdc2 regulates not only entry to mitosis but also regulates G1 and S phase cell cycle transitions and DNA replication, functions previously assigned to Cdk2. In this context it would be interesting to assess the Cdc2 kinase activity throughout the cell cycle as it appears that the Cdc2 activity that is necessary for the S phase functions in these cells is below the threshold for the induction of mitosis and therefore appears to be regulated according to the threshold model of Cdc2 kinase activity.

The investigation of mitotic checkpoint proficiency in response to replication arrest with the DNA polymerase inhibitor aphidicolin revealed a more complex situation. The timing of checkpoint failure in Chk1-deficient DT40 cells differed according to their position in the cell cycle: Those cells that were in G2 phase with 4N DNA content at the time of aphidicolin treatment did initially enter mitosis similar to control cells until the respective G2 cell population was depleted. This observation indicates that aphidicolin can actually induce a mitotic delay specifically in G2 cells. This delay is dependent on Chk1 function as demonstrated by the fact that G2 phase Chk1<sup>-/-</sup> cells enter mitosis without any delay comparable to untreated control cells. The exact mechanism of this aphidicolin-induced G2 delay remains to be determined; it appears however that the activity of the DNA polymerase might still be required even after DNA replication has been completed, for example it could be involved in post-replication DNA repair or recombination. This would distinguish the aphidicolin-induced G2-delay from the DNA damage IR-induced G2/M arrest.

In marked contrast to the Chk1<sup>-/-</sup> G2 phase cells, those cells that were in G1/S phase at the time of aphidicolin treatment did not enter mitosis for an extended period of time and only after this prolonged mitotic delay did cells start to enter mitosis with 2N DNA content. This difference in the timing of cells entering normal 4N or premature 2N mitosis was not simply due to their position in the cell cycle as was shown using purified G1/S cell populations. Compared to elutriated Chk1<sup>-/-</sup> cells that progressed into mitosis with damaged DNA at the same rate as control cells, the aphidicolin-treated Chk1<sup>-/-</sup> cells entered mitosis only at much later times after many hours of delay. The entry to mitosis was premature as cells had not completed the replication of their DNA. This distinctive phenotype with prolonged mitotic delay in response to replication arrest was also observed in BE cells treated with the Chk1 inhibitor UCN-01, indicating that it is a fundamental property of vertebrate cells. Taken together, these results suggest that some other factors contribute to the timing of premature mitosis in the absence of Chk1.

The biochemical analysis of regulatory changes on Cdc2 and cyclinB2 revealed that the phosphorylation of cyclinB2 accumulated during the initial mitotic delay of the aphidicolin-treated Chk1<sup>-/-</sup> cells similar to control cells and was not suppressed as during the mitotic delay in checkpoint-proficient cells. As the phosphorylated isoform of cyclinB2 has been associated with Cdc2 nuclear translocation at the onset of prophase (Gallant and Nigg, 1992), this observation indicated that although the presence of the mitotic phosphorylated cyclinB2 isoform might be necessary it is not sufficient to induce mitosis. Hence, the mitotic delay in the presence of a potentially active Cdc2/cyclinB2 complex might be attributed to other additional factors responsible for restraining Cdc2 activity. The obvious question that therefore arises is: what mechanism times the onset of premature mitosis in the G1/S phase Chk1<sup>-/-</sup> cells?

There are several possibilities that could account for restricting Cdc2 activity, for example the sequestration of the protein into the cytoplasm which has been mentioned above when discussing the Cdc2 kinase activity in checkpoint proficient cells in response to DNA damage. Also, Cdc2 could be associated with another cyclin, such as cyclinA. Alternatively, binding of Cdc2 to another protein for example could restrain the mitotic kinase activity. In order to identify the mechanism of Chk1-independent mitotic delay the characteristics of premature and normal mitosis were compared.

A previous study by Zachos et al. suggested an approximate correlation between the collapse of replication structures and the onset of premature mitosis (Zachos et al., 2005). However, results presented here indicate that the timing of premature mitosis does not

strictly correlate with the loss of chromatin-bound PCNA, which is a DNA polymerase processivity factor and was therefore used as a marker for DNA synthesis and viable replication structures. While PCNA was found to dissociate from the chromatin and disperse into the cytosol before chromatin condensation of any normal mitosis, this was not the case for cells that entered mitosis prematurely. Instead, it appears that Chk1<sup>-/-</sup> cells eventually enter mitosis in the presence of chromatin-bound PCNA. The discrepancies between the PCNA staining patterns between the two studies, by Zachos et al. and here, might be due to different staining protocols, for example, a more stringent buffer could have displaced chromatin-bound PCNA from the nucleus in the earlier study. Nevertheless, the question is raised of whether PCNA staining can be used as a reliable marker for viable replication structures or whether it might also persist on collapsed replication structures, as was seen in the Chk1<sup>-/-</sup> cells that entered mitosis prematurely.

It seems likely that although the initial prolonged mitotic delay in response to replication arrest is Chk1-independent and due to some other checkpoint signal, Chk1 function is still indirectly required for the long-term maintenance of the mitotic delay. So far one cannot exclude the possibility that mitosis with chromatin-bound PCNA is only a transient phenomenon in these cells, also it is unclear whether DNA replication could resume in these cells once the drug is removed or whether these cells have lost all viable replication structures although PCNA is still associated with them. As Chk1 function is generally required for the stabilisation of stalled replication structures (Zachos et al., 2005), it seems probably most likely that the stalled replication structures eventually collapse and that these cells enter mitosis even in the continued presence of PCNA, however to clarify this question further investigation is required.

Taking all these observations into account, the hypothesis was made that the onset of mitosis might be dependent on the completion of DNA synthesis and that the replication machinery itself might initiate a checkpoint signal either while normal DNA replication is ongoing but also when the replication machinery is stalled.

The idea of a connection between DNA replication and mitosis is not completely new. The existence of an intrinsic relationship between the process of DNA replication and the onset of mitosis has previously been introduced based on observations from cell fusion experiments performed by Rao and Johnson (Rao and Johnson, 1970). These experiments demonstrated that when a nucleus from an S phase cell was fused with a nucleus of a G2 phase cell, the progression of the heterokaryon to mitosis was dependent on the completion of DNA replication in the S phase nucleus. Certainly, the cells in these experiments were

checkpoint-proficient, but as Chk1 should hardly be activated under these conditions, it seems likely that these observations indicate the functioning of an intrinsic link between DNA replication and mitosis.

Further evidence for a potential intrinsic mitotic delay mechanism comes from studies in ATR and ATM/ATR knock-out cell lines that are able to effectively delay mitosis in response to replication block although Chk1 activation was severely compromised (Brown and Baltimore, 2003). This study suggested that the ATR knock-out cells can block entry to mitosis by inhibiting Cdc2/cyclinB by some other mechanism than the maintenance of inhibitory Cdc2 phosphorylation. This idea of an ATR-independent replication checkpoint is also supported here because DT40 WT cells were able to sustain a functional S/M-delay even in the presence of the ATM/ATR-inhibitor caffeine. Another study revealed how many, though not all, cell lines differ in their sensitivity to the ATM/ATR-inhibitor caffeine with an indication that most untransformed cell lines can delay mitosis after DNA synthesis inhibition independently of ATM/ATR (Florensa et al., 2003). All these observations strongly suggest that cells can delay mitosis in response to replication arrest in more ways than the conventionally known mechanisms via ATM/ATR.

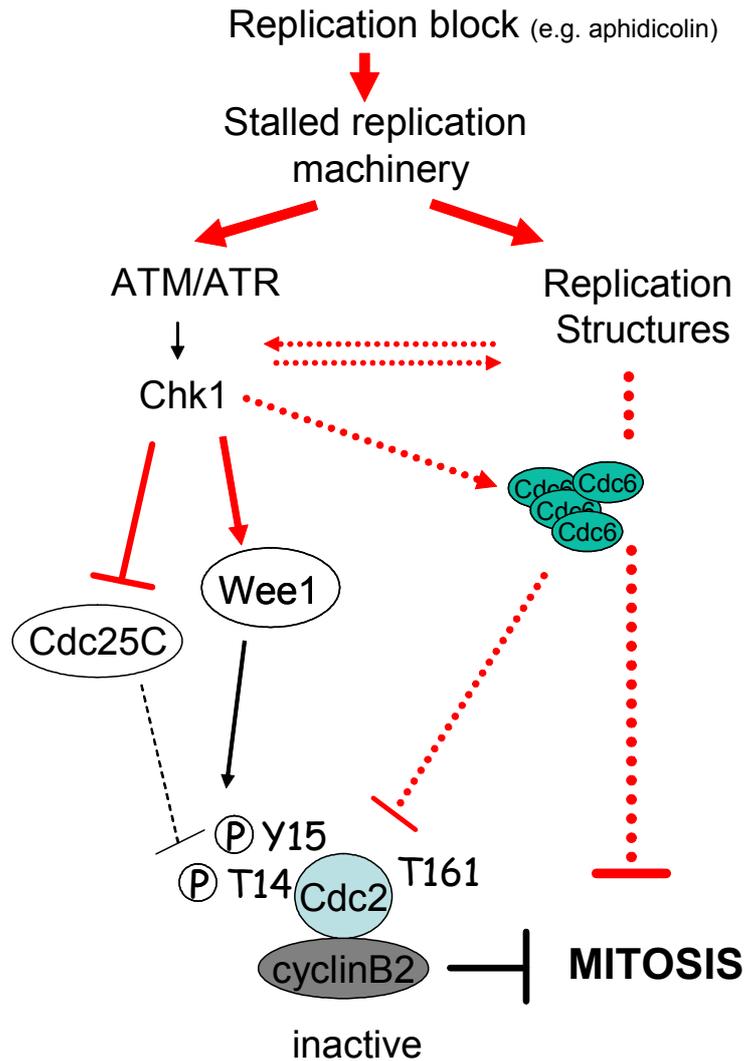
A potential candidate for the link between DNA replication and mitosis would ideally be a protein that is involved in DNA replication as well as checkpoint signalling and maybe even linked to mitotic control mechanisms. As previously mentioned, several replication proteins, such as fission yeast Cdc18/Cdc6 and Orp1 have been implicated to some extent in Cds1 activation during replication checkpoint responses and they can also interact with cyclin dependent kinases (Lopez-Girona et al., 1998; Murakami et al., 2002). Especially Cdc6, which is an essential factor for the initiation of DNA synthesis, has emerged as a prime candidate to be involved in replication checkpoint signalling. So far, a number of studies have addressed potential Cdc6 checkpoint function: For example, one study showed that the over-expression of human Cdc6 in G2 phase cells resulted in a mitotic delay associated with Chk1 phosphorylation (Clay-Farrace et al., 2003). Another study demonstrated that the actual presence of Cdc6 during S phase is required for Chk1 activation in response to replication block in *Xenopus* (Oehlmann et al., 2004). And only very recently Rad26 has been shown to bind directly to Cdc18 and that this interaction can aid the recruitment of Rad3 to the chromatin when DNA replication is stalled (Hermand and Nurse, 2007). In conclusion, these studies have implicated Cdc6 upstream of Chk1 in the replication checkpoint response as the over-expression of Cdc6 was associated with Chk1 phosphorylation.

However, there is also the potential for Cdc6 to interact directly with Cdc2 as several studies have shown that Cdks can bind to Cdc6/Cdc18 in a cyclin-dependent manner (Lopez-Girona et al., 1998; Mimura et al., 2004). This scenario could possibly put Cdc6 downstream of Chk1 in the replication checkpoint response, as Chk1 is involved in the stabilisation of replication structures and thus might affect replication components.

Overall, most of the cited studies on Cdc6 have used ectopically expressed and tagged Cdc6 proteins. The results from the study presented here further confirm a checkpoint function for Cdc6; however, unlike previous studies the results here show that the accumulation of endogenous Cdc6 protein is promoted in a Chk1-dependent manner during DNA replication arrest. To further test whether Cdc6 functions upstream or downstream of Chk1 in the replication checkpoint response, Cdc6 was over-expressed in control BE cells and Chk1-depleted cells. The results from these intervention experiments give preliminary evidence that ectopic expression of Cdc6 can block or at least greatly reduce the mitotic entry of cells in both checkpoint-proficient as well as checkpoint-deficient BE cells. If it is true that Cdc6-overexpression can block mitosis in the Chk1-depleted cells, than this suggest that Cdc6 functions downstream of Chk1 in the S/M checkpoint response.

Obviously, further experiments are required to sufficiently address this issue, such as for example the use of an inducible Tet-On system for Cdc6 in the DT40 WT and the Chk1<sup>-/-</sup> cells which would enable the controlled expression of Cdc6 in the checkpoint-proficient as well as the checkpoint-deficient background. Also, the exact mechanistical details of Cdc6-involvement in mitotic delay are still unclear, but one question to be addressed is the potential sequestration of Cdc2 by Cdc6 bound to the replication machinery.

Taken results from this study into account, Figure 7.2 shows a model including both conventional and other potential mitotic delay mechanisms in response to DNA replication block in vertebrate cells. On one side, stalled replication structures can lead to the activation of Chk1, presumably through ATR via ATRIP-bound single-stranded DNA or other sensor proteins. Activated Chk1 can then contribute to the inhibition of mitosis via the conventional mechanisms of Cdc25C inhibition and Wee1 activation as described above for the DNA damage checkpoint response. On the other side, the replication machinery itself might generate a checkpoint signal leading to mitotic delay which is especially important in the absence of Chk1 or ATM/ATR.



**Figure 7.2: Model for mitotic delay mechanisms in response to DNA replication block**

Replication arrest, such as induced by DNA polymerase inhibition with aphidicolin, results in stalled replication structures. The presence of these structures can result in the activation of a signalling cascade involving ATM/ATR and Chk1 and lead to the inhibition of mitosis by the conventional mechanisms of maintaining the inhibitory phosphorylation of Cdc2 on T14 and Y15. In addition, alternative mitotic delay mechanisms might originate from the replication machinery and block the onset of mitosis independently of ATM/ATR and Chk1. The replication protein Cdc6 appears to accumulate in response to replication arrest in a Chk1-dependent manner and functions in the signalling pathway responsible for blocking the onset of mitosis.

It is likely that these potential pathways generally interact and overlap, for example Chk1 activation might promote Cdc6 accumulation which could block the onset of mitosis maybe directly but most likely by inhibiting Cdc2.

Further studies will be required to address the exact mechanistical details of checkpoint regulation of Cdc6. Many more potential regulatory proteins involved in checkpoint control still remain to be identified. Overall, the investigation of both intrinsic control mechanisms as well as the checkpoint-regulated processes will further improve our understanding of cell cycle control.

In view of cancer therapy, many components of the cell cycle checkpoint machinery have been identified as potential drug targets. Especially in recent years, much attention has been focused on the development of small molecule inhibitors targeting specific proteins of the cell signalling machinery, for example Chk1. It is thought that by inhibiting specific components of the checkpoint pathways in combination with conventional therapeutic approaches it should be possible to further enhance cell death and therefore improve the success rate of the treatment. From recent studies as well as observations made in this study, it appears that under some circumstances Chk1 might indeed be a suitable drug target, as for example Chk1-deficient DT40 tumour cells which do not express functional p53 are more sensitive to killing by IR (Zachos et al., 2003). However, in order to successfully apply the knowledge gained from these studies it will be important to consider both the complexity and interplay between checkpoint mechanisms as well as individual characteristics of each therapeutic case.

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